Validation of *ante mortem* TB tests in Camelids

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TB Research Group

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EXECUTIVE SUMMARY

- The performance of blood-based ante-mortem diagnostic tests to diagnose bovine tuberculosis in South American Camelids has been assessed;
- Four serological tests (Chembio TB StatPak and Dual Path Platform – DPP - lateral flow tests, plus IDEXX and ENFERplex antibody ELISAs) as well as an interferon-gamma (IFNγ) release assay were studied;
- Performance characteristics (relative sensitivities and specificities) of these tests have been defined: *'More tools in the toolbox'*;
- Informed, evidence-based policy discussions between stakeholders are now possible;

Specific results:

- IFNγ test can be applied with high sensitivity, but one would need to accept lower specificity;
- Animals with *M. microti* infection will give a positive IFNγ result to PPD;
- Antibody tests can provide high specificity and good sensitivity, but they are dependent upon skin test or on animals having severe/advanced pathology;
- Combination of IFNγ and antibody tests maximises detection of infected animals;
- Application of the blood tests as individual animal test plays to their strengths: clean-up of infected herds, tracing, trade/movement testing.
- Surveillance use (herd test): Blood tests are probably not applicable at present;
- Potential for future research has been discussed.

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Breakdown herds tested before this current study began, contributing more than half of the infected/diseased alpaca data, without which this study would not have reached its target for diseased animal numbers.

Di Summers, Mike Birch & Gina Bromage

Volunteer TB-free alpaca owners

Supply of TB-free alpacas. A huge thank-you to all those who came forward and volunteered their animals for this study, we could not have produced the data in it without them.

Abbreviations

BSA	Bovine Serum Albumin
DPP	Dual Path Platform (quantitative lateral flow antibody test)
EC	ESAT6/CFP10 peptide cocktail and specific antigen
ELISA	Enzyme-Linked ImmunoSorbent Assay
HRP	Horse Radish Peroxidase (enzyme used in IDEXX antibody ELISA)
IDEXX	Antibody ELISA kit (supplied by IDEXX company)
IFNγ	Interferon-gamma
IgG	Immunoglobulin-G (antibody type)
OD	Optical Density (unit readout system for IFNγ and IDEXX ELISAs)
PBMC	Peripheral Blood Mononuclear Cells (essentially, white blood cells)
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative (tuberculin)
PPDA	PPD produced from *Mycobacterium avium*
PPDB	PPD produced from *Mycobacterium bovis*
PWM	Pokeweed Mitogen (positive control for IFNγ test)
RLU	Relative Light Units (unit readout system for DPP antibody test)
ROC	Receiver Operator Characteristics (data analysis method)
SOP	Standard Operating Procedure
STAT-PAK	Qualitative lateral flow antibody test

Glossary

(a) Antigen: is any substance that causes the immune system to produce antibodies (serology) or T cell responses (IFNγ, skin test) against it.

(b) Culture-positive: *M. bovis* or *M. microti* could be cultured from the samples collected at *post mortem*

(c) Cell-mediated immunity: is an immune response that does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen

(d) False-positive rate: Proportion of test-positive animals that are not infected (B/B+D, or 1 minus specificity)

(e) False-negative rate: Proportion of test-negative animals that are infected (C/A+C, or 1 minus sensitivity)

(f) Herd-level test: Test is designed to detect herds with infection, or to assign disease-free-status to a herd (for example OTF). Examples: Tuberculin skin test for cattle used for surveillance operations, herd certifications

(g) Individual animal test: Test is designed to detect the maximal number of infected individuals (maximise sensitivity as defined by the OIE and EU directives for cattle). Examples: Clear-up of infected herds to remove sources of infection, pre-movement trade test, post-movement trade test, tracing test

(h) Interferon-gamma (IFNγ): IFNγ, or type II interferon, is a cytokine (immunological messenger molecule, similar but not identical to a hormone) that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. IFNγ is secreted by T helper cells (specifically, T_h1 cells), cytotoxic T cells (T_C cells) and NK cells.

