Application of the Enfer chemiluminescent multiplex ELISA system for the detection of *Mycobacterium bovis* infection in goats

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ABSTRACT

A study was conducted to optimise a multiplex serological immunoassay for use in identification of goats infected with *Mycobacterium bovis*. To assess assay specificity, 31 goats with a history of being free from *M. bovis* infection were used. To determine assay sensitivity, 180 Single Intradermal Comparative Tuberculin test (SICTT) positive goats were recruited. Additionally, 286 SICTT negative goats classed as potentially exposed animals present in the same positive herds were also included in the study. The results of the assay demonstrated a specificity of 100%. The multiplex assay detected 57/60 SICTT (95.0%) positive animals in one *M. bovis* infected herd and 120/120 (100%) in a second herd. In a separate experiment, 28 *M. caprae* culture confirmed infected goats from Spain were assayed, of which 24 (85.7%) were found positive in the test. The results show that inclusion of an antibody based assay can improve the ability to identify *M. bovis* and *M. caprae* infected goats. With further development and validation the multiplex assay may prove to be a useful tool for control of *M. bovis* and *M. caprae* infection in goats.

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1. Introduction

In Ireland over 5000 goats are farmed for milk and dairy production in a small industry that is growing and becoming economically important. In contrast to the strict regulations relating to treatment of bovine milk, unpasteurized goat milk is regularly used. Goats sharing the same environment are potentially at risk of exposure to *M. bovis* where tuberculosis (TB) is present in livestock and wildlife reservoirs, and in the absence of comprehensive surveillance disease may go undetected. While the use of the Single Intradermal Comparative Tuberculin Test (SICTT) is standardised and well described for cattle (European Commission, 2004) the same is not true for goats. Efforts in Ireland to adapt the SICTT has proven difficult in young dairy goats with thin skin (Shanahan et al., 2011), thus increasing the potential for missing infected animals. A study carried out on a goat herd with a history of both *M. caprae* and *M. avium* subsp. *paratuberculosis* (*Map*) infection (Álvarez et al., 2008) showed the low sensitivity of SICTT (42.7%) and revealed that 9.2% of infected animals were not detected by cell mediated immune response (CMI) tests, reinforcing the need to develop improved tests to maximise detection. Until recently, the potential for replacing or supplementing CMI tests with antibody based assays was limited. Use of

single antigen based assays has shown that antibody activity for different antigens develops at different times during the course of infection (Kwok et al., 2010). This observation highlights the need to develop methods to simultaneously detect antibody activity for multiple antigens for all stages of infection. To achieve this objective we developed a multiplex assay designed to detect/analyze activity to multiple antigens in a single well of a 96-well plate (Whelan et al., 2008). Thus far, sensitivity between 77.1% and 86.5% with specificity between 79.6% and 100%, have been achieved for TB diagnosis in cattle (Whelan et al., 2010). The objective of the present study was to determine if the multiplex TB assay had potential for optimisation in goats.

2. Materials and methods

2.1. Samples

Sera were obtained from blood samples from 497 goats in Ireland, aliquoted and stored at −20 °C until used. Thirty-one of these were obtained from two historically TB free, SICTT negative herds (17 from one herd and 14 from the second). These animals were used to assess the multiplex assay specificity. One hundred and eighty sera, obtained from two herds (60 and 120 respectively) confirmed positive by the SICTT, histopathology, and culture, were used to compare the performance of the multiplex assay with the SICTT. The remainder of the serum samples (286) were obtained from SICTT negative goats present in the same herds as the SICTT positive goats. These animals were considered as being at high risk of exposure to *M. bovis* infection. This investigation was undertaken in response to an outbreak of tuberculosis in ruminant animals, listed under the Irish Diseases of Animals Act, 1966, by S.I.101/2008 (Irish Statute Book, 2008) and for which there is no internationally validated and approved test and interpretation methodology. Animal testing and sampling for disease control purposes under S.I.101/2008 (Irish Statute Book, 2008) was conducted by Veterinary Inspectors of the Irish Department of Agriculture, Fisheries and Food authorised under the Irish Diseases of Animals Act, 1966 (Irish Statute Book, 1966), and complied with the code of Professional Conduct (Veterinary Practitioners) of the Veterinary Council of Ireland. Sera from 28 goats were obtained from Centro de Vigilancia Sanitaria Veterinaria in Spain. These animals were culled at the slaughterhouse and tested for tuberculosis using histopathological and bacteriological methods (*M. caprae* culture was carried out). The goats included in these studies were not experimental animals. Handling of the animals, sampling and euthanasia were performed by veterinarians in accordance with Spanish legislation.

