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Papers

Evaluation of single reactor bovine tuberculosis breakdowns based on analysis of reactors slaughtered at an Irish export meat plant

OPEN ACCESS

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The ‘Singleton Protocol’ was adopted by the Irish Department of Agriculture Fisheries and Food (DAFF) in 1996 to address the incomplete specificity of the single intra-dermal comparative tuberculin test (SICTT) used in Ireland for the detection of animals infected with bovine tuberculosis (bTB). The protocol allows the early restoration of disease-free status to herds with a single reactor breakdown, where the herd was not confirmed as infected with *Mycobacterium bovis* by epidemiological investigation, by postmortem examination or by further test. The current study examines the ability of the Singleton Protocol to identify false-positive reactors. It investigates the subsequent herd-reactor rate following single reactor removal and analyses the factors leading to a positive postmortem lesion outcome and a positive reactor retest result. Postmortem lesion results were obtained for 371 reactor animals from single reactor breakdowns that were killed at an export meat plant over a 19-month period. Epidemiological and test data for these animals and their herds were obtained from DAFF databases and analysed by univariate and multivariate statistical analysis. Singleton candidates had an 18.7 per cent lower lesion rate than single animal breakdowns not meeting the singleton criteria. No significant difference was found between Singletons and non singletons in the subsequent reactor retest results. Skin thickness at the SICTT is the most significant determinant of a positive lesion result. The area bTB history was shown to be a significant variable in producing a positive reactor retest result.

BOVINE tuberculosis (bTB) is a zoonotic disease of cattle caused by *Mycobacterium bovis*. It has been the subject of an eradication programme in Ireland since 1954, based on an antemortem single intradermal comparative tuberculin test (SICTT) and a postmortem carcase veterinary inspection. The SICTT uses bovine and avian tuberculin in combination, administered at separate sites, by intradermal injection in the middle third of the neck. The skin reactions at the injection points are compared after approximately 72 hours to determine the cell-mediated response of the animal, in accordance with EU Directive 64/432/EEC. Herds that have one or more animals reacting positively to the test have their disease-free status removed until they have two clear tests 60 days apart. Infected animals go directly for slaughter and postmortem examination. Study results, examining SICTT sensitivity under Irish conditions, vary from 90.9 (Costello and others 1997) to 80 per cent (de la Rua-Domenech and others 2006) and even as low as 56.8 to 67.5 per cent (Clegg and others 2011). Specificity has been estimated to range from 99.8 to 99.9 per cent (O'Reilly 1993) to 99.5 per cent (Clegg and others 2011). Postmortem factory surveillance, conducted by veterinary inspectors in accordance with the legislative requirements of EU Regulation 854/2004, is based on the palpation, incision and inspection of a defined range of lymph nodes and other tissues (Frankena and others 2007). This postmortem examination is a routine assessment of fitness for human consumption and not specifically designed to identify all bovine cases of tuberculosis (Good and Duignan 2007). Though lacking the sensitivity of the SICTT, postmortem surveillance has a role in bTB eradication and accounts for between 27 to 46 per cent of new herd breakdowns annually (Good and More 2006).

The SICTT serves to differentiate between responses from exposure to *M bovis* and other non-specific mycobacteria. However, it is recognised that a percentage of breakdowns are in fact not due to infection with *M bovis* but rather a reflection of the shortfall below 100 per cent in tuberculin test specificity (Martin and others 1992). This is a particular concern with single reactor breakdowns. The causes of such non-specific reactions include bovine paratuberculosis, infection with or previous exposure to *Mycobacterium avium*, skin tuberculosis and exposure to environmental mycobacteria or related organisms (Monaghan and others 1994). This shortfall is recognised in Directive 64/432/EEC paragraph 3A Annex A1, which allows for the suspension of disease status and a rapid restoration of status in situations where disease is assessed as being unlikely from an epidemiological perspective and is not confirmed on postmortem examination. Based on a specificity of 99.8 to 99.95 per cent (O'Keeffe 1993, O’Reilly 1993) of the approximately 10 million animal tests administered annually, 5000 to 20,000 may be false positives. In Ireland, between 20,000 and 30,000 reactors have generally been identified annually (although this has progressively fallen in recent years, to 20,211 in 2010 and 18,531 in 2011) suggesting that false positives are likely to be nearer to the lower estimate (O'Keeffe and Crowley 1995b). In 1996, to address this shortfall in test specificity, the Irish Department of Agriculture Food and the Marine (DAFM) introduced the ‘Singleton Protocol’.