(i) *M. bovis*: *Mycobacterium bovis*, major causative agent of tuberculosis in cattle and other animals

(j) *M. microti*: *Mycobacterium microti*, so-called vole bacillus, also member of the TB Complex, causative agent of TB in rodents, cats and SAC

(k) Sensitivity: Proportion of infected animals classified as test-positive (i.e. true positives) (A/A+C, see Table 1)

(l) Specificity: Proportion of non-infected animals identified as test-negatives (i.e. true negatives) (D/B+D, see Table 1)

(m) TB Complex: family of pathogenic mycobacteria causing tuberculosis in mammals, including *M. bovis, M microti* and *M. tuberculosis* (the human TB bacterium). In addition to *M. tuberculosis*, many are zoonotic (i.e. they cause TB in animals that can be naturally transmitted to humans)

(n) T helper cells (T_h cells): are a sub-group of lymphocytes, a type of white blood cell, that play an important role in the immune system. They help other immune cells to function. They produce IFNγ.

(o) Tuberculin: Extracts of *Mycobacterium tuberculosis*, *M. bovis*, or *M. avium* that is used in skin testing in animals and humans to identify a tuberculosis infection. Several types of tuberculin have been used for this, of which purified

protein derivative (PPD) is the most important. PPD is a poorly-defined, complex mixture of antigens.

(p) VL / NVL: Animals with visible lesions typical of TB (VL), or no visible lesions (NVL) at *post mortem*

TABLE 1: Calculations for the characteristics of diagnostic tests

Test Result	**Infected**	**Non-infected**	**Total**
Positive	A (true positives)	B (false positives)	**A+B**
Negative	C (false negatives)	D (true negatives)	**C+D**
Total	**A+C**	**B+D**	

INTRODUCTION

There are around 30,000 alpacas (www.bas-uk.com) and between 2000-4000 llamas (www.britishllamasociety.org) currently registered in Great Britain. Numbers of non-registered South American Camelids (SAC) in GB are not known. Camelids are still viewed as exotic species and currently do not come under the general livestock regulations. Hence no compulsory animal identification tagging, or movement recording is required, although in Wales keepers are now being asked to keep such records under the Tuberculosis (Wales) Order 2011 (www.wales.gov.uk/bovinetb).

SAC are susceptible to tuberculosis (TB) caused by both *Mycobacterium bovis* (*M. bovis*, the agent of bovine TB) and *M. microti* (originally identified as a pathogen of small rodents and shrews, but now also known to cause TB in other species, like cats and SAC) (Lyashchenko *et al*., 2011; Rhodes *et al*., 2011; Smith *et al*., 2009). The latest information available from AHVLA suggests that 11 separate SAC premises were under movement restriction at the end of 2011 following a report of suspected TB at post-mortem examination, or an incident of TB caused by *M. bovis* infection culture-confirmed. Four new incidents of *M. bovis* TB were disclosed in 2011 in GB, all involving alpaca herds in the Southwest of England.

Surveillance for TB in SAC is primarily by post-mortem examination of routine casework or carcases of suspect clinical cases. The disease is notifiable to AHVLA. There is no regular TB testing programme for SAC herds in GB. *Ante mortem* TB testing of SAC usually takes place only for export certification purposes and in response to TB breakdowns that are confirmed by positive culture of *M. bovis*. In GB, the rest of the EU and many other countries, the tuberculin skin test remains the primary official test for TB in SAC, but though highly specific this test has a low sensitivity (studies summarised by Alvarez *et al*., 2011 and unpublished observations from affected SAC herds in GB). However, one positive spin-off from the skin test appears to be a boosting effect of antibody responses in TB-infected animals, even if the animal is skin-test-negative. Initial collaborative studies with Chembio USA to trial the then new diagnostic rapid antibody lateral flow STAT-PAK test in a British llama TB-breakdown herd showed a relative test sensitivity of 69.2% that could be increased to 84.6% by taking serum samples at least 10 days after the tuberculin skin test (Dean *et al*., 2009). The STAT-PAK was first applied to SAC herd in GB in 2009, as a recommended ancillary test for TB in known infected herds. Blood samples are taken between 10-30 days post-skin test to capture any antibody-boosting effect. This was one of the factors responsible for the increased identification of *M. bovis* culture-positive alpacas for that year (www.defra.gov.uk/statistics).

