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Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity

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ABSTRACT

Bovine tuberculosis (BTB) is a major animal health problem with zoonotic implications. Current control programs are based on test and slaughter strategies utilizing skin tests with tuberculins as antigens. The low specificity and associated operative difficulties of these tests have driven the search for new antigens and diagnostic assays. In this multicenter study, using herds from Argentina, Mexico and Northern Ireland, we selected skin test positive and negative animals from herds with different prevalence's of BTB and compared tuberculin (PPDB) and ESAT-6 + CFP10 as antigens *ex vivo*. In low prevalence herds, crossreactivity of PPDB was apparent since up to 60% of the PPDB skin test and *ex vivo* positive animals did not responded to ESAT-6 + CFP10 *ex vivo*. The superior specificity of ESAT-6 + CFP10 was confirmed in a *Mycobacterium avium* sp. *paratuberculosis* infected herd where several of the animals had strong crossreactivity to PPDB and PPDA but not to ESAT-6 + CFP10. In high prevalence herds 85% of the skin test-positive animals, were confirmed *ex vivo* using either PPDB or ESAT-6 + CFP10 as antigen. However, within this group 60% of the skin test negative animals were PPDB and ESAT-6 + CFP10 positive *ex vivo* indicating that the skin test can in some herds yield a significant number of false negative results. In conclusion, the *ex vivo* test is recommended as an ancillary test to accelerate BTB eradication. In high prevalence herds, PPDB or ESAT-6 + CFP10 can be used as antigen whereas in low and medium prevalence herds ESAT-6 + CFP10 is the preferred choice.

1. Introduction

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* (*M. bovis*) represents a significant economical issue and an important health problem and is ranked among the top seven zoonotic threats to humans by WHO (WHO, 2005). Bovines are the principal maintenance hosts among domestic animals (O'Reilly and Daborn, 1995). The disease presents a major trade barrier for animal-related products and livestock production, causing significant losses in rural

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Table 1
Summary of the background for herds enrolled in this study.

Farm country	Herd type	Expected prevalence[a]	Herd size	Animals tested[b]	Animals positive by	
					PCR[c]	Lesions[c]
Alejo Ledesma[d] Argentina	Dairy	High	600	14 (2.3)	6/14	14/14
Morteros[d] Argentina	Dairy	High	500	40 (8)	25/40	ND
Ezeiza Argentina	Dairy	Low	40	23 (57)	12/23	ND
V. Casares Argentina	Dairy/breeding	Low	60	29 (48)	19/29	ND
Bavio[d] Argentina	Dairy	High	50	24 (48)	16/24	6/11
Saliqueló Argentina	Beef	Low	500	32 (6.4)	23/32	ND
Lincoln Argentina	Dairy	High	250	32 (12.8)	13/28	ND
Quemú-Quemú Argentina	Beef	Medium	600	32 (5.3)	18/32	ND
La Paz Argentina	Dairy	Medium	500	32 (5.3)	14/30	ND
Zapotlanejo[d] Mexico	Dairy	Low	74	74 (100)	20/39	15/20
Zapotlanejo Mexico	Dairy	Medium	60	60(100)	15/40	17/20
Zapotlanejo Mexico[d]	Dairy	Medium	53	53 (100)	10/20	14/20
Zapotlanejo Mexico	Dairy	High	137	63 (46)	15/29	14/20
Armagh N. Ireland	Dairy	Low	200	74 (37)	ND	ND
Tyrone N. Ireland	Dairy	Low	400	96 (24)	ND	ND
Antrim N. Ireland	Dairy	Low	150	97 (65)	ND	ND
Rafaela Argentina	Dairy	None	600	33 (5.5)	0	ND
Florida Uruguay	Dairy and beef	None	580	20 (3.4)	0	ND
Mérida Mexico	Dairy	None	1300	33 (2.5)	0	ND
Down N. Ireland	Dairy	None	75	45 (60)	ND	ND
Topilejo Mexico[e]	Dairy	None	82	42 (51)	ND	ND

[a] High >5%, medium 1–5% and low 0–1%. None indicate that these are negative control herds free of bovine TB.
[b] Number of animals tested and the percentage of the total herd in parentheses.
[c] *x*/*y* means *y* animals were tested and *x* were positive.
[d] Indicate farms were animals were sacrificed, necropsied and found as true TB infected in previous work (data not shown).
[e] Herd infected by *M. avium* subsp. *paratuberculosis*.

economies worldwide (EEC, 1964). The zoonotic implications of BTB are especially serious in developing regions (Cosivi et al., 1998) because of the high incidence (de Kantor and Ritacco, 1994) but epidemiological studies show that *M. bovis* infection in cattle is increasing in several developed countries (Krebs, 1997).

In spite of widespread control programmes, based on a test and slaughter strategy, BTB remains biologically present, even in countries officially considered free of the disease, and it appears very difficult to eradicate. The diagnostic assay employed, known as intradermal or skin test, uses purified protein derivative (PPD) tuberculins, and is generally accepted as evidence of infection (Abernethy et al., 2006). The antigen is prepared by precipitation of heat-killed cultures of mycobacteria and consists of an ill-defined mixture of proteins (Paterson, 1948). This can explain the low specificity of the test and the variations in specificity and sensitivity from batch-to-batch. Another drawback of the skin test is that each animal needs to be restrained twice, first for the application of the test, and second, for the reading of the results.

