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**An evaluation of the Irish Single Reactor Breakdown Protocol for 2005 to 2008
inclusive and its potential application as a monitor of tuberculin test
performance**

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Abstract

Under the Irish Bovine Tuberculosis (bTB) Eradication Programme all herds are subjected to at least one test per annum. The Single Intra-dermal Comparative Tuberculin Test (SICTT) is used in Ireland for the detection of cattle infected with *Mycobacterium bovis*. There have been concerns regarding the specificity of the SICTT, notably by farmers, and particularly in herds where the detection of a single positive animal in the absence of an obvious source of (bTB) infection could be perceived as a “false” positive. To address this issue the so-called ‘Singleton Protocol’ was established as part of the Irish bTB eradication programme. This protocol allows for the early restoration of free trading status to herds where a single positive animal was detected and where the herd was not confirmed as infected with *M. bovis* by epidemiological investigation, by *post mortem*, by laboratory examination, or by

further test. This paper presents data from the 2005 to 2008, inclusive, bTB programmes on the number of herds that were assessed, which qualified for inclusion under the 'Singleton Protocol' and the outcome for qualifying herds up to and including having status restored early as a consequence of inclusion in that programme. The outcome of this protocol reaffirms the reliability of the SICTT at current levels of infection. Furthermore it is advocated that the 'Singleton Protocol' be continued as a monitor of herds in which a single positive animal is disclosed, and as overall infection levels of bTB fall the outcome may be used as one means to assess progress towards bTB eradication in Ireland.

Keywords: Bovine tuberculosis; *Mycobacterium bovis*; SICTT reactor; tuberculin test

Introduction

All members of the closely related phylogenic grouping of *Mycobacteria* in the *M. tuberculosis* complex cause tuberculosis in cattle – bovine TB (bTB) - in Ireland the most important is *Mycobacterium bovis*. Tuberculin tests, which avail of a cell-mediated response, to *Mycobacteria*, have been used for the diagnosis of tuberculosis in man and animals for more than 100 years (Monaghan et al., 1994). All herds in the Republic of Ireland are subjected to an annual test for bTB using the Single Intradermal Comparative test (SICTT). The SICTT uses bovine and avian tuberculin PPDs in combination to assess, measure and compare the response at 72+/-4hrs following intra-dermal injection so as to determine the infection status of the animal and herd (European Commission, 1964). Herds in which an animal responds

1 positively to the SICTT i.e., are identified as a ‘reactor’, are said to be experiencing a
2 TB breakdown.

3 In herds where infection has been established the use of the so-called ‘severe’
4 interpretation, which lowers the cut-off points for an animal to be declared a reactor,
5 enhances the sensitivity (Se) of the SICCT over the normal ‘standard’ interpretation.
6 Test Se, (the ability of a test to correctly identify infected animals) and ‘specificity’
7 Sp, (the ability of a test to correctly identify non-infected animals) is a function not
8 just of the test itself and particularly the potency of the tuberculin used (Good et al., in
9 preparation) but also of the environment in which it is used. O’Reilly (1992) assessed
10 the Se of the SICTT under Irish conditions as 91 and 98% Se (standard and severe
11 interpretation respectively). Costelloe et al., (1997) repeated the study and obtained
12 similar results 90.9% Se (89.6 and 91.2 – standard and severe interpretation
13 respectively). Monaghan et al., (1994) acknowledge that experiments to establish test
14 Se and Sp for a particular environment are expensive and labour intensive thus few
15 studies involve slaughter of all, including the non-reacting, cattle. The O’Reilly
16 (1992) and Costelloe et al., (1997) studies slaughtered and examined all animals (221
17 and 353 respectively) involved. In a review of techniques for ante-mortem diagnosis
18 of tuberculosis in bovines de la Rua-Domenech *et al* (2006) affirms that SICCT Se
19 lies between 75% and 95.5% at standard interpretation according to studies, using
20 potencies of bovine and avian tuberculins as in the current U.K. and Irish bTB
21 programmes (Bovine tuberculin PPD 30,000 I.U./ml; Avian tuberculin 25,000 I.U./ml
22 as supplied by Lelystad Biologicals B.V.). Herd level Se (HSe) is a function of the
23 within-herd bTB prevalence and the number of animals tested. The presence of a
24 single test positive animal, regardless of herd size, determines the status of the herd,
25 consequently the HSe will rapidly increase to its maximum level (100%) even when

1 the within-herd bTB prevalence is low and the animal level Se is imperfect (Martin *et*
2 *al.* 1992).

