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The comparative performance of the single intradermal comparative tuberculin test in Irish cattle, using tuberculin PPD combinations from different manufacturers

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Abstract

Ireland currently obtains its avian and bovine tuberculin purified protein derivatives (PPDs) from a single source. Because problems of supply or quality cannot be discounted, it is prudent that Ireland identify alternative supplier(s) as part of a broad risk management strategy. Therefore, the aim of this study was to compare the performance of a number of different tuberculin combinations (that is, pairings of bovine and avian PPD; with different manufacturers) in the single intradermal comparative tuberculin test (SICTT), as currently performed in Ireland. The study was randomised, controlled and double-blinded. A total of 2,172 cattle were used in the study. Each animal was tested using two SICTTs, the first based on the tuberculin combination in current use, and the second using one of six trial tuberculin combinations. Analyses were conducted to compare both reactor-status and skin increase. For each control/trial tuberculin combination, there was good agreement between the control and trial reactor-status. Differences in skin increases were mainly confined to animals categorised as either negative or severe inconclusive. However, the measured differences were minor, and unlikely to have a significant impact on the actual test outcome, either for individual animals or for herds. In conclusion, while further studies determining sensitivity and specificity in Ireland would have to be done in the event of a change in tuberculin PPD there should be minimal disruption of the national

programme if alternative tuberculin PPDs meeting WHO, OIE and EU regulations were used. In this study, the precision of the guinea pig bio-assay to assess tuberculin potency was low and therefore Ireland should maintain its practice of periodically assessing potency in naturally infected cattle, even though this is not currently required under WHO, OIE or EU Regulations.

Key words: Ireland, Bovine tuberculosis, tuberculin, diagnosis, *Mycobacterium bovis*, single intradermal comparative tuberculin test

1. Introduction

The single intradermal comparative tuberculin test (SICTT) to detect tuberculosis (TB) in cattle is in routine use as part of the bovine TB eradication programme in Ireland (Good et al., 2007). This test is conducted by comparing the separate immunological cell-mediated response in each animal to avian and bovine tuberculin purified protein derivative (PPD) (Monaghan et al., 1994), used in accordance with the protocols laid down in Directive 64/432/EEC (European Commission, 1964). When one or more animals in a herd show a positive response to the test, herd-level statutory controls are applied.

In Ireland, ID-Lelystad BV (Institute for Animal Science & Health, Lelystad, The Netherlands) currently supplies all of the avian and bovine tuberculin PPD used in the programme. Because problems of supply or quality cannot be discounted, it is prudent that Ireland identify alternative supplier(s) as part of a broad risk management strategy. There are a number of national TB eradication programmes in the Europe Union (Caffrey, 1994; Reviriego Gordejo and Vermeersch, 2006). As yet, however, no work has been reported on the impact of SICTT performance, using tuberculin PPD from different suppliers on these programmes. Therefore, the aim of this study was to compare the performance of a number of different tuberculin combinations (that is, pairings of bovine and avian PPD; with comparable potency and similar avian/bovine potency differentials but with different manufacturers) in the SICTT as currently performed in Ireland.

2. Materials and methods

2.1 The Single Intradermal Comparative Tuberculin Test

a. The test

Detailed information about the SICTT, to diagnose tuberculosis in cattle, is available elsewhere (Monaghan et al., 1994; de la Rue-Domenech et al., 2006). Briefly, the test is conducted by separately injecting avian and bovine tuberculin intradermally into defined sites on the neck of cattle. The test is read 72 hours later, by comparing the relative millimetre increase in skin fold thickness (an *in-vivo* cell mediated response to each tuberculin) at each injection site. The preparation, potency testing and labelling of each batch of tuberculin PPD must conform to the provisions of the standards laid down in the European Pharmacopoeia monographs for tuberculin PPDs, (European Pharmacopoeia, 2007) the OIE manual for diagnostic tests and vaccines for terrestrial animals (World Organisation for Animal Health, 2009), WHO requirements (World Health Organization, 1987) and the standards for the manufacture and use of bovine tuberculin as laid down in European Commission Directive 64/432/EEC (European Commission, 1964). According to WHO Technical Report Series No. 384 (World Health Organization, 1987), and as referenced in the OIE Terrestrial manual (World Organisation for Animal Health, 2009), potency testing should be performed in the animal species, and under the conditions, in which the tuberculins will be used in practice. It goes on to say that periodic testing in tuberculous cattle is necessary however, this is not mandatory under any of the above.