In view of the need to improve BTB diagnostic methods, the *ex vivo* gamma interferon (IFN-γ) assay appears as an interesting option. It has shown promise to detect various infectious disease conditions in cattle (Weynants et al., 1995; DeBey et al., 1996; Barbuddhe et al., 1998; Lunden et al., 1998; Kalis et al., 2003; Parida et al., 2006; Faldyna et al., 2007) and when applied to BTB using PPD from *M. bovis* (PPDB) as antigen the *ex vivo* assay could detect infections earlier than the skin test (Rothel et al., 1990; Buddle et al., 1995). However, since cattle are likely to be exposed to environmental mycobacteria following birth, the level of IFN-γ responses in non-infected animals can be quite high because of cross-reactions to PPDB resulting in lower specificities. For this reason a large number of purified or synthetic *M. bovis* proteins have been tested for antigenicity and specificity to be potentially applied in this test (Rhodes et al., 2000; Cockle et al., 2002; Mustafa et al., 2002; Aagaard et al., 2003). These studies have identified ESAT-6 and CFP10 as the most promising candidates. Several studies in humans (Pai et al., 2004; Menzies et al., 2007) suggest that the IFN-γ assays based on either of these antigens have higher specificity than the PPD based skin test, and is unaffected by previous Bacillus Calmette-Guérin (BCG) vaccination (Brock et al., 2001; Mori et al., 2004; Harada et al., 2006). The assay has been approved for human diagnosis and is commercially available in a whole blood assay format (QuantiFERON TB GOLD®, Cellestis Ltd., Melbourne, Australia). In addition, their use in cattle has been approved in several countries, as an ancillary tool to skin testing, and has proved efficient in the identification of tuberculous cattle (de la Rua-Domenech et al., 2006).

In a previous study based on a panel of antigens with higher sensitivity and specificity than PPDB we found that the combination of ESAT-6 and CFP10 yielded the best results among the tested combinations (Aagaard et al., 2006). In the present three-center study we increased the number of animals and compared immune responses against ESAT-6 + CFP10 and PPDB in blood samples from skin test negative and positive animals from herds with different BTB prevalence's. Furthermore, we investigated how the infection with *M. avium* subsp. *paratuberculosis*

(MAP) affected the diagnostic performance of the antigens.

2. Materials and methods

2.1. Selection of study herds and animals

A total number of 948 cattle from 21 herds in Argentina, Mexico and Northern Ireland (Table 1), were included in this study. Prior to study animals were skin tested either by the single intradermal comparative cervical skin test or the single caudal fold test, in accordance with official national guidelines for each country. This does introduce a variable in the comparison of results between countries. However, it was the only way the project was practically feasible since veterinarians are trained in the procedure used in their own country. The term skin test positive used in the manuscript refers to the test results performed by the local authorities and used to legally condemn animals if positive. In Northern Ireland, animals were considered positive if the single intradermal cervical comparative test showed a PPDB bias (compared to PPDA) greater than 4 mm, in Mexico the PPDB bias had to be greater than 4 mm, and in Argentina animals were considered positive if the caudal fold skin test with PPDB alone showed an increase larger than 5 mm in skin thickness. 775 animals were from herds with a recent history of bovine tuberculosis and were grouped per country, and assigned to one out of 3 prevalence groups: high (>5%), medium (1–5%) or low (<1%), based on historical incidence of BTB (Table 1). Herd prevalence was defined as the percentage of animals that had been positively diagnosed with bovine tuberculosis by PPD-based skin testing at the most recent herd skin-test control. The remaining 173 animals were considered negative for BTB according to two inclusion criteria: (i) they belonged to herds with no history of tuberculosis during the previous 5 years, or (ii) they had a minimum record of two consecutive negative PPD skin tests (Table 1). The negative control animals from Argentina ($n = 33$) and Mexico ($n = 42$) were from herds maintained at university or research institute facilities. These herds were under strict biosafety control measures to ensure freedom from a range of infectious diseases, including BTB and paratuberculosis. Due to difficulties in finding herds officially free of BTB in Argentina, an additional 20 negative control animals cattle were selected from a herd in a region of Uruguay where BTB is absent. Negative animals in the Northern Ireland control group ($n = 44$) came from farms with no history of BTB within the previous 5 years. Additional blood samples from a second Mexican control group ($n = 42$) were included. These samples were negative to BTB (by skin test), but in contrast to the other control herds, this herd tested positive for MAP using a commercial ELISA assay (Parachek, Pfizer), and some animals presented clinical signs of paratuberculosis.

