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PLENARY PAPER

MOLECULAR AND MATHEMATICAL EPIDEMIOLOGY OF BOVINE MASTITIS

Y.H. SCHUKKEN[1], R.N. ZADOKS, L. WHITE AND D.D. DÖPFER

SUMMARY

In this presentation, some recent developments in the molecular and mathematical epidemiology of bovine mastitis are used as examples to show the progress made in combining molecular and mathematical approaches to relevant research questions. Three examples are highlighted in some detail. Firstly, the arguments leading to the conclusion that in certain herds *S. uberis* infections may behave as a contagious form of mastitis are outlined. Secondly, the pathobiology of chronic coliform intramammary infections is discussed in some detail. The data appear to indicate that some reservoir of coliform bacteria in the mammary gland is necessary to be able to give rise to the observed data. Finally, the population dynamics and interaction of major and minor pathogenic bacterial species are examined. It is concluded that widespread infections of minor pathogens may lead to a reduction in transmission potential of major pathogens. Finally, the contribution of epidemiology and molecular microbiology to the better understanding of the pathobiology of intramammary infections is discussed.

INTRODUCTION

Epidemiological research in bovine mastitis has been performed for many years with very early studies relying mostly on clinical observations and linking certain pathogenic bacteria to the clinical signs that were typically produced in the cow by these bacteria (e.g. *Streptococcus agalactiae* and *Arcanobacterium pyogenes*). Subsequently, bacteria were grouped according to their typical behaviour in animal populations, which gave rise to the 'contagious' and 'environmental' bacterial groups (Smith et al., 1985). More recent studies have used statistical techniques to associate certain risk factors with bacteria or groups of bacteria. For example, use of teat disinfection in low somatic cell count herds was associated with an increased risk of coliform mastitis (Schukken et al., 1991; Lam et al., 1996a) and cows with very low somatic cell counts were more likely to get clinical mastitis or severe clinical mastitis compared with cows with somewhat higher cell counts (Green et al., 1996; Suriayasathaporn et al., 2000; Peeler et al., 2001). As expected with such risk factor studies, not all studies result in associations in the same direction, making the cause and effect arguments somewhat controversial. Additionally, risk factor studies are often cross-sectional, so that the time order of events and causal relationships are impossible to prove.

In the last decade, two additional techniques have been added to the toolkit of the mastitis epidemiologists: mathematical modelling and molecular diagnostics. These new tools have added a lot of opportunities to contribute to a better understanding of the pathobiology of intramammary infections in the dairy cow. Molecular diagnostic tools allow the distinction

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between strains in the same bacterial species (e.g. Wang et al., 1999; Zadoks et al., 2000). Amongst other things, this does allow for more precise longitudinal follow up studies of infection occurrences in populations. Mathematical models have contributed especially in the area of contagious disease dynamics (Anderson & May, 1985; De Jong, 1995), both in terms of explaining observed infection (and/or disease) frequencies and predicting or simulating infection frequencies under a given set of preventive measures (De Jong, 1995).

The objective of this presentation is to review a number of recent studies attempting to answer mastitis research questions using molecular and mathematical methods. Using these examples, we will also attempt to formulate some general experiences and suggestions that we have come to appreciate during the execution of these studies. The three areas of research are the epidemiology of *Streptococcus uberis* infections, the chronicity of *Escherichia coli* intramammary infections and the impact of minor pathogens on the risk of new infections with major pathogens.

EPIDEMIOLOGY OF *S. UBERIS* INFECTIONS

The underlying question with regard to the epidemiology of *S. uberis* infections was whether they originate from other cows or whether they originate from the environment of the cow. The origin of infections must be known so that appropriate control measures for mastitis prevention in dairy herds can be chosen. The question was approached with mathematical and molecular tools. The null-hypothesis that was tested in the mathematical approach to this study was that the number of new infections of *S. uberis* in a population does not depend on the number of existing shedders in that population. In statistical terminology, the infection dynamics are described by: $I_{\Delta t} = \theta P_{t-1}$, where I = incidence, P = prevalence, Δt = a given period of time, t-1 = beginning of time period, θ = regression parameter. The null hypothesis assumes $\theta = 0$. The alternative hypothesis indicates that $\theta > 0$ (possibly $\theta <> 0$). A similar model was previously used by Lam et al. (1996a) to model the dynamics of *Staphylococcus aureus* infections in a dairy herd.

Data were from a dairy farm with 95 ± 5 lactating animals (mean ± s.d.) where an outbreak of *S. uberis* infections was observed (Zadoks, 2002). Data were collected during an 18-month observation period with 27 farm visits at 3-week intervals. These data are summarised in Fig. 1. Initially, a low prevalence and very low incidence of *S. uberis* infections was evident. At approximately sampling period 10, the start of an exponential growth of new infections could be seen. This outbreak halted around sampling 16. The prevalence remained high for a while, but then dropped to a much lower level. Towards the end of the observation period, the prevalence was still approximately three times as high as at the start of the study.

To model these data in a biologically meaningful way, it was necessary to assess whether spread of one specific strain has occurred. It has been described that multiple strains of *S. uberis* co-exist within a given dairy herd (Wang et al., 1999; Douglas et al., 2000). An outbreak consisting of cases from multiple molecularly distinguishable strains would indicate that spread did not occur from cow to cow, that is, would favour the null hypothesis. Typing of the strains in the outbreak using random amplified polymorphic DNA (RAPD) fingerprinting techniques resulted in strong evidence for clonal spread (Zadoks et al., 2003). One large clonal outbreak with the strain named ‘B’ was observed (Fig. 2).

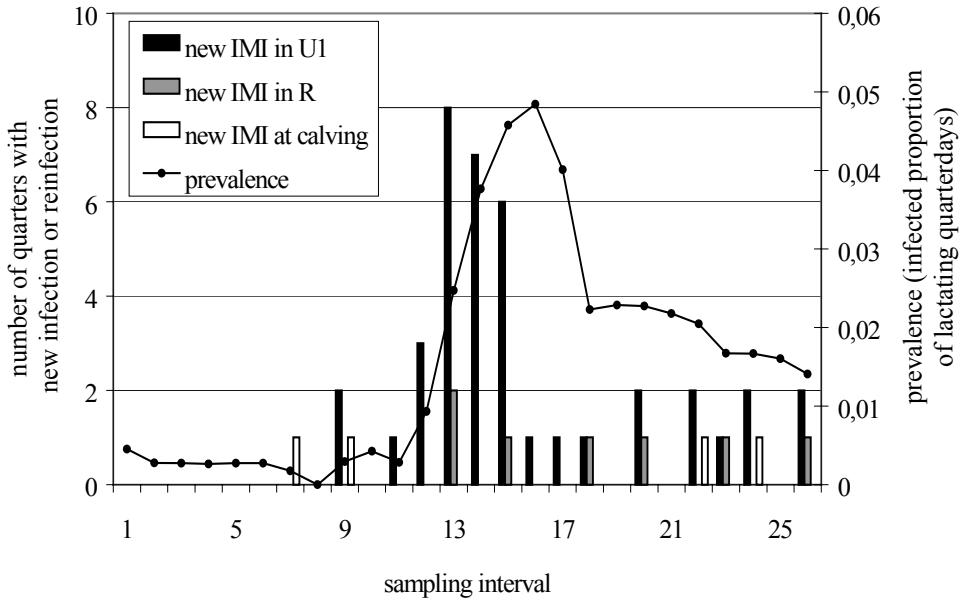

Fig. 1 Outbreak of *Streptococcus uberis* infections in a dairy herd. U1 are previously uninfected quarters, R are quarters that have already experienced a *S. uberis* infection (reinfection). All quarters of all cows were sampled approximately every 3 weeks.

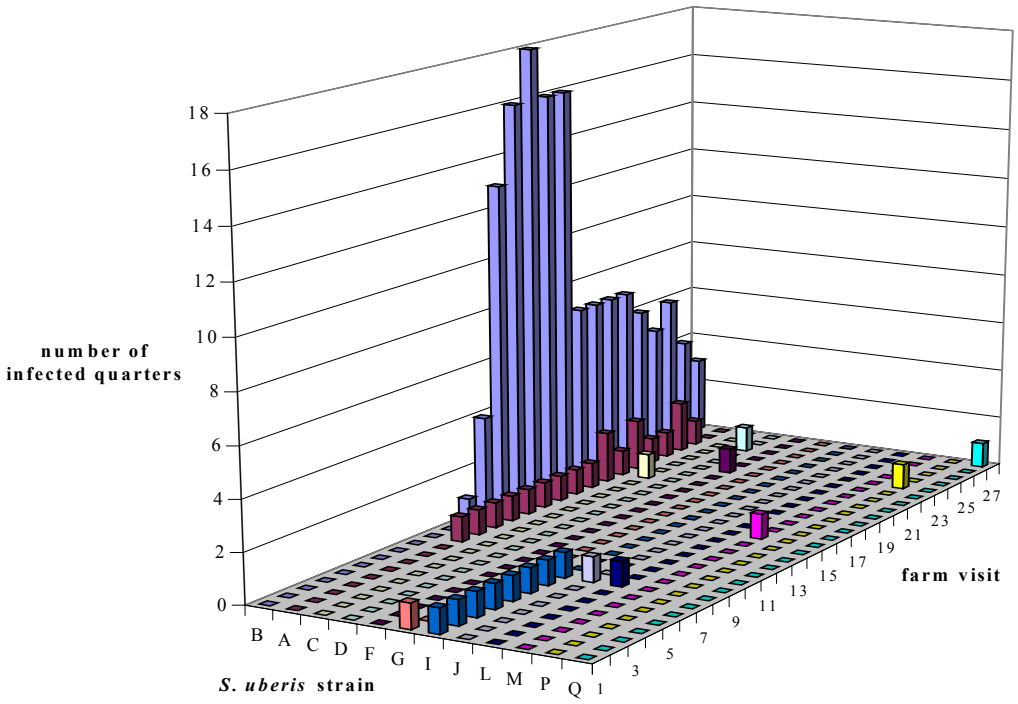

Fig. 2 Frequency of infected quarters by farm and strain of *Streptococcus uberis*. Strains were typed by random amplified polymorphic DNA (RAPD) fingerprinting.

In addition, a number of single infections with a variety of different strains were observed on the farm. The data were modelled in a Poisson logistic regression model:

$$\varepsilon\,[\ln(IMI)] = \ln(\beta') + \ln(S/N) + \theta_1 * \ln(I) + \theta_2 * y + \theta_3 * U_m \quad (1)$$

where, ε = expected value, IMI = number of new intramammary infections with *Streptococcus uberis* in current time interval, β' = transmission parameter for model with ln(S/N) as offset, S = number of quarter-days susceptible in current time interval, N = total number of quarter-days in current time interval, I = number of quarter-days infected in preceding time interval, y = dummy variable for phase (y = 0 for early phase of the study up to and including the outbreak, y = 1 for the late phase of the study), U_m = dummy variable for compartment (U_m = 0 for R, U_m = 1 for U_1) and θ_i = regression coefficients. The estimates, standard errors and *P*-values for ln(β') and the three regression coefficients are shown in Table 1. Of specific interest is parameter θ_1 because this parameter tests the hypothesis that existing shedders contribute to the incidence of new infections.

Table 1. Estimates, standard errors and *P*-values for ln(β') and regression coefficients. U_1 = never before infected with *Streptococcus uberis*; R = recovered from infection with *S. uberis*

Model	Parameter	Coefficient	Estimate	Standard error	*P*-value
U_1 vs. R	ln(β')		0.11	0.78	0.8793
	ln(I)	θ_1	0.68	0.15	< 0.0001
	study phase	θ_2	-1.55	0.35	< 0.0001
	compartment	θ_3	-2.06	0.42	< 0.0001

The regression results indicated that θ_1 was significantly different from 0, and hence the initial null hypothesis was rejected implying that the number of new infections was not independent of the number of existing infections. Therefore, the alternative hypothesis, indicating contagious transmission of *S. uberis*, appeared to best fit the data. Additional arguments that favour cow-to-cow transmission and that indicated possible mechanisms of transmission were 1) finding identical strains of *S. uberis* in the milking liner up to 3 cow-milkings after milking of a *S. uberis* shedding cow, 2) finding only new infections with strain 'B' in lactation (in contrast to other strains that infect also during the dry period), and 3) the relative long duration of infection with strain 'B'. Survival analysis of observational data showed that infections with strain B lasted significantly longer than infections with other strains. Thus, infections with strain B would have a larger window of opportunity for spread, most likely occurring during the milking process, than other strains. The molecular, mathematical and observational data analysis can be complemented by pathogenesis studies. *In vitro* studies have shown differences between *S. uberis* strains in their ability to adhere to and invade mammary epithelial cells (Almeida et al., 1999). If strain B had a higher ability to adhere and invade than other strains, that ability could hypothetically result in a longer duration of infection and hence more time for contagious transmission, which would in turn lead to the phenomena that were observed using mathematical, statistical and molecular tools.

CHRONICITY OF *E. COLI* INTRAMAMMARY INFECTIONS

In recent publications, the occurrence of chronic *E. coli* intramammary infections was reported (Lipman et al., 1995; Döpfer et al., 1999; Döpfer et al., 2000). Using DNA fingerprinting, the presence of indistinguishable isolates from repeated cases of clinical mastitis in the same quarter of the same cow was shown. An example of such an infection is shown in Fig. 3. The isolates in lanes 2-14 were repeated cases of clinical coliform mastitis from the same quarter of the same cow and the isolates in lanes 16 to 19 came from a different quarter in the same cow.

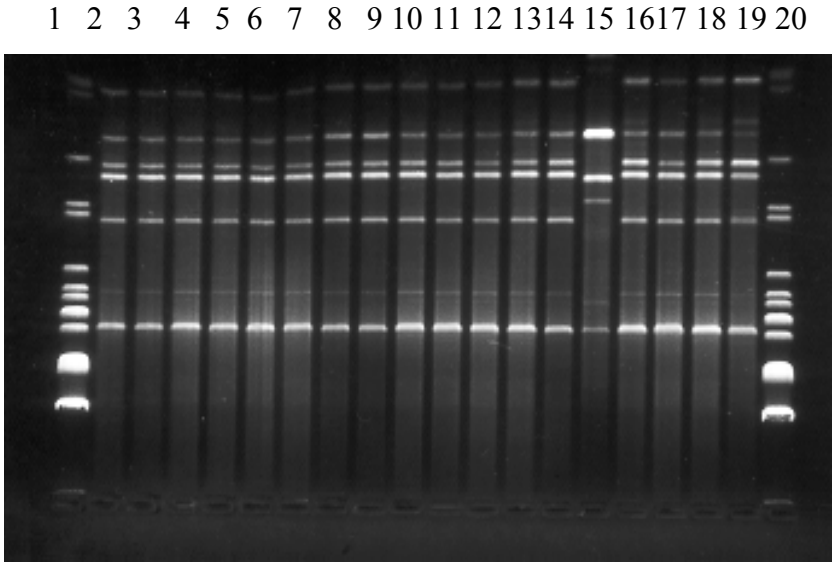

Fig. 3 Genetically indistinguishable isolates (evaluated by DNA fingerprinting using PCR-REP and ERIC primers) from recurrent clinical *E. coli* mastitis cases. Lanes 1 and 20: molecular size marker. Lanes 2-14: Cow 1, quarter A; Lane 15: Cow 2; Lanes 16-19: Cow 1, quarter B.

Because of the high number of *E. coli* strains in the dairy environment, it is unlikely that recurrent isolation of one strain from the same quarter was the result of recurrent new infections. However, it is not impossible. To determine whether the infection was really persistent, longitudinal data on a single chronically infected cow were collected. Colony forming units and somatic cell concentrations from the infected quarter are shown in Fig. 4. In colony forming units and somatic cell concentrations, an apparent inverse cyclicity (with a Pearson correlation of -.36, $p<0.05$) was observed.

These data, although relatively sparse, provide initial evidence that milk leukocytes and bacteria show a dynamic behaviour. Similar behaviour has been suggested for *S. aureus* (Daley, 1991). Such dynamic behaviour may be modelled using 'predator-prey' type modelling techniques:

$$dR/dt = rR(1-R/K) - ENR^2/(R_0^2 + R^2) \quad (2)$$

$$dN/dt=N[-d+cER^2/(R_0^2 + R^2)] \quad (3)$$

where:

R = *E. coli* density in cfu per ml, t = time, r = growth rate of *E. coli* (logistic growth), K = carrying capacity of the system, E = somatic cell count (SCC) saturation level of 'consuming' *E. coli,* N = SCC density in cells per ml, R_0 = half-saturation density of *E. coli* (where $R > R_0$), d = per capita rate at which SCC die out when no *E. coli* are present, c = 'conversion efficiency' of *E. coli* to SCC - this may be considered as feeding efficiency.

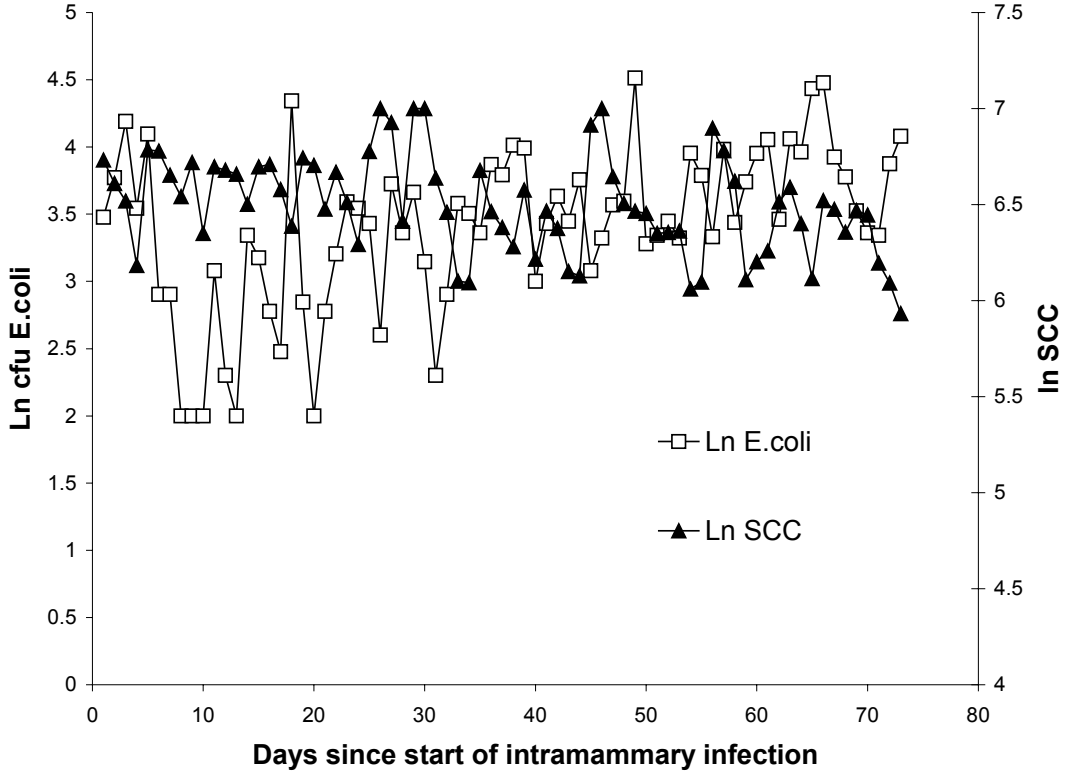

Fig. 4 Time series of a chronic intramammary coliform infection. Daily observations on bacteria counts and somatic cell counts (mostly polymorphnuclear cells) in a chronically infected quarter (Döpfer, 2000)

To incorporate the concept of an intracellular reservoir for the bacteria, the model may be extended by including an additional state variable, Z, governed by the ODE's. Equations 4 and 5 are identical to equations 2 and 3, except for equation 4, where ϕZ, the release of bacteria from the third state, Z, at a rate ϕ per day, is added to and πR, the intracellular invasion of bacteria, R, at a rate π per day, is subtracted from dR/dt.

$$dR/dt = rR(1-R/K) - ENR^2/(R_0^2 + R^2) + \phi Z - \pi R \quad (4)$$

$$dN/dt=N[-d+cER^2/(R_0^2 + R^2)] \quad (5)$$

$$dZ/dt= - \phi Z + \pi R \quad (6)$$

Figure 5 shows the results of a simulation model using the above set of equations. Parameter estimates were obtained from the literature or from the data. The model with the intracellular reservoir fitted the data considerably better compared with a model without such a reservoir. Several potential reservoirs (i.e. dormant bacteria) may be envisaged. For example, leukocytes that contribute to the somatic cell count (SCC) ingest *E. coli* and a subsequent failure to kill the coliform bacteria, creates a reservoir. Studies have also shown that mammary epithelial cells may act as a reservoir (Almeida et al., 1999; Döpfer, 2000).

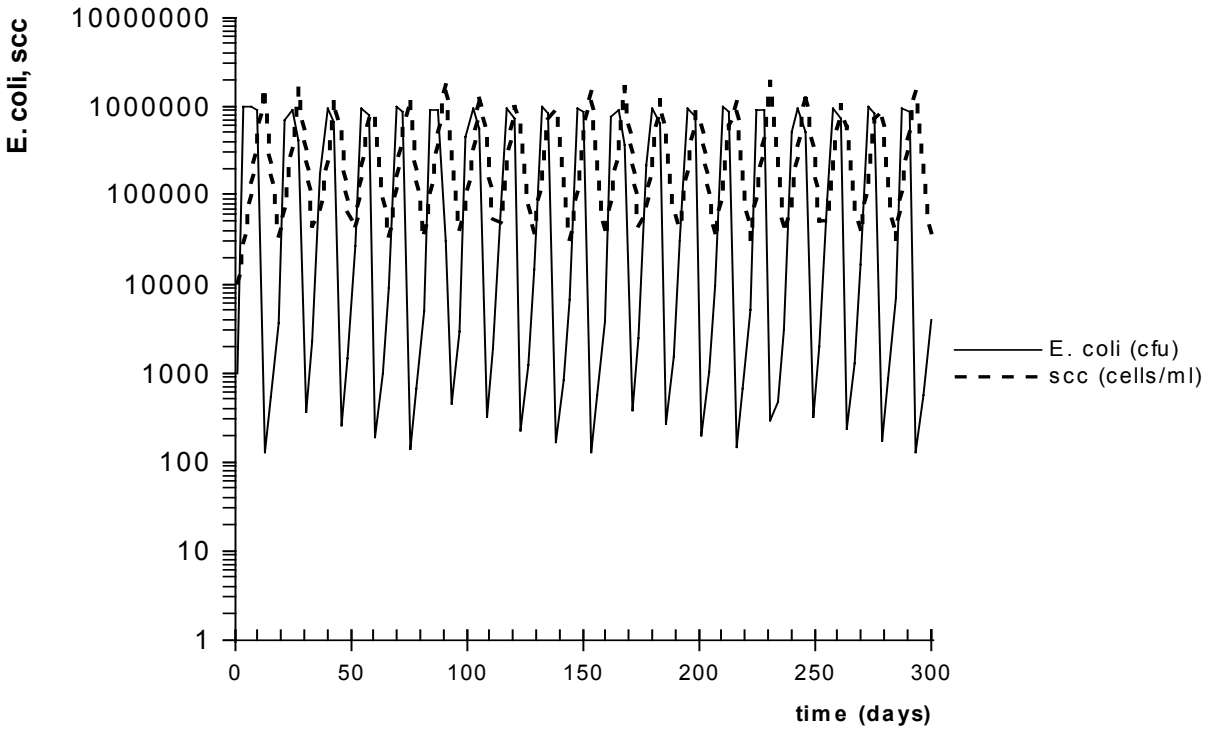

Fig. 5 Simulated data for *Escherichia coli* concentration and somatic cell count (SCC) in a chronically infected quarter based on predator-prey model with an intracellular reservoir of bacteria.

Chronicity of *E. coli* infections would theoretically open up the potential of contagious spread of this pathogen that has hitherto always been considered as an environmental pathogen. Recent field studies in the United Kingdom, where multiple chronic and partly subclinical infections were observed in dairy herds, also suggest that *E. coli* may be evolving to a more cow adapted and possibly contagious organism (Bradley & Green, 2001). Further research in terms of collection of field data will be necessary to obtain better quantitative estimates for the model parameters. Stochastic models will also aid our understanding of chronic infection versus clearance of infection. Initial Monte Carlo simulations of the model presented above do support the conclusion of an *E. coli* reservoir in the mammary gland as a prerequisite for chronic infections. Molecular methods have provided the arguments that chronic coliform infections do exist. The mechanisms behind bacterial persistence are not clear at this point in time. As for the *S. uberis* example, further experimental studies and additional modelling work will be required to elucidate the biological mechanisms further.

IMPACT OF MINOR PATHOGENS ON THE RISK OF NEW INFECTIONS WITH MAJOR PATHOGENS

The bacterial pathogens responsible for infection of the mammary gland may be split into two main categories, major and minor. Infection with major pathogens generally results in clinical illness or strong inflammatory responses and reduced milk yields, whereas minor pathogen infection is usually subclinical with a less severe SCC increase or yield loss. Previous investigations have considered the transmission of major and minor pathogens independently. Experimental evidence has in some cases shown cross-protection between species of pathogens. A mathematical model for the transmission of both major and minor pathogens along with their interaction via the host was developed by White et al. (2001a; 2001b; 2002) to consider various methods for controlling the incidence of major pathogen infection (Fig. 6). A stability analysis of the model equilibria provide explanations for observed phenomena. Previous modelling results focused on one bacterial species only. However, this multi-species model structure has provided a basis for quantifying the extent of cross protection between species and for assessing possible control strategies against the disease.

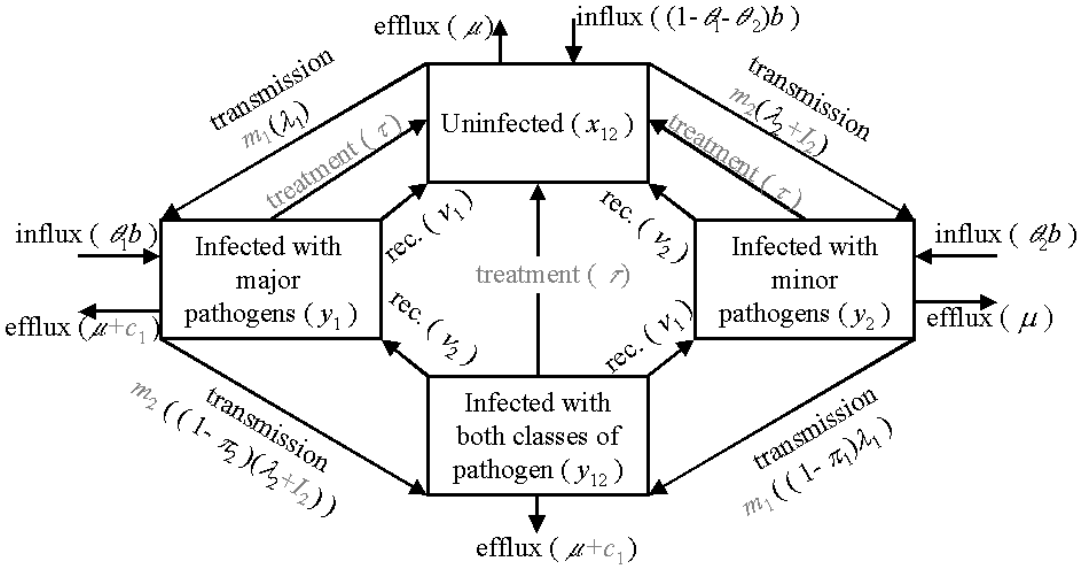

Fig. 6 Multi-species infection transmission model (White et al., 2001b). The variables and transmission parameters are identified in Table 2.

This analysis extends the work of Lam et al. (1996a) who modelled mastitis transmission in cattle using SIS (susceptible-infectious-susceptible) models that were fitted to prevalence and incidence data from herds of dairy cows. The results suggested some interaction in the transmission of the different pathogen species. The results indicated that, where both minor and major pathogens were being transmitted, the basic reproduction number of *S. aureus* (a major pathogen) decreased during the course of an outbreak of mastitis. This result could not be explained using decoupled (no interaction between species) models.

The objective of the extended work was to develop a simple multi-species model, where there is some cross-protection provided by infection by one class of pathogens (minor pathogens) against infection by another class (major pathogens) and to examine the dynamic consequences of the interaction (White et al., 2001a). The multi-species model that was used is

presented in Fig. 6. Other symbols in Fig. 6 represent interventions: m_i represents a decrease in transmission by post milking teat disinfection, c_i additional culling, τ treatment of infections and I_i inoculation of quarters.

The model was fitted to the data observed by Lam et al. (1996a) using the computer package, Facsimile. The raw data were in the form of spreadsheets for each of eighteen samplings. The number of colony forming units of each pathogen for each quarter of each cow was given.

Table 2. Variables and parameters in the multi-species model.

	Symbol	Units	Definition
variables	x_{12}	normalised	Proportion of the lactating population not infected with either class of pathogens.
	y_1	normalised	Proportion of the lactating population infected with major pathogens.
	y_2	normalised	Proportion of the lactating population infected with minor pathogens.
	y_{12}	normalised	Proportion of the lactating population infected with both classes of pathogens.
	λ_1	day^{-1}	Force of infection for major pathogens.
	λ_2	day^{-1}	Force of infection for minor pathogens.
parameters	μ, b	day^{-1}	Average turnover of lactating cows in the herd.
	θ_1	normalised	Proportion of individuals entering the lactating herd already infected with major pathogens.
	θ_2	normalised	Proportion of individuals entering the lactating herd already infected with minor pathogens.
	θ_{12}	normalised	Proportion of individuals entering the lactating herd already infected with both classes of pathogens.
	ν_1	day^{-1}	Average recovery rate from major pathogen infection.
	ν_2	day^{-1}	Average recovery rate from minor pathogen infection.
	β_1	capita^{-1}day^{-1}	Transmission rate of major pathogens.
	β_2	capita^{-1}day^{-1}	Transmission rate of minor pathogens.
	π_1	normalised	Level of cross-protection against major pathogen infection provided by a minor pathogen infection.
	π_2	normalised	Level of cross-protection against minor pathogen infection provided by a major pathogen infection.

The system equations (without intervention) are given by:

$$\left.\begin{aligned}
\dot{x}_{12} &= (1-\theta_1-\theta_2)b - (\lambda_1+\lambda_2+\mu)x_{12} + \nu_1 y_1 + \nu_2 y_2 \\
\dot{y}_1 &= \theta_1 b + \lambda_1 x_{12} + \nu_2 y_{12} - ((1-\pi_2)\lambda_2 + \nu_1 + \mu)y_1 \\
\dot{y}_2 &= \theta_2 b + \lambda_2 x_{12} + \nu_1 y_{12} - ((1-\pi_1)\lambda_1 + \nu_2 + \mu)y_2 \\
\dot{y}_{12} &= (1-\pi_1)\lambda_1 y_2 + (1-\pi_2)\lambda_2 y_1 - (\nu_1+\nu_2+\mu)y_{12}
\end{aligned}\right\} \qquad (7)$$

where:

$$\left.\begin{aligned} 1 &= x_{12} + y_1 + y_2 + y_{12} \\ b &= \mu \\ \lambda_1 &= \beta_1 (y_1 + y_{12}) \\ \lambda_2 &= \beta_2 (y_2 + y_{12}) \end{aligned}\right\} \tag{8}$$

Steady state analysis has produced a 'cross-protection curve' (Fig. 7) that has a similar form to those produced from other multistrain/species models (Gupta et al., 1994; Andreasen et al., 1997; Feng & Velasco-Hernandez, 1997; White et al., 1998). A similar analysis on the model equations extended to include various control procedures has given some theoretical insight into their possible effects.

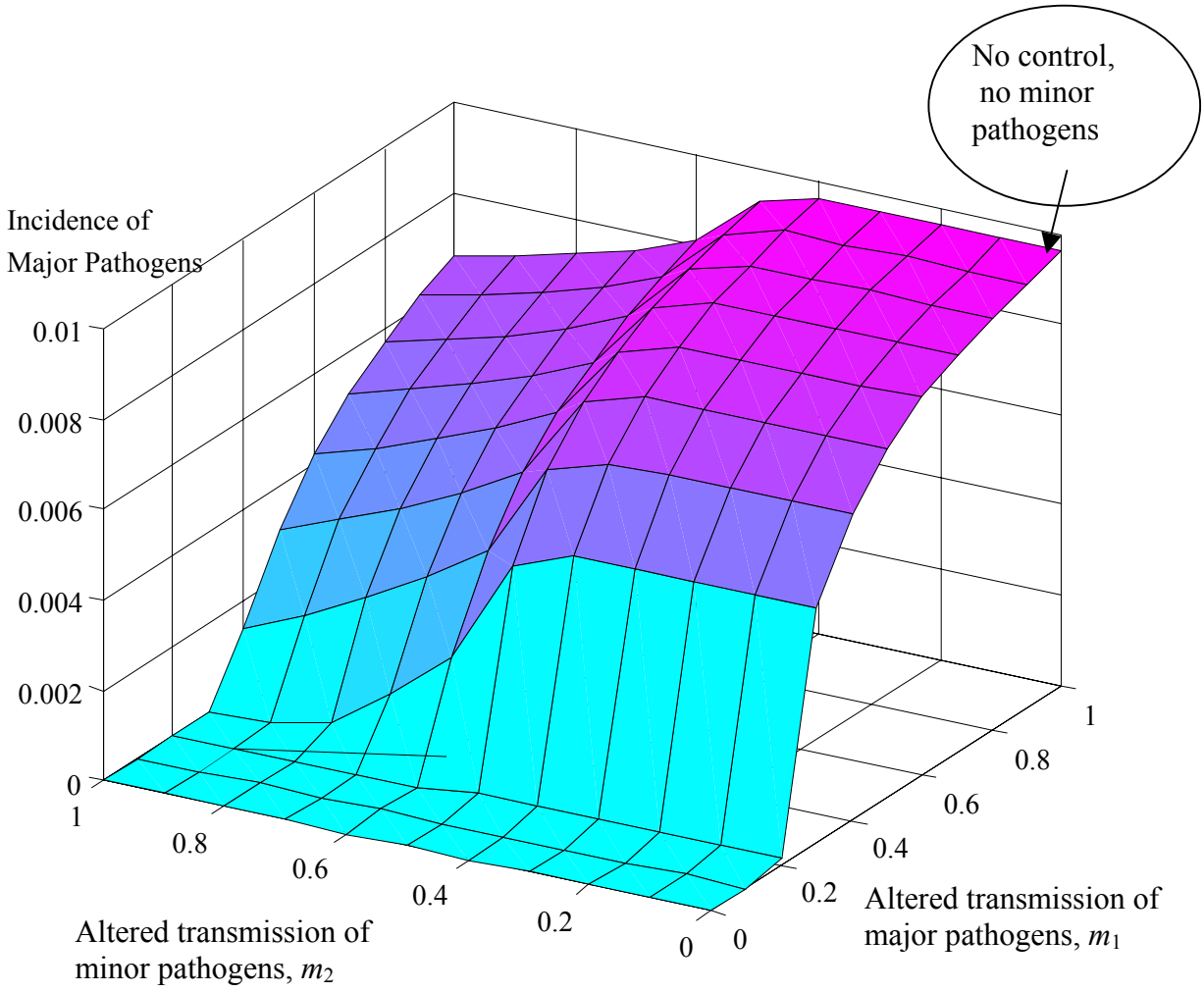

Fig. 7 Predicted impact of transmission intervention on the incidence of mastitis with major pathogens. Transmission of 0 indicates fully successful intervention (no transmission) while transmission of 1 indicates no intervention at all.

Figure 8 shows the boundary in parameter space between where the minor pathogen only equilibrium is stable and where it is unstable (and therefore the equilibrium where both major and minor pathogen classes are present is stable). The axes represent the basic reproduction numbers, R_{01} and $R_{02,}$ for major and minor pathogens respectively. The narrow black line of the graph in Fig. 8 shows the starting point of the comparison, a situation where no control procedures are in place. The cross (X) indicates a particular (uncontrolled) system with given basic reproduction numbers for major and minor pathogens. The cross is above the line, implying that major pathogens should be able to invade the system and persist at equilibrium. When treatment is included in the model, the boundary is shifted upwards, therefore decreasing the likelihood of invasion of major pathogens into the herd. In the example illustrated by Fig. 8, the treatment cure rate was high enough to move the boundary above the cross and would therefore successfully eliminate major pathogens from the herd (given that pathogen spread is predominantly contagious, not environmental).

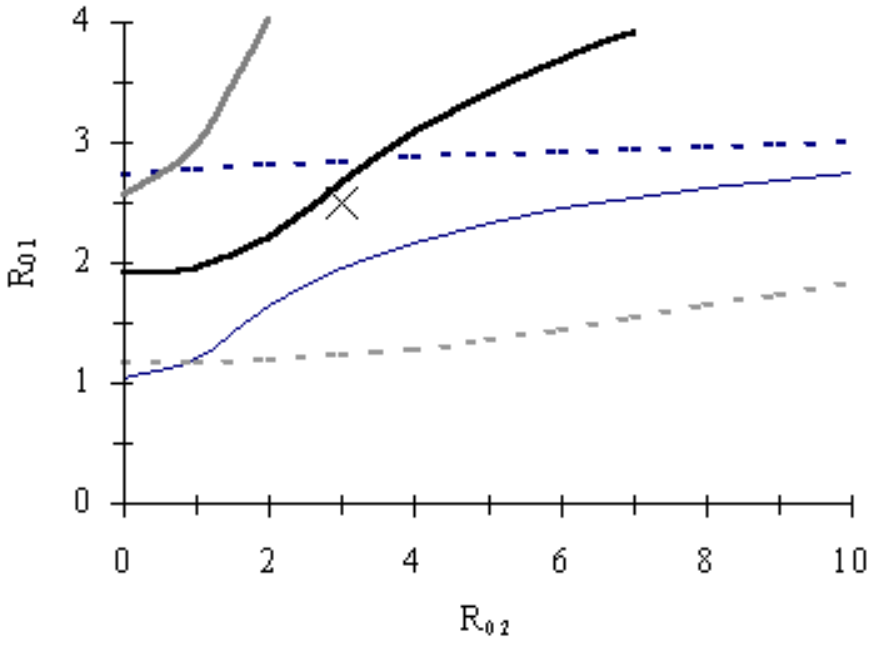

Fig. 8 Graphs showing boundaries in (R_{02}, R_{01}) space between where the coexistence equilibrium is stable (above the line) and where the minor-pathogen-only equilibrium is stable (below the line). The axes represent the basic reproduction numbers, R_{01} and $R_{02,}$ for major and minor pathogens respectively. The narrow black line shows the boundary for the parameter set θ_1=0.0 day^{-1}, θ_2=0.5 day^{-1}, ν_1=ν_2=0.01 day^{-1}, μ=0.0015, π_1=0.7 and π_2=0.0 where the model includes no control programmes (i.e. τ=$c1$=I_2=0 and m_1= m_2=1). Bold black line: treatment (at rate τ=0.01). Bold grey line: culling of major pathogen infected cows (at rate c_1=0.017). Grey dotted line: postmilking teat disinfection (with parameters m_1=0.9 and m_2=0.2). Black dotted line: inoculation of cows with minor pathogens (at rate I_2=0.1).

The culling of cows infected with major pathogens (at the same rate as the treatment cure rate) had a more pronounced effect moving the boundary much higher for increasing values of R_{02}. As shown in Fig. 8, inoculation of cows with minor pathogen species would enhance the herd immunity against major pathogen infections (black dotted line). However, there must be a sufficiently high level of natural cross-protection against major pathogen infection provided by infection with the minor pathogens for them to out-compete the major pathogens. Although this is a theoretically feasible option, it is logistically easy not to infect animals with minor pathogens without at the same time increasing the risk of infection with major pathogens. Novel application systems would need to be developed to make this a feasible option.

DISCUSSION

The known udder pathogenic bacterial species show such a large variability within species that valid modelling of observed events is only possible with knowledge of the particular clones present in the data. This was shown to be important in the analysis of the observed *S. uberis* outbreak in a dairy herd. Several of the strains obtained from infected cows showed behaviour typical of single isolated infections without transmission between animals. However, one of the strains showed a very different epidemiology, with abundant evidence for clonal spread according to the laws of mass action. Modelling of these data was very helpful in providing quantitative evidence for contagious behaviour of this *S. uberis* strain. Using statistical testing, a formal argument can be made that this particular strain showed a rate of new infection that was dependent on the number of shedders. The argument still continues, because it is not impossible that a surge of growth of this particular *S. uberis* strain occurred in the environment of the cows. Several quantitative and non-quantitative arguments favour the contagion hypothesis but the undisputed proof of that may turn out to be impossible. It is interesting to note that the same arguments are used to 'prove' the contagious nature of spread of *S. aureus*, and for reasons beyond the realm of reason, the contagiousness of *S. aureus* does not seem to be a matter of debate. Using molecular fingerprinting techniques, it was shown beyond doubt that chronic coliform infections occur in dairy cows. The observed data suggest a predator-prey type of cyclical system. Using fairly simple Lotka-Volterra type models, the observed data were reasonably well reproduced. Modelled somatic cell counts were relatively stable compared with the large fluctuation observed in the modelled bacterial count data. The addition of an intracellular reservoir somewhat dampened the fluctuation in predicted bacterial counts. Therefore, the initial models suggest that an intracellular reservoir explains the observed data slightly better than a model without an intracellular reservoir of bacteria. Further biological data will need to be collected to further elucidate the location, if any, of reservoirs and the pathogenesis of this infection.

The multi-species model is an extension of previous modelling work. It extended the specific modelling work of Lam et al. (1996a; 1996b; 1997a; 1997b; 1997c) on the transmission of mastitis pathogens as well as providing some validation of a standard multi-species model structure (Lipsitch, 1997). The multi-species model could be used to design an effective control strategy if its parameters were identified. More over, the dynamical output of the model is consistent with the data from the biological system. Rather then looking at individual cows or quarters which is the typical approach in evaluating species competition, it is of much greater value to focus on ecological interactions between pathogen species (and strains) because they can have important influences on transmission dynamics. Infections with contagious organisms not only affect the infected individual, but they have an important impact on the population as well. When complex relationships between species exist, modelling is virtually the only option

to look at the ecology of the organisms in the population. Using this multi-species modelling approach, it has become clear that competition between species may be an important control option with regard to the transmission of clinically important pathogens (White et al., 1998; Ferguson et al., 1999). Such interactions can greatly enhance or reduce the effect of efficient control measures (McLean, 1995; White et al., 1998; Ferguson et al., 1999). The premise of this applied population biology exercise is that a relatively small number of characteristics (e.g. major versus minor pathogens) suffice to account for major patterns in infection occurrence. Clearly these initial studies and models should be expanded further to better reflect the complexity of the biology. This study has not utilised any molecular diagnostics. More precise identification of minor pathogen species (e.g. Corynebacteria are probably different in their protective effect compared to coagulase negative Staphylococci) and identification of major pathogen strains are warranted so as to improve the understanding of herd dynamics. There is evidence that strains within a species differ in transmission potential (R_0 value) under a given set of circumstances (e.g. Middleton et al., 2001 for *S. aureus*; Zadoks et al., 2002 for *S. uberis*).

Some important developments for modern epidemiology are becoming evident when the results of these population studies on mastitis in cows are combined. The use of molecular methods is becoming a prerequisite for precise epidemiological studies on intramammary pathogens. Without a confirmation that clonal spread occurs through a population of animals, it is difficult to be persuasive in novel arguments (paradigm shifts) about the epidemiology of strains (Zadoks et al., 2002; 2003).

Similarly, the use of generic species grouping to study epidemiological behaviour in populations should be used with care. As shown in these examples, some strains do not follow the conventional paradigms of bacterial species behaviour. Most *S. uberis* strains would have an environmental reservoir (Wang et al., 1999), but as we have shown, animal reservoirs may exist. Most coliform infections in the mammary gland are clinically severe and short-lived (Smith et al., 1985), but as shown, chronic infections do occur.

Use of mathematical modelling to further explain the epidemiology and pathogenesis of intramammary infections has great potential. The examples presented all show an important additional understanding of the biology of infection in the population or in the host due to the additional tool of mathematical modelling.

Modelling of mastitis is challenging mainly due to the difference in dimensions between the biological system and the available data. The dimension and complexity of deterministic (mechanistic) models describing mastitis in dairy cows can be increased *ad infinitum*. There are several overlapping and interacting levels of organization in the host (quarter, cow, herd, within cow, between cow) and parasite (species, strains) populations, not to mention all the external influences of the environment. Including random or structured variation in the proposed model would reduce its dimension, as would biologically justifiable assumptions which simplify the model. The models that were used in the current examples are all either deterministic or stochastic compartmental models using differential equations. Koopman et al. (2001) recently described these as the first (simplistic) steps toward models that truly reflect field data. The next generation of models would include individual event history models and dynamic network models. For use in mastitis epidemiology, individual event history models are particularly appealing. The infection history and risk factor constitution (i.e. teat lesion, age, production level, *etc.*) of an individual animal or preferably individual quarter are often known from data gathered in field studies, but this factors cannot be incorporated in the current generation of infection dynamics models. Multiple data sets including results from molecular work and

model-induced experimental work, as well the combined data from sets of observational studies, would increase the dimension of the data. This is one of the challenges over the next few years.

The combination of knowledge of the biology of infection, precise data from observational studies, molecular fingerprinting techniques and mathematical modelling provides an excellent basis for precise understanding of disease pathogenesis or pointing the way to additional hypotheses and research questions. At the same time, these tools can be utilised to understand the population impact of control strategies. An essential aspect of epidemiology is that the whole is more than the sum of its parts. This is true for populations that are more than the sum of many individuals, and for the epidemiological toolkit, that is more than the sum of the individual techniques. It is through combined use of multiple approaches and through collaboration of experts in the different techniques that most progress in understanding of epidemiology and control of animal disease can be made.

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TUBERCULOSIS

A QUANTITATIVE RISK ASSESSMENT FOR BADGER TO CATTLE TRANSMISSION OF *MYCOBACTERIUM BOVIS*

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SUMMARY

A Quantitative Risk Assessment (QRA) model has been developed to estimate the risk of transmission of *Mycobacterium bovis* from badgers to cattle. Within this paper, results are presented relating to two parts of the QRA, namely the exposure assessment and the dose response component. The aim of the model was to estimate the number of cattle infected in a herd if badgers excreting *M. bovis* were also present. The time until the first cow was infected with *M. bovis* was also estimated. The effects of potential control strategies, namely a reduction in the level of infection in the badger population, the herd size and the restriction of cattle from badger latrines, on the model outputs were investigated.

The model indicated that in the presence of a single excreting badger, the first cow in a herd of 74 animals will become infected after a mean of 102 days. This was reduced to a mean of 89 days in the presence of 7 excreting badgers. However, if the herd size was reduced to 60 cattle, the time until first infection was increased to 124 days in the presence of 1 excreting badger and 116 days in the presence of 7 excreting badgers. The mean number of cattle infected per year in an average sized herd ranged from a mean of 2.5 to 2.7 in the presence of 1 or 7 excreting badgers, respectively. This estimate is reduced to a range of 1.5 to 2.2 in the presence of 1 or 7 excreting badgers respectively, if the herd size is reduced to 60 cattle. Furthermore, by restricting cattle access to badger marking areas and latrines the number of cattle infected per year was reduced to a range of 1.5 and 1.6 in the presence of 1-7 excreting badgers respectively.

One of the most important outputs from this model was the identification of crucial areas of data deficiency. However, it was vital that the quantitative estimates from the model were plausible, in order that sensitivity analysis could be performed to identify such deficiencies. Examples of areas of data deficiency identified within the exposure assessment include quantification of the levels of excretion of *M. bovis* at different stages of disease by the different routes of excretion. Data relating to the length of time each stage of disease lasts are also unavailable. In addition, studies are required to determine the existence of intermittent excretion, establish a definition for the term and provide quantitative data for the levels of excretion when it does occur. It was also highlighted that studies are required to investigate the levels of exposure, particularly with reference to the different types of badger excreta.

Furthermore, quality data relating to the dose response of *M. bovis* in cattle are very limited. Therefore, dedicated dose response experiments to investigate the relationship between infection and exposure via inhalation and/or ingestion are required.

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The data limitations or deficiencies identified by the model will be used to target future research in the field of *M. bovis* in badgers. The identification of areas where our knowledge is currently lacking is vital in driving policy decisions and enabling policy makers to target future research requirements in an efficient and economic way.

INTRODUCTION

Mycobacterium bovis is the aetiological agent of bovine tuberculosis. In 1971, the badger was identified as a potential reservoir for *M. bovis* in Great Britain (GB), when a badger carcass infected with *M. bovis* was found on a farm which had recently suffered a tuberculosis breakdown in its cattle herd. Consequently, the hypothesis that badgers could, and were transmitting *M. bovis* to cattle became established. Although this hypothesis has not as yet been formally proven, strong circumstantial evidence exists to prove an association between *M. bovis* infection in badgers and in cattle.

In 1997, as part of an independent government commissioned review, 'Tuberculosis in cattle and badgers' (Krebs, 1997), recommendations were made to focus on investigating the badger to cattle transmission hypothesis. In response to these recommendations, a Quantitative Risk Assessment (QRA) model has been developed in order to estimate the risk of transmission of *M. bovis* from badgers to cattle in localised areas and to investigate the effect of control strategies on these risk estimates. The QRA model which has been developed is stochastic in nature and uses Monte – Carlo simulation to describe the uncertainty associated with the final outputs. These estimates may, however, be considered secondary, as the most important output from this and many other QRA models, is considered to be the identification of data gaps in our current knowledge. The identification of areas where our knowledge is currently lacking is vital in driving policy decisions and enabling policy makers to target future research requirements in an efficient and economic way.

The QRA model has three inter-related modules: a release assessment, an exposure assessment and a dose-response assessment. The release assessment estimates the probability that a randomly selected infected badger within a parish in a specified localised area is excreting *M. bovis*. This part of the model has been described previously and will not be discussed here (Gallagher et al., 2000). The exposure assessment estimates the probability of exposure of cattle to *M. bovis* in the excreta of badgers and finally, the dose-response assessment determines the probability of cattle infection, given exposure.

In this paper, the exposure model is described in detail and is then combined with the dose-response model to generate preliminary illustrative results. Together with these results, crucial areas of data deficiency are highlighted and future work on the model is discussed.

MATERIALS AND METHODS

Exposure model overview

To characterise exposure, a lattice based simulation model has been used. More specifically, the model describes a simplified random field that has been spatially represented by 2 lattice grid structures, the first of size $n \times n$ and the second of size $m \times m$. Such an approach has been used previously to describe, for example, the spread of *Listeria monocytogenes* in silage bales (Kelly et al., 2000).

The first lattice represents a grazing area, the size of which has been derived by considering the average field size within the south west of England and the number of 'meals' consumed by a dairy cow per day. In particular, the average field size (defined as the area grazed by cattle) is estimated as 39 hectares and the number of meals per cow per day is taken to be 5 meals each resulting in the consumption of 79.2m^2 of area grazed field per meal per cow. Each cell within the lattice equates to one cow meal. In total, the grazing lattice is made up of 4,875 cells n= 69.8 (rounded to 70). Note that this field size also approximates to the average size of badger territory in this area of the country (Hutchings & Harris, 1999).

Behavioural studies have indicated that badgers do not deposit excreta randomly across a field but will preferentially defaecate and urinate at field boundaries as a means of territorial marking and also at dedicated latrine sites within the field (Hutchings & Harris, 1997). A badger latrine is represented by the second lattice structure. Using data from Hutchings and Harris (1999), the mean size of a badger latrine is 4m^2 with a density of 0.56 per hectare giving a total area of approximately 87m^2 of latrine for the average field size. Within the latrine lattice, each cell represents an estimated area of investigatory contact of 0.016m^2, therefore m= 72 and thus the total number of cells in the latrine lattice is 5,184. The lattices are joined to represent the field boundary. Together they give a structure made up of :

$$N= n^2+ m^2 \tag{1}$$

cells. It is assumed that badgers will preferentially excrete within the latrine lattice while cattle will graze the grazing lattice.

The model evolves over time, in 24-hour time steps. Each day, the following activities are simulated:

- Badgers enter the field and infected badgers excrete *M. bovis*, preferentially on the latrine lattice;
- The population of *M. bovis* declines as a result of environmental factors;
- Cattle graze the field.

The first two activities result in a population of *M. bovis* on each cell, at the end of each day. The population on cell (i,j) within the grazing lattice is defined by $x_{ij}(t)$ and is given by:

$$x_{ij}(t) = f(x_{ij}(t-1) + \alpha_{ij}(t)) \qquad i, j = 1..n \tag{2}$$

while the population on cell (i,j) in the latrine lattice at the end of day t defined by $y_{ij}(t)$ and is represented by:

$$y_{ij}(t) = f(y_{ij}(t-1) + \gamma_{ij}(t)) \qquad i, j = 1..m \tag{3}$$

Here f(.) is a decay function and $\alpha_{ij}(t)$ and $\gamma_{ij}(t)$ are the numbers of *M. bovis* excreted onto cell (i,j) during day t for the grazing and latrine areas, respectively. Note that excretion and

decay are assumed to be sequential events, that is, *M. bovis* is excreted and then the population decays.

When cattle graze a particular cell on a particular day, they are assumed to be exposed to all $x_{ij}(t)$ or $y_{ij}(t)$ organisms. They then become infected according to the dose-response model which generates the probability of infection given the dose $x_{ij}(t)$ or $y_{ij}(t)$.

The processes of excretion, decay and infection are outlined as follows.

Excretion of *M. bovis* by badgers

The model assumes that there will be a population of $B=7$ badgers within the locality of the field and, because these animals are nocturnal, that each will potentially enter the field during any night. The value of 7 is the mean number of adult badgers in a social group (Hutchings & Harris, 1999). A proportion of the badgers (p_I) are assumed to be infected and excreting *M. bovis*. This proportion has been estimated for selected localised areas (Gallagher et al., 2000; Gallagher et al., 2002a). However, for purposes of this paper, proportions illustrative of different levels of infection control are investigated.

Studies have indicated that badgers infected with *M. bovis* excrete the organism in their urine, faeces, bronchial secretions and in exudate from infected bite wounds. Excretion of *M. bovis* via these routes has been found to occur by each route individually, or by a combination of these routes. In addition, excretion of *M. bovis* by badgers via any of these routes is considered to occur intermittently, until the final stage of disease (Delahay, 2001, pers. comm.). For the purposes of this paper, we have considered intermittent excretion in urine only.

At the beginning of each 24-hour time step, each infected badger in the population is assigned a probability of *M. bovis* excretion for that particular time step. This probability is defined as p_E and represents intermittent excretion, it has been assumed that for a given day an infected badger has an equal probability of excreting or not excreting. The model then simulates the urination of *M. bovis* onto cells within the 2 lattice structures (the latrine lattice and the grazing lattice). In particular, a badger is assumed to urinate within the latrine lattice with probability p_L= 0.6 and within the grazing lattice with probability p_G=(1- p_L)= 0.4. Urination on any particular cell within each of the lattice structures occurs with equal probability, thus, considering any one urination event, the probability that this will occur on cell (i,j) of the grazing lattice is given by:

$$\beta_{ij} = \frac{p_G}{n^2} \qquad i, j = 1..n \tag{4}$$

while the probability that it will occur on cell (i,j) of the latrine lattice is described by:

$$\lambda_{ij} = \frac{p_L}{m^2} \qquad i, j = 1..m \tag{5}$$

The number of *M. bovis* organisms excreted by infected badgers per urination event (μ) is estimated from the amount of urine excreted and the concentration of *M. bovis* in the urine. Derivation of these parameters is based on published data (MAFF, 1979) and is combined with expert opinion gained from an expert opinion workshop and gives a mean value of μ = 70,500.

In addition, the mean number of urination events per badger, per night is estimated as U = 22 (Brown, 1993).

Using these assumptions and parameters, the number of *M. bovis* excreted onto cell (*i,j*) of the grazing lattice at time step *t* is given by:

$$\alpha_{ij}(t) = \sum_{z=1}^{B\times U} \mu\delta_{ij} \qquad i, j = 1..n \tag{6}$$

where δ_{ij} is a Bernoulli random variable with probability $p_I p_E \beta_{ij}$. In other words, for all $B\times U$ urination events, μ organisms are added to cell (*i,j*) if the urine comes from an excreting badger (with probability $p_I p_E$) and falls on cell (*i,j*) (with probability β_{ij}). Similarly, the number of organisms excreted onto cell (*i,j*) of the latrine lattice is:

$$\gamma_{ij}(t) = \sum_{z=1}^{B\times U} \mu\varepsilon_{ij} \qquad i, j = 1..m \tag{7}$$

where ε_{ij} is a Bernoulli random variable with probability $p_I p_E \lambda_{ij}$.

<u>*M. bovis* decay</u>

After excretion of *M. bovis*, the model considers the survival of the organism within the environment. In particular, decay is assumed to follow an exponential model, the parameter of which (*s*) is derived from published data (Gallagher, 1998). Thus, the number of *M. bovis* on cell (*i,j*) of the grazing lattice at the end of day *t* is given by:

$$x_{ij}(t) = [x_{ij}(t-1) + \alpha_{ij}(t)]e^{-s} \qquad i, j = 1..n \tag{8}$$

and the number on cell (*i,j*) of the latrine lattice is:

$$y_{ij}(t) = [y_{ij}(t-1) + \gamma_{ij}(t)]e^{-s} \qquad i, j = 1..m \tag{9}$$

In both cases, *s*= 1.0027 and was estimated by the method of maximum likelihood.

<u>Exposure of cattle to *M. bovis*</u>

Cattle are assumed to graze the field according to the number of meals they will consume per day. It is assumed that cattle will not graze latrine areas due to the presence of badger faeces (Benham & Broom, 1991). However, cattle will undertake investigations of badger latrines. Studies have indicated that investigatory contact with latrines occurs with the probability q_G= 0.3 for each cow meal (Hutchings & Harris, 1999). By reducing the value of q_G to 0, the effect of control in the form of restricting cattle access to latrines is investigated. If an animal grazes cell (*i,j*) and this cell is contaminated, exposure to all $x_{ij}(t)$ or $y_{ij}(t)$ organisms is assumed to occur. The number of cattle grazing the field, *C,* is modified to reflect the effect of reducing the herd size.

M. bovis dose–response model

The dose response relationship is described by fitting an exponential model to data derived from unpublished experimental work (Vordermeier, 2002, pers. comm.). Currently, these data are the only available relating to the intra tracheal infection of calves with 5 doses of *M. bovis*. Doses of $5x10^3$, $5x10^4$, $4x10^5$, $5x10^5$ and $1x10^6$ with 0/2, 2/2, 4/6, 2/2 and 6/6 cattle becoming infected respectively. The probability that a cow is infected due to exposure to *M. bovis* in the grazing lattice is:

$$P_{\inf}(x_{ij}(t);r) = 1 - e^{-rx_{ij}(t)} \tag{10}$$

The probability that cattle are infected due exposure to *M. bovis* in the latrine lattice is:

$$P_{\inf}(y_{ij}(t);r) = 1 - e^{-ry_{ij}(t)} \tag{11}$$

In both cases, the value for $r = 4.1795x10^{-5}$ and was obtained by the method of maximum likelihood.

Implementation of the model

The model is a discrete event simulation and is implemented using Visual Basic for Applications® (Microsoft Corp.). Multiple runs (1,000 iterations) of the model represent the heterogeneity in excretion, decay and infection patterns. On each iteration, dynamics are simulated for a period of 1 year. It is assumed that at t=0, the field is clear of *M. bovis*, thus $x_{ij}(t)$=0, $y_{ij}(t)$=0 $\forall i, j$, and that the herd has just had a clear tuberculin test. The model is therefore representative of a herd in an annually tested parish. The total number of cattle infected and the time until the first infection is generated on each run of the model.

Table 1. Parameter sets used for investigation of control strategies

Parameter set	Number of excreting badgers ($7*p_I$)	Herd size (C)	Probability cow investigates a latrine during a meal (q_G)
1	1	74	0.3
2	2	74	0.3
3	3	74	0.3
4	7	74	0.3
5	1	60	0.3
6	2	60	0.3
7	3	60	0.3
8	7	60	0.3
9	1	74	0
10	2	74	0
11	3	74	0
12	7	74	0

To consider the effect of potential control strategies, results are generated for the parameter sets shown in Table 1. The particular control options investigated consider the level of infection in the badger population (represented by p_I), the herd size (represented by *C)* and the restriction of cattle to latrines (represented by q_G= 0). All other parameters are set to the values outlined above.

RESULTS

Estimates of risk

Results for the mean number of cattle infected are shown in Fig. 1 and for the mean time until infection in Table 2.

Figure 1 illustrates the mean number of cattle infected per year in an average sized herd in the presence of 1 excreting badger (parameter set 1) is estimated as 2.5. This number is increased to 2.7, in the presence of 7 excreting badgers (parameter set 4). The results indicate that by reducing the cattle herd to 60 cattle the number of cattle infected per year reduces to 1.5 in the presence of 1 excreting badger (parameter set 5), and to 2.2, in the presence of 7 excreting badgers (parameter set 8). By restricting cattle access to badger marking areas and latrines, the number of cattle infected per year is reduced to 1.5 in the presence of 1 excreting badger (parameter set 9) and 1.6 in the presence of 7 excreting badgers (parameter set 12).

From Table 2, in the presence of a single excreting badger, the first cow in a herd of 74 animals will become infected after a mean of 102 days. This is reduced to a mean of 89 days in the presence of 7 excreting badgers. However, if cattle are prevented from accessing latrines, the time until first infection is increased to 124 days in the presence of 1 excreting badger and 116 days in the presence of 7 excreting badgers.

Table 2. Mean number of days until first cattle infection for different numbers of excreting badgers, and prevention of access to badger latrines by cattle

Parameter set	Mean Days until infection
1	102
2	99
3	98
4	89
9	124
10	123
11	121
12	116

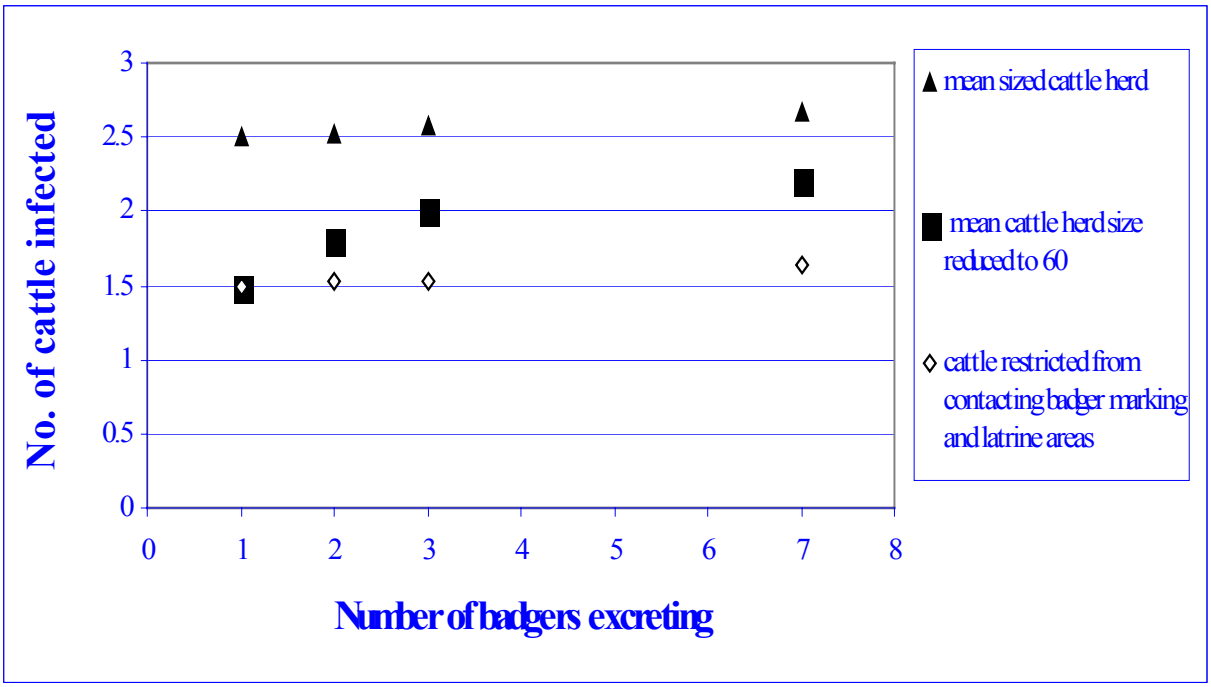

Fig. 1 Mean number of cattle infected (▲) in a mean sized cattle herd (n= 74), (■) if size of cattle herd is to 60 cattle and (◊) if cattle are restricted from contacting badger marking and latrine areas, per year given the presence of 1, 2, 3 or 7 excreting badgers.

DISCUSSION

Several mathematical models have been developed to study the transmission of *M. bovis* in badgers and cattle (Anderson & Trewhella, 1985; Bentil & Murray, 1993; White & Harris 1995; Swinton et al., 1997; Kao et al., 1997). The use of QRA *per se* is, however, relatively novel in this area. This is in contrast to other areas of animal and human health, where risk assessments are widely accepted as useful tools for decision making (Jones et al., 2001; Jones et al., 2002). In this paper, we have presented a QRA model which investigates the risk of cattle infection from badgers. The model considers the dynamics of cattle exposure to *M. bovis* over a 1 year time period. It is stochastic in nature and, in comparison to some QRA models, is based on very few parameters.

Although one of most important outputs from QRA models is the identification of crucial data deficiencies, it is vital that the quantitative estimates are plausible, so that sensitivity analysis can be performed to identify such deficiencies. Here, assuming an average herd size of 74 and cattle access to latrines, the model estimates that the mean number of days until first infection ranges from 102 if 1 excreting badger is present, and to 89 days if 7 excreting badgers are present. Studies undertaken by Little et al. (1982) placed experimentally infected badgers in a barn with uninfected cattle. In the presence of 13 infected badgers, the first cow was infected after approximately 4 – 6 months. However, this measurement was taken as days until a positive tuberculin skin test reaction had occurred, which is in contrast to the model estimate (days of infection). Studies have indicated that a reaction to the tuberculin skin test may take some time to develop. This would indicate that the number of days until infection estimated by the model is broadly acceptable.

For all scenarios relating to herd size and access restriction, the estimate for the number of cattle infected per herd per year remains fairly similar, irrespective of whether 1 or 7 excreting badgers are present. Within a field, there is a finite grazing area and therefore there will be a maximum number of times a cow will be exposed to badger excreta. It then follows that there will be a maximum quantity of *M. bovis* that the cow will be exposed to within the year and therefore a maximum number of cows that would be infected. The results presented here assume that cattle are exposed to 100% of the organisms on the cell. If this were the case, then the number of bacteria excreted by a single badger would be great enough to infect the maximum number of cattle and thus represent a worst-case scenario.

Within the model, the number of cattle infected is a function of both the proportion of bacteria that cattle are exposed to when cattle contact a cell containing *M. bovis* and the dose response relationship. The dose response relationship described here uses data from experiments where cattle are infected via the intra-tracheal route. This has been described as highly sensitive route for cattle infection (Vordermeier M, 2002, pers. comm.). Therefore, the dose response model also describes a worst-case scenario.

Cattle testing data from 1969 - 2002 indicate that the mean number of cattle infected per herd breakdown is 3.4 (Mitchell, 2002, pers. comm.). These data include other modes of cattle infection such as cattle to cattle transmission and direct contact between badgers and cattle. Comparing these data with the results given in Fig. 1 suggest that the model estimates are plausible.

The control strategies investigated within the model indicate that reducing herd size increases the time until first infection and decreases the number of cattle infected. This reduction may be due to the reduced grazing pressure on the field, which reduces the probability of cattle exposure to infected badger excreta. In addition, by restricting cattle access to badger marking areas and latrines, the mean number of infected cattle per herd per year is reduced by approximately 50%. This is expected as badgers have been shown to urinate 60% of the time on recognised marking and latrine areas. Therefore, by restricting access to these areas, cattle exposure is reduced and in turn the number of cattle infected will be reduced.

It is recognised that the need for policy development is often far ahead of the scientific data available and scientific knowledge is, and will always be, imperfect and incomplete (Crawford-Brown & Cothern, 1987). During the development of this model, certain parameters were identified for which no data were currently available. In order to construct the model, estimates for these parameters were obtained using expert opinion, a recognised discipline within risk analysis (Nauta et al., 2001). In particular, an expert opinion workshop was undertaken using standardised methodology (Gallagher et al., 2002b) and with recognised experts in the field of badger pathology. The values generated from this workshop give initial estimates which can be refined should further data become available.

Areas of data deficiency identified within the exposure assessment relate specifically to the excretion of *M. bovis* by badgers. Quantification of the levels of excretion of *M. bovis* at different stages of disease by different routes of excretion do not currently exist. Data relating to the length of time each stage of disease lasts are also unavailable. Although it is widely accepted that intermittent excretion of *M. bovis* by badgers occurs at least in the early and mid stages of disease, with continuous excretion occurring in the latter stages, a definition of intermittent excretion does not exist. Studies are therefore required to determine the existence of

intermittent excretion, establish a definition for the term and provide quantitative data for the levels of excretion when it does occur.

Within the model, exposure of cattle to *M. bovis* is assumed to be to 100% of the bacteria on a cell that a cow grazes. This may be an over-estimate as urine containing *M. bovis* may soak into the soil or evaporate hence cattle may be exposed to only a proportion of the bacteria in the urine. It is therefore highlighted that studies are required to investigate the levels of exposure, particularly with reference to the different types of badger excreta.

As well as data gaps for estimating exposure, quality data relating to the dose response of *M. bovis* in cattle are very limited. The data used here to describe the dose response relationship is small scale and not from a dedicated dose response experiment. In addition, the data relate to intra-tracheal inoculation, where in reality, cattle infection is considered to be due to inhalation and/or ingestion of *M. bovis*. Therefore, dedicated dose response experiments to investigate the relationship between infection and exposure via inhalation and/or ingestion are required.

Currently, analysis is being undertaken to identify which of the data deficient parameters have most effect on the model outputs. Following this, the uncertainty associated with these parameters will be fully quantified, as appropriate. By identifying these parameters and ranking them, the model will assist policy makers in the effective and efficient targeting of scientific research.

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ASSOCIATION BETWEEN MOLECULAR TYPE AND THE EPIDEMIOLOGICAL FEATURES OF *MYCOBACTERIUM BOVIS* IN CATTLE

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SUMMARY

We hypothesised that the molecular type of *Mycobacterium bovis* is associated with the pathology and epidemiology of the disease in cattle. Analyses are based on spoligotyping (n = 11,703 isolates) and variable number tandem repeat typing (VNTR; n = 1,824 isolates), and epidemiological features are calculated from herd, skin test, abattoir and culture data. Spatial confounding was reduced using either polynomial regression on map co-ordinates, or blocks having diverse spoligotypes. The continuous responses, prevalence of non-visible lesions in the cattle population, incident duration and size of the reaction to avian tuberculin were significantly affected by spoligotype, while prevalence of non-visible lesions in the cattle population was affected by VNTR. The binomial responses, proportion of all reactors detected at the disclosing test (DT), proportion of reactors at DT that were inconclusive and proportion of reactors at DT that had visible lesions, were significant in at least five contrasts between molecular types. The effects were consistent with molecular type affecting the specificity of the skin test.

INTRODUCTION

Effects of strain of *Mycobacterium* spp. on epidemiology

Although phenotypic differences amongst isolates of *Mycobacterium tuberculosis* complex bacilli have long been suspected, molecular definition of strains has been possible for little more than a decade. The phenotypic characteristics of isolates may affect the epidemiology (e.g. transmissibility, virulence and immunogenicity) and the diagnosis of infection (e.g. immunogenicity and growth characteristics) (Murray & Nardell, 2002).

Infection in domestic and wild animals: Effects of molecular type of *M. bovis* on the characteristics of disease in animals are rarely described, even though *M. bovis* is a member of the *M. tuberculosis* complex. It is possible to find descriptions of differences between more diverse taxa of *Mycobacterium*, for example between *M. avium* and *M. bovis* in pigs, but that is not relevant to the present study.

Human infection: The W-Beijing family of strains of *M. tuberculosis* has a distinct pathology and epidemiology, characterised by rapid spread and a tendency to demonstrate antibacterial resistance. This family has an enhanced ability to grow within human

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macrophages (Zhang et al., 1999). It is not a new strain but has increased in significance in recent decades with the rising prevalence of human immunodeficiency virus (Qian et al., 1999).

Murray and Nardell (2002) stressed the importance of distinguishing between immunogenicity and virulence, citing a small epidemic of strain CDC1551 in rural United States of America in which a *larger* than normal proportion of contacts gave positive skin tests, whereas lung tubercles in infected rabbits were *smaller* than usual.

Techniques used for the molecular epidemiology of *M. bovis* in Great Britain (GB)

The two techniques routinely used in GB, largely by the Veterinary Laboratories Agency (VLA), rely on amplification of regions of the genome using the polymerase chain reaction (Durr et al., 2000).

Spoligotyping: The direct repeat (DR) region of the genome of *M. tuberculosis* complex strains is composed of many virtually identical DRs of DNA, each 36 base-pairs long, alternating with distinctive spacer sequences of DNA. At least 94 spacers have been identified (van der Zanden et al., 2002), but conventionally 43 are used for differentiating strains by spoligotyping. In GB, twelve spoligotypes account for over 99% of the cattle isolates observed in this paper (VLA, 2002, unpublished). None of the remaining 17 spoligotypes comprise of more than 0.2% of the total numbers typed.

Although the DR region is too small to code for significant genes, the spacer oligonucleide pattern can distinguish clones that differ in other parts of the genome. Spoligotyping is known to distinguish between phenotypically different strains. The loss of six spacers between the two most prevalent *M. bovis* spoligotypes in GB (S9 and S17) is associated with the absence of enzymes involved with fatty acid metabolism (S.V. Gordon, 2002, pers. comm.). The only *M. bovis* spoligotype recorded in GB cattle that has 33 spacers, the BCG-like S35, is the only type possessing the gene glgY (glycogen phosphorylase [EC:2.4.1.1]). The W-Beijing family of strains have characteristic spoligotype signatures (Qian et al., 1999; van der Zanden et al., 2002).

Variable number tandem repeat typing (VNTR): In several loci of the genome in *M. tuberculosis* complex, short sequences of DNA may be repeated end-to-end (in tandem). Frothingham and Meeker-O'Connell (1998) identified six loci that are suitable for typing. Variation of number of repeats at each locus can define epidemiologically informative types, in particular when used in combination with spoligotyping (Durr et al., 2000). In *M. bovis*, the greatest diversity of VNTR types is within spoligotype 9.