b. Test interpretation

In accordance with Directive 64/432/EEC, as amended (European Commission, 1964), the reaction at an individual injection site (either bovine or avian) is determined and considered *negative* 'if only limited swelling is observed, with an increase of not more than 2 mm without clinical signs such as

diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes'; *inconclusive* 'if no clinical signs as mentioned (previously) are observed and if the increase in skin-fold thickness is more than 2 mm and less than 4 mm'; or *positive* 'if clinical signs such as mentioned (previously) are observed or there is an increase of 4 mm or more in the thickness of the fold of skin at the injection site'.

In the current study, each animal was given a 'reactor-status', based on the results of the SICTT:

- *A standard reactor*, if the bovine reaction was both positive and exceeded the avian reaction by more than 4 mm;
- *A standard inconclusive,* if the bovine reaction was either positive or inconclusive, 1 to 4 mm greater than the avian reaction, and the criteria for a standard reactor were not met;
- *A severe inconclusive* if the bovine reaction was either positive or inconclusive, the avian reaction exceeded the bovine reaction by 2 mm or less, and the criteria for a standard reactor or standard inconclusive were each not met; or
- *Negative,* in all other cases.

2.2 The trial

The trial was conducted in Ireland over a number of months during 2006. Cattle of mixed age, breed and sex were gathered from a wide range of holdings of origin (in excess of 1,300) into a unit, which routinely 'finishes' animals for slaughter, over a period of 1-4 months, as part of a commercial enterprise. The animals being finished for slaughter included cows being culled from the diary industry at the end of their productive milking lives, and beef or dairy/beef cows from suckler enterprises. The heifers, bulls and steers in the study included ones with dairy dams and dairy sires; dairy dams and beef sires, and beef dams and beef sires. A proportion of the animals in this unit, chosen based on convenience, were selected for inclusion in this study. The trial was conducted, with animals being tested in batches shortly before slaughter.

Each study animal was tested using two SICTTs (that is, a control and a trial test), which were administered and read concurrently. Each animal was tested using the tuberculin combination in routine use in Ireland (the control test). In addition, each animal was tested using a trial tuberculin combination (the trial test), selected randomly from a pool of six tuberculin combinations, which included:

- The tuberculin combination currently in use in Ireland;
- Four alternative tuberculin combinations, sourced from three different companies; and
- One further tuberculin combination, equivalent to the control tuberculin combination, apart from the type of dye (Ponceau 4R substituted for Ponceau 2R to comply with EU Regulations on the use of ingredients determined as safe for injection into food producing animals) added to the avian tuberculin.

Each tuberculin in each combination was sourced from a single production batch. The potency of each avian and bovine tuberculin was assessed in TB-sensitised guinea pigs in accordance with annex B to Directive 64/432/EEC, as amended (European Commission, 1964), both by each manufacturer during production, and also by ID Lelystad, as blinded samples prior to the start of the study. The potency of the bovine tuberculin was also assessed in naturally infected tuberculous cattle, as described previously (Haagsma, 1997), by one of the manufacturers during production, and for each bovine tuberculin at the Central Veterinary Research Laboratory, Ireland, prior to the start of the study (Table 1).

A single veterinary practitioner conducted the field aspects of the trial. Prior to the trial, the tuberculin in each combination was decanted into sterile vials of uniform size and shape, then coded using one of two letters (for example, the combination from manufacturer A was coded using either F or M; Table 1). The administering veterinarian was blinded to the identity of the trial tuberculin combinations, and also to the fact that the control and one trial tuberculin combination were identical.

