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Vordermeier

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# Evaluation of Gamma Interferon and Antibody Tuberculosis Tests in Alpacas

Shelley Rhodes,<sup>a</sup> Tom Holder,<sup>a</sup> Derek Clifford,<sup>a</sup> Ian Dexter,<sup>a</sup> Jacky Brewer,<sup>a</sup> Noel Smith,<sup>a</sup> Laura Waring,<sup>b</sup> Tim Crawshaw,<sup>c</sup> Steve Gillgan,<sup>d</sup> Konstantin Lyashchenko,<sup>e</sup> John Lawrence,<sup>f</sup> John Clarke,<sup>g</sup> Ricardo de la Rúa-Domenech,<sup>h</sup> and Martin Vordermeier<sup>a</sup>

Animal Health and Veterinary Laboratories Agency (AHVLA)—Weybridge, Addlestone, Surrey, United Kingdom<sup>a</sup>; AHVLA—Langford, Bristol, United Kingdom<sup>b</sup>; AHVLA—Starcross, Staplake Mount, Starcross, Exeter, Devon, United Kingdom<sup>c</sup>; AHVLA—Worcester, United Kingdom<sup>d</sup>; Chembio Diagnostic Systems, Inc., Medford, New York, USA<sup>e</sup>; Idexx Laboratories, Inc., Westbrook, Maine, USA<sup>f</sup>; Enfer Scientific, Unit 7, Newhall, Naas, Kildare, Ireland<sup>g</sup>; and AHVLA-TB Programme, Defra, London, United Kingdom<sup>h</sup>

We describe the performance of cell-based and antibody blood tests for the antemortem diagnosis of tuberculosis (TB) in South American camelids (SAC). The sensitivity and specificity of the gamma interferon (IFN- $\gamma$ ) release assay, two lateral flow rapid antibody tests (Stat-Pak and Dual Path Platform [DPP]), and two enzyme-linked immunosorbent assay (ELISA)-based antibody tests (Idexx and Enferplex) were determined using diseased alpacas from *Mycobacterium bovis* culture-confirmed breakdown herds and TB-free alpacas from geographical areas with no history of bovine TB, respectively. Our results show that while the sensitivities of the IFN- $\gamma$  and antibody tests were similar (range of 57.7% to 66.7%), the specificity of the IFN- $\gamma$  test (89.1%) was lower than those of any of the antibody tests (range of 96.4% to 97.4%). This lower specificity of the IFN- $\gamma$  test was at least in part due to undisclosed *Mycobacterium microti* infection in the TB-free cohort, which stimulates a positive purified protein derivative (PPD) response. The sensitivity of infection detection could be increased by combining two antibody tests, but even the use of all four antibody tests failed to detect all diseased alpacas. These antibody-negative alpacas were IFN- $\gamma$  positive. We found that the maximum sensitivity could be achieved only by the combination of the IFN- $\gamma$  test with two antibody tests in a “test package,” although this resulted in decreased specificity. The data from this evaluation of tests with defined sensitivity and specificity provide potential options for antemortem screening of SAC for TB in herd breakdown situations and could also find application in movement testing and tracing investigations.

There are around 30,000 alpacas ([www.bas-uk.com](http://www.bas-uk.com)) and between 2,000 and 4,000 llamas ([www.britishllamasociety.org](http://www.britishllamasociety.org)) currently registered in Great Britain. The numbers of South American camelids (SAC) not registered with the respective breeder societies in Great Britain are not known ([www.alpacatb.com/news](http://www.alpacatb.com/news)), and so the above figures are likely to underestimate the actual numbers of SAC kept in Great Britain. Camelids are still viewed as exotic species and currently do not come under many of the general livestock regulations that apply to other farmed species. Hence, no compulsory animal identification tagging or movement recording is required, although in Wales, owners are now required to keep such records under the Tuberculosis (Wales) Order 2011 ([www.wales.gov.uk/bovinetb](http://www.wales.gov.uk/bovinetb)).

Camelids are susceptible to tuberculosis (TB) caused by both *Mycobacterium bovis* (the agent of bovine TB) and *Mycobacterium microti*, which was originally identified as a pathogen of small rodents and shrews but is now also known to cause tuberculosis in other species, like cats and SAC (9, 14, 16). The first documented case of *M. bovis* infection in SAC in Great Britain was reported in 1999 in llamas (2). In 2011, six new breakdowns of TB caused by *M. bovis* were disclosed in Great Britain, all involving alpaca herds located in geographical areas of the country where TB is endemic in cattle and wildlife reservoirs (notably badgers). Fifteen new breakdowns were recorded in 2010, again all in alpaca herds in the west of England and Wales. However, the introduction of infection associated with the movement of animals between herds means that TB should also be considered outside such areas.

Surveillance for TB in SAC is primarily by postmortem examination of routine casework or carcasses of animals exhibiting suspected clinical signs of TB. The suspicion of disease in carcasses is

notifiable to the Animal Health and Veterinary Laboratories Agency (AHVLA). Unlike cattle herds, there is no regular testing program for SAC herds in Great Britain. Antemortem TB testing of SAC usually takes place only for export certification purposes and in response to TB breakdowns that are confirmed by culture of *M. bovis*. In the European Union and many other countries, the tuberculin skin test remains the primary official test for TB in SAC. Although highly specific, this test has low sensitivity in these species (studies summarized in reference 1). However, one positive consequence of the skin testing appears to be a boosting effect of antibody responses, known as an anamnestic antibody response. Thus, the sensitivity of TB-specific antibody tests can be increased by a prior skin test, whether the individual animal is skin test positive or negative. In 2006 and 2007, we began to use in Great Britain the then new diagnostic rapid antibody lateral flow Stat-Pak test (Chembio Diagnostic Systems, Inc., Medford, NY). This test already had a published track record in the detection of TB-infected camelids that had failed to react to the tuberculin skin test (8, 22). When the Stat-Pak test was deployed in a British llama herd with culture-confirmed *M. bovis* infection, we obtained a relative test sensitivity of 69.2% that could be increased to 84.6%