2.2. Infection status

All herds selected for this study were located on private premises and postmortem data were not always available. Confirmation of disease status in skin test positive animals (Table 1) was achieved by necropsy followed by observation of lesions and culture of *Mycobacterium bovis*, or by *M. bovis* diagnostic PCR in nasal swabs (Zumarraga et al., 2005). *Mycobacterium bovis* infection was confirmed by PCR from nasal swabs of 205 animals and 80 animals were confirmed infected by observation of lesions in postmortem examinations. For the remaining animals the skin test result, based on the bovine and avian PPDs, along with official BTB status, was taken as an indicator of true infection status. In Mexico and Argentina the animals tested was undergoing a sanitation program.

2.3. IFN-γ whole blood test and antigens

IFN-γ release tests were performed on whole blood from 14 to 74 animals per herd from BTB-positive or negative herd in Argentina and Mexico. From herds under a BTB sanitation program all skin test positive animals were bleed if possible or selected at random. Depending on weather and operative conditions IFN-γ release was measured 27–32 days after skin testing. PPDB, PPDA (PPDs prepared from *Mycobacterium bovis* and *Mycobacterium avium*, respectively) or ESAT-6 + CFP10 was used as antigens (Aagaard et al., 2006). Analysis was done 8–12 h after blood collection. Recombinant forms of ESAT-6 and CFP10 were prepared in an *Escherichia coli* expression system and purified as previously described (Aagaard et al., 2006). For each of the individual recombinant antigens, a final concentration of 2 μg/ml was used, giving a combined total antigen concentration of 4 μg/ml for the ESAT-6 + CFP10 mixture. PPD's provided with the Bovigam® kit, was used at a final concentration of 20 μg/ml. Each antigen was titrated to determine its optimal concentration in the IFN-γ assay using blood from *M. bovis* experimentally infected animals, (data not shown). Negative control wells, to which only PBS was added, were included for each animal tested, as well as positive controls containing 1 μg/ml pokeweed mitogen (Sigma–Aldrich, UK), Only blood samples that yielded an ODI > 2.0 upon incubation with pokeweed mitogen were given for further consideration. Results were expressed as optical density (OD450) indexes (ODI = optical density in antigen-stimulated wells/optical density in PBS-incubated wells). To get comparable results the same protocol was followed at the three test sites and the same batches of antigens, buffers, kits and plates were distributed to all groups. Animal experimentation, for the purpose of antigen titration, was performed in compliance with Queen's University Belfast Research Ethics Committee.

2.4. Statistical analysis

For a general statistical description of the non-Gaussian ODI data, median values and 5th and 95th percentiles were used. To set antigen-specific cut-off values, ODIs were $\log_{10}$ transformed and 95% confidence intervals (CI) established from the negative control groups, for each antigen pool. The 95% CI values were used as cut-off values to interpret data from infected herds. Using the $\log_{10}$ transformed data, statistical differences between mean values in inter- and intra-group comparisons were determined by the Bonferroni multiple comparison test after analysis of variance (one-way ANOVA) had identified significant differences in