Under the Singleton Protocol (Fig 1), single reactor herds may be considered candidates for early health status restoration if they meet certain epidemiological and test criteria. Such herds must have no history of bTB in the previous three years, no contiguous herd breakdowns within the previous two years (indicating a relatively low area history for bTB) and the reactor animal must show a skin reaction reading (bovine > avian) of less than 12 millimetres without reaction oedema. If these singleton candidates have no lesions at postmortem (both gross and laboratory analysis), they are deemed Singleton Animals (SA). If the herd of such an animal had a clear reactor retest, it was then deemed a Singleton herd, having met each of the criteria in the Singleton Protocol. Singleton herds are removed from movement control and regain clear herd status (DAFF 2010). Non-singleton single reactor (NSSR) herds remain restricted for a minimum of 120 days as they do not meet the criteria of the Singleton Protocol. In 2010, there were 1700 herds with single reactors (8.4 per cent of the total number of reactors). Of these, 866 were considered Singleton Candidates, including 313 that attained the herd status of Singleton herds having met the subsequent postmortem and reactor retest requirements. Therefore, Singleton herds represented 18.4 per cent of herds with single reactors. Collectively, the Singleton qualifying criteria represent epidemiological evidence reducing the possibility of animal infection with *M bovis*. However, given the absence of a gold standard reference test (Good and Duignan 2011), it is inevitable that some herds that have their status restored early are actually infected and not false positives. Such cases were shown by Olea-Popelka and others (2008) to have minimal impact on the effectiveness of the overall programme. These authors demonstrated that single reactor animals, regardless of lesion presence, were unlikely to cause problems into the future. Determining the factors most closely associated with lesion development could help us refine the singleton protocol, thereby improving its effectiveness.

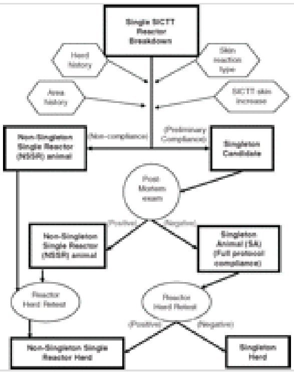

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Fig 1
Singleton Protocol

This study has two objectives: to compare animal lesion rate among singleton candidate animals and NSSR animals and to identify factors associated with lesion rate in the study population and to compare the herd reactor retest breakdown rate among herds with a Singleton animal (SA) and herds with a NSSR animal and to identify factors associated with herd breakdown rate in the study population.

Materials and methods

Data collection

Study population

The study population included TB reactors, either standard or double inconclusive reactors, from single reactor breakdowns, that were killed at a defined Irish export meat plant between June 1, 2009 and December 31, 2010. In Ireland, a standard reactor is defined as an animal positive at the SICTT when the positive bovine reaction is more than 4 mm greater than the avian reaction. An inconclusive reaction is one with a positive bovine reaction between 1 mm and 4 mm greater than the avian reaction. A Double Inconclusive Reactor animal gives an inconclusive result to SICTT at two consecutive tests and is deemed a single reactor animal.

Glossary of terms

Single reactor breakdown: A bTB breakdown, in a herd with a disease-free status, comprising of one standard reactor animal or an animal giving two inconclusive results following two consecutive SICTTs.

Singleton candidate: A single reactor breakdown animal that meets the criteria of the Singleton Protocol before postmortem examination.

NSSR: A single reactor breakdown animal that does not meet the epidemiological or postmortem criteria of the Singleton Protocol.

Singleton animal (SA): A single reactor breakdown animal that meets the epidemiological and postmortem criteria of the Singleton Protocol.

Singleton herd: A herd that contained a Singleton reactor and which re-attained its disease-free status following a clear reactor retest result.

NSSR herd: A herd containing a single reactor breakdown animal that does not meet the epidemiological, postmortem or reactor retest criteria of the Singleton Protocol.

District electoral division (DED): These are the smallest legally defined administrative areas in the Republic of Ireland for which small area statistics are available in the national census.

Animal and herd classification

As highlighted in Fig 1, the study animals were considered as either candidate animals for the Singleton Protocol or NSSR (Fig 1). Veterinary inspectors conducted factory postmortem surveillance for bTB on all study animals, with lesion results being recorded based on observation at veterinary inspection points on the slaughter line. The lesion results of these two animal classes were compared.