2.2. SICTT

The SICTT was applied to goats in this study as specified for cattle in Directive 64/432/EEC (European Commission, 2004). Briefly, the SICTT was performed by separately injecting avian and bovine purified protein derivative (PPD) tuberculin intradermally into defined sites on the neck of goats. The test was read 72 h later, by comparing the relative increase (mm) in skin fold thickness (an *in vivo* cell mediated response to tuberculin) at each injection site. Because the herds were confirmed infected with *M. bovis* the interpretation of the skin test used in this study was classed as 'severe' and were deemed positive if the bovine reaction was >2 mm and exceeded the avian reaction. Follow up SICTTs were conducted according to this protocol.

2.3. IFN-γ

The Bovigam IFN-γ assay was performed under standard conditions as previously described (Gormley et al., 2006). The results and interpretation criteria used were the same as those used for cattle. The IFN-γ assay results were only available for 299/497 of the Irish animals.

2.4. Multiplex assay

The multiplex assay Enferplex (Enfer Scientific, Newhall, Ireland) was carried out as previously described (Whelan et al., 2008). The antigens were deposited as separate spots in a single well of a 96 well plate so that there was no overlap of signal from antibodies in the sera that contained antibodies to one or more of the antigens. The assay was optimised for use with goat sera as follows. Serum samples were diluted 1:1000 into sample dilution buffer and mixed. A 50 μl sample dilution was added per well. The plates were incubated at room temperature with agitation for 90 min. The plates were washed six times and aspirated. The detection antibody (Anti-goat IgG peroxidase conjugate, Sigma) was prepared to a dilution of 1:40,000 in detection antibody dilution buffer. After addition of 50 μl of the detection antibody to test wells, the plates were incubated at room temperature for 30 min with agitation (900 rpm). The plates were washed as above and 40 μl of substrate (50:50 dilution of substrate and diluent) was added to each well. The chemiluminescent signals of each antibody antigen complex were captured and data were extracted and analyzed as previously described (Whelan et al., 2008). The antibody-reactivity was assessed by running the same assay with serial dilutions of serum from 1:1000 to 1:128,000.

2.5. Statistical analysis

For the statistical analysis the Kappa agreement statistic was calculated for the three different tests, using Minitab version 15.

2.6. Bacteriology

Identification of mycobacterial infection in the Spanish goats was performed as described by Álvarez et al. (2008). Briefly, smears from the tissue samples were stained with phenolated auramine (Smithwick, 1976) and observed by transmission fluorescence microscopy. A pool of samples (lung and retropharyngeal, mediastinal and bronchial lymph nodes) from each animal was decontaminated with 0.35% hexadecylpyridinium chloride (HPC) (Corner and Trajstman, 1988) and cultured onto Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (bioMér-

Table 1
Apparent specificity and level of agreement between SICTT and Multiplex Assay/IFN-γ for SICTT positive animals in two Irish herds (ND, not determined).

	Apparent specificity (%)	Agreement between SICTT and Multiplex/IFN-γ (%)		Overall agreement (%)
	$n = 31$	Herd 1 ($n = 120$)	Herd 2 ($n = 60$)	$n = 180$
SICTT	100.0%	100.0%	100.0%	100.0%
Multiplex	100.0%	100.0%	95.0%	98.3%
IFN-γ	100.0%	100.0%[a]	ND	100.0%

[a] $n = 61$ for IFN-γ, no data on 59 animals was available.

ieux España and Biomedics, Madrid, Spain). Isolates were identified by specific PCR amplification of *Mycobacterium* genus-specific 16S rRNA fragment and MPB70 sequence (Wilton and Cousins, 1992), and then characterised as *M. caprae* using spoligotyping (Kamerbeek et al., 1997). Additionally, smears of the ileocaecal valve and mesenteric lymph nodes were stained by Ziehl-Neelsen for a presumptive detection of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). A pool of tissues from each animal were processed with HPC (Greig et al., 1999) and inoculated onto selective media (de Juan et al., 2006).