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Address correspondence to Shelley Rhodes, [Shelley.Rhodes@ahvla.gsi.gov.uk](mailto:Shelley.Rhodes@ahvla.gsi.gov.uk)

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by taking serum samples at least 10 days after the tuberculin skin test (3). Thereafter, the Stat-Pak test continued to be used on an experimental basis in *M. bovis*-infected SAC herds in Great Britain as a voluntary, ancillary test for TB to be applied between 10 and 30 days post-skin test. This combined application of skin and anamnestic serological testing led to the increased identification of *M. bovis* culture-positive alpacas for that year ([www.defra.gov.uk/statistics](http://www.defra.gov.uk/statistics)).

In 2009, we investigated the potential for a cell-based gamma interferon (IFN- $\gamma$ ) release assay in SAC that, unlike the antibody response, would not be dependent on the completion of a prior skin test. Such an assay would be based on the *in vitro* stimulation of blood cells, similar to the IFN- $\gamma$  assays that have become commonly used in the diagnosis of TB in cattle and humans in the last 15 years (7, 17, 24, 25). The initial in-house data for the camelid IFN- $\gamma$  assay for TB (using alpaca and llama samples [data not shown]) looked promising, and a study funded by the British alpaca and llama industry bodies took place at AHVLA during 2011 to 2012 to evaluate the relative performance of this IFN- $\gamma$  assay, the Stat-Pak antibody test, and other new diagnostic antibody tests for TB in SAC.

This report presents the results from that study. It compares the cell-based IFN- $\gamma$  test with four different antibody tests (Chembio Stat-Pak and Dual Path Platform [DPP] lateral flow rapid tests and two antibody ELISAs, one from Idexx Laboratories, Inc., Westbrook, ME, and a new multiplex ELISA, Enferplex, from Enfer Scientific, Kildare, Ireland) as applied to both tuberculous alpacas from confirmed TB-infected (*M. bovis* culture-positive) herds and presumed TB-free alpacas from herds with no history of TB located in low-TB-incidence regions of Great Britain. The results provide relative sensitivity and specificity values for the individual tests and suggest test combinations to maximize TB testing efficiency in different disease scenarios.

## MATERIALS AND METHODS

**Study alpaca populations.** (i) **Diseased/infected alpacas.** As there is no routine surveillance testing of alpacas in Great Britain, infected herds in this study were typically identified following the voluntary postmortem of an alpaca after an untimely death. Following *M. bovis* culture confirmation from this initial animal, tuberculin skin tests would be applied to the herd, with any positives being removed (none in the case of herds in this study, to the authors' knowledge). The rest of the herd would then be subjected to blood tests. All diseased alpacas in this study, therefore, were skin test negative.

For the purpose of this study, diseased alpacas were defined as having typical gross visible lesions (VL) of TB (5) at routine postmortem examination carried out in an AHVLA regional laboratory. A total of 59 skin-test-negative VL alpacas were identified from 10 herds suffering from culture-confirmed *M. bovis* infection, by applying the IFN- $\gamma$  and Chembio Stat-Pak rapid antibody tests (see below). Most VL alpacas, therefore, were positive to one or the other of these tests at the outset. A minority were test negative using these two tests but were slaughtered as direct contacts and found to be VL positive at *postmortem*.

In the study overall, not all tests were carried out on all alpacas (dependent upon the tests agreed to by the owner and AHVLA); 55 VL animals were tested with the IFN- $\gamma$  test, 52 were tested with the various antibody tests, and 48 were tested with both the IFN- $\gamma$  and antibody tests.

(ii) **TB-free alpacas.** (a) **TB-free alpacas in GB.** Heparinized and clotted blood samples were collected from all 257 alpacas volunteered from 17 distinct herds across 10 counties of England known to have a historically very low incidence of TB in cattle (Bedfordshire, Berkshire, Cambridgeshire, Cumbria, Hertfordshire, Kent, Northamptonshire,

Northumberland, Nottinghamshire, and North Yorkshire). Data on the sex and age of the sampled alpacas was not provided by all owners. However, of the data collected, there were 61 females with a mean age (plus standard deviation) of  $6.2 \pm 0.5$  years and 84 males with a mean age of  $4.4 \pm 0.4$  years. The initial tests carried out were the IFN- $\gamma$  and Stat-Pak antibody tests. Alpacas showing a positive response to either of the purified protein derivatives (PPDs) in the IFN- $\gamma$  test or a positive response to the Stat-Pak test were noted, and 2 test-positive alpacas per submission, or 5% of the animals in the herd (whichever was greater), were removed for postmortem examination and mycobacterial culture to establish their true infection status and to estimate the proportion of false-positive test results. These alpacas were subjected to a detailed postmortem examination that included all visible lymph nodes and major organs. Any visible lesions were cultured. Where alpacas had no visible lesions suspicious of TB, samples of tracheobronchial, mediastinal, and retropharyngeal lymph nodes were taken for culture.

(b) **TB-free alpacas from the United States.** Serum samples from 49 alpacas in presumed TB-free herds (based upon epidemiological data) in the United States were included for antibody tests in this study (a gift from Idexx Laboratories, Westbrook, ME). No gender or age information was available for this group of alpacas.

All of the VL alpacas in the present study had received a skin test 10 to 30 days prior to sampling for blood tests, while none of the TB-free alpacas had received a skin test. Overall, a total of 306 TB-free alpacas were available for antibody testing, while 257 TB-free alpacas were tested with both the IFN- $\gamma$  and antibody tests.