Potential relationships between genetic variation and phenotype: Phenotypic characteristics themselves need not point to a single or long-lived genomic feature. Distinctively, genetic variation in the resistance to the antimicrobial, pyrazinamide, can be the result of substitutions, insertions and deletions in a wide variety of locations in the genome (Hou et al., 2000). Typing systems used in epidemiology (for tailoring disease control to distinctive pathological or immunogenetic types or for tracing chains of transmission) would be better served by methods that can identify clones stable over a number of years. Spoligotype and VNTR patterns are known to have relatively stable geographical clustering, making them suitable as epidemiological markers.

The need for spatial analysis

If molecular types really do vary in their effects on the host, geographical clustering of types would be reflected in geographical clustering of effects. But there may be other reasons why characteristics of a disease vary geographically. For example, differences in the climatic and geochemical environment, in the locally prevailing husbandry system, or in the nature of the bovine host such as genotype, or immunological experience to *M. bovis* and environmental mycobacteria. Types should therefore be compared in as consistent an environment as possible.

MATERIALS AND METHODS

Molecular typing

In the last seven years (1996 to 2002), 9,380 cultures of *M. bovis* isolated from field cases were spoligotyped; these represented 85% of all isolates during this period. Some isolates from earlier years (1988 to 1995) were also spoligotyped (n = 2,323), which represented 41% of isolates during these years. Analysis by VNTR was performed on 2,753 of these spoligotyped samples. However, only 1,824 of these isolates were successfully VNTR typed at all of the six loci.

Data on herds and animals

Epidemiological data were collected routinely by staff of by the State Veterinary Service and entered into their animal health database, which contains information on herds, tuberculin tests, bovine tuberculosis (TB) incidents and abattoir inspections of animals culled in the TB control programme. Isolations of *M. bovis* were given a unique identifier that was linked to VLA's molecular typing database.

Data analysis

Logistic regression (LG) and general linear model (GLM) procedures were used for analysis as the observations were either binomial (e.g. lesioned *vs*. none) or quasi-continuous (e.g. incident duration, number of reactors). To facilitate reporting of differences between strains, some binomial responses were also analysed by GLM. Where more than one spoligotype was observed in a single incident (this occurred in 409 herds), data relating to individual animals (e.g. abattoir data, or inconclusive reactors [IR] *vs*. reactors {R]) were analysed separately for each spoligotype. Where observations related to the whole herd (e.g. incident duration), herds were classified by the major spoligotype, where appropriate. For analysis of the effects of VNTR, results were classified as they had been for spoligotypes.

Response variables (hypothetical effects of the molecular type): These are defined within Table 1.

Table 1. Response variables as defined by measurement level and type of analysis

Measurement level	Analysis	Variable name	Definition
Animal	LG	TotIR_dt / R_IRx1dt	Proportion of positive (IR or larger) skin test results at the disclosing test (DT) that were inconclusive (IR)
Animal	LG	R_VL_dt / RVLNVLdt	Proportion of skin test reactors (R) at the DT that had visible lesions on abattoir inspection
Animal	LG	Tot_R_dt / nR_tot	Proportion of skin test reactors detected at the DT (a possible indicator of test sensitivity)
Animal	LG	InfDCIR / Inf_cull	Proportion of infected animals removed at the disclosing test that were not skin test reactors (they were IRs or dangerous contacts [DC])
Animal	GLM	MmAv_R	Reactors' avian skin test reactions (mm increase between 0 h and 72 h)
Animal	GLM	MmBov_R	Reactors' bovine skin test reactions (mm increase between 0 h and 72 h)
Animal	GLM	MmB_A_R	Reactors' skin test reaction differences (bovine skin test reactions [mm increase between 0 h and 72 h] minus avian skin test reactions [mm increase between 0 h and 72 h])
Incident	LG	VE_6M	Proportion of incidents that were initiated at the skin test 6 months after withdrawal of movement restrictions resulting from a previous confirmed incident
Incident	LG	RecurYr1	Whether herd had a subsequent incident within one year after restrictions were removed
Incident	LG	RecurYr2	Whether herd had a subsequent incident in the two years after restrictions were removed
Incident	GLM	Ln_n_R	Geometric mean number of skin test reactors (at all tests)
Incident	GLM	Len_d, ln_Len_d	Duration of restrictions (days) and its logarithm
Geographical	GLM	NVL_prev	Proportion of all cattle that were not visibly lesioned using a 5 × 5 km grid and Gaussian kernel smoothing
Geographical	GLM	VL_prev	Proportion of all cattle that were visibly lesioned, similarly calculated (used as a covariate when estimating NVL_prev)

Independent variables

Molecular type: The molecular type had been determined by spoligotyping and (where sufficient data were available) by VNTR. Observations were weighted according to the number of isolates tested for spoligotype or VNTR, as appropriate. For each of the response variables, molecular type appeared as a categorical (class) variable in the GLM analyses.

General linear model: Analyses were performed for each response variable using molecular type as a categorical variable. The three models used (a) the spoligotype alone, (b) a combination of spoligotype and VNTR type, and (c) the latter plus the spoligotype × VNTR interaction.

Logistic regression: Molecular type appeared as single degree of freedom comparisons in a series of logistic regression analyses. Dummy variables representing the comparisons between types were constructed as follows. For the spoligotypes (S9 … S35, Table 2) these were: S17 *vs*. S9, the 29-spacer types (S10 … S13 and S22) *vs*. S9, many-spacer types (S21, S25 and S35) *vs*. S9, few-spacer types (S15 and S20) *vs*. S9, S15 *vs*. S20, 33-spacer types (S25 and S35) *vs*. S21, S25 *vs*. S35, S11 *vs*. other 29-spacer types, mean of S10, S12 and S22 *vs*. S13, mean of S10 and S22 *vs*. S12, and S10 *vs*. S22. For VNTR within spoligotype 9, the comparisons were 6 *vs*. 7-8 (repeats) of target A, 7 *vs*. 8 of target A, 5 *vs*. 2-4 of target C, 2 *vs*. 4 of target C, 4 *vs*. 5 of target D. For VNTR within S17, the comparison was 4 *vs*. 5 repeats of target B.

Table 2. Phylogenetic relationships between the twelve spoligotypes common in Great Britain, on the assumption that individual spacer sequences can be lost but never gained. Successions should be read downwards in columns

Number of spacers	*21, 25*	*35*	*10*	*11*	*12*	*13*	*22*	*20*	*15*	*17*
35	(BCG) [a]	(BCG)								
34	**S21**	…								
33	**S25**	**S35**								
30			**S9** [b]	**S9**	**S9**	**S9**	**S9**	**S9**	**S9**	**S9**
29			**S10**	**S11**	**S12**	**S13**	**S22**	(S63)	… [c]	…
26								**S20**	(S59)	…
25									**S15**	…
24										**S17**

[a] Identifiers in parentheses are of spoligotypes less commonly observed in cattle.
[b] No *M. bovis* precursor for Spoligotype 9 has been found in Great Britain.
[c] Three dots denote that no intermediate spoligotype has been identified in Great Britain.

Other independent variables (to adjust for characteristics of farms and localities): Continuous variates include National Grid co-ordinates (Easting, Northing), herd size and its logarithm, number of reactors (where appropriate), time since previous test, parish testing interval and its square, sex and age (where known). Dummy variables include whether it was a beef herd, a dairy herd, a slaughterhouse case or an incident commencing with an IR. Classification variables include 'block' of contiguous map squares with similar diversity indices (converted to dummy variables for logistic regression) and herd type (not available for logistic regression).

Spatial analysis: The aim of the analysis was to remove geographical effects rather than to acquire geographical information. Two separate methods were used to minimise the effect of location. Firstly, spatial variation was removed by fitting an increasingly complex polynomial based on the Ordnance Survey Easting (E) and Northing (N) co-ordinates. The following polynomials were fitted: no map co-ordinates, then the first, second, third and finally the fourth

order polynomial. The latter comprised E, N, E^2, E.N, N^2; E^3, E^2.N, E.N^2, N^3; E^4, E^3.N, E^2.N^2, E.N^3 and N^4. Analyses in which the significance of the effect of molecular type vanished with increasing complexity were rejected.

The second method of geographical analysis was to confine analyses to contiguous blocks of GB in which the spoligotype diversity was high. There were three stages. Firstly, the distribution of each of the 12 main spoligotypes in 5 × 5 km squares was calculated and smoothed using a Gaussian kernel of 10-km standard deviation. Then, diversity was calculated using the formula:

$$\text{Diversity Index (DI)} = [\ \sum(\sqrt{n_i})\ ] \div [\ \sqrt{(\sum n_i)}\ ] - 1 \qquad (1)$$

where n_i is the frequency of spoligotype *i*. Finally, contiguous blocks of 5 × 5 km squares were chosen so that squares had a diversity index (DI) of at least 0.7 and a radius of no more than 50 km.

RESULTS

Statistical significance of responses

In most of the continuous responses, spoligotype had a statistically significant effect when analysed without spatial correction (Table 3), but only a few of the responses continued to be significant when the data were spatially corrected (by polynomial or blocking). These response variables were 'prevalence of not visibly lesioned (NVL) reactors adjusted for visibly lesioned (VL) prevalence', 'incident duration', 'reaction to avian tuberculin', and 'proportion of reactors at the disclosing test' and 'total number of reactors'. These were analysed further. A significant response to VNTR was seen only with 'prevalence of NVL reactors adjusted for VL prevalence' (Table 4).

The binomial effects investigated by logistic regression included 11 comparisons between spoligotypes and six comparisons between VNTRs. 'Proportion of reactors detected at the disclosing test' (Table 5) was significant for five of these comparisons, and the effect of spoligotype was also significant with GLM (Table 3). In addition, 'IRs as a proportion of reactors and IRs' was significant for seven of the comparisons and 'VLs as a proportion of VLs and NVLs' was significant for five. Contrasts between spoligotypes or VNTRs did not significantly affect the four remaining responses analysed by GLM (Table 3) or by logistic regression (Table 5). Many estimates of odds ratio (OR) for these four response variables were unstable, with values approaching zero or in the millions, suggesting that data were unbalanced or inadequate.

Odds ratios for responses

Odds ratios for the three binomial response variables that were significantly affected by spoligotype (and sometimes VNTR) are shown in Table 6. The difference between the two major spoligotypes in GB (S17 vs. S9) was significant for all of the three responses: spoligotype 17 had a greater proportion of IRs in disclosing tests and a smaller proportion of VLs in inspected reactors. Additionally, a smaller proportion of the reactors detected in incidents was found at the disclosing test. The comparison S21 *vs*. 34 spacers (S25, S35) was large in

magnitude for two of the responses: S21 had about 40% fewer IRs in the 'reactors + IRs' and a 40% lower VL proportion in the abattoir. S35 itself had a much greater proportion of IRs than S25.

Two VNTR comparisons within Spoligotype 9 significantly affected the proportion of VLs at the abattoir and the proportion of reactors found at the disclosing test: 7-8 vs. 6 repeats of target A, and 5 vs. 2-4 repeats of target C (Table 6).

Estimates of responses

Effects of individual spoligotypes estimated by GLM are shown in Tables 7 and 8 along with their standard errors. Table 7 gives values for four continuous responses and Table 8 the values for binomial responses treated as if they were continuous variables.

Table 3. Variance ratios for the effects of spoligotypes on continuous response variables

Response variable	Means of correction for spatial variation, with statistical significance and number of observations		
	Uncorrected	Polynomial (4^{th}-order)	Blocks
NVL prevalence (adjusted for VL prevalence)	63.42**** (3420)	55.93**** (3420)	63.25**** (2199)
Incident duration (days)	4.28**** (3341)	3.26**** (2241)	4.27**** (2143)
Bovine inc, mm (reactors)	1.86* (465)	1.56† (465)	1.54^{ns} (302)
Avian inc, mm (reactors)	2.25** (465)	2.15** (465)	3.14*** (302)
Bovine – avian difference	2.64** (465)	2.07* (465)	1.36^{ns} (302)
No. disclosing reactors	4.28**** (3316)	4.12**** (3316)	3.67**** (2135)
Number of reactors (total)	3.37**** (3420)	2.63**** (3420)	3.28**** (2199)
Binomial variables treated as if continuous:			
IR as prop'n of IR + R [a]	2.02** (3801)	1.32^{ns} (3801)	1.46† (2470)
VL reactors as proportion of VLs + NVLs[1]	1.70* (3738)	0.56^{ns} (3738)	0.75^{ns} (2435)
Proportion of incidents at 6-month tests	2.46**** (3864)	1.32^{ns} (3864)	1.24^{ns} (2199)
Proportion of all reactors at disclosing test	2.11*** (3864)	1.74* (3864)	2.73**** (2514)
Infected DCs+IRs, prop'n of total infected culls[1]	0.58^{ns} (3106)	0.41^{ns} (3106)	0.92^{ns} (2029)
Recurrence in year 1	1.34^{ns} (1681)	1.04^{ns} (1681)	1.57† (989)
Recurrence in year 2	2.51**** (1681)	1.53† (1681)	1.61† (989)

[a] At the disclosing test only. ^{ns} = not statistically significant ($P > 0.1$)
† = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$

Table 4. Variance ratios for the effects of VNTR and the Spoligotype × VNTR interaction on continuous response variables

Response variable (n)	Variance ratio of effect and correction for spatial variation			
	VNTR		Spoligotype × VNTR interaction	
	None	Polynomial (4^{th}-order)	None	Polynomial (4^{th}-order)
NVL prevalence (839)	12.39****	9.89****	1.12^{ns}	0.73^{ns}
Duration [days] (809)	0.88^{ns}	1.01^{ns}	0.49^{ns}	0.52^{ns}
Skin test reactions of reactors:				
Bovine [mm] (163)	1.01^{ns}	0.61^{ns}	ND [a]	ND
Avian [mm] (163)	1.74†	1.52^{ns}	ND	ND
Bov.–Av. Difference (163)	1.34^{ns}	0.90^{ns}	ND	ND
No. disclosing reactors (816)	3.35****	0.97^{ns}	1.76†	0.81^{ns}
Total number of reactors (839)	3.40****	0.91^{ns}	1.73†	0.93^{ns}

[a] Indeterminate; ^{ns} = not statistically significant; † = $P < 0.1$; **** = $P < 0.0001$

Table 5. Number of significant between-type comparisons for binomial response variables, estimated by logistic regression

Response variable	Number of comparisons that were significant [a]		Number of comparisons with extreme ORs [b]	
	Spoligotypes	VNTRs	Spoligotypes	VNTRs
IRs as a proportion of reactors and IRs	7	0	0	3
VLs as a proportion of VLs + NVLs	3	2	0	2
Proportion of incidents disclosed at 6-month tests	0	0	3	5
Proportion of all reactors detected at disclosing test	2	3	0	0
Infected DCs and IRs as a proportion of all culled at the disclosing test	0	0	1	2
Recurrence in year 1	0	0	5	5
Recurrence in year 2	0	0	4	4

[a] Where analyses for both 4^{th}-order spatial polynomial and blocking were significant ($P<0.05$).
[b] Where odds ratio (OR) < 0.2 or OR > 5.0.

Table 6. Odds ratios for comparisons between spoligotypes and VNTRs (LG analysis). This Table shows analyses where both 4th-order spatial polynomial and blocking were significant.

Dependent variable, comparison	Means of correction for spatial variation		
	Uncorrected	Polynomial (4th order)	Blocks
IRs as a proportion of reactors and IRs at the disclosing test			
S17 vs. S9	1.060** (2352)	1.055* (2352)	1.117**** (1599)
Many spacers vs. S9	0.958^{ns} (1595)	1.090* (1595)	1.159** (827)
S21 vs. 34 spacers	0.763*** (239)	0.575** (239)	0.523** (103)
S35 vs. S25	1.592**** (199)	1.628* (199)	3.407**** (68)
S11 vs. 29 spacers	1.011^{ns} (958)	0.860** (958)	0.659**** (558)
S12 vs. S10 + S22	1.069^{ns} (525)	1.564**** (525)	1.402** (474)
VLs as a proportion of VLs + NVLs at the disclosing test			
S17 vs. S9	0.904**** (2312)	0.946* (2312)	0.920** (1578)
29 spacers vs. S9	1.072*** (2272)	1.063** (2272)	1.059* (1263)
S21 vs. 34 spacers	0.951^{ns} (236)	0.708* (236)	0.581** (102)
S9, target A: 6 vs. 7-8	0.991^{ns} (309)	0.661*** (309)	0.716† (177)
S9, target C: 5 vs. 2-4	1.477*** (141)	2.006*** (141)	1.805* (65)
Proportion of all reactors detected at disclosing test			
S17 vs. S9	0.950** (2180)	0.931** (2180)	0.910*** (1486)
S22 vs. S10	1.012^{ns} (358)	1.935**** (358)	1.832**** (327)
S9, target A: 6 vs. 7-8	0.725**** (296)	0.716**** (296)	0.511**** (173)
S9, target C: 5 vs. 2-4	0.786** (131)	0.583*** (131)	0.513*** (60)
S17, target B: 5 vs. 4	1.530* (147)	4.043**** (147)	1.500* (139)

^{ns} =not statistically significant; †=P<0.1; *=P<0.05; ** =P<0.01; *** =P<0.001; **** =P<0.0001

Table 7. Mean values for five continuous response variables corrected for geographic variation (mean of estimates using 4th order polynomial and blocking, with standard errors in parentheses)

Spoli-gotype	NVL prevalence per 10^5 cattle [adjusted for VL prevalence]	Duration [days] [a]	Reactors' bovine increase [mm]	Reactors' avian increase, [mm]	Number of disclosing test reactors [a]
9	32.5 (1.1)	207 (32)	12.8 (1.3)	2.2 (0.5)	2.66 (0.89)
10	24.5 (1.2)	201 (32)	10.6 (2.5)	2.6 (0.9)	3.04 (1.07)
11	30.0 (1.1)	183 (33)	13.6 (1.7)	3.5 (0.6)	3.07 (1.05)
12	27.5 (1.2)	178 (31)	18.1 (5.6)	3.2 (2.0)	3.55 (1.31)
13	33.3 (1.8)	223 (53)	Insufficient data		1.44 (0.78)
15	32.4 (1.1)	212 (35)	10.8 (2.0)	2.8 (0.7)	4.74 (1.65)
17	34.8 (1.1)	242 (37)	12.0 (1.5)	2.0 (0.5)	2.86 (0.96)
20	29.2 (1.3)	188 (35)	11.5 (4.3)	1.0 (1.6)	3.85 (1.49)
21	36.6 (1.3)	250 (44)	13.9 (4.5)	4.4 (1.6)	2.77 (1.06)
22	36.4 (1.1)	232 (38)	15.9 (1.6)	2.6 (0.6)	2.98 (1.01)
25	32.3 (1.2)	196 (35)	13.5 (5.3)	1.5 (1.9)	1.73 (0.65)
35	32.4 (1.2)	268 (44)	13.5 (1.7)	0.8 (0.6)	5.18 (1.83)

[a] Geometric mean

Table 8. Mean percentage values for four binomial response variables calculated by GLM corrected for spatial variation (mean of estimates using 4th order polynomial and blocking, with standard errors in parentheses)

Spoligo-type	IR as proportion of IRs + Rs	VL reactors as proportion of VLs + NVLs	Proportion of all reactors at disclosing test	Recurrence in 2 years
9	24.5 (7.9)	75.4 (9.3)	78.1 (14.7)	37.9 (14.0)
10	24.3 (8.3)	71.8 (9.8)	84.6 (15.4)	53.6 (15.5)
11	23.3 (8.1)	75.5 (9.5)	86.9 (15.0)	43.0 (14.5)
12	19.5 (8.6)	81.8 (10.2)	88.3 (16.0)	37.2 (15.9)
13	57.7 (12.4)	84.8 (14.6)	65.1 (23.0)	8.4 (26.0)
15	24.7 (8.2)	77.1 (9.7)	76.5 (15.2)	25.2 (14.9)
17	26.9 (7.9)	75.4 (9.4)	71.6 (14.7)	42.1 (13.9)
20	26.2 (8.9)	71.4 (10.5)	78.4 (16.5)	34.3 (18.4)
21	29.6 (9.0)	76.4 (10.6)	65.0 (16.7)	42.2 (17.0)
22	25.3 (8.0)	73.7 (9.5)	82.5 (14.9)	26.8 (14.3)
25	17.2 (8.7)	80.5 (10.3)	93.7 (16.1)	30.4 (17.0)
35	22.2 (8.4)	79.8 (9.9)	79.3 (15.5)	17.7 (18.2)

Table 9. Correlation coefficients between mean characteristics of spoligotypes and the local NVL prevalence

Response variable	Results without spatial correction	Results using 4th order spatial polynomial	Results within high-diversity blocks
Logarithm of duration	+0.52†	+0.62*	+0.63*
Duration, days	+0.34	+0.43	+0.55†
IR / (R + IR) at disclosing test [a]	+0.48	+0.26	+0.24
DC + IR / culls [a]	-0.20	+0.12	+0.29
VL / (VL + NVL) [a]	-0.24	+0.12	+0.16
Whether revealed by six-month tests [a]	-0.11	+0.02	-0.11
Avian SICCT reaction in reactors	+0.19	+0.02	+0.09
Bovine SICCT reaction in reactors	-0.03	+0.01	+0.45
Bovine – Avian SICCT in reactors	+0.19	+0.00	+0.41
Number of reactors (total)	+0.18	-0.04	-0.01
Number of reactors at disclosing test	+0.01	-0.18	-0.23
Recurrence in 24 months [a]	-0.41	-0.27	-0.44
Recurrence in 12 months [a]	-0.41	-0.60*	-0.55†
Prop'n of reactors at disclosing test [a]	-0.35	-0.64*	-0.26

[a] These would normally be analysed using logistic regression; † = $P < 0.1$; * = $P < 0.05$

Table 10. Distribution of spoligotypes and VNTR types in 1,754 isolates in which both typing methods were successfully applied

Major VNTR type [a]	Spoligotype number											
	09	10	11	12	13	15	17	20	21	22	25	35
3-3-5-4*-3-3.1	…	…	…	…	…	…	…	…	…	…	…	36
3-5-5-4*-3-3.1	…	…	…	…	…	…	…	…	18	…	…	…
6-5-5-4*-2-3.1	…	…	…	…	…	…	…	…	…	…	47	…
6-5-5-4*-3-3.1	238	…	…	…	…	…	…	…	…	…	…	…
7-3-5-3*-3-3.1	…	…	…	…	11	…	…	…	…	…	…	…
7-4-5-4*-3-3.1	…	…	…	53	…	…	…	…	…	…	…	…
7-4-5-5*-3-3.1	…	…	…	…	…	…	15	…	…	…	…	…
7-5-2-4*-3-3.1	85	…	…	…	…	…	…	…	…	122	…	…
7-5-4-5*-3-3.1	42	…	…	…	…	…	…	…	…	…	…	…
7-5-5-4*-3-3.1	110	32	164	…	…	114	…	57	…	…	…	…
7-5-5-5*-3-2.1	93	…	…	…	…	…	…	…	…	…	…	…
7-5-5-5*-3-3.1	…	…	…	…	…	…	302	…	…	…	…	…
8-5-5-5*-3-3.1	88	…	…	…	…	…	…	…	…	…	…	…
Others	59	4	5	9	4	3	21	8	…	7	5	2
Total	715	36	169	62	15	117	338	65	18	129	52	38

[a] Major types are those found in at least 10 isolates and at least 4% of each spoligotype.

Correlations between responses

The response variable having the largest variation due to spoligotype was NVL prevalence (Table 3). The correlations between this and other response variables are shown in Table 9. For some responses, controlling spatial variation increased the magnitude of correlation to significance ($P < 0.05$) or near significance ($P < 0.1$). These responses were 'duration' (especially its logarithm), 'recurrence in the following 12 months', and 'proportion of reactors at the disclosing test' (although not when blocked analysis was used). Correlation coefficients for other responses were smaller in magnitude.

For most spoligotypes, there was one major VNTR type (Table 10), except for spoligotype 9 in which there are six such types, and for spoligotype 17, with two major VNTR types. Diversity indices for VNTR varied within spoligotype: for spoligotype 9 it was 2.23 and for the other 11 common spoligotypes it varied between 0.0 and 1.0.

DISCUSSION

Statistical adequacy

It had been suspected that many of the observed associations between spoligotype and disease characteristics were in fact accidental and could be removed by correction for spatial trends. Indeed, removal was achieved by spatial correction for skin test reactions (Table 3), proportion of disclosure by six-month tests, and recurrence (Tables 2 and 4). On the other hand, some of the comparisons between molecular types yielded unusually high or low odds ratios that were not significant (Table 5). It was concluded that the data were not sufficient in number or precision to confirm the existence of effects or allow them to be estimated.

The nature of the spatial correction was examined. In general, fourth-order polynomials formed a smooth curving surface, which was likely to represent the pattern of background levels of the response variable. Removing spatial variation using polynomials contributed a similar sum of squares to the analysis of variance as molecular type itself. Using blocks on the other hand often accounted for a larger proportion of variation than molecular type; this seemed to have been associated with an excessive range of means for blocks. It seems that the use of polynomials is a more robust means of analysis, as suggested to some extent in Tables 3, 6 and 9.

The magnitude of effects

In odds ratios calculated by logistic regression, polynomial or blocking correction for spatial variation generally gave similar effects (Table 6). Even though few of the responses were significantly affected by more than one molecular type comparison, effects were occasionally disparate. For example, the odds ratios for the responses, 'VLs as a proportion of VLs plus NVLs' and 'proportion of reactors detected at the disclosing test' for the contrasts 'S(poligotype)9 vs. S17' and '6 vs. 7-8 repeats of VNTR target A in S9' were similar to one another. For the contrast '5 vs. 2-4 repeats of VNTR target C in S9', the odds ratios for the same two responses were of different magnitudes. This suggests that molecular variation has effects in more than one dimension.

Immunogenicity

It is rare that clinical cases of TB are reported. The closest that one comes is the slaughterhouse case detected on the basis of visible lesions. Otherwise, detection of 'cases' depends on the result of a delayed-type hypersensitivity (DTH) test administered by a field veterinarian. One might distinguish a number of aspects of immunogenicity such as production of an unambiguous skin test response (leading to test 'sensitivity'), the appearance of DTH reactions in animals exposed to but not infected with the disease (leading to reduced 'specificity') and production of DTH at early stages of the disease ('early response').

A high test sensitivity would be expected to detect a large proportion of the infected animals at the disclosing test (some animals may be found so early that relatively few would be lesioned), generate few IRs, lead to short incidents, and reduce recurrence by minimising the probability that infected animals remain in the herd. A small proportion of the animals having visible lesions of culturable *M. bovis* in the abattoir would be DCs or IRs. Early response to DTH would have similar effects, except that in an explosive incident there may be relatively high numbers of IRs.

A high test specificity implies a reduced number of 'falsely positive' reactions. Hence one would expect the number of DTH reactors to be slightly reduced (particularly in the later tests), a decreased number of NVL reactors and a reduced probability of incidents (the unconfirmed ones) in the months following the incident. Avian reactions tend to be incompatible with specificity. On the other hand, very poor specificity can cause a prolongation of incidents and a reduced proportion of reactors detected at the disclosing test, since false positives would continue to be detected after all infected animals have been removed.

Virulence and transmissibility

Virulence in tuberculosis could manifest itself in a number of host responses, but the present data are inadequate to differentiate between them. However, with a highly virulent strain one would expect lesions to be easily detected, and that infected animals would be relatively infectious, resulting in the detection of large numbers of reactors at such breakdowns.

High transmissibility would tend to produce a large number of reactors. In large herds an increased proportion of these may be found at short interval rather than the disclosing tests. Incidents would tend to be explosive, affecting the distribution of IRs and VLs. If the disease is present in wildlife or in neighbouring herds, transmissibility may increase the probability of recurrence.

Table 11 shows the possible effects of these five phenotypic characteristics. The responses for each spoligotype (Tables 6 and 7) were standardised, to produce a response profile. This was then matched with the hypothetical response profiles in Table 11.

The results are necessarily speculative. Sensitivity was not strongly associated with spoligotype: S20 (common in Cornwall) may bring about the highest sensitivity and S13 (W. Sussex) and S17 (Herefordshire to Gloucestershire) the lowest. S11 (Devon) and S20 (Cornwall) both behaved as if the response to the tuberculin test was early. S12 (Cornwall) and S25 (Staffordshire) appeared to induce the highest specificity, and S17 and S21 (Somerset) the lowest. S12 and S10 (Gloucestershire) appeared to have the highest virulence; S9 (widespread), S22 (Herefordshire) (and to some extent S17 and S21) the lowest. Transmissibility appeared to be high with S15 (Cornwall) and S17 and low with S25.

There are known molecular differences between spoligotypes that may help to account for these differences. S21, S25 and S35 appear to be related to *M. bovis* BCG, having lost one or two spacers (Table 2). Similarly, S17, S10-13 and S22 have lost single sequences of spacers with respect to S9, the commonest and presumed ancestral type in GB. S20 may have lost spacers in two events. However, the loss of spacers was not consistently associated with any effect on immunogenicity or pathogenicity.

Table 11. Hypothetical effects of (a) sensitivity, (b) early detection, (c) specificity, (d) virulence and (e) transmissibility of *M. bovis* on response variables

Response variable	Immunogenicity			Pathogenicity	
	(a) Sensi-tivity	(b) Earli-ness	(c) Speci-ficity	(d) Viru-lence	(e) Trans-missibility
Proportion of all reactors detected at the disclosing test	+ +	+ +	+	±	- -
Duration of incident	- -	- -	- -	+	+
Proportion of IRs at the disclosing test	-	+	-	+	+
Proportion of infected DC + IR amongst infected culls at the disclosing test	-	-	-	+?	+
Proportion of VL in reactors slaughtered at the disclosing test: VL / (VL+NVL)	-	- -	+	+ +	-
Proportion of incidents revealed by six-month tests	-	±	-	±	+
Probability of recurrence in the 12 months after ending restrictions	-	±	-	±	+
Probability of recurrence at in the 24 months after ending restrictions	-	±	-	±	+
Avian skin test reactions	±	±	- -	±	±
Bovine skin test reactions	±	±	±	±	±
Differences between bovine and avian skin test reactions	±	±	+	±	±
Number of reactors at the disclosing test	+ +	+	-	+	+ +
Total number of reactors in the incident	+	±	-	+ +	+ +

CONCLUSIONS

There is evidence that the molecular type of *M. bovis* significantly affects the prevalence of NVLs for a given prevalence of VLs, the duration of incidents, avian skin test reactions and number of reactors. It also affects various proportions: the proportion of reactors that were found at the disclosing test, the proportion of IRs and reactors at the disclosing test that were IRs, and the proportion of the reactors that had visible lesions. These in turn suggest that

immunogenicity with respect to the skin test, and possibly the transmissibility, are affected by molecular type.

Tailoring the skin test to the locally prevalent spoligotype is likely to increase the accuracy of interpretation of the skin test in GB and to suggest areas of the country in which additional tests, such as the gamma interferon blood test, could usefully be introduced.

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THE DESIGN OF TEST AND CLEARANCE PROGRAMMES

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SUMMARY

Two mathematical models are presented to illustrate the impact of controlling infectious disease through transmission reduction rather than susceptibility reduction (or immunisation). The general effect of these interventions is to reduce the basic reproduction number (R_0), however, it does make a difference whether it is a reduction in transmissibility (e.g. contact) or duration of infectiousness. At the herd-level, the efficacy of test and clearance programmes are determined by a number of characteristics, which are discussed in terms of equilibrium and dynamic results. A further feature of these programmes is their economic cost, and the fact that costs are determined by the success of the intervention. This can lead to more complex dynamics, where the intervention can fail if it becomes overwhelmed. Applications and further refinements are discussed.

INTRODUCTION

Attempts to control infection rest on reducing the number of individuals infected by those already infected. Control can be achieved by either reducing the infection opportunities of the infected or by reducing the susceptibility of the uninfected. Given the choice, immunisation is frequently chosen over reduction in infection rates, largely because it is less intrusive and usually cheaper. However, where vaccines do not exist, then the only means available to control infection are to reduce the rate of contact between individuals or to reduce the infectious period. The infection dynamic consequences of immunisation are relatively well understood (e.g. Woolhouse et al., 1997; Edmunds et al., 1999), but alternative interventions have historically received less attention, although there are examples of recent research aimed at specific infections (e.g. Barlow et al., 1998; White et al., 2001; Groenendaal et al., 2002; Perez et al., 2002). The aim of this paper is to explore some of the general issues that arise in population dynamics when infection transmission is reduced.

Non-immunisation interventions include such measures as isolation (quarantine), movement restriction, chemotherapeutic cure and culling. Such measures can be applied in conjunction with diagnostic tests aimed to detect those infected. For example, in the 2001 foot and mouth disease (FMD) epidemic, blanket movement restrictions were applied (i.e. all animal movement was reduced) and herds/flocks were culled on the basis of diagnostic tests. Note that the term diagnostic test can be used loosely to include field veterinary suspicion as well as biologically based assays. In contrast, current control of bovine tuberculosis in the UK relies on imposing movement restrictions after diagnosis of a herd breakdown. The herd is then cleared of infection

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by selective culling of infected individuals, the choice of which depends on individual, rather than herd, diagnosis.

The use of diagnosis to impose interventions, rather than blanket application, has an important epidemiological effect. Where as blanket controls reduce transmissibility, controls based on diagnostics act to shorten the infectious period.

The first section of this paper uses a simple model to explore the different consequences of reduction in contact and reduction of the infectious period. The perspective is taken to be transmission within a population of individuals. The second section considers both aspects simultaneously, in the context of managing a population of herds, that is, a herd is either infected or not, and the presence of infection in a herd poses a risk to other herds. The idea that the control programme itself is determined by the epidemiology is also introduced.

Throughout the paper, the basic reproduction number (R_0) is referred to as a measure of the transmissibility of the infection. It can be defined as the number of individuals/herds that would be infected if one individual/herd became infected in a population of uninfected individuals/herds. For the infection to invade and persist, R_0 must be greater than one.

WITHIN HERD CONTROL OF INFECTIOUS DISEASE

Control of infectious disease falls broadly into three categories, immunisation, shortening the infectious period and reducing transmissibility. Immunisation works by changing host immune status to create resistance to infection. Not all hosts need to be immunised to produce a significant reduction in disease and make immunisation cost-effective (Woodhouse et al., 1997; O'Callaghan et al., 1999). Immunisation does not alter the propensity for infection to be transmitted, that is, if immunisation is stopped then infection patterns will revert to those pertaining pre-immunisation. In other words, immunisation does not alter R_0.

Both shortening the infectious period and reducing transmissibility reduce R_0. For example, halving the average number of contacts between animals will halve R_0. Halving the average duration of infectiousness will generally reduce R_0, but by less than half (depending on the influence of death from other causes). A further difference between these interventions is that the timescale of infection dynamics is set by the infectious period, so that reducing this will produce 'faster' changes over time.

These comparisons are illustrated in Fig. 1. A simple susceptible-infected-resistant (SIR) compartmental model is used with the following equations (where s, i and r are the proportions of individuals that are susceptible, infected and resistant/immune, respectively). Here β is the transmission coefficient that includes components of contact and transmissibility, γ is the rate of recovery from infection and μ is the death rate. The proportion of new entrants (births) into the population that are immunised is denoted v.

$$\begin{aligned} \dot{s} &= \mu(1-v) - \mu s - \beta s i \\ \dot{i} &= \beta s i - \gamma i - \mu i \\ \dot{r} &= \mu v + \gamma i - \mu r \end{aligned} \qquad (1)$$

The basic reproduction number (R_0) for this system is:

$$R_0 = \frac{\beta}{\gamma + \mu} \tag{2}$$

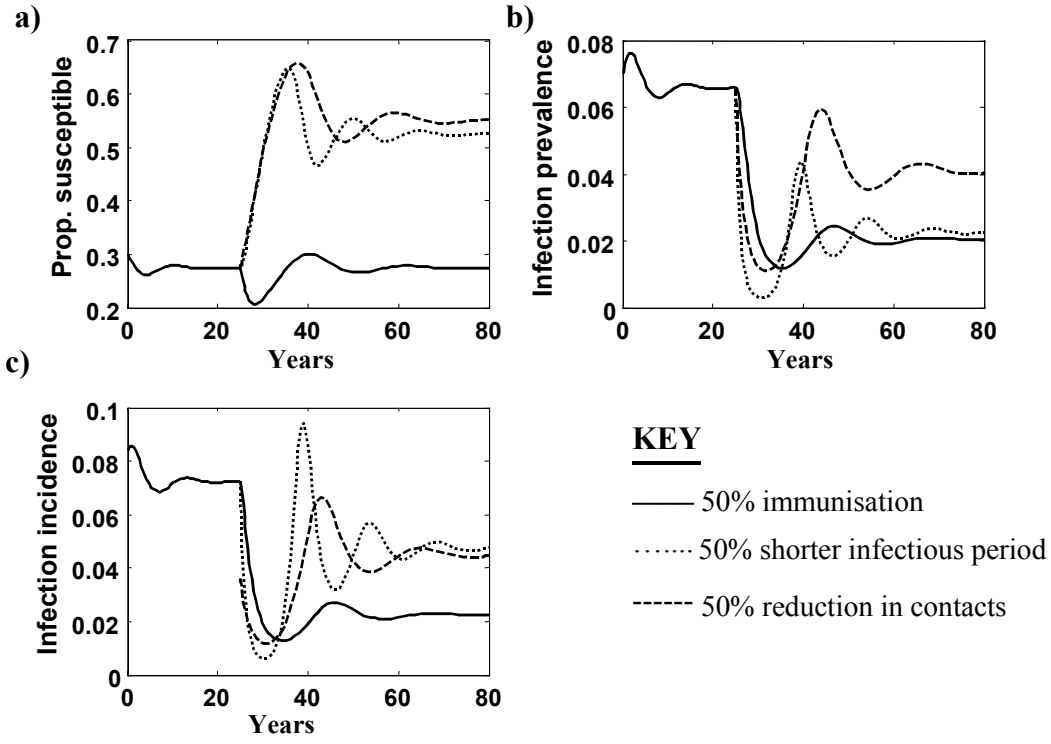

Fig. 1 An example of the effect of three different interventions is shown on three different measures of infection over time. The interventions are: immunising 50% of individuals at birth (v is changed from 0 to 50%), shortening the infectious period by 50% (γ is increased from 1 to 2) and reducing contact by 50% (β is reduced from 4 to 2). The interventions are introduced at year 25. The death rate is maintained at 0.1 throughout. a) The proportion susceptible is not altered by immunisation, but is increased by reduction in R_0. b) The infection prevalence is reduced by all interventions, but less so by reducing contact. c) The infection incidence is reduced by all interventions but more so by immunisation.

All three measures can eliminate infection from a herd. Immunisation can eliminate infection by reducing susceptibility to below that required for the condition to maintain itself within the herd. This threshold is given by (e.g. Woolhouse et al., 1997):

$$v \geq 1 - \frac{1}{R_0} \tag{3}$$

Elimination of infection by reduction in transmissibility is achieved by depressing R_0 below one (that is, so that each infection is on average unable to produce one other infection), even if the whole population is susceptible. For reduction in contact, there is a simple relationship so that reducing contact to 25% of its normal level will eliminate all infections with $R_0 \leq 4$. For

reduction in the duration of the infectious period, this relationship is complicated by the loss of infectiousness due to removal from the herd. If a large proportion of individuals is lost from the herd while still infectious (i.e. recovery is less important in determining the infectious period), then changing recovery rates will have a limited effect.

Figure 1 demonstrates that although each intervention reduces infection, the effectiveness varies depending on the outcome measure used. Immunisation reduces susceptibility, and the dynamic interaction between prevalence and incidence of infection means that immunisation does not increase the proportion susceptible (provided that immunisation does not eliminate infection), but it does have substantive effect on prevalence and incidence. An additional benefit is obtained because even individuals that are not immunised have a reduced chance of infection before they leave the herd. Measures that reduce R_0 also increase the level of susceptibility. This has a counteractive effect on prevalence, as although there is less contact, more of those contacts will result in successful transmission, so the effect on incidence is less marked. However, when shortening the duration of infectiousness reduces R_0, prevalence has an additional fall (since infectious individuals are around for a shorter time). Note that introduction of the intervention produces transient dynamics (oscillations), and that curtailing the infectious period gives these oscillations a higher frequency.

The three interventions have different effects on various outcome measures and also different effects on the short-term dynamic responses to the introduction of the interventions. Choosing between intervention types is influenced principally by the availability of appropriate vaccines, logistics and economics.

BETWEEN HERD CONTROL BY TEST AND CLEARANCE

In order to investigate the herd level dynamics of test and clearance programmes, a mathematical model was developed. The assumptions, structure and behaviour of the model are described.

Model Assumptions

The following assumptions were used to develop a mathematical description of the infection dynamics in a population of herds.

- Herd level model, with herds being either infected (y) or uninfected and susceptible (x) (a susceptible-infected-susceptible (SIS) framework).
- A fixed number of herds, N.
- Transmission from infected to uninfected herds assuming mass action, i.e. the rate of infection experienced by an uninfected herd is linearly related to the proportion of infected herds. The underlying assumption is that all herds contact all other herds at a constant rate.
- Herds can loose infection spontaneously at a constant rate. This can be due, for example, to herd management decisions that control the infection, but without the official intervention.
- Herds are tested at a constant rate, D, so that the average duration between tests is 1/D. This assumes that tests are carried out at random. For example, if there are 1,000 herds to be tested every four years, then 250 are tested per year, but the 250 are drawn at

random every year. In reality, testing is scheduled so that it is more evenly spaced than assumed within the model.

- The herd-level test has a given sensitivity, which is the proportion of tests on infected herds that gives a positive result. The infected herds that are revealed as such on testing, are moved into a class (z).
- The herd-level test has a given specificity, which is the proportion of tests on uninfected herds will give a true negative result. If there is no better test available for confirmatory testing, these herds will then be treated as if they are really positive. The class of falsely positive herds is given the symbol, w.
- All positive herds (including the false positives) undergo a clearance programme designed to remove infection from the herd. In an extreme case, this would include slaughter of the whole herd (e.g. FMD). Less drastically, presumed positive individual animals would be removed according to results of individual testing (e.g. tuberculosis). This clearance procedure lasts $1/\lambda$ years.
- During the clearance procedure, all positive herds (including false positives) are subject to movement and other restrictions designed to reduce infectiousness to other herds. The effectiveness of these restrictions is a number (α) between zero and one, which measures the infectiousness of restricted herds relative to unrestricted herds. False positive herds are also protected from becoming infected by the restrictions. This assumes that the restrictions apply equally to moving animals into and out of the herd.
- At the end of the clearance procedures, a false positive herd returns to be uninfected and at risk. A true positive herd may be declared uninfected but actually be infected with probability, p. If the same test used to determine infection status of infected herds is utilised to determine infection status of herds of unknown status, then this probability is the same as the sensitivity of the original test.

Model Structure

The parameters of the model are listed in Table 1. These assumptions can be encoded into the following set of ordinary differential equations, each of which describes the rate of change of one of the four herd classes:

$$\begin{aligned}
\dot{x} &= -\frac{\beta x}{N}(y + \alpha z) - D(1 - S_P)x + \gamma y + \lambda p z + \lambda w \\
\dot{y} &= \frac{\beta x}{N}(y + \alpha z) - D S_E y - \gamma y + \lambda(1 - p)z \\
\dot{z} &= D S_E y - \lambda z \\
\dot{w} &= D(1 - S_P)x - \lambda w
\end{aligned} \tag{4}$$

Model Equilibrium Properties

In the absence of any control (e.g. D = 0, and consequently, z = w = 0), R_0 is the usual ratio between infectiousness and duration of infectiousness:

$$\hat{R}_0 = \beta / \gamma \tag{5}$$

For the system defined in Eq. 4 with control operating, R_0 is:

$$R_0 = \frac{\beta(\lambda + S_E D\alpha)}{\lambda(\gamma + S_E Dp)} \tag{6}$$

and equilibrium numbers can be found as follows:

$$\begin{aligned} x^* &= \frac{N}{R_0} \\ y^* &= \frac{N\lambda}{S_E D + \lambda}\left(1 - \frac{1}{R_0} - \frac{(1-S_P)D}{\lambda R_0}\right) \\ z^* &= \frac{S_E D}{\lambda} y^* \\ w^* &= \frac{(1-S_P)D}{\lambda} x^* \end{aligned} \tag{7}$$

Note that in Eqs (6) and (7), S_E always appears with D. This implies that deficiencies in test sensitivity can always be overcome by increasing the detection rate, assuming that the test itself does not alter the sensitivity. Consequently, $S_E = 0.7$ is used throughout, but remember when varying D similar effects could be obtained by varying sensitivity.

Table 1. Model Parameters

Parameter symbol	Description	Base value	Comments
Epidemiological Parameters			
β	Transmission coefficient	0.5	Effective contact rate per farm is 0.5 farms per year
γ	Recovery rate	0.1	Recovery from infection without intervention is rare
N	Number of herds	1000	-
Control Programme Parameters			
D	Testing rate	0.25	Testing every 4 years on average
λ	Duration of movement restrictions	2	Average duration of restriction is 0.5 years
p	Probability that an infected, restricted herd is cleared of infection	0.9	-
α	Relative infectiousness of a restricted herd	0.05	-
S_E	Herd test sensitivity	0.7	There is a 70% chance for detecting a true positive
S_P	Herd test specificity	0.9	There is a 90% chance that true negatives will return a negative result

The assumptions create two herd statuses, z and w, that are special in the sense that they are refugees from further interference. Those herds that are infected and detected are undergoing clearance, and their effect cannot be further reduced. If they are able to transmit infection ($\alpha>0$) then increasing the time to clearance will potentially enhance transmission. In a similar vein, those herds that have been falsely designated as positive, w, are protected from infection (by movement restrictions). Consequently, the infection will be eliminated if:

$$(1-S_P)\frac{D}{\lambda} > R_0 - 1 \tag{8}$$

This is simply due to the fact that susceptible farms are put on movement restrictions and protected from infection. This is usually considered an unwanted side effect of test and clearance programmes, since attempts are made to increase specificity and decrease duration of restriction. Using tests with poor sensitivity and increasing duration of restriction moves towards blanket movement restriction.

The desired effect of a detection and clearance policy is to eliminate the infection by reducing R_0 below one. This criterion can be rewritten from Eq. (6) as:

$$S_E D > \frac{\lambda\left(\hat{R}_0 - 1\right)}{\frac{\lambda p}{\gamma} - \alpha\hat{R}_0} \tag{9}$$

If movement restrictions are perfect (i.e. $\alpha = 0$) then Eq. (9) reduces to:

$$\frac{S_E D}{\gamma} > \frac{\left(\hat{R}_0 - 1\right)}{p} \tag{10}$$

The right hand side of Eq. (10) is always greater than one. The left-hand side is the ratio between the effective detection rate and recovery rate, or equivalently, the ratio between the average duration of infectiousness and the average interval between tests. Consequently, to have any chance of controlling the infection, the average duration between tests must be less than the natural duration that a herd is infectious. In other words, the test and clearance programme must shorten the average infectious period.

Further, a necessary (but not sufficient) condition from Eq. (9) for control is that:

$$\frac{\lambda}{\gamma} > \frac{\alpha}{p}\hat{R}_0 \tag{11}$$

which imposes constraints on the duration, success and effectiveness of movement restrictions.

Model Dynamics

The numerical values for the parameters used in the model are given in Table 1. The dynamics are illustrated in Fig. 2. This shows variation in the success of a test and clearance programme due to the testing effort with constant test characteristics, movement restrictions and clearance effectiveness. The timescales are relatively long and largely set by the natural duration of infection within a herd (γ). The introduction of a higher testing effort has the effect of increasing the number of herds placed under movement restrictions (reaching a maximum of just over 10% of all herds). As the infection is controlled, the proportion of restricted herds that have falsely tested positive increases. At this point (10 years since stricter control was introduced), the temptation might be to relax the effect. However, this results in returning R_0 to be greater than one, and an epidemic ensues, although again, this is over a long timescale.

The model suggests that even for modest values of R_0 the number of infected herds is large. Again, this is because the duration of infection is long. However, it is also a function of the assumptions relating to contact. Introducing heterogeneity into the contact pattern (i.e. a small proportion of herds having a large proportion of contacts) will generally reduce this prevalence. Nonetheless, if R_0 is greater than one, then the proportion of herds known to be infected (z) will always be a small fraction of the herds infected.

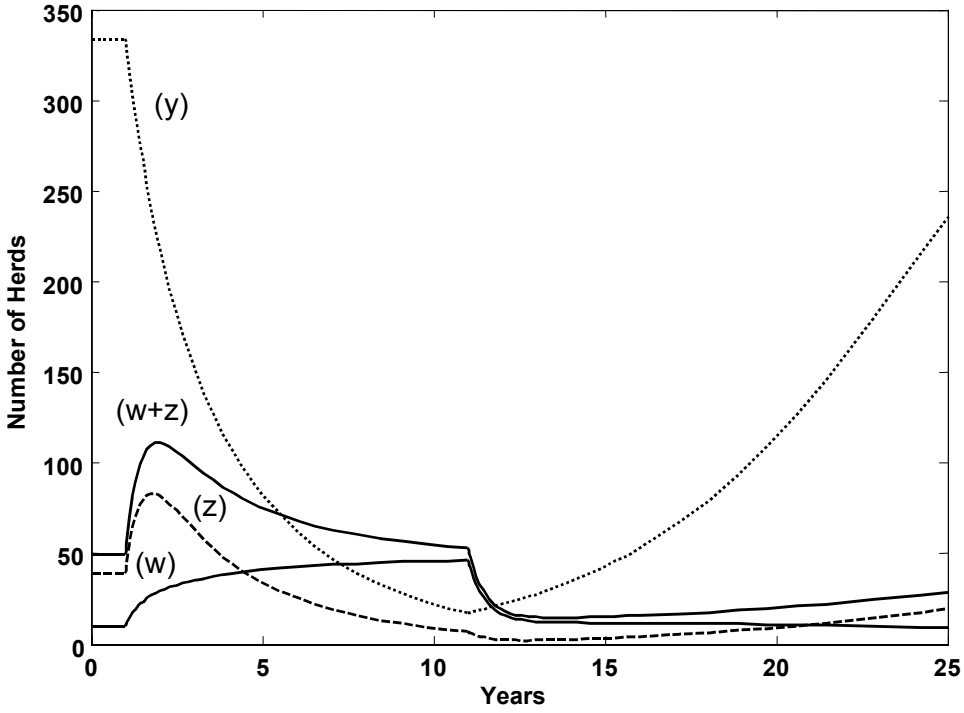

Fig. 2 An illustration of the dynamic consequences of changing the testing frequency. The dotted line is the number of unrestricted infected herds (y); the dashed line is the number of restricted infected herds (z); the lower solid line is the number of restricted uninfected herds (w); and the upper solid line is the total number of restricted herds (w+z). The equilibrium with D = 0.333 (i.e. testing every 3 years on average) is run for one year (other parameters as Table 1; R_0 = 1.6223, x^* = 616.4, y^* = 334.3, z^* = 39.0, w^* = 10.3). At 1 year, the testing interval is reduced to 1 year (D = 1), which is sufficient to eliminate infection. At year 11, the testing frequency is increased to 4 years (D = 0.25).

MODELS WITH CONSTRAINTS ON CONTROL

So far, only the situation where the control programme operates without logistical or financial constraints has been considered. However, this is a very unlikely scenario. More commonly, the test and clearance programme will have constraints, both economic and logistical (e.g. the number of staff available to conduct the programme). In order to illustrate the effect of this constraint, it is assumed that the frequency of testing is linearly related to the number of farms on restriction:

$$\frac{1}{D} = \frac{1}{D_0} + (z + w)A \tag{12}$$

where D_0 is the base line clearance rate (set to annual testing, $D_0 = 1$) and A is a parameter showing the effect of a unit increase in restricted premises (set to 30/365, i.e. each premise under restriction increases the average time between tests by one month).

Figure 3 shows the effects that occur when the control programme efficacy is negatively related to prevalence. Generally, the effect is to create a bistable system, that is, where the control programme is able to eliminate infection provided that the challenge is not too great. If the infection levels cross a boundary, then the control programme is no longer able to cope and the infection becomes endemic. Confounding the control programme with the epidemiology means that R_0 is now a function of the prevalence of infection, since it depends on the testing interval.

In Fig. 3, starting the model with different initial numbers of infected herds (20 and 50) shows this effect. The testing interval is initially 1 year in both cases, and R_0 is less than one (i.e. the programme should eliminate the infection). However, when starting with 50 infected herds, the numbers under restriction rapidly rise, consequently increasing the testing interval and pushing R_0 above one. The epidemic becomes endemic with a large proportion of the herds infected. The resources required to deal with 40 restricted herds means that the test interval is 4 years rather than 1 year. In contrast, when starting with 20 infected herds, the numbers on restriction do not impose the same constraint on testing and R_0 remains below one and the infection is eliminated.

DISCUSSION

The models presented show that the dynamic patterns generated by interventions which change R_0 can be very different from those generated by immunisation. Further, there are two general approaches to reducing R_0. Firstly, an overall (blanket) reduction in transmissibility and/or contact ('biosecurity'), and secondly, intervening to reduce transmission from individuals who have already been infected. Both approaches are widely used in veterinary and human public health. When vaccines are not available (which is the case for the majority of infections), then these interventions are the sole approaches.

Reduction of the infectious period can be achieved by many means (collectively termed 'clearance' within this paper). These include use of antimicrobials (e.g. in contact tracing of humans infected with sexually transmitted infections), isolation or quarantine (e.g. isolation wards in hospitals) or selective culling (generally not an available option in a human setting). In

the herd level model, these interventions return a herd to an uninfected, but susceptible, state. Consequently, these interventions work against themselves, since they create further opportunity for transmission of infection. In contrast, immunisation reduces the possibility for further infection. It is likely that this effect is important in making immunisation a more efficient means of control (Fig. 1).

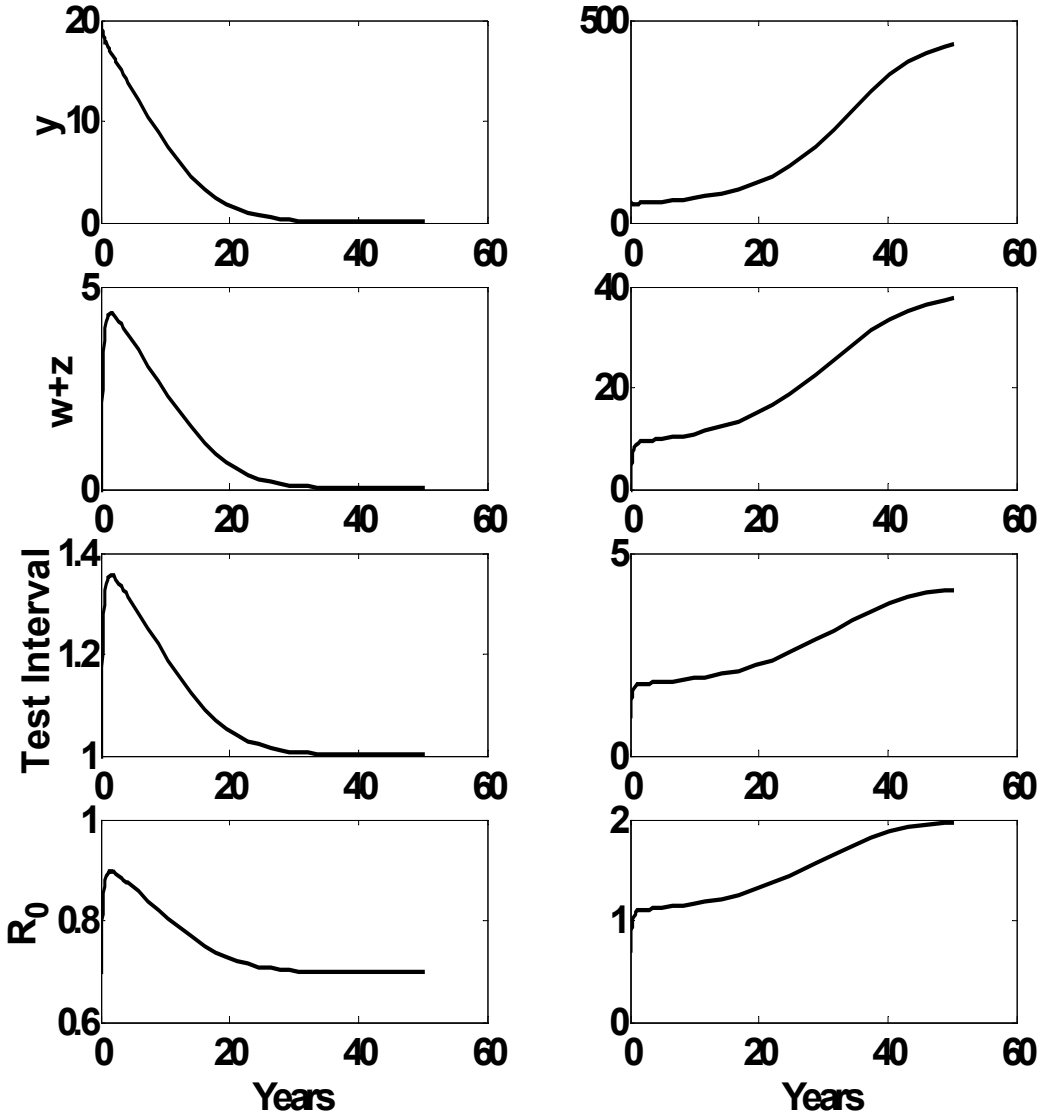

Fig. 3 An illustration of the effect of constraints on infection dynamics. The model is run over 50 years with an initial number of infected farms of 20 (left column) and 50 (right column). The top row is the number of infected herds (y). The second row is the number of restricted herds (w+z). The third row is the average test interval (1/D – see Eq. (12)). The bottom row is the basic reproduction number (R_0). Other parameters are detailed in Table 1, but with $S_P = 1$ (perfect specificity).

These alternative approaches also require two technologies: a diagnostic test and a means of clearance. The absence of a good diagnostic with high sensitivity is a barrier to these approaches (e.g. Woodroffe et al., 1999). Theoretically, low sensitivity might be overcome with

increased frequency of testing (Eq. (9)), but usually the testing procedure bears a high economic cost. Perhaps surprisingly, a test with low specificity, when combined with movement restrictions, is beneficial to the control programme. Equation (8) demonstrates that falsely testing herds as positive and consequently protecting them from infection according to the model assumptions can eliminate infection. Again however, this comes at an economic cost to the herds.

The between herd model described in Eq. (4) captures the overall patterns, but in order to be applied to specific infections requires further refinement. In particular, the contact patterns between herds are (host) species specific, and will influence the quantitative results. The within herd dynamics of a given infection will also play an important role in determining the duration of infection in a herd (without any intervention), its infectiousness and the sensitivity / specificity of herd-level diagnostics. Adding the economic components allows such models to be used to design cost-effective control programmes (e.g. Mukhebi at al., 1999).

In an immunisation programme, the operational economic and logistical costs can be calculated before the programme starts, and are relatively fixed. Within a test and clearance programme, however, the costs can vary substantially with the number of herds that test positive. Budgets for interventions are almost always constrained, so that the control programme does not necessarily scale with the prevalence of infection. This has the effect of tying in the effectiveness of control to the prevalence (which is itself determined by the control programme), which creates an extra dynamic interplay on top of the non-linearities in infectious disease epidemics. The example given here (Fig. 3) shows the sensitivity of such a system. Inclusion of stochastic (chance) effects allows prevalence to vary and, may by chance alone, result in failure of the control programme (Cooper et al., 2003).

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MASTITIS

THE USE OF MARKOV CHAIN MONTE CARLO WITH GIBBS SAMPLING TO INVESTIGATE BOVINE QUARTER SOMATIC CELL COUNT PATTERNS AND THE RISK OF CLINICAL MASTITIS

M.J. GREEN*, P.R. BURTON, L.E. GREEN, Y.H. SCHUKKEN, E.J. PEELER, A.J. BRADLEY AND G.F. MEDLEY

SUMMARY

Quarter milk samples were collected for somatic cell count estimation from three commercial dairy herds at monthly intervals for 12 months. Patterns of quarter somatic cell counts (QSCC) were compared between quarters with and without clinical mastitis. A Bayesian generalized linear mixed model (Markov Chain Monte Carlo with Gibbs sampling) was used to estimate parameters. Preliminary results indicated that quarters with intermediate levels of QSCC (in the approximate range 40,000 – 150,000/ml) had the lowest risk of clinical mastitis in the three months after the QSCC recording was taken. A protective effect of mid-range QSCC may or may not be causal. That is, the somatic cells themselves may provide protection against clinical mastitis, or they may be associated with other immune pathways or events that give protection. A greater understanding of the immune mechanisms behind these findings may lead to improved methods of preventing clinical mastitis.

INTRODUCTION

Over the last decade, there has been debate on whether a low concentration of leukocytes in bovine milk, measured as the somatic cell count (SCC), can increase the risk of clinical mastitis (CM). Leukocytes are known to play an important role in mammary defences following bacterial invasion (Sordillo et al., 1997) but it is uncertain whether the concentration of cells in milk at the time of infection, as well as the speed of migration to the mammary gland, is important in determining the outcome of infection (Hill et al., 1979; Sordillo et al., 1997).

Experimental bacterial challenge studies carried out on quarters have indicated that a relatively low SCC may be associated with increased risk or severity of intramammary infection (Shuster et al., 1996; Schukken et al., 1999; van Werven, 1999). Similarly, a recent field study reported that quarters with a quarter somatic cell count (QSCC) of 1,000 - 5,000/ml were at approximately twice the risk of clinical *Escherichia coli* mastitis in the next month, compared with QSCC of 6,000 - 200,000/ml (Peeler, 2001). However, another field study in three Dutch dairy herds with bulk milk somatic cell counts of 200,000 – 300,000/ml reported that low QSCC

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were not a risk for subsequent clinical mastitis caused by *Streptococcus uberis* or *Staphylococcus aureus* (Zadoks et al., 2001).

The aim of this study was to investigate whether QSCC was associated with an altered risk of CM for up to four months after the QSCC recording.

MATERIALS AND METHODS

Data were collected from three commercial dairy herds, each with 100 - 180 Holstein/Friesian cows, and these have been described previously (Peeler, 2001). During the study, the incidence rate of clinical mastitis was 55 quarter cases per 100 cow years and the monthly bulk milk SCC ranged from 60,000 - 200,000/ml.

Quarter milk samples were collected at a morning milking, every 4 – 6 weeks, for 12 months (Peeler, 2001) and submitted to an accredited, commercial company (On Merit, Newbury, Berkshire, UK) for automated somatic cell count determination. Cases of CM were identified by trained herds-persons on each farm on the basis of visual changes to the milk (clots or watery secretion) or changes to the mammary gland (heat, redness, swelling). Data were entered into a customised Microsoft Access® database (Microsoft Corp, USA) which has been previously described (Peeler, 2001).

Patterns of QSCC were compared between quarters with and without clinical mastitis, whilst controlling for the complex correlation structure within and between quarters and cows. Since difficulties have been reported in accounting for correlations within multidimensional data of this type (Zadoks et al., 2001), a Markov Chain Monte Carlo (MCMC) method based on Gibbs sampling was used to estimate parameters using WinBUGS v1.3 (Spiegelhalter et al., 2000).

Quarters that had CM were compared with unaffected quarters in all cows. When a quarter had a second case of CM, this was defined as a separate mastitic quarter. The QSCC in quarters before CM were compared with the QSCC in unaffected quarters over four consecutive monthly readings. These monthly QSCC readings were labelled as m = +1 (being at risk of CM in the next month), m = +2 (being at risk of CM between one and two months later), and so on to m = +4. When QSCC readings were not available, because CM occurred too soon after calving, those that were available were used, and earlier recordings incorporated as ‘unobserved’. Quarters with CM and no QSCC reading after calving, were omitted (n = 67). The QSCC were initially grouped into the following nine categories for modelling purposes (000’s/ml); 0 - 20, 21 - 40, 41 - 60, 61 - 80, 81 - 100, 101 - 150, 151 - 200, 201 - 400 and > 400.

Modelling was based upon a Bayesian generalised linear mixed model (GLMM) (Breslow & Clayton, 1993; Burton et al., 1999) with CM as a binary response variable. The hierarchical model structure reflected variation between repeated measures of QSCC within quarter over time (level 1), between quarters within cow (level 2) and between cows (level 3). Model exploration was performed with QSCC in nine categories and these categories were combined in the final model based on a similar risk of CM. The fixed covariates farm, parity, quarter position (i.e. left fore, left hind, right fore or right hind), month of lactation and month of year were tested in the model to control for their possible confounding influence.

The GLMM for the binary response was specified in a standard manner (Zeger & Karim, 1991; Burton et al., 1999) as:

$$\text{logit}(\mu_{ijk}) = \alpha + \beta'_{1ijk}X_{1ijk} + \beta'_{2jk}X_{2jk} + \beta'_{3k}X_{3k} + v_k + u_{jk} + e_{ijk} \quad (1)$$

where,
the subscripts i, j and k denote the i^{th} QSCC reading, the j^{th} quarter and the k^{th} cow respectively
CM_{ijk} = the binary response variable, with Bernoulli distribution, [1 = CM, 0 = not CM] denoting response in the period following the i^{th} QSCC reading in the j^{th} quarter of the k^{th} cow.
μ_{ijk} = the fitted probability of CM following QSCC reading i in quarter j of cow k.
α = regression intercept
X_{1ijk} = vector of covariates associated with QSCC reading i of quarter j of cow k.
β'_{1ijk} = transposed vector of coefficients for $X_{1ijk.}$
X_{2jk} = vector of quarter-level exposures for quarter j of cow k.
β'_{2jk} = transposed vector of coefficients for $X_{2jk.}$
X_{3k} = vector of cow-level exposures for cow k.
β'_{3k} = transposed vector of coefficients for $X_{3k.}$
v_k = random effect reflecting residual error between cows.
u_{jk} = random effect reflecting residual error between quarters, within cows.
e_{ijk} = residual error between repeated SCC measurements, within quarter.

Non-informative prior distributions were specified for unbounded fixed effects (~Normal distribution; mean=0, variance=10^6) and for random effect variances (~Gamma distribution; mean=0.001, variance=10^3). Three simultaneous Markov chains were run to enable the monitoring of both mixing and convergence (Gilks et al., 1995). Chains were updated using Gibbs sampling (Gilks et al., 1995). Convergence of the chains was considered to have occurred when the Gelman Rubin statistic approached 1.00 for all parameters (Brooks & Gelman, 1998), and when visually the traces were stable (Gilks et al., 1995). The iterations in the chain up to this point were 'burn-in' and not used for parameter estimates (Gilks et al., 1995). Chains were then run for a further 70,000 iterations each and the posterior means and credibility intervals of parameters were derived from these iterations. The MCMC error was checked to assess the reliability of parameter estimates (Spiegelhalter et al., 2000).

The model parameter distributions were examined using kernel density plots. Model fit was assessed graphically by plotting Pearson Residuals against fitted values (McCullagh & Nelder, 1989) and by calculating the mean of aggregations of these residuals, where:

Pearson residual = {Observed value – fitted value} / {$\sqrt{}$(fitted value x (1 – fitted value))}

PRELIMINARY RESULTS

A total of 12,696 QSCC readings were used from 446 cows. Sixty-seven cases of CM occurred before the first QSCC recording of lactation and therefore were not available for modelling. The remaining 124 cases of clinical mastitis were used in the model.

Having accounted for the effect of farm, time of year, stage of lactation, position of quarter and the effects of clustering of QSCC readings, the level of QSCC systematically influenced the risk of CM for the following three months. Preliminary findings indicated that quarters with

41,000 - 100,000/ml had a significantly reduced risk of CM in the following month (m = +1), compared to quarters with ≤ 40,000/ml. Quarters with > 200,000/ml were at greatest risk of CM in the following month. Quarters with 81,000 - 150,000/ml had a significantly reduced risk of CM between one and two months later (m = +2), compared to quarters with ≤ 80,000/ml. Quarters with 61,000 - 150,000/ml were at significantly reduced risk of CM between two and three months later (m = +3), compared to quarters with ≤ 60,000/ml.

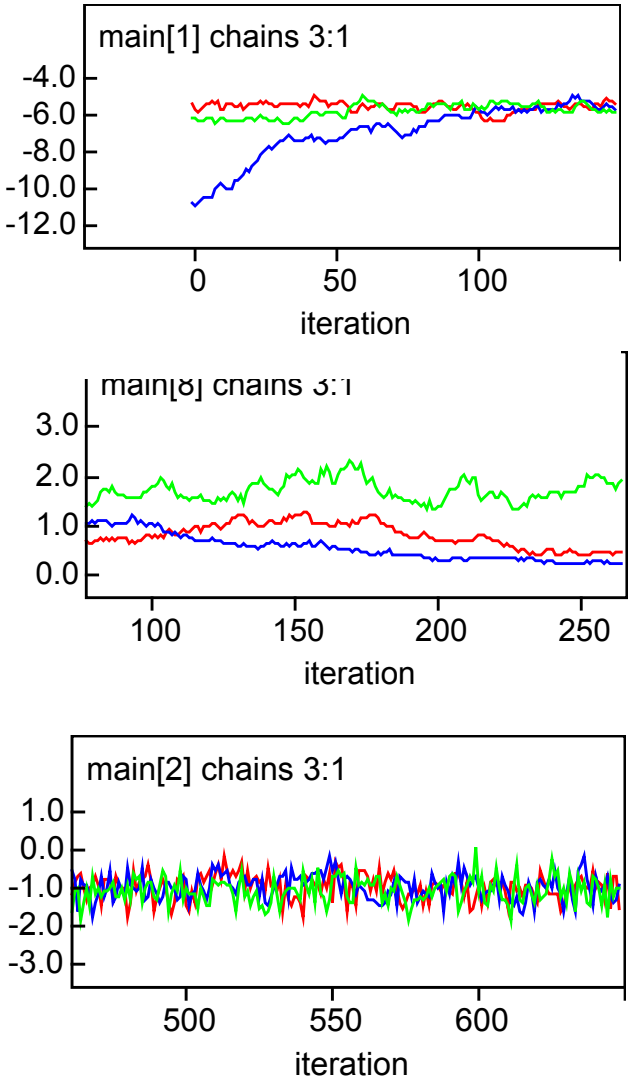

Fig. 1 Examples of MCMC traces for three model parameters. 'Main[1]' illustates three concurrent Markov Chains for the intercept parameter while 'burning in'. The second trace, 'Main [8]', shows an example of 'poor mixing'. The third trace, 'Main[2]' illustrates three chains for a model parameter, once convergence has occurred.

Variation in the underlying risk of CM between quarters within cows (variance = 0.11) was approximately equal to that between cows (variance = 0.12). The Markov Chains estimating these variances did not mix well initially but this improved when the quarter-level variance was sampled from distributions that allowed negative values. To further check that fixed parameter estimates were robust to assumptions about the covariance structure, the final model was repeated with no higher level (quarter or cow) random terms included in the model and also with

the random terms deliberately forced to take values at the upper 97.5th percentile, as estimated in the initial model. Both of these models resulted in the fixed parameter estimates being virtually unchanged, therefore suggesting that they were robust. Illustrations of some Markov Chain features are shown in Fig. 1.

Kernel densities for the posterior distribution of fixed model parameters were Normally distributed and examples of these density plots are presented below (Fig. 2).

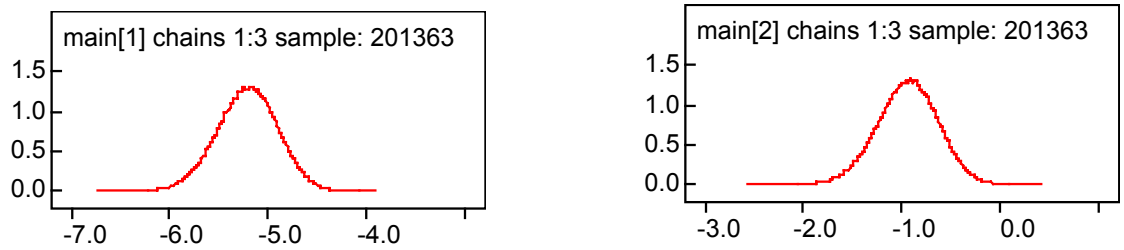

Fig. 2 Examples of kernal densities for two model parameters, after convergence has been reached. These densities summarise the posterior distribution for these parameters calculated from approximately 200,000 iterations of Gibbs sampling.

A graph illustrating the plots of aggregated Pearson Residuals is shown in Fig. 3. The groups of residuals approximate to zero and show no systematic relationship with the fitted value. This is consistent with a good model fit.

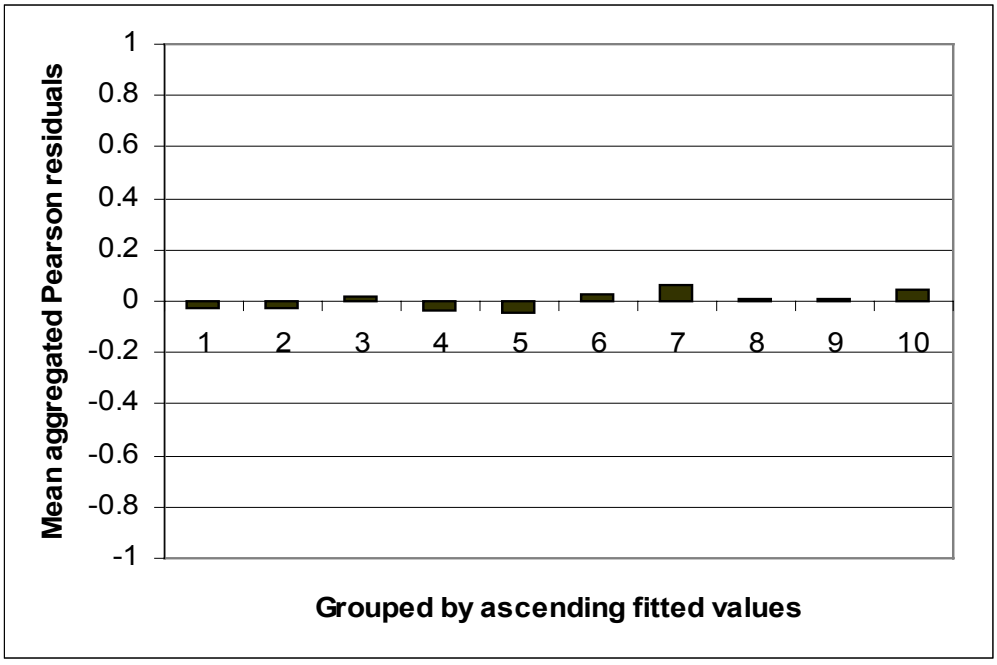

Fig. 3 Graph of Pearson Residuals aggregated in ten groups in order of increasing fitted value, for the Bayesian GLMM. Residual aggregates are close to zero with no systematic variation indicating a good model fit.