As prescribed in Directive 64/432/EEC (European Commission, 1964), the injection sites for each tuberculin combination were located in the middle third of the neck: avian tuberculin was injected

about 10 cm from the crest of the neck and bovine tuberculin about 12.5cm lower on a line roughly parallel with the line of the shoulder. For logistical reasons, the control and trial tuberculin combinations were each administered on the same side of the neck of each animal: the control tuberculin combination at the border of the anterior and middle third of the neck, and the trial tuberculin combination at the border of the middle and posterior third of the neck. The trial tuberculin combination was administered to animals in sequential order, randomised at study start. An individual McClintock 20-dose syringe was supplied for exclusive use for each tuberculin code. The skin-fold thickness at each injection site was measured using sliding calipers (Pan Veterinary, Co. Kildare, Ireland) with broad jaws designed to distribute an even, manually applied pressure. Measurements rounded up to the nearest millimetre were made at 0 hours, and all responses to tuberculin injection were re-measured and assessed at 72 hrs +/- 4 hrs, as required in the Directive. Results were recorded onto a hand-held computer operating software approved by the Department of Agriculture and Food.

Microbiological and/or histological confirmation of tuberculosis was not conducted as part of this study.

The study was randomised, controlled and double-blinded, and has been reported in accordance with the STARD initiative (Bossuyt et al., 2003).

2.3 Statistical analysis

The results from each trial and control test were compared, using methods suitable for paired data.

Animals were assigned a trial and a control reactor-status, according to the definitions given earlier, and these data were compared using Cohen's kappa (Dohoo et al., 2003). In addition, we used McNemar's test to compare the proportion of animals allocated to each reactor-status, based on trial and control test results. Since, the number of discordant pairs was small (<10), an exact p-value for the

McNemar's test was used (Breslow and Day, 1980, page 165). We accounted for multiple comparisons by reactor-status by applying a Bonferroni adjustment to the alpha value.

For each animal, we recorded the skin increases (in mm) at each bovine and avian site (trial bovine, trial avian, control bovine, control avian). We then calculated the difference between the two paired measurements (for each animal, a trial and a control bovine-avian [B-A] differential). A positive B-A differential indicated that the bovine measurement was greater than the avian measurement. For each animal, we also calculated the difference between the trial and control bovine measurements (bovine difference), the trial and control avian measurements (avian difference), and the trial and control B-A differentials (B-A differential difference). Each of these results was positive if the trial measurement was larger than the control measurement. Each animal was then allocated to a reactor-status category based on the control test result. For each reactor-status within each trial/control test combination, we identified the minimum, median and maximum bovine difference, avian difference and B-A differential difference. These differences were compared, overall and within each trial/control test combination, using the Kruskall-Wallis and Wilcoxon signed-ranks tests, respectively.

3. Results

3.1 The study animals

The SICTT was performed on 2,172 cattle of mixed breeds, including 28 tested twice at an inter-test interval exceeding 60 days. The number of animals tested using each tuberculin and the animal type is presented in Table 2. Cattle from in excess of 1,300 herds were included in the study and none were already known to be infected with *M. bovis*. All cattle had been tested with negative results during the 12-months prior to entering the finishing unit, and at time of entry to the unit none were from herds known to be infected with, or under official control for, tuberculosis.

3.2 The SICTT results

a. Reactor-status

In some animals there were discrepancies in the classification of reactor-status, based on results from the trial and control tests (Table 3; discrepancies highlighted in grey). Generally, a control standard reactor was also considered at least an inconclusive reactor in the trial test. However, one control standard reactor animal was negative in each of three trial tests (F, H and J). Similarly, each of the trial standard reactors were also considered non-negative in the control test, except for 2 standard reactors identified using SICTT F and one using SICTT G. There was moderate, but significant ($p<0.001$), agreement between the results from the control and each trial test, as measured using Cohen's kappa (Table 3).

The percentage of animals in each trial/control test combination that were classified to each reactor-status category, based on trial and control test results, is presented in Table 4. No significant differences were detected (McNemar's test, with a Bonferroni adjusted significance level of 0.0125 to account for the four comparisons made within each control/trial test combination). There was also no significant difference in the level of agreement (measured using Cohen's kappa) between each trial/control test combination, by reactor-status.

b. Skin increase

The median (minimum, maximum) bovine difference, avian difference and bovine-avian differential difference, by reactor-status and trial/control test combination, is presented in Table 5. Among all animals positive to the control test, there was no significant difference in either the bovine (Kruskall-Wallis test: $p = 0.106$) or avian ($p = 0.202$) difference, nor in the bovine-avian differential difference ($p = 0.532$).