3 The 1975 O'Reilly and Mac Clancy (1978) trial, in advance of the replacement of
4 human with bovine tuberculin in the Irish bTB programme, showed that some 7% of
5 cattle were positive to the single intradermal test but not to the SICTT. Other
6 pathogenic mycobacteria e.g. *Mycobacterium paratuberculosis* subsp. *avium*, and
7 non-pathogenic environmental *Mycobacteria* such as *M. hiberniae*, abundant in the
8 Irish environment, cause non-specific sensitisation to bovine tuberculin PPD
9 (O'Reilly and Mac Clancy 1978; Cooney *et al.*, 1997) and thus, in order to have an
10 acceptable test Sp, the SICTT was chosen as the screening test for the Irish bTB
11 eradication programme. It is not possible, however, to determine test Sp with a high
12 degree of accuracy except in a tuberculosis-free environment. Irish field experience
13 indicates that the actual percentage of false positive reactors to the SICTT on a
14 national basis is only a fraction of 1% (O'Reilly and Mac Clancy 1978). O'Reilly,
15 (1992) calculated test specificity as 99.8-99.9% and O'Keeffe (1992), demonstrated
16 mathematically that, the Sp of the SICTT, as performed in Ireland in a non-disease-
17 free population, must be at least 99.95% otherwise far more positive animals would be
18 identified. However, when test Sp is less than 100%, as the number of animals tested
19 increases, the probability of at least one false-positive animal increases and thus the
20 herd level Sp decreases. This is of particular relevance to farmers who may have their
21 TB and trading status withdrawn due to detection of a SICTT reactor. The predictive
22 value of a positive test (PPV) is directly related to disease prevalence (Thoen and
23 Steele 1995) and the higher the population disease prevalence, the more likely it is
24 that a positive test is predictive of the disease. The shortfall in test specificity means
25 that a percentage of positive SICTT responses are false positive (Martin *et al.*, 1992).

1 To take the above issues into consideration the ‘Singleton Protocol’ was
 2 incorporated into the bTB eradication programme in 1996 (O’Sullivan 1997). This
 3 protocol acknowledges the shortfall in test specificity and is compatible with
 4 Directive 64/432/EEC (European Commission, 1964) paragraph 3A of Annex AI,
 5 which allows for a rapid status-restoration possibility where disease is not confirmed
 6 following appropriate epidemiological, post mortem and laboratory examinations.
 7 BTB breakdown herds, with no specific indicators of probable infection with *M. bovis*
 8 on epidemiological evaluation may qualify for the ‘Singleton Protocol’. The
 9 ‘Singleton Protocol’ qualifying criteria are: only one reactor identified at the most
 10 recent test; the bovine minus avian difference on reading day $\leq 12\text{mm}$; there was no
 11 oedema present at the bovine site; TB not diagnosed in the herd within the last three
 12 years or in any of the neighbouring herds within the last two years. ‘Singleton
 13 Protocol’ qualifying herds, are placed under movement control and may, subject to
 14 non-confirmation of infection at slaughter and laboratory followed by a clear SICTT
 15 at least 42-days after the removal of the reactor animal, then have their trading status
 16 restored earlier than TB infected herds.