The herds of these single reactor breakdowns were then classified as herds containing a SA (herds that meet the epidemiological and postmortem criteria of the Singleton Protocol) and herds containing a NSSR (herds not meeting the Singleton criteria). The reactor retest results of these herd classifications were compared.

Data analysis

Overview

Data, considered to be risk factors, were collected on each study animal, using the Animal Health Computer System. Herd size, length of time a herd was bTB free, slaughter age and area bTB history were considered as both continuous variables and as categorical variables divided into quartiles. Data analysis was conducted using the statistical software package 'Statistical Package for the Social Sciences (SPSS 17.0)'.

The following risk factors were determined:

Risk factors assessed
Reactor animal type: Singleton candidates or NSSR. Following postmortem examination, the non-lesioned singleton candidate was considered to be a SA.

Reactor herd type: Herd with SA or herd with NSSR.

Reactor type
Herd size, the length of time a herd was free from bTB, measured in days. Herds without a recorded bTB breakdown and 'New Herds' which had not shown a bTB breakdown were assigned a previous breakdown date of 01/01/1989 for data-analysis purposes.

Age of the animal at slaughter, measured in days. Animals without registered dates of birth (ie, animals born before January 1, 1998) were assigned a birth date of the January 1, 1998 for data analysis purposes.

SICTT skin increase, divided into four groups: 0 to 5 mm, 6 to 8 mm, 9 to 11 mm and greater than or equal to 12 mm.

The local area history of bTB, determined by the number of reactors per 1000 animal tests within the district electoral division in 2009.

The herd enterprise type, classified as Beef, Dairy or Suckler.

The bTB field test type, classified as annual test, six-month check test following a restriction, private test, contiguous test or inconclusive retest. All herds tested had a bTB clear status.

Lesion result, following postmortem examination, was classified as positive or negative.

Lesion rate
A Pearson's chi-squared test was carried out to determine if there was a significant difference in lesion rate between NSSR animals and singleton candidate animals and between each of the other independent variable's (listed above).

A logistic regression model, using backward stepwise selection, was constructed to model the probability of an animal having a lesion at slaughter, using SPSS 17.0. Backward stepwise regression analysis was used, the analysis began with a full or saturated model, and variables were eliminated from the model based on a likelihood ratio test ($P>0.05$). With the exception of lesion result, the effect of each risk factor listed above was tested within the model. The authors assessed any two-way interactions which were deemed as biologically plausible. The continuous variables were considered in the model as both continuous and categorical variables split into quartiles. Whether to treat variables as continuous or categorical was determined by examining whether the relationship between the log odds of the outcome and the variable of interest was approximately linear and by comparing the Akaike Information Criteria (AIC) of univariate models. An assessment of the goodness-of-fit was obtained by examining residuals and a Hosmer-Lemeshow test.

Reactor retest breakdown rate
The reactor retest results of herds with a Singleton animal were compared with those of single reactor herds not meeting the epidemiological or postmortem requirements of the Singleton protocol (ie, reactor type), using Pearson's chi-squared test to determine if there was a significant difference in reactor retest result. Similarly, a univariate analysis was carried out to determine whether each of the other variables were associated with the reactor retest result.

A logistic regression model of the probability of having a positive reactor retest result was conducted incorporating each of the nine independent variables described previously. Lesion result was included in this model. The model selection and validation procedure was the same as described above for the lesion rate model.

Results

Lesion rate

Animals from 371 single reactor breakdown herds, from 17 of the 28 district veterinary offices (DVO) divisions, were slaughtered over the 19-month study period. The lesion rate among study animals was 43.2 per cent and 61.9 per cent, for singleton candidates and NSSRs, respectively. These rates were significantly different ($P<0.001$) (Table 1).

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Table 1
Lesion results for each single reactor type and the subsequent reactor retest result for each single reactor herd classification

There was a significant difference in lesion rate by farm type ($P=0.045$) and SICTT result ($P<0.001$), while a borderline significant difference was recorded for slaughter age ($P=0.062$) (Table 2).

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Table 2
Univariate statistics of each risk factor in relation to lesion identification results and reactor retest result

The final logistic regression model of the probability of having a lesion at slaughter included the SICTT skin thickness result (P<0.001) and age at slaughter (P=0.046) (Table 3).