Among animals with non-negative results, there was a significant difference between the bovine and avian difference in each trial/control combination, except G/control (bovine difference: $p = 0.536$; avian difference: $p = 0.829$). These differences mainly relate to animals classified as severe inconclusives. There was no significant differences in the B-A differential (with a Bonferroni adjusted significance level of 0.01 to account for the five comparisons made within each control/trial test combination). Among animals with negative results, there were significant differences in the bovine difference (L/control combination), the avian difference (all combinations) and the B-A differential difference (all combinations).

4. Discussion

As part of the Irish programme, all cattle are assigned a reactor-status (of standard reactor, standard inconclusive, severe inconclusive or negative) on the basis of results from each SICTT result. Therefore, the effect of different tuberculin PPD combinations on reactor-status is of particular importance. For each control/trial tuberculin combination, we found good agreement between the control and trial reactor-status in this study (Table 3). Further, the level and pattern of agreement between the control and trial combinations G and L (each using the tuberculin PPD combination currently in use in Ireland) was similar to that observed with each other control/trial combinations. The level of agreement was also similar (kappa: 0.49 to 0.77), and differences almost invariably non-significant, when each category of reactor-status was considered separately (Table 4). Note, however, that the number of animals in some categories may have been too small to detect any difference, if present. Only a limited number of reactors were identified in the study, which reflects the very low animal-level incidence of tuberculosis in Ireland (More and Good, 2006; ~0.4% annually). We could have identified a greater number of reactor animals, but at considerable cost in time and materials.

The study also provided insights into the effect of different tuberculin combinations on skin reactivity to the avian and bovine tuberculin PPD. Among all non-negative animals (standard reactors, standard inconclusives, severe inconclusives), there were no significant differences between the control and

each trial combination in the B-A differential difference (Table 5). The B-A differential (that is, the bovine skin increase minus the avian skin increase) is used to categorise animals into a reactor-status. Therefore, we are confident that similar field results will have been achieved, with each of the tuberculin combinations under investigation. Based on the detailed information presented in Table 5, we can identify some subtle differences in the performance of the different tuberculin combinations. With each of the control/trial combinations, there were significant differences in both the bovine and avian difference (that is, the difference between the trial and control skin increases at the bovine and avian sites, respectively). In most cases, the control (as compared to trial) skin increase was greater, at both the avian and bovine sites. We believe that these differences are the result of site effects, noting that the control and trial tests were conducted at sites on the anterior and posterior neck, respectively. Although it would have been preferable to use equivalent sites on each side of the neck, this was not possible due to concerns relating to access and operator health and safety. Latin-square designs are used in the cattle bio-assays specifically because sensitivity is known to be greater at the anterior compared with the posterior cervical area (E. Costello, pers. comm.). In a practical sense, this study has shown that it is the relative – rather than the absolute – location of the avian and bovine sites that is of greatest importance. Although a location at the border of the middle and anterior third of the neck is recommended (European Commission, 1964), the A-B difference will not significantly alter if sites anterior or posterior to this are chosen. However, to ensure equivalent skin sensitivity at both the avian and bovine sites, it is important that these sites are both located on a line that is parallel to the angle of the shoulder.

The observed differences in skin reactivity to the avian and bovine tuberculin PPD at the control and trial sites were mainly confined to animals categorised as either negative or severe inconclusive (Table 5). However, the measured differences were minor, and as such unlikely to have a significant impact on the actual test outcome, either for individual animals or for herds. In Ireland, herd control would only be initiated following the detection of at least one standard reactor or an animal that had tested standard inconclusive on two consecutive occasions. Some of these discrepancies may have occurred

following the rounding-up of skin measurements, as required in the Directive (European Commission, 1964).