17 The national *post mortem* visible lesion detection rate (VLR), in SICTT reactors
 18 and in routinely slaughtered cattle, demonstrates significant annual fluctuation as
 19 shown from 1988 to 2008 in Figure 1. Statistical analysis of DAF data (unpublished)
 20 carried out in conjunction with the Veterinary Epidemiology and Economic Research
 21 Unit (VEERU), at Reading University, has shown that if the annual variation in lesion
 22 detection were the product of random events, annual rates would vary by less than 1%
 23 (such are the numbers involved). However, the same analysis failed to find any
 24 significant correlation with any component of the eradication programme itself, to
 25 explain the annual variation. The analysis went on to conclude that changes in the rate

at which visible lesions are found *post mortem* cannot be used as a guide to changes in disease prevalence or the success or otherwise of the eradication programme. More significantly, visible lesion rate cannot be used directly as an indicator of tuberculin test reliability (O'Reilly 1992). Herd incidence is also included in Figure 1 for the same period and, clearly, since the incidence rate of new herd bTB breakdowns has fallen steadily between 2000 (8.2%) and 2008 (5.88%) and the average number of reactors removed in the 5-year period 2002-08 was, at 26,127, 26% lower than in the preceding 5-year period, there is no consistency between lesion detection data in reactors, clear cattle, reactor numbers or herd incidence and thus measurements and analysis of these parameters is neither a satisfactory monitor of tuberculin test reliability or progress towards bTB eradication.

The objective of this paper, is to evaluate the performance of the 'Singleton Protocol', to determine if it reaffirms the reliability of the SICTT in Ireland at current levels of infection, and while accepting that this reliability will decrease as bTB prevalence falls, to assess the possibility of using the outcome of a continued 'Singleton Protocol' as an alternative and more satisfactory monitor of progress towards bTB eradication than other parameters previously considered.

Materials and methods

All test results in the bTB programme are processed and recorded on the Animal Health Computer System (AHCS). The AHCS data was analysed for the years 2005 to 2008 inclusive for all bovine Tuberculosis breakdowns in the Republic of Ireland in order to determine the number of breakdowns that commenced with a single animal being detected as reactor to the SICTT. From this sub-set of breakdown herds, the

number, which met the epidemiological qualifying conditions for the Singleton Protocol, was extracted and the subsequent outcome for these herds was assessed to determine how many confirmed as infected or had status restored early under the protocol.

Results

Herds presenting initially with a single reactor represented 34.1%, 33%, 35.1% and 33.9% of breakdowns in years 2005 to 2008 respectively. The outcome for these single reactor breakdowns is presented in Table 1. The reactor animals from herds that qualified for the Singleton Protocol were subjected to post-slaughter examination. If no visible lesions were detected, the head and thoracic lymph glands were submitted for histology and laboratory culture. Table 2 presents the outcome for these 'Singleton Protocol' participating herds during 2005 to 2008 inclusive, as numbers and percentages.

Discussion

There was little, if any change in the level of infection and a very similar distribution of bTB breakdown types between the years, 2005 to 2008 with 6% of the total breakdowns fulfilling all the Singleton Protocol criteria i.e. these herds did not confirm with bTB and had trading restrictions lifted earlier than those herds with confirmed TB.

Identifying the true health status of a herd with bovine tuberculosis is facilitated by the fact that tuberculosis is 'communicable' and therefore one can expect, though