(iii) **Alpaca blood samples.** One heparinized blood sample (for the IFN- $\gamma$  test) and one clotted blood sample (for the serum antibody tests) were drawn from each alpaca (apart from those from the United States and also some TB-infected herds in Great Britain where the keepers only gave permission for antibody testing to take place). Heparinized blood samples for IFN- $\gamma$  testing were packed into temperature-controlled delivery boxes (DGP, United Kingdom) and delivered to the laboratory by overnight courier. This was done to avoid extremes of temperature that could impair the viability of blood leukocytes. Serum samples were packed and couriered to the laboratory separately.

(a) **Preparation of PBMCs for IFN- $\gamma$  testing.** Preliminary studies to develop a camelid IFN- $\gamma$  assay using whole heparinized blood (as used for cattle IFN- $\gamma$  testing) resulted in IFN- $\gamma$  responses too low to be of any use in a diagnostic assay. However, by separating the peripheral blood mononuclear cells (PBMCs) out of the whole blood and using these in the assay, we were able to show significant IFN- $\gamma$  responses. PBMCs were isolated from heparinized whole blood using density gradient (Histopaque 1077; Sigma, United Kingdom) centrifugation ( $800 \times g$ , 40 min at room temperature). PBMCs were then washed in Hanks balanced salt solution (HBSS; Life Technologies, United Kingdom) and resuspended in culture medium (RPMI 1640 with Glutamax [Life Technologies, United Kingdom] supplemented with 10% fetal calf serum [Sigma], penicillin and streptomycin [Sigma], nonessential amino acids [Life Technologies], and 2-mercaptoethanol [Life Technologies]), and the number of viable PBMCs was counted and set to  $2 \times 10^6$ /ml in culture medium.

(b) **Preparation of serum.** Serum samples were allowed to clot in the collecting tube and then were centrifuged ( $800 \times g$ , 40 min at room temperature) in order to separate the serum fraction. Serum was decanted into a clean tube and frozen at  $-20^\circ\text{C}$  until required for antibody testing.

**Mycobacterial culture and genotyping.** Culture of postmortem tissues was done on a limited basis as per the standard AHVLA protocol: following the initial *M. bovis* isolate from a TB herd breakdown, not all of the subsequent VL alpacas from the same herd were submitted for culture.

Postmortem samples submitted for mycobacterial culture were treated according to AHVLA's standard operating procedure BA.385 ("TB diagnosis: TB culture and processing"). Briefly,  $\sim 20$  g of tissue was ground using a pestle and mortar, decontaminated with oxalic acid, and centrifuged, and the pellet was resuspended in sterile phosphate-buffered saline (PBS) and centrifuged again. The homogenates were then resus-

pended in PBS and sown onto solid and liquid culture media. Cultures were read at 6 weeks of incubation (tissue samples from known *M. bovis*-infected herds and from TB-free herds) and again at 14 weeks (samples from TB-free herds only) in order to allow sufficient time required for both *M. bovis* (6 weeks) and *M. microti* (14 weeks) to grow in culture if present in the tissue sample. Positive cultures were harvested and heat killed before genotyping. *M. bovis* and *M. microti* were identified on the basis of colony morphology and genotyping.

Genotyping is a combination of two PCR techniques—spacer oligonucleotide typing (spoligotyping) and variable number tandem repeat (VNTR) typing. Spoligotyping targets the direct repeat (DR) region of the genome, which contains many repeats of a small, virtually identical, sequence separated by a series of unique sequences (spacers). Spoligotype patterns are polymorphic because of the presence or absence of spacer sequences, and each spoligotype pattern is given a unique identification number. VNTR typing is a minisatellite-like typing technique that targets several different loci found throughout the genome. Each target locus contains a variable number of short, tandemly repeated DNAs. VNTR amplifies these target loci and calculates the number of repeats present from the length of the amplified DNA (16).

**IFN- $\gamma$  test. (i) Stimulation of PBMCs with mycobacterial antigens.** PBMC stimulation was carried out in 96-well culture plates (Life Technologies) in a volume of 200  $\mu$ l/well. For each alpaca, duplicate wells of PBMCs were stimulated with bovine and avian tuberculin (PPDB and PPDA, respectively, both at a 1:100 final dilution) (Prionics, Lelystad, The Netherlands) and the ESAT6-CFP10 (EC) peptide cocktail. EC antigens are found mainly in the pathogenic mycobacteria, such as *M. tuberculosis* and *M. bovis* (not in *M. microti*), and are used in national cattle IFN- $\gamma$  testing in Great Britain, where a higher specificity is required (<http://www.defra.gov.uk/animal-diseases/a-z/bovine-tb/animal-keepers/testing/gamma-interferon/>). Sample positive and negative controls were pokeweed mitogen (PWM; Sigma) and medium only (unstimulated cells), respectively, used to provide a maximum and minimum measure for each animal and in the case of PWM to show that the cells were viable and able to respond in the assay. Cells were cultured for 3 days at 37°C in 5% CO<sub>2</sub> in a humidified incubator, after which cell-free supernatants were harvested and the duplicate wells were pooled. Samples were stored at -20°C until tested for IFN- $\gamma$  content by ELISA.