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Table 3
Statistical model indicating the factors associated with tuberculosis lesion rate in reactor animals‡

Reactor retest breakdown rate

There was no significant difference between the reactor retest results for the SA herds and NSSR herds (P=0.812) (Table 1).

The reactor retest result was significantly different by test type (P<0.001) and local area prevalence of bTB (P=0.021) using univariate analysis (Table 2). The other variables assessed were not significantly associated with the reactor retest result.

The logistic regression model of the reactor retest result (Table 4) had one significant variable at the final step; local area prevalence of bTB. The presence of a lesion at slaughter was included in the model but was not significantly associated with a subsequent herd breakdown at the reactor retest (P=0.672).

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Table 4
Statistical model indicating the factors associated with herd reactor retest breakdown rate§

Discussion

The initial objective of this project was to identify if there was a significant difference in detected lesion rate between single reactor animals from herds qualifying for the Singleton Protocol and those that did not. The subsequent reactor retest result of the study herds containing SAs was examined to see if it differed significantly regarding breakdown rate, compared with herds containing NSSRs. Criteria included in the Singleton Protocol such as herd bTB history, area bTB history and SICTT skin thickness result, together with factors shown to be associated with bTB in previous studies were assessed in this study to determine their association with animal lesion occurrence and herd reactor retest result. In the Irish bTB eradication scheme, there is a difference in the period of herd disease-free status withdrawal from Singleton herds and NSSR herds. This has financial implications for farmers. Therefore, the Singleton Protocol, if effective in identifying false-positive reactors, has an important role in cost-effective bTB disease eradication.

This study showed candidate animals for the Singleton Protocol had a lesion rate of 43.2 per cent compared with a rate of 61.9 per cent for single reactors animals that were not candidates. The difference was significant (P<0.001). The qualifying criteria for the Singleton Protocol reduces the possibility of the singleton reactor being infected with *M bovis* and showing lesions and therefore the possibility of false-positive reactors in Singleton herds is greater. O'Keeffe (1993) suggested that it would be reasonable to expect that the majority of false-positive animals would occur singly in herds. He proposed that, with the small average herd size in Ireland, false positives, due to a shortfall in test specificity, occur at random. However, a reactor, in which no visible lesions of bTB are detected, cannot legitimately be called false positive or even an NVL due to failure to detect lesions. Corner (1994) in his study of postmortem-examination procedures demonstrated that discrete single lesions could be missed in up to 53 per cent of reactors at postmortem. Many cattle reacting positively to the skin test are NVL and fail to have *M bovis* isolated on TB culture (Goodchild and Clifton-Hadley 2001). NVL reactions are due to a variety of reasons. Their significance depends on the intrinsic specificity of the screening test, the stage of the bTB eradication campaign, thoroughness of examination of reactors at slaughter, time since infection, prevalence of bTB and the number of reactors found in the screened herd (Pollock and Neill 2002). It is erroneous to assume that postmortem examination and bacteriological culture are the 'gold standards' against which one should assess the accuracy of immunodiagnostic TB tests (De La Rua Domench and others 2006). However, routine abattoir 'fitness for human consumption' examination defined in EU Regulation 854/2004, although a relatively crude diagnosing device, accounts for 27 to 46 per cent of new herd breakdowns in any year (Good and More 2006). While the presence of a lesion is strongly suggestive of bTB, and laboratory confirmation provides proof of bTB, the converse is not the case (O'Keeffe and Crowley 1995a).

After accounting for all known confounding variables, this study shows that the Singleton criteria have an impact on determining whether a reactor had detectable lesions at slaughter. The determining factors incorporated in the Singleton Protocol, herd bTB history, area bTB history and skin reaction size, together with other variables such as reactor age, farm enterprise type, herd size and test classification, were examined to determine association with lesion occurrence. Logistic regression modelling showed that skin thickness result was the most significant variable in determining the presence of a lesion, even more so than whether the animal was a 'singleton' animal or not. The risk of a lesion among animals with a SICTT reaction difference of greater than or equal to 12 mm was 7.6 times higher compared with animals with a skin-reaction difference of 1 to 5 mm. The odds ratio of showing lesions at postmortem increased with increasing skin-reaction thickness. The Singleton Protocol recognises this increased risk of lesion with increased skin thickness and has established skin thickness as one of its qualifying criteria. This finding reaffirms the finding of O'Keeffe and Crowley (1995a) on the association of skin thickness to lesion rate. Farm enterprise type, which was significant at the univariate analysis, was not found to be significant when the skin increase was included in the model. Costello and others (1998) showed a greater incidence of lesions in cows than in younger stock. Animal age is a significant risk factor for the detection of lesions at slaughter: animals over six years having a greater probability of a lesion compared with animals less than two years of age.