DISCUSSION

Markov Chain Monte Carlo can be a helpful technique for the analysis of complicated data. Advantages of the technique include:

- It is useful for complex, multidimensional models, when parameter estimation based on maximum likelihood (or related approximations) may be inaccurate or impossible;
- It is flexible and therefore allows the biology, rather than mathematical constraints, to lead the modelling;
- It allows implementation of Bayesian principles, including use of prior knowledge of the parameters when available;
- Unobserved variables can be accommodated;
- Visual assessment of the Markov Chains helps to investigate model suitability (e.g. mixing and convergence) and therefore in the assessment of whether model parameters are appropriate.

Disadvantages of MCMC technique include:

- The fitting of complex models can give the illusion that anything is possible and will work! It is easy to fit an inappropriate model;
- Model checking is essential e.g. for poor mixing and to check chains are not trapped in the wrong parameter space. Checking can be carried out, for example, by repeating models with simulated data-sets;
- It is computationally expensive and models can take a long time to run;
- Investigation of models, by trying different parameter combinations, is therefore a slow process, i.e. model exploration is cumbersome;
- Software is more complicated than other 'off the shelf' statistical packages.

The current research focused on the relationship between SCC and CM by observing QSCC before CM occurred. Preliminary results suggest that QSCC in the range 41,000 - 100,000/ml were associated with the lowest risk of CM in the next month and a QSCC of 81,000 – 150,000/ml was associated with the lowest risk of CM between one and two months later. A QSCC of 61,000 – 150,000/ml was associated with a further reduced risk of CM between two and three months later. The protective effect of mid-range QSCC may or may not be causal. That is, the somatic cells themselves may provide protection against CM, or they may be associated with other immune pathways or events that give protection. A greater understanding of the immune mechanisms behind these findings may lead to improved methods of preventing clinical mastitis.

These initial results are in broad agreement with experimental studies conducted in quarters (Schukken et al., 1994; Shuster et al., 1996; Schukken et al., 1999; van Werven, 1999) that report that relatively low QSCC are associated with a greater risk or severity of CM, compared to intermediate QSCC. However, our findings contrast with another longitudinal, observational

study of QSCC, that reported no change in risk of CM from low QSCC in the month before CM (Zadoks et al., 2001). There were notable differences between this previous field study and the current study that may account for the contradictory results. Differences include the magnitude of bulk milk SCC in the herds studied, the mastitis pathogens encountered and the methods of analysis used.

A limitation of such studies on SCC, is that the data from cases of CM that occur soon after calving are missed. It is therefore uncertain whether the relationships we describe also apply to CM immediately after calving and this requires further investigation.

Field investigations of somatic cell counts to date suggest that the relationships between QSCC, cow SCC (a pooled sample from all quarters), herd bulk milk SCC (a pooled sample from all cows) and CM may vary between different herds and with different mastitis pathogens (Peeler, 2001; Zadoks et al., 2001; Beaudeau et al., 2002). That said, our findings, and the findings of a number of experimental studies are entirely consistent with the suggestion that quarters with a low SCC may not be at the lowest risk of CM. It will clearly be important to undertake more investigations in this area and, in particular, into which characteristics of middle range SCC render these quarters at reduced risk of CM. Possible differences between pathogens and farms also need further investigation.

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MASTITIS IN SMALLHOLDER DAIRY FARMS IN TANZANIA: FROM RISK TO INTERVENTION AND KNOWLEDGE TRANSFER

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SUMMARY

An initial rapid rural appraisal of the importance of clinical and subclinical mastitis in smallholder, crossbred dairy herds in two regions of Tanzania (Tanga and Iringa) indicated that farmer recognition of the disease and knowledge of control measures was poor. This was followed by a cross-sectional study of 400 randomly selected farms (100% compliance) which indicated 14.2% (95% Confidence Interval (CI)=11.6-17.3) of cows had developed clinical mastitis over the previous year. The point prevalence of subclinical mastitis, defined as a quarter positive by the California Mastitis Test (CMT) or by bacteriological culture, was 46.2% (95% CI=43.6-48.8) and 24.3% (95% CI=22.2-26.6), respectively. A Mastitis Training Course for farmers and extension officers was held and knowledge gained and use of different methods of dissemination were assessed over time. A subsequent randomised controlled trial of various combinations of dissemination methods (village meeting/video/handout) showed strong associations between knowledge gained and both the individual question asked and the combination of dissemination methods used. All combinations of dissemination methods were shown to be significantly more effective than the control group. Surprisingly, the study also identified the handout method to be significantly better than village meetings at improving mastitis knowledge, with no detectable advantage in combining this material with video and/or a village meeting. A longitudinal disease study in Iringa showed the incidence of clinical mastitis to be 31.7 cases per 100 cow-years and the reduction in milk yield following a clinical incident to be 3.5 litres/day. This was followed by randomised intervention trials comparing intramammary antibiotics and teat dipping with untreated controls. Antibiotic treatment significantly reduced the proportion of bacteriologically positive quarters in the short term (14 days post-infusion). Other risk factors were identified from both the cross-sectional and longitudinal studies using multi-level models (e.g. three level: quarter, cow and farm). These included, from the cross sectional study, a greater risk of mastitis associated with the Boran breed (Odds ratio (OR)=3.40, 95% CI =1.00-11.57 for clinical mastitis, and OR=3.51, 95% CI=1.29-9.55 for a CMT positive quarter). In contrast, the practice of residual calf suckling from the longitudinal study was protective for a bacteriologically positive quarter (OR=0.63, 95% CI=0.48-0.81, P= <0.001) and for a CMT positive quarter (OR=0.69, 95% CI=0.63-0.75, P<0.001).

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INTRODUCTION

Information on the aetiology, prevalence and incidence of clinical mastitis in Tanzania is limited, especially for the smallholder dairy sector. In most studies of mastitis in Africa and in other developing countries, contagious pathogens have been shown to predominate over the environmental pathogens (Hamir et al., 1977; Kinabo & Assey, 1993; Omore et al., 1996). In large-scale dairy farms in Tanzania, the annual clinical incidence of mastitis was reported to range from 1.5 to 3.2 cases per 100 cows (Kinabo & Assey, 1993; Kambarage et al., 1996), while the prevalence of subclinical mastitis, based on isolation of pathogens from clinically normal quarter milk samples in Ethiopia, was reported to be 39% (Abdella, 1996), and 33-39% in Kenya and Ethiopia by the California Mastitis Test (CMT) (Kassa et al., 1999; Mulei, 1999). Large epidemiological studies to identify risk and protective factors for mastitis in smallholder dairy cattle have not been carried out in Tanzania. This information is required to inform current and future management practices for mastitis and to optimise milk production in this sector.

Advice to farmers about livestock and crop systems in developing countries is mostly available through extension services which are often funded by government or non-governmental organisations. However, in Tanzania there is lack of knowledge about the most appropriate methods of delivery of such services (MOAC et al., 1998). The importance of verbal and visual representations to learning has been previously investigated and it is thought that humans have separate verbal and visual processing systems (Clarke & Paivio, 1991, cited by Mayer, 1999) and that connection with existing knowledge is important in enabling understanding of new experiences (Alber & Hasher, 1983, cited by Nuthall, 1999). The current project aimed to transfer knowledge about mastitis to smallholder dairy farmers in Tanzania and to assess the success of different methods of providing this information.

MATERIALS AND METHODS

Rapid rural appraisal

A rapid rural appraisal was carried out in Iringa and Tanga regions of Tanzania over a five-week period in October and November 1998 using a number of methods including informal discussion, semi-structured interviews, wealth ranking, matrix ranking and benefit-cost analysis.

Cross-sectional study

A cross-sectional study was carried out in the same two regions between January and April 1999. A total of 200 smallholder dairy farms from each region, with a maximum herd size of 10 crossbred dairy cattle, were randomly selected. All farmers were interviewed about farm and animal level information relating to general management, mastitis, milking practices, and animal movements, using a structured questionnaire. Quarter milk samples from all lactating cows and heifers were taken for bacteriological culture and for CMT to identify subclinical mastitis. The CMT results were classified as negative, 1+, 2+ or 3+. Culture of milk samples and identification of bacterial species to genus and species level was performed using standard bacteriological procedures.

Longitudinal study and intervention trials

A longitudinal study was carried out in Iringa region between August 1999 and July 2001. Monthly field visits took place to examine the dynamics of farm practices and intramammary infections. Questionnaires were completed by interviews with farmers. The cows were examined clinically and quarter milk samples were taken for CMT (monthly) and bacteriology (every second month). Animal and farm hygiene and management were also monitored. Two intervention trials were nested within the longitudinal study. These were initiated between April and May 2000, and involved a total of 160 smallholder dairy farms with 247 lactating cows. Animals in the intervention trials were studied for a period of one year. These studies aimed to evaluate the effectiveness of two mastitis control practices: firstly, a single antibiotic infusion during lactation and secondly, post-milking teat dipping. Phasing the first intervention and randomisation of both interventions ensured that both treatments could be compared with time-matched controls. Randomisation was carried out at the level of the farm.

For the first intervention trial, Tetra-Delta LC™ (Pharmacia & Upjohn Animal Health Ltd, UK) intramammary antibiotic was used to treat lactating cows irrespective of intramammary infection status. The aim of this study was two-fold. Firstly, to assess the short-term effect of intramammary infusion on subclinical infection and secondly, to reduce the infection rate in the population of cattle prior to the second (teat dip) trial. All cows on farms in the 'treatment' group were infused with the antibiotic on day 0, and cows in the 'control' group were left untreated. Seven and 14 days after infusion, CMT was performed and quarter milk samples for bacteriology were also collected from both treated and control cows. Prior to the second intervention trial, all cows in the control group were also infused with Tetra-Delta LC™ intramammary antibiotic. All cows, regardless of their treatment status in the first trial, were randomly allocated to the second intervention 14 days after infusion thus ensuring that all cows entering the second trial had been treated identically prior to randomisation.

For the second intervention trial, sodium hypochlorite teat disinfection was randomly allocated to 'prevention' and 'control' farms. Farms in the prevention group received a teat dipping cup and were supplied regularly with post-milking teat dip throughout the longitudinal study period. The control farms did not receive hypochlorite and continued with their routine milking practices as carried out prior to the intervention trial.

Knowledge dissemination

Knowledge dissemination within communities of smallholder dairy farmers in Iringa was carried out by holding a Mastitis Training Course (MTC), to which extension officers, village based animal health officers and key community individuals (termed 'farm motivators'), were invited. During the MTC, participants developed their own 'action plans' for treatment and control of mastitis in collaboration with the UK project team, and practical classes were held on recognition of clinical signs of mastitis and diagnosis of subclinical disease by CMT. Project-specific materials were prepared to deliver the principal messages thought to be important and relevant to Tanzanian smallholder dairy farmers. These materials included diagrammatic handouts, posters, videos and pens with 'mastitis' logos. The participants of the MTC were asked to disseminate the materials provided and information gained to farmers in their locality. This indirect method of dissemination was then assessed by questionnaire interview of farmers and by direct observation of the hygiene of their milking equipment and cowsheds in the short-term (one month post-dissemination) and in the longer term (16 months post-dissemination).

A subsequent study designed to assess direct knowledge dissemination to a defined number of smallholder dairy farmers in Tanga, based on a three level cluster design (random selection of farmers, dissemination method, village), was performed from an original sampling frame including all villages containing ten or more farmers. The cluster design was blocked according to village type classification (urban, peri-urban or rural) and twelve villages from each block were then randomly selected, resulting in selection of a total of 36 villages. Within each block, a 'control' group and the five combinations of dissemination methods ('village meeting', 'village meeting and video', 'village meeting and handout', 'village meeting, video and handout' 'handout') were randomly assigned. Ten farmers per village were randomly selected for interviewing both before and after dissemination.

Statistical analysis

Data collected were entered in Epi Info databases and analysed using S-PLUS, MLwiN and EGRET (MathSoft, 1999; Gogte et al., 1999; Rasbash et al., 2000). Univariable associations were explored using contingency tables and chi-squared tests and multivariable relationships were examined using generalised linear mixed effects models. The multi-level models considered the nested hierarchy of quarter, animal and farm (and visit for longitudinal studies). The estimation procedure used the second order penalised quasilikelihood (PQL) and restricted iterative generalised least squares (RIGLS) methods. Animal and farm level variables were screened individually by entering them into three-level models. A final multivariable three-level model for each of the two outcome variables of subclinical mastitis in Iringa region, and subclinical mastitis in Tanga region, were fitted using a forward stepwise procedure with a critical probability of 0.05. For the intervention trials, the effect of treatment was examined by considering treatment and time as fixed effects in multi-level models.

For the knowledge dissemination studies, multi-level models were constructed to examine the relationship between 'volunteering mastitis facts post-dissemination' (outcome variable) and the different dissemination methods employed (explanatory variables) to which respondents had been exposed. In Iringa, it was possible to interview the same respondent in both 1999 and 2000 on only a small proportion of farms, hence two different models were constructed to look at the overall knowledge of the populations in 1999 and 2000; the first used responses obtained in 1999 (one month after the dissemination programme) and the other used responses obtained in 2000 (sixteen months after the dissemination programme). As binary outcome responses were involved, a generalised linear model with a logit link function was used and the random effects were village and farm and individual question.

For respondents exposed to direct dissemination methods in Tanga, multi-level models were constructed to examine the relationship between 'volunteering mastitis facts post-dissemination' (outcome variable) and the different dissemination methods employed (explanatory variables). Two nested hierarchical structures were used, the first comprising of individual question clustered within farm clustered within village, and the second comprised of individual question (administered to a single respondent as above) clustered within question number (i.e. the question administered to all respondents) clustered within question category. The individual question, therefore, refers to an individual respondent being asked a single question, whilst question category refers to the category of question e.g. 'signs of mastitis'. Cross-classified models considering non-nested random effects were not considered.

RESULTS

Rapid rural appraisal

The rapid rural appraisal indicated that illiteracy was identified as a problem especially amongst cowboys and women in rural areas. Disease knowledge was generally poor with the exception of the tick-borne diseases, especially East Coast Fever. Farmer awareness of clinical mastitis and its treatment and control was poor and they were unaware of subclinical mastitis. The majority of dairy farmers were ranked in the medium wealth group in rural areas. Dairying was seen as a good source of year-round income but the benefit-cost analysis indicated poorer returns on investment compared to other agricultural enterprises. Income from dairying was used as a source of collateral often for school and medical fees.

Cross-sectional study

Twenty-three percent of the farms reported observing clinical mastitis in their cows in 1998, affecting 14.2% of lactating cows in that year. The proportion of farms that had recorded a case of clinical mastitis during 1998 was significantly higher in Iringa (28.6%, 95% CI=22.1-35.7) than Tanga (16.8%, 95% CI=11.7-22.9) (P=0.006), as was the proportion of cows affected by clinical mastitis in Iringa (18.5%, 95% CI=14.3-23.3) than Tanga (10.1%, 95% CI=6.9-14.0) (P=0.003). The most common signs reported by respondents in the two regions who had seen clinical mastitis on their farms during 1998 were a painful udder, udder swelling, presence of clots or flakes, and pus in milk. Signs recognised less frequently were presence of blood in the milk and watery milk. Whilst all farmers with cases of mastitis treated their cows in Tanga, only 81% of farmers with clinical mastitis cases in Iringa treated the disease in 1998.

In both Iringa and Tanga regions, the milk yield was reported to be significantly decreased by approximately 3.5 litres/day following the mastitis case (P<0.001). After treatment, normal milk yield was not restored to the level seen prior to mastitis, with the yield after a case of mastitis being 1.5 litres less than the production before clinical signs were seen, a difference that was statistically significant (P<0.001).

During the cross-sectional study, the prevalence of subclinical mastitis, defined by a CMT positive sample, at the cow and quarter levels was 75.9% (95% CI=71.3-80.2) and 46.2 (95% CI=43.6-48.8%), respectively. There was no significant difference in either measurement between the two regions. The prevalence of subclinical mastitis, defined by a significant bacteriological isolation, at cow and quarter levels was 43.8% (95% CI=39.1-48.6) and 24.3% (95% CI=22.2-26.6), respectively. A significantly higher proportion of bacteriologically positive quarters were found in Iringa (27.3%, 95% CI=24.2-30.7), compared to Tanga (21.4%, 95% CI=18.5-24.5) (P=0.008).

Mastitis pathogens isolated from quarter milk samples are shown in Fig. 1. The most common isolates in both regions were *Staphylococcus aureus* (23.4%), *Staphylococcus epidermidis* (23.0%), other *Staphylococcus* spp. (17.3%), *Micrococcus* spp. (12.7%) and *Streptococcus* other than *Streptococcus agalactiae* (10.0%). Other isolates were coliforms (5.4% which included *Escherichia coli*, *Klebsiella* spp. and *Proteus* spp.), *Arcanobacter pyogenes* (3.5%) and *S. agalactiae* (3.3%).

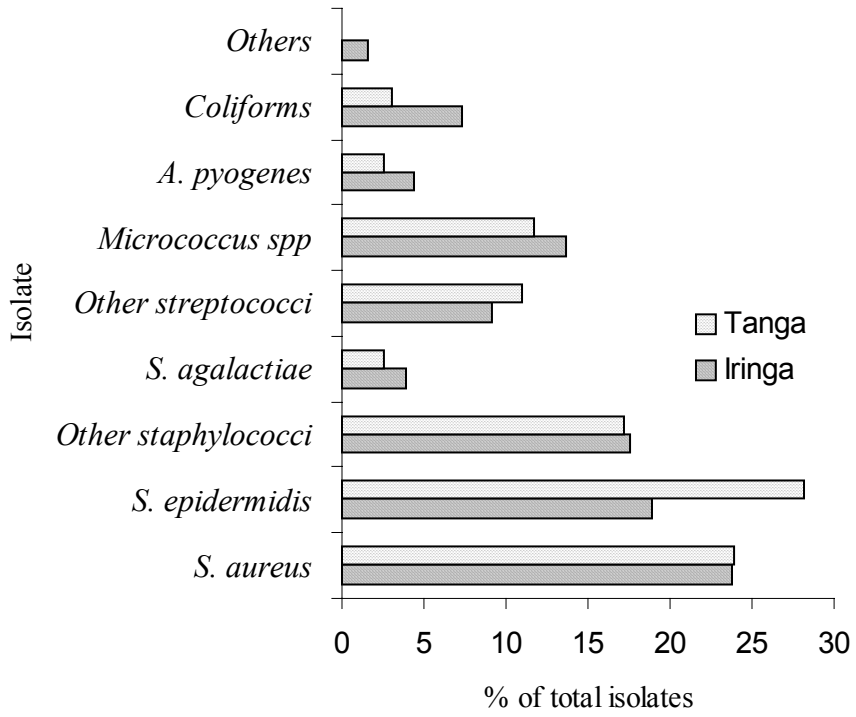


Fig. 1 Bacterial isolates (%) from quarter milk samples in Iringa and Tanga regions

The cross-sectional study identified a number of factors associated with the occurrence of clinical and subclinical mastitis. For example, clinical and subclinical mastitis in Iringa was positively associated with the Boran breed (clinical mastitis OR=3.4, 95% CI=1.0-11.6, P=0.05; CMT positive quarter OR=3.51, 95% CI=1.29-9.55, P=0.005) and the practise of leaving one quarter unmilked (clinical mastitis OR 2.1, 95% CI=1.0-4.2, P=0.04). Herd size was negatively associated with clinical mastitis in Iringa region (β –0.19, 95% CI=-0.33- -0.44, P=0.010).

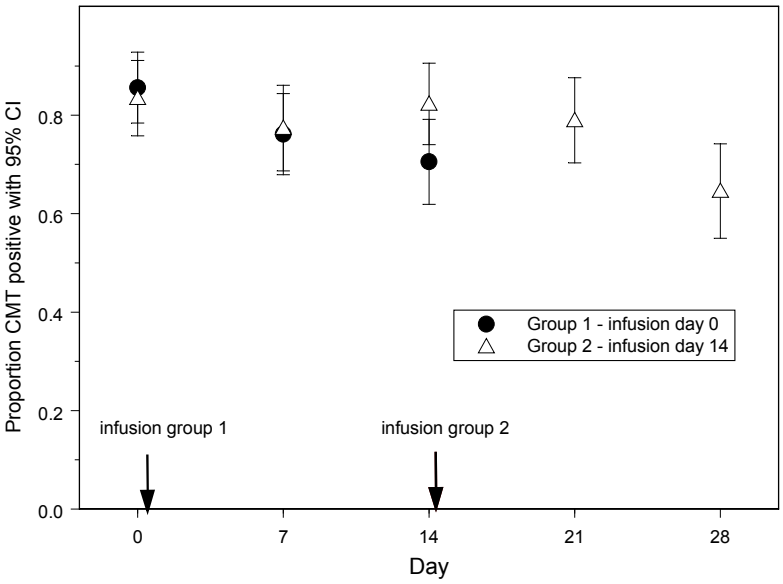
Intramammary antibiotic infusion intervention trial

Figure 2 shows the proportion of cows with at least one quarter positive for CMT (a) and bacteriology (b) in the 'treatment' and 'control' groups. Although there was a decline in the proportion that was CMT positive in both groups after therapy, this was small and not significantly different from the time-matched controls. In contrast, the proportion of cows with at least one bacteriologically positive quarter declined sharply 7 days after infusion in both groups and this was then followed by a slight increase 14 days after infusion. The difference between treatment and time-matched controls was significant when analysed at both the cow and quarter level (P<0.001) but there was no significant interaction between time and treatment (P=0.08).

Hypochlorite post-milking teat dipping intervention trial

There was no significant difference between cows in the prevention and control groups for either clinical mastitis or subclinical mastitis defined by CMT or bacteriology. Table 1 shows the relationships between prevention and the two subclinical mastitis outcome variables.

(a) CMT results



(b) Bacteriological results

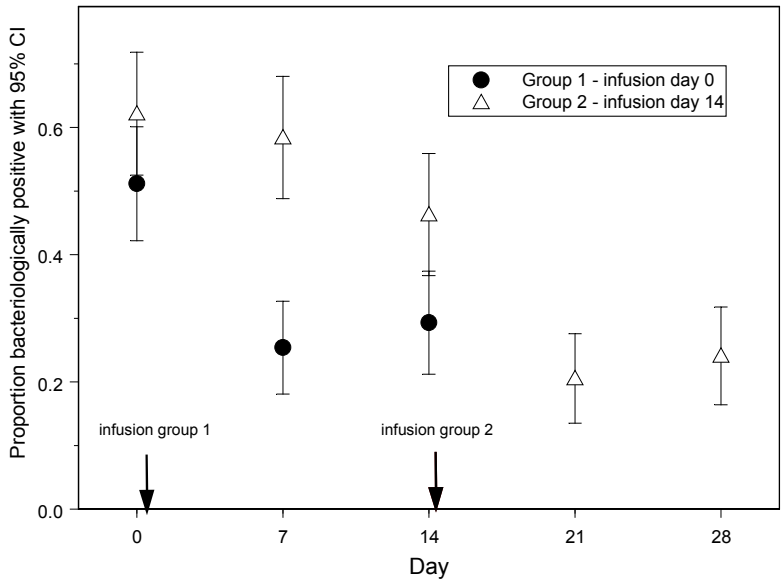


Fig. 2 Proportion of infected cows with at least one quarter positive for CMT (a) and bacteriology (b) before and after intramammary antibiotic infusion. Infusion group 2 provided the time matched controls for comparison with group 1.

Additional findings from the longitudinal study

During the 24 months of the longitudinal study, an average of 230 cows per month were studied giving a total of 454.4 cow-years at risk during which 144 clinical mastitis cases were reported. The average incidence of clinical mastitis was estimated to be 31.7 cases per 100 cow-

years. The incidence of clinical mastitis in cows which received an intramammary infusion was significantly lower than in all other cows involved in the longitudinal study (20.2 cases cf. 40.8 cases per 100 cow years, P=0.007).

Table 1. The relationship between hypochlorite teat dipping and CMT and bacteriology positive quarters, adjusted for time (sampling occasion included as a fixed effect), farm, cow and quarter (as random effects).

Variable	Coefficient (standard error)	Odds ratio (95% CI)	P value
Outcome variable = Bacteriologically positive			
Prevention vs control group	-0.02 (0.2)	0.98 (0.7-1.4)	0.92
Variance estimates:			
Farm	0.39 (0.20)		
Animal	0.72 (0.23)		
Quarter	0.06 (0.18)		
Outcome variable = CMT positive			
Prevention vs control group	0.25 (0.20)	1.3 (0.9-1.9)	0.22
Variance estimates:			
Farm	0.77 (0.26)		
Animal	1.77 (0.26)		
Quarter	1.12 (0.10)		

The practice of residual calf suckling was associated with a reduced risk of subclinical mastitis. After adjusting for confounding by variables including days in milk, lactation number, breed and the occurrence of mastitis in the previous month, the OR for residual calf suckling was 0.7 (95% CI=0.6-0.8, P<0.001) for the outcome 'CMT positive quarter' and 0.6 (95% CI=0.5-0.8, P<0.001) for the outcome 'bacteriologically positive quarter'.

Knowledge dissemination

Observational studies conducted in Iringa during 1999 and 2000

Mastitis knowledge, defined as 'volunteering mastitis facts post-dissemination', revealed positive associations with the following methods by which respondents stated they had learned about mastitis: 'mastitis training course' (MTC) (OR=2.3, 95% CI=2.2-2.5, P≤0.001), 'video' (OR=1.3, 95% CI=1.2-1.4, P=0.004), 'pen' (OR=1.3, 95% CI=1.2-1.5, P=0.004), and 'extension officer' (EO) (OR=1.2, 95% CI=1.1-1.3, P=0.024). Significant negative associations were seen when the respondent was a 'cowboy' (OR=0.8, 95% CI=0.8-0.9, P=0.048) or a 'relative' (OR=0.6, 95% CI= 0.5-0.7, P=0.016) of the householder, when compared to the 'husband' of the household. The final multi-level model for mastitis knowledge in 2000, defined as above, included significant positive associations only with the methods, 'MTC' (OR=1.6, 95% CI=1.4-1.7, P≤0.001), and 'EO' (OR=1.3, 95% CI=1.2-1.4, P=0.004).

Randomised trial of dissemination methods conducted in Tanga in 2001

All of the five methods were effective at disseminating mastitis knowledge compared to the control population. The final multi-level models for mastitis knowledge in Tanga, defined as 'volunteering mastitis facts post-dissemination' showed that all methods were significantly and

positively associated with better mastitis knowledge compared to the control group. When village and farm were included as random effects, the following results were obtained: of the five dissemination methods, the 'handout' method (OR=3.5, 95% CI=3.1-4.0) was the most effective, whilst the combination methods 'village meeting and video' (OR=3.2, 95% CI=2.8-3.6), 'village meeting and handout' (OR=3.3, 95% CI=2.9-3.7) and 'village meeting, video and handout' (OR=3.3, 95% CI=2.9-3.8) methods all showed very similar effects. The 'village meeting' method (OR=2.6, 95% CI= 2.3-3.0) was the least effective ($P<0.0001$ in all cases). When all dissemination methods were compared with 'village meeting', all were associated with significantly greater levels of mastitis knowledge ($P<0.0001$). Similar results were obtained when question-related variables were considered as random effects.

In addition to the randomised dissemination methods, mastitis knowledge was associated with other dissemination events and methods. Mastitis knowledge was positively associated with learning from a previous farmer-training course, another training course or a pamphlet. The OR for these volunteered methods of learning about mastitis were much lower (<2), compared to those seen for the five dissemination methods used in the current study. There were also positive associations with respondents who had received 'form' (secondary) or 'higher' level education, compared to 'standard' (primary) level.

The variance estimate for 'farm' (0.05, SE=0.02) was greater than 'village' (0.02, SE=0.01) although both were very small indicating that most of the residual variation was attributable to the individual question administered to the respondent (level 1). When question-related variables were considered as random effects, the variance estimate for question number (2.0, SE=0.4) was greater than question type (0.4, SE=0.4). This suggested that most of the residual variation was between the responses to individual questions (level 1), with the remaining variation largely attributable to the question number (level 2) with little contribution from question category (level 3).

DISCUSSION

Mastitis, in both its clinical and subclinical forms was found to be common in Tanzania and contagious pathogens were found to be more common than environmental pathogens, with *Staphylococcus aureus* being isolated most frequently. The prevalence of subclinical mastitis in the current study, defined by CMT positive cows and quarters, was high (76% and 46%, respectively). Other recent studies reported a slightly lower prevalence of 62% of cows positive by CMT in Tanzania (Malole, 1998), and 33% of quarters positive by CMT in Kenya (Mulei, 1999). The prevalence of subclinical mastitis, defined by bacteriologically positive cows was 44%, which is higher than that reported previously (9% to 26%) in the Southern Highlands of Tanzania (Mahlau & Hyera, 1984; Phiri et al., 1998). The majority of farms studied previously belonged to either medium or large scale sectors rather than smallholder dairy farms, although all three sectors had common sources of foundation stock. The difference in disease prevalence may, therefore, be attributed to management factors at farm level.

The main impact of the intervention trial with intramammary antibiotic was the reduced proportion of infected cows, assessed by bacteriological culture of quarters 7 and 14 days after infusion. However, there was relatively little effect of infusion on the proportion of CMT positive quarters during the trial. When all infused cows (i.e. all those that took part in the intervention trials) were compared with all other cows in the longitudinal study, a significantly lower proportion developed clinical mastitis. This finding should be treated with caution as the

cows were no longer randomly allocated to infusion groups, however, the large difference between the two groups warrants further investigation. The average incidence rate for clinical mastitis of 31.7 cases per 100 cow-years at risk in the current study is lower than the incidence reported from Kenya for a similar smallholder dairy farming system (Gitau et al., 1994; Omore et al., 1996).

Risk factors for clinical and subclinical mastitis during the cross-sectional study included the Boran breed with this breed being 3.4 times more likely to have clinical mastitis, and 3.5 times more likely to have a CMT positive quarter, than cows of the Tanzanian Shorthorn Zebu (TSHZ) breed. This may be explained by an indirect effect of Boran breed on mastitis mediated through an increased milk yield, compared to the TSHZ breed (Mchau, 1991). Alternatively, there may be a real association, as yet unexplained, between the Boran breed and mastitis. Protective factors for subclinical mastitis during the longitudinal study included residual suckling by a calf post-milking. It is possible that suckling protects the udder from infection by facilitating complete emptying of the gland, or that the presence of antibacterial factors in calf saliva may reduce infection of the teat ducts.

Results of the knowledge dissemination study showed that the MTC, video, pen and EO have been successful in transferring short-term knowledge to farmers, but that only the MTC and EO were successful methods in the longer term. For direct dissemination of knowledge, the 'handout' method was extremely successful in transferring information to respondents and the combination methods of video/village meeting/handout did not show any additional benefit over handout alone. This result contradicts the findings of some previous studies where leaflets were shown to have high levels of exposure but low levels of knowledge dissemination (Mitchell et al., 2001). The success of the handout in the current study may be due to the inclusion of important design features as a result of extensive consultation with Tanzanian colleagues and pre-testing with the target population. The individual question was shown to be an important source of variation indicating that comprehension of different subject areas may be involved and that particular attention to designing appropriate questions is important in similar studies.

The combination of rapid rural appraisal, cross-sectional, longitudinal and intervention studies has improved our understanding of the impact and awareness of mastitis in smallholder dairy farms in Tanzania. Through training courses and workshops, this information has been disseminated to farmers and animal health workers resulting in marked improvements in knowledge of the disease and its control. Clearly this knowledge must persist and become the basis of mastitis prevention so as to achieve a sustainable improvement in udder health in this important dairy farming system.

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THE TRANSMISSION OF CLINICAL AND SUBCLINICAL MASTITIS IN DAIRY COWS: A THEORETICAL APPROACH

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SUMMARY

A multispecies model that incorporated the transmission of both major and minor mastitis pathogens, as well as the interaction between them via co-infection of a quarter, suggested that major and minor pathogens can occasionally interact in a counter-intuitive way which has implications for the control of clinical mastitis. The key finding was that in a herd with major and minor pathogens, a delay in culling of cows with major pathogen infections of more than 40 days post-infection could result in higher levels of major pathogen infections. However, early culling would reduce the levels. A theoretical exploration of current control strategies was carried out, informed by parameters estimated from the model and data. The results at each stage allowed the suggestion of areas requiring further research. This work has further highlighted the potential for mathematical modelling to be incorporated into the overall research of veterinary disease dynamics.

INTRODUCTION

Mastitis in dairy cows has significant economic and animal welfare implications for the dairy industry. The bacterial pathogens responsible for infection of the mammary gland may be split into two main categories, major and minor pathogens. Infection with major pathogens generally results in clinical illness or pronounced inflammatory responses and reduced milk yields, whereas infection with a minor pathogen is usually subclinical. For the purposes of this paper, the class 'major pathogens' comprises of *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae*. The class 'minor pathogens' comprises of *Corynebacterium bovis* and *coagulase-negative Micrococcaceae*.

The understanding of the transmission and control of infectious disease has been aided by the use of mathematical models, which has been illustrated recently the high profile FMD work of Keeling et al. (2002). Generally, the mathematical models that have been applied to data have not included antigenic diversity, since the inclusion of multiple strains and/or species of microparasites in such models results in high order dynamical systems which require data of greater detail than are normally available (White et al., 1998). However, a body of theoretical work on these model structures suggests that antigenic diversity can explain certain dynamical behaviour (Andreassen et al., 1997; Gupta et al., 1998; Gomes & Medley, 2000) and vaccine

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induced strain replacement (McLean, 1995; Gupta et al., 1997; White et al., 1998) in host-multiparasite systems.

Mathematical modelling of mastitis transmission and control in dairy cows has been performed previously. Deterministic Susceptible-Infectious-Resistant (SIR) type structures were used to consider the disease dynamics of mastitis (Lam et al., 1996a; White et al., 2001a; White et al. 2001b; White et al., 2002) and to measure the effects of certain controls (Lam et al., 1996a; Lam et al., 1997a; Lam et al., 1997b; White et al., 2001b; White et al., 2002).

The models employed were for the transmission of two classes of pathogens and included an interaction between the classes via co-infection of the individual. These models were therefore similar to multistrain models previously published (Lipsitch, 1997) and are, to the authors' knowledge, the first of this category of models to be applied to data. This paper builds on previous work by using the same model but applies it to data from seven herds simultaneously. This allows for increased confidence in global parameter estimates and for the comparison of quantities that vary in value between herds.

In addition, a comprehensive exploration of possible control measures against clinical mastitis was carried out where postmilking teat disinfection (PMTD), antibiotic treatment, quarter drying and culling were considered.

MATERIALS AND METHODS

The mathematical transmission model (White et al., 2001a) was extended to include control measures. Traditional control measures comprised of PMTD, quarter drying, culling and antibiotic treatment (lactation and dry cow therapy). PMTD was modelled as a proportional reduction in the susceptibility of quarters to major and minor pathogens by factors of m_1 and m_2, respectively. Quarter drying was modelled as a removal of those quarters infected with major pathogens from the lactating herd at a rate, q_1 day^{-1}, for the duration of lactation. Culling was modelled as occurring at rate, c_1 day^{-1}. Alternatively, we define the time between a cow becoming infected with major pathogens and being culled as $(c_1)^{-1}$ days. It was assumed that culled cows were replaced and that only one quarter of each cow was infected with major pathogens with the other three quarters being distributed between the uninfected (x_{12}), minor pathogen only infected (y_2) and quarter dried (q_d) states as in the whole herd. Lactation therapy (the antibiotic treatment of major pathogen infected quarters during lactation) was modelled as the flow from states y_1 (infected only with major pathogens) and y_{12} (infected with both classes of pathogens) to x_{12} at rate, τ day^{-1}. The rate, τ, may be considered as the product of the efficacy of antibiotic treatment and the inverse of the time between infection of the quarter and its recovery after treatment. Dry cow therapy was modelled as a reduction in the proportion of quarters entering the herd infected with major pathogens by a factor, d.

$$\dot{x}_{12} = (1 - d\theta_1 - \theta_2 - d\theta_{12})(\mu + 4c_1(y_1 + y_{12})) - \left(m_1\lambda_1 + m_2\lambda_2 + \mu + 3c_1\left(\frac{y_1 + y_{12}}{x_{12} + y_2 + q_d}\right)\right)x_{12} + (\nu_1 + \tau)y_1 + \nu_2 y_2 + \tau y_{12} \quad (1)$$

$$\dot{y}_1 = d\theta_1(\mu + 4c_1(y_1 + y_{12})) + m_1\lambda_1 x_{12} + \nu_2 y_{12} - ((1 - \pi_2)m_2\lambda_2 + \nu_1 + \tau + \mu + c_1 + q_1)y_1 \quad (2)$$

$$\dot{y}_2 = \theta_2(\mu + 4c_1(y_1 + y_{12})) + a_2 m_2 \lambda_2 x_{12} + \nu_1 y_{12} - \left((1-\pi_1)m_1\lambda_1 + \nu_2 + \mu + 3c_1\left(\frac{y_1 + y_{12}}{x_{12} + y_2 + q_1}\right)\right)y_2 \quad (3)$$

$$\dot{y}_{12} = d\theta_{12}(\mu + 4c_1(y_1 + y_{12})) + (1-\pi_1)m_1\lambda_1 y_2 + (1-\pi_2)m_2\lambda_2 y_1 - (\nu_1 + \tau + \nu_2 + \mu + c_1 + q_1)y_{12} \quad (4)$$

$$\dot{q}_d = q_1(y_1 + y_{12}) - \left(\mu + 3c_1\left(\frac{y_1 + y_{12}}{x_{12} + y_2 + q_1}\right)\right)q_d \quad (5)$$

where

$$\lambda_1 = \beta_1(y_1 + y_{12}) \quad (6)$$

$$\lambda_2 = \beta_2(y_2 + y_{12}) \quad (7)$$

The parameters are defined as follows. μ is the rate of turnover of cows in the lactating herd, ν_1 is the rate of recovery from major pathogen infections, ν_2 is the rate of recovery from minor pathogen infections, θ_1 is the proportion of the quarters entering the lactating herd already infected with major pathogens only, θ_2 is the proportion of the quarters entering the lactating herd already infected with minor pathogens only, θ_{12} is the proportion of the quarters entering the lactating herd already infected with both classes of pathogens, β_1 is the transmission rate of major pathogens (that is, the product of the probability of an uninfected quarter becoming infected with major pathogens after a contact event with another major pathogen infected quarter and the contact rate [number of times in a day quarters make a potentially infectious contact]), β_2 is the transmission rate of minor pathogens, π_1 is the level of cross protection against major pathogen infections given by infection with minor pathogen infection, π_2 is the level of cross protection against minor pathogen infections given by infection with major pathogen infection.

The original data were collected from seven dairy herds during a study that investigated the efficacy of PMTD (Lam, 1996). The purpose of PMTD is to remove bacteria from the exterior of the teat, thus reducing the likelihood of pathogen invasion into the mammary gland, which would cause inflammation and/or elevated somatic cell counts in the milk. In Lam's study, the same two teats of each cow were disinfected after milking and the remaining two teats left as untreated controls for the duration of the study.

The model defined in White et al. (2002) was fitted to the data using the computer package, Berkeley Madonna (Macey et al., 1999), which minimises the root mean square deviation (RMSD) of the model output from the data using the simplex method (Press, 1992). The raw data were in the form of spreadsheets for each of eighteen samplings. The number of colony forming units $(0.01\ \text{ml})^{-1}$ of each pathogen for each quarter of each cow was given. The data set for all the samplings from all of the herds was pre-processed to produce time series that corresponded to the state variables.

Due to the amount of noise in the data, the number of parameters to be estimated were reduced by estimating as many as possible from the data and other sources. The details of this process can be found in White et al. (2001a). Using the model and the data from the seven dairy

herds under study, the remaining parameter values were estimated using least squares minimisation. The β_1 and β_2 were different for each herd, π_1 and π_2 were the same for each herd and m_1 and m_2 were the same within subgroups of herds.

RESULTS

Parameter Estimation

The average rate of turnover of cows in the lactating herd and the recovery rates from minor and major pathogen infections were fixed from a previous study (White et al., 2001a). Table 2 shows these fixed values.

Table 2. Fixed parameter values for rate of turnover of cows in lactating herd (μ), rate of recovery from major and minor pathogen infections (ν_1 and ν_2), proportion of the quarters entering the lactating herd already infected with a major pathogen, minor pathogen and both pathogens (θ_1, θ_2 and θ_{12}, respectively)

Parameter	Units	Herd						
		1	2	3	4	5	6	7
μ	day^{-1}	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045
ν_1	day^{-1}	0.01	0.01	0.01	0.01	0.01	0.01	0.01
ν_2	day^{-1}	0	0	0	0	0	0	0
θ_1	-	0.011	0.010	0.049	0.023	0.045	0.030	0.017
θ_2	-	0.559	0.414	0.338	0.503	0.545	0.384	0.514
θ_{12}	-	0.031	0.059	0.013	0.023	0.045	0.026	0.017

The herd groupings for the parameters m_1 and m_2 were determined by a preliminary fit of these parameters for each herd, and then equating similar values and refitting whilst the RMSD value to four significant figures remained unchanged.

Table 3. Fitted parameter values for transmission rates of major and minor pathogens (β_1 and β_2), effects of PMTD (m_1 and m_2) and cross-protection levels (π_1 and π_2) for the seven herds

Parameter	Units	Herd						
		1	2	3	4	5	6	7
β_1	day^{-1}	0.0625	0.0677	0.0078	0.0207	0.0840	0.0117	0.0098
β_2	day^{-1}	0.0068	0.0038	0.0015	0.0037	0.0052	0.0033	0.0061
m_1	-	0.063	0.063	**2.1**	**2.1**	0.063	**2.1**	**2.1**
m_2	-	0.19	**2.8**	**2.8**	**2.8**	0.42	**2.8**	$2.2X10^{-5}$
π_1	-	0.864	0.864	0.864	0.864	0.864	0.864	0.864
π_2	-	-2.72	-2.72	-2.72	-2.72	-2.72	-2.72	-2.72

The values in bold (Table 2) indicate values where the effect of PMTD is to enhance the transmission of major or minor pathogens. That is, when m_1 or m_2 is greater than unity. This is predicted for herds 3, 4, 6 and 7 with respect to major pathogens and herds 2, 3, 4 and 6 for minor pathogens.

The following figure shows the plots of the fit of the model to all seven herds.

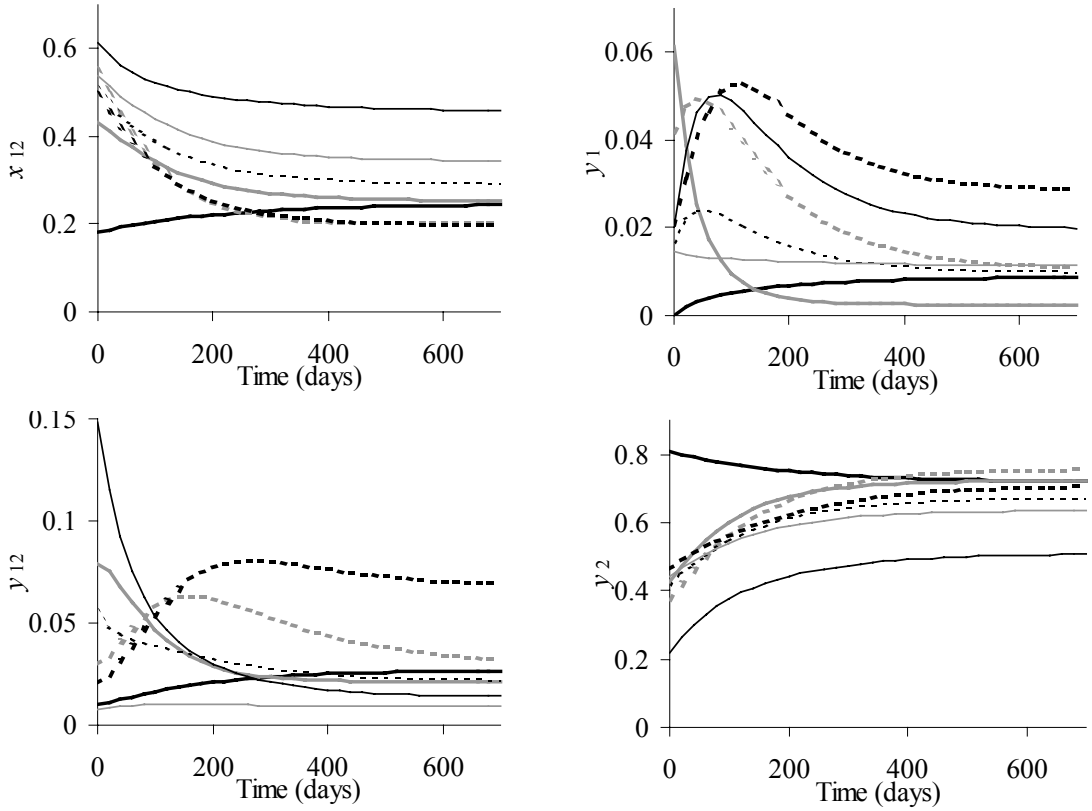

Fig. 1 Plots of the global fit to herds 1 to 7. Proportions of the quarters uninfected (x_{12}; top left), infected with major pathogens (y_1; top right), infected with minor pathogens (y_2; bottom right) and infected with both classes of pathogens (y_{12}; bottom left) for each herd. Herds: 1) bold solid black line; 2) bold solid grey line; 3) solid black line; 4) bold dashed grey line; 5) bold dashed black line; 6) dashed black line; 7) solid grey line.

The basic reproduction number (R_0) of a class of pathogens is defined as the average number of secondary infections of quarters caused by the presence of a single infected quarter in an uninfected lactating herd (Kermack & McKendrick, 1927). It is given by the equation:

$$R_{01} = \beta_1/(\nu_1 + \mu) \tag{8}$$

for major pathogens and similarly for minor pathogens. A value less than unity for a class of pathogens would imply that infection would not persist in the herd if there was no influx of infected quarters and no enhancement of susceptibility due, for example, to the presence of another class in the herd. Table 4 shows the basic reproduction numbers for minor (R_{02}) and major (R_{01}) pathogens for each herd calculated from the fit of the model to the data.

Table 4. Estimated basic reproduction numbers

Parameter	Units	Herd						
		1	2	3	4	5	6	7
R_{01}	-	4.32	**0.46**	**0.54**	1.43	5.81	**0.81**	**0.68**
R_{02}	-	1.52	**0.84**	**0.35**	**0.84**	1.17	**0.74**	1.37

The values in bold show basic reproduction numbers of value less than unity. This occurs for major pathogens in herds 2, 3, 6 and 7. Also, the basic reproduction number of major pathogens in herd 4 is close to unity, thus a basic reproduction number clearly greater than unity is predicted only for herds 1 and 5. For minor pathogens, the basic reproduction number is less than unity in herds 2, 3, 4 and 6 and greater than, but close to unity, in the remaining herds.

Control

The model given by Equations (1-7) was run to equilibrium with the control parameters set such that they have no effect (Fig. 1). The values of the variables at equilibrium were set as initial conditions and then the model was rerun for an array of values for each control parameter. The percentage decrease in the proportion of quarters infected with major pathogens with or without minor pathogens was calculated for each herd. Thus a percentage decrease of 100% would imply the total eradication of major pathogens, a percentage decrease of 0% would imply that the control in question had no effect whatsoever and a negative percentage would imply that use of the control increases the equilibrium proportion of major pathogen infected quarters. The bold lines refer to herds 1, 2 and 5 in the Figures hereafter.

Postmilking teat disinfection was estimated as having very different effects in the seven herds (see m_1 and m_2 values in Table 3). The graph below shows how different values of m_1 and m_2 can affect the prevalence of major pathogens in the herds.

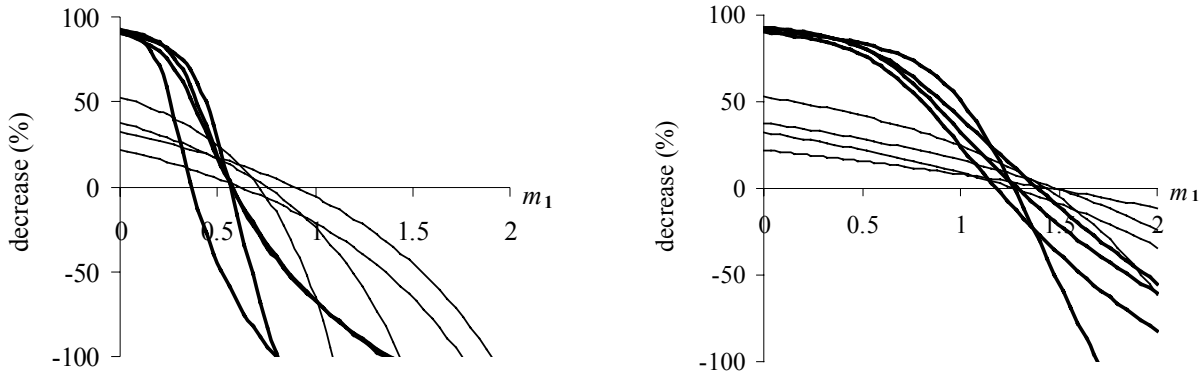

Fig. 2 The effects of PMTD. The percentage equilibrium decrease for values of m_1 between 0 and 2 and the fitted values of m_2 0.19 (left) and 2.8 (right).

The plots visually separate the herds into two groups: those that move from high positive (near 100%) to negative effects sharply and those that move from intermediate positive (between 20 and 60%) to negative effects more gradually. Herds 1, 2 and 5 display the former behaviour. For the remaining herds, that show the latter behaviour, PMTD was most effective in herd 4 followed by herds 6, 3 then 7. The effects of PMTD on the equilibrium prevalence of minor pathogens are not illustrated here, but remain constant for the range of values of m_1 and alter the equilibrium prevalence by ± 50%.

Quarter drying was considered for rates between 0 and 0.1 day^{-1} (Fig. 3). This means that the diagnosis time, i.e. the time between the initial infection and drying off of a quarter, was greater than 10 days.

The plots in Fig. 3 (left) predict that quarter drying has a similar effect in all herds, reducing prevalence of major pathogens by 90 to 100% for high values of q_1. The highest percentage decreases were for herds 1, 2 and 5 followed by herds 4, 6, 3 and 7. The slope of the percentage decrease was reduced dramatically for values of q_1 greater than about 0.02 to 0.04 day^{-1} (diagnosis times from 25 to 50 days). In Fig. 3 (right), the equilibrium proportion rapidly peaks at values of q_1 between 0.006 and 0.01 (diagnosis times between 100 and 170 days) for herds 2, 5 and 1 and approaches its constant value at q_1~0.04 day^{-1} (diagnosis time roughly 25 days). For the remaining herds, increasing q_1 results in an increasing proportion of dried quarters towards a constant value. The magnitude of the maximum proportion ascends in order for herds 3, 6, 4 and 7. Quarter drying also consistently acts to reduce the equilibrium proportion of minor pathogen infected quarters (not shown) in all herds by between 2 and 30%.

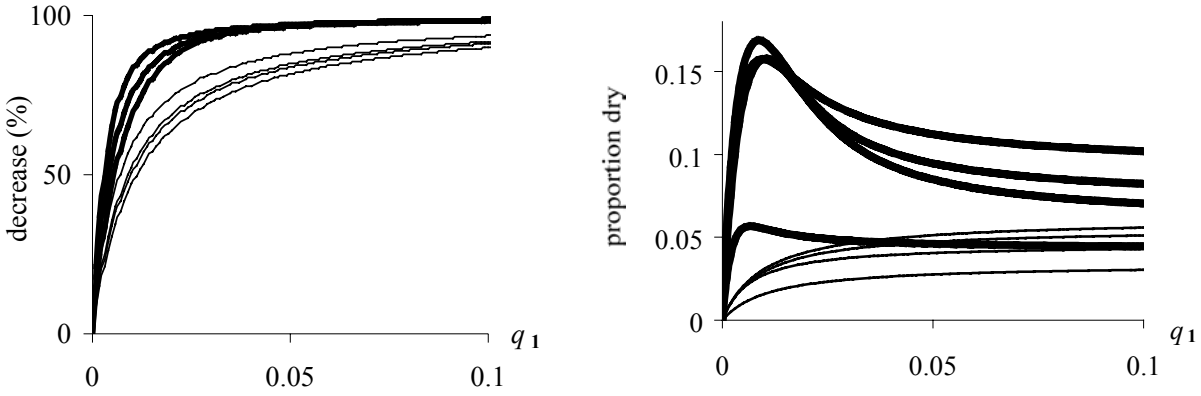

Fig. 3 The effects of quarter drying. The predicted percentage decrease (left) and the equilibrium proportion of dried quarters (right) for each herd against quarter drying rate.

Culling cows with a major pathogen infected quarter was considered for effectiveness in reducing prevalence as well as the proportion of cows requiring to be culled. The rate c_1 is the inverse of the time between initial infection and culling. The cost of culling will correlate closely with the proportion of cows requiring to be culled. We also considered the equilibrium frequency of culling where we predicted the average number of days per culling event assuming a herd size of 50 cows. The percentage decrease in major pathogens (Fig. 4 left) was negative for herds 1, 2 and 5 for values of c_1 less than 0.025 day^{-1} (diagnosis time greater than 40 days). That is, under these conditions, the predicted effect of culling was to increase the prevalence of major pathogens. It then increased towards a high constant value for values of c_1 greater than 0.04 day^{-1} (diagnostic time less than 25 days). For the remaining herds, the percentage decrease was positive and increases towards a high constant value for values of c_1 greater than 0.02 day^{-1} (diagnostic time less than 50 days). The frequency of culling (for a herd size of 50 cows) is illustrated in Fig. 4 (right). The lowest average period between culling was about 3 days (for herd 5), whereas the highest was over 300 days. Culling also consistently acted to reduce the equilibrium proportion of minor pathogen infected quarters (not shown) in all herds by between 2 and 30%.

Lactation therapy was considered for effectiveness in reducing prevalence as well as the proportion of quarters requiring treatment. The rate τ was a product of the cure rate (Craven, 1987) and the inverse of the sum of the times between infection and treatment and between treatment and recovery. The cost of lactation therapy will correlate closely with the number of quarters requiring treatment. Lactation therapy is most effective against herds 1, 2 and 5 (particularly herd 1) where the percentage decrease approaches its maximum value more rapidly than in the other herds for increasing τ. Given a cure rate of 50% (Craven, 1987) and a recovery time of 5 days, the time between a quarter becoming infected and beginning treatment should be less than a week in order for the treatment rate, τ, to be greater than 0.04. Antibiotic treatment of lactating cows infected with major pathogens also consistently acts to reduce the equilibrium proportion of minor pathogen infected quarters (not shown) in all herds by between 2 and 30%.

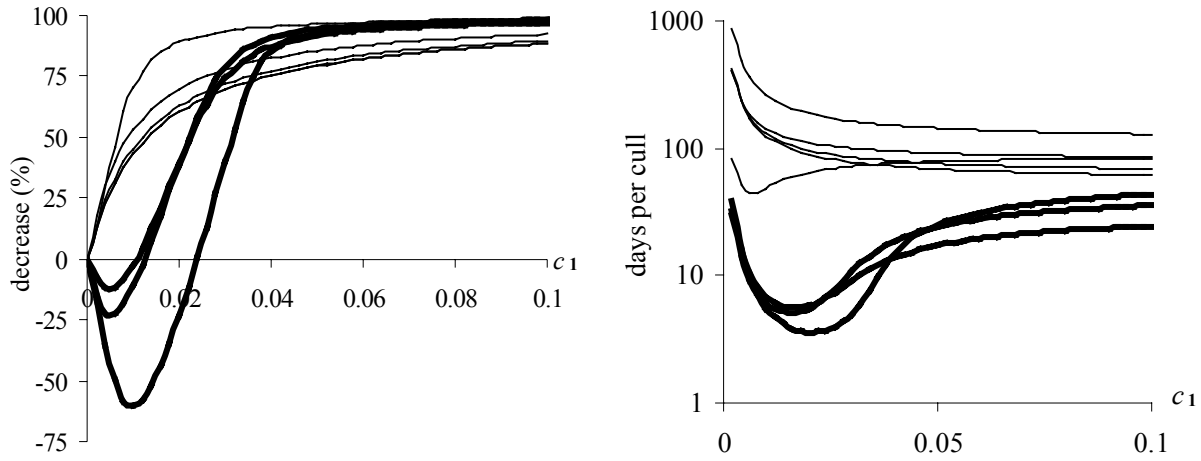

Fig. 4 The effects of culling. The decrease in equilibrium prevalence of major pathogens (left) and the predicted mean number of days between culls, on a logarithmic scale, (right) against c_1.

Dry cow therapy was considered in a relatively simplistic way, since only the dynamics of the lactating herd was dealt with in the models presented. Thus, we assume that the proportion of quarters entering the herd after the dry period infected with major pathogens can be reduced by a factor d. This value is a product of (1 - efficacy of the treatment) and the average proportion of infected quarters that were diagnosed successfully. Apart from herds 1, 2 and 5, increasing d from 0 to 1 roughly linearly decreases percentage change from 100 to 0%. The percentage decrease remains below 20% for herds 1, 2 and 5. Antibiotic treatment of cows at the end of their dry period also consistently acts to reduce the equilibrium proportion of minor pathogen infected quarters (not shown) in all herds by less than 10%.

DISCUSSION

Eight of the fourteen basic reproduction number estimates in Table 4 were less than unity. This indicates that in over half of the herds, major and/or minor pathogens were unable to sustain themselves through contagious transmission alone, and their presence was due to the influx of pre-infected cows into the lactating herd. An explanation of this is that the estimates of the basic reproduction numbers were artificially low due to either the farmers employing

prevention/treatment methods that were not included in the model or the presence of the environmental pathogen *E. coli* (also not included in the model). Another possibility is that in these herds, the disease really is being driven by infections picked up during the dry period. We could suggest that there is an environmental (linear) component to the force of infection of these pathogens which, since it is not included in the model, has been absorbed into the (linear) influx proportion estimates (θ values). However, the influx proportions were fixed from the data and so were not fitted, so this is unlikely.

Figure 2 illustrates the counter-intuitive outcome of the discontinuation of PMTD of a drop in the prevalence of major pathogen infections. PMTD is intended to reduce the incidence of clinical mastitis and thus its discontinuation should precipitate an increase in the prevalence of the pathogens that generally cause this disease. This result reflects the results presented in Table 4 of Lam et al. (1997a) where herds 2, 4, 6 and 7 showed a higher number (by 2 or more) of clinical cases of either *Staph. aureus*, *Strept. uberis* and *Strept. dysgalactiae* in quarters undergoing PMTD compared with controls. However, these results do not coincide with estimates of m_1. We would expect from these estimates that major pathogens should increase in herds 3, 4, 6 and 7 since m_1 (= 2.1) is estimated to be greater than unity indicating an enhancement of susceptibility of dipped quarters to major pathogens. We suggest that PMTD has a complex nonlinear effect on the incidence of clinical mastitis dependent on (1) its individual effect on the susceptibility of the quarters to major and minor pathogens, (2) the transmissibility of both classes of pathogens and (3) the interaction between these classes. These effects seem to be independent of whether the spraying (herds 1, 4 and 6) or dipping methods (herds 2, 3, 5 and 7) were used (Lam et al., 1997a). If PMTD has a strong effect on minor pathogens (m_2 low) (Fig. 2, left), then in order to achieve a reduction in major pathogen prevalence at equilibrium, PMTD must also reduce the susceptibility of quarters to major pathogens by a factor of four. For higher values of m_2, PMTD can be more effective against clinical mastitis. We also predict that for certain combinations of values for m_1 and m_2 PMTD can increase the prevalence of major pathogens thus having a negative effect, more than doubling the prevalence in some cases. This transition from positive to negative effects was particularly pronounced in those herds where the basic reproduction number of major pathogens was greater than unity (1, 2 and 5). In these herds, the predicted positive effects were far greater, but the negative effects were far worse. The swift transition between these effects indicates that a slight variation in the efficacy of PMTD against major pathogens (m_1) could result in a large outbreak of clinical mastitis. The gradient of this transition decreases and occurs at higher values of m_1 as the value of m_2 is increased. Thus a teat dip which enhances the susceptibility of quarters to minor pathogens while reducing the susceptibility to major pathogens would be an effective way of controlling clinical mastitis, although this would result in increased prevalence of subclinical mastitis caused by minor pathogens.

For those herds where the basic reproduction number is greater than unity, the diagnosis time must be kept well below 20 days to ensure that culling major pathogen infected animals has a highly positive effect (Fig. 4 left). If the diagnosis time was greater than 40 days, then the major pathogens in the quarter of the cow were present long enough to infect others, whereas the minor pathogens (which are less transmissible) in her other quarters were not. Culling the cows at this point provides a niche for invasion of major pathogens into the rest of the population due to the simultaneous removal of minor pathogens. A diagnosis time of less than 20 days also locally maximises the frequency of culling at equilibrium (Fig. 4 right). Then the most economically viable approach is to cull cows within 20 days of infection, which would involve culling a cow every 25 to 130 days depending on the basic reproduction number of the major pathogen class in a particular herd.

From the left graph of Fig. 3, we can deduce that a time of 25 to 50 days between the quarter becoming infected and it being dried off was required to achieve the maximum effect. Diagnoses any sooner than that will not improve the percentage decrease in prevalence of major pathogens. The right graph of Fig. 3 shows that for herds 1, 2 and 5 the proportion of quarters of lactating cows that are dry at any time was highest for diagnosis times between 25 and 170 days. Therefore, in herds where the basic reproduction number was greater than unity, an average diagnosis time of less than 25 days would minimise losses to productivity by minimising the proportion of dry quarters at any time to within the range of 5 to 15%.

We predict that lactation therapy at a treatment rate, τ greater than 0.04 day^{-1} would ensure the maximum effect and the minimum cost by maximising the number of days between treatments. Dry cow therapy was predicted as having the most variable effect on the prevalence of major pathogens. For herds where the basic reproduction number of major pathogens was less than unity (herds 3, 4, 6 and 7), dry cow therapy can be very effective depending on the cure rate of the treatment. This is because in these herds the disease is being maintained by the influx of major pathogen infected quarters into the lactating herd and therefore restricting this route produces a high percentage drop in equilibrium prevalence. However, for those herds whose major pathogen basic reproduction number was greater than unity (herds 1, 2 and 5), dry cow therapy even at 100% detection and cure rate would only reduce major pathogen prevalence by a maximum of about 20% (herd 1). It has been reported that the use of antibacterial drugs has the effect of reducing the efficacy of treatment (Myllys et al., 1994). This translates as a reduction in the values of τ and/or d in the context of the model presented. This would mean that more animals would require treatment, which would reduce them further. This is another incentive for swift detection, since minimising the use of antibiotics should slow down this process. Recent studies of the epidemiology and control of major pathogens (including coliforms) during the dry period (Bradley & Green, 2003a; Bradley & Green, 2003b; Green et al., 2003) show that new infections acquired in this period make a significant contribution to the incidence of clinical mastitis in those herds. A model of the dynamics of infection during the dry period as well as the lactation period would be more appropriate, since dry cow therapy could have a nonlinear effect, which is not modelled here.

The seven dairy herds from which the data originate had similar sizes, breeds of cow, management and milking procedures and low bulk somatic cell counts (Lam, 1996). However, the estimates for the basic reproduction numbers of major (and to a lesser extent minor) pathogen infection and the influence of PMTD were variable. Herds 1, 2 and 5 clearly had different predicted responses to controls from the other herds even though the time series did not visually indicate this (Fig. 3). These were the three largest herds in the study of sizes 62, 78 and 70 cows respectively compared to 40 to 54 cows in the other herds (Lam et al., 1996b). There was no correlation between the predominant species of major (Lam et al., 1997a) or minor pathogens (Lam et al. 1996a) in each herd and the groupings of the herds from parameter estimation.

The possible increase in prevalence of major pathogens was not the only negative aspect of control. The economic aspects of lactation therapy were explored in Craven (1987) where it is pointed out that it is relatively easier to calculate the costs of a therapy than to calculate the probable losses if the therapy was not carried out. This transmission and control model could be useful for this purpose, as well as finding ways to minimise cost as was demonstrated for quarter drying, culling and lactation therapy. As stated in Myllys et al. (1994), another side effect of controlling mastitis is the risk of creation of a ‘bacteriological vacuum’. The transmission and

control model can be extended to include other species in order to assess the likelihood of replacement due to different strategies.

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EQUINE EPIDEMIOLOGY

RISK FACTORS FOR MUSCULOSKELETAL INJURIES IN TWO-YEAR-OLD THOROUGHBRED RACEHORSES

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SUMMARY

Musculoskeletal injuries (MSI) have been identified as the most common cause of training days lost and weeks spent resting at pasture. Several training-, track- and horse-related risk factors have been identified, but there is no clear understanding of the inter-relationship between these factors. The aim of this longitudinal cohort study was to investigate risk factors for MSI in 2-year-old Thoroughbred racehorses. Australian Thoroughbred trainers were convenience sampled and enrolled in 26-month longitudinal cohort study. The study population consisted of all horses, trained by a participating trainer, that were born in the 1998 foaling season. Horses were followed from the time of enrolment until the completion of the study or until they were lost to follow-up. Trainers were visited at approximately 14-day intervals to collect training and injury data. Training days were categorised as fast days if the maximum speed during training exceeded 800 m/minute. For each horse, the first training period that included one or more fast days was analysed. The association between continuous variables and the MSI was investigated using 2 sample t-tests. Chi-square analyses were performed to determine the association between categorical variables and MSI. Variables with a $P \leq 0.30$ were entered into a multiple logistic regression model and the model made using backward elimination. Data from 274 horses trained by 14 trainers at 5 racetracks were analysed. Forty percent of the horses sustained a MSI during their first fast preparation. In a logistic model the following training related risk factors were significantly associated with MSI: percentage of fast days from the first fast day until the end of the preparation, the average distance worked at speeds $\geq$800 m/minute and the total distance worked at speeds $\geq$890 m/minute. The relationship between these training variables and MSI was altered by gender and previous MSI. There was no effect of training centre in the multivariable model.

INTRODUCTION

In Thoroughbred racehorses musculoskeletal injuries (MSI) have been identified as the main cause of lost training days and weeks spent resting at pasture (Rossdale et al., 1985; Kobluk et al., 1990; Lindner & Dingerkus, 1993; Bailey et al., 1997b). An Australian study found that the most common types of MSI to affect two- and three-year-old Thoroughbreds were shin soreness (42%) and fetlock problems (25%) (Bailey, 1998). This study also found that 40% of the horses that suffered from shin soreness developed the problem for a second or third time, whilst the recurrence rate for fetlock problems was 48%.

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The causes of MSI are likely to involve a number of different factors (Caine et al., 1996; Brunker et al., 1999). Some risk factors will affect the way in which the skeletal tissue is loaded or its ability to respond to the loading. These factors are normally divided into intrinsic or extrinsic factors. Intrinsic risk factors are characteristics of the horse whilst extrinsic factors are characteristics of the environment.

Intrinsic risk factors include conformation (Axelsson et al., 2001), sire index (Gaustad et al., 1995), age (Robinson et al., 1988; Mohammed et al., 1991; Wilson et al., 1996; Williams et al., 2001), previous injury (Cohen et al., 1999; Hill et al., 2001) and gender (Estberg et al., 1996b; Carrier et al., 1998; Hernandez et al., 2001). Extrinsic factors may include factors relating to the training center or the training program. A number of studies investigating risk factors for racing MSI have reported that differences in the injury rates between tracks (McKee, 1995; Wilson et al., 1996; Bailey et al., 1997a). The importance of track-related factors is further highlighted by studies that have reported a reduction in injury rates following track reconstructions (Evans and Walsh, 1997, Oikawa et al., 1994). Some of the track related risk factors for racing MSI that have been identified are track geometry (Clanton et al., 1991, Hill et al., 1986, JRA, 1991, Oikawa et al., 1994, Peloso et al., 1994, Wilson et al., 1996), under banking and inadequate use of transition curves (Evans & Walsh, 1997), the type of surface (Hill et al., 1986, JRA, 1991, Mohammed et al., 1991, Mohammed et al., 1992, Mundy, 1997; Williams et al., 2001) and the condition of the surface (Bailey et al., 1998). A prospective investigation of risk factors for shin soreness found that training surface was a risk factor for shin soreness (Moyer et al., 1991; Moyer & Fisher, 1992). However, this study did not control for differences in training programs that may also have accounted for these differences. Another study found that the risk of shin soreness increased as the average distance worked at speeds of approximately 660 m/minute increased (Boston & Nunamaker, 2000). It was also found that the risk of shin soreness decreased as the average distance worked at speeds of approximately 900-960 m/minute increased. In the USA a case-control study found that two-year-old racehorses were at an increased risk of fatal MSI when, in a two-month period, they accumulated more than 3,400 meters during races and official timed workouts (Estberg et al., 1995).

When examining individual risk factors it is important to consider not only their independent effects but also how they interact with other risk factors. At present, there is very little information relating to the inter-relationship and relative importance of the training-, track- and horse-related risk factors. The aim of this study is examine the training and track related risk factors for MSI in young Thoroughbred racehorses. The study also aims to evaluate the effect of previous MSI, gender and age. This paper reports on the analysis of potential risk factors for MSI in the first preparation where the horse worked at speeds $\geq$800 m/minute.

MATERIALS AND METHODS

Study Design and implementation

The risk factors for MSI were investigated using a longitudinal cohort study design. In February and March 2000 consultations were held in New South Wales with a number of Australian racetrack veterinarians and racing officials to determine trainers that might be willing to participate. Following these discussions the trainers were contacted through their veterinarian, and invited to participate in the study. At least one follow-up visit was made to ensure that the trainer, or nominated person, understood the necessary commitments and was still willing to

supply the required training and injury data. Trainers were enrolled in the study between June and August 2000 and data collection continued until the 1st August 2002.

The study population consisted of all horses born in the 1998 foaling season that were trained by participating trainers. Horses were followed from the time of enrolment until the completion of the study or until they were lost to follow-up. Horses were lost to follow-up if the trainer left the study, or the horse changed trainers, was sold overseas, sent to stud or died.

Data collection

One investigator (Naomi Cogger) visited all participating stables at approximately 14-day intervals to collect daily training and injury data. The data were collected using a standardised form that was maintained on a daily basis or completed at the time of the visit, using stable records. The training data included information relating to the intensity of training on each day, the distance worked at speeds ≥800 m/minute and <890 m/minute and the distance worked at speeds ≥890 m/minute.

During the fortnightly visit the trainer, or the nominated person, was interviewed to confirm the injury status of the horses currently in the stable. If the horse had a disorder involving the musculoskeletal system, the anatomical location of the problem and the affected leg(s) were recorded. Additional information relating to the nature of the problem was also collected. The trainer supplied the information relating to the nature of the injury and advised whether a veterinarian had made the diagnosis. However, the diagnosis was not subsequently confirmed with the veterinarian.

The trainer also informed the investigator if any horses either previously enrolled in the study, or eligible for enrolment in the study, had entered the stable between visits. If the horse had not previously been enrolled in the study then information relating to the previous exposure to training, previous injuries, sex and either the breeding or racing name of the horse was collected. The breeding or racing name was used to search the Australian and New Zealand studbooks for the exact date of birth.

Classification of training data

Every day that the horse was enrolled in the study and in the stable was referred to as a training day. Training days were classified into a number of categories depending on the activity undertaken on that day. The different classifications of training days are shown in Table 1. Training days were grouped together into units referred to as preparations. A preparation was defined, as a period of time in which the horse did not leave the stable for more than seven days. If the preparation included one fast day it was referred to as a fast preparation. The first fast day in the preparation marked the division of the preparation into slow and fast portions. Where the slow portion was the time from the start of the preparation until the first fast day and the fast portion refers to the time from the first fast day until the end of the preparation.

Data analysis

The outcome of interest in this analysis was any problem involving the musculoskeletal system that resulted in the horse leaving the stable for a period of more than 7 days. The analysis was conducted on the horse's first fast preparation, during the two-year-old racing season. A

horse was excluded from the analysis if its first fast day was prior to enrolment in the study. The prevalence of all MSI and the different types of MSI was calculated. Tables 2 and 3 show the categorical and continuous variables that were considered as potential risk factors. The association between continuous variables and MSI was investigated using 2 sample t-tests. Chi-square analysis was used performed to determine the association between categorical variables and MSI.

Variables with a p-value $\leq$0.30 were considered in a multivariable logistic regression model. The model was built using a method of backward elimination. Variables were retained in the final effects model if removing them resulted in a significant change (P<0.05) in the deviance. In the final model the shape of the relationship between the continuous variables and the outcome variable was evaluated using the method described in Hosmer and Lemeshow (2000). If the relationship was non linear then the continuous variable was recoded as a categorical variable.

Table 1. Categories of training days

Categories	Definition
Rest day	A training day where the horse's only activity undertaken was walking and/or swimming or the horse was box rested
Slow day	A training day where the horse's maximal training speed did not exceed approximately 650 m/minute.
Fast day	A training day where the horse's maximal training speed exceed 800 m/minute.
Non-gallop fast day	A fast day where the maximal training speed was less than approximately 890 m/minute.
Gallop-fast days	A fast day where the maximal training speed was greater than or equal to 890 m/minute.
Trial day	A training day where the horse completed a barrier trial
Race day	A training day where the horse completed a race

RESULTS

Data from the first fast preparation for 274 horses trained by 14 trainers at 3 metropolitan and 2 provincial racetracks were analysed. The mean (s.e) duration of each preparation was 56.9 (1.9) days (Range: 7-227), with the fast portion 37.6 (1.7) days (Range: 1-192) and the slow portion 19.30 (0.90) (Range: 1-113). During this preparation, 40% of horses sustained a MSI. The most common injures to affect horses were shin soreness (24%), fetlock problems (6%) and knee problems (5%).

Tables 2 and 3 show the association between categorical and continuous variables and MSI. In the multivariable logistic regression model sex, previous injury, percentage of days in the fast portion of the preparation that were fast, the average distance worked fast on fast days and the total distance galloped were found to be significantly associated ($P\leq$0.05) with MSI. Analysis of the continuous variables demonstrated that the relationship between the percentage of fast days in the fast portion of the preparation and the total distance worked at gallop pace and the outcome variable were not linear.

Table 2. The association between categorical variables and musculoskeletal injuries in 274 horses during their first fast preparation.