**(ii) IFN- $\gamma$  ELISA.** The IFN- $\gamma$  ELISA was carried out using reagents cross-reactive for bovine, ovine, or equine IFN- $\gamma$  (catalog no. 3115; Mabtech, Sweden). ELISA plates (Nunc Maxisorp; Life Technologies) were coated overnight (4°C) with 50  $\mu$ l/well of the kit's coating antibody (7.5  $\mu$ g/ml in carbonate coating buffer, pH 9.6 [Sigma]). The wells were emptied and then blocked for 1 h at room temperature using 200  $\mu$ l/well of 4% bovine serum albumin (BSA) diluted in phosphate-buffered saline (4% BSA-PBS). ELISA plates were washed 3 times using 0.1% Tween 20-PBS wash buffer. Wells were emptied, and samples were added. For each alpaca, duplicate wells of 50  $\mu$ l of unstimulated, PPDB-, PPDA-, EC-, and PWM-stimulated supernatants were added to the ELISA plate. ELISA-positive (10 ng/ml bovine recombinant IFN- $\gamma$ ) and -negative controls were also added in duplicate (50  $\mu$ l/well). ELISA plates were incubated at room temperature for 1 h. Plates were washed, and 50  $\mu$ l/well of the kit secondary/detection antibody, diluted 1:2,000 in 1% BSA-PBS, was added. Plates were incubated at room temperature for a further hour and washed, and 50  $\mu$ l/well streptavidin-alkaline phosphatase (Mabtech, 3310-10), diluted 1:500, was added for 1 h at room temperature. Plates were finally washed, and 100  $\mu$ l/well 1-step *p*-nitrophenyl phosphate (PNPP) substrate (ThermoFisher Scientific) was added for 30 min at room temperature. Plates were then read at 405 nm (wavelength) on an ELISA reader. Data were transferred to Excel spreadsheets for analysis.

**(iii) IFN- $\gamma$  test interpretation. (a) PPDB and PPDA.** For a positive response, the optical density (OD) of the mean PPDB response should be 0.1 OD unit greater than the OD for the mean PPDA response (PPDB-PPDA > 0.1), thus showing a differential bias/recognition of *M. bovis* antigens over environmental mycobacteria (represented by PPDA).

Schiller et al. (15) showed that this criterion applied to cattle provided a specificity of 96.5% and a sensitivity of 90.9%, while in TB-free cattle herds in Great Britain, the same cutoff criterion provided a specificity of 96.7% (<http://www.defra.gov.uk/animal-diseases/a-z/bovine-tb/animal-keepers/testing/gamma-interferon/>).

**(b) EC peptide cocktail.** For a positive response, the mean OD reading for EC should be 0.1 OD unit greater than the mean OD of unstimulated cells (EC-nil > 0.1). Vordermeier et al. (19) showed that this cutoff criterion applied to cattle provided a specificity of 98.8% and a sensitivity of 72.7%, while a combination of EC positivity and PPDB positivity provided a higher specificity of 99.2% (<http://www.defra.gov.uk/animal-diseases/a-z/bovine-tb/animal-keepers/testing/gamma-interferon/>).

Additionally, for a test result to be considered valid, the mean positive sample control (PWM) had to be greater than 0.45 OD unit.

**TB Stat-Pak lateral flow antibody test.** The TB Stat-Pak antibody test (VetTB Stat-Pak; Chembio Diagnostic Systems, Inc., Medford, NY) uses selected mycobacterial antigens (MPB83, ESAT6, and CFP10) immobilized on a nitrocellulose strip and a blue latex signal detection system for rapid detection of antibodies as previously described (8). The test was carried out according to the manufacturer's instructions. One test (cassette) was used per alpaca. Tests were carried out at room temperature. Thirty microliters of serum was dispensed into the sample well of the cassette and allowed to soak into the wick. Three drops of sample buffer (included in the kit) was then added to the sample well. The test was incubated at room temperature for 20 min, and the results were noted. For a valid test, a complete blue band must appear across the control line site (C-band). For a positive test, a complete blue band must also appear at the test line site (T-band). The Stat-Pak test, together with the IFN- $\gamma$  test, was used in the first instance to identify infected alpacas from *M. bovis*-confirmed TB breakdown herds for this study. Such animals were slaughtered and subjected to postmortem examination.

**DPP rapid antibody test.** The Dual Path Platform (DPP) test (Chembio Diagnostic Systems, Inc.) consists of two nitrocellulose strips inside a cassette that allows independent delivery of the test sample and antibody-detecting reagent to the antigens (MPB83 and MPB70) and the test control (6). One cassette was used per alpaca. Tests were carried out at room temperature. Five microliters of serum was dispensed into the sample well followed by 3 drops of sample buffer. After 5 min, a further 4 drops of sample buffer was added to the buffer-only well. Cassettes were incubated for a further 20 min. Test results were obtained by inserting each cassette into a hand-held optical reader device (Chembio), measuring reflectance in relative light units (RLU). An RLU numerical value for the control band (to show the test was valid) and the test band was provided by the reader. As the DPP test was carried out retrospectively on the samples, it was not used to identify positive/negative alpacas in the first instance. Test-positive/negative cutoff values were established by receiver operator characteristic (ROC) analysis.

**Idexx antibody ELISA.** The Idexx ELISA for bovine TB (Idexx Laboratories, Inc., Westbrook, ME) was carried out according to the manufacturer's instructions, but with modification to detect camelid (not bovine) antibodies. ELISA plates precoated with mycobacterial antigens (MPB83 and MPB70) were supplied by Idexx, together with positive and negative controls and a secondary antibody to bovine IgG antibody. The kit secondary antibody, while retained for the kit-positive and -negative bovine plate controls, was replaced with a goat anti-llama IgG (Novus Biologicals, United Kingdom) coupled to horseradish peroxidase (HRP) for detection of alpaca antibody in the serum samples. The anti-llama secondary antibody was titrated in preliminary experiments to optimize the concentration to be used in this study. Briefly, serum samples were diluted 1:50 in kit sample diluent. Samples (100  $\mu$ l/well in duplicate) were then added to the ELISA plate together with plate positive and negative controls, and ELISA plates were incubated for 1 h at room temperature. Plates were washed with wash buffer (supplied with the kit) and drained. Anti-llama IgG-HRP diluted 1:50,000 in 1% BSA-PBS was then added (100  $\mu$ l/well) to all serum sample wells. The kit anti-bovine IgG-HRP, diluted as instructed,

was added to plate control wells. Plates were incubated for 30 min at room temperature and then washed again. Substrate (supplied with kit) was added to each well (100  $\mu$ l/well), the plates were developed for 15 min at room temperature, and the reaction was stopped by adding 100  $\mu$ l/well of stop buffer (supplied with kit). Plates were read at 450 nm on an ELISA reader, and the data were transferred to Excel spreadsheets for analysis. As the Idexx test was carried out retrospectively on the samples, it was not used to identify TB-positive/negative alpacas in the first instance. Test positive/negative cutoff values were established by receiver operator characteristic (ROC) analysis.