An outlier was identified in the control/G tuberculin combination, with one animal achieving a bovine difference of 84 mm. Based on the control test, the animal was negative, and on the trial test, very strongly a standard reactor. Note that the bovine tuberculin PPD was identical in the control and G tuberculin combinations. This difference is unexplainable beyond postulating that it may have been an inaccurate intradermal injection of bovine tuberculin PPD at the anterior site which serves only to highlight the issue of test repeatability and the necessity for two consecutive tuberculin tests clear before restoring disease-free status to a herd as is required under the Directive.

A number of steps were taken during this study to minimise a range of potential biases. The study was conducted in a commercial fattening unit where cattle of mixed age, breed and sex from throughout Ireland are assembled. These animals will each have been tested using the SICTT at some point during the 12 months preceding their entry into the unit, and it was anticipated that at least some would have been exposed under natural field conditions to *M. bovis* infection prior to acquisition by the enterprise. For logistic reasons, the study animals were selected using convenience sampling; essentially whole batches of cattle shortly before slaughter. We have no reason to believe that the study animals are not representative of the general Irish cattle population. A number of steps were taken to minimise measurement bias. The tuberculin test is a subjective diagnostic test, which can be affected by a range of operator-related factors, including care and accuracy associated with the intradermal injection of tuberculin and the measurement of the skin response. Significant inter-operator variability has been observed previously. Further, Wahlström (2004) reported that the measured thickness of a 'standard' skin fold was a subjective measurement personally set by each veterinarian. As long as the veterinarian is consistent, such differences should not affect test accuracy. A single veterinary practitioner conducted all field aspects of this study specifically to minimise the potential for measurement bias. In compliance with international norms (Bossuyt et al., 2003), the study was randomised and controlled. Further, the field veterinary practitioner and ID Lelystad were blinded to

306 the identity of the trial tuberculin combinations and the tuberculin PPDs, respectively. The practitioner
307 was also not aware that the control and one trial tuberculin combination were identical. Although the
308 study was conducted over a period of 8 months, we do not believe that time of year will have
309 adversely influenced the SICTT results. As part of the national TB eradication programme, the SICTT
310 is routinely conducted in Ireland throughout the year. When comparing the rate of lesion disclosure
311 among cattle with varying SICTT responses, Towey and O'Keeffe (1996) found some evidence of
312 seasonal differences in multiple animal breakdown herds, but not in single animal breakdown herds.
313 Any temporal effect of skin reactivity is believed to be related to a seasonal risk in exposure rather
314 than seasonal changes in immune response (Martin et al., 2001).
315
316 In this study, the potency estimates from the guinea pig bio-assay were imprecise. Assay repeatability
317 is in part due to the inherent variability of tuberculin PPD. Bovine tuberculin PPD has been described
318 as a poorly defined, complex mixture containing more than 100 individual components in various
319 stages of denaturation (Pollock *et al.*, 2001), and is known to vary widely both in protein content and
320 antigenic profile (Tameni et al., 1998). This may explain, at least in part, the variation in estimates of
321 the potency of the ID Lelystad bovine tuberculin PPD that were obtained in this facility during
322 production and in association with the trial (Table 1). However, our results also point to substantial
323 imprecision in the guinea pig bio-assay, for reasons unrelated to the material under evaluation. Widely
324 varying potency estimates (14,950 and 32,180 IU; Table 1) were obtained from duplicate PPD samples
325 of ID Lelystad bovine tuberculin PPD tested in the same laboratory at the same time. In addition, we
326 also found limited agreement between the guinea pig and cattle bio-assays. Using the above-
327 mentioned tuberculin PPD, a potency of 45,003 IU was estimated in the cattle bio-assay. Similar
328 concerns about these bio-assays have been expressed previously (Dobbelaer et al., 1983; Bakker et al.,
329 2005), and it is acknowledged that biological variation is a feature of *in vivo* models. In recognition of
330 this problem, relevant regulations require the fiducial limits of error (P=0.95) to be not less than 50%
331 and not more then 200% of the estimated potency, and the estimated potency not less than 75% and
332 not more than 133%, and not less than 66% and not more than 150%, of the stated potency of 20,000
333 IU/ml for avian and bovine tuberculin, respectively (European Commission, 1964). To reduce