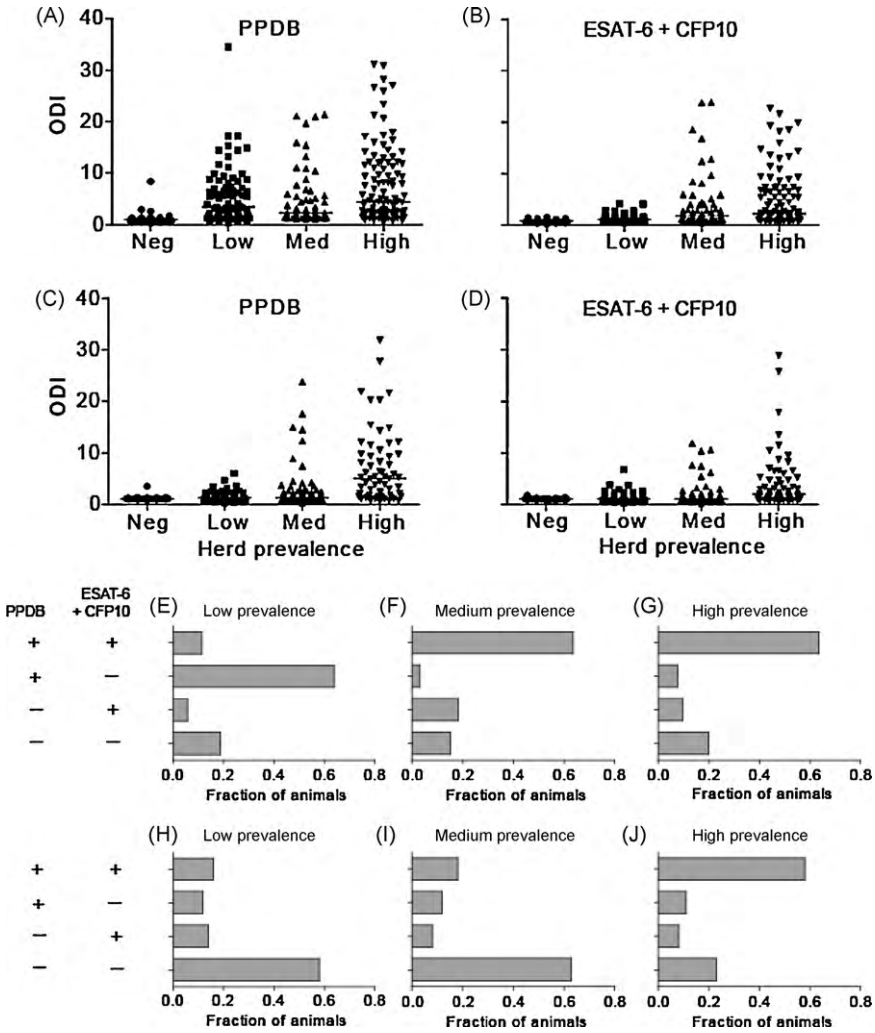


Fig. 1. Antigen recognition in PPD skin test positive animals from herds with different BTB prevalence rates. Blood samples from Argentinean (A and B) and Mexican (C and D) were tested for recognition of PPDB (A and C) and ESAT-6 + CFP10 (B and D). Results are presented as ODIs (sample OD/PBS control OD) and median values are indicated with a line. The antigen recognition pattern was investigated in detail for low (E and H), medium (F and I) and high (G and J) prevalence herds. +/− indicate a positive or negative response after stimulation with an antigen. Cut-off values were calculated separately for each antigen based upon 95% CI's for logarithmic transformed data from bovine tuberculosis free herds.

Table 2
Response distribution and percentage of *in vitro* assay confirmed BTB skin test positive animals in herds from Argentina and Mexico in herds of low, medium and high TB prevalences and control herds.

Country	Expected prevalence	*n*	PPDB[a]		PPDB-A[a]		ESAT-6 + CFP10[a]		Percent confirmed[b]	
			Median	5–95th percentile	Median	5–95th percentile	Median	5–95th percentile	PPDB (2.5)	ESAT-6 + CFP10 (1.5)
Argentina	Low	84	3.5	0.7–15.2	1.97	−0.02 to 11.9	1.1	0.7–2.8	59.5	15.5
	Medium	64	2.3	1.1–20.7	0.76	−0.9 to 18.5	1.8	0.8–18.3	46.0	51.6
	High	110	4.4	1.0–26.2	1.82	−0.4 to 12.0	2.3	0.7–18.4	67.3	66.1
	Control	53	1	0.5–2.5	−0.03	−0.72 to 0.3	0.8	0.4–1.5	–	–
Mexico	Low	74	1.3	0.5–3.5	−0.13	−2.06 to 0.69	1.1	0.4–3.1	6.8	5.4
	Medium	113	1.3	0.7–9.9	−0.11	−1.13 to 6.64	1.0	0.6–6.6	17.2	16.6
	High	63	5.0	0.7–21.8	2.10	−0.82 to 15.4	2.0	0.6–17.0	67.8	66.7
	Control	33	1.1	0.9–2.0	−0.11	−1.77 to 0.10	1.1	0.8–1.5	–	–

[a] Whole blood IFN-γ assay. Cut-off values for specificity and *in vitro* confirmation was calculated after $\log_{10}$ transformation of the readings from the BTB negative herds and was identical to the upper limit for the 95% confidence interval. It was calculated by the mean + 1.96 × the standard deviation.

[b] Calculated cut-off values are given in brackets as ODIs and is based on the combination of the two negative control groups (n = 86 in total). The specificity of the assay was 94.3% and 100% for PPDB and ESAT6 + CFP10 respectively in the Argentinean samples and 98.7 for both antigens in the Mexican samples.

data distribution among the groups. P values <0.05 were taken as significant (Cleveland, 1984).

3. Results

3.1. Ex vivo assay in skin test positive animals from low, medium, and high prevalence herds

Based upon previous results in cattle and humans, a combination of two recombinant *M. bovis* proteins – ESAT-6 and CFP10 – was selected for comparison with PPDB as a diagnostic marker for tuberculosis in cattle. Blood drawn from animals coming from low, medium or high prevalence herds in Argentina and Mexico (Table 1) was used to detect IFN-γ release as a marker for a cellular immune response. Comparison of the results between the two countries revealed some differences despite using the same protocol and reagent batches. First we analyzed samples coming from animals that had all been scored as skin test positive (Fig. 1). In the Argentinean population a large number of samples from low prevalence herds gave strong responses to PPDB and the strength of the response, reflected in the ODI range, was similar to what we found for the samples from the medium prevalence herds. The high responders in the low prevalence group also responded strongly to PPDA which might suggest some kind of crossreaction. In contrast, stimulation with the ESAT-6 + CFP10 proteins resulted in minimal responses in the low prevalence group and these were statistically significantly different from the PPDB responses ($p < 0.01$). For ESAT-6 + CFP10 there was a progressive increase in both responder frequency and strength going from low to high prevalence herds (Fig. 1B, Table 2) whereas that was only observed with PPDB in the medium and high prevalence groups. In the Mexican samples the frequency of strong responders was lower in all three prevalence groups for PPDB and ESAT-6 + CFP10 than in the Argentinean samples (Table 2). For both antigens there was a clear correlation between responder frequency and prevalence grouping and the response range also increased with prevalence grouping (Fig. 1C and D and Table 2).