**Enferplex antibody ELISA.** The Enferplex (multiplex) ELISA for bovine TB (Enfer Scientific, Co. Kildare, Ireland) was carried out at the Enfer Scientific Laboratories as previously described (23) with some modifications for the detection of TB in camelids. The seven antigens used in this multispot assay were PPDB, ESAT6, CFP10, Rv3616c, MPB83, MPB70, and an MPB70 peptide. Briefly, serum samples were diluted 1:500 in sample dilution buffer (Enfer buffer G; Enfer Scientific). Fifty microliters of the diluted sample was added per test well. The microtiter plates were then incubated at room temperature with agitation for 1 h. The plates were washed with 1 $\times$  Enfer wash buffer (Enfer Scientific) and aspirated. The detection antibody (protein G coupled to HRP at a 1:5,000 dilution in Enfer buffer H [Enfer Scientific]) was added (50  $\mu$ l/well), and the plates were incubated at room temperature for 30 min with agitation. The plates were washed as described above, and 50  $\mu$ l of substrate (50:50 substrate and diluent; Enfer Scientific) was added per well. Signals as relative light units (RLU) were captured, and data were extracted and analyzed as previously described (23). For a positive test readout, a sample should give signals of greater than 3,000 RLU on at least two of the seven antigens.

**Data analysis.** Receiver operator characteristic (ROC) analyses were performed using Prism 4 (Graph Pad, San Diego, CA) software. ROC analysis uses data from diseased and disease-free individuals in a graphical plot of test sensitivity versus false-positive (1-specificity) proportions, and provides the test positive/negative cutoff required to achieve the desired paired sensitivity and specificity values.

## RESULTS

**Animals. (i) Diseased/VL alpacas.** Culture data were available for 43/59 VL alpacas. (As per the standard AHVLA protocol, following the initial *M. bovis* isolate from a TB herd breakdown, not all of the subsequent VL alpacas from the same herd were submitted for culture.) The *M. bovis* genotypes within the study VL pool of alpacas for which we had data included genotypes 17:a (25 alpacas across 2 herds), 25:a (2 alpacas from one herd), and 11:a (1 alpaca). *M. microti* (spoligotype 34) was isolated from one alpaca. Seven VL alpacas submitted for culture were culture negative. (Of these 7, histology was not done on 4 alpacas, while 3 alpacas were reported as “histology not typical of TB.”) In total, 59 VL alpacas from 10 distinct herds were entered into this study. The numbers of VL alpacas from each herd (plus the index case *M. bovis* genotype for each breakdown in parentheses) are as follows: 30 (17:a), 9 (17:a), 5 (11:a), 5 (17:a), 3 (25:a), 3 (25:a), 1 (11:a), 1 (32), 1 (9), and 1 (11:a). Gender information was available for 42 of the VL alpacas: 28 VL alpacas were female, and 14 VL alpacas were male. No age data were available.

**(ii) TB-free alpacas.** Of the 257 TB-free alpacas initially tested with IFN- $\gamma$  and the Chembio Stat-Pak rapid test, 19 were positive by either test and slaughtered for postmortem examination and mycobacterial culture. Of these, 8 were VL animals and 11 were non-VL (NVL). Of the 8 VL test-positive alpacas, 2 cases of *M. microti* infection were identified (one spoligotype 34 and one spoligotype 19).

Acid-fast bacilli were identified in both *M. microti* cases. In one of these 2 cases, mineralized liver and mandibular lymph node

TABLE 1 Observed IFN- $\gamma$  test sensitivity and specificity values

Test result <sup>a</sup>	VL alpacas (n = 55)			TB-free alpacas (n = 257)		
	n/55	% sensitivity	95% CI	n/257	% specificity	95% CI
PPD <sup>+</sup>	35	63.6	50.9–76.3	28	89.1	85.3–92.9
EC <sup>+</sup>	27	49.1	35.9–62.3	26	89.9	86.2–93.6
EC <sup>+</sup> PPD <sup>-</sup>	8	14.5	5.2–23.8	21	91.8	88.4–95.2
PPD <sup>+</sup> EC <sup>+</sup>	19	34.6	22.0–47.2	5	98.1	96.4–99.8
PPD <sup>+</sup> or EC <sup>+</sup>	44	80.0	69.4–90.6	49	80.9	76.1–85.7

<sup>a</sup> PPD, PPDB – PPDA; EC, ESAT6-CFP10 peptide cocktail.

lesions were also noted, while the other cases had more extensive lymph node lesions (hepatic, mesenteric, ileocecal, and prescapular) with a single lung lesion.

In the other 6 alpacas classified as VL in this group, small calcified liver lesions appeared common, and one of them showed similar lesions were also present in the mandibular and mesenteric lymph nodes. However, none of these lesions had histopathology typical of TB or contained acid-fast bacilli. *M. bovis* could not be cultured from any of these alpacas. An unclassified mycobacterium was isolated from one of them.

We decided to leave *M. microti*-positive alpacas in this study for two reasons: (i) because taking them out made no difference at all to the ROC analysis and test parameters (neither by discarding the individual *M. microti*-positive animal nor by discounting the whole herd [TB-free] if it contained one *M. microti*-positive animal), and (ii) because it was felt that their presence truly reflected the biology of these alpaca herds.