Variable	Number		P Value
	Uninjured	Injured	
Gender			
Female	95	55	0.15
Male	65	54	
Previous Preparation			
No	117	72	0.30
Yes	47	38	
Previous injury			
No	158	97	0.009
Yes	6	13	
Training centre			
1	8	4	0.009
2	7	8	
3	28	15	
4	51	55	
5	70	28	
Barrier trial			
No	106	65	0.35
Yes	58	45	
Race			
No	141	99	0.32
Yes	23	11	

DISCUSSION

MSI were common events during the first fast preparation, affecting 40% of horses. A number of training-, track and horse-related variables were considered as potential risk factors for MSI during this preparation. The training related variables describe frequency, duration and cumulative effects of exposure to exercise at speeds ≥800 m/minute. The frequency of exposure in this analysis was described by the percentage of days in the fast portion of the preparation that were fast. The average distance worked at speeds greater than or equal to 800 m/minute on fast days described duration of exposure. The cumulative exposure was described with the by the total distance worked at speeds ≥800 m/minute and the total distance worked at speeds ≥890. In the final effects model the variables for the frequency and duration of exposure and the total distance worked at speeds ≥890 m/minute were significantly associated with MSI. These results support previous research in the USA that found an association between distances worked at races and official timed workouts and fatal MSI (Estberg et al., 1995; 1996a; 1997; 1998). This is one of the first times the relationship between distances worked in daily training sessions and non-fatal MSI has been described.

The relationship between the training variables and MSI was altered by both gender and previous MSI. This is supported by previous research using multivariable techniques that has found previous injury (Cohen et al., 1999; Hill et al., 2001) and gender (Estberg et al., 1996b; Carrier et al., 1998; Hernandez et al., 2001) are risk factors for MSI.

Table 3. The association between continuous variables and musculoskeletal injuries in 274 horses during their first fast preparation

Variable	Mean (S.E)		P-value
	Uninjured	Injured	
Age at the start of the two-year-old racing season (years)	1.85 (0.01)	1.84 (0.01)	0.33
Age at the commencement of training (years)	2.06 (0.02)	2.05 (0.02)	0.76
Age at the start of the first preparation (years)	2.08 (0.03)	2.16 (0.03)	0.32
Total number of fast days			0.34
Total number of non-gallop fast days	5.66 (0.30)	5.71 (0.36)	0.93
Total number of gallop fast days	3.72 (0.34)	4.45 (0.35)	0.15
Duration of preparation (days)	57.16 (2.74)	56.60 (2.49)	0.89
Duration of the slow portion of the preparation (days)	18.57 (1.16)	20.39 (1.43)	0.33
Duration of the fast portion of the preparation (days)	38.59 (2.51)	36.21 (2.23)	0.50
% of days in the fast portion of the preparation that were fast	63.32 (1.92)	61.45 (2.13)	0.28
Average distance worked at speeds $\geq$800 m/minute on fast days (meters)	545.01 (13.32)	649.31 (13.50)	<0.001
Total distance worked at speeds$\geq$800 m/minute (meters)	5502.9 (382.32)	6670.2 (458.79)	0.04
Total distance worked at speeds $\geq$890 m/minute (meters)	1442 (148.34)	1804 (162.70)	0.11

There was a significant association between training centre and MSI. Although, in the multivariable model there was no significant association between the MSI and the proportion of horses injured at each training centre. This suggests that the differences in injury rates between training centres were the result of differences in exposure to training- and horse-related factors. However, the analysis did not determine if certain types of injuries were more likely to occur at a particular training centre. Furthermore, the data analysed represents a small portion of the training data collected during the study. Therefore, more detailed analysis of the first fast preparation and all of the training data collected is required before concluding that training centre is not a risk factor for MSI.

Whether the horse raced or barrier trialled during the first fast preparation were also considered as potential training-related risk factors. Previous research suggests that the high number of health problems, of which MSI were the most common, was the main reason for the high proportion of unraced two-year-old racehorses (Bailey, 1998). This analysis found that horses with MSI were no less likely to race or barrier trial during their first fast preparation, suggesting that during the first fast preparation the decision not to race a horse may be based on factors other that the presences of an MSI.

Age was the only other horse-related variable considered in this analysis. The age of each horse reflected the age in relation to a fixed date, the 1st August 2000, the commencement of training and the start of the first fast preparation. There was no association between any of the age variables and MSI. This would suggest that delaying exposure to training speeds $\geq$800m/minute in the two-year-old racing season does not reduce the risk of MSI. This is supported by Wilson (1996) who reported that two-year-olds commencing racing earlier in the two-year-old season were at no greater risk of MSI than those that started later in the season (Wilson et al., 1996).

The duration of the preparation and the duration of the fast portion of the preparation were also considered as potential risk factors. However, neither were significantly associated with MSI. Future risk analysis of the first fast preparation should involve the use of proportional hazards models to account for differences in duration of follow-up.

In this analysis multiple logistic regression was used to examine the relationship between these variables and MSI. However, when the outcome of interest affects more than 5% of the population that the estimates of risk generated by the logistic model can be imprecise. MSI were found to affect 40% of the horses during their first fast preparations, therefore, adjusted estimates of risk were not calculated because they would not reflect the true risk. Instead the analysis focused on determining those variables that were associated with MSI. Future analysis should consider the use of Poisson and proportional hazards models as both can give a reliable estimate of risk when the outcome of interest is common (Callas et al., 1998). Another advantage of the proportional hazards model is that time dependent covariates could be included, allowing the transient effect of some risk factors to be evaluated (Cumming et al., 1990; Eisen, 1999). In addition, recent advances in proportional hazards models allow the inclusion of multiple end points and repeat events. The proportional hazard models would be of benefit in the more complex analysis risk factors for different types of MSI and multiple MSI within a single horse.

The first fast preparation was selected for analysis because based on a survey of trainers (Buckingham & Jeffcott, 1990) and anecdotal evidence it was thought to represent the first time that a horse would be at risk of MSI. However, it was found that 7% of horses had sustained a MSI in a previous preparation. Therefore it would appear that horses are at risk of MSI from the time training commences.

In conclusion MSI were found to be a common health problem during the first fast preparation. In the first fast preparation the variables gender, previous MSI, total distance worked at speeds greater than 890 m/minutes, average distance worked at speeds greater than or equal to 800 m/minute on fast days and percentage of days during the fast portion of the preparation that were fast were associated with MSI. Further analysis of the first fast preparation and other data collected during this study are required before these findings can be used to estimate risk and make recommendations for the training and management of young Thoroughbred racehorse.

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A CASE-CONTROL STUDY INVESTIGATING FACTORS ASSOCIATED WITH PELVIC AND TIBIAL STRESS FRACTURES IN THOROUGHBRED RACEHORSES IN TRAINING

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SUMMARY

Little scientific information is available on musculoskeletal injury occurring in Thoroughbred racehorses in training, although it is the major cause of wastage in the racing industry. We recently conducted a large-scale study in the UK to investigate the incidence of and risk factors for such injuries, fractures in particular. Thirteen racehorse trainers provided data on horses in their care, with daily recording of training information and provision of details on any musculoskeletal injuries incurred. Data collection covered two consecutive flat racing seasons, 1999 and 2000. This paper describes findings from a nested case-control study investigating factors associated with pelvic and tibial stress fractures. Cases were identified from the main study with randomly selected controls being matched on date of fracture in the case. Age and gender of the horse, its training history and training surfaces used were examined as risk factors. Training intensity was quantified by calculating cumulative distances cantered and worked at high speed in 30- and 60-day periods prior to date of fracture. In the 30-day period, the risk of pelvic or tibial stress fracture increased with increasing distance cantered, reaching a peak at around 250 furlongs, after which the risk reduced. This trend was not obvious in the 60-day period, with no significant differences in risk of injury for the different distance categories. Use of one particular type of all-weather surface was related to an increased risk of pelvic or tibial stress injury (OR=8.41, 95%CI=1.15-61.29, P=0.04). Trainer was associated with differences in fracture risk after adjusting for exercise distances and surface but age and gender were not.

INTRODUCTION

Musculoskeletal injury is the major cause of wastage and days lost from training in Thoroughbred racehorses worldwide (Jeffcott et al., 1982; Rossdale et al., 1985; Lindner & Dingerkus, 1993; Olivier et al., 1997; Bailey et al., 1999). In addition to their economic impact on the racing industry, these injuries raise animal welfare concerns and fuel campaigns to abolish horseracing. With official requirements to report and record injuries and fatalities occurring on racecourses, numerous studies have been conducted using these data-sources. Although racecourse accidents are a highly visible part of the sport about which there is considerable public disquiet, the majority of injuries actually occur during training (Bathe, 1994; Pickersgill et al., 2000; Verheyen et al., unpublished observations). However, no industry requirements exist in the UK to report injuries occurring in horses away from the racecourse and the lack of readily available data combined with the complexity of obtaining reliable training information means few investigators have addressed this area.

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In contrast to the UK, training of racehorses in the USA occurs on the racetracks and records are kept of officially timed workouts, which form part of the horses' training programme. These training data have been included in studies investigating risk factors for both fatal and non-fatal musculoskeletal injury sustained during racing and training (Estberg et al., 1995; 1996a; 1996b; Carrier et al., 1998; Cohen et al., 2000). Predisposing factors identified include age, gender and training history. Boston and Nunamaker (2000) studied the training programme of two-year-old Thoroughbred racehorses to identify components associated with fatigue injury of the third metacarpal bone ('bucked shins'). They found regular short-distance exercise at high speed to be related to a reduced incidence of bucked shins whereas long distances worked at lower speeds were associated with an increased risk of the disease.

We recently conducted a large-scale study in the UK to estimate the incidence of musculoskeletal injury and investigate associated risk factors in Thoroughbred racehorses in training (Verheyen et al., 2000). Results from this study suggested an overall fracture incidence of 1.2 per 100 horses per month (95% Confidence Interval = 1.0 to 1.4). Training intensity was related to risk of fracture with horses both cantering and galloping for longer distances found to be at increased risk. There were a wide variety of fracture types and bones involved, with stress fractures accounting for 58% of the total. Stress fractures are believed to occur as a result of continued repetitive loading during bone remodelling in response to physical exercise (Burr, 1997). Repeated overuse at the resorption stage of the remodelling process may result in microdamage that can no longer be repaired and ultimately lead to fracture.

Training of racehorses in the UK occurs on a wide variety of surfaces, including grass or 'turf', woodchip and synthetic, so-called 'all weather' gallops. Training surfaces vary between trainers and training centres and it is important that any differences in injury risk associated with specific surfaces be identified in order to advise on construction of training gallops. Very little scientific information is available on the risk of musculoskeletal disease in relation to training surfaces used. Moyer et al. (1991) found the incidence of bucked shins to be higher in horses training on a dirt track compared to those using a wood fibre surface. The study by Pickersgill et al. (2000) suggested a protective effect from training on an equitrack surface, with cases of fracture having spent less time exercising on this type of track than controls.

The aim of the current study was to identify factors associated with the occurrence of pelvic or tibial stress fractures in Thoroughbred racehorses in training in the UK. These types of injury were a common cause of lameness in our study population, together accounting for nearly one third of fractures diagnosed (unpublished data). They were grouped together to provide an adequate number of cases for investigation, although we acknowledge that risk factors may vary for the two injury types. However, both are stress injuries affecting the hindquarters, so we considered it reasonable to group them together. Predisposing factors investigated included age and gender of the horse, its training intensity and training surfaces used. To our knowledge, no other published work describes risk factors associated with pelvic or tibial stress fractures in racehorses in training.

MATERIALS AND METHODS

Study design and period

A nested case-control design was used with cases and randomly selected controls matched on date of fracture of the case. Cases of pelvic or tibial stress fracture were identified from our

large prospective observational study of injuries occurring in British racehorses in training (Verheyen et al. 2000). Data collection covered two flat racing seasons, starting in the autumn of 1998, when yearling horses were entering the yards to start their training, and ending in October 2000.

Study population

Thirteen racehorse trainers, based throughout the UK and willing to co-operate, were recruited for the project. The study population consisted of horses being trained to race 'on the flat', typically two- and three-year-olds, although a proportion of older horses were studied. The number of study horses per yard was variable and largely depended on the extra amount of work involved for yard staff in recording the required data. Six trainers were willing to have all of their horses on the project, mainly because some method of recording training information was already in place or the total number of horses in the yard was not excessive. The remaining seven trainers were asked to choose the horses they wished to participate on the basis that they should be representative of the yard structure and likely to remain in the yard for at least one racing season. Where only a limited number of horses were being studied, new horses were supplemented at the end of year one to keep numbers adequate.

Data collection

Horse information: Information on the horses in the study included their name, age, gender, identity of dam and sire and date of birth. When male horses were castrated during the course of the study the date of the operation was noted and gender amended accordingly.

Training data: Daily records were kept of each study horse's activity for each day it was enrolled in the project, even if it was not being exercised. When in full training, information included the main type of exercise performed on each day, although all cantering was recorded. For all cantering exercise and faster work, distance and training surface was recorded. Training intensity was quantified as cumulative distances exercised at different speeds during different time periods prior to fracture[1]. When horses spent periods away from the yard, this was not included in the 'time at risk', unless the horse's activity was known and recorded. Horses were also deemed not to be at risk when they were recovering from fracture, i.e. from the date of fracture up to the start of an ascending exercise program (usually unridden walking exercise after a period of box rest).

Fracture data: Fracture information was obtained from the trainers' veterinary surgeon using a standardised form and copies of accompanying veterinary reports were received. Case details included animal identification, date of fracture, training gallop where the fracture occurred (if known) and type of exercise the horse was performing at the time of injury. Diagnostic procedures were recorded and the fracture was described in detail, including site and treatment. If known, it was also noted what the eventual outcome was for the animal (e.g. return to training, retired or sold).

[1] Exercise distances in horseracing are commonly measured in furlongs, with one furlong equalling 220 yards or 1/8 of a mile, or approximately 200 metres.

Data processing

All data were recorded in a dedicated database[1]. Consistency checks were performed using location, gallops and distances. Validation of data was carried out wherever possible, based on additional yard records and using the online Racing Post horse database (http://www.racingpost.co.uk/horses).

Case definition

A case was defined as any study animal diagnosed with a pelvic or tibial stress fracture confirmed by ultrasound (Shepherd & Pilsworth, 1994), nuclear scintigraphy and/or radiography (Pilsworth & Webbon, 1988; Pilsworth et al., 1993). Horses with suspected fractures that were not confirmed through routine diagnostic procedures were not included as cases.

Control selection and matching

Controls were randomly selected from the study population of horses at risk on date of fracture in the case. Matching on date of fracture was performed because of the seasonality of flat racing and the variable risk periods for horses in the sample. Cases could serve as controls before they became a case or when they were back at risk after recovery from fracture. Because stringent exclusion criteria were applied (see below), a case:control ratio of 1:5 was chosen.

Exclusion criteria

For the 30-day period prior to fracture, cases and controls were excluded if i) they had been on the study for less than 27 days, ii) they had more than 3 days of activity 'not recorded' or iii) they spent more than 3 days not at risk either because they were recovering from fracture or were absent from the yard and their activity was uncertain. The same exclusion criteria were applied for the 60-day period, although the numbers of days were 52, 8 and 8 respectively. Exclusions were applied to avoid biased results with regard to training intensity. Although the chosen cut-off points were arbitrary, they were based on the amount of missing data for the cases in this study, so as to maximise the number of cases included.

Assignment of training surface variable

In order to assess training surface as a risk factor, total cumulative distances exercised in 30- and 60-day periods prior to injury were divided according to distances covered on specific surfaces. Where a particular surface was used for at least 70% of the total cumulative distance in the specified time period, this surface was assigned to that particular horse. Where no one surface was used for ≥70% of exercise distance, surface was recorded as a mixture. The same procedure was repeated for distances covered at a high speed only (galloping or racing). For the 30-day period, horses were not assigned a surface if they had covered <50 furlongs in total and <20 furlongs at high speed. For the 60-day period, these figures were 100 and 40 furlongs respectively. Exercise distances below these cut-of points were deemed not sufficient to warrant assignment of a training surface variable.

[1] Microsoft SQL Server 7.0 with Access 97 front end

Statistical methods

Descriptive statistics included numbers of horses, number and site of fractures, age and gender distributions and description of continuous variables. The randomly selected controls were compared to the total population of non-cases in terms of age, gender and trainer distribution to check they were representative of the population from which the cases were drawn.

Univariable analyses: Conditional logistic regression was used to assess the main exposure variables and potential confounders in relation to risk of pelvic or tibial stress fracture. Each matched case-set included five controls, randomly selected from horses at risk on date of fracture in the case. All variables and their categorisation are summarised in Table 4.

Multivariable analyses: Distance variables were tested 'a priori' in multivariable conditional logistic regression models, adjusting for trainer and surface. Age and gender were also tested for inclusion in the various models. Following results from univariable analyses, inclusion of quadratic terms in a polynomial model was assessed, as well as biologically plausible interactions. The association between distance variables and the probability of fracture was examined graphically to assess functional relationships. Variables were retained in the model if the Wald-test p-value was ≤0.05 and/or the variable significantly improved model fit (likelihood ratio statistic ≤0.05). All statistical calculations were performed using the software package STATA. The level of statistical significance was set at P=0.05.

RESULTS

Descriptive results

Twenty cases of pelvic stress fracture and 21 diagnoses of tibial stress fracture were identified from the main dataset, giving a total of 41 cases. The majority (88%) of pelvic and tibial fractures occurred during training, with only 5 cases (12%) occurring on the racecourse. The total number of horses studied per yard and cases of pelvic and tibial stress fractures are summarised in Table 1. Site, age and gender distributions of cases are shown in Table 2.

After applying exclusion criteria, the 30-day sample included 39 cases and 177 controls and the 60-day sample 37 cases and 160 controls. The randomly selected group of all controls was representative of the study population in terms of age, gender and trainer distribution (data not shown). Six cases were also selected as controls at a different point in time, three before the occurrence of their injury and three when they were considered to be back at risk after recovery from fracture.

Description of continuous variables is shown in Table 3. On average, horses exercised around 5.5 furlongs per day or around 40 furlongs per week in both the 30- and 60-day periods. The majority of exercise consisted of cantering, although slightly faster work (such as 'half speed' or 'strong canter') is included in this figure. The average amount of high-speed exercise was around 4 furlongs per week. It should be noted that flat racing is a highly seasonal sport, with the racing season starting around April and closing early October. Horses would generally only work at high speed during these months, hence the low average high-speed distance. Note also that the distribution of high-speed exercise was heavily skewed to the right, with 74 of 216 horses (34%) in the 30-day sample and 55 of 197 animals (28%) in the 60-day sample having done no fast exercise at all.

Univariable analyses

Results from univariable conditional logistic regression analyses are shown in Table 4.

Table 1. Numbers of horses studied, pelvic and tibial stress fractures by trainer

Trainer	Location	Total number of horses	Number of pelvic stress fractures	Number of tibial stress fractures	Total
1	Newmarket	30	0	0	0
2	Newmarket	200	6	4	10
3	Newmarket	145	1	1	2
4	Newmarket	38	0	4	4
5	Newmarket	26	0	1	1
6	Epsom	70	1	0	1
7	Epsom	63	2	0	2
8	Berkshire	166	0	2	2
9	Wiltshire	42	2	1	3
10	Lambourn	28	0	0	0
11	Lambourn	37	1	3	4
12	Middleham	272	5	2	7
13	Coverham	61	2	3	5
Total		1,178	20	21	41

Table 2. Description of pelvic and tibial stress fractures by site, age and gender

		Pelvis	Tibia	Total
Site	Left	12	11	23
	Right	7	8	15
	Bilateral	1	2	3
Age	2-year-old	7	8	15
	3-year-old	11	11	22
	Older	2	2	4
Gender	Female	10	8	18
	Entire male	8	10	18
	Gelding	2	3	5

Multivariable analyses

30-day Model: When adjusting exercise distance for trainer, the total distance exercised in 30 days was significantly associated with risk of fracture. When analysing canter and fast distances separately whilst adjusting for trainer, it appeared that this effect was largely due to the cumulative distance cantered. Statistically, there was no departure from linear trend, suggesting a common odds ratio of 2.21 (95% CI=1.25-3.90, P=0.006) from one canter distance category to the next. However, when examining the odds ratios visually, the linear trend seemed to level out in the highest distance category (ORs 3.63, 8.17 and 9.15 respectively). Therefore, modelling canter distance as a quadratic term in the model with canter distance as a continuous variable

provided a better description of the data (Table 5). The risk of fracture increased with increasing distance cantered, peaking at just over 250 furlongs, after which the risk seemed to reduce again, although confidence intervals for the higher canter distances were very wide. High-speed exercise distance was not significant in the model, nor was an 'a priori' test for interaction between high-speed and canter distances.

The effect of trainer was highly confounded by exercise distance but it was interesting that strong associations still existed between trainer and risk of fracture after adjustment. It was thought possible for trainer to be a proxy measure for training surface, but adjusting exercise distance for surface instead of trainer did not result in significant associations. Age and/or gender were not significant when added to the model shown in Table 5 and did not improve model fit. The final 30-day model is summarised in Table 5.

60-day Model: The patterns for the distance variables were similar to those in univariable analysis when adjusted for trainer, although associations were not statistically significant. When also adjusting for training surface, horses exercising between 300 and 400 furlongs and over 400 furlongs in total seemed to be at similar risk and were therefore grouped together. In this model, one type of all-weather surface was associated with an eightfold increase in fracture risk compared to a mixture of surfaces (OR=8.41, 95%CI=1.15-61.29, P=0.04). Trainer effects were confounded by surface, but most confounding occurred in the negative direction, with odds ratios being higher when adjusting for surface. Incorporating only surface in the model without trainer did not result in significant associations, suggesting trainer was an important confounder in the association between surface and fracture risk.

Table 3. Description of age and distance variables

Variable	Mean	Median	Range
Age (years)	2.9	3	2 – 8
Total distance exercised in 30 days*	168.9	167.8	0 – 416
Total distance cantered in 30 days*	151.5	138	0 – 401
Total distance high-speed exercise in 30 days*	17.5	13	0 – 77.5
Total distance exercised in 60 days*	324.7	326	0 – 724
Total distance cantered in 60 days*	293.5	271.5	0 – 692
Total distance high-speed exercise in 60 days*	31.3	24	0 – 126.5

*in furlongs (1 furlong = 220 yards ~ 200 metres)

As in the 30-day model, fast work did not seem to be significantly associated with fracture risk, on its own or in combination with cantering work over the 60-day period prior to fracture. Age and gender did not improve the model fit, with neither exposure variable being related to risk of pelvic or tibial stress fracture. The final 60-day model is summarised in Table 6.

Table 4. Summary of univariable conditional logistic regression analyses: association between main exposure variables and potential confounders and risk of pelvic or tibial stress fracture

Variable		Cases (n1 (%))	Controls (n2 (%))	Crude Odds Ratio	95% Confidence Interval	P-value
Age group	2-year olds	15 (38)	83 (45)	1		
	3-year-olds	22 (55)	71 (38)	1.72	0.83 – 3.54	0.15
	Older	3 (8)	32 (17)	0.51	0.14 – 1.84	0.30
Gender 1	Female	18 (45)	61 (33)	1		
	Entire male	18 (45)	99 (53)	0.63	0.31 – 1.30	0.22
	Gelding	4 (10)	26 (14)	0.53	0.16 – 1.72	0.29
Gender 2	Female	18 (45)	61 (33)	1		
	Male	22 (55)	125 (67)	0.61	0.31 – 1.21	0.16
Total distance* 30 days	0 - <100	5 (13)	43 (24)	1		
	100 - <150	6 (15)	34 (19)	1.56	0.45 – 5.46	0.48
	150 - <200	13 (33)	40 (23)	2.78	0.92 – 8.39	0.07
	200+	15 (38)	60 (34)	2.02	0.69 – 5.91	0.20
Canter distance* 30 days	0 - <100	7 (18)	54 (31)	1		
	100 - <150	11 (28)	49 (28)	1.75	0.63 – 4.83	0.28
	150 - <200	9 (23)	29 (16)	2.31	0.76 – 7.02	0.14
	200+	12 (31)	45 (25)	1.95	0.72 – 5.32	0.19
High speed Distance* 30 days	0	11 (28)	63 (36)	1		
	1 - <20	11 (28)	51 (29)	1.29	0.51 – 3.26	0.59
	20+	17 (44)	63 (36)	1.63	0.68 – 3.92	0.27
Total distance* 60 days	0 - <200	9 (24)	35 (22)	1		
	200 - <300	6 (16)	37 (23)	0.67	0.22 – 2.04	0.48
	300 - <400	12 (32)	40 (25)	1.28	0.49 – 3.34	0.61
	400+	10 (27)	48 (30)	0.82	0.31 – 2.19	0.69
Canter distance* 60 days	0 - <200	10 (27)	42 (26)	1		
	200 - <300	9 (24)	47 (29)	0.87	0.33 – 2.29	0.77
	300 - <400	11 (30)	36 (23)	1.38	0.53 – 3.58	0.51
	400+	7 (19)	35 (22)	0.81	0.28 – 2.34	0.70
High speed Distance* 60 days	0	9 (24)	46 (29)	1		
	1 - <25	10 (27)	35 (22)	1.52	0.56 – 4.16	0.41
	25 - <50	13 (35)	36 (23)	2.01	0.75 – 5.42	0.17
	50+	5 (14)	43 (27)	0.65	0.19 – 2.18	0.49
Surface 30d (total distance)	Mixture	18 (49)	69 (49)	1		
	'AW surface A'	9 (24)	28 (20)	1.09	0.41 – 2.91	0.86
	'AW surface B'	10 (27)	44 (31)	0.78	0.33 – 1.83	0.57
Surface 60d (total distance)	Mixture	13 (38)	62 (44)	1		
	'AW surface A'	12 (35)	38 (27)	1.39	0.54 – 3.58	0.49
	'AW surface B'	9 (26)	42 (30)	1.02	0.40 – 2.59	0.98
Surface 30d (high speed)	Grass/peat moss	13 (76)	52 (83)	1		
	Other	4 (24)	11 (17)	1.22	0.21 – 6.94	0.82
Surface 60d (high speed)	Grass/peat moss	9 (82)	44 (80)	1		
	Other	2 (18)	11 (20)	1.51	0.12 – 19.77	0.75
Trainer[$]	12	7 (18)	52 (30)	1		
	2	10 (25)	45 (26)	1.67	0.56 – 4.95	0.36
	3	2 (5)	25 (14)	0.59	0.11 – 3.15	0.54
	4	4 (10)	9 (5)	3.38	0.75 – 15.25	0.11
	5	1 (3)	5 (3)	1.05	0.09 – 11.74	0.97
	6	1 (3)	8 (5)	0.94	0.10 – 8.67	0.96
	7	2 (5)	10 (6)	1.19	0.22 – 6.33	0.84
	8	2 (5)	5 (3)	2.37	0.38 – 14.85	0.36
	9	3 (8)	8 (5)	3.04	0.60 – 15.41	0.18
	11	4 (10)	3 (2)	6.73	1.31 – 34.53	0.02
	13	4 (10)	6 (3)	5.06	1.07 – 23.99	0.04

*in furlongs, AW = All-weather, [$]Trainers 1 and 10 excluded from analyses as they contributed no cases

Table 5. Summary of results from conditional logistic regression model: association between cumulative canter distance in 30 days and risk of pelvic or tibial stress fracture, adjusted for trainer

Variable		Adjusted Odds Ratio	95% Confidence Interval	Wald- test P-value	LRS
Canter distance 30 days*		1.03	1.01 – 1.06	0.006	0.001
[Canter distance]^2 30 days		0.9999	0.9998 – 1.0000	0.04	0.02
Trainer$	12	1			0.02
	2	3.68	0.84 – 16.21	0.09	
	3	0.80	0.13 – 4.97	0.81	
	4	5.95	1.03 – 34.54	0.05	
	5	0.98	0.08 – 11.68	0.98	
	6	1.86	0.10 – 33.36	0.67	
	7	7.66	0.90 – 65.16	0.06	
	8	12.20	1.21 – 123.02	0.03	
	9	2.15	0.38 – 12.25	0.39	
	11	19.24	1.82 – 203.30	0.01	
	13	30.84	3.38 – 280.71	0.002	

*in furlongs, $Trainers 1 and 10 excluded from analyses as they contributed no cases, LRS=Likelihood Ratio Statistic

DISCUSSION

To our knowledge, the study reported here is the first to specifically investigate training-related factors associated with pelvic and tibial stress fractures in Thoroughbreds. Studies conducted in the USA have usually described training features in relation to catastrophic injury (Estberg et al., 1995; 1996b; Carrier et al., 1998). Also, the training history assessed in these studies is generally based on high-speed exercise history, ignoring any work performed at lower speed. Comparing studies in this area is difficult, mainly because of different case and control definitions and choice of exposure variables. For example, Estberg et al. (1996b) found that horses that accumulated higher distances of high-speed exercise in a two-month period were at increased risk of fatal racing fracture. In contrast, Cohen et al. (2000) found horses that suffered musculoskeletal injury during a race to have accumulated less high-speed work compared to non-injured controls. However, the case definition in each study was different (fatal fracture vs. musculoskeletal injury) and controls in the study by Estberg et al. consisted of non-fatally injured horses, whereas the Cohen controls were non-injured race participants.

Based on these studies, we examined 30-day and 60-day training periods prior to fracture. Choosing well-defined time periods allowed quantification of training intensity by calculating cumulative distances exercised at different speeds. The risk of pelvic or tibial stress fracture seemed to largely depend on the amount of exercise performed at lower speeds. Not surprisingly, the risk increased with increasing exercise but then reduced again for horses cantering more than 250 furlongs in a 30-day period. However, confidence intervals around these estimates were very wide due to limited numbers of animals in the higher distance categories. In the 60-day period, horses exercising between 200 and 300 furlongs were at lower risk of fracture compared to those working less than 200 furlongs, although again confidence

intervals were wide and differences not significant. The observed reduction in risk for the highest distance categories may be the reflection of a 'healthy horse' effect, with animals less prone to injury performing more exercise. It could be hypothesised that horses doing more cantering exercise would also be doing more high-speed work, with regular bouts of fast work thought to be associated with decreased risk of fatigue injury of the third metacarpal bone (Boston & Nunamaker, 2000) and consequently possibly stress injury at other sites. However, when examining our data, there was no tendency for horses in the highest canter categories to also be the ones performing most fast work.

Table 6. Summary of results from conditional logistic regression model: association between cumulative total exercise distance in 60 days and training surface and risk of pelvic or tibial stress fracture, adjusted for trainer

Variable		Adjusted Odds Ratio	95% Confidence Interval	Wald-test P-value	LRS
Total distance	0 - <200	1.00			0.04
60 days*	200 - <300	0.32	0.07 – 1.40	0.13	
	≥300	1.92	0.54 – 6.77	0.31	
Surface	Mixture	1.00			0.03
	'AW surface A'	8.41	1.15 – 61.29	0.04	
	'AW surface B'	5.16	0.34 – 77.36	0.24	
Trainer[$]	12	1.00			0.04
	2	2.78	0.10 – 76.61	0.55	
	3	0.22	0.00 – 10.42	0.44	
	4	29.57	1.04 – 840.73	0.05	
	5	5.59	0.12 – 255.81	0.38	
	6	14.02	0.33 – 588.94	0.17	
	7	7.61	0.24 – 237.12	0.25	
	8	17.27	0.91 – 326.17	0.06	
	9	21.75	0.66 – 715.97	0.08	
	11	7.20	0.13 – 385.61	0.33	
	13	27.23	1.34 – 551.34	0.03	

*in furlongs, [$]Trainers 1 and 10 excluded from analyses as they contributed no cases, AW=All-weather LRS=Likelihood Ratio Statistic

The method of assigning a surface variable to a horse was purely arbitrary and open to criticism. We felt that a horse using a particular surface for at least 70% of its exercise time could fairly be associated with that surface. The cut-off of 70% gave sufficient variety for surface to be assessed as an exposure variable. Another way of assessing training surfaces would be to investigate cumulative distances exercised on different surface types. However, with the variety of surfaces available, this would result in numerous continuous variables which, in our relatively small dataset, may have lead to difficulties in statistical analyses. This approach will be taken however when including all fractures in our surface analyses.

Our finding that 'All-weather surface A' was associated with increased risk of pelvic or tibial stress fracture should be interpreted with caution. In our approach, all training gallops with this type of surface were grouped together, whereas they may differ widely with regard to for example construction, incline and maintenance. However, when in turn eliminating trainers from the model who regularly exercised their horses on this type of surface, the effect of 'All-weather surface A' remained, suggesting a genuine effect worthy of further investigation.

It was interesting to find significant trainer effects after exercise distances and/or training surfaces had been taken into account. This would indicate unknown factors operating at the trainer level which seem to have an influence on the occurrence of pelvic and tibial stress fractures. Issues including nutrition, riders and management of lame horses for example could be important in this respect. The degree of veterinary involvement in the yard could affect trainer estimates in one of two ways. Stress fractures may be more readily diagnosed in yards where veterinary attention is sought quickly. Alternatively, when the vet is not consulted as often, subtle lamenesses may go unnoticed and ultimately result in fracture. Varying degrees of diagnostic lameness investigations may also have led to cases not being included in this study when a diagnosis of pelvic or tibial stress fracture was suspected but not confirmed. This may have happened for example when the racing season was drawing to a close and trainers were willing to box rest the horse without further investigations. However, because cases in this study could serve as controls before or after their injury, undetected cases would only have introduced misclassification bias if they had been selected as controls in the period that they were recovering from the unconfirmed fracture.

Other biases may have arisen in this study from issues in the study design and analyses. Trainers recruited to participate in the study were a convenience rather than random sample of UK racehorse trainers. This choice can be justified however by the need for long-term compliance, which would have resulted in substantial non-response and inaccuracy in data recording if a random sample had been taken. Trainers were chosen to represent a variety of yard sizes, quality of horses and training patterns. In yards where a limited number of horses were studied, selection of study horses by the trainers could have introduced bias. Trainers may have been more likely to provide sound horses for the study or, alternatively, horses with musculoskeletal problems. We acknowledge that it would have been better to take a random sample of horses in these yards.

Data were being recorded by yard staff and although every effort was made to check their validity and accuracy, it can not be guaranteed that they were free from error. A rapport of trust and confidentiality was encouraged through regular yard visits and interim feedback reports during the course of the study. Due to the large volume of records being collected, double data entry into our database was not feasible.

Allowing horses with a small amount of missing data to remain in the sample could also have influenced the results. However, excluding all horses with an incomplete dataset would have resulted in fewer cases for analyses. Furthermore, due to the nature of the data and the sample, allowing only horses with a full dataset in the analyses may in itself lead to bias, as such animals may not have been representative of the study population.

The relatively small sample size in this study led to wide confidence intervals around the odds ratio estimates. Combined with overall high exposure levels in the controls, the study had low power to detect small but possibly significant differences of practical relevance. For example, with 55% of controls exercising over 300 furlongs in the 60-day period, the study only

had around 40% power to detect a twofold increase in fracture risk for this distance category, which may be of clinical relevance. Repeating the study with a larger number of cases may also allow separate analyses for pelvic and tibial fractures. A retrospective case-control approach could be feasible, although this would be at the expense of accuracy and detail in training information.

Identifying modifiable risk factors for musculoskeletal injury will help in devising scientifically based training regimes for young racehorses in order to reduce their incidence, benefiting both the horse and the racing industry. It would be prudent however not to make firm recommendations based only on the findings in this study. Not only is it based on a limited sample size, it may well be that training regimes devised to reduce pelvic and tibial stress fractures are not necessarily beneficial in reducing other types of musculoskeletal injury. In this respect, further investigations into other injury types are necessary to build up a full picture and advise on training regimes accordingly.

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ANALYSIS OF HORSE RACE VIDEOS TO IDENTIFY RISK FACTORS FOR FATAL DISTAL LIMB FRACTURE

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SUMMARY

The objective of this study was to identify risk factors, during racing, associated with imminent fatal distal limb fracture in Thoroughbreds. One hundred and nine cases of fatal distal limb fracture were identified over a two-year period. Three uninjured control horses were randomly selected from the same race as the case horse. Videos of races in which fractures occurred were viewed using a defined protocol. Fractures in flat races occurred at any time during the race, whereas almost 74% (45/61) of cases in national hunt type races occurred in the second half of races. More than 75% (79/103) of cases were spontaneous, i.e., there was no obvious external influence such as a fall at a fence or collision with another horse. In races run on turf, 77% (69/90) of fractures occurred in straights, whereas in all weather races only 38% (5/13) of fractures occurred in straights. Sixty six percent (44/67) of horses sustaining a forelimb fracture, fractured the forelimb they were using as lead leg at the time of fracture. When case and control horses were compared, horses that were, a) making good progress through the race, b) reluctant to start and c) received encouragement in the final 10 seconds before the time of fracture, were more likely to sustain a fracture.

INTRODUCTION

The majority of previous studies of racing injuries have focused on identification of risk factors associated with events prior to the race in which the injury occurs. Characteristics of the horse, previous racing histories and training regimes have all been shown to be associated with an increased risk of injury (Estberg et al., 1996; 1998; Bailey et al., 1997; Carrier et al., 1998; Cohen et al., 2000; Hernandez et al., 2001). Two previous reports have included details of video analysis of events occurring during racing, but both have their limitations. Ueda et al. (1993) analysed patrol videos of 58 horses that suffered "serious accidents" on racecourses. This study did not include any comparison with uninjured control horses, but did conclude that horses were more likely to injure the lead forelimb than any other limb. They also suggested that injuries were more likely to occur during a change of lead leg and that more injuries occurred in turns, than on straight parts of the course. Cohen et al. (1997) analysed videos of 216 cases of musculoskeletal injury and 532 race-matched controls. They studied the 12-second period prior to fracture and demonstrated that the risk of injury was increased soon after a collision with another horse or soon after a change of lead leg. They also showed that jockeys were less likely to use the whip on case horses and that case horses were more likely to be

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toward the back of the field after the first two furlongs of a race. As far as the authors of the current study are aware there is no previous report that compares injured and uninjured horses, from the start of races to time of fracture. This paper therefore represents the most comprehensive attempt to date at identification of events occurring during racing which are associated with fracture.

MATERIALS AND METHODS

Case and control definitions

One hundred and nine races, which contained a fatal distal limb fracture, were identified from all 59 UK racecourses over a two-year period. A case was defined as a horse which sustained a fracture of the carpus/tarsus and/or distal limb, during racing, which required euthanasia. Cases were confirmed by post-mortem examination. Three controls per case were selected at random from all uninjured horses, in the same race as the case horse, which reached the point in the race where the fracture occurred.

Protocol

All races in the UK are filmed from three or four different views by *Racetech (Raynes Park, London)*. Copies of videos of each race in which a fatal distal limb fracture occurred were purchased. Each view was recorded on to a separate cassette so that all views could be watched simultaneously. Races were watched on four monitors and synchronised using frame counters on the films. A single remote control was used to advance all four videocassette recorders together.

The race viewer (TP) was more familiar with the names of the case horses than the control horses, so in order to reduce the risk of observer bias, case and control horses were identified by the colours worn by the jockeys, without reference to the horse's names. Race cards, which included all the jockey's colours, were obtained from *Racing Post (MGN Ltd., London)*. All views of the case race were synchronised at the start of the race. Films were viewed frame by frame until the point in the race where the fracture occurred. Races were then re-viewed from the start of the race in real time.

Variables measured

Frame by frame: The galloping horse has a gait that consists of a stance phase and a suspension phase. The stance phase starts as the trailing hindlimb (left hind when on right fore lead) contacts the ground and ends when the leading forelimb leaves the ground. The suspension phase is the period when there is no limb contact with the ground. The gallop is normally transverse in that, when on a right fore lead the order of limb contact with the ground is left hind, right hind, left fore and right fore (Dalin & Jeffcott, 1994). For each case and control horse, the lead forelimb used at the start of the race, was identified. The time of all changes of lead leg and the lead leg at the time of fracture were also recorded. From these data the number of changes of lead leg and total time spent on each lead leg were calculated.

The position of the study horses with respect to the rest of the field was recorded every 20-seconds. Horses were placed in one square of a 3×3 grid giving relative position both front to

back and inside to outside of the study horses. From this and rank position data, further variables giving a measure of progress through the race were produced. The progress of each horse over the whole race and in the final 60 seconds before the time of fracture, was defined in one of three different ways:

- Good – consistent improvement from back to front of the field.
- Bad – consistent decline from front to back of field.
- Level – consistent position throughout the race.

The mode position throughout the race, front to back and inside to outside, was also identified for each horse.

Real time: The time to the point of fracture and the time taken by the winning horse to complete the race were recorded. The Jockey's use of the whip and actions to encourage or hold back his or her horse were noted. Other than use of the whip, actions determined to be encouragement were defined as "niggling" (small hand movements) or "pushing" (much larger hand movements up the neck of the horse). The behaviour of the horse immediately prior to and during the race was recorded. Events surrounding the moment of fracture were described and the exact location of the fracture on the course was marked on racecourse plans.

Statistical Analysis

Case comparisons: The Wilcoxon signed rank test was used to identify significant trends in the percentage of race completed before fracture. The Chi-squared test was used to detect associations between the side of fracture and variables associated with the lead leg and direction of running. Logistic regression enabled the univariate relationships between independent variables and side of fracture to be estimated. Each variable was included in a model individually with side of forelimb fracture as the dependent variable. A multivariable logistic regression model was fitted, using forward stepwise procedures. All variables, regardless of the outcome from the univariate analysis, were available for inclusion in the model.

Case-control comparisons: The variable relating to rank progress through the whole race was analysed as both a categorical and continuous variable. The categorical variables rank progress through the race, progress compared to the whole field for the whole race and for the final minute and variables relating to the mode position of the horses, were analysed as a series of binary variables. This enabled each category to be compared to all other categories together rather than each category being compared to a defined reference category. A composite variable relating to jockey behaviour was created in order to identify jockeys that were encouraging their horses by niggling, pushing or by use of the whip.

The Mantel-Haenszel matched procedure, for obtaining point estimates of the odds ratios and Mantel-Haenszel chi square tests, were used to screen for univariate relationships between all independent variables and the likelihood of fracture (Schlesselman, 1982). A multivariable conditional logistic regression model was then fitted, using forward stepwise procedures. Variables that had *P*-values less than or equal to 0.2 were considered for inclusion in the final model. Inclusion of both of the variables identifying good progress through the whole race (rank position and position with respect to the whole field) prevented convergence of the model as they were highly correlated. Good *rank* progress was therefore used in the final model, as this variable appeared more strongly associated with the risk of fracture in the univariate analysis. Substitution of the good rank progress variable with the good field progress variable did not significantly alter the fit of the final model. Variables were retained in the model if they

significantly reduced the level of deviance of the model (likelihood ratio statistic, *P*-value < 0.05). The statistical packages S-Plus 2000 (MathSoft, Inc.), Epi-info 2000 (CDC, USA) and EGRET (Cytel Software Corporation, USA) were used.

RESULTS

Case Descriptions

Percentage of race completed: It was possible to identify the exact time of fracture in 94% (103/109) of races. The percentage of the race completed before fracture (time to fracture/time of winner) is shown in Fig. 1. In flat races 57% (24/42) of fractures occurred in the second half of races. In contrast 74% (45/61) of fractures in national hunt type races (hurdle, steeplechase and national hunt flat) occurred in the second half of races. The median percentage of race completed before fracture was significantly greater than 50% in national hunt type races (Wilcoxon signed rank test; $P = 0.002$), but not flat races (Wilcoxon signed rank test; $P = 0.26$).

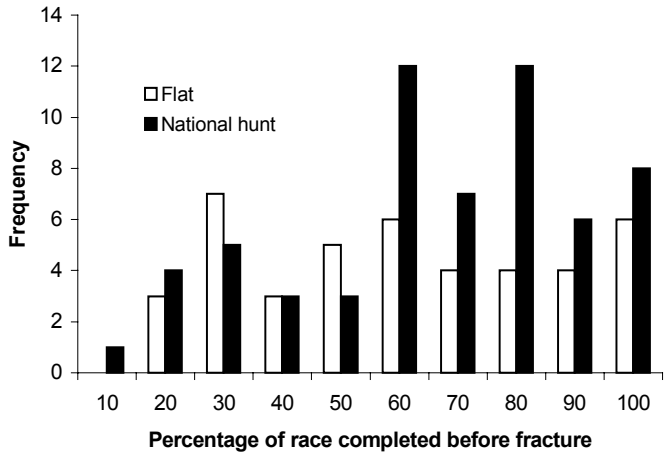

Fig 1 The stage of the race at which the fracture occurred

Position on the racecourse: In turf races (Flat, Hurdle, Steeplechase and NHF) between 57% and 82% of fractures occurred in straights, whereas in all weather flat racing 62% (8/13) of fractures occurred in corners.

Fracture event description: Eighty-nine percent of cases (92/103) resulted in the horse being pulled up. Overall 77% (79/103) of cases did not involve any obvious extrinsic risk factor such as an obstacle. Forty four percent (24/54) of cases in jump races occurred at an obstacle. Of the cases which involved a hurdle, 22% (2/9) were associated with a fall and of those which occurred at a steeplechase fence, 53% (8/15) were associated with a fall. One fracture occurred as the case horse collided with an uninjured horse, which had fallen at a fence in a steeplechase race.

Leading leg: It was possible to gather data relating to lead leg for 79% (68/86) of forelimb cases. Table 1 shows the association between right and left forelimb fractures and the lead leg used at the start of races, the most used lead leg and the lead leg at the time of fracture (end lead leg). It was not possible to gather complete sets of data for all 68 cases. Sixty-six percent

(44/67) of fractures affected the leg that the horse was leading on at the time of fracture (Chi square = 7.52: P < 0.01). Forty percent (27/67) of fractures affected the start lead leg and 50% (33/66) of fractures affected the most used lead leg.

Table 1. The number of forelimb cases that started, used most and ended on right and left forelimb leads

Fractured limb	Start lead leg		Most used lead leg		End lead leg	
	Right	Left	Right	Left	Right	Left
Right fore	17	22	18	19	22	16
Left fore	18	10	14	15	7	22

Direction of running: Ninety-two percent (99/108) of the unilateral cases occurred in races which were either left or right handed. Of the forelimb fractures, which occurred in left or right handed races, 57% (44/77) affected the inner limb. In hindlimb cases, 59% (13/22) affected the inner limb. There was no significant association between side of fracture and direction of the race for all cases ($P = 0.10$), forelimb cases ($P = 0.17$) or hindlimb cases ($P = 0.85$). Seventy-five percent (36/48) of horses on left-handed tracks used their left fore as lead limb most often and 69% (18/26) of horses on right-handed tracks used their right fore as lead limb most often. There was a strong association between most used lead forelimb and direction of running (Chi-squared = 13.5: $P < 0.001$).

Relationship between independent variables and side of forelimb fracture

Univariate analysis: Table 2 shows that there was no significant association between side of fracture and most used lead leg or direction of running. There was however a significant association between side of fracture and lead forelimb at the time of fracture (Odds ratio = 4.22, $P = 0.006$). There was also a weak association between start lead leg and side of fracture (Odds ratio = 0.43, $P = 0.1$).

Table 2. Univariate relationship between independent variables and side of forelimb fracture

Variable	Odds ratio	95% C.I.	P value
Lead leg			
• Start	0.43	0.15 – 1.18	0.10
• Most used	1.01	0.38 – 2.73	0.96
• At time of fracture	4.22	1.47 – 13.03	0.006
Direction of running	1.9	0.75 – 4.9	0.17

Multivariable analysis: A simple two variable model was produced (Table 3.). Both start and final lead leg were associated with the risk of forelimb fracture. Case horses were more likely to start leading with the forelimb that remained unaffected (O.R. 0.37, $P = 0.039$). At the time of fracture, case horses were 6 times more likely to fracture their lead forelimb than their non-lead forelimb ($P = 0.002$).

Table 3. Multivariable logistic regression model of side of forelimb fracture

Variable	Odds ratio	95% C.I.	P value
Lead leg			
• Start	0.37	0.14 – 0.95	0.039
• At time of fracture	6.04	1.95 – 18.7	0.002

Case-control comparisons - Relationship between independent variables and distal limb fracture

Univariate analysis: The results of the univariate screening analysis are listed in Table 4. Variables associated with progress through the whole race and in the final minute before case horse fracture, mode position, jockey and horse behaviour and changes in lead leg in the final three seconds before case horse fracture, were available for inclusion in the multivariable model.

Multivariable analysis: A simple three variable model was produced using a forward stepwise procedure (Table 5). Case horses were 2.5 times more likely to have been making good progress through the race ($P = 0.015$) and 2.29 times more likely to have been reluctant to start ($P = 0.048$) than control horses. Case horses were also 2.3 times more likely to have been encouraged by the jockey at some time during the 10 seconds prior to the time of fracture ($P = 0.016$).

Table 5. Multivariable conditional logistic regression model of variables during racing and fatal distal limb fracture

Variable	Odds ratio	95% C.I.	P value
Good rank progress through the race	2.50	1.20 – 5.22	0.015
Reluctant to start	2.29	1.01 – 5.21	0.048
Encouraged in final 10 seconds	2.30	1.17 – 4.55	0.016

DISCUSSION

This paper has identified previously unreported risk factors for fatal distal limb fracture in racing Thoroughbreds. This was done using a case-control study design applied to the analysis whole races from start to the point of fracture. Analysis of videotapes of racing accidents carried out in Japan suggested that horses were more likely to sustain an injury when they changed lead legs, moved obliquely or when jockeys used whips (Ueda et al., 1993). Injuries were also reported to affect the lead forelimb most often and to occur more frequently in corners. This analysis, however, did not compare the frequency of events in non-injured control horses from the same races. Cohen et al. (1997) carried out case-control video analysis of races in which horses sustained injuries and also demonstrated that a change of lead leg was associated with catastrophic injury. In contrast to the report by Ueda et al. (1993), Cohen et al. (1997) suggested that the use of a whip was associated with a lower risk of injury. This work mainly concentrated on the 12-second period prior to the time of fracture.

Table 4. Univariate relationships between independent variables during racing and fatal distal limb fracture

Variable	Odds ratio	95% C.I.	P value
Rank progress through race (continuous) [a]	0.62	0.38 – 1.00	0.052
Rank progress through race (categorical)			
• Good	2.33	1.24 – 4.38	0.03
• Bad	0.63	0.25 – 1.56	0.21
• Level	0.83	0.36 – 1.92	0.49
Progress compared to all horses:			
a) Through whole race			
• Good	1.96	1.12 – 3.44	0.06
• Bad	0.89	0.47 – 1.70	0.73
• Level	0.87	0.48 – 1.59	0.63
b) In final minute before case horse fracture			
• Good	1.09	0.53 – 2.27	0.84
• Bad	0.51	0.20 – 1.29	0.08
• Level	1.59	0.87 – 2.93	0.21
Mode position:			
a) Front to back of field			
• Front third	0.69	0.39 – 1.21	0.12
• Mid third	1.05	0.64 – 1.72	0.95
• Back third	1.40	0.88 – 2.25	0.21
b) Inside to outside of field			
• Inner third	1.35	0.83 – 2.18	0.33
• Mid third	0.77	0.44 – 1.36	0.28
• Outer third	0.74	0.38 – 1.46	0.30
Jockey behaviour:			
a) During the whole race			
• Niggle	1.79	1.02 – 3.31	0.06
• Push	1.68	0.81 – 3.48	0.26
• Whip	1.23	0.64 – 2.34	0.65
• Encourage	2.26	1.29 – 3.97	0.01
b) In final 10 sec before case horse fracture			
• Niggle	1.93	0.92 – 4.06	0.17
• Push	1.07	0.50 – 2.31	1.00
• Whip	1.50	0.76 – 2.95	0.33
• Encourage	2.11	1.16 – 4.33	0.02
Horse behaviour			
• Reluctant to start	2.47	1.21 – 5.03	0.03
• Pulling during the race	1.29	0.68 – 2.43	0.59
Lead leg changes before case horse fracture			
• Total no. of lead leg changes	0.69	1.54 – 3.12	0.34
• Change at all in final 3 seconds	1.83	0.98 – 3.40	0.10
• Change at all in final 10 seconds	1.28	0.73 – 2.23	0.47
• Change at all between final 3 - 10 seconds	0.84	0.44 – 1.61	0.48

[a] 1=good, 2=level, 3=bad.

The precise time and location of injury was recorded for 94% of cases. The data were analysed separately for flat and national hunt type races, as there were clear differences between

the two types of race. Fractures were relatively evenly distributed throughout flat races. In contrast, the majority of fractures in national hunt type races, tended to occur in the second half of races (74%: 45/61), indicating that the speed of the horse may be an important factor in the development of fractures. National hunt races are longer and are often run at a slowly increasing pace, such that horses only reach a true racing speed in the second half of races. In contrast, flat races tend to be run at a greater and more consistent speed throughout, which may explain the more even distribution of fractures. Measurement of sectional speeds would help identify the true speed of the horses at the time of fracture, however this was not possible as distance markers are only used in the final few furlongs on UK racecourses. Oikawa et al. (1994) also concluded that there was an association between increasing speed and risk of injury. By remodelling a racecourse in Japan, introducing a steeper incline in the finishing straight, race times were significantly reduced. Although only monitored for one year after remodelling, Oikawa et al. (1994) claimed that this reduction in speed significantly reduced the incidence of injuries. The potential relationship between injury and speed may be explained by the observation that there is a linear relationship between load on the limb and speed (Rubin & Lanyon, 1982; Nunamaker, 1986; Davies et al., 1993). Obviously the difference in speed between racehorses in the same race is not great, but it may be that a small difference in speed at or near maximal race speed, may be sufficient to produce failure of a bone, which is already enduring close to maximal load. Clanton et al. (1991) showed that more than 50% of musculoskeletal breakdowns occurred in the final 2 furlongs of races, but identified the final corner as the exact site of the majority of these breakdowns. They therefore concluded that it was a particular feature of that corner and not increasing speed, which was associated with the risk of injury. McKee and Clarke (1993) reported that fatalities were evenly distributed throughout races, suggesting no association between speed or fatigue and injury: However their data refers to all fatalities on racecourses and does not differentiate between flat and national hunt type races.

The lack of distance markers on UK racecourses meant that the defined half way point of races in the current study was actually the point where 50% of the total race time had been completed, rather than the actual halfway mark by distance. This would result in a greater distance being covered in the "second half" of races run at increasing pace. If the risk of fracture was simply a function of distance covered one would therefore expect to see a greater number of fractures in the "second half" of races. Future analysis will attempt to identify the quarter and half distance points, from racecourse maps and race videos, to address this potential source of bias.

Overall there were a greater number of fatalities (72%) located on the straight parts of the racecourses, which may simply represent the time spent at risk in the different parts of the racecourses. Comparisons can however be made between the different race types. The percentage of fractures occurring on straights is greatest in hurdle (82%) and steeplechase (81%) racing. This can be explained by the fact that jumps are rarely placed in corners, therefore the greater risk in jump racing, in part attributable to the obstacles, is predominantly present on the straight parts of the course. It was only in all weather flat racing that corners appeared to be associated with a greater risk. Comparison of the two types of flat race showed that there was a significant association between all weather races and the likelihood of fracture in a corner. This is unlikely to reflect the differences in track configurations, which may have resulted in a slightly greater percentage of time spent in corners in all weather races. It is more likely to be associated with differences in ground-hoof interactions. Clanton et al. (1991) reported that 77% (17/22) of breakdowns at one dirt racetrack occurred while the horse was either entering or leaving the final turn. Fredricson et al. (1975) have demonstrated that a lack of adequate camber

in turns leads to a loss of gait symmetry which results in rotation of the joints of the lower limb, most particularly the fetlock. They also demonstrated greater thermographic measurements in inner forelimbs, which are adducted to the greatest extent when negotiating a turn. Taken as evidence for greater strain Fredricson et al. (1975) suggest that inadequate camber in corners results in a greater potential for injury of the distal limb. Pratt (1984) showed that the hoof tends to slide further on all weather or dirt compared to turf tracks. Greater slippage of the inner hoof underneath the body of the horse in inadequately cambered turns, would accentuate the rotation of the lower limb joints observed by Fredricson et al. (1975), therefore increasing the risk of injury on all weather/dirt surfaces. Accurate assessment of the degree of camber in turns on the 59 UK racecourses was not possible in this study, but these results would suggest that further research, with emphasis on the design of corners on all weather tracks should be a priority.

Logistic regression analysis demonstrated a strong association between affected forelimb and lead leg at the time of fracture. Ueda et al. (1993) found a similar association, with more than 70% of cases injuring their lead forelimb. Ratzlaff et al. (1990) showed that when galloping, peak vertical forces were greatest for the lead forelimb followed by the non-lead forelimb, lead hindlimb and non-lead hindlimb, respectively. These results suggest that the greater propensity to fracture the lead forelimb is associated with the increased strain under which it is placed. The lack of association between most used lead leg and affected forelimb shows that horses were not favouring or protecting one or other forelimb, by using it less often as the lead leg during a race. Second, this result suggests that there was no immediate cumulative effect of increased strain on an individual limb, which was used more often as the lead leg over the course of a race. This implies that it is the extra force experienced in the few strides prior to fracture that may contribute to the greater number of lead fore fractures. At the start of a race it is possible that each forelimb has an equal likelihood of sustaining a fracture or that one forelimb is more likely to fracture due to pre-existing pathology. Either way, the limb that fractures is more likely to do so under the extra forces experienced as the lead leg.

The multivariable model developed for forelimb cases not only identified an association between the fractured limb and lead leg at the time of fracture, but also showed that there was an association with the lead leg used at the start of the race. Horses were more likely to fracture the forelimb, which they used as the non-lead leg for the first few strides of a race. It might be that case horses were favouring that leg at the start of races because it was more painful, possibly due to pre-existing pathology. If this were the case, assuming that painful, pre-existing pathology predisposes to fracture, we would have expected to see an association between the side that fractured and least used (protected) lead leg, throughout the whole of the race. Information on most used and start lead legs during training gallops would be useful to identify if certain horses consistently used one or other lead leg more often.

Associations between lead leg and side of hindlimb fracture were not identified partly due to the lower incidence of hindlimb fracture resulting in less statistical power. But also, as shown by Ratzlaff et al. (1990), the vertical forces experienced by the hindlimbs are 25 to 30% lower than for the forelimbs. It is therefore possible that other factors are more important in determining which hindlimb is likely to fracture. Such factors will however, take a lot longer to identify, as the incidence of hindlimb fractures is comparatively low.

There was no association between direction of running and side of fracture for either fore or hindlimb cases. It had been hypothesised that, as horses tend to lead on the inner forelimb while running round a turn, greater stress would be experienced by the left forelimb on left handed tracks and vice versa on right handed tracks. The fact that there was a strong association

between most used lead leg and direction of running, indicates that horses were not compensating for excessive use of the inner limb as lead leg in corners, by using the outer limb as lead leg, more often in the straight parts of the course. We can therefore conclude that any extra strain placed on the inner forelimb, used more often as lead leg due to the direction of running, does not result in a greater risk of fracture for that limb.

Progress during a race was measured, in two different ways, first by putting the four study horses in rank position (1-4) and second by recording which third of the field (front to back) each of the four horses were in, i.e., measuring the horses' progress against the rest of the field. Analysis of these variables demonstrated that horses that were running faster or making good progress were more likely to suffer a fracture compared to those horses that were either remaining level or going backwards. In addition, those horses going slowest in the final minute before fracture appeared to be at least risk of fracture. These results add credence to our earlier findings with respect to the percentage of race completed before fracture and the suggestion that greater speed increases the risk of fracture. The apparent protective effect of horses making bad progress in the final minute was excluded from the final multivariable model but good rank progress remained a significant risk factor.

None of the individual measures of encouragement were significantly associated with fracture. A composite variable, which identified any form of encouragement, was therefore created. Horses that were being encouraged at any time in the race (not including the final 10 seconds) or specifically in the final 10 seconds were more likely to suffer a fracture. In the final multivariable model the variable identifying encouragement at any time in the race was excluded, since it was confounded by horses that were reluctant to start. There was a strong association between those that were reluctant to start and those receiving encouragement. Eighty eight percent (30/34) of horses that were reluctant to start received encouragement during the race, compared to only 40% (120/302) that were not reluctant to start. Encouragement in the final 10 seconds remained in the final multivariable model. This suggests that jockeys may have been aware that their horse was slowing down, but were unaware that this may have been due to discomfort or pain. This contradicts the findings from Cohen et al. (1997) who observed that jockeys on injured horses were less likely to use the whip in the final 12 seconds before any injury. They explained the apparent protective effect of the whip by suggesting that jockeys may have been aware of a problem prior to the apparent stride where the injury occurred and were therefore reluctant to use a whip. The reason for the difference between the two studies may lie in the case definitions. Injuries were defined as catastrophic (requiring euthanasia) or career ending (no training for greater than 6 months) by Cohen et al. (1997). There were major differences in the anatomical locations of injuries for the two different categories. For example 73% (98/135) of catastrophic injuries were fractures whereas only 46% (48/104) of career ending injuries were fractures. The catastrophic injury definition therefore more closely resembles the one used in the current study and when analysed individually Cohen et al. (1997) showed that there was no association between whip use and catastrophic injury. The association between career ending injury and whip use remained. It is possible that soft tissue injuries, more common in the "career ending" definition used by Cohen et al. (1997), are more insidious in onset and that horses pull up more quickly when sustaining a fracture. Jockeys may therefore have more time to react to signs of pain coming from horses with soft tissue injuries and therefore stop using the whip.

Reluctance to start at the beginning of races was weakly associated with an increased risk of injury in the final multivariable model. A possible explanation is that horses were in some pain or discomfort. If this were the case though we would have expected to see horses favouring one

or other lead leg through the race, unless of course, pain was bilateral. It would be interesting to note if certain horses were also reluctant to start in previous races, but race reports are not detailed enough to extract this information. Similarly it would be interesting to note if these horses were also reluctant to work during training. This information was not available from the questionnaires conducted with trainers for this study.

As jockey and horse behaviour variables were recorded after initial frame by frame analysis there was a potential for bias as the race viewer was no longer blinded to which horse was going to suffer injury. The collection of cases is continuing and it is proposed that when the next set of videos are viewed the ones used from this two year study will be re-viewed in real time in order to validate these behaviour related findings.

In conclusion, as identified by the different times that fractures occur at in different types of race and by the association with horses making good progress, greater speed appears to be a significant risk factor for fatal distal limb fracture. Obviously it is not possible to advise trainers to train horses so that they run more slowly, but as shown by Oikawa et al. (1994) it may be possible to make alterations to racecourses to reduce the overall speed of races and thus reduce the likelihood of fatality.

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SURVIVAL ANALYSIS

SURVIVAL ANALYSIS OF FATAL INJURIES DURING RACING: A COMPARISON OF DIFFERENT APPROACHES

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SUMMARY

Survival analysis was used to assess risk factors for fatal injuries to horses on UK racecourses. This allowed assessment of variation due to temporal horse-level effects, including previous racing intensity and historical distribution of race types, as well as race-level factors. Comparisons were made between measuring survival time as number of days and as number of races to injury from the first race. Two related models were presented for time as number of races to injury: a Cox regression model fitted using partial likelihood, with the Efron approximation to handling ties, and a discrete-time logit model fitted using maximum likelihood. The latter approach had the advantages of being computationally more efficient and enabling the testing of different functional forms for the dependence of hazard on time. Retrospective data were available from all race starts on the 59 courses in Britain from 1990 to the end of 1999, as analysed by Wood et al. (2000, 2001). The analysis was conducted on the 47,424 horses that had started racing in the UK. Horses starting racing abroad were excluded, but some included horses would have raced abroad at some stage during their racing career. The results for the selected models were broadly consistent with each other and with previously published studies. The risk factors for fatal injury included race type, firmness of surface, age, race distance and previous racing intensity. The main difference between the models for time as number of days and number of races concerned the role of age: age at race was identified as the more important factor in the latter model, whereas age at first race was more significant in the former model.

INTRODUCTION

Understanding the causes of severe injuries to horses, in particular during racing, is an important issue for horses and for racing. The majority of deaths on racecourses (70-80%) are due to musculoskeletal injuries, although the nature of the injuries varies between race types (Williams et al., 2001). Previous studies have found that the rates of fatal injury per race start are much higher in jump races than flat races (McKee, 1995; Bailey et al., 1998; Wood et al., 2000; Williams et al., 2001). Other risk factors identified include race age, age at start of racing career, firmness and type of racing surface, race distance, season and previous racing intensity (Mohammed et al., 1991; Bailey et al., 1998; Wood et al., 2000).

Many studies have used ordinary logistic regression methods to identify factors associated with injury. In general, these models treat each race start as independent and so ignore the

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hierarchical structure of the data. Some researchers have taken a horse level approach in order to account for the effect of previous race-speed exercise distances (Estberg et al., 1995; Cohen et al., 2000). Hierarchical, multilevel models have been used by Wood et al. (2001), for fatal injuries, and Pinchbeck et al. (2002a, 2002b), for falls, to estimate the contribution of clustering at different levels including race, course, trainer and jockey. Their results suggested that most of the variation was at the start level, although significant proportions of variation were found at higher levels.

The purpose of this study was to explore the use of survival analysis techniques for identifying risk factors for fatal injuries to horses in UK races. Survival analysis involves taking a horse-level approach and enabled us to assess how temporal effects (e.g., 'wear and tear' from previous races) contributed to the risk of fatal injury over the racing career of a horse, independent of and in interaction with race-level risk factors. The data included all races held in Britain from 1990 to 1999 inclusive (Wood et al., 2000; 2001). Comparisons were made between different approaches, including sample selection, representation of time to injury and model specification.

MATERIALS AND METHODS

Data

The study utilised retrospective data from all UK race starts held under Jockey Club rules from 1/1/1990 to 31/12/1999 (Wood et al., 2001). Data were provided by Weatherbys Ltd. in electronic format and imported into SAS for data cleaning and analysis; they were checked for validity against independent data sources. Information was available for four principal race types: flat races, hurdles (races which involved jumping a flexible hurdle), chases (including both hunter chases and steeplechases, both of which were races over solid 'brush' fences) and 'National Hunt flat races' (races over hurdle courses when the hurdles had been removed). Race starts were excluded if horses withdrew prior to the race start. Variables available for analysis included horse related variables (such as age at race, age at first race, gender, previous racing intensity and weight carried) and race level variables (such as date, type and distance, firmness of racing surface ('going'), number of runners and winning speed).

A horse was classified as a case if it died or was euthanased on the racecourse after it had started in a race. The majority of horses were euthanased after severe injury by a member of the veterinary team, but some died as a direct result of the injury. Horses that survived their final race were classified as controls and their survival times censored at this point. No information was available on the reasons for censoring in control horses, excepting reaching the end of 1999.

In order to study factors influencing racehorse survival, it is clearly desirable to have information on every race that a particular horse has run. To reduce the potential bias introduced by horses with incomplete UK racing histories, 2,076 horses were excluded as they had a missing date of first race, as were 9,939 that had raced before 1990 (out of a total of 63,754). In addition, 4,315 horses with a date of first race after 1/1/1990, where that race was abroad were also excluded. It was impossible to detect horses that only raced abroad after their first race.

Statistical analysis

Representation of time dimension: Time to fatal injury was initially measured as the number of days between the first race and the date of injury. Horses were excluded from the

risk set between races, as data were only available about races. Leaving a horse in the risk set between races would have exaggerated the risk attached to frequent racing. Also, it made no intuitive sense to treat race-level variables, such as firmness of going, as fixed between races.

The effect of removing horses from the risk set on days when they were not at risk is equivalent to modelling a non-linear transformation of time as the number of races to injury. Our second approach to representing the time dimension was to measure time explicitly as the number of races to injury.

Survival models: Exploratory analyses were carried out to assess the relationships between time to fatal injury and potential risk factors using plots similar to Kaplan-Meier curves, but for multiple time dependent variables (Venables & Ripley, 1997). Factors were considered individually and after adjusting for other factors using a multivariable model (see below).

Models for the time to fatal injury, with time measured as both the number of days and number of races, were fitted using Cox regression (Cox, 1972). The models take the form

$$\log h(t) = f(t) + \beta_1 X_{1t} + \ldots + \beta_p X_{pt} \qquad (1)$$

where $h(t)$ is the hazard function at time t, $f(t)$ is the logarithm of the baseline hazard, $X_{1t}, \ldots X_{pt}$ are values of the covariates at time t and $\beta_1, \ldots \beta_p$ are parameters that are independent of time. In this study the covariates included fixed factors such as age at first race and time dependent variables such as race type, going and race age.

Two methods of handling ties were employed: the less computationally expensive Efron approximation (Efron, 1977) was used for selecting models for both definitions of time, but comparisons were made with the more appropriate discrete method (Cox, 1972) for time as the number of races and little difference was found.

It can be shown that under the discrete method of handling ties, the Cox regression model can be described as a proportional odds model (Cox, 1972). The model then takes the form

$$\log\left(p_i/(1-p_i)\right) = f(i) + \beta_1 X_{1i} + \ldots + \beta_p X_{pi} \qquad (2)$$

where p_i is the probability of a fatal injury in (discrete) time interval i, and $X_{1i}, \ldots X_{pi}$ are values of the covariates in time interval i. The standard approach to estimating this model is to use partial likelihood to estimate the parameters β_i, treating the parameters of $f(i)$ as nuisance parameters.

An alternative approach to estimating the discrete-time model is to treat it as a binary regression model and use maximum likelihood. Standard logistic regression software can be used to fit the model, if the data are structured such that there is one record for each discrete time interval that each horse was observed (Allison, 1995). Advantages of using the logit model over partial likelihood are improved computational efficiency (especially when there are many ties and/or time-dependent covariates) and that the effect of time is now modelled explicitly (i.e., $f(i)$ is an explicit function of time). Possible functions $f(i)$ include a linear function $f(i) = \alpha + \gamma i$, a quadratic function $f(i) = \alpha + \gamma i + \delta i^2$ or a logarithmic function $f(i) = \alpha + \gamma \log i$ where α, γ and δ are parameters to be estimated. If the number of time

intervals is small relative to the number of observations then a possible function is $f(i) = \alpha_i$ (ie a separate parameter for each time point). Comparisons were made between the two methods of estimating the discrete-time model when time was measured as the number of races.

Smoothing splines were used to explore the functional form of the relationship between time to injury and the continuous variables (age, distance, number of runs in last 12 months, weight carried, number of runners) in the Cox regression models (Therneau and Grambsch, 2000). Multivariable models were used to account for confounding from other variables. The nonparametric spline functions were plotted against the logarithm of the hazard function in order to help suggest possible polynomial functions of the continuous variables to represent the relationships in the final models. The continuous variables were first centred by subtraction of the sample means to reduce the possible effect of multicollinearity (Kleinbaum et al., 1992).

Variables were selected for inclusion in the multivariable models by first screening univariable models; variables with $P<0.25$ were considered for inclusion in subsequent models. The multivariable models were built using a backwards elimination procedure with variables retained in the model if they improved the fit (likelihood ratio chi-square $P<0.05$) or were significantly associated with the outcome (Wald $P<0.05$). Biologically meaningful two-way interaction terms were tested between all main effects variables. Hazard ratios and associated 95% confidence intervals were calculated for continuous-time Cox regression models. Adjusted odds ratios (OR) and associated 95% confidence intervals (CI) were estimated from the discrete-time logit models. The quality of model fit for the logit models was assessed using the Hosmer-Lemeshow statistic (Hosmer & Lemeshow, 1989).

The smoothing splines were fitted and the exploratory plots obtained using S-Plus (S-plus 2000 Mathsoft Inc) and all other models were fitted using SAS/STAT (SAS v.8, SAS Institute).

RESULTS

Descriptive statistics

A total of 63,754 horses made 719,099 starts in UK races between 1990 and 1999 resulting in 2,015 fatal injuries. Jumping races were associated with the highest risk of fatal injury, with 6.7 deaths per 1,000 starts for steeplechases and 4.9 per 1,000 starts in hurdle races. In flat races, the rate was 0.9 deaths per 1,000 starts. Excluding horses with incomplete racing histories (apart from races run abroad after the first race) made little difference to the fatality rates. The 16,330 excluded horses made a higher proportion of starts in hurdle and chase races than the 47,424 selected horses (34% and 30% of starts for the excluded horses were in hurdle and chase races respectively, compared to 21% and 9% respectively for the selected horses).

Exploratory analysis

Effect of age at start of racing career: Previous analyses of this data set have suggested the importance of the age at first race as a risk factor for fatal injury (Wood et al., 2000). The temporal effect of age at first race (i.e., age started racing) on time to injury is displayed graphically in the plots in Figure 1. Separate plots are shown for time measured as the number of days to injury and time measured as the number of races to injury. For each measure of time, a plot is shown before and after adjustment for potential confounders, such as the race age of the

horse, with a multivariate model. The plots indicate that most of the effect of age at first race is explained by the confounding variables, for both definitions of survival time. Further testing, by systematically removing variables from the multivariable model, showed that actual race age had the largest confounding effect. Similar plots were used to explore the relationships between time to injury and other risk factors (results not shown).

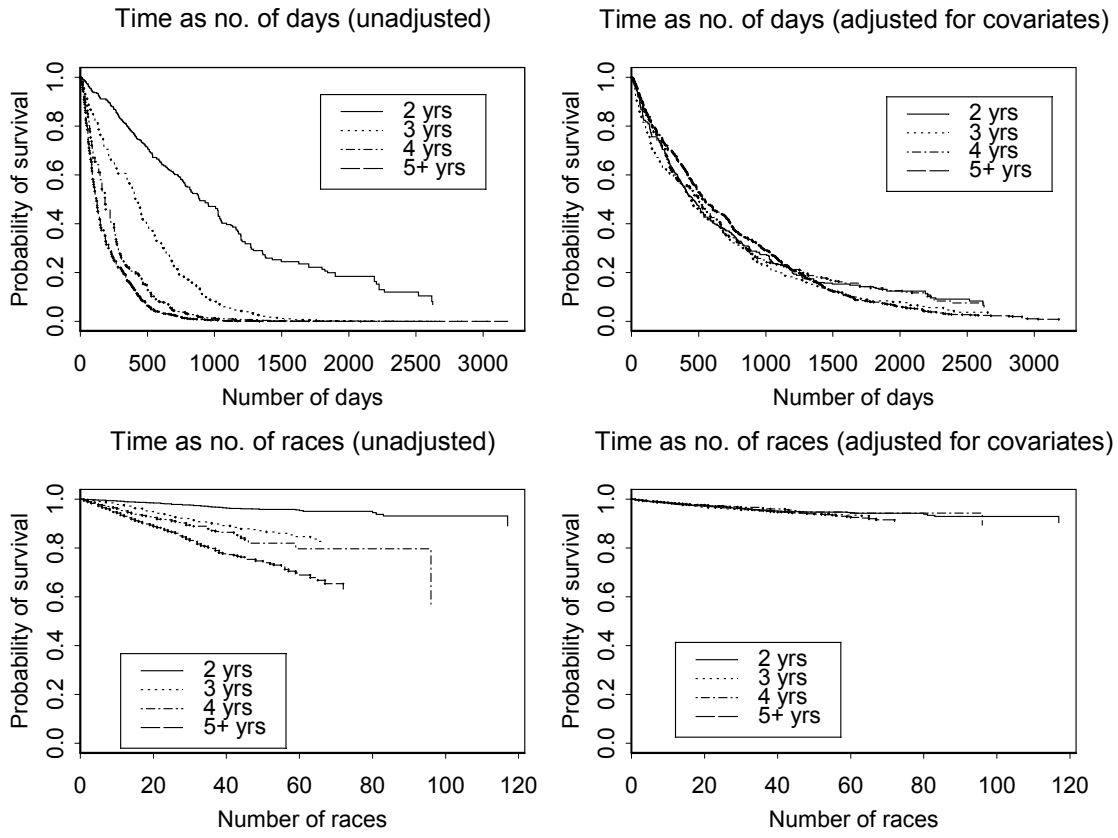

Fig. 1 Graphs showing the relationship between age started racing and time to injury for the two measures of survival time (before and after adjusting for covariates), with the age at first race categories shown in the legend.