experimentally induced skin reactions, which can interfere with the bio-assay, Cobb et al. (2001) propose the use of hairless guinea pigs. As a quality control measure on a number of occasions annually Ireland routinely assays the potency of a selection of the normal tuberculin supplied for use in bovines naturally infected with *M. bovis*. The requirement to check potency in the bovine bio-assay was necessitated in the original Directive 64/432/EEC (European Commission, 1964) and has also previously been recommended in WHO technical reports (including World Health Organization, 1987). However, there is considerable expense and logistic effort associated with routine use of this assay in sourcing, holding and handling a sufficient number of artificially or naturally infected bovine animals. The requirement was initially modified and made the responsibility of designated community laboratories and later removed when Annex B of Directive 64/432/EEC was updated in 2002 (European Commission, 2002) and is thus now rarely conducted. Moreover, repeated use of the guinea-pig bio-assay, for essentially the same product batch during the manufacturing or licensing process does not appear to be justified, given the above-mentioned problems of assay imprecision.

5. Conclusion

Despite the limited nature of this study, it provides some reassurance to Irish policy-makers. In the event of a change in supply, further studies to determine the sensitivity and specificity of alternative tuberculin PPDs in the Irish environment would undoubtedly be needed. However, it would appear that there should be minimal disruption of the national programme if it were necessary to use alternative tuberculin PPDs that comply with WHO, OIE and EU Regulations. The effect of differing potency combinations (avian/bovine) in the detection of actual infected cattle should be assessed. Further, we advise the ongoing use of the bovine bio-assay as a quality check on bovine tuberculin PPD supply remains advisable.

6. Acknowledgements

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7. Conflict of Interest Statement

The authors have no conflict of interest.

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438 Table 1. The source and potency of the avian and bovine tuberculin purified protein derivative (PPD)
439 in each tuberculin combination
440

Tuberculin combination	Manufacturer	Potency (mean IU) of the:						
		Avian tuberculin PPD			Bovine tuberculin PPD			
		Guinea pig		Cattle	Guinea Pig		Cattle	
		Prod.[a]	Trial[b]	Prod.[a]	Prod.[a]	Trial[b]	Prod.[a]	Trial[c]
F(M)	A	25,000	16,500	nd	27,812	13,980	nd	25,900
G(R)[d,f]	ID Lelystad	nd	27,750	nd	26,070	32,180	nd	45,003
H(T)	B	38,250	31,500	19,800	19,180	24,500	nd	33,868
J(N)	C	14,175	10,250	nd	28,350	5,850	nd	11,552
K(S)	B	19,500	9,250	nd	11,200	22,750	36,550	28,747
L(P)[e,f]	ID Lelystad	21,780	24,500	nd	26,070	14,950	nd	45,003

a. As assessed by the manufacturer

b. As assessed by ID Lelystad, using blinded samples prior to the start of the study

c. As assessed by the Central Veterinary Research Laboratory in Ireland, prior to the start of the study

d. Identical to the control tuberculin combination, except Ponceau 4R substituted for Ponceau 2R in the avian tuberculin PPD

e. Identical to the control tuberculin combination

f. The bovine tuberculin PPD in tuberculin combinations G(R) and L(P) was identical. Therefore, only a single potency estimate is available from the manufacturer's guinea pig model. Further potency estimates, using the guinea pig model, were conducted using duplicate samples of the bovine tuberculin PPD; each result was then randomly allocated to one of the two tuberculin combinations. The potency of the bovine tuberculin PPD was only assessed on a

single occasion using the bovine model.

nd = not done

441

442 Table 2. Number of animals tested, by trial test and sex. All animals were tested using both a trial and
443 control test
444

Trial test	Number of animals			
	Total	Females		Males
		Heifers	Cows	
F	399	85	63	251
G	333	131	42	160
H	407	99	43	265
J	276	89	34	153
K	393	93	-	300
L	392	166	22	204
Total	2,172	663	204	1,305