For a more detailed comparison of the data we calculated specificities and the percentage of animals that were identified as positive in the *ex vivo* test (Table 2). To partly compensate for PPDB's sensitivity to environmental mycobacteria exposure cut-off values were determined separately for each antigen based on 95% confidence intervals (CI's) calculated after logarithmic transformation of the ODI results from the control animals ($n = 86$). Based on these criteria the calculated specificities were between 94.3% and 100% (Table 2, footnote). None of the control animals were positive against both antigens and only one animal in each country was strongly positive, in both cases after PPDB stimulation (Fig. 1A and C). The discrepancy between stimulation with PPDB and ESAT-6 + CFP10 was again evident in animals from Argentinean low prevalence herds. The positive skin test was supported by PPDB stimulation in almost 60% of the samples whereas stimulation with ESAT-6 + CFP10 only confirmed 15.5% of the samples (Table 2). In samples from medium and high prevalence herds the results were much more alike for the two antigens and the skin test positive status was confirmed in 46% and 52% of the samples in the medium prevalence group and for 66–67% of the samples by PPDB and ESAT-6 + CFP10 *ex vivo* (Table 2). In the Mexican low prevalence herds the percentage of *ex vivo* positive animals was very low for both antigens (6.8% and 5.4%). In the medium prevalence group the percentage of confirmed cases was higher for both antigens (17.2% and 16.6%) but still approximately 3-fold lower than the corresponding group in Argentina. In the high prevalence group both antigens again gave very similar results (67.8% and 66.7%) which were almost identical to the Argentinean high prevalence group (Table 2).

Comparison of the individual animals' recognition patterns of PPDB and ESAT-6 + CFP10 clearly illustrates some major differences among PPDB skin test, prevalence grouping and country (Fig. 1E–J). For all three prevalence groups there was a relatively large fraction of animals that did not respond to any of the two antigens (−,−) despite being PPDB skin test positive. In the Argentinean samples the percentage of non-responders was almost 20% regardless of the prevalence grouping (Fig. 1E–G) whereas in Mexico it was around 60% in the low and medium prevalence groups (Fig. 1H and I). For the medium and high prevalence groups in Argentina more than 60% of the animals were positive to both PPDB and ESAT-6 + CFP10 *ex vivo*. In the low prevalence group more than 60% responded to PPDB but not to ESAT-6 + CFP10. In Mexico it was only in the high prevalence group that almost 60% of the animals were confirmed by both antigens *ex vivo* whereas in the medium and low prevalence groups only 17–19% responded to both antigens. For the low prevalence group the level is quite similar to what we found in the corresponding Argentinean group (14%).

3.2. IFN-γ responses in tuberculin PPDB skin test negative animals from tuberculosis herds

From each of the Argentinean BTB infected herds we also drew blood from PPDB skin test negative animals and tested for reactivity towards PPDB and ESAT-6 + CFP10 (Fig. 2 A and B). For both antigens the overall responses were as expected lower than in the skin test positive animals and there was a correlation between responder frequency and prevalence group (Fig. 2A and B). Samples from the low prevalence herds all ($n = 15$) tested negative with both PPDB and ESAT-6 + CFP10, confirming the animals skin test negative status (Fig. 2A and C) and suggesting that prior skin testing did not sensitize the animals to a degree that influenced IFN-g readout one month later. In the medium prevalence group ($n = 29$), 21 (72%) of the animals were negative against both antigens (−,−), 4 (14%) were positive against both (+,+), 3 (10%) were positive against PPDB but not against ESAT-6 + CFP10 (+,−), and 1 (3.5%) was only positive after ESAT-6 + CFP10 stimulation (−,+). In animals from high prevalence herds ($n = 17$) 10 (59%) of the animals were positive against both antigens (+,+), none of the animals responded only to PPDB (+,−), 2 (12%) responded singularly to ESAT-6 + CFP10 and only 5 (29%) of the animals were negative to both antigens (−,−). Despite all animals were skin tests negative there was a decrease in the frequency of animals being non-responders