Functional form of continuous variables: Figure 2 shows selected results from the multivariable Cox regression models for time as the number of races to injury with smoothing splines for the centred continuous variables (with means: age=4.6 years, age at first race=3.1 years, distance=12.0 furlongs, number of runs in the last 12 months=5.9, number of runners=12, weight carried=133 pounds). Three continuous variables, age at race, race distance and number of runs in the last 12 months, had a significant non-linear effect on survival time to injury (P<0.05), after adjustment for confounding variables. Addition of linear and quadratic polynomial terms to the multivariable survival models for these variables led to an improvement in fit (P<0.05). The graphs suggested the possibility of cubic relationships for distance and number of runs in the last 12 months, but the addition of cubic terms did not improve the model fit (P=0.14 & 0.08 respectively). None of the other continuous variables were significantly associated with risk of injury after adjusting for confounders (number of runners, P=0.13).

Survival models

Analysis of time as number of days: Table 1 shows the final Cox regression model for risk factors associated with fatal injury when survival time was measured as the number of days to injury. Horses running in a hurdle, chase or NHF race had about 1.5 times the hazard of a fatal injury relative to horses running in a flat race. The risk increased with the firmness of racing surface. Races run in the early part of the season (Feb to May) were associated with an increase in risk. Females had a lower risk of fatal injury. Horses switching to a new race type for the first time were at increased risk of injury. Race distance had a quadratic relationship with risk of injury. Race age was replaced by age at first race of this type (with a quadratic relationship). Number of runs in the last 12 months had a negative linear association with risk (quadratic term non-significant, P=0.27).

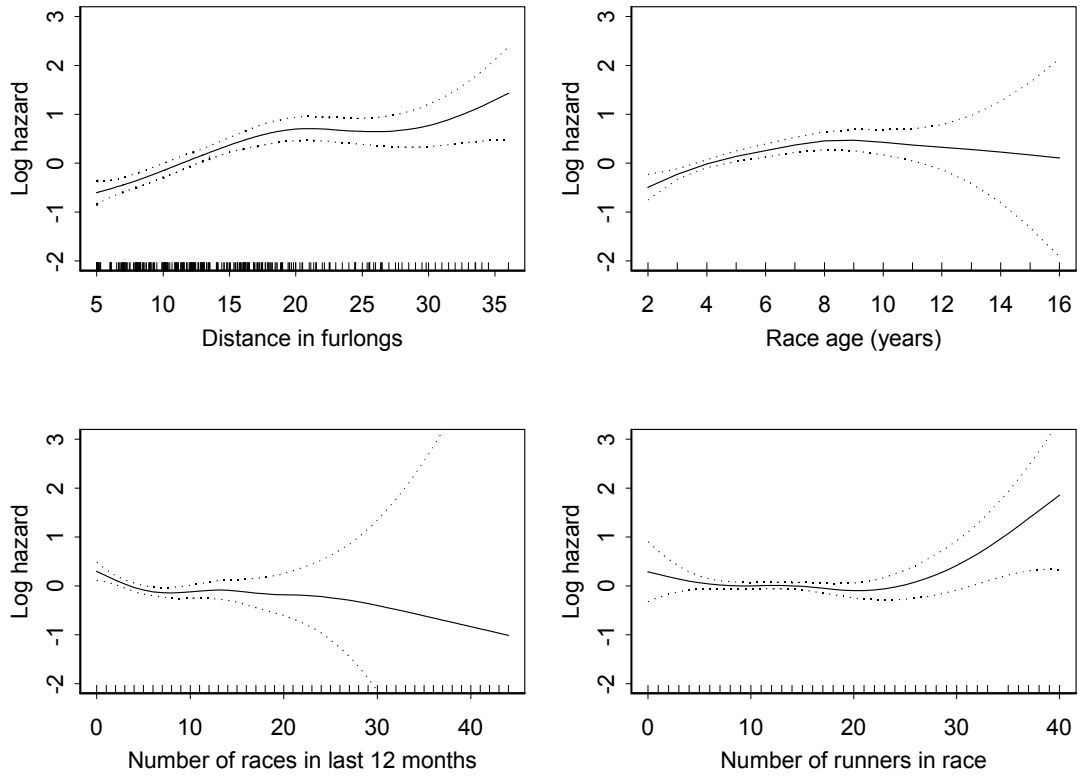

Fig. 2 Graphical representation of the functional form of the continuous variables modelled using smoothing splines in a multivariable Cox regression model for risk of fatal injury with time measured as the number of days to injury. The plots show the smoothed, fitted log hazards and 95% confidence intervals, with rug plots to represent the number of race starts on the x-axis.

Analysis of time as number of races: Table 2 shows the selected models for risk factors associated with fatal injury when survival time was measured as the number of races to injury: first, a Cox regression model fitted using partial likelihood and, secondly, a logit model fitted using maximum likelihood estimation. The two versions of the model gave very similar coefficients; the Cox regression model does not include estimates for the intercept and the time-related variables (number of races and an indicator for first race in the UK) because these are factored out into the baseline hazard.

Table 1. Final Cox regression model for risk factors associated with fatal injury in all race types when time was measured as number of days

	COEFF.	STD ERROR	P-VALUE	RELATIVE HAZARD	LOWER CI	UPPER CI
Type of race						
Flat	0.0000			1.000		
NHF	0.3757	0.2060	0.0682	1.456	0.972	3.041
Hurdle	0.5423	0.1699	0.0014	1.720	1.233	4.979
Chase	0.3869	0.2068	0.0614	1.472	0.982	3.143
Going						
Hard/Firm	0.3010	0.1215	0.0132	1.351	1.065	2.437
Good-to-firm	0.1246	0.0758	0.1005	1.133	0.976	1.446
Good	0.0000			1.000		
Good-to-soft	-0.1730	0.0925	0.0613	0.841	0.702	0.599
Soft	-0.3995	0.1092	0.0003	0.671	0.541	0.307
Heavy	-0.9821	0.2364	<0.0001	0.375	0.236	0.055
All Weather	0.4250	0.1331	0.0014	1.530	1.178	3.518
Season						
Feb-May	0.2739	0.0912	0.0027	1.315	1.100	2.250
Jun-Aug	0.2126	0.1211	0.0790	1.237	0.976	1.876
Sep-Nov	0.0649	0.1014	0.5223	1.067	0.875	1.212
Dec-Jan	0.0000			1.000		
Sex						
Female	-0.1997	0.0736	0.0067	0.819	0.709	0.554
Male	0.0000			1.000		
Racing history						
1st race of altered type	0.3992	0.1246	0.0014	1.491	1.168	3.260
Mixed racing types	0.1232	0.1007	0.2211	1.131	0.929	1.440
All races of same type	0.0000			1.000		
Distance (furlongs)	0.0764	0.0151	<0.0001	1.079	1.048	1.254
Distance^2 (furlongs)	-0.0029	0.0009	0.0019	0.997	0.995	0.992
Age at first race of current type (years)	0.2481	0.0569	<0.0001	1.282	1.146	2.084
Age at first race of current type^2	-0.0259	0.0099	0.0088	0.974	0.956	0.926
Number of races in last 12 months	-0.0235	0.0083	0.0044	0.977	0.961	0.933

Table 2. Final Cox regression and discrete-logit models for risk factors associated with fatal injury in all race types when time was measured as number of races

	COX MODEL			LOGIT MODEL		
	COEFF.	SE	P-VALUE	COEFF.	SE	P-VALUE
Intercept				-6.6447	0.1611	<0.0001
No of races to date (NRACE)				-0.0166	0.0057	0.0037
Type of race						
Flat	0.0000			0.0000		
NHF	0.5022	0.3051	0.0998	0.5121	0.3139	0.1028
Hurdle	0.5766	0.1842	0.0017	0.5614	0.1823	0.0021
Chase	0.3874	0.2222	0.0813	0.4081	0.2199	0.0635
Going						
Hard/Firm	0.3229	0.1200	0.0071	0.3219	0.1203	0.0075
Good-to-firm	0.1298	0.0750	0.0834	0.1341	0.0751	0.0744
Good	0.0000			0.0000		
Good-to-soft	-0.1674	0.0917	0.0679	-0.1682	0.0919	0.0672
Soft	-0.3987	0.1085	0.0002	-0.3975	0.1087	0.0003
Heavy	-0.9760	0.2355	<0.0001	-0.9783	0.2358	<0.0001
All Weather	0.4071	0.1324	0.0021	0.4142	0.1324	0.0018
Season						
Feb-May	0.2782	0.0902	0.002	0.2772	0.0903	0.0022
Jun-Aug	0.2160	0.1195	0.0706	0.2175	0.1197	0.0692
Sep-Nov	0.0690	0.0999	0.4897	0.0688	0.1001	0.4918
Dec-Jan	0.0000			0.0000		
Sex						
Female	-0.2014	0.0733	0.006	-0.2028	0.0735	0.0058
Male	0.0000			0.0000		
Racing history						
1st race in UK	0.0000			0.1325	0.1426	0.3525
1st race of altered type	0.5164	0.1242	<0.0001	0.5251	0.1240	<0.0001
Mixed racing types	0.1831	0.0986	0.0632	0.2132	0.0942	0.0236
All races of same type	0.0000			0.0000		
Distance (furlongs)	0.0694	0.0154	<0.0001	0.0694	0.0154	<0.0001
Distance^2 (furlongs)	-0.0023	0.0009	0.0121	-0.0024	0.0009	0.0118
Race age (years)	0.2136	0.0409	<0.0001	0.2174	0.0403	<0.0001
Race age^2	-0.0280	0.0068	<0.0001	-0.0281	0.0067	<0.0001
Number of races in last 12 months	-0.0321	0.0123	0.0089	-0.0259	0.0108	0.0162
Number of races in last 12 months^2	0.0025	0.0011	0.0202	0.0020	0.0010	0.0456
Race type x NRACE						
NHF x NRACE	-0.0380	0.1271	0.7651	-0.0530	0.1349	0.6947
Hurdle x NRACE	0.0081	0.0060	0.1749	0.0085	0.0059	0.1480
Chase x NRACE	0.0199	0.0069	0.0039	0.0186	0.0066	0.0049

Table 3. Log-likelihoods for discrete-logit models with different restrictions on time component

MODEL	-2 LOG LIKELIHOOD	EXTRA PARAMETERS	P-VALUE[a]
No time component	16,317.14		
Logarithmic	16,305.69	1	0.00071
Linear	16,297.58	1	0.00001
Quadratic	16,297.05	2	0.00004
Categorised into deciles	16,284.13	9	0.00013

[a] calculated for likelihood test of improvement in fit over model without time component

The discrete-time logit model provided a reasonable fit to the data (Hosmer-Lemeshow statistic, P=0.20). The sensitivity and specificity were 75% and 68% respectively (i.e. 75% of injured horses and 68% of non-injured horses were correctly classified), when a cut-off probability of 0.0023 was selected (the observed risk was ~0.0023). One of the advantages of the discrete-time logit model is that it gave direct estimates of the effect of time on hazard. The maximum number of race starts per horse was 162, so there were a total of 162 tied event times. The high number of distinct race times together with the small number of horses reaching the higher number of starts (90% of horses made 27 or fewer race starts) made it computationally unfeasible to fit an unrestricted model (with 1 parameter per race time). Therefore, different ways of restricting the relationship between hazard and the effect of time were compared. Table 3 shows the log-likelihoods for models containing linear, quadratic and logarithmic functions of time, in addition to the fixed covariates. The final comparison shows the effect of categorising the number of races into deciles. Significance was assessed by comparing the log-likelihood from each restricted model, relative to a model with no time component. Although all models with restricted time effects gave a significant improvement in fit over the model with no time components, the linear model was selected as the best fit on the basis of having the lowest p-value (linear model shown in Table 2).

Further modelling of the effect of time on risk of fatal injury was conducted by considering interactions between number of races and the covariates. The only significant interaction was between race type and the number of races, with the number having significantly less effect on risk for steeplechases than flat races (Wald test, P=0.0049).

DISCUSSION

In this study we have explored the use of survival analysis for identifying risk factors for fatal injuries to horses in UK races. An important aspect of the problem was the choice of how to measure the time until fatal injury, given that each horse was only at risk during the races it ran, which were at discrete, unequally spaced time points. Two approaches were taken. First, survival time was defined as the number of days before injury occurred from the date of the horse's first race, horses being removed from the risk set between races. Second, survival time was defined as the number of races until injury, focusing on the cumulative time at risk. In the latter, race age was included as a covariate to take account of the unequal elapsed intervals between races. Time (as number of days) since last race was also tested as a covariate

One of the attractions of measuring time as the number of races is that it reduced the noise from irregular intervals between races, whilst still enabling the effect of racing intensity to be quantified as a covariate. An advantage of measuring time in days is that it was probably less sensitive to missing races, such as races abroad. When time is measured as the number of races, missing race data will clearly lead to underestimation of the time to injury, in addition to biasing the time-dependent covariates such as measures of previous racing intensity. Despite this, the overall results were broadly comparable whether time was measured as the number of days to injury or the number of races to injury. The main difference between the two models concerned the relative importance of the age at first race and the race age (see below).

Survival models were fitted using Cox regression for the two definitions of time to injury. Time in days was treated as a grouped continuous measurement, with tied event times being handled using Efron's approximation, to make the computations feasible. Little difference between the Efron approximation and the exact method of handling ties (also called averaged likelihood) has been reported for large tied data sets (Therneau and Grambsch, 2000). Time as the number of races to injury is a discrete-time process and so was most appropriately treated using Cox's model for discrete-time data. The discrete and continuous data models are distinct: a linear logistic hazard with log-odds ratio parameters versus a log-linear hazard with log-relative risk parameters respectively (cf. equations 1 and 2). However, Efron's method also provided a good approximation to the exact (discrete) partial likelihood for this data.

An alternative way of estimating the discrete-time model is to use logistic regression, fitted using standard maximum likelihood. The discrete-time logit model gave very similar results to the Cox regression model (for time as number of races), but was computationally more efficient and allowed time to be modelled explicitly, rather than factored out through partial likelihood. A logit model could have been used when time was measured as the number of days to injury, as observations occurred at regular intervals (days), but this is really an example of grouped continuous-time data and so a discrete-time model was conceptually inappropriate.

Logistic regression is frequently used for modelling binary data, but maximum likelihood estimation can give biased estimates of the probability of rare events, such as fatal injury in racing. An alternative approach is exact logistic regression (ELR), but this is rarely used in practice (King & Ryan, 2002) and would have been computationally exorbitant in this case.

In the analyses presented in this paper, one global model was constructed for each horse regardless of race type (with race type as a time-dependent covariate). We compared risk factors for flat and jump racing by fitting interactions between race type and other covariates. The only significant interaction was with number of races. Separate models were also constructed for each race type and compared with the overall model (results not shown).

The similarity between the Cox regression and discrete-time logit models suggests that the choice of modelling technique was less important than sample selection and sources of missing data. To build an unbiased picture of how risk of fatal injury evolves over time, it is necessary to have a complete record of a horse's racing career and the lack of information on races run abroad between 1990 and 1999 was a limitation of this study.

Previous analyses of these data have used separate ordinary logistic regression and mixed-effect models for each race type (Wood et al., 2000; 2001). The logistic regression models were of a similar form to the discrete-time logit models used for separate race types (not shown), apart from the lack of explicit estimates for the effect of time (denoted by f(i) in equation 2). The

exclusion of 16,330 (out of 63,754) horses in this study restricted the power to detect the effect of some covariates, but did enable us to assess temporal effects on risk of injury.

The main risk factors in the multivariable models were broadly consistent with those from previous studies. Flat races had a lower risk of injury than hurdle, chase and National Hunt flat races. Risk increased with the firmness of racing surface and race distance (with the relationship steeper in shorter flat races) and decreased with the number of races run in the last 12 months. Horses running their first race of a new type were at higher risk of injury, with some suggestion that horses that had previously run more than one type of race were at higher risk. There was a seasonal effect with races between February and May being significantly riskier, although this effect was not observed in the separate flat or chase models. Female horses were at lower risk.

Age played an important part in almost all the fitted models, but some discrepancies were found as to how it influenced risk of injury. One of the hypotheses of interest *a priori* was whether the race age or the age at which a horse started racing was more influential. We considered age at first race both as age at which a horse began racing and age at which the horse first ran in the current race type. With time as the number of races, race age had a quadratic relationship with risk of injury, with peak risk occurring at about 9 years old, with age at first race not being significant. With time in days, age at first race in the current race type had a quadratic relationship with risk. With this variable, the effect of race age was not significant.

Fitting the discrete-time logit model has enabled us to produce explicit estimates of the effect of time on risk of injury. The results for the overall model suggested that risk of fatal injury decreases over time (as measured by number of races). However, unobserved heterogeneity tends to produce hazard estimates that decrease with time, even when the true hazard is constant (Heckman & Singer, 1985). Thus, it is not clear whether there is a genuine decrease in risk over the racing lifetime of the horse, having adjusted for covariates such as age at race, or whether the negative coefficient is due to unobserved heterogeneity (i.e. the "better quality" horses tend to survive longer, so the observed risk decreases with time). However, Gail et al. (1984) suggested that tests of coefficient departure from zero remained valid, albeit with attenuation of coefficients, providing the unobserved heterogeneity is independent of the measured covariates. With the heterogeneity being undefined in this study, this was not clear.

Informative censoring, sometimes called non-independent censoring, arises if, conditional on the covariates at each event time, the censored horses are not representative of those at risk of fatal injury in subsequent races (i.e. they have hazards that are systematically higher or lower than those that are not censored) and can lead to biased parameter estimates (Collett, 1994). Informative censoring may have occurred in this study as no information other than fatal injury was available on why horses stopped racing. Horses would have been censored if they reached the end of the study period (31/12/1999), if they were retired to stud, died during training or were retired from racing due to injury or lack of ability. Horses with chronic musculoskeletal injuries might have been at increased risk of fatal injury if they were to race again. A crude method of assessing potential informative censoring is to refit the model under different assumptions (Allison, 1995). A sensitivity analysis was conducted to assess the impact of the extreme assumption that censored horses would have experienced a fatal injury in their next race. Substantial changes in the magnitude and direction of the coefficients in the selected discrete-time logit model were observed, but the new censoring assumption was totally unrealistic. We conclude that the results from the survival modelling should be interpreted with some caution and this issue may have implications for similar studies of racing injuries where horse-level effects are considered.

The analyses in this study could be extended by taking account of the hierarchical nature of the data using models with random effects for higher levels such as racecourse (e.g. frailty models instead of Cox regression models: Therneau and Grambsch, 2000 and multilevel logistic models in place of discrete-time logit models: Goldstein, 1995). However, this is unlikely to have a large effect on the results as previous studies (Wood et al., 2001, consistent with Pinchbeck et al., 2002a & 2002b, for horse falls) suggest that most of the variation in fatal injuries resides in the start level variables.

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ESTABLISHING TRENDS IN ANTIBIOTIC RESISTANCE BY SURVIVAL ANALYSIS

J.A. STEGEMAN*, J.C.M. VERNOOIJ, O.A. KHALIFA, J. VAN DEN BROEK AND D.J. MEVIUS

SUMMARY

In this study we applied survival analysis to compare the resistance of *Enterococcus faecium* isolates randomly collected from Dutch broilers against Ciprofloxacin, Erythromycin and Virginiamycin in 1998, 1999 and 2001. In the survival analysis, inhibition of bacterial growth was the event and, time to event was replaced by concentration of antibiotic to event. As a consequence changes in the growth of bacteria can be tested over an entire range of concentrations and no cut off value for resistance has to be determined. Bacterial growth was tested in a range of concentrations of the antibiotics that increased stepwise in a two-fold way. The minimal inhibitory concentration (MIC) is the lowest concentration of the antibiotic that still inhibits the growth of the bacterium. However, due to the fixed concentration steps, ties (samples with equal MIC value) will occur. As a consequence, we performed the survival analysis by use of a Cox logistic model instead of the more common Cox Proportional Hazards model. Instead of a ratio for the increase of the basic hazard rate (h_o), the outcome of the Cox logistic model is an odds ratio (OR) for the increase of $h_o/(1+h_o)$. In 2001 the survival of *E. faecium* in the presence of Virginiamycin and Erythromycin was reduced in comparison to 1998. The ORs associated with inhibition of bacterial growth over the range of concentrations tested were 2.88 (95% confidence interval (CI) 2.21-3.76) for Erythromycin and 2.11 (1.80-2.49) for Virginiamycin. The reason for this change is most likely the ban on Virginiamycin and Macrolide antibiotics in broiler feeds that was implemented between the sample collections of 1998 and 1999. The findings of the survival analysis are in agreement with the results of the logistic regression analysis using the internationally agreed cut off values for the MICs to determine an isolate susceptible or resistant. However, in contrast to the survival analysis, the logistic regression also showed a significant decrease in the resistance of *E. faecium* against Ciprofloxacin. This decrease was smaller than the decrease of the resistance against Erythromycin and Virginiamycin. The reason for the differences between the results of the survival analysis and the logistic regression analysis are probably because most changes in the MICs of the antibiotics in this study included the cut off value and logistic regression specifically identifies those changes.

INTRODUCTION

Antibiotic resistance of bacteria in farm animals is not only important because it results in therapy failure in animals, but also because it might lead to antibiotic resistance in man. Because of this public health hazard, several European countries have recently implemented surveys to trace increases of antibiotic resistance in farm animals (Danmap, SVARM, Norway, Mevius et

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al. 2000). In these surveys a bacterium is considered resistant against an antibiotic if its minimal inhibitory concentration (MIC) exceeds an internationally accepted cut off for bacterial resistance. As a consequence of the binary nature of this classification, trends in antibiotic resistance are usually established by comparing the proportions of resistant bacteria in animal populations longitudinally. However, increases in the MICs below the cut-off cannot be detected in such a comparison. Nevertheless, it might be relevant to detect such changes. A reason is that the MIC's of some antibiotics shift gradually or stepwise towards the cut off concentration. In addition, antibiotics may be used at concentrations below the cut off value.

The MIC of an antibiotic is established by examining the growth of a bacterium in the presence of a series of increasing concentrations of the antibiotic. The MIC is the lowest concentration of the antibiotic that still inhibits the growth of the bacterium. The frequency distributions of the resulting MICs, are often not normally distributed (see for example the distribution of the MICs of Virginiamycin in *Enterococcus Faecium* in Figure 3). Moreover, censoring of data occurs, because a bacterium might still grow at the highest concentration tested. A method to handle this censoring is survival analysis. Normally, we use survival analysis when the outcome of interest is time until some event occurs. However, if we replace time until event by concentration until inhibition of bacterial growth, survival analysis seems a possible method to test trends in MIC's statistically.

In this study, we applied survival analysis to compare the resistance of *E. faecium* isolated from Dutch broilers against Ciprofloxacin, Erythromycin and Virginiamycin in 1998, 1999 and 2001. *E. faecium* is part of the indigenous flora of broilers and considered to be a good indicator of the selection pressure exerted by antibiotics used in that population and for resistance problems to be expected in pathogens (Lester et al., 1990). Of the antibiotics included in this study, Virginiamycin has been banned from use as a growth promoter in broiler feed between the first and second sample collection. In addition, Tylosin and Spiramycin, two antibiotics that belong to the same group of Macrolide antibiotics as Erythromycin, were banned at the same time. Induction of resistance against one of the antibiotics in this group results in cross resistance against the others (Brander et al., 1982). Ciprofloxacin was included as a reference antibiotic, because we had no indications for a distinct change in the use of this antibiotic during the study period. In addition, we compared the results of the survival analysis by the results of a logistic regression analysis, using the cut off concentrations to determine whether bacteria were resistant or not.

MATERIALS AND METHODS

Collection and testing of samples

Six slaughterhouses for broilers were randomly selected in The Netherlands. Once a day, at each slaughterhouse, a caecum sample was collected from one broiler from a randomly selected flock. In this way 314, 234 and 285 samples of caecal contents were collected in the years 1998, 1999 and 2001 respectively. The distribution of the numbers of samples over the slaughterhouses was proportional to the distribution of the numbers of broilers slaughtered.

The samples were suspended in buffered peptone solution with 20% glycerol and stored at -20 ^{o}C pending analysis. *E. faecium* was isolated on Slanetz and Bartley agar by inoculating the plates with 50 µl of serial dilutions of the sample in saline with a spiral plater. A colony with typical morphology was pure cultured and typed biochemically. The MIC was determined using

the micro broth dilution test according to the NCCLS guideline (M7-A3), using Sensititre trays[1] with a custom made panel of antibiotics. In this panel, Ciprofloxacin was included at concentration ranging from 0.031 – 32 μg/ml and Erythromycin and Virginiamycin were included at concentrations ranging from 0.06 – 64 μg/ml. Within these ranges, the concentrations increased at two-fold steps. Next, the MIC was defined as the lowest concentration that inhibited visible bacterial growth. However, the growth of some isolates was not inhibited at the highest concentration tested. Finally, we used ATCC strains to examine the quality of the Sensititre trays.

Analysis of data

Our data consisted of growth or inhibition of growth at each concentration tested. Because of the stepwise two-fold increase of the concentrations, we performed a 2log transformation on the concentration in order to enable a clear graphical representation of the data. However, to avoid the occurrence of values below 0, all concentration steps were first multiplied by 32. Furthermore, when bacterial growth was not inhibited at the highest concentration step, the sample was qualified as censored at that concentration step. We used the statistical program R, version 1.6.1 (Ihaka and Gentleman, 1996) for the analysis of data.

Survival Analysis: For the survival analysis we first considered the Cox Proportional Hazards (PH) model, because it is a robust method (Kleinbaum, 1996). In our analysis, inhibition of bacterial growth is the event and, furthermore, we analysed concentration (c) of antibiotics to event, instead of time (t) to event. In the Cox PH model the hazard function equation is:

$$h(c, X) = h_0(c)e^{\sum_{i=1}^{p} \beta_i X_i} \quad (1)$$

where $h_0(c)$ is the unspecified baseline hazard rate, X are the explanatory variables and (X_1, .., X_p) and β_i are the coefficients that quantify their effects. The explanatory variables included in the analysis were antibiotic, and the interaction between year and antibiotic. Furthermore, because in each sample the MIC values of three antibiotics were established, frailty (repeated measures within a sample) was added to the model for correlations between the MIC values of the three antibiotics.

However, the Cox PH model assumes that the hazard ratio is constant over the range of concentrations (PH assumption) and that the concentration can have any value in that range. The data of this study do not meet the assumption of continuous data, because the inhibition of bacterial growth can only occur at fixed concentration steps. As a consequence, ties (samples with equal MIC value) will occur. Moreover, graphical inspection showed that the PH assumption was inappropriate. Furthermore, although parametric survival models do not have the assumption of proportional hazards, they assume continuous concentrations, and consequently also do not fit the data.

[1] manufactured by Accumed LTD, UK

To take the discrete concentration steps and the occurrence of ties into account, we transformed the Cox PH model into a logistic model for hazards (Therneau and Grambsch, 2000). The equation for that function is:

$$\frac{h(c,X)}{1+h(c,X)} = \frac{h_0(c)}{1+h_0(c)} e^{\sum_{i=1}^{p} \beta_i X_i} \quad (2)$$

Consequently, the logistic model for hazards estimates log odds ratios (ORs) instead of the log hazard ratios in the PH model. When the hazard is very small, i.e., maximum one event per concentration (no ties), equation 2 transforms back into the Cox PH model. Also in this model we included antibiotic and the interaction between antibiotic and year as explanatory variables and added frailty to the model.

Logistic regression: On the basis of the MIC cut off values that have been internationally agreed upon, isolates of *E. faecium* were either considered susceptible or resistant to each of the three antibiotics included in this study. These cut off values were: >2 μg/ml for Ciprofloxacin, >4 μg/ml for Erythromycin and >4 μg/ml for Virginiamycin and after the transformation of the data they become >6, >7 and >7 , respectively. The data were analysed by use of a Generalised Linear Mixed Model using a binomial distribution. The dependent variable was resistance; i.e., present (y = 1) or absent (y = 0). In the analysis we used a logit function to link the random and systematic components of the model. Antibiotic, and the interaction between antibiotic and year were included as explanatory variables. Finally, sample was included in the model as a random effect, to take into account that all three antibiotics were tested in the same sample.

RESULTS

In Figures 1-3, the upper graphs show the relative frequency distributions of the log transformed MIC values of Ciprofloxacin, Erythromycin and Virginiamycin, respectively. The frequency distribution of Ciprofloxacin was uni-modal, with the cut off value for resistance somewhat right to the middle of the curve. Furthermore, figure 1 shows only slight changes in the frequency distribution of the log(MIC) of Ciprofloxacin from 1998 – 2001. The relative frequency distribution of the log(MIC)s of Erythromycin demonstrates that the growth of a high percentage of the *E. faecium* strains was not inhibited at the highest concentration of the antibiotic included in the study. However, besides these highly resistant strains, the other *E. faecium* strains were all below the cut off value for resistance. Moreover, the percentage of Erythromycin resistant strains decreased between 1998 and 2001. Furthermore, the relative frequency distribution of log(MIC) of Virginiamycin, clearly shifted to the left from 1998 – 2001, indicating a decreasing resistance of *E. faecium* isolates. Moreover, the frequency distribution of the log(MIC)'s of Virginiamycin was bimodal, with the cut off for resistance between the two peaks of the distribution.

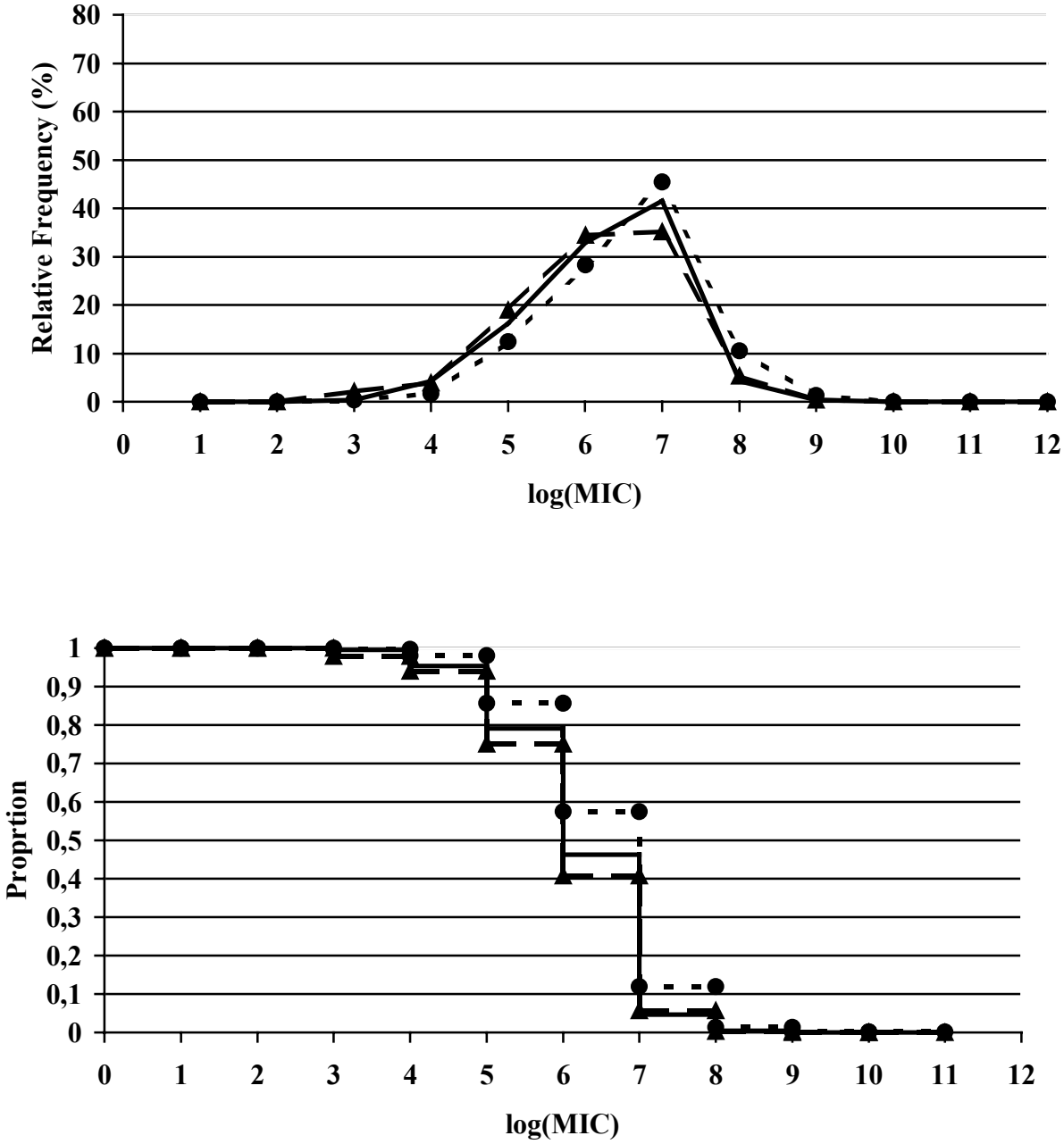

Fig. 1 Relative frequency distribution (above) and survival curve (below) of the log(MIC) values of Ciprofloxacin in *E. faecium* isolates from Dutch broilers in 1998 (--●--), 1999 (——) and 2001 (—▲—). Log(MIC)>6 are considered resistant.

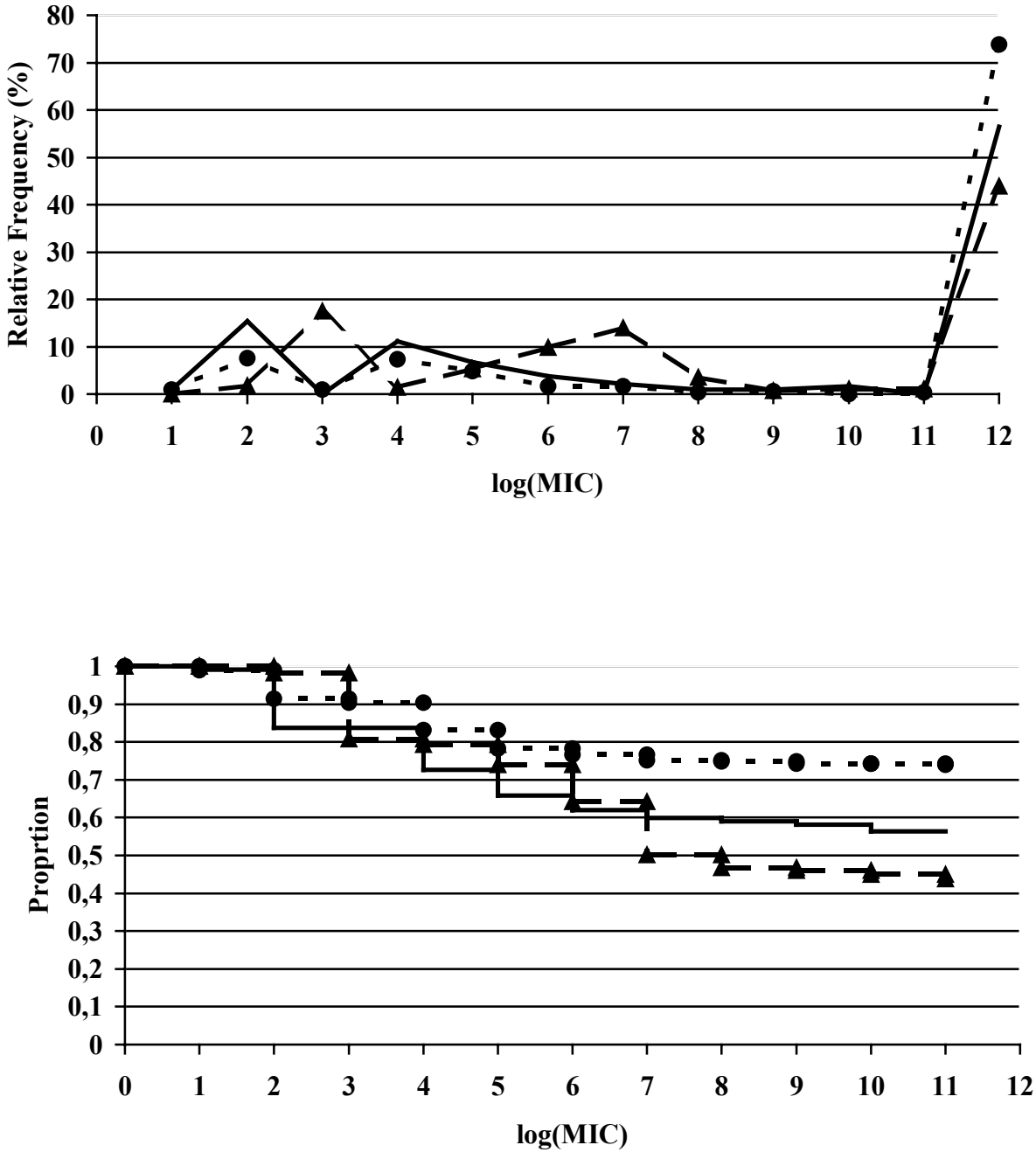

Fig. 2 Relative frequency distribution (above) and survival curve (below) of the log(MIC) values of Erythromycin in *E. faecium* isolates from Dutch broilers in 1998 (--●--), 1999 (——) and 2001 (—▲—). Log(MIC)>7 are considered resistant.

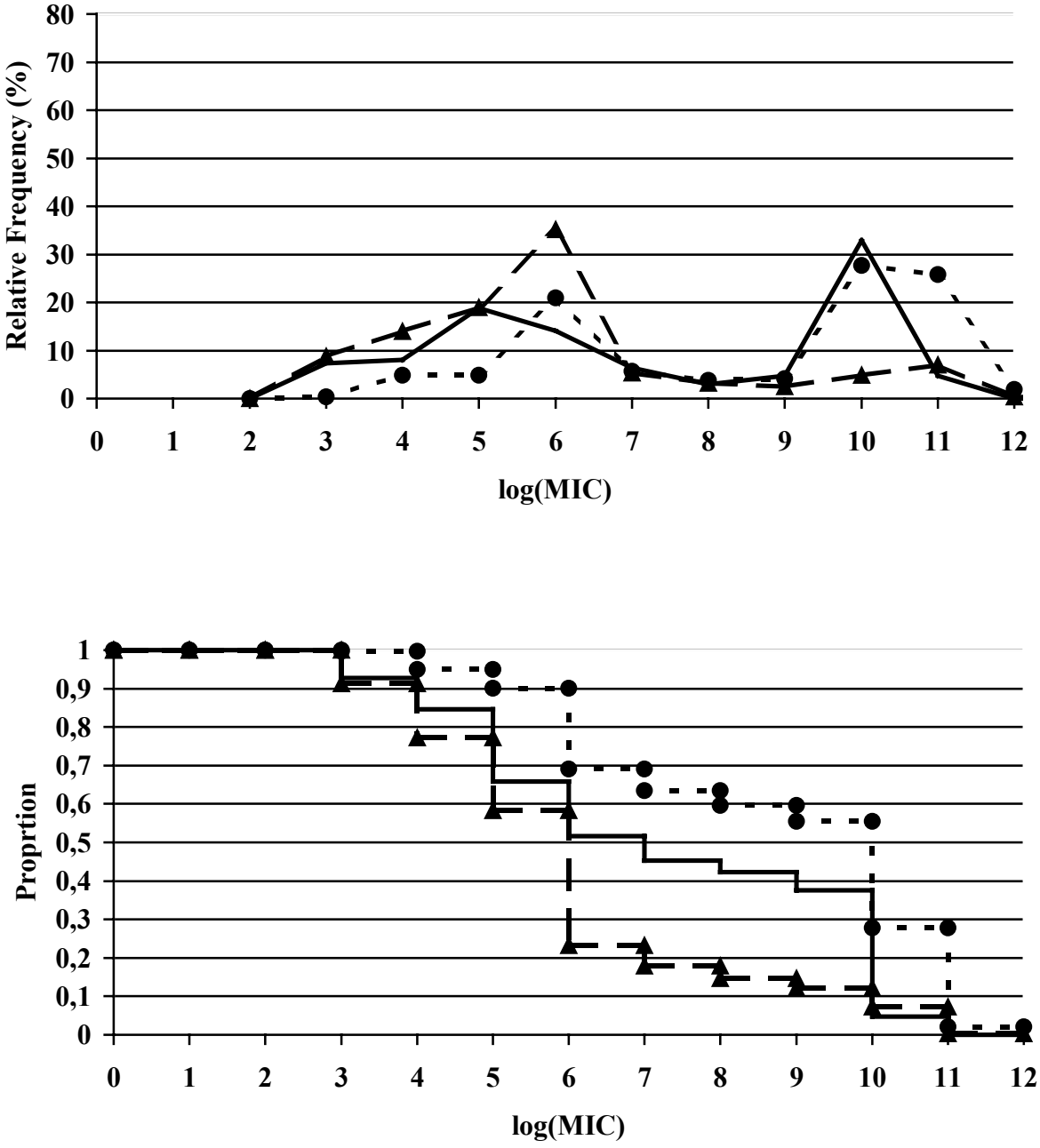

Fig. 3 Relative frequency distribution (above) and survival curve (below) of the log(MIC) values of Virginiamycin in *E. faecium* isolates from Dutch broilers in 1998 (--•--), 1999 (——) and 2001 (—▲—). Log(MIC)>7 are considered resistant.

The proportions of resistant bacteria according to the cut off MIC of the antibiotics are shown in table 1. This tables shows a reduction of the proportion of resistant isolates between 1998 and 2001 for all three antibiotics. However, the fraction of *E. faecium* isolates resistant against Virginiamycin showed the largest reduction and the fraction of *E. faecium* isolates resistant against Ciprofloxacin showed the smallest reduction.

Table 1. Proportion of *E. faecium* isolates resistant to Ciprofloxacin, Erythromycin and Virginiamycin in 1998, 1999 and 2001.

ANTIBIOTIC\YEAR	1998	1999	2001
Ciprofloxacin	0.58	0.46	0.41
Erythromycin	0.75	0.60	0.52
Virginiamycin	0.62	0.45	0.18

Survival analysis: The survival curves for E. faecium exposed to increasing concentrations of the three antibiotics are also shown in Figures 1, 2 and 3. Similar to the frequency distributions, we see few differences between the years for Ciprofloxacin. However, we see that none of the *E. faecium* isolates survived the highest concentrations of Ciprofloxacin. This is quite different for Erythromycin, where a large proportion of the isolates survived the highest concentration. Nevertheless, Figure 2 shows that this fraction of highly resistant strains decreased from 1998 to 2001. Although none of the *E. faecium* isolates of 1998 survived the highest concentration of Virginiamycin tested, bacterial survival is generally lower in 1999 and 2001 than in 1998 (figure 3). Consequently, the survival curves suggest that *E. faecium* isolates became more susceptible to Virginiamycin and Erythromycin during the period of the study. This was confirmed by the results of the logistic Cox model, as shown in table 2. Ciprofloxacin was the reference antibiotic in the model. Apparently, *E. faecium* generally survived higher concentrations of Virginiamycin, and especially Erythromycin, than of Ciprofloxacin. In addition, for Erythromycin, as well as for Virginiamycin, the hazard for inhibition of bacterial growth increased during the period of the study, indicating that on average, growth of the bacteria was inhibited at lower concentrations of these antibiotics.

Table 2. Odds ratios of survival analysis (logistic Cox model) and of logistic regression of the resistance of *E. faecium* isolates for Ciprofloxacin (Cip), Erythromycin (Ery) and Virginiamycin (Vir) in 1998, 1999 and 2001 (Ciprofloxacin is the reference antibiotic).

EXPLANATORY VARIABLE	SURVIVAL ANALYSIS (95% CI)	LOGISTIC REGRESSION (95% CI)
Ery	0.12 (0.09-0.15)	0.38 (0.03-0.75)
Vir	0.57 (0.48-0.67)	0.67 (0.32-1.03)
Cip year 1999 versus 1998	1.12 (0.94-1.32)	1.72 (1.35-2.10)
Cip year 2001 versus 1998	1.17 (1.00-1.38)	2.24 (1.88-2.60)
Ery year 1999 versus 1998	2.21 (1.66-2.94)	2.33 (1.94-2.74)
Ery year 2001 versus 1998	2.88 (2.21-3.76)	3.76 (3.38-4.14)
Vir year 1999 versus 1998	1.41 (1.19-1.68)	2.44 (2.06-2.82)
Vir year 2001 versus 1998	2.11 (1.80-2.49)	11.64 (11.23-12.06)

Logistic regression: The results of the logistic regression model are also shown in Table 2. In general, the results are similar to the results obtained by the survival analysis. However, Virginiamycin did not differ significantly from Ciprofloxacin and logistic regression suggested a sharper decrease in resistance against Erythromycin than survival analysis. Furthermore, in contrast to the results of the survival analysis, the results of the logistic regression model indicated a decrease in the resistance of *E. faecium* for Ciprofloxacin between 1998 and 2001. Finally, the results of logistic regression model indicated a sharper decrease in the resistance of *E. faecium* for Erythromycin and Virginiamycin than the results of the survival model.

DISCUSSION

In this study we applied survival analysis to compare the resistance of *E. faecium* isolates from Dutch broilers against Ciprofloxacin, Erythromycin and Virginiamycin in 1998, 1999 and 2001. From the results we conclude that the resistance against Erythromycin and Virginiamycin decreased during the study period. These findings are in agreement with the results of the logistic regression analysis using the internationally agreed cut off values to determine an isolate susceptible or resistant. However, in contrast to the survival analysis, the logistic regression also showed a significant decrease in the resistance of *E. faecium* against Ciprofloxacin. This decrease was smaller than the decrease of the resistance against Erythromycin and Virginiamycin.

The differences in the results of logistic Cox model and the logistic regression are remarkable. Although survival analysis is able to detect changes in MICs that do not include the cut off value, it did not indicate a significant change in the resistance of *E. faecium* against Ciprofloxacin and, for both Erythromycin and Virginiamycin, the OR's resulting from the survival analysis were lower than the ones from the logistic regression analysis. Apparently, in this study, most changes in the MIC's include the cut off value, which is demonstrated by the Figures 1-3. To detect changes that involve the cut off value, the logistic regression analysis has a higher power than the survival analysis, because it specifically examines these changes, whereas survival analysis investigates changes over the whole range of concentrations.

A plausible explanation for the decreasing resistance of *E. faecium* against Erythromycin and Virginiamycin is the ban on a number of antibacterial growth promoters in broiler feeds between the first and second sample collection. The ban on the use of Virginiamycin removed the selection pressure on bacteria that were resistant for Virginiamycin. Furthermore, because there is cross resistance of bacteria for Macrolide antibiotics, the ban on the use of Tylosin and Spiramycin, did more or less the same for Erythromycin. However, we have no clear explanation for the decrease in resistance against Ciprofloxacin as observed by the logistic regression analysis.

In conclusion, we demonstrated the use of survival analysis to establish trends in antibiotic resistance. Survival analysis was able to detect a decrease in the resistance of Erythromycin and Virginiamycin by examining changes in MICs over the entire range of antibiotic concentrations tested. However, in this study the changes in the MIC's predominantly included the internationally accepted cut off values. As a result, survival analysis did not detect differences between years that were not detected by logistic regression analysis. However, changes below the internationally accepted cut off value can be detected by survival analysis and not by logistic regression. As a consequence, survival analysis could be a useful tool in addition to logistic regression analysis.

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SURVIVAL ANALYSIS OF 124 CASES OF CANINE PERICARDIAL DISEASE: CENSORING AND PROGNOSTICATION

S.H. BINNS[1], M.S. JOHNSON, M.W.S. MARTIN AND M.J. DAY

SUMMARY

Methods of survival analysis developed for human clinical data are unable to deal effectively with animals euthanased due to the disease in question, a form of 'informative censoring'. This paper describes a retrospective analysis of data from 124 dogs with pericardial disease in which more than half failed to survive, of which a high proportion were euthanased. Survival analysis was carried out using three classifications of animals reaching the endpoint. When animals dying naturally were defined as 'failures' the analysis lacked power. Inclusion of euthanased animals as 'failures' increased power, and the ranking of estimates between strata tended to be conserved, but some prognostic indicators for death became less significant. Whichever method is used to classify euthanased animals for survival analysis, the definitions used must be carefully reported and inferences drawn with caution. Analytical methods for datasets involving informative censoring need to be developed and made readily accessible.

INTRODUCTION

Problems in the application of methods of survival analysis in companion animal epidemiology have been described by Hosgood and Scholl (2001). In particular, methods developed for the analysis of human clinical data are unable to deal effectively with the problems posed by animals euthanased due to the disease in question. Observations from euthanased animals may be deleted from the dataset, right-censored at the time of euthanasia (right censoring occurs when survival time is known only to exceed a certain value (Leung et al., 1997)), or included as cases meeting the endpoint criterion ('failures'). The problem is that euthanasia is not an endpoint determined by disease pathology alone, but by a complex interplay of factors relating to the disease, the animal, the owner, financial constraints, and the veterinary surgeon. This gives rise to a form of 'informative censoring'. Therefore, all of the common methods of accounting for euthanased animals will result in biases in the estimation of survival time. These problems are compounded by other common features of longitudinal datasets: interval censoring (animals are not continuously observed, but only examined at certain intervals); a proportion of animals are generally 'lost to follow-up'; others may die or be euthanased due to other diseases; or the cause of death or euthanasia may be unknown.

Pericardial diseases account for approximately one percent of all cardiovascular diseases affecting small animals. Most common are the diseases causing pericardial effusion and cardiac tamponade (the decompensated phase of cardiac compression), especially benign idiopathic

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pericardial effusion and neoplastic and infectious pericarditis (Miller & Sisson, 1995). Pericardial effusion develops most commonly as a result of neoplasia, but idiopathic pericarditis is relatively common in large-breed male dogs. Dogs with effusion secondary to neoplasia are reported to have a poorer prognosis for long-term survival (Dunning et al., 1998*)*.

This study involved a retrospective analysis of the records of 124 dogs with pericardial disease, which provided a good example of the problems highlighted by Hosgood and Scholl (2001): a high proportion of the dogs failed to survive until the end of the study period, and of these most were euthanased.

MATERIALS AND METHODS

Cases of canine pericarditis for this study were selected from the records of 115 adult dogs referred to the Veterinary Cardiorespiratory Centre, Kenilworth, between January 1994 and January 2002, together with 28 cases from other referral centres in the UK. Only cases with primary pericardial disease were included in the analysis; dogs with pericardial effusion secondary to other causes, such as dilated cardiomyopathy or hypoproteinaemia, were excluded.

A total of 143 cases of pericarditis were identified, of which 124 were defined as having primary pericarditis. The records were examined retrospectively and additional information obtained by telephone contact with the owners and their veterinary surgeons. For many of the analyses, cases were categorised into those in which a mass was seen on echocardiography and those in which no mass could be identified. In addition, idiopathic pericarditis cases were defined as those with no mass detectable on echocardiography and satisfactory follow-up (survival longer than 12 months) or histological confirmation. Remaining cases were categorised as 'indeterminate' or infectious.

Data were recorded in a MS Access 97 database (Microsoft Corporation), and survival analysis was carried out using Stata version 7 (Stata Corporation). Twenty-nine variables related to signalment, clinical signs and examination findings were evaluated for their prognostic usefulness using Kaplan-Meier analytical methods: calculation of the survival function, log-rank tests (using a significance level of $P<0.05$) and calculation of univariable hazard ratios. This was followed by forward stepwise Cox proportional hazards regression including a test of the proportional hazards assumption for the final models. Interactions were tested in the final models developed with each dataset. Models were developed initially using only presenting signs and then using both presenting signs and the diagnostic category or the results of echocardiography.

All dogs lost to follow-up and those alive at the end of the study were right-censored at the time of last examination. The survival analysis was carried out using three datasets with different classifications of the endpoint ('failure'):

1. All dogs dying naturally due to any cause (all euthanased dogs right-censored) (*n = 18)*
2. All dogs dying naturally or euthanased due to any cause ($n = 81$)
3. All dogs dying or being euthanased due to pericarditis (dogs with other or unknown cause of death/euthanasia right-censored) ($n = 52$).

RESULTS

In total, 81 of the 124 primary pericarditis cases available for analysis (65%) were known to fail to survive to the end of the study period, and of these a high proportion (63/81; 78%) were euthanased. The cause of death was recorded to be pericarditis for 12 of the 18 dogs that died naturally; the cause of death for the other six was unknown. Of the 63 dogs that were euthanased, the reason was recorded as pericarditis for 40 dogs, as another known cause for four dogs, and the cause was unknown in 19 cases. Of the 124 cases analysed, 42 were diagnosed with a mass by echocardiography or histopathology, 56 were diagnosed with idiopathic pericarditis, 25 were classified as indeterminate and one as infectious pericarditis; the latter two categories were combined in the analysis of diagnostic category.

Table 1 shows a selection of the results of the univariable analysis using the three datasets. When euthanased dogs were right-censored rather than considered to reach the endpoint (dataset one) the analysis lacked power and frequently failed to reach estimates of median survival time, especially for variables of more than two categories, such as gender. However, the ranking of the estimates for median survival time, particularly for dichotomous variables, were generally conserved between all the datasets. Tables 2 and 3 show the results of the Cox proportional hazards regression analysis. No interaction terms were significant in the final models.

The presence of an identifiable or suspicious mass on echocardiography was an important prognostic factor (Fig. 1) in all three datasets. Various presenting signs (particularly weakness, ascites and weak pulse) were also useful prognostic indicators. Weakness was associated with decreased survival (Fig. 2), whereas the presence of ascites (Fig. 3) or a weak pulse was associated with increased survival.

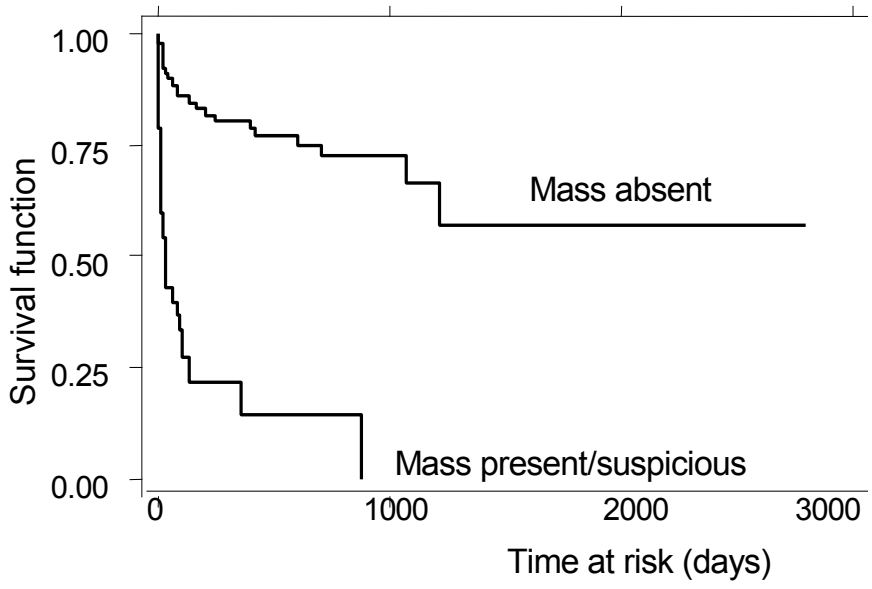

Fig. 1 Kaplan-Meier estimates of survival function, by presence or absence of a mass on echocardiography (dataset 3)

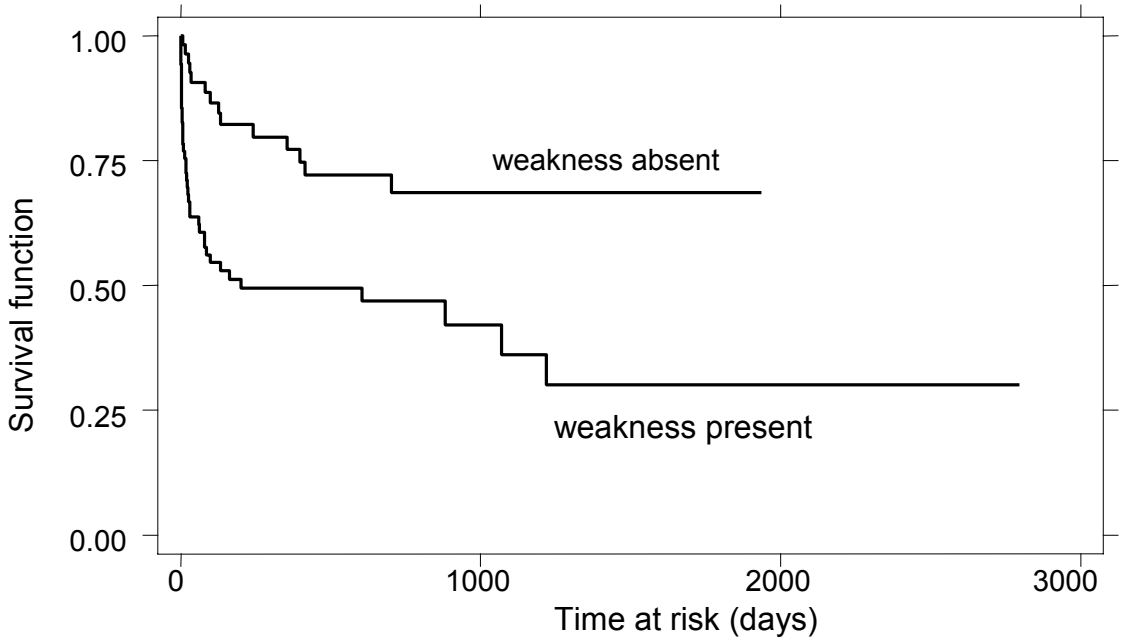

Fig. 2 Kaplan-Meier estimates of survival function, by presence or absence of weakness on presentation (dataset 3)

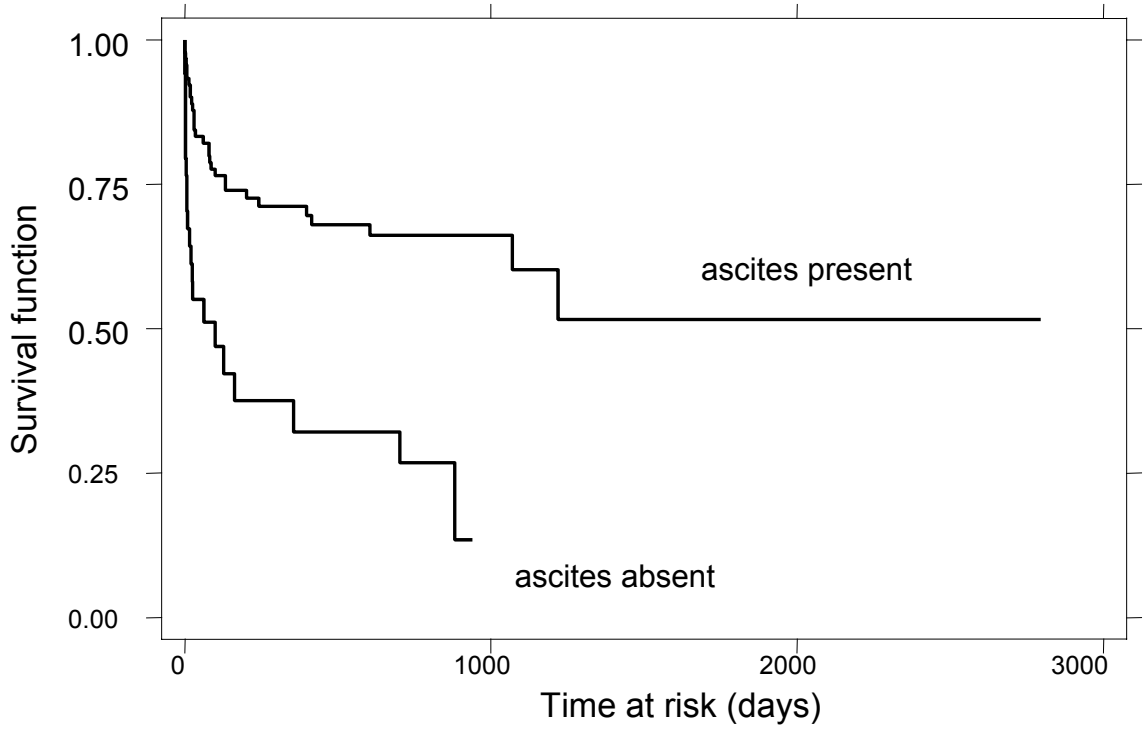

Fig. 3 Kaplan-Meier estimates of survival function, by presence or absence of ascites on presentation (dataset 3)

Table 1. Kaplan-Meier analysis of selected presenting signs and diagnostic category using three datasets with different definitions of endpoint (failure) (124 cases)

Endpoint:	All died (n = 18)			All died and euthanased (n = 81)			Died or euthanased due to pericarditis (n = 52)		
Variable	MST[a] (95% CI)	Rate ratio (95% CI)	P-value[c]	MST[a] (95% CI)	Rate ratio (95% CI)	P-value[c]	MST[a] (95% CI)	Rate ratio (95% CI)	P-value[c]
Weakness	2216 (1218-)	3.4 (1.1-14.1)	0.04	115 (31-296)	2.0 (1.3-3.4)	0.003	202 (60-1218)	2.6 (1.4-5.2)	<0.001
No	-			1205 (355-)			-		
Cough	2796	0.2 (0.01-1.0)	0.01	421 (98-1824)	0.9 (0.5-1.5)	0.60	- (126-)	0.7 (0.3-1.5)	0.4
No	2216 (1218-)			242 (104-731)			1069 (242-)		
Ascites	2216 (1218-)	0.2 (0.1-0.7)	0.001	631 (296-1218)	0.3 (0.2-0.4)	<0.001	- (1069-)	0.2 (0.1-0.3)	<0.001
No	-			45(8-115)			100 (9-702)		
Weak pulse	2796	0.26 (0.1-0.9)	0.01	882 (134-1935)	0.5 (0.3-0.9)	0.03	- (882-)	0.4 (0.2-0.8)	0.03
No	2216 (1218-)			184 (80-414)			605 (126-)		
Collapse	- (162-)	1.8 (0.4-5.6)	0.4	30 (8-104)	2.8 (1.7-4.5)	<0.001	31 (8-202)	3.9 (2.1-7.0)	<0.001
No	2216 (1218-)			631 (242-1205)			1218 (882-)		
Mass on echo[b]	-	1.8 (0.2-7.8)	1.0	28 (8-80)	12.1 (7.5-19.2)	<0.001	30 (8-87)	20.1 (11.2-36.6)	<0.001
No	2216 (1218-)			1069 (605-1824)			- (1069-)		
Category:									
Pericarditis	2216 (1218-)	Baseline	0.005	1218 (1069-1941)	Baseline	<0.001	- (1218-)	Baseline	<0.001
Tumour	-	2.5 (0.7-9.4)		28 (8-60)	12.3 (7.1-21.2)		30 (8-87)	29.4 (13.0-66.9)	
Indeterminate	414 (115-)	9.1 (3.3-25.2)		115(60-202)	12.5 (6.9-22.7)		242 (81-414)	20.9 (8.4-51.8)	

[a]Median survival time; days, with 95% confidence interval where possible

[b]Mass seen or suspected on echocardiographic examination

[c]P-value calculated from log-rank test

Table 2. Cox proportional hazards regression analysis of selected variables and presenting signs using three datasets with different definitions of endpoint (failure) (124 cases)

Endpoint:		All died (n = 18)			All died and euthanased (n = 81)			Died or euthanased due to pericarditis (n = 52)		
Variable		Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood[b]	Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood[b]	Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood[b]
Age		0.8 (0.7-1.0)	0.03	-65.1	1.1 (1.0-1.2)	0.03	-327.8	1.1 (1.0-1.2)	0.2	-226.2
Sex:	F	Baseline			Baseline			Baseline		
	FN	0.5 (0.1-3.2)	0.5		1.3 (0.6-2.9)	0.5		1.2 (0.5-2.8)	0.7	
	M	0.5 (0.1-1.9)	0.3		0.6 (0.3-1.2)	0.1		0.5 (0.2-1.0)	0.05	
	MN	0.2 (0.02-2.1)	0.2	-66.6	0.7 (0.3-1.7)	0.5	-327.7	0.6 (0.2-1.5)	0.3	-223.3
Ascites		0.2 (0.1-0.6)	0.003	-63.8	0.3 (0.2-0.5)	<0.001	-321.6	0.3 (0.2-0.5)	<0.001	-219.4
Weak pulse		0.2 (0.04-0.8)	0.02	-63.9	0.6 (0.4-0.9)	0.03	-328.7	0.5 (0.3-0.9)	0.03	-224.9
Weakness		3.2 (1.0-10.2)	0.05	-65.4	2.1 (1.3-3.4)	0.003	-326.6	2.8 (1.5-5.1)	0.001	-221.6
Ascites		0.2 (0.1-0.6)	0.003		0.3 (0.2-0.5)	<0.001		0.3 (0.2-0.5)	<0.001	
Weakness		3.3 (1.0-10.2)	0.04	-61.5	2.1 (1.3-3.4)	0.03	-316.8	2.8 (1.5-5.2)	0.001	-213.4
Weakness		7.4 (1.8-31.2)	0.006		2.3 (1.4-3.7)	0.001		3.3 (1.7-6.2)	<0.001	
Ascites		0.08 (0.02-0.3)	<0.001		0.3 (0.2-0.5)	<0.001		0.2 (0.1-0.4)	<0.001	
Weak pulse		0.08 (0.01-0.4)	0.01 (0.98[c])	-54.8	0.5 (0.3-0.8) (0.56[c])	0.007	-312.8	0.4 (0.2-0.8)	0.006 (0.60[c])	-209.1

[a]P-value for inclusion of variable in model (Wald test)

[b]Log likelihood for model

[c]Test of proportional hazards assumption for final model

Table 3. Cox proportional hazards regression analysis including diagnostic category variables or results of echocardiographic examination (124 cases)

Endpoint:	All died (n = 18)			All died and euthanased (n = 81)			Died or euthanased due to pericarditis (n = 52)		
Variable	Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood	Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood	Hazard ratio (95% CI)	P-value[a] (Phtest[c])	Log-likelihood
Categ: Pericarditis	Baseline			Baseline			Baseline		
Tumour	2.1 (0.5-8.6)	0.3		9.2 (5.2-16.4)	<0.001		17.9 (7.7-41.7)	<0.001	
Indeterminate	6.6 (2.1-21.0)	0.001	-62.8	7.3 (3.9-13.9)	<0.001	-295.4	9.1 (3.5-23.4)	<0.001	-195.5
Mass on echocardiography	1.0 (0.2-4.8)	1.0	-67.6	6.4 (3.9-10.6)	<0.001	-301.3	8.0 (4.4-14.5)	<0.001	-204.4
Categ: Pericarditis	Baseline			Baseline			Baseline		
Tumour	1.0 (0.2-4.4)	1.0		6.9 (3.8-12.4)	<0.001		12.7 (5.3-30.2)	<0.001	
Indeterminate	6.5 (1.9-22.4)	0.003		6.9 (3.6-13.2)	<0.001		8.8 (3.4-22.8)	<0.001	
Weakness	16.1 (2.7-97.3)	0.002		2.2 (1.3-3.6)	0.003		2.8 (1.4-5.3)	0.002	
Ascites	0.1 (0.01-0.3)	0.001		0.4 (0.3-0.7)	0.004		0.4 (0.2-0.7)	0.002	
Weak pulse	0.1 (0.01-0.5)	0.006 (0.84[c])	-50.1	0.6 (0.4-1.0)	0.07 (0.004[c])	-285.43	0.5 (0.3-1.0)	0.04 (0.04[c])	-185.3
Mass on echocardiography	0.4 (0.1-2.2)	0.3		4.5 (2.7-7.6)	<0.001		5.3 (2.8-10.0)	<0.001	
Weakness	8.0 (1.9-34.2)	0.005		2.0 (1.2-3.3)	0.007		2.6 (1.3-4.9)	0.004	
Ascites	0.07 (0.02-0.3)	<0.001		0.4 (0.3-0.8)	0.003		0.4 (0.2-0.7)	0.004	
Weak pulse	0.07 (0.01-0.4)	0.003 (0.3[c])	-54.2	0.5 (0.3-0.9)	0.02 (0.3[c])	-292.5	0.5 (0.2-0.9)	0.02 (0.2[c])	-195.6

[a]P-value for inclusion of variable in model (Wald test)

[b]Log likelihood for model

[c]Test of proportional hazards assumption for final model

DISCUSSION

As reported by other authors (Dunning et al., 1998), analysis of all three datasets in this study indicated that the presence of a neoplastic lesion, usually identified as a mass lesion on echocardiography, was significantly associated with a shorter survival time. Aggressive neoplastic conditions frequently result in bleeding into the pericardial sac, with the rapid accumulation of pericardial fluid and cardiac tamponade (Miller & Sisson, 1995).

Some clinical presenting signs, particularly ascites, weakness and weak pulse, were also significantly associated with survival time. Ascites and weak pulse were positive prognostic indicators, possibly due to an association with more benign cardiac disease, often even when diagnostic category or the presence of a mass lesion was included in the regression model. The finding that dogs with ascites on first presentation were significantly more likely to survive was also noted by Dunning et al. (1998). Slow accumulation of pericardial fluid is more likely to occur in chronic disease and to be characteristic of a less aggressive disease process. When pericardial fluid accumulates slowly, the pericardium is able to accommodate to the fluid without initially decreasing cardiac output, so that the dog gradually develops the signs of right-sided congestive heart failure (Miller & Sisson, 1995). Weakness and/or collapse were associated with a poorer prognosis, perhaps because they were more likely to indicate an acute onset of pericardial effusion.

Age and gender were not important prognostic indicators in any dataset, but there was some suggestion that female dogs with pericarditis were more likely to have a poorer prognosis, probably because of the association of the more benign idiopathic pericarditis with male dogs (Miller & Sisson, 1995).

Datasets one and two were used to compare alternative methods of handling data from euthanased animals. Observations from euthanased animals may be deleted from the dataset (White, 1991), right-censored at the time of euthanasia (as in dataset one), or more commonly, included as cases meeting the endpoint criterion (as in datasets two and three) (Hammer et al., 1995; Dunning et al. 1998). Whichever method is chosen, it is important to describe the method of accounting for euthanased animals in a survival analysis (Hosgood & Scholl, 2001). Exclusion of euthanased animals from the current dataset was not attempted, as this would have resulted in deletion of half of the observations. In addition, the deletion of censored observation has been demonstrated to result in underestimation of survival time and reversal of the ranking of point estimates (Hosgood & Scholl, 2001).

When euthanased dogs were right-censored rather than being considered to reach the endpoint (dataset one) the analysis lacked power and frequently failed to reach estimates of median survival time because the probability of survival did not decrease below 0.5. Similarly, the regression models obtained using this dataset often failed to converge or gave very high standard errors. This was especially noted when the variable representing the presence of a mass was included in the model, which is presumably due to the fact that most animals in which neoplasia was diagnosed were euthanased rather than being allowed to die naturally. Only two of the 40 animals in which a mass was diagnosed reached the endpoint of natural death, whereas 16 were euthanased. This problem is common in veterinary studies of neoplastic disease, where animals tend to be euthanased rather than dying naturally (Hosgood & Scholl, 2001).

Censoring should be carried out under the assumption that time of censoring is independent of survival time (Leung et al., 1997). This is likely to be true of right-censoring due to study termination, but not necessarily true of right-censoring due to loss to follow-up, and is certainly not true of right-censoring due to euthanasia for the disease in question (Hosgood & Scholl, 2001). If censoring is non-independent, and hence informative (censoring provides more information than simply that survival exceeded a certain time) then standard Kaplan-Meier methods will give biased estimates (Leung et al., 1997). If the survival and censoring time are positively correlated then Kaplan-Meier methods tend to overestimate the survival function, and vice versa.

Methods for testing the assumption of independent censoring and analysis of datasets with informative censoring do exist (reviewed by Leung et al., 1997), but are not yet readily available in widely-used statistical packages. Such techniques include modelling the joint distribution of the survival and censoring times (Moeschberger, 1974), and a semiparametric model called the 'cone model' which includes a scalar parameter which reflects the degree to which censoring affects survival (Lagakos & Williams, 1978). It has also been suggested that it is possible to estimate the bounds of the survival function without assuming any censoring mechanism or parametric distribution for the survival time (Peterson, 1976). Alternatively, the survival function can be estimated under a nonparametric 'stretch/contract' model in which censoring coincides with an event that alters subsequent survival times by a known scale parameter (Fisher & Kanarek, 1974). It is also possible to generalise the Kaplan-Meier estimator in informative censoring situations where certain measures of the dependence of survival and censoring times are known (Slud & Rubenstein, 1983; Klein & Moeschberger, 1988). Most of the above methods assume that the censored cases are either all informative or all non-informative, which is not always the case, and rely on the assumption of a fixed model parameter, which will be rarely known in practice. Alternatively, if covariates are available, it may be possible to identify a surrogate variable measured on the censored subjects and use it to predict residual survival time (Cox, 1983).

When euthanased animals were treated as reaching the endpoint (datasets two and three) a more complete analysis was possible, and unlike the datasets analysed by Hosgood and Scholl (2001), the ranking of the estimates of median survival time for strata tended to be conserved whichever dataset was used. However, some variables, such as the presence of a cough, were significant prognostic indicators for natural death, but were less so when euthanased cases were also considered to be 'failures'. Because euthanasia is not an endpoint determined solely by disease pathology, equating observations from animals that die with those that are euthanased will result in biased estimates of the survival function and median survival time (Hosgood & Scholl, 2001). The methods and conclusions of such an analysis need to be reported with care, and comparability between studies will be reduced.

The classification of animals dying or being euthanased due to unknown or other causes is also important, and such cases are often right-censored, as in dataset three and the paper by Dunning et al. (1998). The results of the analysis of the current data were not substantially different between datasets two and three, but the classification of animals according to cause of death may become more important when the disease under consideration has a lower attributable mortality. Alternatively, animals dying of unknown causes may be included with those reaching the endpoint if it is suggested from clinical considerations that their death is likely to have been associated with the disease in question (Schwarz et al., 1991).