In 2009 we investigated the potential for a cell-based interferon-gamma (IFNγ) release assay in SAC that, unlike the antibody response, would be independent of the skin test as it uses a different boost brought about by the *in vitro* stimulation of blood cells in the laboratory, similar to the IFNγ assays that have become commonly

used in the diagnosis of TB in cattle and humans in the last 15 years. The initial in-house data for the IFNγ test looked promising and a potential study was outlined by AHVLA to compare the performance of this IFNγ test with the STAT-PAK antibody test, and other new antibody tests becoming available.

The present study compares the cell-based IFNγ test with four different antibody tests as applied to both diseased alpacas from confirmed TB-breakdown (*M. bovis* culture-positive) herds and presumed TB-free alpacas from herds with no history of TB (based on TB-non-endemic regions of GB). The results provide relative Sensitivity and Specificity values for the individual tests and suggest test combinations to maximise testing efficiency in different herd scenarios.

METHODS

1. Study alpaca populations

(a) Diseased/Infected alpacas

For the purpose of this study, diseased alpacas were defined with visible lesions (VL) at post mortem and originating from known infected herds in which *M. bovis* had been isolated in laboratory cultures. A total of 59 skin-test-negative VL alpacas were identified from 10 culture-confirmed *M. bovis* herd breakdowns by applying the IFNγ and Chembio STAT-PAK rapid antibody tests (see below). Most VL therefore were test-positive to one or other test at the outset, a minority were test-negative using these two tests but slaughtered as dangerous contacts and found to be VL at *post mortem*.

In the study overall, not all tests could be carried out on all alpacas (dependent upon the tests agreed by the owner and DEFRA); **55 VL were tested with the IFNγ test**, **52 VL were tested with the various antibody tests** and **48 VL were tested with *both* the IFNγ and antibody tests.**

(b) TB-free alpacas

(i) TB-free herds in GB

Heparinized and clotted blood samples were collected from a total of 257 alpacas volunteered from 17 distinct premises across 10 counties known to have a historically low incidence of TB in cattle (Bedfordshire, Berkshire, Cambridgeshire, Cumbria, Hertfordshire, Kent, Northamptonshire, Northumberland, Nottinghamshire and North Yorkshire). Data on sex and age of the alpacas was not provided by all owners. However *of the data collected* there were 61 females with a mean age of 6.2±0.5 years, and 84 males with a mean age of 4.4±0.4 years, suggesting more males tested and of significantly lower age than females ($p<0.01$). Only alpacas >6 months old were recruited into the study. Initial tests carried out were the IFNγ and STAT-PAK antibody tests. Alpacas showing a positive response

to either PPD (purified protein derivative, or, tuberculin) in the IFNγ test (see below, 4(b) IFNγ ELISA, test positivity) or a positive to the STAT-PAK test were noted. Up to 2 test-positive alpacas per sampled herd, or 5% of the animals in the herd (whichever the greater) were removed for *post mortem* examination and mycobacterial culture to establish their true infection status and estimate the proportion of false positive results. **Both the IFNγ and the antibody tests were carried out on all 257 alpacas**.

(ii) TB-free herds in the USA

Serum samples belonging to **49 alpacas** from presumed TB-free herds (based upon epidemiological data) in the United States of America and supplied by IDEXX were included **for antibody tests** in this study. No gender or age information was available for this group of alpacas.

Overall, a total of **306 TB-free alpacas were therefore available for antibody testing,** while **257 TB-free alpacas were tested with *both* the IFNγ and antibody tests.**

(c) Alpaca blood samples

One heparinized (for IFNγ test) and one clotted (for serum antibody tests) blood sample was drawn from each alpaca (apart from some TB breakdown cases where only one or other [IFNγ or antibody] test had been requested). Heparinized blood samples for IFNγ testing were packed into temperature-controlled delivery boxes (DGP, UK) and delivered by overnight courier to AHVLA-Weybridge. Temperature-control of the sample is important to avoid extremes of temperature that could affect the viability of blood leukocytes (white cells) required for the IFNγ test. Serum samples do not require such temperature control, and could be packed and couriered to AHVLA-Langford (STAT-PAK test) or AHVLA-Weybridge (all other antibody tests). All blood samples were collected by AHVLA staff.