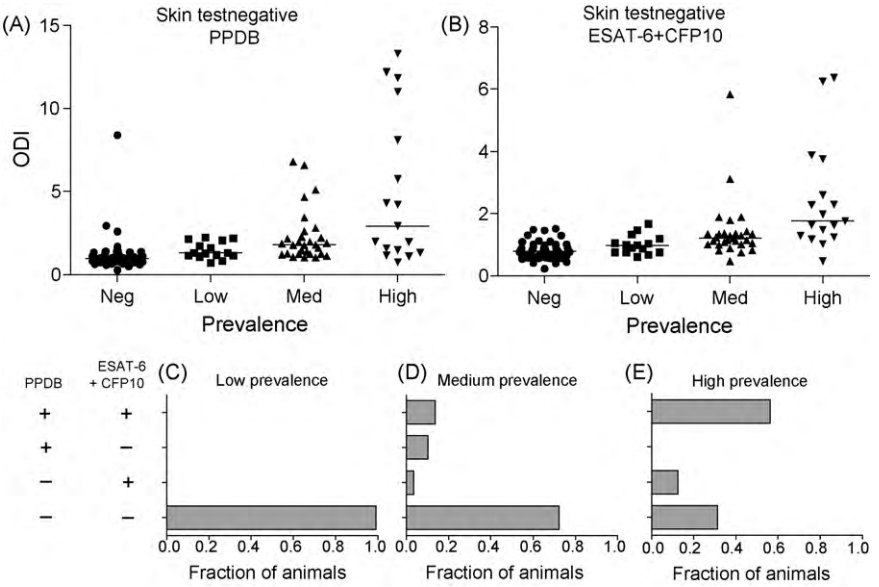


Fig. 2. Recognition of antigens in skin test negative animals. PPDB (A) and ESAT-6 + CFP10 (B) were tested for T cell recognition in blood samples from Argentinean skin test negative animals from herds with low, medium and high prevalence of bovine tuberculosis. Results are given as ODIs and the median values for each group are indicated with a line. For each of the low (C), medium (D) and high (E) prevalence groups, the recognition of PPDB and ESAT-6 + CFP10 was compared. +/− indicate a positive or negative response after stimulation with an antigen. Cut-off values used for positivity were identical to those in Fig. 1.

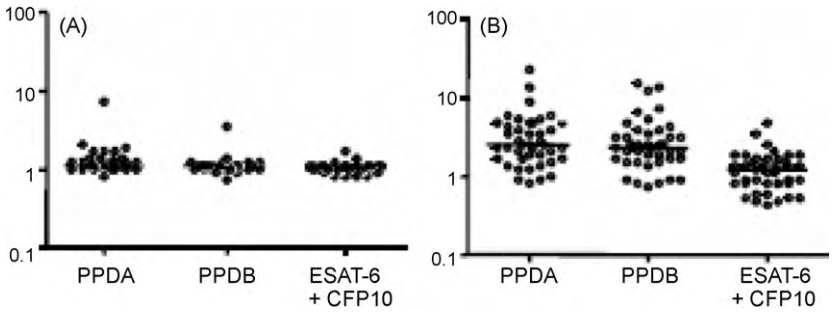
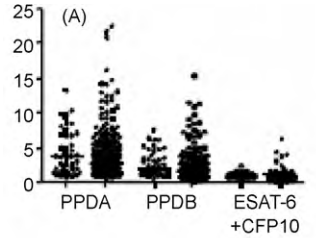


Fig. 3. The influence of paratuberculosis on antigen recognition in low or null prevalence herds. Two groups of skin test negative animals from farms with no prior history of tuberculosis were tested for recognition of PPDA, PPDB and ESAT-6 + CFP10. One farm did not have MAP infections among the animals (A) whereas the other did (B). Antigen recognition results are given as ODI values and medians are indicated with a line.



	Animal number									
	1	2	3	4	5	6	7	8	9	10
PPDA	4.1	22.7	7.4	16.4	4.5	14.3	22.0	14.4	2.5	1.3
PPDB	3.8	11.6	8.8	9.9	3.9	8.3	9.2	15.5	3.1	1.4
ESAT-6 +CFP10	4.5	1.1	1.0	0.9	4.2	0.9	1.0	1.1	2.5	3.1
ST*	N	N	N	N	N	N	N	N	N	N

Fig. 4. Antigen recognition in animals from Northern Ireland. For each antigen the results from non-infected control animals (left) and animals from farms with very low prevalence of bovine (right) (A) are given as ODIs and the median values for each group are indicated with a line. ODI results for individual animals with values above the cut-off values for PPDB or ESAT-6 + CFP10 are given on black background. The PPD based skin test is given and the results for the 3 skin test positive animals identified are given on gray background.

to PPDB and ESAT-6 + CFP10 (100% → 72% → 29%) and an increase in frequency of animals that responded to both antigens (0% → 14% → 59%).

3.3. ESAT-6 + CFP10 diagnosis is not influenced by a herd paratuberculosis infection

To investigate how a very common non-tuberculosis mycobacterial infection influenced the IFN-γ readout depending on the antigen we compared PPDA, PPDB and ESAT-6 + CFP10 specific responses in animals from two herds with no recent history of tuberculosis. All animals were PPDB skin test negative. One group ($n = 33$) came from a herd with no evidence of any mycobacterial infection (Fig. 3A) and the other group ($n = 42$) came from a herd known to be infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Fig. 3B). ESAT-6 + CFP10 stimulation of samples from the non-infected herd resulted in a median ODI value of 1.06 and a 95th percentile of 1.48 ODI's. In the same herd the median ODI value for the PPDB was 1.09 and 1.18 for PPDA, and the corresponding 95th percentile ODI's were 1.98 and 3.71. In samples from the MAP infected herd the ODI median after stimulation with ESAT-6 + CFP10 increased to 1.16 ODI's and the 95th percentile to 2.01 ODIs. For the same samples stimulated with PPDB or PPDA the medians more than doubled (ODI's of 2.27 and 2.41, respectively) and the 95th percentile increased to 13.3 and 12.4 ODI's. Statistical comparison of the logarithmic transformed results for the two herds revealed no statistical difference in the recognition of ESAT-6 + CFP10 whereas the PPDA and PPDB responses were statistical different between the herds ($p < 0.001$). Doing inter-herd comparisons we found a statistical difference between ESAT-6 + CFP10 and PPDB responses in the MAP infected herd ($p < 0.001$) whereas there was no differences in samples from the non-infected herd.