445

446 Table 3. Comparison of animal reactor-status, based on control and trial test results

447

Trial test and reactor status, based on these results		Reactor-status, based on results from the control test				Total	Cohen's Kappa (95% C.I.)	P-value[c]
		Negative	Severe inconc.[a]	Standard inconc.[b]	Standard reactor			
F	Negative	342	11		1	354		
	Severe inconc.[a]	17	12			29		
	Standard inconc.[b]	1	4	3	2	10		
	Standard reactor	2		1	3	6		
	Total	362	27	4	6	399	0.48 (0.36 – 0.61)	<0.001
G	Negative	305	5			310		
	Severe inconc.[a]	5	7			12		
	Standard inconc.[b]	1	1		2	4		
	Standard reactor	1	1	1	4	7		
	Total	312	14	1	6	333	0.59 (0.44 – 0.75)	<0.001
H	Negative	359	14		1	374		
	Severe inconc.[a]	7	17		1	25		
	Standard inconc.[b]		3	2		5		
	Standard reactor			1	2	3		
	Total	366	34	3	4	407	0.61 (0.48 – 0.73)	<0.001

J	Negative	239	7		1	247		
	Severe inconc.[a]	6	10			16		
	Standard inconc.[b]	3	4	2		9		
	Standard reactor		1	1	2	4		
	Total	248	22	3	3	276	0.56 (0.42 – 0.71)	<0.001
K	Negative	352	13			365		
	Severe inconc.[a]	9	9			18		
	Standard inconc.[b]	3	2		2	7		
	Standard reactor				3	3		
	Total	364	24	0	5	393	0.46 (0.31 – 0.61)	<0.001
L	Negative	337	9			346		
	Severe inconc.[a]	16	13			29		
	Standard inconc.[b]	2	5	5	1	13		
	Standard reactor		1	1	2	4		
	Total	355	28	6	3	392	0.54 (0.42 – 0.66)	<0.001

a. Standard inconclusive result

b. Severe inconclusive result

c. Significance test of the level of agreement between the control and respective trial SICTT

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449

450

451 Table 4. The percentage of animals in each control/trial test combination that were classified to each reactor-status category, based on control and trial test

452 results

453

Reactor-status[a]		Control/trial test combination					
		Control/F	Control/G	Control/H	Control/J	Control/K	Control/L
All non-negative results[b]	Control % +ve	9.3	6.3	10.1	10.1	7.4	9.4
	Trial % +ve	11.3	6.9	8.1	10.5	7.1	11.7
	P-value[c]	0.215	0.774	0.134	1.000	1.000	0.122
	Kappa	0.57	0.71	0.67	0.67	0.53	0.64
	(95% C.I.)	(0.43, 0.70)	(0.55, 0.86)	(0.55, 0.80)	(0.52, 0.81)	(0.36, 0.69)	(0.51, 0.76)
Standard reactors	Control % +ve	1.5	1.8	1.0	1.1	1.3	0.8
	Trial % +ve	1.5	2.1	0.7	1.5	0.8	1.0
	P-value	1.000	1.000	1.000	1.000	0.500	1.000
	Kappa	0.49	0.61	0.57	0.57	0.75	0.57
	(95% C.I.)	(0.14, 0.84)	(0.29, 0.92)	(0.13, 1.00)	(0.12, 1.00)	(0.41, 1.00)	(0.13, 1.00)

Standard inconclusives	Control % +ve	1.0	0.3	0.7	1.1	0	1.5
	Trial % +ve	2.5	1.2	1.2	3.3	1.8	3.3
	P-value	0.070	0.375	0.625	0.070	0.016	0.039
	Kappa	0.71	0.77	0.71	0.54	0.66	0.68
	(95% C.I.)	(0.51, 0.91)	(0.56, 0.99)	(0.44, 0.98)	(0.27, 0.82)	(0.38, 0.94)	(0.48, 0.89)
Severe inconclusives	Control % +ve	6.8	4.2	8.4	8.0	6.1	7.1
	Trial % +ve	7.3	3.6	6.1	5.8	4.6	7.4
	P-value	0.860	0.774	0.108	0.238	0.307	0.858
	Kappa	0.57	0.71	0.68	0.68	0.53	0.64
	(95% C.I.)	(0.44, 0.71)	(0.55, 0.86)	(0.56, 0.81)	(0.54, 0.83)	(0.36, 0.69)	(0.51, 0.76)

a. The reactor-status is based on the results from the control SICTT

b. Standard reactors, standard and severe inconclusives

c. The significance of the measurement differences was tested using McNemar's test