3. Results

3.1. Sensitivity and specificity of the multiplex assay

The multiplex assay was first optimised for the sensitive detection of *M. bovis* infection. Among the SICTT positives (bovine PPD reaction >2 mm and >avian PPD reaction) the multiplex assay detected 95.0% (57/60) and 100.0% (120/120) from the two herds investigated (Table 1). In the herd containing 120 SICTT positives, 61 goats were also tested with the IFN-γ assay and were determined as positive, animals from the second herd (60) were not tested with the IFN-γ assay.

The specificity of the multiplex assay was determined using 31 animals from two herds with histories of being TB free (Table 1). These animals were negative in the SICTT and

Bovigam IFN-γ assays. None of the animals were positive in the multiplex assay giving a test specificity of 100%.

Data for all three tests were available for 299 animals (31 negative controls, 61 SICTT positive and 207 SICTT negative potentially exposed). The results were compared using the Kappa agreement statistic index. The kappa agreement for the SICTT versus the Bovigam IFN-γ was 0.957, the agreement between the SICTT and the Enferplex system was 0.963. Agreement between the Bovigam IFN-γ and the Enferplex system was 0.973. The kappa indexes indicate that all three tests were highly comparable for these samples.

3.2. Multiplex assay on SICTT negative goats from known infected herds

SICTT negative animals originating from two known infected herds were tested on the multiplex assay. In herd 1, out of a total of 236 SICTT negative animals tested, two were multiplex positive. In this herd 157 animals were tested by the Bovigam IFN-γ assay and four were positive. These four animals were multiplex negative. In the second herd, a total of 50 SICTT negative animals were assayed. From the second herd 39/50 samples were negative on both assays, 7/50 samples were positive on both tests, while 2/50 were positive on the multiplex assay only and 2/50 were positive on the IFN-γ assay only.

In a follow up study on goats from the group of 236 SICTT negative animals, six additional animals were found
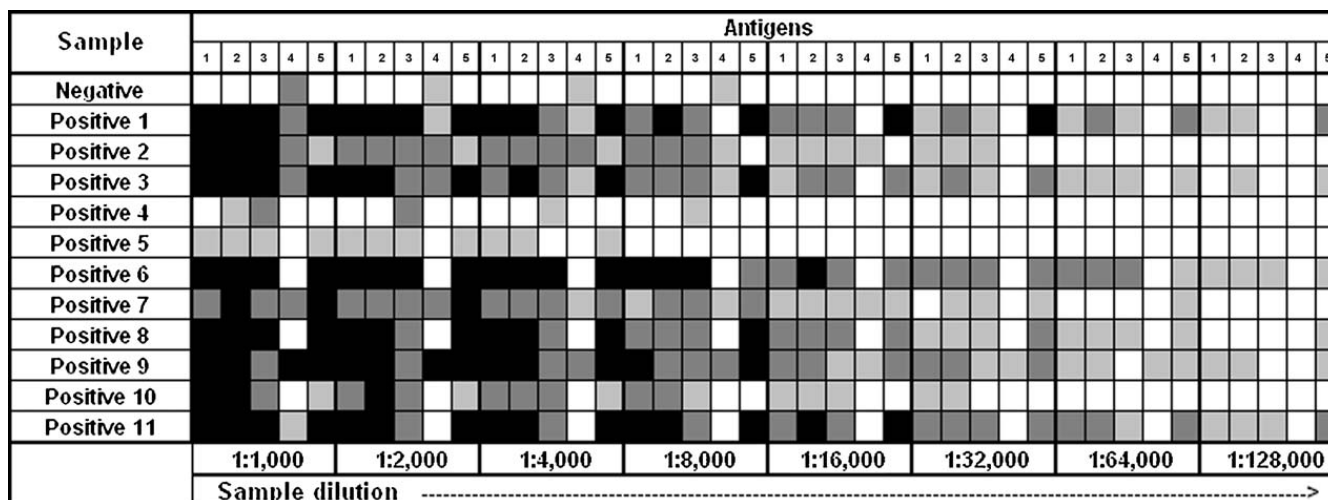
Fig. 1. Profile of sero-reactivity of SICTT positive goats on the Multiplex assay. Antigens shown were selected from the multiplex system and are as follows: 1: MPB70 peptide preparation; 2: MPB70; 3: MPB83; 4: Rv3616c; and 5: CFP-10. Serial dilutions of the serum were prepared and tested on the multiplex from 1:1000 to 1:128,000. Sample 1 is a control negative and 11 positive samples are representative positive samples that were randomly selected. Shading key for signals from the multiplex system in relative light units (RLU): (▨) 1500–10,000 RLU; (▦) 10,000–30,000 RLU; (■) >30,000 RLU.