3.4. Specificity problems in a very low prevalence setting

The specificity problem due to animal exposure to other mycobacterial species was clearly illustrated in herds from Northern Ireland where a prevalence of BTB < 1% was expected (Fig. 4A). In accordance with the prevalence three of the 267 study animals were skin test positive whereas all 45 control animals were negative. *Ex vivo* responses against PPDB were quite significant in the negative control group with a median ODI value of 2.06 and a 95th percentile of 4.21 ODI's and combined with even higher values for PPDA (median value of 3.61 ODI's and a 95th percentile of 5.13 ODI's) it is most likely that many of the animals had been exposed to other mycobacterial species prior to testing (Fig. 4A). In the 267 study animals the PPDA and PPDB responses were weaker than in the control group for both PPD's but the overall responses were again stronger for PPDA than for PPDB with median ODI values of 2.81 for PPDA and 1.52 for PPDB and 95th percentile values of 4.41 and 2.53 ODI's, respectively. In contrast, the ODI's for ESAT-6 + CFP10 were very low in both control and field animals with median ODI values of 0.94 and 0.95 and 95th percentile values of 1.07 and 1.08 ODI's (Fig. 4A). In order to identify animals that could potentially be infected, a cut-off value was calculated for PPDB and ESAT-6 + CFP10 as previously described. Among the 267 animals tested, 15 were positive to either PPDB or ESAT-6 + CFP10 (Fig. 4B, animal nos. 1–15). All nine PPDB positive animals responded strongly to PPDA stimulation (ODI's between 7.4 and 22.7) whereas only one responded to ESAT-6 + CFP10. This discrepancy raises doubts about the specificity of PPDB. Seven animals were positive after ESAT-6 + CFP10 stimulation and those animals had relatively low PPD responses (1–4.5 ODI's) with PPDA/PPDB ratios between 0.8 and 1.2. Combined, these results suggest that the animals had been exposed to *M. bovis* infection and were in a very early stage of infection. However a cutoff at the 95th percentile will statistically give 6 false positives from a sample size of 267 and since the expected prevalence was less than one 1% (<3 animals) it is possible that several of the 7 responders were uninfected and therefore false positives. None of the 3 skin test positive animals were confirmed by the IFN-γ assay regardless of the antigen used (animal nos. 16–18 in Fig. 4B). All three situations clearly illustrate the diagnostic specificity problem we are facing.

4. Discussion

This is the first multicenter evaluation of the bovine *ex vivo* IFN-γ test using PPDB or purified proteins as antigens for their comparison as diagnostic antigens in herds with different disease prevalence's. Because the skin test status of each animal was known, the *ex vivo* results for the two antigens were comparable in both skin test positive and negative animals.

In herds expected to have high or medium bovine tuberculosis prevalence's we found a good correlation between a positive skin test and a positive *ex vivo* test. 60–65% of the skin test positive animals were confirmed by both PPDB and ESAT-6 + CFP10 *ex vivo* and an additional 20–25% were positive to at least one of the antigens *ex vivo* (Fig. 1). However, in the skin test negative animals from high prevalence herds we found little correlation between skin test and *ex vivo* test. Our data imply that almost 60% of the skin test negative animals were false negatives. Since the sensitivity of the *ex vivo* assay has been reported to be 73–100% with specificities from 85% to 99.6% (Ryan et al., 2000; Vordermeier et al., 2004; de la Rua-Domenech et al., 2006; Aagaard et al., 2006) the majority of these animals are likely to be infected with bovine tuberculosis. The four most likely explanations for why an animal can have a negative PPDB skin test and one month later be PPDB positive *ex vivo* are: (i) the nature of the tests are quite different, *in vivo* vs. in vitro, with completely different types of readout and length of incubation time, (ii) PPDB varies not only from producer-to-producer but also batch-to-batch (Buddle et al., 1995), (iii) sensitization by PPDB in the skin test, (iv) exposure to environmental mycobacteria or newly infections in the time between tests. We do not believe sensitization is a problem because a similar "conversion" was not observed in low prevalence herds. Exposure to environmental mycobacteria or a recent infection cannot completely be rule out but given that the animals are coming from different herds from two countries we find this unlikely as a general explanation. Based on our data we

therefore conclude that the negative predictive value of the PPDB skin test in high prevalence herds is low and infected animals will be missed during routine surveillance by skin test. To make faster progress towards eradication of bovine tuberculosis, problematic herds with high prevalence's should be tested with both assays using PPDB or ESAT-6 + CFP10 as antigen *ex vivo*. If positive in either test the animals should be removed from the herd.