Problems may arise when the probability of euthanasia is associated with the type of disease. For example, dogs in which a mass lesion was detected upon echocardiography were more likely to be euthanased than to die naturally. Similarly, the type of disease may be associated with survival time itself, as was the case with the diagnostic category (neoplasia-related, idiopathic or 'indeterminate' pericardial disease) in this dataset. The designation of an idiopathic pericarditis case was partly determined by a longer survival time. This may have been a factor in the significance of the test of the proportional hazards assumption, indicating that the assumption of proportional hazards was violated in models including diagnostic category when euthanased animals were treated as reaching the endpoint (datasets two and three).

In conclusion, the use of observations from euthanased animals as though they had reached the endpoint, as in most studies, results in biased estimates of survival time. However, right-censoring these observations may result in a lack of power in the analysis and will also result in biased estimation if methods to account for informative censoring are not used. It is to be hoped that robust and accessible methods will soon be developed for the analysis of survival data in which a large proportion of the observations undergo informative censoring. Until this occurs, some consensus must be reached on the method of classification of euthanased animals in survival analysis so that consistency and comparability between studies can be improved.

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FOOT AND MOUTH DISEASE

THE EFFECT OF SPEED OF ANIMAL SLAUGHTER ON INFECTED PREMISES AND THE INTENSITY OF CULLING ON OTHER PREMISES ON THE RATE OF SPREAD OF FOOT AND MOUTH DISEASE

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SUMMARY

During the foot and mouth disease outbreak in the UK in 2001, the main control policies were rapid case finding, killing of animals on infected premises (IPs) and the culling of animals on dangerous contact (DC) premises. DCs can be divided into two groups, premises contiguous to an IP (CPs) and non-contiguous premises (NonCPs). The largest clusters in England were in Cumbria, Wessex (Somerset/Devon/Cornwall) and Settle. For each, estimated dissemination rate (EDR), average time from first lesion to slaughter lagged by one week (FLtoSLag), overall DC culling intensity (DC:IP ratio), CP culling intensity (CP:IP ratio) and NonCP culling intensity (NonCP:IP ratio) were calculated on a weekly basis. Linear regression was used to investigate any effect of FLtoSLag, DC:IP, CP:IP and NonCP:IP on EDR. FLtoSLag has a statistically significant effect in 2 of the 3 clusters and NonCP:IP in one. CP:IP did not show a significant effect in any of the 3 clusters. Monitoring FLtoSLag and NonCP:IP in real time during an epidemic would be a valuable tool for evaluating and directing control efforts.

INTRODUCTION

The foot and mouth disease (FMD) epidemic in the United Kingdom (UK) in 2001 was one of the most severe outbreaks seen in a country normally free of FMD. The widespread initial seeding of the disease before the first IP was confirmed led to a rapid rise in the number of cases during the first weeks in widely separated geographical areas. This gave the impression of an epidemic out of control which, together with the suspicion that FMD was spreading unseen through sheep flocks, led to the adoption of new control policies based on the output from mathematical models (Anderson, 2002). The contiguous premise (CP) culling policy was the most important and novel aspect of these. Under this policy, all premises contiguous to an IP were regarded as DCs (and therefore all susceptible stock culled) unless a very strong case could be made that this should not be carried out.

At the same time as this policy emerged, the State Veterinary Service was working to reduce the time between report of disease and killing of livestock on confirmed IPs (i.e. the speed of culling). This is known to be important in the control of the disease (Northumberland, 1969). The longer diseased livestock remain on a farm, the greater the likelihood that the virus will 'escape' to neighbouring and more distant farms. This risk will be a function of time and the

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numbers and species of animals shedding virus. Many epidemiologists have observed, particularly in cattle, that there is an increase over time in the prevalence of animals with clinical lesions, which is the period when maximum virus shedding occurs (Donaldson, 1987; Alexandersen et al., 2002). Reducing time from first lesion to slaughter will decrease the potential spread of the virus from a farm both by shortening shedding time and by decreasing maximum virus output.

Premises where susceptible livestock were judged to have been exposed to infection (via the movement of animals, people, vehicles, fomites, *etc.*) were subject to culling as dangerous contacts (DCs). This decreased the potential spread by removing premises that may be incubating the disease. Culling of stock on premises contiguous to an IP (CPs) is a particular form of DC culling.

From this description, the separate effects of speed of slaughter on IPs and DC culling on the spread of the epidemic would be seen at different times. Culling of DCs should have had a rapid effect by removing already infected farms which would have become clinically affected IPs in the next few days. Speed of IP slaughter would have a more delayed effect by preventing the spread of infection to other farms which would become IPs after a full incubation period plus time from first lesion to report.

The Estimated Dissemination Rate (EDR) is defined as the number of cases occurring in one week divided by the number of cases in the previous week (Miller, 1976; Gibbens et al., 2001). In this paper, the number of cases used is the number of IPs with a first lesion date during that particular week. If the EDR is >1, this indicates that the number of cases is rising i.e. there is an upward trend to the epidemic curve and the disease is 'out of control'. An EDR of <1 indicates a declining epidemic curve. An effective control method should result in a decline in EDR and when all control methods are combined, EDR must be <1 if the disease is to be eradicated. The lower the value of EDR, the more rapidly this should be achieved.

The analysis in this paper looks for a relationship between EDR and the two major control policies which were the speed of slaughter as measured by days from first lesion to end of slaughter of IPs and the intensity of DC culling as measured by numbers of farms culled per IP reported during that week.

The three clusters included were the largest in England. Cumbria was by far the largest, as well as being one of the earliest. The Wessex cluster (Devon, Cornwall and Somerset) was the second largest cluster, also starting early in the outbreak, but peaking earlier at a lower number of IPs per day. The Settle cluster started later in the outbreak and was the third largest; it includes the original area around Settle which then spread down the Ribble valley towards Clitheroe.

The ways in which control policies were applied in each cluster and their occurrence and duration in relation to these was different. In Wessex, the outbreak started in late February and CP culling was significant from 29 March 2001, when the CP culling policy was announced. In Cumbria, the outbreak started at the end of February but, due to resource constraints, there was relatively little DC culling before 20 April 2001. The CP culling policy was amended on 27 April 2001 to allow additional exemptions based on veterinary judgement; so most CP culling in Cumbria was undertaken when this amended policy was in force. The Settle outbreak began in May and there was CP culling throughout the outbreak, unlike Cumbria and Wessex.

In Cumbria, there was an additional form of cull in which all sheep, goats and pigs within 3km of an IP were culled. These have been excluded from this analysis because they are not related to any individual IP, geographically or temporally. Additionally, of around 1,600 premises from which animals were removed in this cull, 90% were left with susceptible stock because most farms that had sheep also stocked cattle.

MATERIALS AND METHODS

The following data were extracted from the Department for Environment, Food and Rural Affairs Disease Control System (DCS) and Final Epidemiology Report databases for all IPs and DC culls in three geographically separated outbreaks during the UK outbreak (Cumbria, Wessex [Cornwall, Devon and Somerset] and Settle [Settle/Clitheroe]). First lesion date was calculated in the databases by subtracting the age of the oldest lesion reported from the report date. Each DC cull was classed as either contiguous (CP) or non-contiguous (NonCP).

For each IP:	First lesion date, report date, species with oldest lesions, confirmation date, slaughter completion date,
For each DC cull:	Authorisation date, category of DC cull (CP / NonCP)

From this information, the following parameters have been calculated on a weekly basis for each cluster, starting from the date of the first lesion date of the first IP in the cluster.

Estimated Dissemination Rate (EDR)	$\frac{\text{Number of IPs by date of first lesion}}{\text{Number of IPs by date of first lesion in previous week}}$
First lesion to slaughter (FLtoS)	$\frac{\text{Sum of days from first lesion to end of slaughter}}{\text{Number of cases with first lesion}}$
FltoSLag	FLtoS in the previous week
DC:IP ratio (All DC culls)	$\frac{\textit{Total number of premises culled}}{\text{Number of confirmed report cases}}$
CP:IP ratio	$\frac{\text{Number of CP culls}}{\text{Number of confirmed report cases}}$
NonCP:IP ratio	$\frac{\textit{Number of NonCP premises culled}}{\text{Number of confirmed report cases}}$

The effect of FLtoSLag, DC:IP, CP:IP and NonCP:IP on EDR has been explored using linear regression (Statistix® 7.0, Analytical Software). Although EDR cannot be less than 0, it has no theoretical maximum. The same is true for the various culling ratios and FLtoSLag. The level for statistical significance was set at 95% confidence. For each cluster, only weeks with data for all parameters have been used in the regression analysis. Common data transformations were used in an attempt to obtain an improved fit (e.g. log transformation, square root), but the results obtained were no better than those obtained with no data transformation.

Additionally, seven day rolling averages of EDR, FLtoSLag, DC:IP, CP:IP and NonCP:IP have been calculated using totals over a seven day period centred on the day for which the value was calculated. These were used to plot smoothed graphs.

RESULTS

Table 1 summarises the numbers of IPs, species composition of affected animals and culls in each cluster. In both Cumbria and Settle, cattle were the commonest species with oldest lesions on a farm, but in Wessex there was a more or less 50:50 split between cattle and sheep. Cumbria had the lowest DC:IP ratio overall with Wessex and Settle being markedly higher. This was mainly because of a higher CP:IP ratio, although Wessex also had a higher NonCP:IP culling ratio. It is clear that the three clusters are different in terms of the species involved and the DC culling employed.

Table 1. Numbers of IPs, DC, CP and NonCP culls per cluster

	Cumbria	Wessex	Settle
No of IPs	892	185	101
% IPs, Cattle with oldest lesions	66.9%	52.4%	70.3%
% IPs, Sheep with oldest lesions	33.1%	47.6%	29.7%
DC culls (CP + NonCP)	953	855	446
CP culls	662	590	388
NonCP culls	291	265	58
Overall DC:IP ratio	1.07	4.68	4.42
Overall CP:IP ratio	0.74	3.19	3.84
Overall NonCP:IP ratio	0.33	1.49	0.56

Figures 1, 2 and 3 show seven day rolling averages of EDR and FLtoSLag by date for each cluster. In each, there is a visual impression that the two tend to vary together, with EDR falling as FLtoSLag falls. This is by no means always the case and there are occasions when one increases markedly whereas the other changes little or not at all. One example of this was in Cumbria in late April / early May where FLtoSLag showed a large spike with little increase in EDR. This was due to an IP discovered from serology during July, in sheep where the first lesion date was estimated to be 85 days previously when the last active disease was seen in the vicinity of this later IP. This had a significant effect on rolling averages but had less effect on the figures for seven day periods because the effect of that single IP was diluted.

Figures 4, 5 and 6 show seven day rolling averages of EDR and DC:IP and CP:IP ratios for the Cumbria, Wessex and Settle clusters. Note that DC:IP and CP:IP are shown on an inverted axis as the logical effect of a higher culling ratio would be a lower EDR. The DC:IP and CP:IP curves were very similar because CP culls were the majority of all culls, although this was less the case in Wessex where NonCP culling was more prominent. There is little visual impression that EDR varies as DC:IP or CP:IP ratio although there is some indication of this for DC:IP ratio.

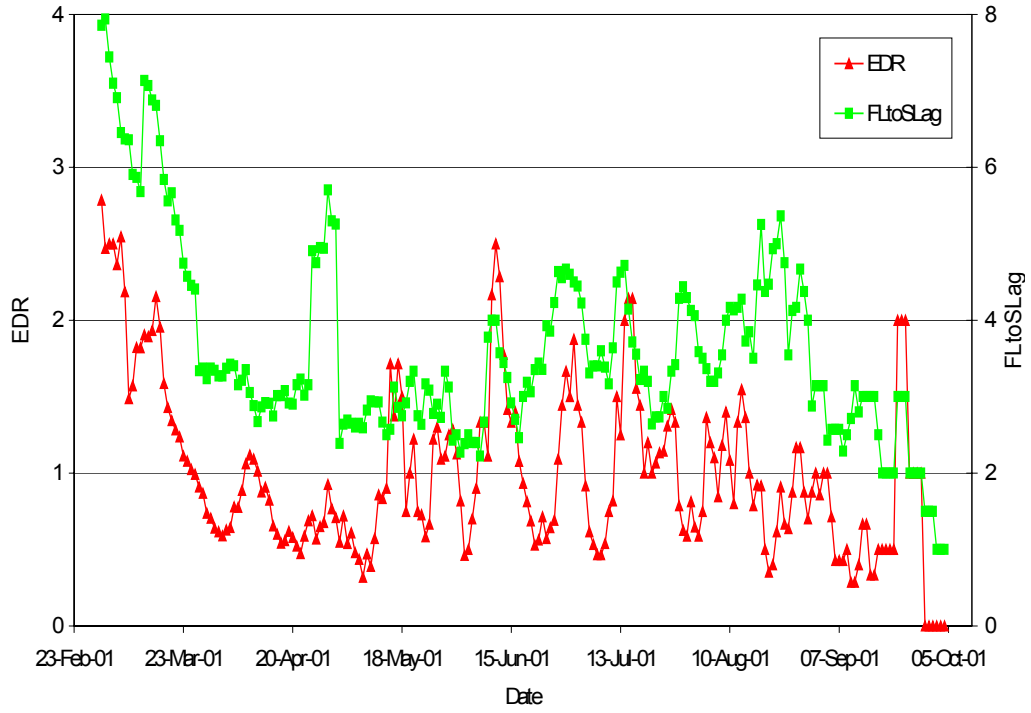

Fig. 1 EDR and FLtoSLag by date in Cumbria

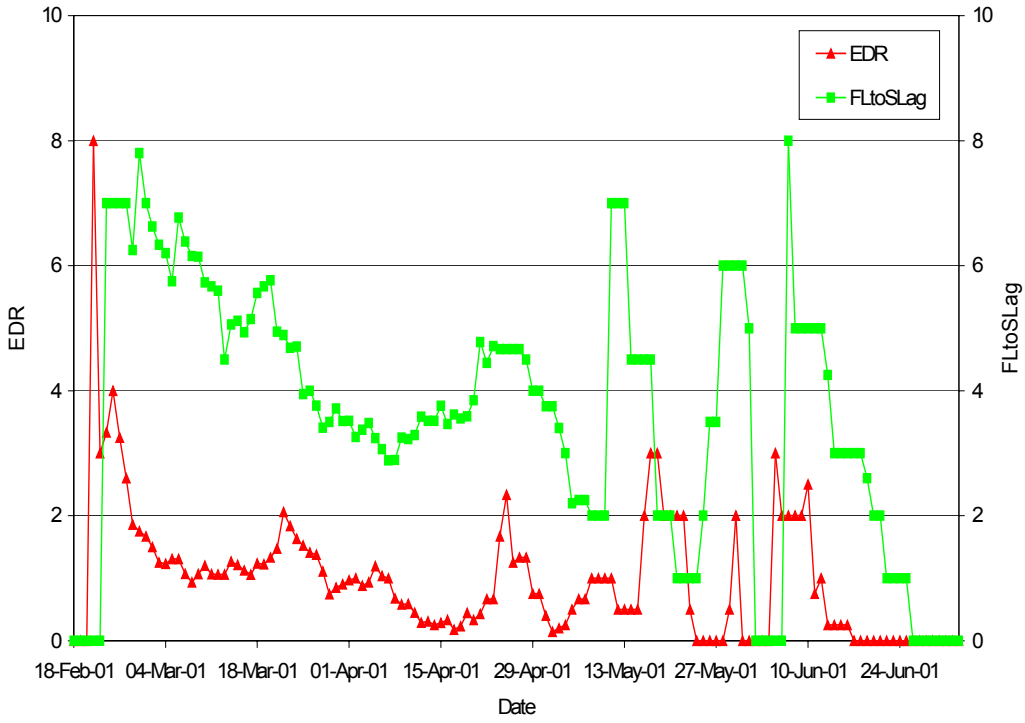

Fig. 2 EDR and FLtoSLag by date in Wessex

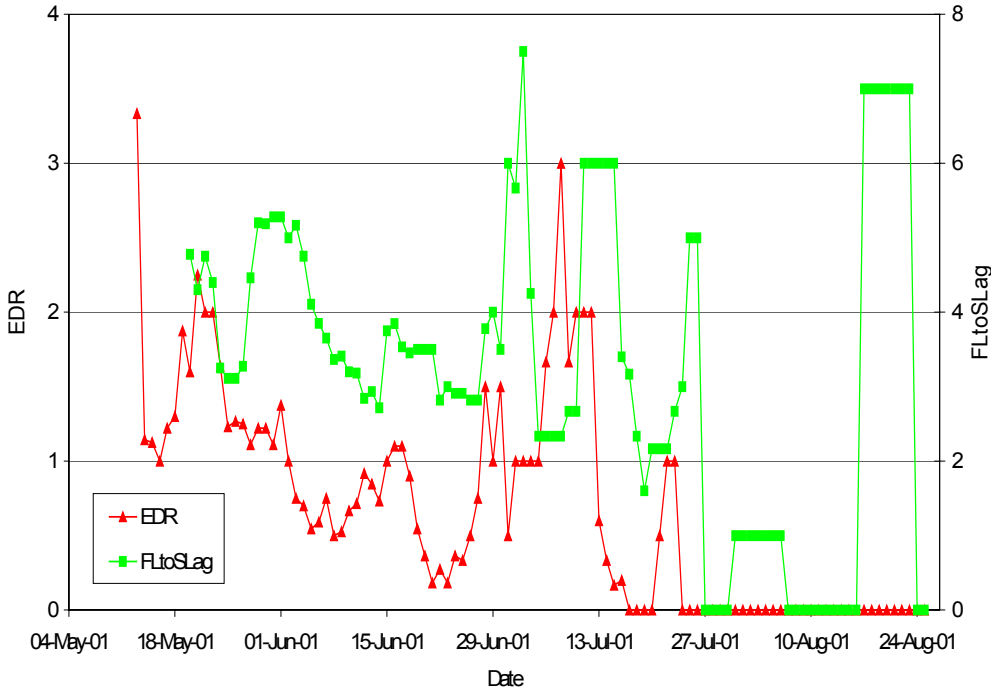

Fig. 3 EDR and FLtoSLag by date in Settle

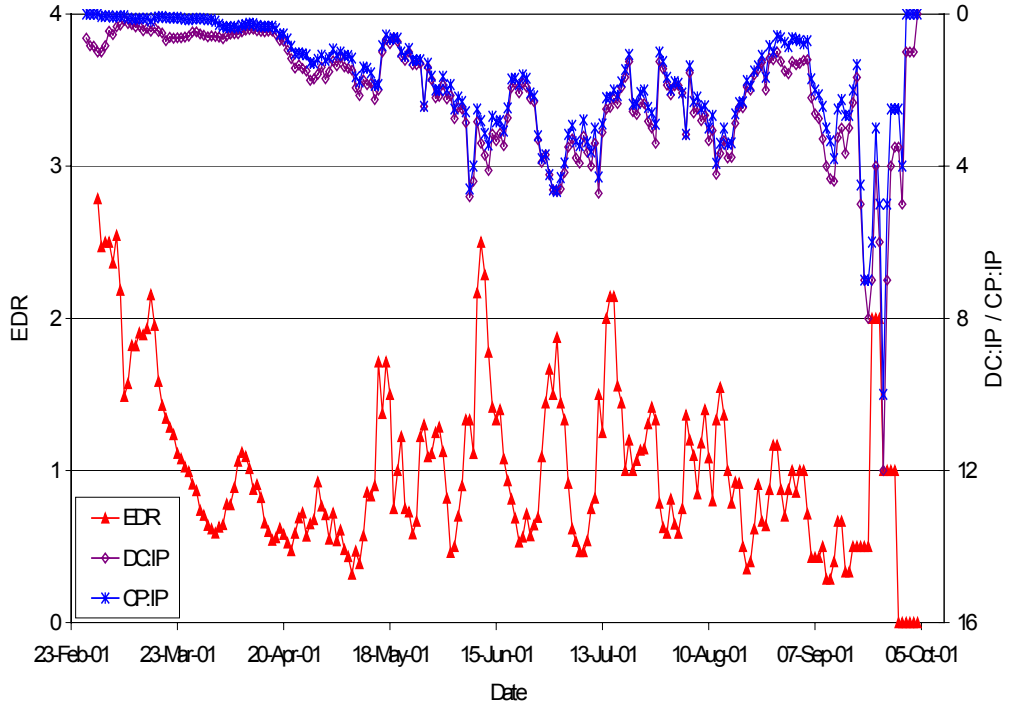

Fig. 4 EDR, DC:IP and CP:IP by date in Cumbria

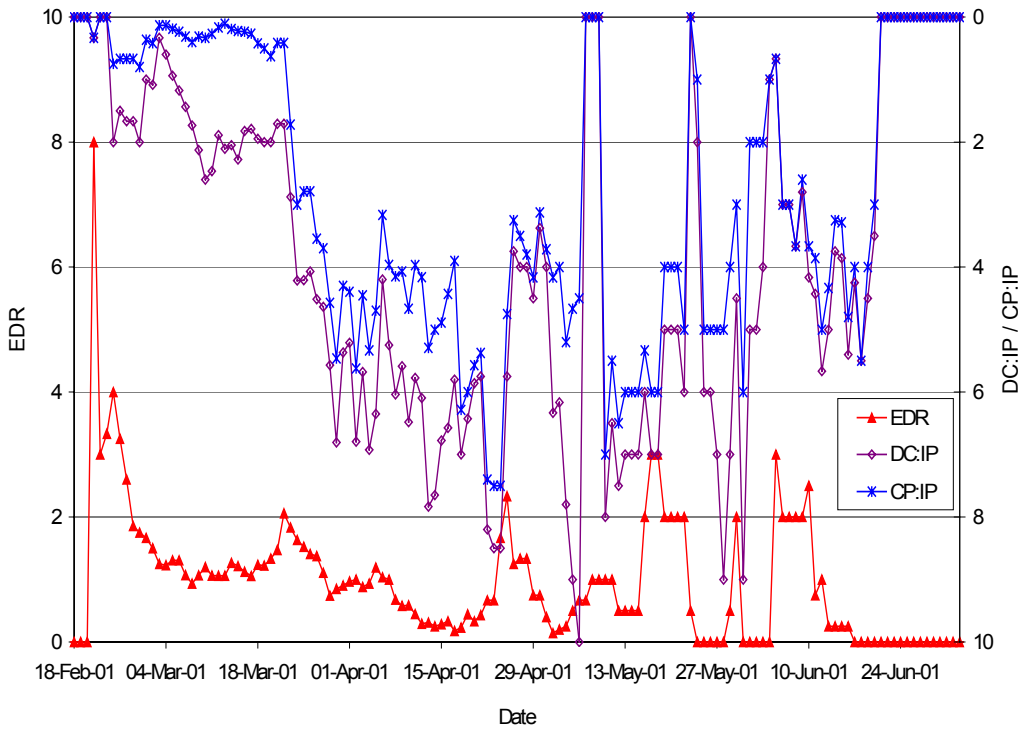

Fig. 5 EDR, DC:IP and CP:IP by date in Wessex

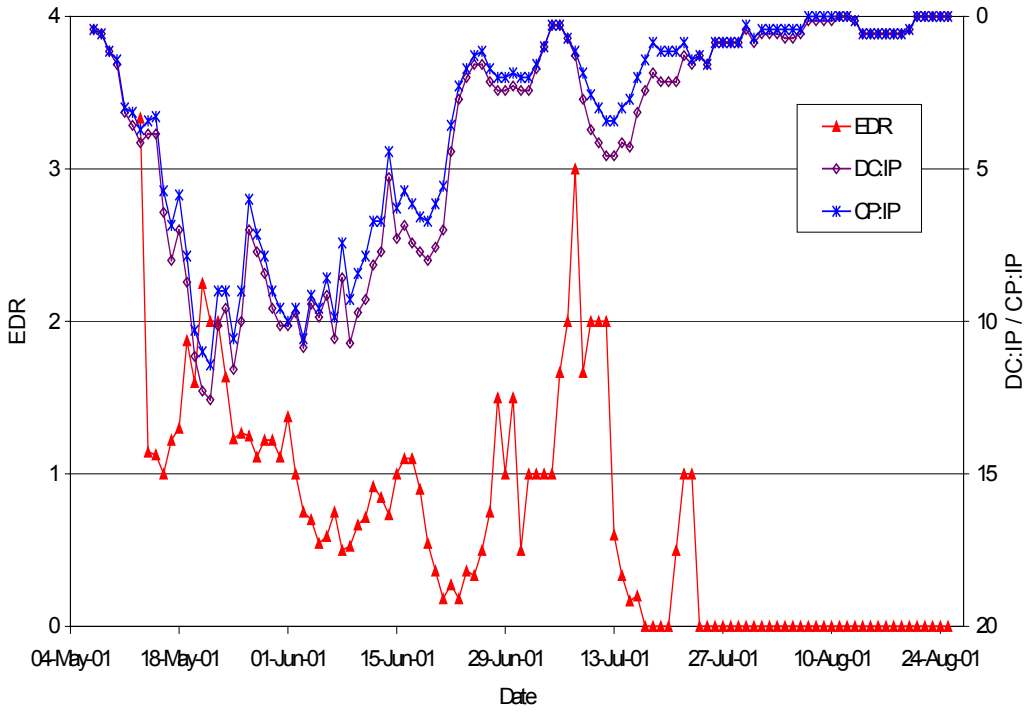

Fig. 6 EDR, DC:IP and CP:IP by date in Settle

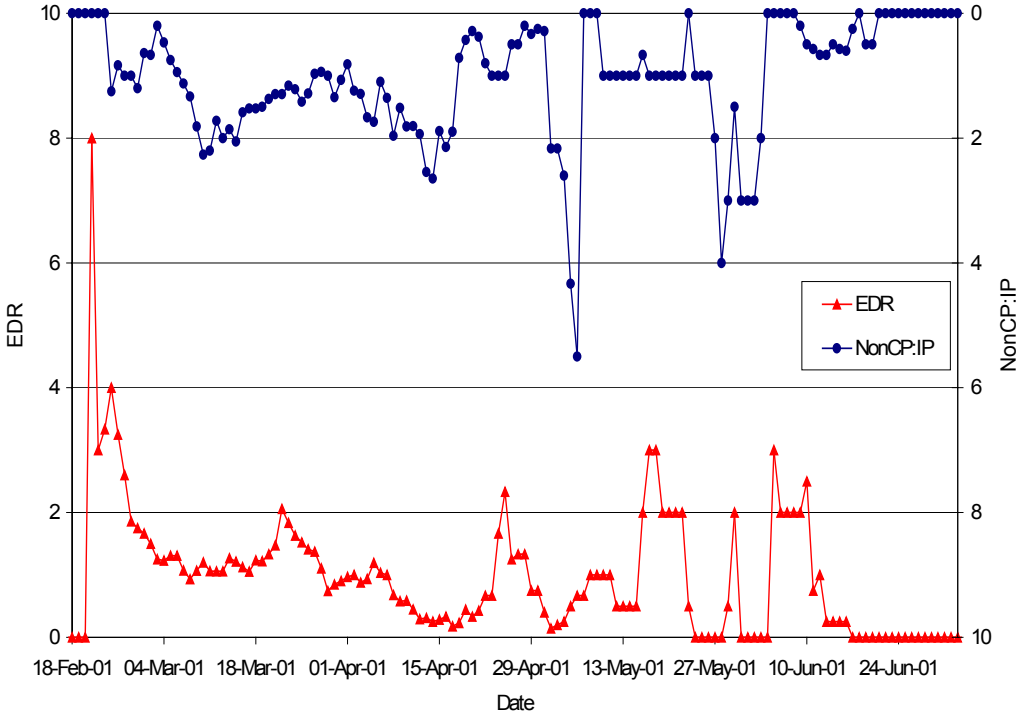

Fig. 7 EDR and NonCP:IP by date in Wessex

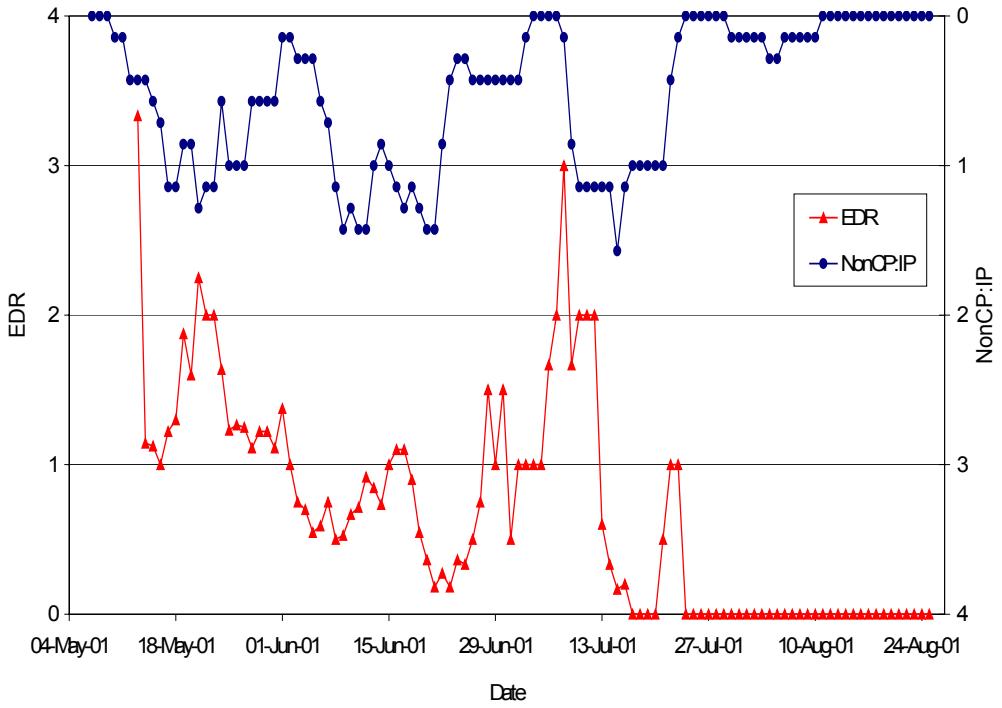

Fig. 8 EDR and NonCP:IP by date in Settle

Figures 7 and 8 show seven day rolling averages of EDR and NonCP:IP ratios for the Settle and Wessex clusters with an inverted axis for the cull ratio. Again, there is no strong visual

impression of EDR being affected by NonCP:IP ratio, although there may be some evidence for this in Wessex.

Table 2 summaries the range of seven day values for EDR, FLtoSLag and culling intensities used in the regression analysis. This again shows the differences between the clusters and that many of these values were not normally distributed. Cumbria had the lowest overall culling intensity but the highest maxima and range for DC:IP and CP:IP ratio. Settle had the narrowest range of EDR and FLtoSLag. It also had the highest minimum CP:IP ratio, which would be expected as the CP culling policy was in place before the first case was identified in this cluster.

Table 2. Summary of seven day parameters for the three clusters

Parameter	Cluster	n	Minimum	Median	Maximum
EDR	Cumbria	31	0.43	0.91	2.79
	Wessex	16	0.18	1.03	3.00
	Settle	10	0.00	1.06	2.00
FLtoSLag	Cumbria	31	2.00	3.20	7.86
	Wessex	16	1.00	4.04	8.00
	Settle	10	2.17	3.40	6.00
DC:IP	Cumbria	31	0.31	1.73	12.00
	Wessex	16	1.08	4.60	7.83
	Settle	10	2.00	4.36	8.17
CP:IP	Cumbria	31	0.00	1.64	10.00
	Wessex	16	0.24	3.58	6.00
	Settle	10	2.00	3.75	6.50
NonCP:IP	Cumbria	31	0.09	0.32	2.00
	Wessex	16	0.00	1.05	3.00
	Settle	10	0.00	0.68	3.50

Table 3 shows the results of the regression analysis including the separate components of the equation. The general form of the equation is:

$$\text{EDR} = \text{Constant} + (\text{Coefficient} \times \text{Parameter}) \quad (1)$$

The value r^2 is a measure of the proportion of the variation in EDR that could be due to variation in the parameter used in the analysis. The greater the value of r^2 the more likely it is that variation in the parameter would show a measurable change in EDR under field conditions. In the Cumbria and Settle clusters, FLtoSLag showed a statistically significant relationship (at the 95% level) to EDR and also accounted for a biologically significant amount of variation.

In Wessex, a statistically significant relationship was seen between EDR and NonCP:IP ratio, again accounting for a biologically significant proportion of the variation in EDR. None of the other regression calculations showed a statistically significant result at the 95% level although NonCP:IP was close to this threshold in Settle and DC:IP in both Wessex and Settle. The CP:IP ratio did not show a statistically or biologically significant relationship in any of the three clusters.

Table 3. Results of regression analysis for the three clusters (EDR as the dependent variable)

Control Measure	Cluster	n	Constant	Coefficient.	r^2	p-value
FLtoSLag	Cumbria	31	0.05	0.26	0.48	<0.0001
	Wessex	16	0.50	0.15	0.15	0.1392
	Settle	10	-0.23	0.32	0.32	0.0169
DC:IP	Cumbria	31	1.16	-0.06	0.07	0.1455
	Wessex	16	1.90	-0.15	0.21	0.0742
	Settle	10	1.98	-0.20	0.38	0.0583
CP:IP	Cumbria	31	1.16	-0.08	0.08	0.1300
	Wessex	16	1.49	-0.09	0.07	0.3250
	Settle	10	1.82	-0.21	0.18	0.2192
NonCP:IP	Cumbria	31	1.09	-0.18	0.02	0.4970
	Wessex	16	1.72	-0.44	0.27	0.0378
	Settle	10	1.35	-0.37	0.36	0.0644

The outbreak in Cumbria can be split into two periods, before and after 20 April 2001. It was from this time that a significant intensity of DC culling started to be undertaken as shown by Fig. 4. However, as shown in Figs 1 and 4, by this time both EDR and FLtoS had fallen substantially from their highest levels and any analysis after this time included only around 50% of the range of values in these two parameters over the whole length of the outbreak. No statistically significant relationships were seen in the period after 20 April 2001. In the period before 20 April 2001, there was a highly significant relationship between EDR and FLtoS ($n = 7$, constant –0.45, coefficient = 0.37, $r^2 = 0.89$, $p = 0.0015$). A statistically significant relationship was also seen between CP:IP and EDR ($n = 7$, $r^2 = 0.65$, $p = 0.0283$) but the full equation indicated that this is unlikely to be biologically plausible (constant = 2.56, coefficient = -6.21) as culling even one CP per IP would lead to a highly negative value for EDR.

DISCUSSION

The values shown in Tables 1 and 2 demonstrate that the three clusters were distinctly heterogeneous in composition and the application of control measures as well as their geographical location. This illustrates the need for data from different clusters to be analysed separately; they should not be aggregated.

In both Cumbria and Settle clusters, there was a statistically significant relationship between FLtoSLag and EDR, with EDR falling as FLtoSLag was shortened. This emphasises the known importance of early case detection and rapid slaughter of infected premises (Northumberland, 1969).

However, in Wessex, there was no statistically significant relationship between FLtoSLag and EDR although the seven day rolling average (Fig. 2) would seem to offer a reasonable fit. The reason for this may be that this cluster contained a high proportion of IPs on which sheep were reported as being the species with the oldest lesions (Table 1). This may have weakened the relationship between EDR and FLtoSLag because sheep produce less total virus (Sellers,

1971; Alexandersen et al., 2002) and so farms where sheep had the oldest lesions may have been less infectious to other farms.

The effect of DC culling on EDR is less clear. In Cumbria, there was no significant effect of culling ratios, overall or split into CP and NonCP. In both Wessex and Settle, overall DC:IP came close to significance at the 95% level. When this was split in to CP and NonCP culling ratios, it was the latter that had a significant effect in Wessex and it also seemed most important in Settle. There was no statistically significant effect of CP culling ratio on EDR in any of the three clusters.

It could be said that as the CP culling policy was not announced until 29 March 2001 and not implemented to any real intensity until 20 April 2001 in Cumbria (Fig. 4), analysis of CP:IP ratio should only have been carried out after these dates. There is some validity to this argument. However, it was also the case that, particularly in Cumbria but also in Wessex, most decrease in EDR was achieved during the earlier period. Three quarters of the IPs in Cumbria occurred before 20 April 2001, yet EDR fell to below 1 before this date. Woolhouse et al. (2001) reported that a related value, R_0, fell to below 1 in Cumbria during the week commencing 28 March 2001 which indicated that the epidemic in Cumbria was under control.

The analysis of the field data suggests that speed of slaughter and culling of known dangerous contacts can reduce EDR to below 1 and so control the disease. The need for a CP cull is not supported by the analysis presented in this paper. Why might there be this difference between the predictions of the models (Ferguson et al., 2001a; Ferguson et al., 2001b; Keeling et al., 2001) and the analysis of the field data?

To assess this requires a detailed knowledge of how the models were constructed, which is not always easily gleaned from the published papers. However, we would suggest several possible weaknesses in the models which may have contributed to this. Farm to farm infectivity was assumed to start early after the arrival of the virus on an IP and to be constantly maximal from then until slaughter. Disease was assumed to spread equally radially. The models were developed using data from early in the outbreak and therefore possibly were not able to take in to account the degree to which FLtoS was falling from the start of the outbreak in the existing clusters. There were also resource constraints in the entry of data into the DCS system during the early weeks of the epidemic, which will have affected the completeness of the data available for use in the models.

Anderson (2002) highlighted the shortcomings in data gathering and processing during the FMD outbreak, in particular in the first few weeks. Complete data were not available, nor were there staff to enter and analyse the information available. Decision makers did not have timely, accurate and relevant information about what was happening on the ground. In future, it will be important that 'real time' collection and analysis of data are available. Simple statistics such as EDR, FLtoSLag, DC:IP and NonCP:IP ratio can be easily calculated if basic data are recorded for each IP. This is best done locally as shown by the heterogeneous nature of the clusters and reinforced by the fact that data are best 'cleaned' as close to the point of collection as possible, both geographically and temporally. Interestingly, a simple DOS-based computer programme dating from the mid-1980s and simulating control of an exotic disease (Exotica) uses figures very similar to EDR and FLtoS as measures of evaluating disease control and may be a valuable training tool.

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QUANTIFICATION OF THE RISK OF FOOT AND MOUTH DISEASE ASSOCIATED WITH PROXIMITY IN SPACE AND TIME TO OTHER INFECTED PREMISES

N.M. TAYLOR*, N. HONHOLD, A.D. PATERSON AND L.M. MANSLEY

SUMMARY

This paper presents a spatio-temporal analysis which estimates the risk of disease for premises in the initially affected part of Cumbria during the foot and mouth disease epidemic in the United Kingdom in 2001. The analysis uses data derived from the Department for Environment, Food and Rural Affairs disease control system. The importance of proximity as a risk factor is quantified in terms of attributable risk. It is estimated that the spread of disease to just under half of the cases (48%) may be directly attributable to the fact that a recently infected premises was within 1.5km of the case farm.

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious disease spread by direct and indirect contact between infected and susceptible animals. The virus may be spread by the movement of infected animals, by short range aerosol transmission between contiguous groups of animals, by longer range airborne spread, by movement of contaminated animal products or by mechanical movement of virus by fomites such as contaminated vehicles and people (Donaldson, 1987). The potential for airborne spread of the strain of FMD virus involved in the 2001 epidemic in the United Kingdom (UK) was considered to be very limited (Donaldson et al., 2001) and Gibbens et al. (2001) reported that airborne spread of virus did not play a significant role in the epidemic.

During the recent UK FMD epidemic, following the controls on movement of livestock imposed on February 23, the disease continued to spread in the localities already infected prior to that date. Most of this spread could have been due to contact between contiguous groups of animals and virus transmission mediated by fomites, though some spread by clandestine movements of livestock or livestock products cannot be ruled out.

Throughout the epidemic, it was not possible to identify with any certainty specific sources of infection for most of the infected premises (Gibbens and Wilesmith, 2002). The term 'local spread' was frequently used in the field whenever an infected premise (IP) occurred within 3km of any previous IP and where no definite contact with a more distant source of infection could be identified. The assumption was that the source of infection was one of the recent nearby IPs.

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By definition, spread between contiguous groups of animals can only take place over a short distance and it is not unreasonable to assume that on average, fomite spread is more likely to occur over shorter, rather than longer, distances. The analysis presented here quantifies the rates of disease among premises at different distances from potential sources of infection and assesses the relative importance of spread, by whatever method, of FMD over different distances.

MATERIALS AND METHODS

A list of premises in Cumbria was obtained from the Geographic Information System (GIS) section at the Carlisle Disease Control Centre (DCC). This list provided:

- Unique identification code for premises (county/parish/holding (CPH) number);
- Location of premises, given by easting and northing (x - y) co-ordinates of the farm buildings.

Premises on this list had been allocated to different zones. These 'special epidemiological zones' (SEZ) were defined during the epidemic, based on 3km protection zones produced using the GIS. A 3km protection zone is the area enclosed by a circle, radius 3km, centred on an IP. In North Cumbria and South Penrith, 3km protection zones overlapped to become confluent and it is these confluent areas which became the North Cumbria and South Penrith SEZs. The South Penrith SEZ was defined to create a zone containing all premises within 3km of IPs involved in the so-called 'tail' of the epidemic during May to September. The North Cumbria SEZ, which adjoined the South Penrith SEZ, contained premises within 3km of most of the IPs involved in the early intense part of the epidemic, during February to mid-May. The analysis reported here is based on a dataset of premises in the North Cumbria SEZ only.

Livestock data collected during the June 2000 census proved inaccurate and did not correctly reflect the situation on farms at the start of the epidemic in February 2001. However, all premises within 3km protection zones were visited for culling or surveillance purposes during the epidemic and the species of stock present or total absence of stock was accurately recorded on these visits. The surveillance and culling data were used in this analysis to divide premises into those not stocked and those stocked during the epidemic. Those stocked were further divided into those having cattle and those not having cattle.

Lists of all premises culled during the epidemic were obtained from the disease control system database of the Department for Environment, Food and Rural Affairs. These lists provide:

- Unique identification code for each premises (CPH number for linkage with GIS list);
- Reason for cull;
- Date of report (for IPs);
- Date of confirmation (for IPs);
- Date of completion of slaughter.

For each IP, the date on which FMD lesions would have first been present in livestock ('first lesion date') was assigned, based on an estimate of the age (in days) of the oldest lesion found when stock on the farm were examined by veterinary inspectors on the scene of each IP. First lesion date was calculated by subtracting the age of the oldest lesion from the report date. First

lesion date was typically two to four days before the date of report, although some more extreme cases did occur.

Livestock on premises were culled for several reasons:

- Because FMD was confirmed (IP);
- Because FMD was suspected on clinical grounds (slaughter on suspicion (SOS) cull), and some of these premises were later confirmed as IPs;
- Where animals on a premises were considered likely to have been exposed to FMD infection, the premises was subject to a dangerous contact (DC) cull. This included culling of stock on premises contiguous to an IP following the introduction of the contiguous premises (CP) culling policy on March 29;
- Sheep and pigs only were additionally culled on many premises as part of the so-called '3km cull', which took place between March 28 and May 19 and covered most of the North Cumbria SEZ and the northern part of the South Penrith SEZ. Where the surveillance data recorded no cattle on a premises, the result of a 3km cull was assumed to be total depopulation, since this was the end result for almost all sheep-only premises in the parts of Cumbria covered by the 3km cull. Where the surveillance data recorded cattle on a farm, a 3km cull did not result in total depopulation since cattle would remain on the premises.

Some premises were subjected to more than one type of cull. For example, a premises may have been subject to a 3km cull of sheep and later had cattle culled as a result of the premises being infected (IP) or as a DC, CP or SOS. Where this was the case, the final status of the premises was based on the last cull on the premises.

For the purposes of analysis, the first lesion date is taken as the date on which a farm becomes an IP. Premises would cease to be at risk of becoming an IP when either:

- FMD lesions appeared in stock on the farm, or;
- All susceptible stock on the farm have been culled, or;
- 14 days after slaughter of the last IP in Cumbria (i.e. October 14, 2001).

Using the list of culls, the final status of all premises in the North Cumbria SEZ and the 'end of at risk' date on which a premises ceased to be at risk of becoming an IP were determined according to the criteria shown in Table 1.

Table 1. Final status of premises and end of at risk date

Final status	Criteria	'End of at risk' date
IP	All infected premises	First lesion date
non-IP depopulation	Any premises subject to either SOS cull, DC cull, CP cull or 3km cull where no cattle were recorded on the farm	Date slaughter completed
Stock standing	Premises escaping all culls or premises subject to 3km cull where cattle remained on the farm	October 14, 2001

A temporal risk window was calculated for each IP. This is the window during which disease would be most likely to appear (as clinical lesions) on any premises to which infection had spread from the IP. It is determined by both the likely period of virus shedding from the IP and the typical range for the interval from virus introduction onto another farm to first lesion date on that farm (incubation period). The likely period of virus shedding from the IP was taken as from one day before lesions first appear (Radostits et al., 2000) until the end of slaughter and preliminary disinfection. The range of incubation period quoted in the literature is from two to 14 days (Garland and Donaldson, 1990). Thus, the temporal risk window opens one day after first lesion date for the IP and closes 14 days after slaughter of the IP.

The eastings and northings data were used to calculate straight line distances between premises using Pythagoras' theorem.

Seven risk categories were then defined to include premises falling within the temporal risk window(s) of different numbers of nearby IPs. For ease of future reference, the risk categories were coded as defined in Table 2. The distance limits of 1.5km and 3km were used so that the association between disease incidence and proximity to IPs could be investigated. The 1.5km limit was chosen as this would include mostly contiguous premises and because a zonal pre-emptive cull of premises within 1.5km of IPs was one of the control options suggested by epidemiological modelling during the epidemic (Ferguson et al., 2001).

Table 2. Categories of risk to which premises in Cumbria were exposed

Risk category	Definition	Comment
R1	Premises not within a temporal risk window of any IP within 3km	IPs occurring in R1 would have no possible source of infection within a 3km radius
R2	Premises within a temporal risk window of one IP between 1.5 and 3km but not within a temporal risk window of any IP within 1.5km	IPs occurring in R2, R3 and R4 would have no possible source of infection within a 1.5km radius
R3	Premises within a temporal risk window of two IPs between 1.5 and 3km but not within a temporal risk window of any IP within 1.5km	
R4	Premises within a temporal risk window of more than two IPs between 1.5 and 3km but not within a temporal risk window of any IP within 1.5km	
R5	Premises within a temporal risk window of one IP within 1.5km	IPs occurring in R5, R6 and R7 would have at least one possible source of infection within a 1.5km radius
R6	Premises within a temporal risk window of two IPs within 1.5km	
R7	Premises within a temporal risk window of more than two IPs within 1.5km	

Using a customised module within a Microsoft Access® database, the total number of premises in each risk category at the start of each day of the epidemic and the number of IPs occurring among premises in each risk category during each day were counted.

For the whole or any part of the period of the epidemic, the total number of IPs in each risk category were calculated and the daily counts of premises in each risk category were added to give the total premises-days-at-risk for each risk category. By dividing the total number of IPs by the total premises-days-at-risk, incidence rates were calculated for each risk category. For convenience, these rates are expressed as IPs per 1,000 premises-days-at-risk.

Since non-IP depopulations had an effect on the population at risk, the number of non-IP depopulations of premises in each risk category was also counted so that non-IP depopulation rates could be calculated.

Rates were also calculated for aggregated risk categories: R1 + R2 + R3 + R4, representing all premises with no possible source IP within 1.5km; R2 + R3 + R4, representing all premises with at least one possible source IP between 1.5km and 3km distant and no possible source IP within 1.5km and R5 + R6 + R7, representing all premises with at least one possible source IP within 1.5km.

For ease of interpretation, incidence rates were converted to risk (probability) of disease over a 17 day exposure period. A temporal risk window of 17 days results from a first lesion to slaughter interval of 4 days, which was a typical interval for IPs in the dataset. This '17 day risk' represents the proportion of premises surrounding an IP, or IPs, which could be expected to become infected during the temporal risk window of the IP(s).

Mathematically, incidence rate, as calculated here, is a true rate, which is not the same as risk, but a true rate can be used to estimate risk using the formula (Giesecke, 1994):

$$risk \text{ over a given time} = 1 - e^{-\text{rate} \times \text{time}} \tag{1}$$

This formula (Eq. (1)) produces an estimate of risk under the assumption that the disease rate is constant over the time period in question.

The importance of proximity as a risk factor was assessed by calculating the population attributable fraction, which is an estimate of the proportion of disease in a population which may be directly attributable to exposure to the risk factor, or, in other words, the proportion by which the incidence rate in the entire population would be reduced if exposure were eliminated (Last, 1988). The population attributable fraction was calculated to estimate the proportion of disease in all premises which may be attributed to premises having at least one possible source IP within 1.5km, using the formula (Eq. (2)) given by Last (1988):

$$PopulationAttributableFraction = \frac{I_p - I_u}{I_p} \tag{2}$$

where I_p is the incidence rate in the whole population and I_u is the incidence rate in the unexposed part of the population (i.e. the premises in risk categories R1 + R2 + R3 + R4).

RESULTS

Of the 2,684 premises holding FMD-susceptible stock in the North Cumbria SEZ at the start of the epidemic, 637 (24%) became IPs, 706 (26%) were subject to non-IP depopulation, leaving 1,341 (50%) with stock remaining at the end of the epidemic. Approximately a third of the farms with stock remaining had lost sheep in the 3km cull, but still had cattle left. Nearly all (99%) of the 637 cases in the North Cumbria SEZ occurred up to and including May 19, with only six further cases occurring sporadically until the final case in this zone on June 21. The analysis of incidence rates here uses data up to and including May 19.

Figure 1 shows the incidence of disease in the North Cumbria SEZ, up to May 19. The bars representing the number of IPs occurring each day (by first lesion date), are partitioned to show the numbers of IPs occurring among premises in each of three groups: risk category R1 and the aggregate categories, R2 + R3 + R4 and R5 + R6 + R7. The superimposed line shows the five-day rolling average of total cases per day.

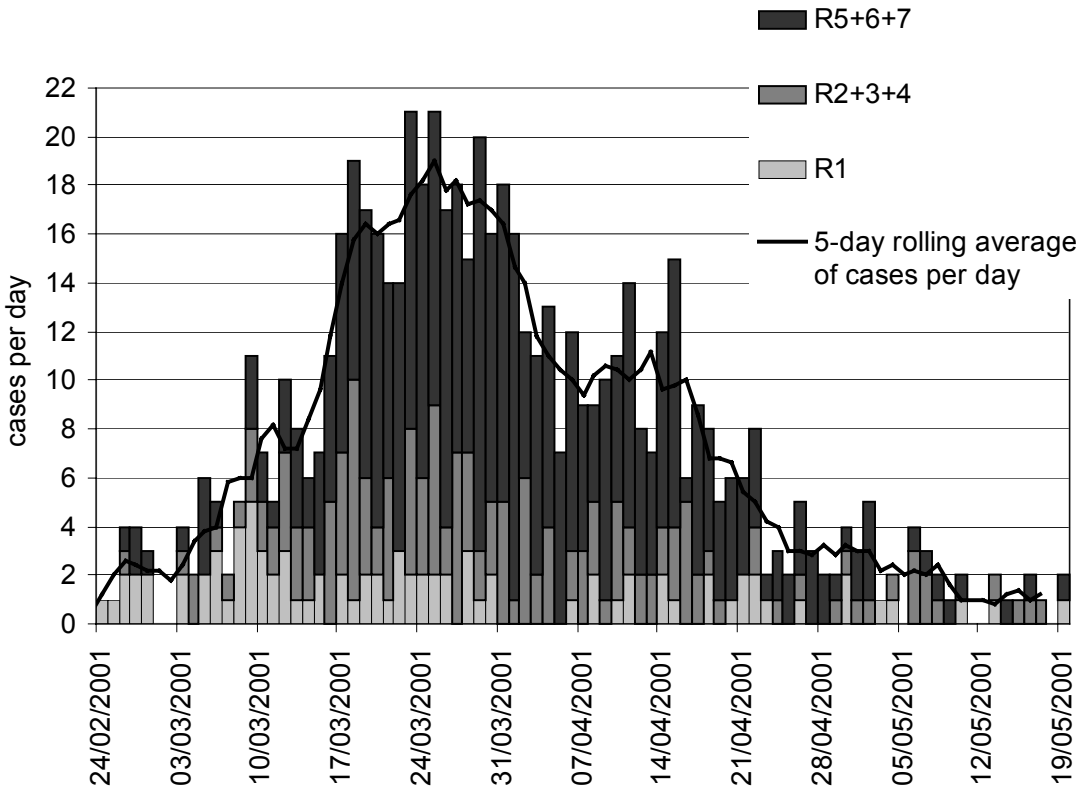

Fig. 1 Incidence of IPs by risk category in the North Cumbria SEZ (February 24 to May 19)

There was a rapid increase in cases per day with a peak of 19 cases per day for the five days centred on March 25. Case incidence fell over the following four weeks. An interruption to the decline in incidence during April was associated with relatively rapid spread of disease among dairy farms in an area around Wigton and Silloth, southwest of Carlisle.

During the early days of the epidemic in North Cumbria, IPs tended to occur at some distance from each other. Infected premises with first lesion dates up to March 9 could have been infected by virus carried onto the farm by infected stock brought in before nationwide animal movement restrictions were introduced on February 23, 14 days earlier. Up to and including this date, 48 IPs had occurred, 25 (52%) of which had no possible source IP within

3km (risk category R1) and 35 (73%) of which had no possible source IP within 1.5km (risk categories R1 to R4). On most days after March 14, at least 50% of new IPs had at least one possible source IP within 1.5km (risk categories R5 to R7; Fig. 3). However, IPs with no possible source within 1.5km continued to occur until the end of the epidemic. The proportions of IPs occurring, that had no possible source within 3km and within 1.5km were 87/631 (14%) and 246/631 (39%), respectively. Sixty-one percent of IP's had a possible source within 1.5km.

Tables 3 and 4 show the disease incidence rates and 17 day risks faced by premises in the different risk categories. The 17 day risks are also calculated for three time periods so that changes in risk over time could be illustrated.

Table 3. Incidence rate or FMD in North Cumbria (February 24 to May 19)

Risk category	Premises days at risk	No. IPs	Incidence rate[a]
R1	82,734	87	1.05
R2	24,352	59	2.42
R3	9,494	30	3.16
R4	11,508	70	6.08
R2+3+4	45,354	159	3.51
R1+2+3+4	128,088	246	1.92
R5	26,927	191	7.09
R6	9,085	94	10.35
R7	6,409	100	15.60
R5+6+7	42,421	385	9.08
Overall	170,509	631	3.70

[a](IP's per 1,000 premises-days-at-risk)

Table 4. 17 day risk of FMD in North Cumbria (February 24 to May 19)

	'17 day risk' (% of premises expected to become IP's)			
Risk category	Overall	Feb 24 - Mar 23	Mar 24 - Apr 20	Apr 21 - May 19
R1	2%	2%	4%	1%
R2	4%	6%	3%	3%
R3	5%	7%	7%	1%
R4	10%	18%	10%	3%
R2+3+4	6%	8%	6%	2%
R1+2+3+4	3%	3%	5%	2%
R5	11%	15%	12%	6%
R6	16%	25%	15%	11%
R7	23%	34%	23%	10%
R5+6+7	14%	19%	15%	7%

The category-specific risks increased through the risk categories R1 to R7. This reflected increasing risk of disease associated with both increasing proximity between possible source IPs and premises at risk, and increasing numbers of possible source IPs nearby a premises at risk. During the period analysed, premises spent an average of 31 days in risk category R1, 17 days in R2, R3 or R4 and 16 days in R5, R6 or R7. This would account for the 24% of premises affected overall.

The population attributable fraction, that is, the proportion of all IP's occurring that may be directly attributable to the fact that a possible source IP was within 1.5km, was 0.48 (i.e. [3.70-1.92]/3.70).

Table 4 shows that the category-specific risks were maximal during the first 28 days. Disease risk in all categories except R1 then fell consistently over the subsequent two periods. The disease risk in category R1 peaked during the second 28 days. At this time, the overall density of IPs on the ground was maximal, so premises in risk category R1 would be closer to more IPs at this time, even though no IPs may have been within 3km.

Figure 2 shows the breakdown of premises by status in North Cumbria up to May 19. The bars representing the whole population of premises each day are partitioned to show the premises at risk in each of the three groups (R1; R2+3+4; R5+6+7) and the premises no longer at risk by virtue of having being culled as IPs ('IP') or subject to a non-IP depopulation ('non-IP depop.').

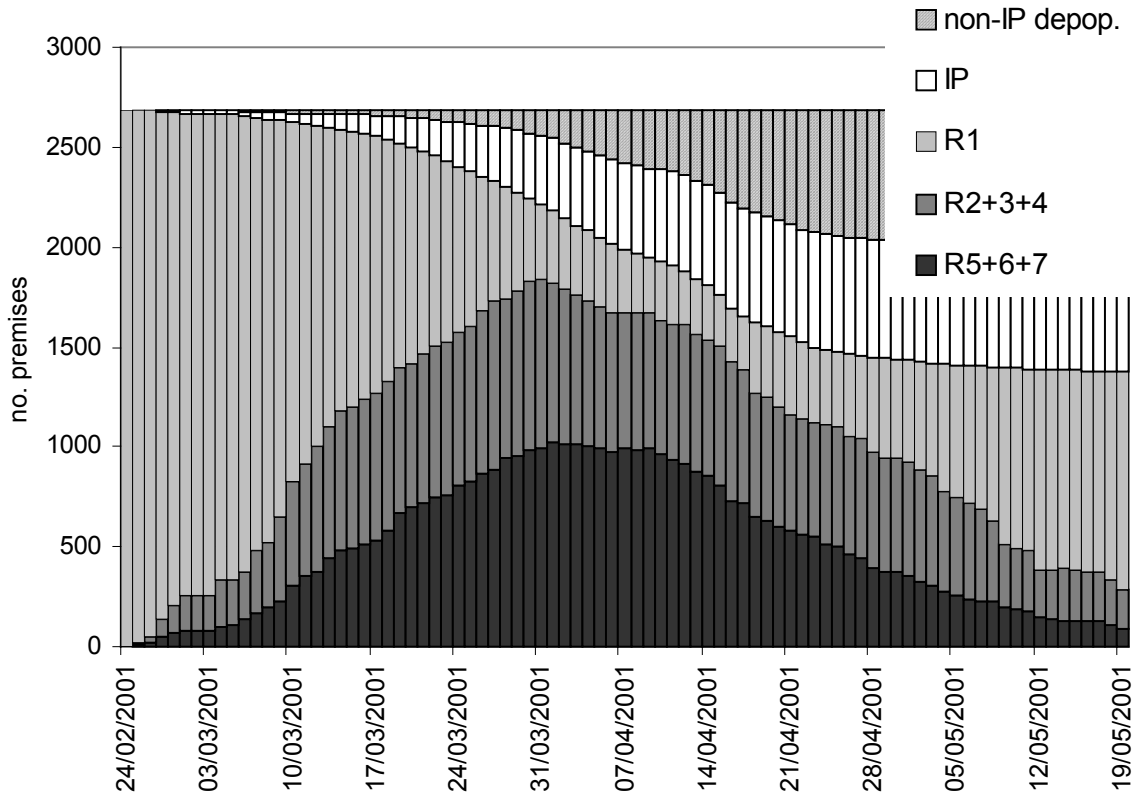

Fig. 2 The population of premises by status in the North Cumbria SEZ

The proportional structure of the population at risk can be seen in Fig. 3. The bars in this figure represent the population-time-at-risk only, calculated on a weekly basis, partitioned to show the proportions of premises in each of the three risk groups. Figure 3 also shows the overall disease incidence rate plotted on a weekly basis.

An impression of how non-IP depopulation may have affected the size and structure of the population at risk may be gained from the risk category-specific non-IP depopulation rates. Figure 2 shows that most non-IP depopulations in North Cumbria occurred between March 24 and April 20, so rates for this period alone have been calculated, as well as rates for the whole time to May 19. These are shown in Table 5.

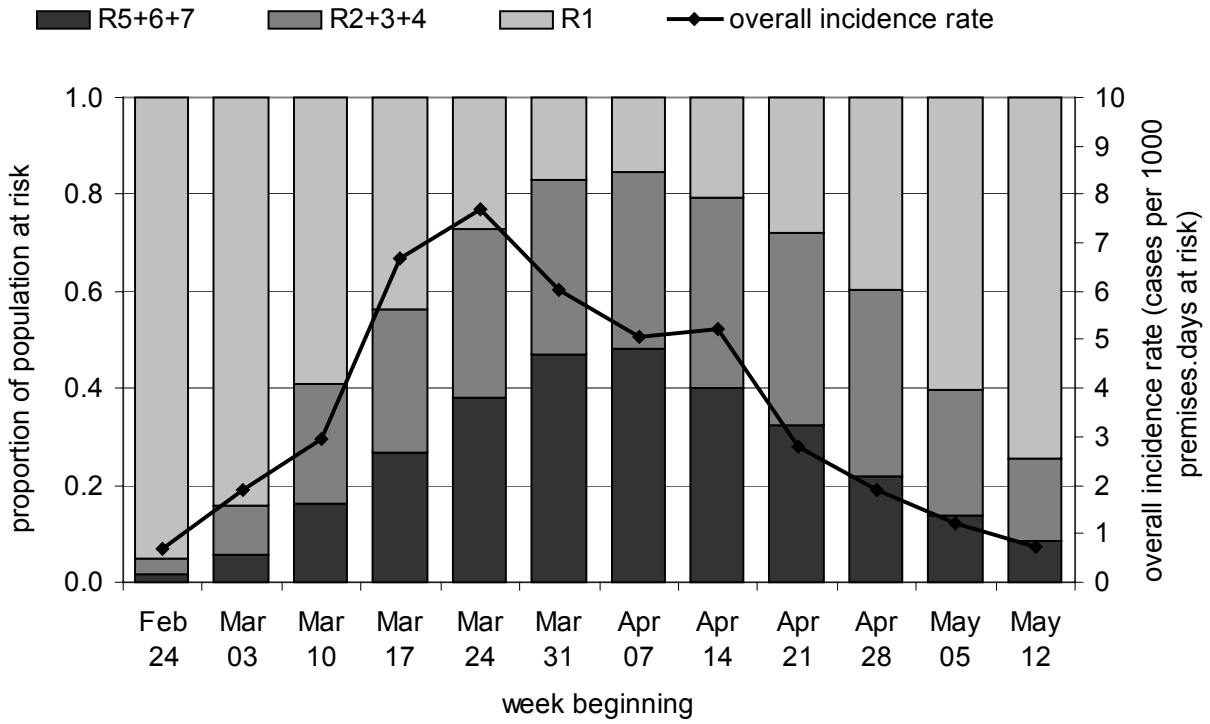

Fig. 3 Structure of the population at risk of FMD and overall disease incidence

Table 5. Non-IP depopulation rates in North Cumbria (February 24 to May 19)

	February 24 – May 19			March 24 - April 20		
Risk category	Premises-days-at-risk	No. non-IP depop.	Non-IP depop. rate[a]	Premises-days-at-risk	No. non-IP depop.	Non-IP depop. rate[a]
R1	82,734	77	0.93	10,975	45	4.10
R2+3+4	45,354	233	5.14	20,321	186	9.15
R5+6+7	42,421	366	8.63	24,941	280	11.23
Overall	170,509	684	4.01	56,237	511	9.09

[a](depopulations per 1,000 premises-days-at-risk)

As the epidemic progressed, the total number of premises at risk declined as a result of IP and non-IP depopulations (Fig. 2). The structure of the population remaining at risk was dynamic (Figs 2 and 3). The numbers of premises in risk categories R2 to R7 (those within 3km

of recent IPs) would be added to as IPs occurred. The numbers of premises in risk categories R2 to R7 would be reduced as a result of IP or non-IP depopulation and as a result of the temporal risk windows of previous IPs closing, thus releasing surrounding premises from the risk of disease originated by them.

During the first six weeks of the epidemic, the number of premises within 3km of an IP was increased by the rising incidence of IPs. By the week beginning March 31, just after the peak of the epidemic, over 80% of the premises at risk had at least one possible source IP within 3km and over half of these had at least one possible source IP within 1.5km (Fig. 3).

Between March 24 and April 20, when most non-IP depopulations occurred, the non-IP depopulation rate among premises in risk categories R5 to R7 was only 1.2 times greater than the overall rate (Table 5), indicating that non-IP depopulations at this time were not strongly targeted on premises closest to recent IP's (i.e. those at highest risk of developing disease). Although the total number of premises at risk was falling steadily after March 24 (Fig. 2), the proportions of the total having a possible source IP within 1.5km and within 3km did not begin to fall until the week beginning April 14 (Fig. 3), three weeks after the peak in disease incidence rate. The timing of this fall coincides with the time when the temporal risk windows of IPs occurring up to the peak of the epidemic had closed while the daily incidence of new IPs, opening new temporal risk windows, was falling, so that the number of premises being released from risk categories R2 to R7 would be greater than the number of premises being added.

DISCUSSION

The analysis presented here provides estimates of the risk of disease faced by farms at different distances from IPs. These estimates are of overall risk, regardless of the methods of spread and as such, they do not attempt to explain actual mechanisms by which disease was spread. It is unsurprising to discover that the risk of disease increased when premises were closer to increasing numbers of IPs. This is an indication of increasing likelihood of direct contact spread and fomite transmission as the density of IPs on the ground increases. However, closer examination of disease incidence in the different risk categories and of the dynamics of the population at risk, can improve our understanding of the way in which the FMD epidemic of 2001 was propagated and controlled in North Cumbria.

Before the nationwide imposition of animal movement controls on February 23, infection had spread to 119 farms, 27 of these premises being in Cumbria, (Gibbens & Wilesmith, 2002). Although the majority of later cases had a possible source within 1.5km, a significant number of cases continued to occur throughout the epidemic which did not. This shows that spread of disease after the imposition of animal movement controls did not only occur from farm to nearby farm, what might be termed 'radial creep', but spread by longer jumps also frequently occurred. Thus, early in the epidemic in North Cumbria, disease occurred in widely scattered locations (IPs in risk categories R1 to R4; Fig. 3). Scattered cases then continued to occur throughout the epidemic, but from mid-March, once the density of IPs had increased, more and more short distance creep of disease occurred, filling in the areas between IPs and causing a further rise in case incidence.

Given that premises in the higher numbered risk categories faced increased risk of disease, it might be expected that as the disease incidence rate increased over the first five weeks of the epidemic, a vicious cycle would have been set up. Figures 2 and 3 show that as increasing

numbers of cases per day occurred, the number and proportion of premises in the higher risk categories increased. This could be expected, in turn, to lead to an ever increasing overall incidence rate until the number of premises at risk is depleted below a certain level. An early model of the epidemic (Ferguson et al., 2001) suggested that up to 79% of farms in Cumbria, Dumfries and Galloway could become infected based on the assumed *status quo* on March 28. However, a fall in overall disease incidence rate began the week beginning March 31. This fall in disease incidence rate cannot be explained by any reduction in the proportion of premises in the higher risk categories, as might be brought about by a high rate of non-IP depopulation in those risk categories, since the proportion of premises in these risk categories was still rising (Fig. 3). This means that the epidemic in North Cumbria did not simply 'run its course', its extent was limited by the increasingly effective control measures applied from the beginning of the epidemic. A reduction in overall incidence rate at a time when the proportion of premises at risk in the higher risk categories was still rising could only result from the falls in the underlying disease risks in each risk category (Tables 3 and 4). This 'across the board' fall in disease risk could only have been brought about by control methods which reduced risk of direct contact and fomite spread, such as rapid culling of IPs and enforcement of biosecurity regulations, and/or culling which was specifically targeted at farms with known dangerous contacts through the movement of animals, people, vehicles *etc.*. Of these, the shortening of time taken to cull IPs is considered to be the most important (Honhold et al., 2003). While more rapid culling of IPs will reduce the temporal risk window, thereby reducing spread of disease to surrounding premises by reducing the exposure time, a reduction in risk calculated over a fixed time period (as observed here in 17 day risk) could only result from a reduction in the average risk of disease per day of exposure in premises surrounding IPs. More rapid culling of IPs could only bring this about if the disease challenge presented by an IP increased over time, for example, because of an increasing prevalence of animals with clinical lesions. Then, slaughtering an IP at two days after first lesions appear instead of four days would prevent two days of higher disease challenge and reduce the average daily disease challenge presented by that IP.

The apparent greater risk of disease among farms close to IPs was used as justification for culling of livestock on premises contiguous to IPs (Anderson, 2002). The population attributable fraction calculated here estimates the proportion of all IPs which could be attributed to the risk factor, 'being within 1.5km of a possible source of infection'. This risk factor is a 'black box' which must include all the individual factors which increase the risk of disease when compared with premises more than 1.5km distant from a possible source of infection. The population attributable fraction of 0.48, implies that the 'black box' of risk factors may have been responsible for infection of just under half of all the IPs. This fraction differs from the 61% of all IPs occurring within 1.5km of a possible source because a proportion of the 61% are attributed to the 'background' risk factors faced by all premises. This result suggests that a control programme should give at least equal priority to controlling longer distance spread by fomites, as to controlling spread to contiguous premises or premises within a certain small radius such as 1.5km. Culling within a certain radius of IPs will inevitably prevent short distance spread by removing susceptible livestock in the local area. A danger of focusing on controlling short distance spread in this way is that, because high numbers of premises are culled for each IP that occurs, if longer distance spread maintains disease incidence at even low levels, the total number of premises culled will increase rapidly and may exceed that which may have been necessary had only IPs been culled, even though the total number of IPs may be greater in the latter case.

These results show the importance and potential value of timely analysis and careful interpretation of field data during an epidemic. Analyses similar to those carried out here could

be usefully applied at local DCC's during an epidemic. The nearest possible source IP, based on temporal risk windows, can be searched for each time an IP occurs and charts similar to Fig. 1 could be plotted on a daily basis. The disease incidence rates for premises in different risk categories could also be calculated on a 'real time' basis, although this requires good data on numbers and location of all premises within an area, data which were not completely available in the early days of the 2001 epidemic. The category-specific incidence rates in fact provide similar information to counts of cases in each risk category (the numerators alone) with the important difference that the size of the denominator (number of premises at risk) is factored in, allowing valid comparisons to be made with risks calculated for other times or places. The results of such analyses would provide an indication of the level of control being achieved over short and longer distance spread of disease and could be used as a guide to varying control measures to react to the local epidemiological picture.

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A QUANTITATIVE INSIGHT INTO 'BIOSECURITY': A CASE-CONTROL STUDY INVESTIGATING THE RISK FACTORS PREDISPOSING CUMBRIAN DAIRY FARMS TO FOOT AND MOUTH DISEASE

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SUMMARY

A case-control study was used to investigate the relationship between a wide range of putative risk factors considered likely to have affected the probability of dairy farms in north Cumbria becoming infected with foot and mouth disease (FMD) virus during the FMD epidemic in the United Kingdom in 2001. One hundred and thirteen case and ninety-eight control farms were interviewed.

This paper describes the results of the initial univariate analysis of those risk factors so far explored. It was shown that the risk of FMD infection was significantly reduced on mixed dairy-beef enterprises, and on premises where the farmer personally supervised the disinfection of vehicles on and off the farm. An increased risk was noted where fields were entered to inspect stock, where farms shared a boundary with an infected premises and where there was more than one vehicle movement on and off the farm per day.

Of the other factors so far investigated, no statistically significant increased or decreased risk effect could be attributed to a many of the presumed risk factors. However, this may have been as a result of the low numbers of farms that reported some of the practices that were of particular interest (such as, the use of relief milkers and the presence of certain species of wildlife), or due to the fact that the case and control farms in the study were particularly uniform with respect to many of their management practices.

INTRODUCTION

Dairy farms became the focus of this study as a result of ongoing, field-based, analysis of attack rates for foot and mouth disease (FMD) throughout the 2001 outbreak in Cumbria. It was revealed that dairy farms appeared to be at a substantially higher risk of contracting FMD than those premises that did not have dairy cattle (the odds ratio (OR) for a Cumbrian dairy becoming an infected premises (IP) compared with a non-dairy premises was 3.15).

A case-control study was initiated with the aim of identifying those factors that may be the most important components of biosecurity at the farm level. In addition to the more usual

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technical, management and husbandry aspects, the study also encompassed aspects of human behaviour. Emphasis was placed on identifying those risk factors that could be managed or altered in an outbreak so as to reduce the likelihood of introduction of FMD onto, or spread from, a dairy farm.

One of the most significant features of the virus type implicated in the 2001 FMD epidemic in the United Kingdom was the limited potential for long distance airborne transmission of the virus (Donaldson et al., 2001). Short distance spread was considered to be of greater significance in this instance (Gibbens et al., 2001). As a consequence, the terms 'local spread' and 'biosecurity' assumed epidemiological importance in terms of a need to disentangle the elements of each of these generic terms.

The various potential methods for the spread of FMD are described by Donaldson (1987). However, in this restricted context of local spread, the term 'biosecurity' focuses on ways of preventing or limiting the movement of fomites both on and off individual farms. As farms become an effective source of virus prior to the appearance of clinical signs, it is important to consider that biosecurity has an important role in keeping virus on case farms, as well as off control farms.

MATERIALS AND METHODS

Sample selection

The outbreak in Cumbria was retrospectively categorised, spatially and temporally, into two areas: North Cumbria (where the initial peak of the Cumbrian epidemic occurred) and the South Penrith area in the east of Cumbria (where the tail of the epidemic occurred). The Wigton area, within North Cumbria, was selected for this study because it was the major dairy area in Cumbria and also, initial investigation had shown that it was more likely to be possible to identify and interview a sufficient number of owners of control premises. The outbreak in this area was well delineated spatially, and similar husbandry was practised throughout.

Case dairy farms (IP farms) and control dairy farms (non-IP farms) were selected using the Department for Environment, Food and Rural Affairs (DEFRA) Disease Control System (DCS) data for Cumbria, by selecting farms identified as dairies, located between easting 308000-340000 and northing 545000-561000. Case premises were defined as those farms milking cattle at the time they were diagnosed as FMD positive (from IP 96 on 7th March 2001, to IP 1600 on 15th May 2001), and control farms were farms milking cattle between 15th February, 2001 and 15th May, 2001 that did not contract FMD. May 15th was chosen as the end point for the period of interest, because this marked the end of the potential epidemiological spread window from the last case in the Wigton area.

Of the 198 case farms and the 214 control farms identified as dairies from DCS, only 154 case premises and 167 control premises complied with the above definition and were eligible to take part in the study. Sufficient Temporary Veterinary Inspector (TVI) manpower resource was made available, and it was possible to visit all willing case and control farms.

An initial 120 case and 120 control farms were randomly selected from the valid premises identified, and then randomly allocated to individual TVIs. A replacement list was generated using the same procedure and TVIs instructed to use these replacements in order, in the event of non-compliance.