(i) Preparation of peripheral blood mononuclear cells (PBMC) for IFN-γ testing

Preliminary studies to develop a camelid IFNγ assay using whole heparinised blood (as used for cattle IFNγ testing) resulted in IFNγ responses too low to be of use in a diagnostic assay. However by separating the PBMC (essentially the white blood cells) out of the whole blood and using these in the assay we were able to show significant IFNγ responses. The separation of PBMC from every blood sample is laborious compared to the culture of whole blood, but this was an essential step for the camelid test. All procedures for the culture of blood cells were carried out in a Class II safety cabinet. PBMC were isolated from heparinized whole blood using density gradient (Histopaque 1077, SIGMA, UK)

centrifugation (800g, 40 minutes at room temperature [RT]). PBMC were then washed in Hanks Balanced Salt Solution (HBSS, Life Technologies, UK), re-suspended in culture medium (RPMI 1640 with Glutamax [Life Technologies, UK] supplemented with 10% foetal calf serum [SIGMA], penicillin and streptomycin [SIGMA], non-essential amino acids [Life Technologies] and 2-mercaptoethanol [Life Technologies]) and the number of viable PBMC counted. PBMC were then set to $2x10^6$/ml in culture medium.

(ii) Preparation of serum

Serum samples were allowed to clot in the collecting tube, and were then centrifuged (800g, 40 minutes at RT) in order to separate the serum fraction. Serum was decanted into a clean tube and frozen at -20^{o}C until required for antibody testing.

2. Mycobacterial culture and genotyping

Samples submitted for mycobacterial culture to AHVLA-Weybridge were treated according to the SOP (standard operating procedure) BA.385 “TB Diagnosis: TB culture and processing”. Briefly, ~20g of tissue was ground using a pestle and mortar, decontaminated with oxalic acid, centrifuged and the pellet resuspended in sterile phosphate-buffered saline (PBS) and centrifuged again. The homogenates were then resuspended again into PBS and sown onto solid and liquid culture media. Cultures were read at 6 weeks (breakdown herds and TB-free herds) and again at 14 weeks (TB-free herds only) in order to cover the time required for both *M. bovis* (6 weeks) and *M. microti* (14 weeks) to grow in culture. Positive cultures were harvested and heat-killed before genotyping.

Genotyping of *M. bovis* is a combination of two PCR (Polymerase Chain Reaction) techniques – Spoligotyping (Spacer Oligonucleotide typing) and VNTR (Variable Number Tandem Repeats). Spoligotyping targets the Direct Repeat (DR) region of the genome. This region contains a small sequence of DNA that is repeated several times throughout this region. Each repeated sequence is separated by a unique sequence of DNA (the spacer sequence). Spoligotyping amplifies this DR region and shows us which of these spacers is present or absent. Each different combination of present/absent spacers is then given a unique spoligotype number. VNTR targets several different loci found throughout the genome. Each loci contains a unique sequence of repeated DNA, with each repeat being directly next to its proceeding sequence (ie a string of repeated DNA sequence is formed). VNTR amplifies these loci and measures the length of the DNA generated. The number of repeated units of DNA can then be calculated to give the number of repeats for each loci (Smith et al., 2009).

3. IFNγ Test

(a) Stimulation of PBMC with mycobacterial antigens

PBMC stimulation was carried out in a sterile environment in 96-well culture plates (Life Technologies) in a volume of 200μl/well. For each individual alpaca, duplicate wells of PBMC were stimulated with bovine and avian tuberculins (PPDB and PPDA, Prionics, Lelystad, The Netherlands, both at 1:100 final dilution), and the peptide cocktail ESAT6/CFP10 (EC). EC antigens are found mainly in the pathogenic mycobacteria such as *M. tuberculosis* and *M. bovis* (but not in *M. microti*) and are used in national cattle IFNγ testing where a higher specificity is required. Sample positive and negative controls were pokeweed mitogen (PWM, SIGMA) and medium-only (un-stimulated cells), respectively, 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^{o}C, 5%CO_2 in a humidified incubator, after which cell-free supernatants were harvested and the duplicate wells pooled. This is a longer culture time than is used for the cattle whole blood IFNγ test, and is due to the difference of the kinetics of the IFNγ response between whole blood and PBMC cultured cells. Samples were stored at -20^{o}C until tested for IFNγ content by ELISA.