1 not rely on, spread – more than one infected animal – and a degree of persistence if
 2 the herd is actually diseased. The VLR for standard reactors removed under the
 3 programme varied between 34% and 39% during the period 2005-2008 (Department
 4 of Agriculture records unpublished), the VLR in the singleton protocol animals was
 5 significantly lower for each year, p -value <0.001 except 2006 when $p=0.0128$.
 6 Considering that herds admitted to the ‘Singleton Protocol’ procedure were assessed
 7 as being unlikely to be infected with bTB the VLR disclosed (Table 2) were
 8 nevertheless surprisingly high. *Post mortem* and laboratory examination diagnostic
 9 limitations, are such that it is not possible to confirm all *M. bovis* infected animals
 10 even in heavily infected environments, or to use lack of confirmation as an absolute
 11 determinant of disease freedom or of a non-specific responder to the SICTT
 12 (O’Reilly and Mac Clancy 1978; de la Rua-Domenech et al., 2006). Moreover, in
 13 Ireland under Regulation 854/2004/EC, (European Commission 2004) a routine
 14 assessment of fitness for human consumption is conducted *post*-slaughter at the
 15 abattoir on animals removed as a result of the SICTT (reactors). This is neither a
 16 detailed *necropsy* nor a bTB diagnostic instrument specifically designed to detect
 17 lesions of bTB. Indeed lesion detection rate is highly variable (Corner et al., 1990;
 18 Whipple et al., 1996; Collins 1997; Frankena et al., 2007). It is accepted that discrete
 19 tuberculous lesions may go undetected in between 47% (Corner et al., 1990) and 53%
 20 (Corner 1994) of reactors with lesions and, in many cases, the only site of infection,
 21 may not be examined (Corner et al., 1990; Whipple et al., 1996). In addition the
 22 relative efficiency of factory surveillance in the disclosure of tuberculous lesions is
 23 variable (Frankena et al., 2007). On the basis of this information the authors have
 24 assumed that visible lesions detected during each successive year of the study
 25 represents only 50% of the expected number of lesioned animals in the ‘Singleton

1 Protocol' group had a detailed necropsy been undertaken. This level of *post mortem*
2 diagnostic failure to detect lesions during abattoir inspection appears most significant
3 in an animal with a single lesion and during one study 66% of tuberculous cattle
4 subjected to a detailed necropsy had only a single lesion (Corner 1994). While the
5 collection, histological examination and/or culturing of glands attempts to reduce this
6 diagnostic deficit it also contributes to the shortfall in confirmation. Under routine
7 conditions at abattoir line speed lymph node collection can only be targeted at a small
8 range of sample sites (head and thoracic nodes) as compared to the various sites
9 where infection is possible. In addition, there is regularly a shortfall in collecting the
10 target number of nodes. Further, Corner *et al.* (1990) reported that, in detailed
11 necropsy findings, 9.8% of single lesions were found in the lung substance. In Ireland,
12 lungs are subject to palpation only and not submitted to any additional examination
13 under the 'Singleton Protocol'. Consequently, some sites of infection are neither
14 examined nor collected and hence never submitted to the laboratory for examination.
15 Thus for all these reasons the total number of animals with actual lesions is expected
16 to be at least double the number found with visible lesions. One might have expected
17 to recover the 50% shortfall in visible lesions by detection and confirmation in the
18 laboratory. However, there is also the unavoidable affect of sample decontamination
19 regimes resulting in reduced sensitivity of laboratory *in-vitro* culture, which,
20 contributes significantly to the inability to confirm infection in all the truly infected
21 reactor animals. As a result less than 50% of the expected shortfall in visible lesioned
22 animals went on to be confirmed as infected. In addition there is also the possibility of
23 culture isolation from lymph nodes that appear normal on gross pathological
24 inspection (Whipple *et al.*, 1996; FSAI 2003), which would be expected to provide
25 additional confirmed cases. However, unless other indicators of bTB are detected in

1 the herd, it is inevitable that some herds which have status restored early are actually
 2 infected and not false positives due to the Sp shortfall of the SICTT. For these
 3 reasons, failure to confirm infection in the 6%, of total breakdown herds, that had
 4 trading restrictions lifted early cannot be taken as proof that the animal or herd in
 5 question was not actually infected.