Identification of risk factors

The risk factors investigated were those that had been identified in discussions conducted both formally and informally throughout the outbreak, with all stakeholders (farmers, DEFRA veterinary staff, TVIs, and slaughtermen, *etc*.). The opportunity was taken to investigate some of the folklore that became established, mainly by the farming community, during the outbreak especially with reference to factors such as wildlife and carcase disposal wagons. Special emphasis was placed on identifying those risk factors that could be managed or altered during an outbreak, to reduce the likelihood of introduction of FMD onto dairy premises. The broad categories of risk factors listed in Table 1 were deemed to be of interest.

Table 1. Risk-factor categories

Farm	Movement	Biosecurity	People
1. Farm size and management	1. Visits to outlying stock	1. Farm gate routine for vehicles	1. Routine personal / family contacts
2. Stock holding	2. Traffic and vehicle movement	2. Farm gate routine for people	2. Social contacts
3. Stock husbandry	3. Visits to and from other stock farm		3. Business contacts
4. The 3 km cull	4. Wildlife		
5. Farm location, fragmentation and buildings			
6. Neighbouring farms			

The pilot study

A preliminary pilot study, with 20 randomly selected IP premises and 20 non-affected control farms, was initiated in October 2001 immediately following the end of the national epidemic, using the same sampling protocol described above. Modifications were made to the original questionnaire design as a result of this study. Results from the pilot study were not included in the main study, nor were the farms revisited.

The final questionnaire

Data were collected using a formal questionnaire that was completed during a single interview with the farmer, the family and other farm staff. The questionnaire consisted of 225 questions, arranged in fifteen sections, and took approximately one-hour to complete. The questionnaires were handed-in at the end of each day and scrutinised to identify problems and ensure consistency of recording. Follow-up interviews were used to clarify points, if required. Data entry was carried out by one trained TVI, using a custom written module in Access 97 (Microsoft) to ensure consistency of data entry, categorisation and interpretation of the written answers.

Data collection

The main study was undertaken during the period from January 2002 to April 2002. Farms previously interviewed in the pilot study were not included in the follow-up study. The majority of the interviews took place over a four-week period from 11 February 2002 to 16 March 2002, approximately 12 months after the period of interest.

Data were collected using personal, pre-arranged visits by a team of ten TVIs who had worked throughout the outbreak and who had detailed knowledge of the area of interest. Each TVI interviewed an approximately equal number of case and control premises. Individuals were aware of the case or control status of individual dairies. In the circumstances, it would have been impossible for TVIs to remain unaware of the status of the farm. Full biosecurity precautions were observed by TVIs on all case and control farms. Standardisation procedures were introduced to ensure that questions were asked in as similar manner as possible, so as to reduce bias. The questions specifically targeted activities during the time period between 15th February 2001 and 30th April 2001, which was considered to be the duration of the Wigton outbreak. IP farms were asked to supply information relevant to the period from February 15th up to the point at which they became FMD positive.

Statistical analyses

The descriptive statistics and data analysis were generated using Access XP (Microsoft), Excel XP (Microsoft), Statistix 7 (Analytical Software), WinEpiscope v2.0 (Thrusfield et al., 2001) and Epi-Info 6.04d. (Dean et al., 1998). Univariate analyses were carried out for each risk factor, to identify those that were associated with a premises becoming a case. The results were reported as the odds ratio for each variable, with the 95% confidence limits stated. Odds ratios (OR) with 95% confidence limits spanning 1.0 were considered not to be statistically significant.

RESULTS

By the end of the study period, all replacement premises had been contacted and it had been possible to obtain data from a total of 113 case and 98 control farms - a response rate of 73% for cases and 59% for controls. The results for the potential risk factors so far analysed, are summarised in Tables 2, 3 and 4.

Factors associated with a protective effect

Table 2. Factors associated with a protective effect (upper 95% confidence limit for OR <1)

Category	Factor	OR	-95% CI	+95% CI
1. Farm size and management	Slurry spreading during the period	0.29	0.15	0.55
2.	Other field work carried out	0.31	0.55	0.99
3. Stock holding	Mixed enterprises with beef	0.02	0.01	0.05
4. The 3 km cull	Removal of sheep in the 3 km cull	0.11	0.05	0.21
5. Visits to and from other stock farms	Visits to other stock farms	0.09	0.00	0.66
6. Traffic and vehicle movement	Carcase lorries on roads adjacent to farm	0.42	0.23	0.79
7. Farm gate routine for vehicles	Certainty of disinfection of milk tanker & hoses upon entry and exit	0.06	0.01	0.26

Factors associated with an increased risk

Table 3. Factors associated with increased risk (lower 95% confidence limit for odds ratio >1)

Category	Factor	OR	- 95% CI	+ 95% CI
1. Neighbouring farms	IP on a farm sharing a boundary	3.22	1.11	9.78
2. Visits to outlying stock	Field entered to inspect stock or carry out work	1.89	1.03	4.05
3. Traffic and vehicle movement	More than one vehicle movement on/off farm per day	2.93	1.07	3.42
4. Farm gate routine for vehicles	Vehicles disinfected on and off the farm	1.19	1.08	3.88
	Knapsack sprayer at the farm gate	1.19	1.08	3.88
	Pressure washer at the farm	1.83	1.07	2.05
	Brush at the farm gate	1.42	1.03	1.87

Factors with no demonstrable association with FMD risk

Variables for which no association can be demonstrated are as interesting in this context as those for which an association is apparent. Of the relationships so far analysed from this large dataset, no significant risk effect could be demonstrated for the variables shown in Table 4.

Farm related factors

Stock holding: a significantly higher proportion of unaffected dairy farms also kept beef cattle. The odds ratio for a dairy-beef mixed enterprise becoming a case was 0.02 ($0.01 < C.I. < 0.05$).

Slurry spreading: there was a protective effect (odds ratio 0.29; $0.15 < C.I. < 0.55$)) for those farms that spread slurry during the period of interest. This is likely to be explained by the movement restrictions imposed on IPs following confirmation, making them less likely to have spread slurry during the period.

Other field work carried out: Similarly, the protective effect of other field work, odds ratio 0.55 ($0.31 < C.I. < 0.99$), is attributable to the fact that the majority of owners of IPs halted all routine work following confirmation as an infected premises.

The 3 km cull: The overall effect of the 3 km cull was not specifically investigated, and it is not possible to draw any valid conclusions from the apparently significant protective effect of the cull (OR = 0.11, $0.05 < CI < 0.21$), because the outbreak in the North Cumbria area was largely over before the first livestock were removed under the cull on 28 March 2001. Thus the majority of the premises remaining with stock to be taken under the cull were by definition control farms.

Table 4. Factors not associated with FMD risk (95% CI for when the OR includes 1) (results considered to be of particular interest are indicated with an "*")

Farm	Movement	Biosecurity	People
1. Farm size and management *Number and type of personnel working on the farm. *Use of relief milkers. *Use of public roads for slurry spreading. *Use of outside help for work. Size (area) of farm. Proportion of arable land. Method of manure storage. *Use of contractors. *Sharing / borrowing equipment. • Stock holding Herd / flock size for all species, except beef cattle. • Stock husbandry *Housing of livestock of all types. Sheep lambing in fields. Strayed stock of any species. Bedding type used. Feed type and source. Type of housing for the milking herd. • The 3 km cull *Contact of dairy cattle with 3 km cull sheep. Farmer or employees assisting with loading sheep for the 3 km cull. • Farm location, fragmentation and buildings Number of geographically separate locations farmed. • Neighbouring farms *Type of boundary with neighbouring farm. *Stock type present on neighbouring farm.	2. Visits to outlying stock *Disinfection between visits to different groups of stock. Type of vehicle used to inspect stock. Separate protective clothing used for each group. Person carrying out the inspection e.g. farmer, living on site, living exclusively off-site. • Traffic and vehicle movement *Type of road at the farm gate. *Type and weight of traffic. *IP animals grazed adjacent to road. • Visits to and from other stock farms Biosecurity. precautions observed when visiting other stock farms. *Visits from other stock farmers.	3. Farm gate routine for vehicles *Milk tanker company. Satisfaction of the farmer with disinfection. Disinfection of fuel and feed lorry hoses. *Frequency of collection. • Farm gate routine for people *Boot dips. *Brush at the farm gate. *Type of disinfectant used. *How frequently disinfectant was changed. • Wildlife *Wildlife species present.	4. Routine personal / family contacts *Children. attending / not attending school Number of people on the farm. *Presence of young adults. • Social contacts All other visitors routines at farm gate. Social contact.

Neighbouring farms: A farm with a neighbour (i.e. sharing a boundary) that was an IP was 3.22 times as likely to become a case than a farm without (1.11 < C.I. < 9.78)

Movement related factors

Entering field with stock: The increased risk associated with entering fields to inspect stock or carry out routine work such as fertilizer spreading is an interesting result because this was part of the standard biosecurity advice issued to farmers and is behaviour that is easy and practical to modify.

More than one vehicle movement onto farm per day: The data for this question were categorised into one movement or less per day and greater than one movement per day, as this would correspond with the cut-off for the minimum vehicle movement possible on a dairy farm which was an average of one movement per day to include milk tanker and feed lorry visits. This indicates that vehicle movement does not have to be prevented altogether to achieve a significant and worthwhile reduction in risk.

Carcase lorries travelling on roads adjacent to farm: The movement of carcase disposal lorries were controlled and co-ordinated to keep them away from uninfected areas on their way to disposal sites, and their movements were of great concern to farmers. The result here shows that there was no significant risk associated with known movements past farms. This result could be followed up by spatial analysis of the known routes.

Visits to other stock farms: Visits by farmers to other stock farms during the risk period had a paradoxical protective effect, which cannot be explained. There was no significant effect of the contrary visits to the farm premises from other stock farmers.

Biosecurity expenditure

A supplementary series of questions were asked at the end of the questionnaire session to give participating farmers the opportunity to express their views and to round off the discussion. Amongst these the farmer was asked to determine expenditure on biosecurity precautions. The results are shown in Table 5.

Table 5: Costs for biosecurity precautions implemented

	Set-up costs			Monthly maintenance		
	Mean	Min	Max	Mean	Min	Max
Case	£219	£0	£3000	£30	£0	£400
Control	£492	£0	£4500	£21	£0	£150

DISCUSSION

A study such as this attempts to investigate the relationship between risk factors and the presence or absence of disease and is fraught with practical problems. It is important to recognise that many risk factors are not binary events, either present or absent; they are present by degree, in a manner that is difficult to categorise objectively, and into this is woven the vagaries of human nature. For example, the presence or absence of a foot bath provides no

information has to whether it was sited correctly, actually used by visitors, or properly maintained by the farmer. In many questions the 'correct' answer is unavoidably obvious, and further compounding this quandary, is the inability to be certain that respondents did what they said, let alone how well it was done.

In this study, no association could be detected between many of the risk factors that seemed to be so anecdotally obvious during the outbreak, and which subsequently fell into the folklore surrounding the events of 2001. The absence of an association between a potential risk factor and a premises becoming a case may be due to one or more of the following reasons:

1. Bias and compliance;
2. Chance;
3. There is no actual association of the risk factor with the occurrence of FMD;
4. The sample population was homogenous with respect to the risk factor;
5. The study had insufficient power to detect an association.

Some of the significant associations identified by the pilot study (such as the risk associated with vehicles crossing areas shared by livestock) were found not to be significant when investigated by the main study.

Bias and compliance are key issues for studies of this type and the lower response rate of the control population (98 from 167 - 59%) compared to the case population (113 from 154 - 73%) was a matter of concern. By the end of the study period, all willing, eligible controls had been interviewed and it was not possible to improve on this figure.

Reasons 4 and 5 above are likely to have been significant in this investigation. The dairy agriculture practised in this area is strikingly uniform, and the relatively low number of farms that complied with the definition for case and control farms probably affected the power.

The paradoxical situation where factors expected to improve biosecurity were shown to be associated with increased risk can be explained in many cases by the fact that the probability of infection was not evenly distributed either spatially or temporally throughout the area of interest. In areas that had the highest concentration of IPs, the farmers were more likely to adopt improved biosecurity, but their farms were subject to greater challenge. Also, farms that became IPs (cases) had less time within which to be exposed to risk factors than controls, importantly, IPs were less likely to have experienced the 3 km cull, to spread slurry or to carry out field work because many of the IPs in the Wigton area had already occurred before the time when these activities took place.

Farm related factors:

Stock holding: Mixed enterprises with beef cattle were found to be significantly less likely to become infected with FMD. Wide discussion with stake holders and the range of analyses so far carried out, have failed to identify any risk components unique to such farms that may be responsible for this surprising result.

Biosecurity related factors:

Personal supervision of disinfection: The highly significant protective effect of farmers who personally supervised the disinfection of the milk tanker and other vehicular movement on and

off the farm is suggestive that there is a type of farmer whose meticulous approach and readiness "to go the extra mile", ensures that the farm is less likely to become infected with FMD, as a result of attention to detail. It may be that this personality trait transcends the details and is a proxy, encompassing many elements of all that constitutes good practice in farm biosecurity.

People-related factors

No significant risk or protective effect could be demonstrated for any of the factors classified in the "people" category that have been analysed.

Farmer co-operation: It is also important to recognise that the ability and willingness of farmers to answer specific questions varied considerably. Specifically, as with many questionnaire surveys, it was impossible to avoid questions prompting obvious "correct" answers, and it is possible that recall bias may have influenced what individual farmers remembered. This exercise was conducted as close to the end of the outbreak in the North Cumbria area as was possible under the practical limitations imposed by the outbreak. It is interesting to note that in the pilot study, carried out in the period immediately after the end of the outbreak, farmers from case farms were the most willing to co-operate, which was contrary to expectations. When the full-scale study was carried out three months later, the opposite was found to be the case – the control farms were the easiest to recruit into the survey.

Factors that have been shown to be significantly associated with becoming a case, will be further analysed using a multi-variable modelling approach.

CONCLUSIONS

Most of the biosecurity measures highlighted during the outbreak would be good standard operating procedures for any farm at all times, to reduce the risk of introducing novel disease. Observations noted by the TVIs, made at the time the interviews were carried out, illustrated how quickly dairy and sheep farmers – surprisingly, even those from infected areas - have chosen to disregard the lessons of biosecurity advocated during the national outbreak, which are everyday practice on modern pig and poultry farms.

Of the factors so far associated with an increased risk, entering fields to check stock and the number of movements on and off the farm can be altered most easily and with minimal cost.

It may seem that many of the measures normally associated with good biosecurity do not appear to significantly enhance the protection of farms from FMD, however, this finding is likely to be related to the observation that the risk was not spread evenly either spatially or temporally and that farmers tended to adopt better biosecurity measures where the risk of infection was higher. It is interesting to note that control farms tended to have spent significantly more on biosecurity set-up costs, but less on maintenance.

Of the factors associated with a protective effect, only the personal supervision of disinfection of every movement on and off the farm is amenable to modification, and as this is likely to be a proxy for a character type reflecting many other management aspects of the farm business, it may not be as easy to manipulate as would first appear to be the case. It is difficult to measure the key effects of this character type using this questionnaire as an instrument, because the effects are those of degree and attention to detail, rather than simple "yes or no" issues.

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OPEN SESSION

A COMPARATIVE STUDY FOR EMERGENCY VACCINATION AGAINST CLASSICAL SWINE FEVER WITH AN E2 SUB-UNIT MARKER VACCINE AND A C-STRAIN VACCINE.

J. DEWULF[1], H. LAEVENS, F. KOENEN, K. MINTIENS AND A. DE KRUIF

SUMMARY

At present, two types of vaccines against CSF virus are available: E2 sub-unit marker vaccines and conventional live C-strain vaccines. To evaluate the potential use of both vaccines in an emergency vaccination scenario, 3 comparable experiments were erformed. In each experiment 2 groups of weaner pigs, vaccinated with a marker vaccine or a C-strain vaccine, were challenged with CSF virus at 0, 7, and 14 days post vaccination (dpv). Using the marker vaccine, virus transmission was totally prevented 14 days post vaccination, resulting in a transmission ratio (R) of 0 (0-1.5). When the challenge occurred at 0 or 7 days post vaccination the Rs were ∞ (1.5-∞) and 3.5 (1.6-10.9), respectively. Using the conventional vaccine, the virus transmission was already totally prevented when the challenge occurred at the same day of the vaccination R = 0 (0-1.5). Therefore, the advantages of the marker vaccine (possibility of differential diagnosis) need to be weighed against the disadvantage of a longer interval between vaccination and onset of immunity.

INTRODUCTION

Since 1980, the control of classical swine fever (CSF) in the European Union (EU) is based on a policy of non-vaccination and stamping-out. However, recent outbreaks have shown that in non-vaccinated populations the control of CSF through stamping out may be very expensive, particularly in areas with high pig densities (Koenen et al., 1996; Meuwissen et al., 1999). This is partially due to the large number of animals that are pre-emptively slaughtered when trying to cope with the virus spread in the neighbourhood of infected herds. Ethically, this strategy has become more and more debatable (Terpstra, 1998).

Although it is still not fully understood which routes of transmission are responsible for neighbourhood infections, it is clear that pre-emptive eradication of the neighbourhood of an infected herd is an effective and even a possibly indispensable measure in the control of a CSF epidemic in areas with high pig densities (Koenen et al., 1996; Elbers et al., 1999). The purpose of this measure is to prevent major within-herd outbreaks in order to reduce the virus infection load in a neighbourhood. This reduced infection load subsequently results in a reduction of the between-herd virus transmission.

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Theoretically, vaccination of neighbouring herds instead of eradication could achieve the same goal. This should result in a decreased infectivity of the infected vaccinated animals and a decreased susceptibility of the vaccinated animals not yet infected (de Jong & Kimman, 1994). To be equally as efficient as the eradication strategy, it is essential that the interval between vaccination and onset of immunity (reduction of infectivity and susceptibility) is as short as possible.

At present there are two types of vaccines against CSF virus commercially available: E2 sub-unit marker vaccines and the conventional live C-strain vaccines. To evaluate the potential use of both vaccines in an emergency vaccination scenario, 3 comparable experiments were carried out in which groups of weaner pigs, vaccinated with a marker vaccine or a C-strain vaccine, were challenged with CSF virus at 0, 7, and 14 days post vaccination (dpv). The clinical protection and the reduction of virus transmission, induced by both vaccines, were compared.

MATERIALS AND METHODS

Animals

In each of the three experiments, 32 clinically healthy, conventional weaner pigs originating from the same herd were used. These animals were checked for the absence of bovine viral diarrhoea (BVD) and CSF antigen and antibodies.

Virus

The isolate used for challenge exposure was originally obtained from the first infected herd of the 1993-1994 Belgian CSF epidemic. The isolate was verified to be free of African swine fever virus and BVD virus. By using a panel of monoclonal antibodies, the isolate was characterised as antigenically similar to an isolate known as the 'souche Lorraine' (Koenen & Lefebvre, 1994), which has been described as a moderately virulent strain (Laevens, 1999). The virus was cultivated on PK_{15} cells and 2 passages were carried out. The titre was 10^3 median tissue culture infective doses ($TCID_{50}$ / ml).

Vaccine

In each experiment 2 vaccines were used:

The marker vaccine used (Porcilis® Pesti, Intervet International BV) consists of the E2 glycoprotein of the CSF virus strain Alfort/Tübingen. The glycoprotein was produced by means of a baculovirus stimulating insect cells to express the glycoprotein.

The conventional vaccine used (Pestiffa®, Merial France) is the so-called Chinese strain or C-strain, which is a modified live vaccine, produced by serial passages in rabbits.

Experimental design

All three experiments were set up in a similar way. Upon arrival, 32 conventional weaner pigs of 12-15 kg were randomly allocated to 4 pens (8 pigs per pen) in 2 separated compartments (2 pens per compartment). After an acclimatisation period of 7-14 days, all pigs of pens 1 and 2 (compartment A) were vaccinated with the marker vaccine, whereas all pigs of

pens 3 and 4 (compartment B) were vaccinated with the C-strain vaccine. At 0 (experiment A), 7 (experiment B) or 14 (experiment C) dpv, 2 randomly selected pigs per pen were challenged with virulent CSF virus by deep intramuscular injection (2 ml). Before the challenge exposure, the selected pigs were moved to a separate pen where they remained until 6 hours after challenge exposure. Before the reintroduction into their respective pens, the challenged pigs were washed with clean water. During the observation period (between challenge exposure and end of the experiment), the health and infection status of the challenged and contact pigs was monitored. After the observation period all remaining pigs were euthanased.

Pens were always visited in the following order: compartment B (pen 4 $\rightarrow$ pen 3), compartment A (pen 2 $\rightarrow$ pen 1). Between visits of pens within the same compartment, gloves were changed and footwear was disinfected. Between the visits to compartments B and A, overalls, gloves and footwear were changed. All materials necessary for blood sampling, rectal temperature monitoring, cleansing of the pens, and feeding of the pigs were provided per pen.

Sample collection and clinical examination

During the acclimatisation period, clotted and heparinised blood samples were collected from all pigs upon arrival, and one week later. In the interval between vaccination and infection, blood samples were collected 3 times a week, and during the observation period blood samples were collected every other day. Simultaneously with sample collection, all pigs were examined clinically. The following signs were recorded: liveliness (apathy), body condition (cachexia), coughing, conjunctivitis, diarrhoea, ataxia, and haemorrhages. Rectal temperature was recorded daily. Tissue samples (tonsil, kidney, spleen, heart, liver) were collected from every pig that died or was euthanased.

Sample analyses

For virus isolation (VI) in blood or leukocytes, 100µl whole blood or 100 µl buffy coat was challenged in duplicate on to a non-confluent monolayer of PK_{15} cells cultured in multiwell plates (24 wells / plate). For VI in tissue samples, one cm^3 of each organ was homogenised into 9 ml minimal essential medium (MEM) and ground with an ultra-Turrax. After centrifugation for 10 min at 4000g, 300µl of the supernatant was challenged in duplicate onto a non-confluent monolayer of PK_{15} cells cultured in multiwell plates (24 wells / plate). After 48 hours, the cells were fixed with isopropanol and stained with a polyclonal fluorescein-conjugated anti-CSF immunoglobulin.

For antibody detection in serum, the virus neutralisation (VN) test using the $Alfort_{187}$ strain and the Herd Check CSFV ELISA (Ab-ELISA) test (IDEXX, Scandinavia, Osterbybruk, Sweden) were used. The discriminating ELISA (D-ELISA) test (Chekit® CSF-Marker, Dr. Bommeli AG, Switzerland), identifying antibodies against the E^{rns} glycoprotein of the CSF virus, was used in the marker-vaccinated pigs to distinguish between antibody response of vaccinated and infected pigs.

Data analyses

The reproduction ratio (R), a measure of transmission of infection, and defined as the average number of new infections arising from one typical infectious case, was calculated numerically using the maximum likelihood estimator.

$$R = \max \prod_{i=1}^{n} F\left\langle X_i, R \middle| N, S_0, I_0 \right\rangle$$

where F (X_i,R | N,S_0,I_0) is the likelihood function for the observed value X_i. where X_i is the total number of pigs that become infected, N, S_0, and I_0 are the total number of animals, the number of susceptible animals and the number of infectious animals at the beginning of the outbreak, respectively (Bouma et al., 1996). 95 % confidence intervals (CI) were constructed around the estimated value of R as described by Kroese and de Jong (2001).

Fever was defined as a rectal temperature >40.0°C. This was the one-sided upper limit (rounded off) of the 95% CI calculated on the average rectal temperature of all weaner pigs during all observations before the challenge exposure. Leukopenia was defined in a similar way. The one-sided lower limit (rounded off) of the 95% CI was equal to 12 000 cells/ml.

Periods during which a given clinical symptom occurred started with the first of at least two subsequent observations of a given clinical symptom, and ended with the first of at least two subsequent observations for which the given clinical symptom was absent. Periods of fever and leukopenia were defined in a similar way.

The duration of the interval between challenge exposure and a first positive blood sample, and the duration of the viraemia, detected with different diagnostic tests, were compared using a paired sample T-test (SPSS 10.0, Chicago, USA).

RESULTS

Clinical symptoms and rectal temperature

Experiment A (challenge = 0 dpv) (Table 1): All challenged pigs developed severe clinical symptoms and fever in the marker as well as in the C-strain-vaccinated pens (Fig. 1). Seven out of the eight challenged pigs died due to the CSF infection. In the marker-vaccinated contact pigs, the clinical symptoms were less severe and were only present in a limited number of the pigs. In the C-strain-vaccinated contact pigs, only one pig developed a number of clinical symptoms and eventually died.

Experiment B (challenge = 7 dpv): In the marker-vaccinated pens, all challenged pigs became clinically ill. The most frequently observed symptoms were: fever, conjunctivitis, cachexia, apathy, and diarrhoea. Eventually 3 out of the 4 challenged pigs died. In the contact pigs, the clinical symptoms were restricted to transient fever (n = 6) and conjunctivitis (n = 3) and no mortality occurred. In the C-strain-vaccinated pens, none of the challenged or contact pigs became clinically ill or died. The average rectal temperature of the challenged and contact pigs in both the marker- and the C-strain-vaccinated groups is presented in Fig. 1.

Experiment C (challenge = 14 dpv): In pen 3, (C-strain vaccine) one pig was euthanased during the acclimatisation period because of a broken leg. Therefore there were only 5 contact pigs in this pen. In the marker-vaccinated pens, 3 out of the 4 challenged pigs developed fever, which lasted on average 4 days. None of the contact pigs developed fever. In the C-strain-vaccinated pigs no fever was detectable in the challenged nor in the contact pigs (Fig. 1). No

other clinical symptoms or mortality were observed in either the marker or the C-strain-vaccinated pens.

Table 1. Summary of clinical symptoms in challenged and contact pigs in experiment A

	Marker vaccine		C-strain vaccine	
	Challenged	Contact	Challenged	Contact
Apathy	1/4 (8 days)*	1/12 (6 days)	1/4 (4 days)	0/12
Cachexia	2/4 (4 days)	1/12 (14 days)	2/4 (9 days)	1/12 (4 days)
Conjunctivitis	3/4 (12 days)	3/12 (13 days)	4/4 (16 days)	1/12 (6 days)
Diarrhoea	1/4 (6 days)	0/12	0/12	1/12 (4 days)
Ataxia	3/4 (8 days)	1/12 (4 days)	2/4 (11 days)	0/12
Mortality	4/4	3/12	3/4	1/12

* Average duration of the clinical symptom.

Virus isolation

In all experiments, the results of VI in whole blood were highly comparable to the results of VI in leukocytes. The average duration of the interval between challenge exposure and first positive sample was 0.26 days shorter, and the average length of the viraemic period was 0.58 days longer when using the VI in leukocytes in comparison to VI in whole blood. Both differences were not significantly different from 0 (n = 31, $P = 0.16$ and $P = 0.09$ respectively). Therefore, only the results of the VI in leukocytes are presented.

Table 2. Results of VI for the different experiments

			VI in leukocytes		
			# positive pigs	First positive sample (SD)*	Average duration (SD)
Exp. A (challenge = 0 dpv)	Marker Vaccine	Challenged (n = 4)	4	4 (0)	15 (2.6)
		Contact (n = 12)	10	13 (1.1)	11.4 (5.2)
	C-strain Vaccine	Challenged (n = 4)	4	4 (0)	27.5 (7.9)
		Contact (n = 12)	0	/	/
Exp. B (challenge = 7 dpv)	Marker Vaccine	Challenged (n = 4)	4	4 (0)	17 (12.9)
		Contact (n = 12)	5	17.6 (2.6)	2 (0)
	C-strain Vaccine	Challenged (n = 4)	0	/	/
		Contact (n = 12)	0	/	/
Exp. C (challenge = 14 dpv)	Marker Vaccine	Challenged (n = 4)	4	5 (1.2)	3.5 (1.9)
		Contact (n = 12)	0	/	/
	C-strain Vaccine	Challenged (n = 4)	0	/	/
		Contact (n = 11)	0	/	/

* Average duration of the interval between challenge and first positive sample in days (SD = standard deviation)

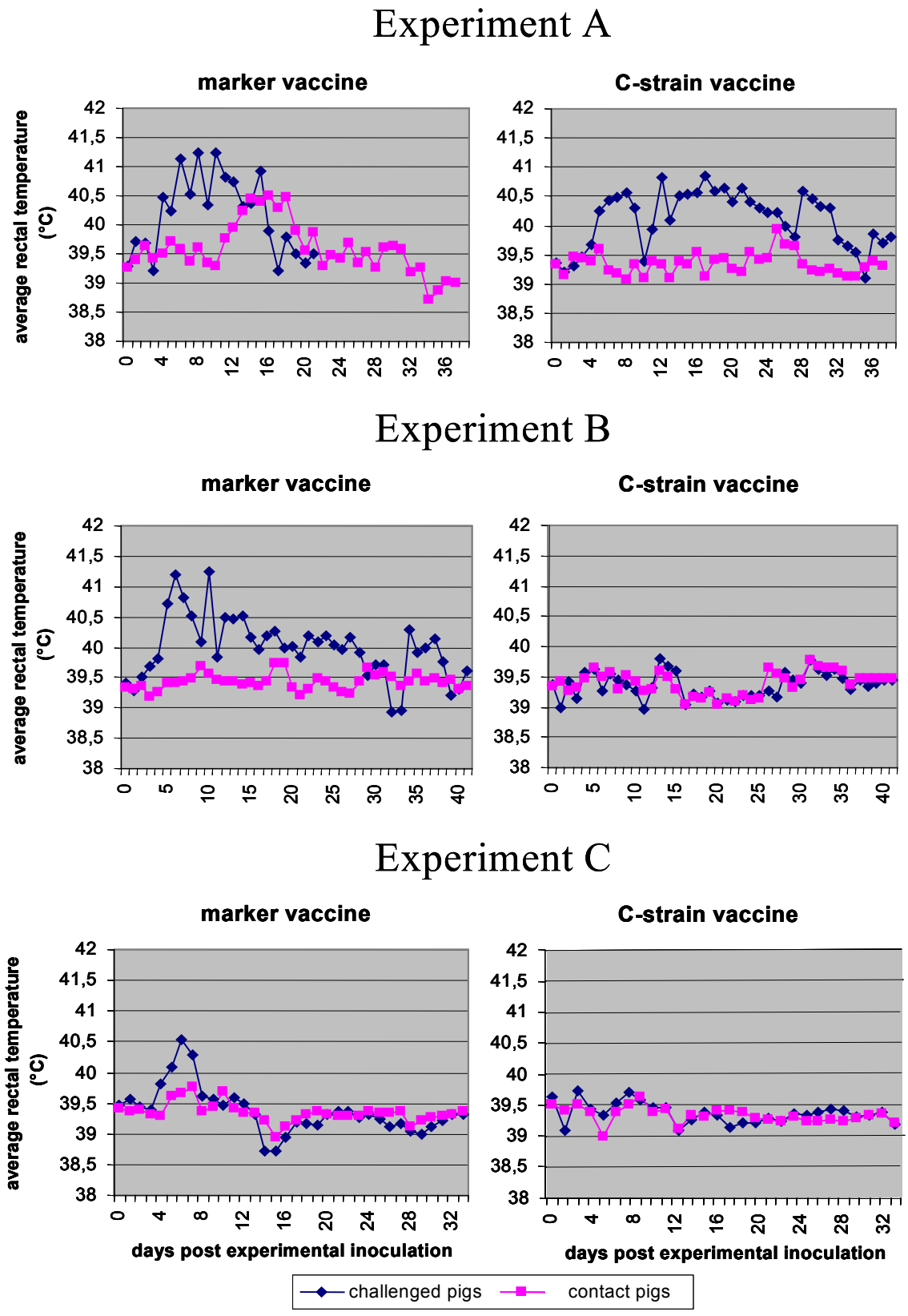

Fig. 1 Average rectal temperature of challenged and contact pigs

Experiment A (challenge = 0 dpv) (Table 2): In the marker-vaccinated pens, all challenged pigs and 10 out of the 12 contact pigs became viraemic. In the C-strain-vaccinated pens, all challenged but none of the contact pigs became viraemic.

Experiment B (challenge = 7 dpv) (Table 2): In the marker-vaccinated pens, all challenged pigs and 5 out of the 12 contact pigs became viraemic. In the C-strain-vaccinated pens, none of the challenged or contact pigs became viraemic.

Experiment C (challenge = 14 dpv) (Table 2): In the marker-vaccinated pens, all challenged pigs and none of the contact pigs became viraemic. In the C-strain-vaccinated pens, none of the challenged or contact pigs became viraemic.

Serology

The results of the VN and the Ab-ELISA were comparable. Using the VN test, positive results were on average 0.63 days (not significantly different from 0, n = 90, $P = 0.41$) earlier in comparison to the Ab-ELISA test. Therefore, only the results of the VN test are presented.

Table 3. Serological results for the different experiments

			Virus Neutralisation		Discriminating ELISA	
			# positive pigs	First positive sample (SD)*	# positive pigs	First positive sample (SD)
Exp. A (challenge = 0 dpv)	Marker Vaccine	Challenged (n = 4)	2	17 days (1.4)	3	14 days (0)
		Contact (n = 12)	12	15 days (2.3)	12	22.7 days (2.6)
	C-strain Vaccine	Challenged (n = 4)	3	13.3 days (2.3)	nd	nd
		Contact (n = 12)	12	13.5 days (2.7)	nd	nd
Exp. B (challenge = 7 dpv)	Marker Vaccine	Challenged (n = 4)	4	10.5 days (5.3)	3	18 days (8.7)
		Contact (n = 12)	12	12 days (4.7)	11	27 days (6.3)
	C-strain Vaccine	Challenged (n = 4)	4	3 days (1.2)	nd	nd
		Contact (n = 12)	12	4.8 days (2.8)	nd	nd
Exp. C (challenge = 14 dpv)	Marker Vaccine	Challenged (n = 4)	4	12 days (6.9)	4	12.5 days (1.9)
		Contact (n = 12)	12	11.8 days (5.9)	0	/
	C-strain Vaccine	Challenged (n = 4)	4	6 days (2.8)	nd	nd
		Contact (n = 11)	11	3.63 days (2.4)	nd	nd

* Average duration of the interval between challenge and first positive sample (SD = standard deviation)

nd: not done

Experiment A (challenge 0 dpv) (Table 3): In the marker- as well as the C-strain-vaccinated pens, some of the challenged pigs did not react positively in the different serological test. This is probably because they died shortly after the infection and before the serological response became detectable. In the marker-vaccinated pens, all contact pigs reacted positively in the VN and the D-ELISA.

Experiment B (challenge 7 dpv) (Table 3): All challenged and contact pigs reacted positively in the VN, in both the marker- and C-strain vaccinated pens.

Experiment C (challenge 14 dpv) (Table 3): All challenged and contact pigs reacted positively in the VN, in both the marker- and the C-strain vaccinated pens. In the D-ELISA, of the marker-vaccinated pigs, only the challenged pigs became positive whereas the contact pigs remained negative.

Reproduction ratio

Experiment A (challenge = 0 dpv) (Table 4): Based on the results of the VI, it was concluded that 10 and 0 contact pigs became infected in the marker- and C-strain-vaccinated pens, respectively, resulting in an R of 2.9 (1.5-10.8) for the marker-vaccinated pens and an R of 0 (0-1.5) for the C-strain vaccinated pens. When the results of the D-ELISA were used to determine the number of infected contact pigs, it was found that in the marker-vaccinated pens all contact pigs became infected. This resulted in an R of ∞ (1.9-∞).

Table 4. Different values of the reproduction ratio's in the different experiments.

			# of contact infected pigs	R per pen (CI)**	Overall R (CI)
Exp. A (0 days)	Marker vaccine (VI)*	Pen 1	4	1.6 (0.5-6.9)	2.9 (1.5-10.8)
		Pen 2	6	+∞ (1.1-+∞)	
	Marker vaccine (D-ELISA)*	Pen 1	6	+∞ (1.1-+∞)	+∞ (1.9-+∞)
		Pen 2	6	+∞ (1.1-+∞)	
	C-strain vaccine (VI)*	Pen 3	0	0 (0-4.6)	0 (0-1.5)
		Pen 4	0	0 (0-4.6)	
Exp. B (7 days)	Marker vaccine (VI)	Pen 1	2	0.8 (0.2-5.8)	1.0 (0.3-3.0)
		Pen 2	3	1.2 (0.3-9.4)	
	Marker vaccine (D-ELISA)	Pen 1	6	+∞ (1.1-+∞)	3.5 (1.6-10.9)
		Pen 2	5	2.4 (0.7-7.5)	
	C-strain vaccine (VI)	Pen 3	/	/	/
		Pen 4	/	/	/
Exp. C (14 days)	Marker vaccine (VI)	Pen 1	0	0 (0-4.6)	0 (0-1.5)
		Pen 2	0	0 (0-4.6)	
	Marker vaccine (D-ELISA)	Pen 1	0	0 (0-4.6)	0 (0-1.5)
		Pen 2	0	0 (0-4.6)	
	C-strain vaccine (VI)	Pen 3	/	/	/
		Pen 4	/	/	/

* test used to determine the number of contact infections

** CI = 95 % confidence interval

Experiment B (challenge = 7 dpv) (Table 4): Based on the results of the VI, it was concluded that 5 contact pigs became infected in the marker-vaccinated pens, resulting in an R of 1.0 (0.3-3.0). In the C-strain-vaccinated pens, neither the challenged nor the contact pigs became infected. Therefore no reproduction ratio could be calculated. Although viraemia was

only detectable in 5 of the 12 marker-vaccinated contact pigs, 11 of them reacted positively in the D-ELISA. When these results were used to calculate the reproduction ratio, the overall R for the marker-vaccinated pens became 3.5 (1.6-10.9).

Experiment C (challenge = 14 dpv) (Table 4): Based on the results of the VI, it was found that all challenged pigs, but none of the contact pigs, became infected in the marker-vaccinated pens. This results in a reproduction ratio of 0 (0 – 1.5). The same result is obtained when the results of the D-ELISA are used to determine the number of infected contact pigs. In the C-strain-vaccinated pens, no viraemia was detectable in the challenged nor in the contact pigs and no R could be calculated.

DISCUSSION

The purpose of this study was to quantify transmission of CSF virus among pigs vaccinated with an E2 sub-unit marker vaccine and pigs vaccinated with a conventional C-strain vaccine at different time intervals after vaccination. No non-vaccinated control groups were included in the experiments. However, this does not influence the interpretation of the results, since the challenge model used was successful in all experiments, illustrated by the fact that in each experiment at least one group of pigs became viraemic.

The horizontal virus transmission was fully prevented (R = 0) by the marker vaccine when the infection occurred at 14 dpv. Due to the limited number of animals in the experiments, this does not result in an R which is significantly smaller then 1 (0, 1.5). The reproduction ratio, calculated on the results of the VI test (R = 1.0 (0.3-3.0)), suggests that virus transmission was already largely reduced at 7 dpv. However, these results are somewhat misleading since the results of the D-ELISA indicate that 11 instead of 5 contact pigs became infected, resulting in an R of 3.5 (1.6-10.9). This leads to the conclusion that CSF virus transmission is not yet sufficiently reduced by the marker vaccine when the challenge exposure occurs at 7 dpv. The difference in number of infected pigs detected by the VI and the D-ELISA may be due to a viraemia with a short duration that can be missed when samples are only take every two days, or a viraemia that remains under the detection limit of the VI technique. In previous experiments it was also found that marker-vaccinated pigs did react positively in the D-ELISA without having had a detectable viraemia in VI (Dewulf et al., 2000; Dewulf et al., 2001). At 0 dpv, no reduction of the virus transmission was observed (R = ∞ (1.9-∞)). The results of these transmission experiments are highly comparable to the results of similar experiments described by Bouma et al. (2000). The clinical protection in pigs vaccinated with the marker vaccine was present 7 to 10 days before the virus transmission was prevented.

In the conventionally vaccinated pens, virus transmission was already fully prevented when the vaccination occurred on the day of the challenge exposure, unless the challenged pigs developed severe viraemia and became clinically diseased. This may be explained by the fact that it takes, on average, 4 days for a challenged pig to become infectious (Terpstra, 1988) and during these 4 days the contact pigs have already developed a sufficient immunity to prevent the infection. On the other hand, it needs to be stressed that the point estimate of R (=0), calculated based upon the results of these experiments is not significantly smaller than 1(0,1.5), which is probably due to the limited number of pigs in the experiments. Besides, it is impossible to confirm the virological results by a serological test, since there is no discriminating test available to make a serological differentiation between conventionally vaccinated pigs and infected pigs. Therefore, it may be that some contact pigs did become infected, and consequently the R we

calculated could be an underestimation of the reality. Indicating that this experiment needs to be repeated to increase the validity of the conclusions. Nevertheless, if there was still a transmission of the virus, this did not lead to a detectable viraemia, nor any kind of clinical symptoms, indicating that it is unlikely that these contact pigs are themselves a source of further infectivity. When the challenge exposure occurred at 7 or 14 dpv, even the challenged pigs did not develop viraemia and, by consequence, there was also no detectable transmission of the virus towards the contact pigs. All pigs vaccinated with the C-strain vaccine in the different experiments were also clinically protected, except the challenged pigs and one contact pig in experiment A. It remains indistinct whether the one clinically diseased contact pig in experiment A was a result of a CSF infection, since there was no detectable viraemia.

The results of these experiments may have important implications for the use of the different vaccines in an emergency vaccination scenario. The length of the interval between vaccination and onset of immunity will determine the time before a reduction of the virus transmission is achieved. If the interval between vaccination and onset of immunity is short, herds can't become infectious anymore shortly after they have been vaccinated, and the first generation of secondary cases may already be prevented. In this case, only a small neighbourhood needs to be vaccinated to be able to stop the between-herd transmission through local spread. If the interval is longer, a herd that is infected shortly before or after vaccination may still become infectious, and only the second or third generation of secondary cases will be prevented. In such a situation, a larger vaccination region is necessary. Therefore, the interval between vaccination and onset of immunity determines the radius of the vaccination area.

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AN EPIDEMIOLOGICAL STUDY OF WHITE SPOT DISEASE IN SHRIMP FARMS OF KARNATAKA, INDIA

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SUMMARY

The farming of marine shrimp is a major contributor to the economies of many maritime tropical countries and to the livelihoods of vast numbers of rural families. Over the last decade shrimp farms have been affected by a serious pandemic of White Spot Disease (WSD) associated with White Spot Syndrome Virus (WSSV). Estimates of the cost of this pandemic vary but all agree it has had a massive social and economic impact. In this paper we present some data from a project studying the epidemiology of WSD in Vietnam and India. We discuss the effect of WSD on productivity using a previously proposed case definition, the risk associated with the presence of WSSV in the young shrimp at stocking, the value of moribund/dead shrimp collected at the side of the pond as a source of data and an evaluation of the farmers' emergency harvest strategy.

INTRODUCTION

Throughout the tropics the culture of marine penaeid shrimps is a major contribution to both national economies and livelihoods of rural communities. Estimated shrimp production from aquaculture in 1998 was 1.8 million tonnes (www.globefish.org). Since the early 1990s shrimp farms have been afflicted by a major pandemic of White Spot Disease (WSD) associated with the White Spot Syndrome Virus (WSSV) provisionally classified in a new virus family, Nimaviridae (Vlak et al., 2002). Existing economic estimates of the impact of this disease are no better than informed guesses (Flegel & Alday Sans, 1998). However, there is no doubt that this disease, now present in all shrimp farming countries both in Asia and the Americas, has had an extremely serious social and economic impact.

Attempts to control the disease have been based on the avoidance of risk factors, identified largely from laboratory-based experiments and practical experience (e.g. Nakano et al., 1994; Chou et al., 1995; Lo et al., 1996; Limsuwan, 1997a; Flegel & Alday Sanz, 1998; Chou et al., 1998; Kanchanaphum et al., 1998; Maeda et al., 1998; Sudha et al., 1998; Withyachumnarnkul, 1999). There is some anecdotal evidence that such strategies have allowed farmers to delay the onset of the disease but the prevalence of outbreaks at a pond level does not seem to have decreased. Vaccination is not an option since shrimp rely mostly on the non-specific immune system and there is no evidence of immunological memory (Thörnqvist & Söderhäll, 1996). In

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the face of an outbreak the farmers' only option is to conduct an emergency harvest to salvage some value from the crop. This practice often pre-empts the expression of an epidemic within the pond.

Epidemiology of farmed shrimp and other aquatic animals is still in its infancy and faces a number of significant challenges but has the potential to make a major contribution to the health management in aquaculture (Georgiadis et al., 2001).

Monitoring the health of the population is practically difficult in shrimp ponds. The young shrimp (post larvae or PL) were stocked in coastal ponds (0.6 to 1.6 ha) at up to 144,260 PL per pond. The nature of the water and the behaviour of the shrimp made them impossible to observe in the pond from around two weeks after stocking and there was no reliable method to monitor survival. While it was possible to obtain samples of animals from the ponds during the production cycle by the use of cast nets; capture and examination of live animals is potentially harmful and individual marking was not a realistic option. Clinical signs in shrimp were widely accepted to be of little diagnostic value and there are relatively few tests available to monitor disease processes (Lightner, 1996). For example, to date all attempts to produce continuous shrimp cell lines have all failed. For these reasons accurate data on population size and prevalence were only available at stocking and harvest.

In this paper we present the effect of WSD on productivity using a previously proposed case definition, an investigation of the risk associated with WSSV in PL, the value of moribund/dead shrimp collected at the side of the pond as a source of data and an examination of the farmers' emergency harvest strategy.

The study reported here was part of larger project funded by the British Government Department for International Development, examining the epidemiology of WSD in Vietnam and India. The objective of the project was to investigate risk factors for outbreaks and to develop practical strategies to reduce the impact of the disease on the productivity of affected farms. Other data from this project have already been published or are in the process of publication (Corsin et al., 2001; 2002; in press; Thakur et al., 2002; Mohan et al., 2002).

MATERIALS AND METHODS

The study was conducted in Kundapur, Karnataka, India, which was selected for its location, 110 km north of Mangalore, where the laboratories for sample analyses were located. The local farming history also made identification and selection of an appropriate sample population possible.

A stratified random sample of 100 of the 150 farmers was selected. These farmers were mostly visited individually and data on the date of stocking gathered. Seventy of the selected farmers were enrolled between September 1999 and January 2000 and a randomly selected pond followed on each farm until harvest, which was completed by the end of April 2000.

Before the pond was stocked with *Penaeus monodon*, wild animals (e.g., shrimp, crabs, fish, etc.) and plankton samples were collected. A structured interview based questionnaire was used to collect data on previous crops and pond preparation practices. At stocking data on source of PL were collected by interviewing the farmer, while PL activity was measured by direct observation. Activity of the PL was determined by the research assistants at the time of stocking by transferring 500 PL sampled from a random selection of the transportation bags, to a bucket,

gently stirring the water and observing the proportion of PL actively swimming against the current. A 3-level scoring system based on the proportion of active PL (>2/3=good; <2/3 and >1/3=average; <1/3=bad) was used. The four research assistants were trained and tested in the use of this technique to improve inter-observer agreement. In the 70 enrolled ponds there were 73 stocking events and a total of 500 PL (8 samples each of 50 PL) were collected prior to each stocking event for PCR analysis. The farmers' opinion of quality of the PL was obtained by interviewing the farmer soon after the stocking had been completed, when they were asked to classify the batch as good, average or bad. No effort was made to influence the method by which the farmer evaluated the quality of the PL, but the criteria were recorded.

From stocking till harvest farmers were supplied with sheets to record daily data on feeding regime, water exchange, other management practices, the presence, number and clinical signs of any moribund/dead shrimp observed at the side of the pond. The farmers removed a pleopod (swimming leg) from each moribund/dead shrimp collected and fixed it in absolute methanol for analyses by PCR. The remainder of the shrimp were placed in a container of 10% formalin for processing and histopathological analyses. The recording sheets were collected and water quality measured during visits at a fixed time every week by the research assistant. During such visits samples of feed and moribund/dead shrimp were also collected.

At harvest, 400 *P.monodon* were collected and fixed for PCR analyses, of these, 100 were also examined for size and clinical signs, and data on the harvest were collected by interviewing the farmer. Other data were recorded but are not reported here.

A PCR laboratory was established at the Fisheries College, Mangalore and samples were processed in order to test for the presence of WSSV. A total of 1340 pooled samples were tested including 382 *P.monodon* PL; 56 moribund/dead *P.monodon*; 105 harvested *P.monodon*. For PCR a rapid DNA extraction was used (Kiatpathomchai, 2001), the samples were then tested by nested PCR for the presence of WSSV DNA (Lo et al., 1996). Samples were recorded as 1 step positive if a PCR product was visible after the first step of amplification and as 2 step positive if re-amplification was necessary to visualise the presence of WSSV DNA. The PCR protocol was subjected to a full series of internal validations and results were also confirmed by parallel testing in other laboratories.

Histopathological sections from moribund/dead and harvested *P.monodon* were also prepared and examined for the presence of WSD pathology and other pathological conditions (Mohan et al., 2002).

A previously developed case definition was used to identify outbreaks of WSD at a pond level. This was: "the observation of 5 or more moribund/dead shrimp at the side of the pond in a single day and the detection of WSSV in the shrimp at harvest by 1 step PCR or histopathology".

Actual harvest data from the farms was compared with a previously developed harvest decision-making tool based on observed mortalities and clinical signs. This simple decision tree allowed cases to be identified with a specificity of 93.8 (lower and upper confidence limits 85.4-100) and a sensitivity of 94.3 (86.6-100) (Turnbull et al., 2002).

Statistical analyses

Means and standard deviation (SD) or medians and inter-quartile (IQ) ranges were used to describe variables. Relative risks (RR) and exact binomial 95% confidence intervals (CI)

around proportions were calculated with Epi Info 6 (Dean et al., 1996). Comparison of means (Student t-test) or medians (Mann-Whitney Rank Sum Test) were performed with SigmaStat© 2.0 (Jandel Corporation, USA). Sensitivity and specificity and upper and lower confidence limites (CL) were calculated using Win Episcope 2.0 (http://www.clive.ed.ac.uk/winepiscope). Statistica© 0.6 (StatSoft, Inc. Tulsa, USA) was used for survival and multiple regression analysis.

RESULTS

WSD and Production

A full set of information and samples from stocking to harvest were collected from 62 of the 70 enrolled ponds. Of the ponds, 31 were WSD cases, 37 ponds were 1 step positive at harvest and 59 ponds were 2 step PCR positive. There was a significant difference in the production between the cases and non-cases but no significant difference in survival (Table 1).

Table 1. Comparison between the production in ponds that were cases and non-cases

	Ponds =WSD cases	Ponds=Non WSD cases	*P*
	Median (IQ range)		
Length of production cycle (days)	79 (64-89)	102 (95-112)	<0.001
	Survival analysis Gehan's Wilcoxan test statistic 3.94$^{\nabla}$		0.00008
	Mean (SD)		
Yield (kg/ha)	668.3 (334.2)	979.7 (571)	0.012
Shrimp weight at harvest (g)	16.1 (6.2)	25.1 (6.7)	<0.001
Survival %$^{\otimes}$	56.3 (16.4)	52.2 (20.8)	0.432

$^{\otimes}$Mean and SD on untransformed data, test performed on Arcsine Square Root transformed data.

Data obtained from PL

PL activity or quality assessed by the farmer was not associated with WSSV by 2 step PCR (Table 2).

Table 2. Farmer and research assistant estimation of PL quality/activity and WSSV 2 step PCR status of PL batch

	WSSV			
RA PL activity	positive	negative	RR: 1.12	
Average	16	16	95% CI: 0.68 – 1.83	p=0.842
Good	17	21		
	WSSV			
Farmer PL quality	positive	negative	RR: 0.97	
Average	17	18	95% CI: 0.61 – 1.55	p=0.910
Good	19	19		

The PCR status of the PL was not significantly associated with production (Table 3). Half of the ponds (31) were stocked with 2 step positive PL and only 3 ponds were stocked with 1 step positive PL. Of these 3 ponds, 2 were both 1 step PCR positive and WSD cases at harvest the other was neither 1 step positive nor a WSD case. Stocking with 2 step positive PL did not increase the risk of an outbreak of WSD (WSD outbreak Relative Risk (RR) 0.86, 95% Confidence Interval (CI) 0.58-1.26) or of harvesting 1 step PCR positive shrimp (WSSV at harvest RR 0.95, CI 0.67-1.36).

Table 3. Comparison between production in ponds stocked with 2 step PCR positive and negative PL

	WSSV +ve PL	WSSV –ve PL	*P*
	Mean (SD)		
Length of production cycle (days)	89.3 (22.2)	85.5 (23.1)	0.495
	Survival analysis Gehan's Wilcoxan test statistic 0.081		0.936
	Median (IQ range)		
Yield (kg/ha)	726.4 (473.8-1001.5)	672.9 (388.9-1010.6)	0.843
Shrimp weight at harvest (g)	22.4 (12.7-26.0)	21.5 (14.6-27.0)	0.618

Use of samples of dead or moribund shrimp to determine health of population

A total of 56 ponds recorded mortalities, 73 samples of moribund/dead shrimp were collected from 50 ponds for histopathology (from 1 to 5 samples per pond and 1 to 13 shrimp per sample) and 55 samples from 44 ponds for PCR analysis (1 to 4 samples per pond and 1 to 20 pleopods per sample). Despite autolytic changes histological examination of samples from dead or moribund shrimp from the side of the ponds revealed the presence of pathognomonic WSSV intranuclear inclusion bodies. Other lesions were also detected including chronic cellular inflammatory lesions typical of chronic bacterial infections.

Table 4. Sensitivity and specificity of data derived from shrimp collected at the side of the pond and from random samples at harvest as a diagnostic test for WSD at a pond level

	Sensitivity % (CL)	Specificity % (CL)
WSSV histopathology	92.9 (83.3-100)	75.0 (53.8-96.2)
White spots under shell	65.5 (48.2-82.8)	94.1 (82.9-100)
1 step PCR	66.7 (47.8-85.5)	85.7 (67.4-100)
2 step PCR	87.5 (74.3-100)	53.8 (26.7-80.9)
White spots under shell in random sample of 400 at harvest	68.3 (54.0-82.5)	81.0 (64.2-97.7)

The sensitivity and specificity was calculated, as diagnostic tests for an outbreak of WSD, for histopathology, PCR, the presence of typical clinical signs (white spots under the shell) from

moribund/dead and the presence of typical clinical signs in a random selection of 400 shrimp at harvest (Table 4).

Emergency harvest strategy

Farmers' decision to harvest was based on a range of parameters including the stage of the production cycle and observations from the pond side. A previously developed economic model (manuscript in preparation) for the farms in the study suggested that the mean economic break-even point was at 70 days post stocking. Only 3 non-cases harvested before 70 days and 2 of those ponds had problems that fully justified harvest. In the ponds that were cases there was a mean delay of 2.3 days (SD 2.0) between the time when the decision making tool (Turnbull et al., 2002) would have suggested harvest and when actual harvest took place. The mean delay before 70 days of culture was 1.8 days and after 70 days was 2.5 days. There was a significant negative association between length of delay and productivity. The delay was converted to a dichotomous variable (greater or less than the mean delay) and included as an independent variable in a multiple regression model with stocking density and days of culture as continuous independent variables and kg/ha as the continuous dependent variable. The association between delay and production but was not significant (Whole model multiple R=0.731, F=10, *P*=0.0001, univariate Days of culture F=25.06, *P*=0.00003; Stocking density F=3.71, *P*=0.65, Delay F=3.55, *P*=0.70).

DISCUSSION

A major challenge in this study was dealing with a disease that was not allowed to progress to a full outbreak. Farmers invariably responded to evidence of an outbreak of WSD by conducting an emergency harvest. Such a strategy is common in aquaculture where the crop may have a market value even at a relatively early stage of the production cycle. A case definition had to differentiate between disease at an individual animal level and an outbreak at a pond level. The case definition proposed incorporated the reporting of 5 or more moribund/dead shrimp at the side of the pond in a single day. This number was not denominator based since it was not possible to accurately estimate the survival or number of shrimp remaining in the pond between stocking when the range was between 10,420 PL per pond and harvest when the range was 3,639 to 66,795. The shrimp at the side of the pond are described as moribund/dead, since determining if a shrimp is recently dead or in extremis is difficult even under laboratory conditions.

WSD and Production

When evaluating the impact of WSD outbreaks in ponds it was only possible to examine the combined impact of WSD and the resulting emergency harvests. The combination of the disease and the farmers' response had a significant effect on productivity and the length of the production cycle. In the earlier stages of the WSD pandemic when farmers were less well informed about WSD, large numbers of ponds were allowed to progress to full outbreak and in these cases very severe mortalities of up to 100% were observed (Nakano et al., 1994; Wongteerasupaya et al., 1995; Anonymous, 1997; Karunasagar et al., 1998; Park et al., 1998; Zhan et al., 1998). The conclusion from these data presented here is that productivity is still significantly reduced by the presence of WSD in the system.

Data obtained from PL

As stated in the introduction, farmers have a limited number of options for controlling WSD, they can attempt to avoid risk factors or conduct an emergency harvest. One of the main risk factors described in the literature is the presence of WSSV in PL at stocking (Limsuwan, 1997a; Flegel & Alday Sanz, 1998; Mushiake et al., 1999; Withyachumnarnkul, 1999). There are a very large number of commercial laboratories throughout the tropics many analysing PL samples for farmers at costs of more than £18 per sample. Such samples are frequently taken with little if any regard for sampling theory, there is very little standardisation between laboratories and there have been problems with quality control.

Assuming that WSSV were a significant risk factor for WSD outbreaks it would be useful if there were a simple pond side indicator for the presence of WSSV in PL but neither of the crude measures of PL quality examined here were associated with the PCR status of the PL. When the 2 step PCR status of the PL was examined it was not found to be significantly associated with outbreaks of WSD, 1 step PCR status at harvest or productivity. There are several possible explanations for this counterintuitive result. The 2 step PCR results in the PL may either have represented non-viable virus or non-infectious viral contamination. If viable virus were present either as an active infection or contamination it may not have had a sufficiently large basic reproductive number to sustain the infection. Even batches of 1 step PCR positive PL were not invariably associated with either a WSD outbreak or a 1 step PCR result at harvest. The main route of transmission of the virus in the pond was thought to be by ingestion of dead shrimp (Chou et al., 1995; 1998; Soto et al., 2001), although the virus can spread via the water (Nakano et al., 1994; Chou et al., 1998). The PL when stocked were approximately 0.01g at a mean of 8 per m^2, and therefore it is quite conceivable that a PL might die of WSD but fail to transfer the infection to another shrimp. One more possible explanation is that the effect of the virus in the PL was masked by other sources of virus during the production system since the ponds in this study had frequent water exchange. Regardless of the explanation there would appear to be little value in testing PL in this farming system although it would be unwise to extrapolate this recommendation to other systems. The prevalence of WSSV within populations of PL may be a significant component of the risk associated with PL and that relationship is currently being analysed.

Use of samples of dead or moribund shrimp to determine health of population

It is practically difficult to obtain regular estimates of survival or disease prevalence from shrimp ponds. It has been accepted for at least 14 years that there was little if any value in samples of moribund/dead shrimp collected from the side of the pond (Bell and Lightner 1988). The rational was that shrimp in common with other poikilotherms have enzyme systems and microflora that are adapted to work over a range of temperatures and therefore even rapid cooling does not delay autolysis. In this study samples of moribund/dead shrimp were collected for both histopathological analyses and PCR. The majority of the farmers were willing to collect and record samples, although the weekly visit by the research assistants probably improved compliance. This simple and inexpensive form of data collection produced a variety of useful information. The samples were non-random and therefore did not produce any indication of population prevalence, however, since they were biased towards the shrimp most likely to be affected by WSD they were potentially more sensitive than a purely random sample.

The samples for histopathology provided evidence of both WSD pathology in individual animals and presence of lesions typical of chronic bacterial infections. Histopathology proved

to be more sensitive than either 1 or 2 step PCR and more specific than 2 step PCR as a diagnostic test for WSD at a pond level. Evidence presented elsewhere (Mohan et al., 2002) suggests that there was no significant deterioration in the WSSV DNA within the moribund/dead samples. It would seem counterintuitive that histological diagnosis of WSD would be more sensitive than specific, since it is well documented that histology is very specific at an individual animal level. However, the cumulative effect of examining larger numbers of animals may well increase the sensitivity and since individuals with WSD are not always indicative of an outbreak at a pond level the specificity for a pond level outbreak would not necessarily be high. Techniques such as histopathology and PCR might produce valuable research data but it would appear that such expensive and often slow techniques are not necessary for detecting the presence of WSD or making a decision to harvest. A previously reported harvest decision-making tool was based purely on the pattern of observed mortalities and was both a sensitive and specific indicator of WSD at a pond level (Turnbull et al., 2002).

The presence of the widely reported white spots under the cuticle in dead or moribund shrimp was found to be highly specific for WSD at a pond level, contrary to common perception. A previous publication by Corsin et al. (2001) reported that white spots were 77.3% sensitive and 77.8% specific for the presence of WSSV by 1 step PCR, based on the presence of white spots and 1 step PCR product in 400 harvested shrimp. Analyses in this study produced similar sensitivity (68.3%) and specificity (81%).

In an article from 1997(b), Limsuwan reported white spots associated with a variety of environmental conditions and pathogens. Based solely on extensive experience of the industry he described four types of clinical picture, the key observation was that the presence of shrimp (<12g) with white spots at the side of the pond was usually associated with WSD. White spots on otherwise healthy shrimp from cast net samples were not usually associated with WSD.

This study based on farmer observation in a semi-intensive Indian system produced findings, which are consistent with our previous study in a Vietnamese rice-shrimp system (Corsin et al., 2002) and those of Limsuwan (1997b) based on the intensive Thai systems. All these studies would suggest that white spots in random samples from the pond are not necessarily a good predictor of WSD but white spots in moribund/dead shrimp from the side of the pond are a highly specific but not sensitive indication of WSD in the population. The lack of sensitivity would suggest that not all affected shrimp demonstrate these signs.

Emergency harvest strategy

Assessment of the harvest strategy used by the farmers in this production system indicated that only one pond was harvested before the estimated break-even point of 70 days post stocking in the absence of a serious health problem. Two other non-cases were harvested before 70 days, both had problems but did not fulfil the case definition. Of the cases most would have harvested earlier if they had followed the harvest decision-making tool and there was an indication that those who delayed by more than the mean (>2.5 days) had a lower yield from their pond, however, this was not a highly significant association. It is not clear why the farmers appeared to delay harvest longer after 70 days of culture. Application of this decision making tool could potentially have reduced the delay between identification of an outbreak of WSD and harvest, although the majority of farmers in this study appeared to already have an adequate system for deciding when to harvest. Such decision-making tools might be of more value to less well-informed farmers or new entrants into the farming system.

CONCLUSION

The aim of this project was to help farmers increase productivity and the preliminary findings were presented to farmers and discussed with a range of stakeholders within a year of the start of sampling. The project has improved our understanding of WSD and we have developed some strategies for conducting population-based investigations on shrimp farms. Much fundamental information is still required to allow more sophisticated control of WSD including prevalence pattern of viral infection and disease in a pond prior to the development of an epidemic within the pond.

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EFFECTS OF SEROPOSITIVITY FOR BOVINE LEUKAEMIA VIRUS, *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*, AND *NEOSPORA CANINUM* ON THE CALVING TO CONCEPTION INTERVAL IN MARITIME CANADIAN DAIRY CATTLE

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SUMMARY

The purpose of this research was to determine the effect of seropositivity for exposure to bovine leukaemia virus (BLV), *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Neospora caninum* (NC) on calving to conception interval (CCI) in dairy cattle in three eastern Canadian provinces. Samples were tested for antibodies against BLV, MAP and NC using commercially available ELISA test kits. The average CCI for seronegative cows for all three infections was 115.58 days. BLV and MAP seropositivity in 1st lactation heifers were associated with a 1.97 (0.29 – 3.65, 95% C.I.) and 7.16 (1.85 – 12.48, 95% C.I.) unit increase in log of CCI compared to seronegative 1st lactation heifers. The hazard (probability of getting pregnant at a particular time) associated with BLV and MAP seropositive heifers (first lactation) was 0.79 (0.65 – 0.95, 95% C.I.) and 0.49 (0.26 – 0.93, 95% C.I.) times that of BLV and MAP seronegative heifers, respectively.

INTRODUCTION

Infectious diseases, that can be subclinically harboured in apparently healthy animals, such as bovine leukaemia, Johne's disease and neosporosis, are undergoing increasing scrutiny and research as a result of new World Trade Organisation regulations concerning animal health and animal movement between countries (World Trade Organisation, 1994). While these diseases lead to clinical manifestations, there are still questions regarding the impacts of subclinical infection by the agents associated with these diseases.

A recent survey done in maritime Canadian dairy herds reported that 70.0%, 16.7% and 78.9% of the herds had at least one seropositive animal for bovine leukaemia virus (BLV) and at least two seropositive animals for *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and *Neospora caninum* (NC). Within herd prevalences in herds seropositive for BLV, MAP and NC were 30.9%, 8.5% and 24.0% (Keefe & VanLeeuwen, 2000; VanLeeuwen et al., 2001). Seroprevalences of these pathogens have been reported from around the world and have been described in detail in many review articles (Pelzer, 1997; Dubey, 1999; Keefe & VanLeeuwen, 2000; Olsen et al., 2002).

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Based on the Maritime Canadian survey, and literature estimates of associated costs, a subsequent study estimated the direct farm costs associated with infection with these organisms for infected Maritime Canadian dairy herds. The costs for BLV, MAP and NC, respectively, were reported to be $806, $2472 and $2304 per infected herd using an average herd size of 50 cows (Chi et al., 2002). However, a direct loss due to an increase in calving to conception days associated with BLV and NC was not included, because there are no consistent results in the literature. Furthermore, the direct loss due to increase in calving to conception interval days (CCI) associated with MAP infection, was estimated using a study done in four high MAP seroprevalence dairy herds in Michigan (Johnson-Ifearulundu et al., 2000). The increase in CCI associated with MAP seropositivity might be quite different in low-prevalence MAP dairy herds in Maritime Canada. The pathophysiology behind the effects of seropositivity for BLV, MAP and NC on CCI is still being researched. There have been some conflicting results regarding the associated effects of seropositivity for BLV, MAP and NC on CCI.

For BLV, the reported reproductive effects associated with seropositivity for BLV ranges from no difference to 0.47 months longer CCI in BLV seropositive cows (Heald et al., 1992). In a herd-level study, BLV seropositive herds had a slight non-significant increase in CCI (Emanuelson et al., 1992). The reproductive effects associated with seropositivity for BLV in dairy cows in high within-herd prevalence herds might vary with lactation number or co-infection with other pathogens (like MAP which also impairs cellular immunity). The impact of BLV seropositivity in a population of randomly selected herds, such as we have studied in Maritime Canada, is unclear.

For MAP, a preliminary study in maritime Canadian dairy cattle reported that the overall 3 year culling risk from decreased milk production, mastitis or reproductive inefficiency was 1.53 (P<0.01) times higher in MAP seropositive cows than seronegative cows after controlling for parity, 305 day milk production and linear score SCC. The odds of being culled because of decreased milk production, mastitis or reproductive inefficiency was 2.90 (P=0.02) times greater in MAP seropositive cows as compared to seronegative cows (Tiwari et al., 2002). Similar results (22.6% versus 3.6%) were reported in subclinically infected (confirmed by fecal culture) cows culled due to mastitis and (68.8% versus 60.2%) due to infertility (Merkal et al., 1975). These studies did not try to quantify impaired reproductive efficiency associated with MAP. Fecal culture positive cows have been associated with 1.73 months greater calving interval (Abbas et al., 1983). In another study, seropositive cows were associated with 28-day increase in days open; however, there was no association for cows that were faecal culture (FC) positive compared to FC negatives (Johnson-Ifearulundu et al., 2000). Seropositivity to MAP seems to have a negative impact on reproductive efficiency but these effects may not apply to low within-herd prevalence herds such as we have in Maritime Canadia. Furthermore, the impact of MAP seropositivity may also vary with lactation number or co-infection with other pathogens.

The main clinical sign observed in NC infected cows is abortion. NC seropositive cows were 3 times more likely to abort than NC seronegative cows. The mean gestation age of NC abortion has been reported to be 5.5 months but abortion can occur throughout the gestation period. Studies in the past (Bjorkman et al., 1996; Jensen et al., 1999) have indicated no relationship between seropositivity for NC and reproductive efficiency. Cows aborting in mid or late lactation might be culled because of being diagnosed with NC, thereby having no effect on CCI (with no subsequent calving). However, cows aborting in the first 3 months of gestation might not be noticed as an abortion (e.g., early embryonic death) and bred again, contributing to increased CCI. Effect of seropositivity for NC on CCI in randomly selected herds having low abortion rates requires further elucidation.

The objective of this study was to determine the effect of seropositivity for infection with bovine leukaemia virus (BLV), *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Neospora caninum* (NC) on reproductive performance (CCI) in dairy cattle in three eastern Canadian provinces, controlling for effects of co-infections with the other tested pathogens. This study was one part of a large research project the aim of which was to determine the impacts and economical losses associated with BLV, MAP and NC in the entire Canadian dairy industry.

MATERIALS AND METHODS

Serum Sample Collection

A stratified two-stage random sampling procedure was employed. During the summer of 1998, participating herds were randomly selected (using computer generated random numbers) until 90 herds were recruited, 30 from each Maritime province: Prince Edward Island (PEI), New Brunswick (NB), and Nova Scotia (NS). Only herds that were enrolled in a monthly, individual cow milk-testing programme through the Atlantic Dairy Livestock Improvement Corporation (ADLIC) were eligible for participation. Approximately 60-70% of the herds in maritimes are on ADLIC. Response rate among randomly selected eligible participants was > 85% for all provinces, producing an externally valid and unbiased sample population of herds. Demographic examination of sampled herds compared to regional industry averages did not identify any significant difference. This study was part of a large study, designed to determine the seroprevalence of BLV, MAP and NC. The sample size formula used to determine the number of required herds, assumed 250 herds per province on ADLIC, a seroprevalence of 10%, an allowable error of 10%, and a confidence level of 95%. Using computer generated random numbers, up to 30 lactating animals were selected for serum collection in each herd. With an average herd size of 45 cows, 30 cattle were needed to be tested in each herd to detect at least 1 infected animal in a herd, based on a within-herd prevalence estimate of 10%, confidence of 90%, and sensitivity of the enzyme linked immunosorbent assay (ELISA) test for MAP of 43.0% (Socket et al., 1992).

Laboratory Analysis and Reproductive Data Collection

The serum samples were stored at -20^{o}C until all the samples were collected. The samples were subsequently assessed for antibody against: BLV using an ELISA[a] (sensitivity 98.5%, specificity 99.9%)(Johnson R., 1991); MAP using an ELISA[b] (sensitivity 43.0%, specificity 99.0%)(Sockett et al., 1992), tested in duplicate; and NC using an ELISA[c] (sensitivity 99.0%, specificity 98.4%) (Bergeron et al., 2000). An animal was considered to be infected with BLV, MAP or NC if the serum-to-positive ratio on the ELISA was ∃0.50, ∃0.25, and ∃0.60, respectively, as recommended by the manufacturers of the various test kits. The BLV ELISA test requires a confirmation of positive tests, using a sample-to-negative host-cell ratio of ∃1.8.

BLV testing was conducted at the national BLV testing laboratory, which is certified to conduct BLV testing for international trade purposes. MAP testing was conducted at Prairie Diagnostic Services, which is certified to have appropriate quality control for MAP ELISA

[a] IDEXX ELISA - IDEXX Corporation - Idexx Laboratories, Westbrook, Maine, USA
[b] IDEXX ELISA - IDEXX Corporation - Idexx Laboratories, Westbrook, Maine, USA
[c] BIOVET ELISA - BIOVET Inc. - St. Hyacinthe, Quebec, Canada

testing by the United States Dept of Agriculture. NC testing was conducted at the BIOVET Inc. laboratory in Quebec.

Statistical Analysis

For each tested animal, the production and reproduction data for each lactation period that started from September 1st, 1995 to December 31st, 2001, were gathered electronically from a central milk-recording database. Calving intervals starting after October 31st, 1999 were excluded in order to provide a minimum of 26 months of follow up for each calving. Calving intervals above 26 months were excluded (less than 1% cow lactations) because they were assumed to be recording errors or outliers. Calving to conception interval (CCI) was chosen to be an overall measure of dairy cow reproductive performance. CCI was estimated from calving interval data by subtracting an average gestation length (284 days). CCIs less than 30 days were excluded (less than 1% cow lactations) because they were assumed to be recording errors or outliers.

CCI was used as the outcome variable for two sets of analyses. First, linear mixed models (using MLwiN, version 1.2) were used to determine the individual and interactive effects of seropositivity for BLV, MAP and NC on CCI, after controlling for province and herd effects, seropositivity to the other two microorganisms, and other possible confounders for which data were available (e.g., parity, milk production, season of breeding). Because restricted maximum-likelihood produces less biased estimates, this modelling option was utilised. Also, variability in CCI was quantified at the lactation, cow and herd levels. Residual analysis was done at the lactation, cow and herd level.

Second, using Weibull models, the associations between seropositivity for BLV, MAP and NC and time from calving to conception were determined, again controlling for a number of possible confounders, including lactation number, province and seropositivity to the other two microorganisms (STATA, version 7). A robust estimate of variance was used to adjust for clustering at the herd level. Diagnostic analysis was also done to assess the overall model fit and to test the assumptions of proportional hazards on the basis of time varying covariates, Schoenfeld residuals and graphical methods, and Cox-Snell residuals.

The equivalence of the results from the Weibull and linear mixed models was further investigated using simulated data. Using a baseline hazard that approximates the one observed in the data and a hazard ratio equal to that observed for MAP, survival times were simulated for 10,000 cows. The effect of MAP on the log of the survival time was then determined.

RESULTS

Overall, 20.8, 2.6 and 20.3% of cattle were test-positive for exposure to BLV, MAP and NC, respectively. Details of these seroprevalence levels have been reported elsewhere (Keefe and VanLeeuwen, 2000; VanLeeuwen et al., 2001). Due to technical difficulties with testing certain serum samples, only 2445, 2395, and 2425 of these cows had laboratory test results in the final database for BLV, MAP and NC, respectively. Due to culling, only 2235 of these cows had a CCI, producing 4410 CCIs for the respective lactations that started from September 1st, 1995 to October 31st, 1999. Average herd size among the 90 sampled herds was 42 milking cows per herd (range of 17-145).

Linear Mixed Model

During the diagnostic analyses of the initial model building process, right skewing of the residuals was evident. Therefore, natural log was selected for to be the transformation of CCI. This was confirmed by Box-Cox transformation to be the best transformation. The average CCI for seronegative cows for all three infections was 115.58 (110.7-120.67, 95%C.I.) days. When no fixed effects were included in the model, the proportion of variance explained at the herd, cow and lactation levels were 8.5%, 10.4% and 81.1%, respectively. These estimates did not change much when the significant fixed effects were added. BLV and MAP seropositivity in 1st lactation heifers were associated with an increase of 1.97 (0.29 - 3.65, 95% C.I.) and 7.16 (1.85 - 12.48, 95% C.I.) unit increase in log of CCI compared to seronegative 1st lactation heifers. NC seropositive cows were not significantly associated with CCI, regardless of parity. Graphical assessment of residuals met the required assumptions.