Table 2
Comparison of post mortem analysis and multiplex assay Enferplex results for the 28 goats confirmed to be infected with *M. caprae*. All animals were negative for *M. avium* subsp. *paratuberculosis* (ileocecal valve, intestine, mesenteric lymph nodes) using bacteriological culture.

Animal	Post-mortem analysis				Enferplex
	TB lesion[a]	TB smear[b]	pTB lesion[c]	pTB smear[d]	
1	+	−	+	−	+
2	+	−	−	D	−
3	+	+	−	−	+
4	+	−	−	D	+
5	+	D	−	−	+
6	+	+	−	+	+
7	+	D	−	−	+
8	+	+	−	−	+
9	+	+	−	−	−
10	+	+	−	−	+
11	+	+	−	−	+
12	+	+	−	D	+
13	+	+	−	D	+
14	+	+	−	−	+
15	+	+	−	−	−
16	+	D	−	−	+
17	−	−	−	−	+
18	+	+	+	−	+
19	+	+	−	D	+
20	+	D	+	−	+
21	+	+	−	−	+
22	+	+	+	−	+
23	−	−	+	+	+
24	+	−	−	+	+
25	+	−	−	+	+
26	+	−	+	+	+
27	+	+	−	D	+
28	+	+	−	−	+−

+, positive; −, negative; +−, borderline;, D, doubtful/inconclusive.
[a] TB lesion, Visible lesions, compatible with tuberculosis (lung and retropharyngeal, mediastinal and bronchial lymph nodes).
[b] Auramine staining.
[c] Visible lesions, compatible with paratuberculosis (pTB) (ileocecal valve, intestine, mesenteric lymph nodes).
[d] ZN staining.

positive by either the multiplex assay or the IFN-γ (two out of the six and four out of the six respectively, no overlap was observed). Three of the four IFN-γ positive animals were classed as non-visible lesion animals (NVL) at slaughter while the remaining animal died before slaughter, no further results were available for these four animals. The two animals that were multiplex positive have remained negative on three subsequent SICTTs. The herds are currently regarded as TB free.

3.3. Sero-reactivity using the multiplex assay

The multiplex assay was used to determine the relative levels of sero-reactive antibodies present in 11 sera randomly selected from the panel of sera obtained from infected animals. The sera were tested against a panel of representative antigens as used in the multiplex assay: 1 = MPB70 peptide preparation; 2 = MPB70; 3 = MPB83; 4 = Rv3616c; and 5 = CFP-10 (Fig. 1). Relatively high levels of specific antibody activity were observed in the sera of infected goats against the *M. bovis* antigens, with over 50% of infected animals tested showing antibody activity to 1:128,000. Titers of antibody activity as high as 1:64,000 were detected against all the antigens used in the assay. Antibody titers of 1:128,000 were detected against four out of the five antigens. For one antigen low signals were

observed out to 1:8000. Control sera were negative at the 1:1000 dilution used in this study.

3.4. Multiplex analysis of M. caprae infected goats

Serum from 28 Spanish goats, confirmed culture-positive for *M. caprae*, were tested on the multiplex assay. The results showed that 24 of the 28 *M. caprae* infected goats (85.7%) were positive on the multiplex assay. TB specific granulomas were found in 26 animals at post-mortem (Table 2). Six animals had lesions in the intestine and/or mesenteric lymph nodes compatable with para-tuberculosis (pTB). An additional three were positive for ZN staining. Two animals (numbers 23 and 26) had visible lesions and were positive for ZN staining also. None of the goats were positive for *Map* in the bacteriology culture.

4. Discussion

The primary immunological response to *Mycobacterium tuberculosis* complex infections in animal hosts is the CMI response. This has been exploited for the development of several *in vitro* tests to measure CMI antigen specific and non-specific responses, including lymphocyte proliferation assays, assays for gamma interferon, granulocyte-macrophage colony-stimulating factor, tumour necrosis

factor, and reactive nitrogen and oxygen intermediates (Muscoplat et al., 1975; Thoen, 2006; Martinez-Romero et al., 2009). The CMI response can also be assessed *in vivo* using the SICTT, this method is currently used in goats and cattle. The specificity of the skin test has been observed up to 100%, while the sensitivity varies from 38.3% to 95% (Cousins and Florisson, 2005). One concern about using the SICTT, especially for younger goats, is related to their thin skin, often <2 mm thick, and the problems of administering intradermal tuberculin injections accurately.