6 Of the 137,763 breakdown episodes (defined as the time interval between
 7 restriction following detection of an infected animal and de-restriction allowing a
 8 return to trading) in Ireland during 1989-2002, some 52,868 (38.4%) episodes were
 9 associated with a single standard reactor i.e., the so-called singleton breakdowns, with
 10 or without a lesion at post mortem (O’Keeffe and White 2005). Detailed work has
 11 shown that such breakdowns are at lesser risk of a future herd breakdown than
 12 breakdowns with at least two standard reactors (with or without lesions) (Griffin et al.,
 13 1993; O’Keeffe 1993; Olea-Popelka et al., 2004). These studies have also shown that,
 14 having had a subsequent clear SICTT, herds with a single reactor animal only,
 15 regardless of visible lesion status, are very unlikely to cause problems into the future
 16 and thus the early release of the herds which had an unconfirmed but actually infected
 17 animal will not have had a negative impact on bTB eradication.

18 Table 2 shows that only 6.3%, 5.5%, 6.0% and 6.5% of all breakdowns in the
 19 period from 2005 to 2008 respectively had their disease status restored ‘early’ under
 20 the ‘Singleton Protocol’ process. This is actually the maximum number of these
 21 breakdowns, which might reasonably be deemed to have been due to a non-specific
 22 response to tuberculin and a consequence of the less than 100% specificity of the
 23 SICTT. It is quite probable, at present levels of bTB, and taking the evidence and
 24 shortcomings cited above into consideration, that up to 50% of the herds where the
 25 status was restored ‘early’, the SICTT positive animal was infected with *M. bovis*.

1 This probability has no detrimental effect on the programme's effectiveness
 2 (O'Keeffe and White 2005). It does however; confirm that the problem of 'NVL'
 3 reactors is not necessarily one of poor SICTT Sp but does reflect inadequate gold
 4 standards to determine true infection status (de la Rua-Domenech et al., 2006).
 5 Therefore, it can be deduced that the outcome from the so-called 'Singleton Protocol'
 6 indicates that, at present levels of infection in Ireland, only circa 3% of total
 7 breakdowns were due to the shortfall in SICTT specificity below 100% and that the
 8 reliability (O'Reilly 1992) of the SICTT is currently in the region of 97%.

9 This estimate of 97% is broadly in agreement with test reliability calculated in the
 10 conventional manner as per O'Reilly (1992) who states that test reliability is a
 11 relatively crude index of the diagnostic ability of a test and is usually expressed as a
 12 percentage and which in turn using Irish figures for sensitivity and specificity
 13 (O'Keeffe, 1992; O'Reilly 1993; Costelloe et al., 1997) results in the equation
 14

$$\begin{aligned}
 \% \text{ Test reliability} &= \frac{\% \text{ sensitivity} + \% \text{ specificity}}{2} \times 100 \\
 \% \text{ Test reliability} &= \frac{(91 \text{ to } 98) + (99.8 \text{ to } 99.95)}{2} \times 100 \\
 &= \frac{190.8 \text{ to } 199.75}{2} \times 100 \\
 &= 95.4 \text{ to } 99.88\% \text{ or} \\
 &\sim 97.64\%
 \end{aligned}$$

15

16 Conclusion

17

1 The results of the Animal Health Computer System data for the Irish bTB
2 eradication programme for the years 2005 to 2008 inclusive analysed and presented
3 here are consistent with the published literature on the sensitivity, specificity and test
4 reliability of the SICCT. The 'Singleton Protocol', including targeting the primary
5 predilection lymph nodes for laboratory examination as heretofore, should therefore
6 continue and be used as one means to monitor of the reliability of the SICCT in the
7 Irish bTB eradication programme. Furthermore, as Ireland progresses towards bTB
8 eradication the percentage of herds that fail to confirm as infected under the
9 'Singleton Protocol' should rise and therefore this may be a useful monitor of
10 progress towards eradication.

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13
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21 22 **Conflict of Interest Statement**

23
24 The authors have no conflict of interest.