The visible lesion rate of 43 per cent among the animals being considered as Singleton candidates is high, which suggests that further risk factors be considered as part of the Singleton criteria. The improvement of qualifying criteria would certainly be helpful, allowing more accurate differentiation of infected (true positive) and non-infected (false positive) Singleton candidates. Irish research has consistently identified several risk factors indicative of genuine infection (as measured by time to re-restriction), including location, past herd bTB history and herd size. Two of these factors are already incorporated into the Singleton Protocol. There is ongoing work towards the development of predictive statistical models for recurrence, both in Ireland (Wolfe and others 2010) and the UK (Karolemeas and others 2011), but with limited success to date.

The study population of Singleton animals decreased from the initial 190 candidate animals to 108 singleton animals as 82 of the initial singleton candidates did not meet the postmortem criteria of the singleton protocol. The positive reactor retest result in herds with SA (8.3 per cent) is not significantly different from the positive reactor retest result in herds with NSSR (7.6 per cent). This low subsequent breakdown rate in all single reactor breakdowns reaffirms the findings of Griffin and others (1993), where such single animal breakdowns were found to pose a lower future risk compared with multireactor herds. In the logistic regression model of a positive result at the reactor retest, local bTB incidence was the final significant factor remaining in the model (log likelihood P value=0.006). Herds within district electoral division areas with bTB incidence rates in the three higher quartiles were found to have over seven times the reactor retest breakdown rate of those herds in the lowest quartile. Olea-Popelka (2004) stated that lesions in cattle are not an important future predictor of bTB breakdowns and this was also shown in this study. Findings in other studies showing increased herd size, a positive history of previous herd bTB, and the age of the reactor (Martin and others 2003, Olea-Popelka and others 2008) resulting in an increased hazard of a future bTB breakdown were not reflected in the present study. This may have been due to the large sample size of these previous studies in comparison with this study and the slightly different populations of animals examined.

At present levels of bTB, approximately 50 per cent of the singleton herds where the status was restored ‘early’ (6 per cent of total breakdowns), the reactor animal was, in all probability, infected with *M bovis* (Good and Duignan 2007). However, having had a subsequent clear tuberculin test, these three per cent of herds are very unlikely to cause problems into the future (Good and Duignan 2011). The occurrence of low numbers of infected animals within herds has led to the belief that bovine TB is characterised by low cattle to cattle transmissibility with low numbers of *M bovis* bacilli shed by infected animals (Palmer and Waters 2006). The minimum infective dose for cattle is determined by the aerodynamic diameter of the infectious particle and has been estimated to be one bacillus when delivered to an alveolus in an aerosol (Pollock and others 2006, Corner and others 2011). Only a small fraction of these fine aerosol droplets contain viable tubercle bacilli capable of initiating infection in the alveolus (Neill and others 2001). Pollock and Neill (2002) found that clinical disease probably occurs only in a relatively small proportion of cattle that are exposed to *M bovis* and that the quantity and frequency of shedding in cattle appeared inversely proportional to the infecting dose.

In conclusion, the Singleton Protocol was designed to address the shortfall in skin-test specificity for bovine tuberculosis by identifying herds with low potential infection rates. Surveillance of the cattle population for bTB using lesions found at slaughter is an essential component of an overall control programme (Good 2006). The postmortem lesion result of this study indicates that the protocol effectively fulfils its role of identifying reactors with the increased possibility of being false positive and increases the viability of farm enterprises by limiting unjustified herd restrictions in herds with low levels of re-infection. However, the Singleton Protocol has no significant influence on reactor retest result. The study does reaffirm the low positive reactor retest result of single reactor breakdowns shown by Olea-Popelka (2004) and the significant influence of area bTB incidence on reactor retest result (Olea-Popelka and others 2008). Analysis of the risk factors effecting lesion rate indicates the important role of skin thickness result in the determination of lesion presence within the criteria of the Singleton Protocol.

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