Antibody-based diagnostic tests can also be used in conjunction with skin tests or *in vitro* cell-mediated tests to identify TB infected animals. There have been reports of the use of antibody based diagnostics in goats. Acosta et al. (2000) reported a high sensitivity of ELISA for anti-MPB70 (93.4%) in goats naturally infected with *M. bovis*, with a specificity of 100% (all animals infected with *Map* and *Corynebacterium pseudotuberculosis* were test negative). The efficiency of using a serum ELISA for diagnosis of caprine paratuberculosis has been described (Rajukumar et al., 2001), indicating that antibody-based assays can be used to differentiate TB and pTB infected animals. The measurement of antibody responses to *M. bovis* infection by a lateral-flow rapid test and multi-antigen print immunoassay shows good potential in multiple species of free-ranging wildlife (Lyashchenko et al., 2008). In addition, high sensitivity and specificity of an anamnestic ELISA based on bovine PPD antigen for detection of *M. bovis*-infected goats has been reported by Gutiérrez et al. (1998). However, the complex nature of the immune response to *M. bovis* infection necessitates the development of antibody based diagnostics that include multiple antigens to detect the full spectrum of the disease, from early infection to advanced clinical disease. The use of multiple antigens may also aid the diagnosis of infection caused by different *M. bovis* strains.

There are several research groups working on the concept of using multiple antigens in ELISA, lateral flow format diagnostics as well as IFN-γ assays for various species, with promising results (Lyashchenko et al., 2000; Amadori et al., 2002; Cockle et al., 2006; Coad et al., 2008; Whelan et al., 2008). The use of the multiplex assay described here has been evaluated in bovine populations (Whelan et al., 2008, 2010, 2011; Clegg et al., 2011). As a follow-up of the encouraging results obtained from cattle, we set out to optimise the multiplex assay for the detection of *M. bovis* infection in goats.

In a group of 31 animals with a history of being TB free, the specificity was determined to be 100%. Although the sample size was small, the absence of any false positives suggests high test specificity. However, validation of this result will require testing of increased numbers of non-infected animals. When comparing the results of the multiplex assay to those of the SICTT, the multiplex assay detected on average 98.3% of the SICTT positive animals from the two herds investigated, indicating that there is potential for the use of this antibody based assay for the diagnosis of *M. bovis* infection in goats. However, the multiplex assay did not detect all SICTT positive animals in herd 1 (57/60), but did detect additional positives, along with Bovigam IFN-γ assay, in the population of animals

that were exposed but SICTT negative, suggesting that both the multiplex assay and Bovigam IFN-γ were detecting additional potentially infected animals in the herds that were missed by the skin test and vice versa. The possibility to use multiple diagnostic methods that are based on different biological methodologies highlight the potential to detect additional infection in animals that would potentially be otherwise left undetected. Overall the multiplex assay detected 98.3% of the SICTT positive animals from the two herds investigated. From the *M. caprae* confirmed animals a total of 85.7% were detected.

The use of multiple antigens in a serologically based assay for the diagnosis of *M. bovis* infection is a key factor in developing an accurate test, as no single antigen has yet been identified that covers all stages of the infection (Kwok et al., 2010). Also, the added sensitivity due to the design and the dynamic range of the multiplex system allows for accurate detection of positive animals with wide ranging levels of circulating antibodies. There was no defined difference in the antigen recognition patterns observed for the *M. caprae* compared to the *M. bovis* infected animals tested. Further work with larger numbers of samples would be needed to investigate this fully.

5. Conclusion

The results show that a serum based assay for the detection of *M. bovis* infection in goats is a feasible alternative or ancillary test to the SICTT test. The Multiplex serum test can be performed with greater frequency than the intradermal tuberculin test since there is no issue with desensitization occurring with short interval tests as there is with the SICTT. Also, there is no requirement for a repeat visit to read the SICTT result. With further development and validation the multiplex test could aid in the eradication of *M. bovis* and *M. caprae* infection from goat herds.

Conflict of interest statement

The authors, WD, MD, AA, JB, EG, AD, and MG declare that they have no financial or personal conflicts of interest. ES, PQ, CW and JC are Enfer employees, SO and HFK are Fusion Antibodies employees, however, neither Enfer nor Fusion Antibodies nor their employees played any role that could have inappropriately influenced (biased) this work.

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