(b) IFNγ ELISA

The IFNγ ELISA was carried out using reagents from MABTECH (Sweden); for our purposes the kit for bovine/ovine/equine IFNγ (cat.no. 3115) was found to be cross-reactive with llama and alpaca IFNγ. ELISA plates (NUNC, Life Technologies) were coated overnight (4^{o}C) with 50μl/well coating antibody (7.5μg/ml in carbonate coating buffer, pH.9.6, SIGMA). The wells were emptied and then blocked for 1 hour at room temperature (RT) using 200μl/well of 4% bovine serum albumin (BSA) diluted in phosphate buffered saline (4%BSA/PBS). ELISA plates were washed 3 times using 0.1%Tween20/PBS wash buffer. Wells were emptied and samples added. For each alpaca duplicate wells of 50μl of un-stimulated, PPDB-, PPDA-, EC- and PWM-stimulated supernatants were added to the ELISA plate. ELISA positive (10ng/ml bovine recombinant IFNγ) and negative controls were also added in duplicate (50μl/well). ELISA plates were incubated at RT for 1 hour. Plates were washed and 50μl/well secondary antibody, diluted 1:2000 in 1%BSA/PBS added. Plates were incubated at RT for a further hour, washed, and 50μl/well streptavidin-alkaline phosphatase (MABTECH, 3310-10), diluted 1:500 added for one hour at RT. Plates were finally washed and 100μl/well 1-step PNPP substrate (ThermoFisher Scientific, USA) added for 30 minutes at RT. Plates were then read at 405nm (wavelength) on an ELISA reader. Data were transferred to Excel spreadsheets for analysis.

Test positivity was initially based upon cattle IFNγ test criteria:

(i) PPDB and PPDA - the optical density (O.D.) of the mean PPDB response should be 0.1. O.D. greater than the O.D. 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* (2009) showed these criteria applied to cattle provided a specificity of 96.5% and a sensitivity of 90.9%, while the GB Cattle Specificity Trial (http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/tb/documents/gifnspecificityreport.pdf) showed a specificity of 96.7%.

(ii) ESAT6/CFP10 (EC) peptide cocktail - the mean O.D. reading for EC should be 0.1 O.D. greater than the mean O.D. of un-stimulated cells (EC-nil>0.1). Vordermeier *et al* (2001) showed that these criteria applied to cattle provided a specificity of 98.8% and a sensitivity of 72.7%, while the GB Cattle Specificity Trial showed that a combination of EC-positivity and PPDB-positivity provided the higher specificity of 99.2%.

For a test to be valid also, the mean positive sample control (PWM) should be greater than 0.45 O.D.

4. TB STAT-PAK lateral flow antibody test

The TB STAT-PAK antibody test (VetTB STAT-PAK, Chembio Diagnostic Systems, Inc., Medford, NY, USA) uses selected mycobacterial antigens immobilised on a nitrocellulose strip and a blue latex signal detection system for rapid detection of antibodies as previously described (Lyashchenko *et al*., 2007).The test was carried out according to the manufacturer's instructions. One test (cassette) was used per alpaca. Tests were carried out at RT. 30μl serum was dispensed into the sample well of the cassette and allowed to soak into the wick. 3 drops of sample buffer (included in the kit) were then added to the sample well. The test was incubated at RT for 20 minutes and the results 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 IFNγ test, was used in the first instance to identify VL alpacas from *M. bovis*-confirmed TB breakdown herds for this study.

5. Dual Path Platform (DPP) lateral flow antibody test

The DPP test (Chembio Diagnostic Systems, Inc.) is based on the same principle as the STAT-PAK test, but consists of 2 nitrocellulose strips inside a cassette that allows independent delivery of test sample and antibody-detecting reagent (Greenwald *et al*., 2009). One cassette was used per alpaca. Tests were carried out at RT. 5μl serum was dispensed into the sample well followed by 3 drops of sample buffer. After 5 minutes a further 4 drops of sample buffer were added to the buffer-only well. Cassettes were incubated for a further 20 minutes. 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 cut-off values were established by receiver operator characteristic (ROC) analysis.