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454 Table 5. The median (minimum, maximum) bovine difference, avian difference and bovine-avian differential difference, by reactor-status and trial/control test
455 combination
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Reactor-status[a]	Median value (minimum, maximum)					
	F/control combination	G/control combination	H/control combination	J/Control combination	K/control combination	L/control combination
All non-negative results[b]						
Number of animals	37	21	41	28	29	37
Bovine difference[c]	-1 (-69, 3)**[d]	0 (-8, 84)	-1 (-9, 2)**	0 (-4, 5)*[d]	-1 (-11, 5)**	-1 (-13, 4)**
Avian difference[e]	-1 (-8, 4)**	0 (-4, 6)	-1 (-4, 11)**	-2 (-4, 9)**	-1 (-5, 3)**	-1 (-9, 4)**
B-A differential difference[f]	0 (-72, 4)	0 (-7, 85)	0 (-11, 5)	0 (-9, 8)	0 (-13, 8)	0 (-9, 9)
Standard reactors						
Number of animals	6	6	4	3	5	3
Bovine difference	-3 (-69, 1)	-0.5 (-8, 32)	-1.5 (-3, 1)	0 (-4, 0)	-4 (-11, 5)	-2 (-13, 1)
Avian difference	1 (-4, 3)	0.5 (-1, 6)	2 (-3, 11)	-2 (-2, 9)	-2 (-5, 3)	-2 (-4, 0)
B-A differential difference	-2 (-72, -1)*	-0.5 (-7, 26)	-4 (-11, 2)	-2 (-9, 2)	-3 (-13, 8)	-2 (-9, 3)

Standard inconclusive reactors						
Number of animals	4	1	3	3	0	6
Bovine difference	-0.5 (-4, 2)	5 (5, 5)	0 (-1, 0)	0 (-1, 2)	-	0 (-1, 3)
Avian difference	-2 (-3, -1)	-1 (-1, -1)	1 (-1, 1)	-2 (-2, 0)	-	-0.5 (-2, 3)
B-A differential difference	1.5 (-1, 3)	6 (6, 6)	-1 (-2, 1)	1 (0, 4)	-	0.5 (-2, 4)
Severe inconclusive reactors						
Number of animals	27	14	34	22	24	28
Bovine difference	-1 (-8, 3)**	0 (-3, 84)	-1 (-9, 2)**	0 (-3, 5)	-1 (-4, 1)**	-1 (-4, 4)**
Avian difference	-1 (-8, 4)**	-0.5 (-4, 3)	-1 (-4, 1)**	-1.5 (-4, 1)**	-1 (-4, 2)**	-1 (-9, 4)**
B-A differential difference	0 (-4, 4)	0 (-3, 85)	0 (-6, 5)	0 (-1, 8)	0 (-3, 4)	0 (-4, 9)
All negative results						
Number of animals	362	312	366	248	364	355
Bovine difference	0 (-6, 8)	0 (-3, 6)	0 (-3, 4)	0 (-4, 8)	0 (-3, 6)	0 (-5, 6)**
Avian difference	0 (-13, 6)**	0 (-10, 33)**	0 (-9, 6)**	0 (-6, 8)**	0 (-8, 11)**	0 (-23, 5)**
B-A differential difference	0 (-6, 16)**	0 (-33, 8)**	0 (-6, 8)**	0 (-8, 6)**	0 (-11, 8)**	0 (-5, 23)**

a. The reactor-status is based on the results from the control SICTT

b. Standard reactors, standard and severe inconclusive reactors

c. The difference in skin measurement (in mm; if positive, trial is larger) at the trial and control bovine sites

d. The significance of the measurement differences was tested using a Wilcoxon signed-rank test.(* $p \leq 0.05$;** $p \leq 0.01$)

e. The difference in skin measurement (in mm; if positive, trial is larger) at the trial and control avian sites

f. The difference (in mm; if positive, trial is larger) between the trial and control bovine-avian differential

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