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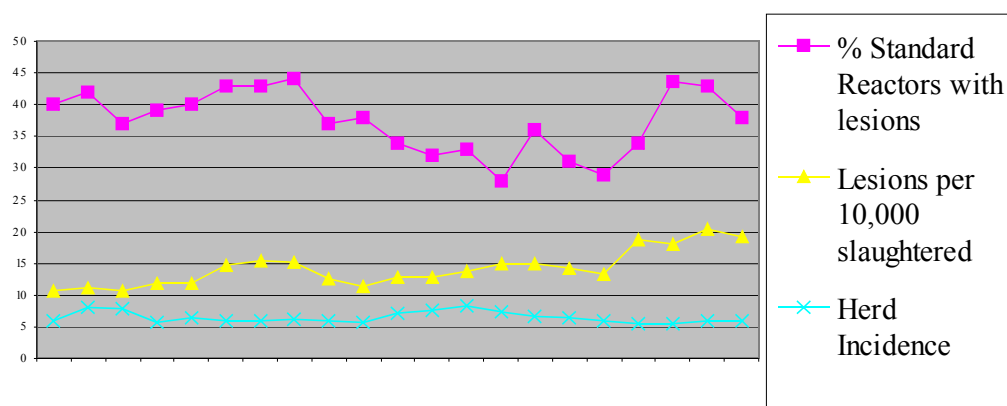
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Figure 1: 1988 to 2008 inclusive % herd incidence bovine TB and national *post mortem* tuberculous lesion detection rate, % in Standard interpretation SICTT reactors, and per 10,000 non-reactor cattle slaughtered routinely.

- 1 Table 1: Evaluation of outcome for herds with a single reactor against the Singleton
 2 Protocol criteria by calendar year for the period 2005-2008 inclusive.
 3

	2005	2006	2007	2008
Herd breakdowns commencing with a single reactor	2,267	2,110	2,471	2,317
Those assessed as probable infected because of:				
Test measurements B-A > 12mm	578	593	698	600
(% Single reactor breakdowns)	(25.5%)	(28.1%)	(28.2%)	(25.9%)
Area/herd history	702	525	615	570
(% Single reactor breakdowns)	(30.97%)	(24.88%)	(24.9%)	(24.6%)
Oedema at bovine site	34	33	36	24
(% Single reactor breakdowns)	(1.5%)	(1.6%)	(1.5%)	(1.0%)
<u>Total excluded</u> for epidemiological reasons	1,314	1,151	1,349	1,194
(% single reactor breakdowns)	(57.96%)	(54.55%)	(54.59%)	(51.53%)
Herds qualifying for 'Singleton Protocol'	953	959	1,122	1,123
(% single reactor breakdowns)	(42.04%)	(45.45%)	(45.41%)	(48.47%)

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2 Table 2: Outcome for herds qualifying for 'Singleton Protocol' in 2005 to 2008; reasons herds were confirmed as bTB infected and numbers

3 with status restored early

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	2005			2006			2007			2008		
		% Single reactor bTB breakdowns			% Single reactor bTB breakdowns			% Single reactor bTB breakdowns			% Single reactor bTB breakdowns	
Herds qualifying for 'Singleton Protocol'		953	42.04%		959	45.45%		1,122	45.41%		1,123	48.47%
Outcome for herds qualifying for 'Singleton Protocol'												
	Number	% qualifying singletons		Number	% qualifying singletons		Number	% qualifying singletons		Number	% qualifying singletons	
Visible bTB lesions post mortem	256	26.86%	11.29%	323	33.68%	15.31%	306	27.27%	12.38%	306	27.25%	13.21%

bTB confirmed in laboratory	131	13.75%	5.79%	152	15.85%	7.20%	230	20.49%	9.31%	191	17.01%	8.24%
Other indicators of bTB in herd	143	15.11%	6.35%	130	13.55%	6.16%	151	14.43%	6.56%	182	16.21%	7.85%
Total of qualifying herds confirmed bTB infected	530	55.72%	23.42%	605	63.09%	28.67%	687	62.30%	28.29%	679	60.46%	29.31%
Herds with status restored early	422	44.28%	18.62%	354	36.91%	16.77%	435	37.70%	17.12%	444	39.54%	19.16%
% Total breakdowns with status restored early	6.30%			5.50%			6.00%			6.50%		