**IFN- $\gamma$  test results. (i) Observed results: sensitivity and specificity.** Fifty-five VL and 257 TB-free alpacas were available for IFN- $\gamma$  testing and entry into ROC analysis. Test positivity was initially determined using test cutoffs employed for cattle IFN- $\gamma$  testing (see Materials and Methods), which the ROC analysis subsequently showed to be an accurate reflection of the sensitivity and specificity at this cutoff for alpaca responses. Using these cattle IFN- $\gamma$  test-positive cutoff criteria (PPDB-PPDA > 0.1 and EC-nil > 0.1), the numbers and percentages of positive responders in the 55 VL alpacas and 257 TB-free alpaca were determined (Table 1), with corresponding sensitivity and specificity values plus 95% confidence intervals (95% CIs).

The sensitivity and specificity values were lower for both PPD and EC as single tests compared to test performance in cattle (test performance of Bovigam [Prionics, Switzerland]): IFN- $\gamma$  test in cattle, PPD sensitivity, 90.9%, and specificity, 96.5% (15); EC sensitivity, 72.7%, and specificity, 98.8% (19).

Our study results suggest the lower specificity in SAC may be due to undiagnosed *M. microti* infection in the TB-free cohort, supported by the culture of *M. microti* from two PPD-positive VL alpacas in this group. *M. microti* is known to induce tuberculous pathology similar to that of *M. bovis* in camelids (11, 13, 26), and as a member of the closely related *Mycobacterium tuberculosis* (MTB) complex, it will induce positive responses to PPD (recently described for *M. microti* infection in cats in reference 14).

In terms of the lower sensitivity of PPD in alpacas, we investigated the potential “masking” of PPDB IFN- $\gamma$  responses by high PPDA responses caused by exposure to other, non-MTB complex mycobacteria. We looked at alpacas that were “test negative” but had both positive PPDB and PPDA responses (i.e., PPDB-nil > 0.1 and PPDA-nil > 0.1). We found that for the TB breakdown herds (from which the 55 VL animals in this study originated),

TABLE 2 Increasing the IFN- $\gamma$  test specificity values

Test result <sup>a</sup>	VL alpacas (n = 55)			TB-free alpacas (n = 257)		
	n/55	% sensitivity	95% CI	n/257	% specificity	95% CI
PPD <sup>+</sup>	16	29.1	17.1–41.1	8	96.9	94.8–99.0
EC <sup>+</sup>	16	29.1	17.1–41.1	8	96.9	94.8–99.0
EC <sup>+</sup> PPD <sup>-</sup>	7	12.7	3.9–21.5	8	96.9	94.8–99.0
PPD <sup>+</sup> EC <sup>+</sup>	9	16.4	6.6–26.1	0	100.0	98.6–100
PPD <sup>+</sup> or EC <sup>+</sup>	23	41.8	28.8–54.8	16	93.8	90.9–96.7

<sup>a</sup> PPD, PPDB – PPDA; EC, ESAT6-CFP10 peptide cocktail.

28.8% of alpacas were deemed “test negative” due to high PPDA responses, while only 16.2% of TB-free alpacas were test negative due to high PPDA responses. Furthermore, of all 55 VL alpacas in this study, half of those that were PPDB negative also had high PPDA responses. This could suggest a higher risk of response masking in TB breakdown herds relative to TB-free herds.

A number of VL alpacas showed positive responses to EC (ESAT6/CFP10) peptide cocktail, two-thirds of which were also positive to PPD. Eight VL alpacas were positive to EC only, suggesting that failure to incorporate EC within any IFN- $\gamma$  testing regimen could miss a significant proportion of infected alpacas (here 14.5%). However, while using positivity to either PPD or EC raises test sensitivity (to 80.0%), there is an associated loss of specificity (80.9%). In contrast, dual positivity to both PPD and EC has the highest specificity (98.1%) but would miss two-thirds of the VL alpacas. Interestingly, in the TB-free cohort, most EC<sup>+</sup> alpacas (~85% of them) were negative to PPD, which could be due to exposure to an environmental mycobacterium, such as *Mycobacterium kansasii* (which expresses these antigens and is known to elicit immune responses cross-reactive with *M. bovis*) (18, 21), or of course, undiagnosed *M. bovis* infection.

The IFN- $\gamma$  data from these 55 VL and 257 TB-free alpacas were then analyzed using the ROC method for defined sensitivity, specificity, and associated test cutoffs. ROC analysis of the observed test data (from Table 1) supported our initial choice of the test cutoffs given above in that, for the same specificities for PPD and EC shown in our alpaca cohorts, the cutoff readouts from the analysis were 0.1003 for PPD and 0.1008 for EC (as opposed to 0.1 for both the PPD and EC tests used above). We refer to these cutoffs as high-sensitivity cutoffs.

(ii) **Increasing the specificity of the IFN- $\gamma$  test.** The use of ROC analysis of the above data to increase the IFN- $\gamma$  test specificity to values closer to those of cattle IFN- $\gamma$  testing (96.7%; GB Cattle Specificity Trial) gave test positivity and sensitivity values, as shown in Table 2.

To achieve the higher 96.9% specificity shown for PPD and EC required increased test cutoffs (PPDB-PPDA > 0.7272 and EC-nil > 0.3797). Application of these high-specificity cutoffs reduced test sensitivity values by ~54% and ~40%, respectively, which were deemed unrealistic, since, particularly in the case of PPD, positive responses could be identifying true MTB complex infections (like *M. microti*).