In low prevalence herds from Argentina the percentage of skin test positive animals that could be confirmed by both antigens *ex vivo* was only 15–18% (Fig. 1). Since a large number of independent studies has established ESAT-6 and CFP10 as *M. bovis* specific antigens with high sensitivity and specificity (Pollock and Andersen, 1997; Vordermeier et al., 1999, 2001; van Pinxteren et al., 2000; Aagaard et al., 2003), it is most likely that the in those herds tuberculin skin test incorrectly identified up to 80% of the animals as being infected. The positive predictive value of the skin test is therefore low in low prevalence herds and tuberculin skin testing will in many cases identify non-infected animals as being positive. The major reason for the high frequency of false PPDB skin test responders is PPDB's lack of specificity, due to exposure of the animals to non-tuberculous mycobacteria (Francis et al., 1978; Amadori et al., 2002; Hope et al., 2005; Gormley et al., 2006). In this study, PPDB's poor specificity was particularly highlighted in low prevalence herds from Argentina where more than 60% of the PPDB skin test positive animals did not respond to ESAT-6 + CFP10 *ex vivo* (Fig. 1E). The high cost of the *ex vivo* test precludes its use in many countries as a primary screening test for BTB. As an alternative it is likely that the specificity of the skin test could be enhanced through the use of *M. bovis* specific recombinant proteins such as ESAT-6 and CFP10 (Weldingh and Andersen, 2008). In countries where it is more important to become BTB free as soon as possible the *ex vivo* IFN-γ assay has been suggested as an additional test to improve detection of diseased animals (Neill et al., 1994; Wood and Jones, 2001) and has been recommended in Australia, New Zealand, USA and by the EU tuberculosis-sub-group task force (Directorate General for Health and Consumer Affairs, DG SANCO). In such settings we propose to use of ESAT-6 and CFP10 as the antigens *ex vivo*.

PPD's specificity problem was further emphasized by comparing its *ex vivo* responses to ESAT-6 + CFP10 in a herd with an ongoing MAP infection. The influence of a MAP infection upon a diagnostic test for bovine tuberculosis is relevant because except for a few countries the prevalence of MAP in cattle and other ruminants is very high worldwide (Li et al., 2005). In the MAP infected herd we found clearly elevated responses to both PPDB and PPDA compared to the responses in a non-infected control herd. These results are quite similar to what have previously been reported in animals with other mycobacterial exposures (Amadori et al., 2002; Hope et al., 2005). Furthermore, it has been reported that in a herd infected with both *M. bovis* and MAP, specific diagnosis of BTB by PPDB and PPDA was compromised and it was concluded that tuberculin reagents might not be appropriate for the eradication of BTB in herds with dual mycobacterial infections (Abernethy et al., 2006). Lately, a decrease in both *ex vivo* and skin test PPDB responses were reported in animals with dual paratuberculosis/BTB infections (Alvarez et al., 2008; Alvarez et al., 2009) resulting in a decrease of sensitivity. In contrast to PPD, the range of responses to ESAT-6 + CFP10 in the MAP infected herd was only marginally influenced by the infection (Fig. 3B) and a possible negative effect on BTB diagnosis should therefore be minimal when ESAT-6 + CFP10 is used as antigen *ex vivo*.

Herds in Northern Ireland have a low prevalence of bovine tuberculosis but in general the animals are highly exposed to environmental mycobacteria with all the complications that creates for tuberculosis diagnostic (Amadori et al., 2002). In agreement with this we found many animals with high background responses to PPD's in the negative control herd. More than 1/3 of these animals had ODI's (OD reading antigen stimulation/OD reading PBS) above 3.0 following PPDB stimulation and 2/3 after PPDA stimulation (Fig. 3C). In contrast, none of the control animals had ODI's above 2 after ESAT-6 + CFP10 stimulation. In the test group the animals had even higher responses to PPDB and PPDA but since the PPDA responses in general were higher than the PPDB response and there were no response to ESAT-6 + CFP10, with the exception of one animal, it is most likely that the majority of the responding animals had been exposed to other mycobacterial species rather than being infected with BTB. In accordance with this, only three animals were skin test positive. However, similar to what we observed in low prevalence herds in Argentina and Mexico the positive status could not be confirmed *ex vivo*. *Ex vivo*, 15 animals came out positive but only one was positive with both antigens. The lack of agreement not only between test forms but also between antigens clearly illustrate the complexity of diagnosis BTB in setting with low BTB prevalence and massive exposure to environmental mycobacteria. The current strategy in the UK is based upon skin testing followed by the *ex vivo* Bovigam® assay (Prionics, Switzerland) to confirm new outbreaks (Coad et al., 2008). We believe that ESAT-6 + CFP10 can be especially useful as antigens in such complex settings and should be included as antigen in at least one of the assays but preferably both of them. In the skin test it should only be included in parallel with PPDB/PPDA. If an animal is tested positive in any of the tests it should be removed from the herd.

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