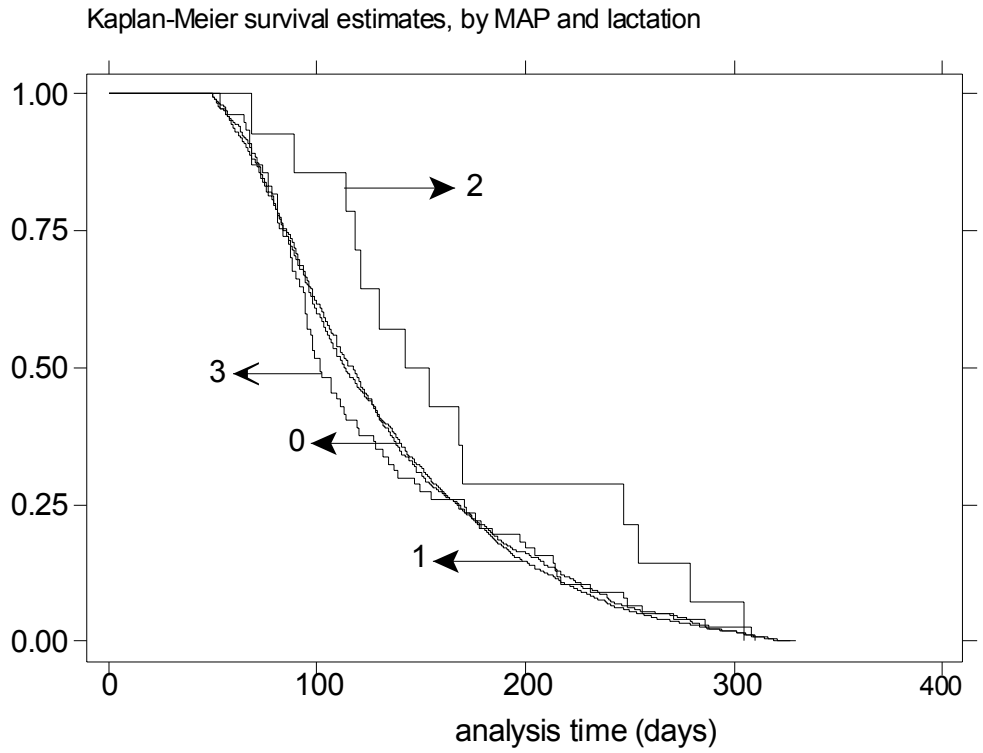

Fig. 1 Survival curves for calving-to-conception interval in first lactation heifers and second plus lactation cows for MAP seropositive and seronegative cows (0 = first lactation MAP seronegative heifers, 1 = second plus lactation MAP seronegative cows, 2 = first lactation MAP seropositive heifers, 3 = second plus lactation MAP seropositive cows.

Weibull model

The hazard (probability of getting pregnant at a particular time) associated with BLV and MAP seropositive heifers (first lactation) was 0.79 (0.65 - 0.95, 95% C.I.) and 0.49 (0.26 - 0.93, 95% C.I.) times that of BLV and MAP seronegative heifers, respectively. NC had no significant effect on hazard of conception. Graphical assessment, including time varying covariates with BLV, MAP and lactation number indicated that the basic assumptions of proportional hazards

were not violated. Cox-Snell residuals and Nelson-Aalen Cumulative hazard (as shown in Figure 2) also indicated a good overall fit of the final model.

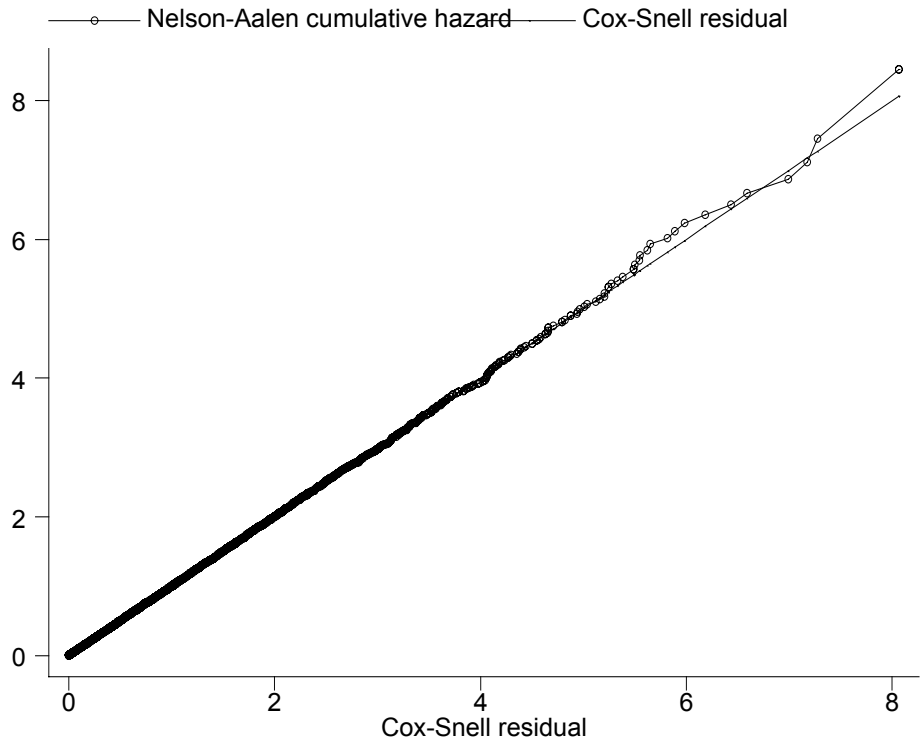

Fig. 2 Nelson-Aalen cumulative hazard of Cox-Snell residuals

Simulation

The simulation study determined that a hazard ratio of 0.47 was associated with an increase of 0.30 units in the log survival time. This was very close to the observed increase found in the linear mixed models.

DISCUSSION

Calving to conception interval was chosen to be an overall measure of dairy-cow reproductive performance due to incomplete breeding records. While on some farms, complete breeding information is usually recorded by the ADLIC technicians, this practice is not 100% consistent among all farms and all technicians. CCI was considered to be a more unbiased estimate of reproductive efficiency compared to days to first service. CCI only requires 2 calving dates, which are very consistently reported whereas days to first service or services per conception require accurate breeding data entry.

Although, lactations within cows were repeated measures, the maximum number of lactations per cow was only four and average number of lactations per cow was 1.97, therefore, correlations among all lactations within each cow were assumed to be equal in linear mixed models.

Linear mixed model and Weibull model approaches were considered for estimating the reduced reproductive efficiency associated with seropositivity for BLV, MAP and NC. The linear mixed model was equivalent to the log normal accelerated failure time model. The covariate estimate acts multiplicatively in survival time. Linear mixed models assume normally distributed errors with zero mean and σ^2 variance, and normally distributed random effects, however, they do not assume proportional hazards.

The Weibull distribution was chosen because of monotonically increasing baseline hazard. The covariate estimate acts multiplicatively in the hazard of conception and assumes proportional hazards. Due to the low variability at the herd and animal level, and given that the selection of these distribution was not obvious, we chose a robust error approach rather than adding a frailty effect to the Weibull model.

Our data met the assumptions of both the linear mixed and Weibull models so there was no obvious reason to choose one over the other. The comparability of the correlation between the observed and predicted survival times from the two models, in conjunction with the results from the simulated data, suggested that the two models produced equivalent results. However, only a small portion of the total variance was accounted for by the statistically significant fixed variables, producing relatively poor model predictability.

Our BLV results provide a better understanding of the conflicting results regarding the impact of BLV infection on reproductive efficiency. In our study, seropositivity for BLV in first lactation heifers was significantly associated with increase in CCI. Brenner et al. (1989) and Herald et al. (1992) did find a similar relationship among all cow parities, but did not look for interactions between parity and BLV seropositivity. We also found that seropositivity for BLV in second plus lactation cows was not significantly associated with increased CCI. Others also (Langston et al., 1978; Huber et al., 1981) found no association when looking at all parities, but again, interactions between parity and BLV seropositivity were not investigated. It is possible that the distribution of first and second plus lactation cows in the study populations in previous studies biased their results toward finding or not finding an impact.

The mechanism by which, BLV infection on reproductive efficiency is not known. However, we do know that BLV infection causes alterations in cell-mediated immune responses (Brenner et al., 1989). Reduced immunocompetence associated with BLV seropositivity might decrease the resistance of cows toward other infectious diseases, possibly resulting in decrease in reproductive and productive efficiency and culling.

Seropositivity for MAP was significantly and non-significantly associated with increase in CCI in first lactation heifers and second plus lactations, respectively, which supports the finding of Abbas et al. (1983) and Johnson-Ifearulundu et al. (2000). In contrast, McNab et al. (1991) found no association. Differences in results might be due to differences in defined positive animals (Abbas et al. (1983) on fecal culture and McNab et al. (1991) on LAM-ELISA) and study designs (culled cows in Abbas et al. (1983) and non-culled cows in herd in McNab et al. (1991)).

Biologicial plausibility for why MAP might lead to increased CCI is somewhat clearer than for BLV. MAP infection causes localised granulomatous lesions in lymph nodes and lamina propria of terminal ileum but granulamatous lesions can be disseminated through out the entire GI tract (Chiodini et al., 1984). The gastroenteritis produces mucosal thickening that results in

reduced absorption of nutrients from intestine and might be able to cause a negative energy balance.

The reason why our findings indicated a preferential increase in CCI for first lactation heifers, which were Map seropositive is less clear. One explanation might be related to negative energy balance, which is likely to be enhanced in first lactation heifers that are still growing. Collins et al. (1996) reported that different diagnostic tests identify different stages of paratuberculosis, resulting in different subsets of test-positive animals. Studies done on cull animals (in more advanced stages of paratuberculosis) and known infected animals or herds might not be representative of other animals in the same herd or other herds. Related to this, we theorise on our findings: First lactation heifers with MAP infection have been shown to produce less milk than similar parity heifers without MAP (VanLeeuwen et al., 2002). However, farmers are often reluctant to cull first calf heifers with a mediocre first lactation result because they are willing to give them a "second chance". Therefore, these MAP seropositive heifers may be rebred, even if it takes more than the average number of breedings, resulting in a longer CCI. Thus, we were able to identify an interaction between MAP seropositivity and lactation number. Second or greater lactation cows that are MAP seropositive are less likely to receive this "second chance" and therefore would be less likely to be persistently rebred, causing them to be culled. These cows would then not have been part of our dataset, and therefore we did not detect a significant difference in CCI among second plus lactation, MAP positive cows. Future analyses will be conducted to test this theory.

Serpositivity of NC was not associated with an increase in CCI, which supports the previous findings of Bjorkman et al. (1996) and Jenson et al. (1999). Moan et al. (1996) also found no failure in cows after an abortion caused by NC. Inability to find an increase in CCI associated with NC could be either because effects in cows aborting in early to middle gestation are very small or cows aborting in middle to late gestation might never be bred again and therefore get culled, thereby being excluded from a CCI study population.

In conclusion, it appears that first calf heifers, seropositive for BLV and MAP have higher log of calving to conception intervals by 1.97 and 7.16 units. Eighty percent of the variance in CCI being at the lactation level, the CCI factors that vary from lactation to lactation (e.g., feed, post-partum diseases, etc.) appear to be responsible for the majority of the variation in CCI. Increase in CCI might have been because of negative energy balance and alterations in cell-mediated immunity that either reduces the cow's ability to express oestrus or increases the number of services required per conception. Results from our research will help veterinarians to advise producers on their culling decisions in cows seropositive for BLV and MAP and can provide direction for future studies.

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PREDICTION OF *CULICOIDES*-BORNE DISEASE RISK IN EUROPE AND NORTH AFRICA USING SATELLITE IMAGERY

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SUMMARY

The OIE list 'A' status disease, bluetongue, is a non-contagious, infectious arboviral disease thought to infect all known ruminant species. Since 1998, an unprecedented epidemic of the disease has occurred in the Mediterranean basin, resulting in the deaths of over 300,000 sheep to date. Numerous countries have been affected, including many without previously recorded outbreaks. Bluetongue virus is transmitted by biting midges of which one species, *Culicoides imicola*, is the major vector in the Old World. In response to the recent epidemic, monitoring of *Culicoides* vectors is being undertaken in six Mediterranean countries, using a standardised light trapping procedure. *Culicoides imicola* was trapped for 2 years at 87 sites across Portugal and models were devloped for predicting the abundance of the midge at these sites. The best models were identified using discriminant analysis from 40 temporally Fourier-processed remotely sensed variables of 1 km spatial resolution. The best model correctly predicted abundance at 76 of the 87 sites, and was then used to predict *Culicoides imicola* abundance elsewhere across Europe and north Africa. The prediction map obtained showed excellent results, given the current knowledge on bluetongue outbreaks and *Culicoides imicola* distribution, and provided indications of environmental conditions that determine the abundance of the species in specific regions. Predicted high abundance of *Culicoides imicola* occurred in the majority of areas affected in the recent bluetongue epizootic, including the Balearics, Sardinia, Corsica, Sicily, areas of mainland Italy, large areas of Greece, western Turkey and northern Algeria and Tunisia.

INTRODUCTION

The infectious, non-contagious arboviral disease, bluetongue (BT), is thought to infect all known ruminant species. Bluetongue virus (BTV) is transmitted between its vertebrate hosts by biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) and there are 24 known serotypes (Mellor et al., 2000). BTV occurs in southern Asia, Africa, the Middle East, Australia and the Americas, and is estimated to cause losses of the order of $3 billion a year (Tabachnick et al. 1996). Such cost has prompted the Office International des Epizootics (OIE) to assign list 'A' status to the disease.

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Depending on climatic factors affecting the vector, BTV can seasonally extend to neighbouring areas causing epidemics as it affects non-immunised ruminant populations (Gibbs & Greiner, 1994). The Mediterranean region has had many examples of such incursions throughout the last 60 years. The most recent of which involves several serotypes of BTV, which have been spreading into the Mediterranean region, resulting in the deaths of over 300,000 sheep since 1998. In the first incursion, several Greek islands reported serotype 9 in 1998, followed by similar findings in northern and central Greece, southern Bulgaria and European Turkey in 1999. Serotypes 4 and 16 were also identified in Greece in August 1999, and in 2000, serotype 16 was isolated in Anatolian Turkey (Baylis & Mellor, 2001). A second incursion began in December 1999, starting with reports of serotype 2 in Tunisia, and then spreading to Algeria, Sicily, Sardinia and Calabria on the Italian mainland by early September 2000. Late September saw BT spread to the Spanish island of Majorca, and by October it had reached the Spanish island of Menorca and the French island of Corsica. Additional outbreaks were reported in Majorca and Menorca in early December, all caused by BTV serotype 2 (Mellor & Wittmann, 2002). Yet more outbreaks in new areas of Europe were seen in 2001. By September new BT cases were reported in western Bulgaria and mainland Greece, and in the following 3 months cases were confirmed in Macedonia, Kosovo, Yugoslavia and southern Croatia. In early 2002 BT was reported in northern Italy as far north as Tuscany (OIE, 2002), and as recently as September, further outbreaks were reported in Bosnia-Herzegovina (International Society for Infectious Diseases, 2002). The majority of the areas affected by the 1998-2002 outbreaks (Algeria, Bosnia-Herzegovina, Bulgaria, Croatia, France (Corsica), Kosovo, Macedonia, the Spanish islands of Majorca and Menorca, Italy (Sardinia, Sicily and various mainland areas), Tunisia and Yugoslavia) have never previously reported any occurrence of BT.

A single species, *Culicoides imicola*, has been implicated as the major vector of BTV in the Old World, including the Mediterranean region (Mellor et al., 2000). This species is also known to transmit several other viruses, including that which causes the OIE list 'A' disease African horse sickness (AHS). However, many of the areas recently affected by BT have been shown to be free of *C. imicola* (Mellor & Wittmann, 2002), suggesting the involvement of novel vector species. These novel vectors are likely to be members of the *C. obsoletus* and/or *C. pulicaris* groups, which are the commonest *Culicoides* species across northern Europe. It is also likely that climate change has, and will, extend areas of the Mediterranean region at risk from BTV, in addition to increasing the severity, duration and likelihood of BT epizootics following virus introduction (Mellor & Wittmann, 2002). The BT outbreak seen over the last 4 years in the Mediterranean region is the largest on record and is likely to be exacerbated if global warming occurs, therefore, it is important that future areas at risk from BTV be identified to effectively develop control strategies and deploy control methods.

With the possibility of more severe BT outbreaks within the Mediterranean region in mind, *Culicoides* surveillance is being undertaken in many countries as part of an EU-funded project. This paper describes work restricted to data from Portugal, as this was the first country to be fully surveyed. This work here represents, therefore, the first output of a much larger programme of surveying and modelling. Surveillance data on *Culicoides* from 87 sites distributed evenly across Portugal were collected in the summers of 2000 and 2001 (Capela et al., unpublished). Remotely sensed imagery, together with this trapping data, were used to develop a model for predicting the abundance of *C. imicola* across Portugal. The predictions of the model were then extended to the rest of Europe and north Africa to identify other areas with suitable environments for *C. imicola* and, consequently, at risk of BT. In an attempt to validate the model, the predictions were compared with the reported distribution of BT and AHS over the

last 50 years. The prediction map was also used to identify areas at potential future risk from *Culicoides*-borne diseases.

MATERIALS AND METHODS

The abundances of *C. imicola* at sites in Portugal were obtained from data collected as part of the EU project. These data were divided into three classes for abundance modelling: zero to low, intermediate and high levels of abundance. The best multivariate model of the allocations to these classes from combinations of 41 remotely sensed environmental variables were then identified using discriminant analysis. This model was then used to predict the abundance of *C. imicola* throughout Europe and north Africa.

Culicoides imicola Catch Data

Eighty-seven sites across Portugal were sampled for *Culicoides* during summer 2000 and 2001. Portugal was divided into forty-five 50 x 50 km quadrats and two farms or livestock holdings within each were chosen according to certain criteria. 'Summer' sampling was carried out between July and October in each year as the period corresponds to *Culicoides* peak abundance in Portugal (Rawlings et al., 1997). Environmental and climatic variations between sampling years were accounted for by randomly allocating each quadrat to two equal groups to be sampled in either summer 2000 or 2001. Each trap site was located using a Garmin GPS 12 receiver and sampled using Onderstepoort-type blacklight traps. Where possible, each site was sampled for two nights and the largest nights' catch used for the predictive modelling. *C. imicola* was not found at 41 of the 87 sites in Portugal. In order to carry out *C. imicola* abundance modelling, each trap site was assigned to a class, dependent upon the number of *C. imicola* caught. Class 1 (zero to low) contained 51 sites, with a range of catches from 0 to 2 *C. imicola*. Class 2 (intermediate) had 18 sites, with a range of 3 to 100, and class 3 (high) had 18 sites, with a range of catches from 101 to 4200.

Remotely Sensed Data

In total, 41 environmental variables were used in the modelling procedure. The altitude of each trapping site was derived from the 1 x 1 km spatial resolution global topography (GTOPO30) digital elevation model obtained from the US Geological Survey (2002). The remaining 40 variables were derived from the Pathfinder Advanced Very High Resolution Radiometer (AVHRR) dataset (Rogers et al., 1997). The 1 x 1 km spatial resolution imagery, covering the period April 1992 to April 1996, was downloaded from the 'Land Processes Distributed Active Archive Center' website (US Geological Survey, 2002).

The remotely sensed imagery was processed to extract four variables with environmental significance: Normalized Difference Vegetation Index (NDVI), Middle Infra-Red Reflectance (MIR), Land Surface Temperature (LST) and Air Temperature (TAIR). The NDVI images were calculated using channels 1 (red reflectance) and 2 (near infra-red reflectance) in the equation NDVI = (Ch2 – Ch1)/(Ch2 + Ch1). The NDVI has a long history of use within the field of remote sensing, and is specifically a measure of chlorophyll abundance, but is also correlated with soil moisture, rainfall and vegetation coverage, biomass and productivity (Campbell, 1996). MIR is recorded by channel 3 of the AVHRR, and is correlated with the surface temperature, water content and structure of vegetation canopies (Boyd & Curran, 1998). The LST images were calculated from AVHRR channels 4 and 5 using a simple 'split-window' algorithm based on radiative transfer theory: LST = Ch4 + 3.33(Ch4 –Ch5) (Price, 1984). Application of this

algorithm for monthly maximum LST determination shows accuracy equivalent to that of spatial interpolation of meteorological data for both tropical Africa (Hay and Lennon, 1999) and temperate Europe (Green and Hay, 2000). Contextual combinations of NDVI and LST (Goetz et al., 2000) were used to derive TAIR images. Such imagery displays estimates of the air temperature a few metres above the land surface. Inference of TAIR is based on an assumption that the radiometric temperature of a fully vegetated canopy is in equilibrium with the ambient air temperature, because of the similar heat capacity of dense vegetation and the surrounding air (Prihodko & Goward, 1997).

Once the raw imagery was processed to provide monthly indices correlated with meteorological variables and vegetation abundance, the data were subjected to temporal Fourier analysis (Rogers et al., 1996). This technique extracts information about the seasonal cycles of the indices in terms of their annual, biannual and triannual cycles, each described by their phase and amplitude. Fourier analysis in this way performs three useful functions. First, it removes noise from the original satellite signal, leaving a smoothed picture of seasonal change. Second, it achieves volume reduction of monthly datasets that often show strong correlations. Finally, it achieves data ordination in a way that has an obvious biological interpretation in terms of seasonal cycles (Rogers, 2000). The results of such temporal Fourier processing were 10 individual variables for each of the environmental and meteorological indices. These were the amplitude and phase of the annual, biannual and triannual cycles (6 variables), and the mean, maximum, minimum and variance (4 variables) of the combined (annual + biannual + triannual) Fourier description of the original signal.

Modelling

The modelling process consisted of identifying the combination of remotely sensed variables that most effectively allocated the 87 sites to abundance classes. This combination of variables (the model) was chosen using discriminant analysis, described in detail by Rogers et al. (1996) and Rogers (2000). Separate within-group covariance matrices are assumed and, from the available 41 variables, selection of those to be included in the model was undertaken using a forward step-wise procedure. The variable inclusion criterion was that the addition of the chosen variable caused the greatest between-group increase in the total Mahalanobis distance. This measure is the covariance-adjusted distance between two multivariate distribution centroids, or from a sample point to a centroid (Rogers, 2000). For the *C.imicola* abundance model, 7 variables were included, decided by the fact that inclusion of further variables produced little or no increase in total Mahalanobis distance.

Internal validation was used to assess the model, i.e. the same data used for prediction are used for assessment. Ideally, the training set should have been divided, with half used to develop the covariance matrices and the other half used to test prediction accuracy. However, with only 87 data points, the entire set was used for training as well as assessment. This has, of course, inflated estimates of the accuracy of the technique, although perhaps only modestly (Randolph, 2000). The overall statistical significance of the model was measured using the kappa statistic (Robinson, 2000), which is commonly used in public health and epidemiology. It estimates the agreement of two variables, while taking into account the degree of overlap expected by chance. It ranges from 0 to 1, with values greater than 0.75 indicating an excellent model fit (Robinson, 2000). Three statistics were use to measure the accuracy of the abundance predictions: sensitivity (equivalent to producer's accuracy in remote sensing literature), specificity and consumer's accuracy. Sensitivity represents the percentage of positive observations predicted

correctly, specificity is the percentage of negative observations predicted correctly, and consumer's accuracy is the percentage of predictions observed to be correct. The terms sensitivity and specificity are more widely used in epidemiological studies (Rogers et al., 1997) than in the field of remote sensing, while producer's and consumer's accuracies are more widely used in remote sensing (Campbell, 1996).

The model was then used to generate predictions for the rest of Europe and for north Africa by generating probabilities that pixels would fall into one of the three abundance classes. The abundance class that corresponded to the largest probability at that pixel was then assigned and taken to be the predicted level of *C. imicola* abundance at that pixel. No prediction was made when the Mahalanobis distance between a pixel and its assigned class was two or more times greater than the maximum distance observed between any one of the 87 sites in Portugal and the classes to which they belonged. This limited prediction to areas where the environment and climate were broadly similar to those observed at the Portuguese trap sites, and lead to fewer predictions being made at great distances from Portugal.

RESULTS

Model statistics

The seven model variables that best allocated the 87 sites to the observed *C. imicola* abundance classes are listed in table 1. The phase of the annual NDVI cycle was the single most important model variable. By taking an average across the trap sites, it was observed that for the sites where *C. imicola* was absent or rare, the annual NDVI cycle peaked in late April. For the intermediate class, the cycle peaked at the start of April, and for the high abundance sites, the peak was in mid-March. Overall, the model correctly predicted the abundance class at 76 of the 87 sites (87.4%), and the kappa statistic for the model was 0.88.

Table 1. The seven variables, ranked in order of importance, which best allocated the 87 Portuguese trap sites to the observed *Culicoides imicola* abundance classes. The kappa statistic of the model is 0.88. (MIR = Middle Infra-Red Reflectance, NDVI = Normalized Difference Vegetation Index, LST = Land Surface Temperature, TAIR = Air Temperature).

Rank	Variable Type	variable
1	NDVI	Phase of annual cycle
2	MIR	Mean
3	LST	Variance
4	NDVI	Amplitude of biannual cycle
5	MIR	Amplitude of biannual cycle
6	TAIR	Amplitude of biannual cycle
7	TAIR	Phase of triannual cycle

Table 2 shows the sensitivities, specificities and consumer's accuracies for the abundance classes. All three classes produced sensitivities of over 85%, indicating a high percentage of positive observations predicted correctly. The model incorrectly predicted one site (Coruche in eastern Portugal) to be in the zero/rare abundance class, four sites (two in the far south-east, two

in the far south-west) in the intermediate class, and six sites (all in the south) to be in the high abundance class. The specificities were uniformly high, indicating good prediction of negative observations. In contrast, the consumer's accuracies showed some class-to-class variation in percentages. Thus, 44 of the 46 predictions that *C. imicola* would be absent or rare (95.7%) were correct, 16 of the 20 predictions that *C. imicola* abundance would be intermediate were correct (80%), and 15 of the 21 that it would be in the high abundance class (71.4%) were correct.

Table 2. Measures of accuracy of the model for the three abundance classes.

	ABUNDANCE CLASS		
	ZERO/LOW	INTERMEDIATE	HIGH
SENSITIVITY	86.3	88.9	88.2
SPECIFICITY	100.0	97.1	94.2
CONSUMER'S ACCURACY	95.7	80	71.4

Prediction Map

The predicted abundance map of *C. imicola* across Mediterranean Europe and north Africa is shown in Figure 1. Figure 1 highlights certain areas as containing high levels of *C.imicola*: Most of the northern edge of north Africa, south-west Iberia, the south-eastern coast of Spain, the Balearic islands, Corsica, Sardinia, the southern half of Sicily, Lazio (central-west coast of Italy), Puglia (the heel of Italy), areas of south-western Italy, the eastern and western edges of mainland Greece, many of the Greek islands, western Albania, the southern Croatian coast, Cyprus, western Turkey and Syria.

DISCUSSION

Availability of detailed data on *C. imicola* distribution in the Mediterranean region is currently limited. However, there are many surveys underway as part of the EU project mentioned earlier, which, once complete, will provide a better test of modelling outputs. In the meantime, provincial and regional level data on reported *C. imicola*-borne diseases are available. Figure 2 shows, at the regional or provincial level, all the areas reported to have been affected by *C. imicola*-borne diseases (BT and AHS) since 1950, and the different virus serotypes involved. Comparison of this map with the prediction map in Figure 1 shows excellent agreement.

The map of predicted *C. imicola* abundance (Figure 1) highlights areas of Europe and north Africa that are known to have experienced *Culicoides*-borne disease in the past. The areas with the closest correspondence between prediction and reported disease include southern Portugal (expected as Portuguese data was used for model development), southern Spain, northern Morocco, northern Algeria, northern Tunisia, Majorca, Menorca, Corsica, Sardinia, Sicily, Calabria (the toe of Italy), Lazio, Tuscany, the eastern and western edges of mainland Greece, Rhodes, Lesbos, western Turkey, Syria and Cyprus. *C. imicola* is known to be present in south-west Iberia (Rawlings et al., 1997), Morocco (Baylis et al., 1997), Lesbos (Boorman & Wilkinson, 1983), Rhodes (Boorman, 1986), western Turkey (Jennings et al., 1983), Cyprus (Mellor and Pitzolis, 1979), Corsica (Zientara et al., 2001), Sardinia (Goffredo et al., 2001) mainland Italy (Scaramozzino & DeLiberato, 2002) and mainland Greece (Anon., 1999).

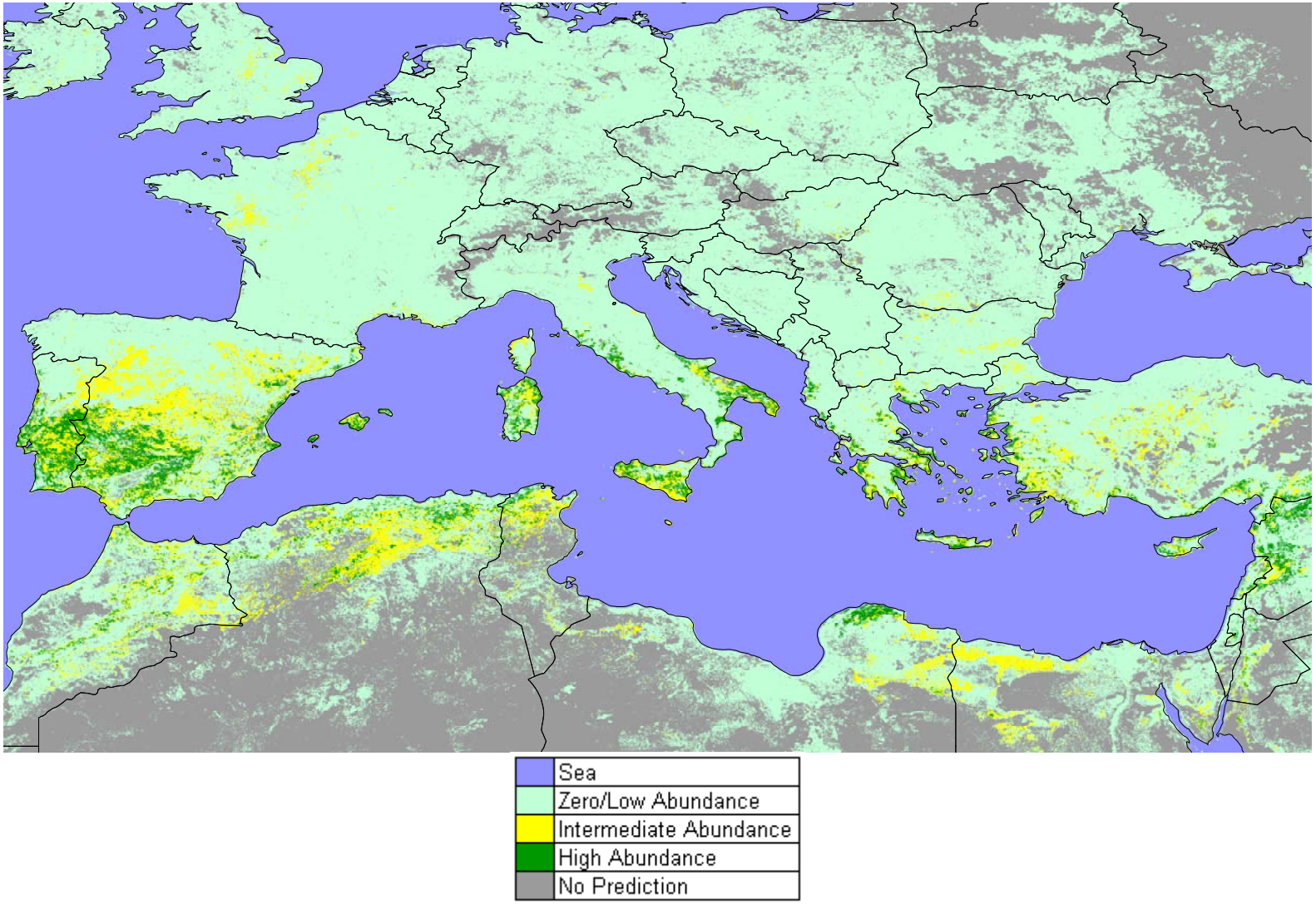

Fig. 1 Abundances of *Culicoides imicola* around the Mediterranean predicted by a model derived from the observed abundances at 87 sites in Portugal.

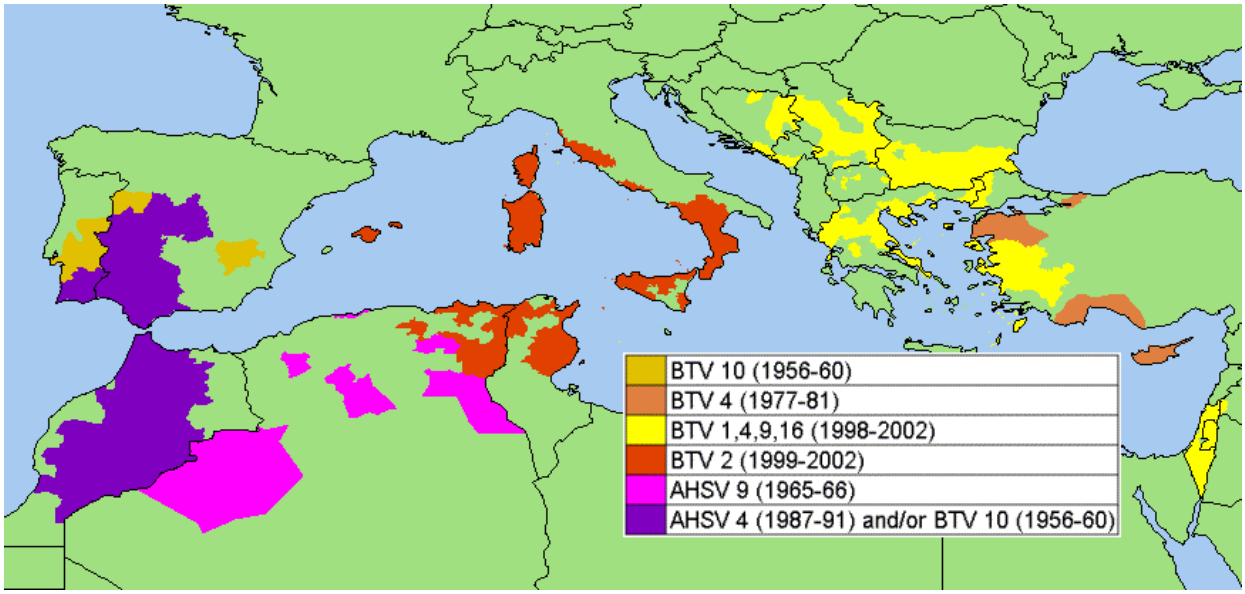

Fig. 2 Provinces or regions affected by bluetongue (BT) and African horse sickness (AHS) since 1950 (BTV = BT virus, AHSV = AHS virus). The key gives the virus, serotype and years of the outbreak. Data for 1956 to 1991 are from Baylis et al. (2001) and data for 1998 to the present are from OIE (2002).

Several areas are identified in Figure 1, where *C. imicola* is predicted to be present in high levels of abundance, but where no disease has previously been reported. However, it is possible for the vector to be present without the disease being reported, as the virus may not have entered the area, there may be few or no susceptible vertebrate hosts or the disease may have been overlooked (Baylis et al., 2001). Nonetheless, such areas should still be considered to be at risk from *Culicoides*-borne diseases in the future. These include the east coast of Spain, the island of Ibiza, the island of Crete and western Albania. Small areas around the border between Bulgaria, Greece and Turkey are also identified by the prediction map as being suitable habitats for the presence, in intermediate numbers, of *C. imicola.* However, recent surveys have failed to find the presence of *C. imicola* here (P.S. Mellor, personal communication). Although failings due to the possible uncertainty inherent in *Culicoides* trapping is one potential explanation, such findings more likely illustrate limitations in the modelling process when extending predictions to areas of higher latitudes and more easterly longitudes. It is conceivable that environmental determinants of *C. imicola* distribution in Eastern Europe are distinct from those in Portugal used to build the model. The inclusion of catch data from Eastern Europe would certainly provide more confidence in the resultant prediction maps for this area.

A similar piece of work to that undertaken in this paper was carried out by Baylis et al. (2001). The best combination of remotely sensed variables that described the abundance of *C. imicola* in the Mediterranean region was identified by discriminant analysis. Differences in the approach of Baylis et al. included the use of data from just 49 trap sites widely distributed across Iberia and Morocco, and 8km spatial resolution satellite imagery. The resultant prediction map produced good agreement with known BT and AHS outbreaks, but failed to predict the presence of *C. imicola* in certain areas where it is known to exist or where BT outbreaks have been reported. These include Corsica, the extreme tip of Calabria (the province of Reggio Calabria), the Spanish island of Menorca and north-western Greece, all of which are predicted to have *C. imicola* present in Figure 1. This suggests that the finer spatial resolution of the remotely sensed

variables, and the increased number of trap sites used to produce the predictions shown in this paper, have, in certain locations, increased the accuracy of the models over that used by Baylis et al (2001). In other locations, e.g. eastern Europe, comparisons are difficult to make as the model used by Baylis et al (2001) made predictions in very few areas.

Although prediction accuracies from the work in this paper appear to have been improved over that of Baylis et al. (2001) in some locations, there still exist areas where BT has recently occurred, but *C. imicola* are predicted to be absent or rare. These areas are focussed around Croatia, Bosnia-Herzegovina, Kosovo, Serbia, Montenegro, Macedonia, Yugoslavia, Bulgaria and the far north-east of Greece. However, light-trap surveys in the Greek and Bulgarian affected areas at the time of the outbreaks failed to reveal the presence of *C. imicola* (Anon., 1999, Georgiev et al., 2001), instead, 90% of the catch was *C. obsoletus* and *C. pulicaris*. These species complexes have for many years been considered to be potential vectors of BTV (and AHSV) (Mellor & Pitzolis, 1979, Jennings & Mellor, 1988, Mellor et al., 1990), but have never been implicated as the major vector in a field outbreak of the disease. Therefore, future prediction modelling of BTV will not only focus on *C. imicola*, but also on *C. obsoletus, C. pulicaris* and other potential vectors.

This paper has shown that the use of freely available remotely sensed imagery, combined with simple mathematical models and insect data from one relatively small country, can be used to identify, predict and explain observed patterns in insect numbers at the continental scale. This can be done more easily, cheaply and effectively than by using a combination of weather station data and large numbers of trap sites. The modelling process can also be used to infer biological understanding of vector-borne diseases and the vectors themselves by identifying the most significant environmental variables in determining vector distribution. This work has shown that the abundance of *C. imicola* in the Mediterranean region is strongly correlated with the annual timing of the vegetation abundance peak, itself related to soil moisture levels. The model developed was used to predict *C. imicola* abundance across Europe and north Africa, and the excellent agreement with actual *Culicoides*-borne disease outbreaks confirms the value and utility of such an approach.

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RISK FACTORS ASSOCIATED WITH EQUINE GRASS SICKNESS RECURRING ON PREVIOUSLY AFFECTED PREMISES

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SUMMARY

Premises suffering cases of equine grass sickness (EGS) between January 1st 1997 and December 31st 2001 were identified by various sources and asked to complete a detailed questionnaire that included sections on disease history and horse, premises and pasture management. Recurrent premises were defined as those that had previous cases during a defined risk period. For each premise the total number of cases excluding the questionnaire case that occurred during the risk period, was calculated. Cases clustered within 30 days were treated as a single case. Data were analysed using Poisson regression analysis and premises with risk periods less than 2 years were excluded. Standard forward stepwise multivariable analysis was conducted following preliminary univariable screening of variables. Of 509 premises contacted during the study, 305 (60%) returned useable questionnaires and 100 of these (33%) were classified as recurrent premises. There was evidence for an increased rate of recurrence with higher numbers of horses, presence of younger animals, studs and livery/riding establishments, loam and sand soils, rearing of domestic birds and mechanical droppings removal. The rate of recurrence apparently decreased with chalk soil, co-grazing ruminants, grass cutting on pastures and removal of droppings by hand. Some results require careful interpretation as several statistically significant interactions were identified. Many of the findings of this study are complementary to the theory that EGS is a toxico-infectious form of botulism. Several of the significant factors identified may relate to soil disturbance and consequent soil contamination of grass, thereby increasing the rate of exposure of grazing horses to the *Clostridium botulinum* bacterium that resides in soil. Results agree with earlier studies that the presence of younger, probably non-immune horses on premises increases the rate of recurrence of EGS.

INTRODUCTION

Equine grass sickness (EGS) or equine dysautonomia is a debilitating and rapidly fatal neurodegenerative disease of horses, the cause of which has still not been definitively identified. The description of this disease as 'grass sickness' undoubtedly arose from the early observation that it occurred predominantly in grazing horses (Begg, 1936; Gilmour & Jolly, 1974). The disease was first recognised in Scotland in the early 1900s (Tocher et al., 1923; Tocher, 1924; Begg, 1936; Greig, 1942; McCarthy et al., 2001) but was also reported from areas of England and Wales around the same time (Greig, 1942; McCarthy et al., 2001). Since then cases have been diagnosed in many countries in mainland Europe (McCarthy et al., 2001) and a clinically

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and pathologically identical disease, *mal seco*, is recognised in South America (Uzal and Robles, 1993; Araya et al., 2002).

Clinical signs of grass sickness are characterised as either acute, sub-acute or chronic according to the duration and severity of signs (Doxey et al., 1991b) and are attributable to histologically evident disruption of the autonomic nervous system, particularly of the gastrointestinal tract (Obel, 1955; Mahaffey, 1959; Barlow, 1969, Scholes et al., 1993; Doxey et al., 2000; John et al., 2001). Clinical presentation of EGS is typically characterised by signs of mild colic, increased heart rate, muscle tremors, patchy sweating, difficulty in swallowing, reduced intestinal motility, weight loss and occasionally sudden death (Tocher et al., 1923; Tocher, 1924; Greig, 1942; Doxey et al., 1991b; Milne, 1996). Horses with the mildest, chronic form may be successfully nursed to recovery (Doxey et al., 1995), but the majority of acute and sub-acute cases are incurable.

There have been several different theories proposed for the cause of EGS, including toxic plants, insects, fungi, filterable viruses and bacterial toxins (Tocher et al., 1923; Tocher, 1924; Begg, 1936; Greig, 1942; McCarthy et al., 2001). Among these, the theory that EGS is a form of toxico-infectious botulism resulting from *Clostridium botulinum* producing toxin locally within the intestinal tract seems to be the currently favoured hypothesis, with both historical and modern evidence to support it. Tocher first proposed botulism as the underlying cause of EGS with his theory being strongly supported by evidence of statistically significant ($P<0.0001$) protection against deaths from the disease by vaccination with an antitoxin neutralised botulinum toxin vaccine (Tocher et al., 1923; Tocher, 1924; Wood et al., 1999). However, Tocher's theory lost favour until recently when a significant association between presence of both the organism and *C. botulinum* type C toxin was shown in histologically confirmed EGS cases compared with apparently healthy controls (Hunter et al., 1999). It has been further shown that EGS cases have significantly lower antibodies to *C. botulinum* and its type C toxin than horses that have either been in contact with EGS or have grazed frequently affected land (Hunter & Poxton, 2001; McCarthy, 2002).

Several analytical studies of the epidemiology of EGS have been conducted. Earlier studies were restricted to Scotland (Gilmour & Jolly, 1974; Doxey et al., 1991a) but later investigations used cases from throughout the UK (Wood et al., 1998; McCarthy, 2002). Studies have consistently shown that the disease is associated with grazing, with peak diagnoses during the spring and early summer, although cases may occur in any month (Doxey et al., 1991a; Wood et al., 1998). Young animals and those that have recently moved premises and/or pasture are at significantly increased risk of disease (Gilmour & Jolly, 1974; Wood et al., 1998). The studies by Gilmour and Jolly (1974) and Wood et al. (1998) both demonstrated a significantly increased risk of EGS associated with premises on which there had been previous cases and that this risk increased the more recently that cases had occurred (Table 1). Wood et al. (1998) also showed a ten-fold reduction in risk for horses in contact with previous cases.

Although EGS recurs on previously affected premises, the factors that influence this have not been previously investigated and, consequently, practical methods of reducing the risk of such recurrence have not been available for horse owners. A questionnaire-based epidemiological study was, therefore, conducted to identify factors significantly associated with recurrence of EGS on premises. Knowledge of these factors may, through modifying certain management practices, help to reduce the frequency of cases on affected premises in the future.

Table 1. Numbers of cases and controls and corresponding odds ratios for categories of time since the last case on the premises in two epidemiological studies of equine grass sickness

	Time since last equine grass sickness (EGS) case			
	No cases before	>5 years*	2-5 years	<2 years
		Gilmour & Jolly (1974)		
EGS cases	44	12	8	26
Controls	1851	242	175	286
Odds ratio	1.0 (ref.)	2.1	1.9	3.8
		Wood et al. (1998)		
EGS cases	78	24	9	24
Controls	166	31	13	16
Odds ratio**	1.0 (ref.)	1.3	2.5	6.7

*or not known **Odds ratios after accounting for matching, taken from Wood et al. (1998)

MATERIALS AND METHODS

Data collection

Information was collected by detailed postal questionnaire from premises that had suffered at least one case of EGS since the beginning of 1997. Affected premises were identified from a variety of sources including the databases of the Animal Health Trust, Equine Grass Sickness Fund, Edinburgh veterinary school and other UK veterinary faculties, laboratories and practices. Data collection started in October 1999 with premises that had suffered cases since 1st January 1997 being recruited retrospectively and premises that had EGS cases after October 1999 being prospectively recruited up to the end of December 2001 when data collection ended.

The questionnaire (available on request from the presenting author) that was used to collect data from affected premises was 11 pages long and divided into 5 sections. Section 1 asked for personal details and Section 2 collected details on the occurrence of EGS, including numbers and dates of previous cases. Section 3 enquired about horse and premise details, including the numbers and ages of horses and type and size of premise, available housing, grazing and water sources. Section 4 collected horse management details including how horses were kept, fed and wormed and Section 5 enquired about pasture management practices including details of droppings removal, harrowing, fertilising, grass cutting, re-seeding and other animals that were kept. Information from the original questionnaires was transferred to a dedicated Microsoft Access database, with different levels of variables being variously coded for future analyses.

Statistical analyses

'Recurrent premises' were defined as having at least one previous case (i.e. excluding the case for which the questionnaire was sent = 'questionnaire case') during a defined risk period. For each premise a risk period was calculated that was taken from the date of completion of the questionnaire back to either an arbitrary start date of 1st January 1990 or the date that the owner took up residence on the premises if this was after the beginning of 1990. For this defined risk period the total number of cases excluding the 'questionnaire case' were calculated from the disease history provided, with cases that occurred within 30 days of each other being treated as a single case. The 'questionnaire case' was excluded from the total number of cases so that the total would equal the number of recurrent cases.

As the structure of these data was events (recurrent cases) over time (risk period) an appropriate statistical approach was Poisson regression analysis (Frome, 1983). However, to avoid bias from overly short follow up periods, 7 premises with risk periods less than 2 years were excluded from further analyses. Each variable (Tables 2 and 3) was individually tested for the significance of its association with the recurrence of disease during the risk period using univariable Poisson regression (Stata software). In order to control for confounding and effect modification between variables, those that were significantly associated with recurrence of disease at $P<0.275$ in univariable analyses were used in multivariable Poisson regression analysis. Multivariable modelling was conducted using a forward stepwise approach (Hosmer & Lemeshow, 1989). Variables were retained if they were associated with disease (Wald χ^2: $P\leq0.05$) or their inclusion resulted in a significant improvement in the overall fit of the model as measured by the likelihood ratio statistic (LRS χ^2: $P\leq0.05$). The final model was examined for biologically meaningful two-way interaction terms that provided a significant improvement in the overall fit of the model as measured by the likelihood ratio statistic (LRS χ^2: $P\leq0.05$).

In addition, overall EGS incidence rates were calculated for each premise and expressed in 'numbers of cases per year'. This was done by dividing the total number of cases, including the 'questionnaire case', by the calculated risk period expressed in 'years'. An adjusted incidence rate, expressed in 'numbers of cases per 100 horses per year', was also calculated with adjustment made for the number of horses on the premises. This was based on the assumption that horse numbers on premises were largely static over time. Statistically significant differences between 'recurrent' and 'non-recurrent' premises in the incidence rate, number of horses and adjusted incidence rate were investigated using the non-parametric Wilcoxon rank-sum test.

RESULTS

Incidence rate estimates and univariable Poisson regression analyses

Of 509 premises contacted during the study, 305 returned useable questionnaires (60% return rate) and of these, 100 premises (33%) were defined as 'recurrent' because they had had at least one EGS case prior to the 'questionnaire case' during the risk period. The median EGS incidence rate for 'recurrent' premises (0.21 cases/premises/year) was approximately double that for 'non-recurrent' premises (0.09 cases/premises/year), a difference that was statistically significant ($P<0.001$). However, examination of the distribution of numbers of horses on premises showed that 'recurrent' premises (median 15.5 horses/premises) had significantly ($P<0.001$) greater numbers of horses than 'non-recurrent' premises (median 5 horses/premises). Median EGS incidence rates adjusted for horse numbers on premises actually showed no statistically significant difference ($P=0.47$) between 'recurrent' (1.9 cases/100 horses/premises/year) and 'non-recurrent' premises (2.1 cases/100 horses/premises/year).

Tables 2 and 3 summarise numbers and proportions of 'recurrent' and 'non-recurrent' premises for different categories of variables and shows rate ratios, 95% confidence intervals and corresponding Wald χ^2 *P*-values from univariable Poisson regression analyses. Among horse and premises related variables (Table 2) there was a clear trend towards increasing rate of recurrence of EGS with increasing numbers of horses and stables on premises. Presence of younger horses was also associated with an increased rate. Livery/riding establishments and stud farms had increased rates compared to farms.

Table 2. Numbers and proportions of 'recurrent' and 'non-recurrent' premises for different categories of premises and horse management variables and rate ratios, 95% confidence intervals and corresponding Wald χ^2 *P*-values from univariable Poisson regression analyses

Variable	Category	Recurrent premises n (%)	Nonrecurrent premises n (%)	Rate Ratio	95% CI lower	95% CI upper	*P*-value
Number of horses on the premises	1-5	21 (17)	104 (83)	referent			
	6-10	12 (21)	45 (79)	1.80	0.97	3.32	0.061
	11-15	17 (47)	19 (53)	7.09	4.25	11.83	<0.001
	16-20	12 (57)	9 (43)	9.03	5.26	15.49	<0.001
	21-40	20 (54)	18 (46)	8.55	5.24	13.97	<0.001
	41+	18 (64)	10 (36)	15.54	9.73	24.81	<0.001
Presence of horses <2 y.o	No	41 (22)	142 (78)	referent			
	Yes	59 (49)	61 (51)	3.41	2.63	4.43	<0.001
Premises type	Farm	27 (29)	67 (71)	referent			
	Livery/Riding	30 (45)	37 (55)	2.87	2.06	4.01	<0.001
	Stud	20 (69)	9 (31)	2.70	1.82	4.00	<0.001
	Rented	8 (20)	32 (80)	0.68	0.37	1.25	0.220
	Other	15 (20)	60 (80)	1.22	0.83	1.79	0.313
Number of stables	None	17 (28)	44 (72)	referent			
	1-5	18 (18)	80 (82)	0.55	0.34	0.91	0.019
	6-10	20 (32)	42 (68)	1.34	0.86	2.10	0.195
	11-20	26 (50)	26 (50)	2.45	1.62	3.70	<0.001
	21+	19 (59)	13 (41)	4.47	3.00	6.69	<0.001
Water source whilst housed	Mains	70 (33)	144 (67)	referent			
	Well	12 (60)	8 (40)	1.40	0.94	2.08	0.095
	Other	12 (27)	32 (73)	0.79	0.55	1.14	0.209
Water source whilst grazing	Mains	43 (28)	113 (72)	referent			
	Stream	12 (38)	20 (62)	0.98	0.62	1.53	0.917
	Well	7 (41)	10 (59)	2.32	1.55	3.48	<0.001
	Mains + stream	24 (46)	28 (54)	1.69	1.24	2.31	0.001
	Other	14 (30)	32 (70)	1.22	0.85	1.75	0.276
Grazing type	Permanent	65 (28)	169 (72)	referent			
	Ley	23 (52)	21 (48)	1.78	1.32	2.41	<0.001
	Hill/moor	11 (42)	15 (58)	1.74	1.21	2.51	0.003
Soil type	Clay	30 (27)	83 (78)	referent			
	Sand	22 (37)	37 (63)	1.14	0.82	1.57	0.433
	Chalk	5 (29)	12 (71)	0.23	0.07	0.73	0.013
	Loam	24 (46)	28 (54)	1.36	0.99	1.88	0.059
	Other	9 (28)	23 (72)	0.49	0.28	0.88	0.017
Supplementary hay fed in spring	No	29 (34)	59 (67)	referent			
	Yes	71 (35)	132 (65)	2.14	1.54	2.97	<0.001
Supplementary hay fed in summer	No	63 (34)	125 (66)	referent			
	Yes	37 (36)	65 (64)	1.71	1.34	2.19	<0.001
Supplementary spring concentrate	No	25 (34)	49 (66)	referent			
	Yes	75 (35)	142 (65)	1.24	0.92	1.67	0.152
Supplementary summer concentrate	No	43 (34)	82 (66)	referent			
	Yes	57 (34)	109 (66)	1.67	1.28	2.17	<0.001
Worming frequency	≥6 monthly	11 (27)	30 (73)	referent			
	3 monthly	43 (32)	92 (68)	2.68	1.54	4.67	<0.001
	2 monthly	35 (35)	66 (65)	3.08	1.76	5.38	<0.001
	<2 monthly	10 (43)	13 (57)	2.88	1.48	5.59	0.002

For pasture management variables (Table 3), although there was an overall increased rate of recurrence associated with removal of droppings from pastures, when this factor was broken down according to whether it was done manually or mechanically, these methods had opposing directions of effect that were each statistically significant. Removal of droppings by hand was apparently protective but mechanical removal was associated with a greater than fourfold increase in rate of recurrence.

Table 3. Numbers and proportions of 'recurrent' and 'non-recurrent' premises for different categories of pasture management variables and rate ratios, 95% confidence intervals and corresponding Wald χ^2 *P*-values from univariable Poisson regression analyses

Variable	Category	Recurrent premises n (%)	Nonrecurrent premises n (%)	Rate Ratio	95% CI lower	95% CI upper	*P*-value
Droppings removed	Not removed	56 (31)	122 (69)	referent			
	Removed	43 (34)	82 (66)	1.54	1.21	1.96	0.001
Method of droppings removal	Not removed	56 (31)	122 (69)	referent			
	By hand	20 (21)	76 (79)	0.53	0.36	0.79	0.001
	Mechanically	23 (79)	6 (21)	4.30	3.30	5.60	<0.001
Pasture harrowed	Not harrowed	49 (30)	117 (70)	referent			
	Harrowed	50 (37)	85 (63)	0.97	0.76	1.24	0.819
Pasture fertilised	Not fertilised	23 (20)	90 (80)	referent			
	Fertilised	74 (40)	113 (60)	2.06	1.53	2.77	<0.001
Fertiliser type	None	23 (20)	90 (80)	referent			
	Nitrogen based	35 (32)	74 (68)	1.85	1.37	2.52	<0.001
	Other	39 (50)	39 (50)	1.67	1.20	2.33	0.002
Pasture cut	Not cut	27 (28)	68 (72)	referent			
	Cut	72 (35)	135 (65)	0.76	0.59	0.98	0.033
Pasture re-seeded	Not re-seeded	50 (27)	132 (73)	referent			
	Re-seeded	47 (40)	70 (60)	1.16	0.91	1.48	0.235
Re-seeded how long ago	Not re-seeded	50 (27)	132 (73)	referent			
	<5 years ago	35 (48)	38 (52)	1.49	1.15	1.93	0.002
	>5 years ago	9 (23)	30 (77)	0.43	0.25	0.74	0.002
Other domestic animals on the pasture	None	29 (28)	75 (72)	referent			
	Ruminants	46 (32)	97 (68)	0.70	0.53	0.93	0.014
	Birds/fowl	11 (48)	12 (52)	1.61	1.08	2.41	0.020
	Other	11 (36)	20 (64)	1.43	0.99	2.08	0.056

When re-seeding of pastures, which was not in itself significantly associated with recurrence, was broken down according to the period since re-seeding was conducted, opposing directions of effect were identified. Re-seeding within the last 5 years was associated with an increased rate of recurrence whereas re-seeding more than 5 years previously was apparently protective. For reasons outlined in the discussion this variable and the related variable of ley pastures compared to permanent grazing, were considered to be likely effects of rather than risk factors for recurrence and were not, therefore, included in multivariable Poisson regression analyses. Similarly, as supplementary feeding of hay and concentrate to grazing horses had been advocated following studies by Gilmour & Jolly (1974), the spring and summer supplementary

feeding variables, were also not included in multivariable modelling as they might be a consequence of recurrence of EGS rather than contributing to it.

Table 4. Final multivariable Poisson regression model including interaction terms for recurrence of EGS on previously affected premises, showing coefficient estimates and their standard errors, rate ratios and their 95% confidence intervals and corresponding Wald and LRS χ^2 *P*-values

Variable	Category	Coefficient	Standard error	Rate Ratio	95% CI lower	95% CI upper	*P* value
Intercept		-2.57	0.28				
Number of horses on the premises	1-5			referent			**<0.0001*
	6-10	0.08	0.37	1.08	0.52	2.22	0.838
	11-15	1.06	0.32	2.90	1.55	5.42	0.001
	16-20	2.46	0.35	11.7	5.91	23.1	<0.001
	21-40	1.27	0.30	3.56	1.98	6.40	<0.001
	41+	1.97	0.31	7.18	3.92	13.2	<0.001
Presence of horses <2 y.o.	No			referent			**0.0001*
	Yes [2YO]	0.53	0.24	1.70	1.06	2.71	0.027
Soil type	Clay			referent			**<0.0001*
	Sand	0.36	0.20	1.43	0.98	2.10	0.067
	Chalk	-1.48	0.61	0.23	0.07	0.76	0.016
	Loam	0.74	0.21	2.11	1.40	3.16	<0.001
	Other	-0.98	0.44	0.38	0.16	0.90	0.027
Method of droppings removal [REM]	Not removed			referent			**<0.0001*
	By hand [1]	-1.71	0.49	0.18	0.07	0.48	0.001
	Mechanically [2]	1.02	0.47	2.76	1.10	6.94	0.031
Pasture cut	Not cut			referent			**<0.0001*
	Cut [CUT]	-2.15	0.32	0.12	0.06	0.22	<0.001
Other domestic animals on the pasture [DOM]	None			referent			**<0.0001*
	Ruminants [1]	-2.22	0.41	0.11	0.05	0.24	0.001
	Birds/fowl [2]	-0.09	0.30	0.91	0.50	1.65	0.760
	Other [3]	-2.37	1.03	0.09	0.01	0.70	0.021
[REM]*[2YO]	[REM 1]*[2YO]	1.22	0.50	3.40	1.27	9.11	0.015
	[REM 2]*[2YO]	-0.54	0.42	0.58	0.26	1.32	0.196
							**0.004*
[REM]*[CUT]	[REM 1]*[CUT]	1.22	0.46	3.39	1.37	8.38	0.008
	[REM 2]*[CUT]	0.26	0.40	1.30	0.59	2.84	0.515
							**0.023*
[DOM]*[CUT]	[DOM 1]*[CUT]	2.03	0.48	7.65	2.99	19.6	<0.001
	[DOM 2]*[CUT]	0.73	0.48	2.07	0.81	5.28	0.128
	[DOM 3]*[CUT]	2.91	1.06	18.3	2.29	147	0.006
							**<0.0001*

*Likelihood ratio statistic (LRS) χ^2 *P*-value

Multivariable Poisson regression analyses

Table 4 outlines the final multivariable Poisson regression model including several statistically significant interaction terms that were identified during model building and which are outlined below in Table 5. Controlling for other variables in the model, there was evidence for an increased rate of recurrence of EGS on premises associated with increasing horse numbers, the presence of younger animals, sand and loam soil types and use of mechanical

droppings removal. There was evidence for a decreased rate associated with chalk and other soil types, removal of droppings by hand, grass cutting and ruminants grazing pasture.

Effect modification (interaction)

Several statistically significant interaction terms (as judged by LRS χ^2 *P*-values) were simultaneously retained in the final multivariable Poisson regression model and the effect of these are summarised in Table 5. The effects of other domestic animals on pasture and methods of droppings removal from pasture were significantly modified according to whether or not pastures were cut and/or there were animals less than two years old present on premises.

Table 5. Details of significant effect modification between variables in the final multivariable Poisson regression model for recurrence of EGS on previously affected premises, with rate ratio interaction term estimates (underscored) applied to baseline rate ratios

Interaction between Variable 1*Variable 2				
		Variable 2		
Variable 1	Categories ↓	Categories →		
Method of droppings removal*presence of horses <2 years old				
		Presence of horses <2 years old		
		No	Yes	
Method of droppings removal	None	referent		1.70
	By hand	0.18	0.18*3.40 =	0.61
	Mechanically	2.76	2.76*0.58 =	1.60
			LRS χ^2 P-value	*0.004*
Method of droppings removal*pasture cutting				
		Pasture cutting		
		No	Yes	
Method of droppings removal	None	referent		0.12
	By hand	0.18	0.18*3.39 =	0.61
	Mechanically	2.76	2.76*1.30 =	3.59
			LRS χ^2 P-value	*0.023*
Other domestic animals*pasture cutting				
		Pasture cutting		
		No	Yes	
Other domestic animals on the pasture	None	referent		0.12
	Ruminants	0.11	0.11*7.65 =	0.84
	Birds/fowl	0.91	0.91*2.07 =	1.88
	Other	0.09	0.09*18.3 =	1.65
			LRS χ^2 P-value	*<0.0001*

LRS = Likelihood ratio statistic

Examination of the modification of effects between different variables showed that there was a decreased effect over that expected (in a multiplicative model) for mechanical droppings removal on premises with animals less than 2 years old. There was a reduced overall protective effect than expected from manual droppings removal when there was pasture cutting and the increased rate associated with mechanical droppings removal increased further rather than

decreasing when pastures were cut. The protective effect of grazing ruminants was only apparent when pastures were not cut. Similarly, there was evidence in the final multivariable model for an increased rate from having domestic birds/fowl on premises but only when pastures were cut.

DISCUSSION

This was the first study of its kind to investigate risk factors for recurrence of EGS on previously affected premises and as such it was deliberately designed to gather as much information as possible on how horses and pastures were managed on EGS affected premises. The association with recurrence of disease for several factors was considered to be more likely to be an 'effect' rather a 'cause' of disease recurring on premises and as such these factors were not further investigated by inclusion in multivariable analyses. These factors included the practice of supplementary feeding during the spring and summer, i.e., during the high risk period for EGS (Doxey et al., 1991a; Wood et al., 1998), which was suggested to be protective in an earlier study by Gilmour & Jolly (1974). It was also believed that re-seeding of affected pastures might have been more likely to be practiced on premises where the disease had recurred and particularly where this was recent, in order to try and reduce future recurrence rather than being a factor contributing to it. This factor was also related to grazing type, as the majority of ley grazing had been re-seeded in the previous 5 years.

Among variables retained in the final multivariable Poisson regression model the majority demonstrated strong associations with recurrence of EGS on premises. As such they may provide novel insights into the pathogenesis of this disease and these aspects are discussed below. However, the identification of several significant interaction terms in the final multivariable model emphasises that considerable caution is required in interpreting the findings of this study and that these observations require specific hypotheses to be generated and subsequently tested in appropriately designed, controlled and randomised intervention studies.

The numbers of horses on premises was an important risk factor for recurrence of EGS. As it is horses and not premises that suffer the disease, it is logical that animals may each act as separate sentinels for EGS occurrence and, therefore, as numbers of horses rise incrementally an increase in rate of recurrence would be expected. It is not clear, however, why there was an upward exaggeration from the trend for increasing rate with increasing numbers of animals for premises with 16-20 animals in the multivariable analysis. There was a similar trend in increasing rate of recurrence with increasing numbers of stables on premises, which would be a proxy measure for numbers of horses. The importance of the number of horses on the premises was also illustrated in that 'recurrent' premises had an equivalent median EGS incidence rate to 'non-recurrent' premises when rates were adjusted for numbers of animals.

During the process of multivariable modelling the effects of different premises types became no longer significant as measured by Wald χ^2 or likelihood ratio statistic χ^2 *P*-values. This indicated that there was undoubtedly confounding of premise type by the variables retained in the final model, particularly by numbers of horses and by presence of younger animals. It could be shown that both these factors had considerable influence on the rate ratio for stud farms, which tended to have larger numbers of horses and particularly younger animals.

Results support earlier findings (Gilmour & Jolly, 1974; Doxey et al., 1991a; Wood et al., 1998) that younger horses are at increased risk of EGS than older animals, because after

controlling for other significant factors there remained an overall increased rate of recurrence of EGS on premises on which horses less than 2 years old were present. There was again evidence for confounding of this factor by horse numbers on premises.

A new finding in this study was that the rate of recurrence of EGS varied significantly between premises on different soil types. Results of multivariable analysis showed that premises on loam and sand soils had increased rates of recurrence compared with premises on clay, whereas premises on chalk and other soil types had significantly reduced rates of recurrence. Sand and loam soils are acidic, light and well-draining. Chalk soils are relatively shallow and compacted and there are severe limitations to the types of plants that will grow in them. Clay soils provide good supplies of plant nutrients, but have high water holding capacity, which fills air spaces within the soil and makes them very 'heavy'. Variation in rates of recurrence of EGS on premises of different soil types may, therefore, be consistent with the theory that the disease is due to toxico-infectious form of botulism (Tocher et al., 1923; Tocher, 1924; Hunter et al., 1999; Hunter & Poxton, 2001; McCarthy, 2002) because *C. botulinum* is a bacterium that resides in the soil. The hypothesis is that characteristics of sand and loam soils permit these soil types to be more easily disturbed and turned-over than clay soils. Therefore, soil inhabitants, such as earthworms and moles, are able to burrow more freely through sand and loam soil types, causing disruption and increasing the rate of soil contamination of grass and hence bringing the bacterium into contact with grazing horses more frequently than with clay, chalk and other soils. The characteristics of chalk soils, which are shallow and impacted, will tend to limit the activity of soil inhabitants compared to other soil types, in addition the reduced mineral content of this soil might decrease numbers of soil inhabitants. Soil type as a significant risk factor for recurrence of EGS on premises is consistent with findings by McCarthy (2002) that disturbance of pasture such as by moles was associated with an increased risk of disease. This theory requires further investigation to determine the validity of the findings of these 2 independent studies. If soil type and soil disturbance are truly associated with risk of recurrence they may help to explain geographical differences in occurrence of EGS and may be an important factor to consider in future predictions of disease occurrence for specific premises or affected premises within particular areas of the UK. It would also be interesting to measure numbers of *C. botulinum* at different depths in different soil types and to assess the extent of contamination of grazing by these bacteria in soil under different management conditions.

Mechanical droppings removal was identified as an important risk factor for recurrence of EGS. This was expected to be a proxy measure for the numbers of horses on premises and for the premises type, as studs and other larger establishments with many horses frequently remove droppings mechanically, usually using paddock sweepers. Therefore, the association between the method of droppings removal and recurrence of EGS was expected to disappear in multivariable analyses when controlled for these other factors. However, the association remained significant in the multivariable analysis although there was some evidence of confounding and effect modification. The size of effect decreased from that found in the univariable analysis and was modified according to whether there were younger horses or grass cutting on premises. In addition there was evidence that removing droppings from pastures by hand actually provided a protective effect. The mechanism by which mechanical droppings removal increased the rate of recurrence of EGS compared to the protective effect of manual removal is unclear. However, an aetiological agent or factor in droppings or soil may be important and the process of sweeping paddocks mechanically may act to disseminate this agent rather than remove it. Therefore, we hypothesise that removing droppings mechanically using paddock sweepers causes soil disturbance as well as dissemination of faecal material that contain Clostridial spp., thereby bringing grazing horses into contact with bacteria such as *C.*

botulinum. Manual droppings removal may avoid soil exposure to horses by discouraging overgrazing of pastures and reducing exposure to faecal material. The hypothesis relating to methods of droppings removal further supports the views of Begg (1936) and Wood et al. (1999) that the aetiological agent is present in the soil rather than the grass and would be consistent with EGS being a toxico-infectious form of botulism.

Having other domestic animals on premises with horses was significantly associated with variations in the rate of recurrence of EGS, although there was significant interaction with pasture cutting, which make interpretation of these factors difficult at this stage. There was evidence that grazing ruminants did convey some protection against recurrence, although it is unclear whether this is simply attributable to consequently reduced grazing intensity by horses or to some indirect mechanism related to removal of toxic material, bacteria or parasites from the pasture. Univariable analyses and inclusion of interactions in final multivariable analyses also demonstrated an increased rate of recurrence associated with the presence of domestic birds or fowl on premises. This finding corresponds with anecdotal observations that recurrence of EGS frequently occurred in areas where intensive rearing of game birds occurred. In addition, birds have long been recognised as a source of botulism and the importation of 'guano' (a nitrogenous fertiliser of avian origin) from south America in the mid/late 19th Century to improve Scottish agricultural land, was soon followed by the first reports of EGS in this part of Britain (K. Miller – personal communication).

Cutting grass on pastures was associated with a significantly reduced rate of recurrence of the disease, although there were apparently significant interactions with droppings removal methods and other domestic animals on pastures. This protective association may again have been attributable to reduced grazing intensity by horses but may also have encouraged more even grazing of pastures and hence reduced overgrazing of specific areas, which might be more likely to expose soil and bring horses in contact with bacteria residing in soil.

Although access to grazing has historically been the major risk factor for EGS (Begg, 1936; Gilmour & Jolly, 1974), contention has since arisen as to the role that grass itself plays in the disease and the possibility that there is some specific aetiological factor within the soil (Wood et al., 1999). The risk factors identified in this study are consistent with the hypothesis that EGS is caused by toxico-infection by *C. botulinum*. As a result of these findings, it is very tempting to suggest possible protective control measures for premises affected with EGS, such as good pasture management, co-grazing of ruminants and the avoidance of pasture sweepers and domestic birds. However, these interventions cannot be entirely justified from this single, retrospective study in which there was evidence of significant effect modification between various factors. The results require corroboration by further, independent studies and in future, specific hypotheses need to be generated and then tested rigorously in carefully designed and conducted intervention studies.

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CONSTITUTION AND RULES

NAME

1. The society will be named the Society for Veterinary Epidemiology and Preventive Medicine.

OBJECTS

2. The objects of the Society will be to promote veterinary epidemiology and preventive medicine.

MEMBERSHIP

3. Membership will be open to persons either actively engaged or interested in veterinary epidemiology and preventive medicine.
4. Membership is conditional on the return to the Secretary of a completed application form and subscription equivalent to the rate for one calendar year. Subsequent subscriptions fall due on the first day of May each year.
5. Non-payment of subscription for six months will be interpreted as resignation from the Society.

OFFICERS OF THE SOCIETY

6. The Officers of the Society will be President, Senior Vice-President, Junior Vice-President, Honorary Secretary and Honorary Treasurer. Officers will be elected annually at the Annual General Meeting, with the exception of the President and Senior Vice-President who will assume office. No officer can continue in the same office for longer than six years.

COMMITTEE

7. The Executive Committee of the Society normally will comprise the officers of the Society and not more than four ordinary elected members. However, the Committee will have powers of co-option.

ELECTION

8. The election of office bearers and ordinary committee members will take place at the Annual General Meeting. Ordinary members of the Executive Committee will be elected for a period of three years. Retiring members of the Executive Committee will be eligible for re-election. Members will receive nomination forms with notification of the Annual General Meeting. Completed nomination forms, including the signatures of a proposer, seconder, and the nominee, will be returned to the Secretary at least 21 days before the date of the Annual General Meeting. Unless a nomination is unopposed, election will be by secret ballot at the Annual General Meeting. Only in the event of there being no nomination for any vacant post will the Chairman take nominations at the Annual General Meeting. Tellers will be appointed by unanimous agreement of the Annual General Meeting.

FINANCE

9. An annual subscription will be paid by each member in advance on the first day of May each year. The amount will be decided at the annual general meeting and will be decided by a simple majority vote of members present at the Annual General Meeting.
10. The Honorary Treasurer will receive, for the use of the Society, all monies payable to it and from such monies will pay all sums payable by the Society. He will keep account of all such receipts and payments in a manner directed by the Executive Committee. All monies received by the Society will be paid into such a bank as may be decided by the Executive Committee of the Society and in the name of the Society. All cheques will be signed by either the Honorary Treasurer or the Honorary Secretary.
11. Two auditors will be appointed annually by members at the Annual General Meeting. The audited accounts and balance sheet will be circulated to members with the notice concerning the Annual General Meeting and will be presented to the meeting.

MEETINGS

12. Ordinary general meetings of the Society will be held at such a time as the Executive Committee may decide on the recommendations of members. The Annual General Meeting will be held in conjunction with an ordinary general meeting.

GUESTS

13. Members may invite non-members to ordinary general meetings.

PUBLICATION

14. The proceedings of the meetings of the Society will not be reported either in part or in whole without the written permission of the Executive Committee.
15. The Society may produce publications at the discretion of the Executive Committee.

GENERAL

16. All meetings will be convened by notice at least 21 days before the meeting.
17. The President will preside at all general and executive meetings or, in his absence, the Senior Vice-President or, in his absence, the Junior Vice-President or, in his absence, the Honorary Secretary or, in his absence, the Honorary Treasurer. Failing any of these, the members present will elect one of their number to preside as Chairman.
18. The conduct of all business transacted will be under the control of the Chairman, to whom all remarks must be addressed and whose ruling on a point of order, or on the admissibility of an explanation, will be final and will not be open to discussion at the meeting at which it is delivered. However, this rule will not preclude any member from raising any question upon the ruling of the chair by notice of motion.
19. In case of an equal division of votes, the Chairman of the meeting will have a second and casting vote.
20. All members on election will be supplied with a copy of this constitution.
21. No alteration will be made to these rules except by a two-thirds majority of those members voting at an annual general meeting of the Society, and then only if notice of intention to alter the constitution concerned will have appeared in the notice convening the meeting. A quorum will constitute twenty per cent of members.
22. Any matter not provided for in this constitution will be dealt with at the discretion of the Executive Committee.

April, 1982
Revised March, 1985; April, 1988; November 1994
Corrected January 1997; April 2002