Interestingly, however, of those remaining EC-positive alpacas (i.e., positive using the higher test cutoffs in Table 2) in the TB-free group, none were now positive to PPD, and so application of a test criterion of positive to “PPD and EC” could now provide 100% specificity. However, the corresponding sensitivity was poor, at 16.4%, and was unlikely to be adequate to identify infected herds. However, then again, considering that all of the VL alpacas in this

TABLE 3 Antibody test sensitivity and specificity values

Test	VL alpacas			TB-free alpacas		
	n/total	% sensitivity	95% CI	n/total	% specificity	95% CI
Stat-Pak	35/52	67.3	54.5–80.8	8/306	97.4	95.6–99.2
DPP	30/52	57.7	44.3–71.1	10/306	96.7	94.1–98.4
Idexx	36/52	69.2	56.7–81.7	8/306	97.4	95.6–99.2
Enferplex	32/48	66.7	53.4–80.0	8/257	96.9	94.8–99.0

study were tuberculin skin test negative (single comparative test), the IFN- $\gamma$  test even at these stringent levels could still present a distinct improvement over the status quo.

**Serological tests for antibody responses.** Serum samples were available from a total of 52 VL and 306 TB-free alpacas. All were used for the Stat-Pak, Idexx, and DPP tests. For the Enferplex test, 48 VL and 257 TB-free alpacas were tested. The Stat-Pak rapid test, being qualitative, is shown as the number and percent positive for the two alpaca cohorts. The Enferplex test also provides a final qualitative positive or negative test outcome. The Stat-Pak and Enferplex tests were therefore not amenable to ROC analysis. Quantitative data for the DPP rapid test and the Idexx ELISA were both subjected to ROC analysis, and the test specificities were set to 96.7% and 97.4% for DPP (cutoff, >9.05) and Idexx (cutoff, >0.34), respectively. The results are shown in Table 3.

All of the antibody tests performed similarly, with high specificity (very few positives in the TB-free group, which had not been subjected to a skin test) and moderate to good sensitivity ranging from 57.7 to 69.2, with overlapping 95% CIs. In our study, the Stat-Pak and Idexx tests performed slightly better than the DPP and Enferplex tests, with an approximate 11% (6 animals) difference in sensitivity from the highest performer (Idexx) to the lowest (DPP).

Of the 48 VL alpacas tested using all 4 antibody tests, 8 of them were negative in all 4 tests (16.7% of VLs). All of these 8 antibody-negative VL alpacas were identified as positive by the IFN- $\gamma$  test using high-sensitivity cutoffs (PPDB-PPDA > 0.1 and EC-nil > 0.1); i.e., 3 PPD<sup>+</sup> EC<sup>-</sup> alpacas, 2 PPD<sup>+</sup> EC<sup>+</sup> alpacas, and 3 EC<sup>+</sup> PPD<sup>-</sup> alpacas.

The combination of more than one antibody test suggested a significant increase in sensitivity at the expense of a small drop in specificity: for example, the sensitivity of the Stat-Pak test could be increased by more than 10% by combining it with the Idexx test, with a corresponding 1.9% drop in specificity (data not shown). However, even in combination, antibody tests alone did not capture all diseased alpacas, leaving 16.7% of infected animals undetected.

**Dual parallel IFN- $\gamma$  and antibody testing for TB breakdown herds.** We assessed the diagnostic performance of the IFN- $\gamma$  test in conjunction with one or two antibody tests. These combinations were carried out on the 48 VL and 257 TB-free alpacas for which all of the tests were carried out. In this way, we sought to illustrate potential parallel testing options for test package application in culture-confirmed TB breakdown situations where the objective is to maximize the sensitivity of antemortem testing.

Table 4 shows the sensitivity and specificity values of the various test combinations. The IFN- $\gamma$  test uses the high-sensitivity cutoffs and incorporates the use of both PPD and EC (PPDB-PPDA > 0.1 and EC-nil > 0.1) shown previously to provide the highest sensitivity of infection detection. The ROC cutoffs for the

TABLE 4 IFN- $\gamma$  and antibody test combinations and sensitivity and specificity values<sup>a</sup>

Test combination	VL alpacas ( <i>n</i> = 48)			TB-free alpacas ( <i>n</i> = 257)		
	<i>n</i> /48	% sensitivity	95% CI	<i>n</i> /257	% specificity	95% CI
IFN- $\gamma$ + Stat-Pak	45	93.8	87.0–100	52	79.2	74.2–84.2
IFN- $\gamma$ + Idexx	44	91.7	83.9–99.5	55	78.6	73.6–83.6
IFN- $\gamma$ + DPP	44	91.7	83.9–99.5	58	77.4	72.3–82.5
IFN- $\gamma$ + Enferplex	45	93.8	87–100	58	77.4	72.3–82.5
IFN- $\gamma$ + Stat-Pak + Idexx	48	100	92.6–100	57	77.8	72.7–82.9
IFN- $\gamma$ + Stat-Pak + DPP	48	100	92.6–100	60	76.7	71.5–81.9
IFN- $\gamma$ + Stat-Pak + Enferplex	47	97.9	93.8–100	60	76.7	71.5–81.9
IFN- $\gamma$ + Idexx + DPP	45	93.8	87.0–100	62	75.9	70.0–81.2
IFN- $\gamma$ + Idexx + Enferplex	44	91.7	83.9–99.5	62	75.9	70.0–81.2
IFN- $\gamma$ + Enferplex + DPP	47	97.9	93.8–100	65	74.7	69.4–80

<sup>a</sup> IFN- $\gamma$  positive, PPDB-PPDA > 0.1 or EC-nil > 0.1.

DPP and Idexx tests were 9.05 and 0.34, respectively, as previously described. The Stat-Pak and Enferplex tests were qualitatively applied.

The data show that combining one antibody test with the IFN- $\gamma$  test offered significant increases in the sensitivity of VL detection over the use of either the IFN- $\gamma$  test alone or an antibody test alone as single tests. These higher sensitivities were achieved at the expense of reduced specificities. The combination of the IFN- $\gamma$  test with two antibody tests (Stat-Pak plus either the Idexx or DPP test) was able to detect all VL animals in this study, providing 100% sensitivity, but this is unsurprising given that the vast majority of VL animals in this study were selected for slaughter on the basis of a positive result from IFN- $\gamma$  or Stat-Pak testing. These data suggest that different *in vitro* tests can detect different groups of *M. bovis*-infected animals and support a combination of antibody and IFN- $\gamma$  testing to maximize the detection of diseased animals.

## DISCUSSION

This report describes the validation of five *in vitro* tests that measure cell-mediated (a new camelid-specific IFN- $\gamma$ ) and antibody (Stat-Pak, DPP, Idexx, and Enferplex) responses for the diagnosis of TB in SAC. Definition of the diagnostic characteristics of these tests for SAC widens the range of potentially available tools for the control of TB in a growing industry. The approach was to determine the sensitivity and specificity of each test by using *M. bovis*-infected and TB-free alpacas, respectively, these being the most abundant species of SAC in Great Britain. Having estimated these parameters, we were then able to combine the different tests in order to maximize the sensitivity of infection detection for TB breakdown herds.

Significantly, the data show that in a breakdown herd situation, both IFN- $\gamma$  and antibody tests could be applied in parallel interpretation with high sensitivity. This would be achieved by accepting lower specificities. Antibody test combinations could increase the sensitivity of VL detection, compared with single antibody tests. However, the combination of antibody tests (Stat-Pak and Idexx or Stat-Pak and DPP) with IFN- $\gamma$  maximized the sensitivity of VL detection in this study.

The data from our IFN- $\gamma$  test evaluation suggest that application of this test should depend on the outcome required. Thus, for TB breakdown herds, high-sensitivity test cutoffs using positivity to either PPD or EC could be employed to identify and remove the maximum number of infected animals from the herd. The use of

the peptide cocktail EC appeared to be vital for the detection of diseased alpacas, a significant proportion of which had no response to PPD. Our data suggested that this IFN- $\gamma$  test option (positivity to PPD or EC), together with two antibody tests (in our study, Stat-Pak and Idexx [or DPP]), maximized the overall sensitivity of detection.

For potential surveillance purposes, our study showed that the IFN- $\gamma$  test could be used at high specificity (100% in this study), but the corresponding sensitivity, at 16.4%, would be too low for a herd-level test.

While the parameters and method of the camelid IFN- $\gamma$  test underwent their initial trial using both llama and alpaca samples (preliminary data not shown in this report), it is possible that small differences in test sensitivity and specificity could exist between the different camelid species. Lyashchenko et al. (9) compared the Stat-Pak and DPP serological tests in TB-infected llamas and alpacas across Great Britain (alpacas), Switzerland (llamas), and the United States (alpacas) and found only small differences in test sensitivity and specificity, with slightly higher sensitivity for both tests and identical (Stat-Pak) or slightly lower (DPP) specificity in llamas compared to alpacas. Our study, by necessity, was carried out using the most populous species of SAC in Great Britain (alpacas), and so we cannot at this stage know the magnitude of any species variation (if any) in Great Britain.

All of the antibody tests performed well in terms of high specificity and moderate to good sensitivity. However, data from cattle have shown that antibody test sensitivity can be boosted by up to 30% in animals with lesions following the single comparative intradermal tuberculin skin test (20). Similarly, a boosting effect of the skin test upon Stat-Pak results in llamas was reported by Dean et al. (3). All of the VL alpacas in the present study had received a skin test 10 to 30 days prior to sampling for blood tests, while none of the TB-free alpacas had received a skin test. The result of this disparity in skin test applications between the two groups of alpacas could therefore provide overestimates of both sensitivity and specificity values of all the antibody tests.

The antibody tests, nevertheless, are relatively cheap and simple to perform. If carried out following a tuberculin skin test with parallel test interpretation, they could offer SAC owners a practical way of screening individual animals for TB prior to a sale or export, before entering a show, or before movements between herds, thus reducing the risk of spreading TB.

Our data for the Stat-Pak test compare well with a recent anal-

ysis of 830 alpacas from Great Britain breakdown herds (summarized in reference 1), where, of the 9.75% Stat-Pak-positive alpacas tested, 76.3% were VL and 23.7% were NVL, and only 9.2% of the VL alpacas were Stat-Pak negative.

Data from the TB-free cohort in our study suggested that the IFN- $\gamma$  test specificity for *M. bovis* infection may be compromised by its detection also of *M. microti* infection, another member of the TB complex that causes clinical TB in SAC (11, 13, 26). In a study of feline TB, positive IFN- $\gamma$  responses to PPD were present in 80% of *M. microti*-infected domestic cats, while positive Stat-Pak tests were found in ~40% of *M. microti*-infected cats (14). While our SAC study included two breakdown herds in which *M. microti* had also been identified (one during the study and one before the study), the literature suggests that the home ranges for *M. bovis* and *M. microti* do not generally overlap (16). Therefore, the presence of coinfecting herds of SAC suggests that either type of infection was introduced into the herd as a result of animal movements. Therefore, not to remove a PPD-IFN- $\gamma$ -positive or antibody-positive animal from a breakdown herd on the basis that it could be *M. microti* (and not *M. bovis*) would be inadvisable. Furthermore, this underlines the importance of mycobacterial culture confirmation for test-positive SAC to differentiate between notifiable *M. bovis* and nonnotifiable *M. microti* infections. Given the zoonotic potential of *M. microti*, the removal of camelids infected with this member of the MTB complex may be a positive consequence of IFN- $\gamma$  testing (4, 10, 12).

In summary, the *in vitro* tests evaluated in this report for the detection of TB in SAC currently lend themselves, we believe, more to individual animal tests than to whole-herd surveillance tests, the latter of which would probably require further improvements in sensitivity and specificity. However, in a confirmed TB breakdown situation, our data provide options for combinations of tests to maximize the detection of infected animals, to accelerate the resolution of TB breakdowns, and thereby to substantially reduce or remove the risk of residual infection, recurrent breakdowns, and further spread.

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