6. IDEXX antibody ELISA

The IDEXX ELISA test (IDEXX Laboratories, Inc., Maine, USA) was carried out according to the manufacturer's instructions, but with modification to detect camelid (not cattle) antibodies. ELISA plates pre-coated with mycobacterial antigens 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, UK) coupled to horse-radish-peroxidase (HRP) for detection of alpaca antibody in the serum samples. The anti-llama secondary antibody was titrated in preliminary experiments to optimise the concentration to be used in this study. Briefly, serum samples were diluted 1:50 in kit sample diluent. Samples (100μl/well in duplicate) were then added to the ELISA plate together with plate positive and negative controls, and ELISA plates incubated for 1 hour at RT. Plates were washed with 0.1%Tween20/PBS and drained. Anti-llama IgG-HRP diluted 1:50,000 in 1%BSA/PBS was then added (100μl/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 minutes at RT, then washed again. Substrate (supplied with kit, 100μl/well) was added to each well and the plates developed for 15 minutes at RT, and the reaction stopped by adding 100μl/well Stop Buffer (supplied with kit). Plates were read at 450nm on an ELISA reader and the data transferred to Excel spreadsheets for analysis. As the IDEXX test was carried out retrospectively on the samples, it was not used to identify positive/negative alpacas in the first instance. Test positive/negative cut-off values were established by receiver operator characteristic (ROC) analysis.

7. ENFERplex ELISA

The ENFERplex (multiplex) ELISA for bovine TB (Enfer Scientific, Co. Kildare, Ireland) was carried out at the Enfer Scientific Laboratories as previously described (Whelan *et al*., 2008) with some modifications for the detection of TB in camelids. Briefly, serum samples were diluted 1:500 in sample dilution buffer (Enfer Buffer G, Enfer Scientific). 50μl of the diluted sample was added per test well. The microtitre plates were then incubated at RT with agitation for one hour. The plates were washed with 1 X Enfer Wash Buffer (Enfer Scientific) and aspirated. The detection antibody (Protein G coupled to HRP, 1:5000 dilution in Enfer Buffer H, Enfer Scientific) was added (50μl/well) and the plates incubated

at RT for 30 minutes with agitation. The plates were washed as above and 50µl of substrate (50:50 substrate and diluent, Enfer Scientific) added per well. Signals as relative light units (RLU) were captured and data were extracted and analysed as previously described (Whelan *et al*., 2008). Positivity was calculated based upon the reactivity to each of the 7 specific antigens in the multiplex ELISA.

8. Data analysis and statistical modelling

(a) ROC Analysis

Data for each individual test, incorporating VL and TB-free cohorts, were analysed by Receiver Operator Characteristics (ROC) using Prism 4 (Graph Pad, San Diego, CA, USA) software. This method provides a graphical plot of test sensitivity versus false-positivity (1-specificity), and provides test positive/negative cut-offs using data from known diseased (VL) and control (TB-free) populations.

(b) Statistical Modelling

The object of this analysis was to evaluate and compare all tests for TB in the absence of a "gold standard" diagnosis. Bayesian analysis was applied to test data (for alpacas in TB-breakdown [i.e. not just VL] and TB-free populations) using WinBUGS software. The models employed assume the tests are conditionally independent and estimates positive and negative predictive values as well as estimates for sensitivity, specificity and prevalence (with 95% Bayesian credible intervals [BCI]).

RESULTS

1. Animals

(a) Diseased/VL alpacas

Culture data was available for 43/59 VL alpacas (as per standard DEFRA protocol, following the initial *M. bovis* culture confirmation of a TB breakdown not all VL alpacas were submitted for culture). The *M. bovis* genotypes within the study VL pool of alpacas for which we had data included spoligotypes 17:A (25 alpacas across 2 herds), 25:A (2 alpacas from one herd), and 11:A (1 alpaca). *M. microti* (Sp.34) was isolated from one alpaca. 7 VL alpacas submitted for culture were culture-negative. In total 59 VL alpacas from 10 distinct herds were entered into this study. The number of VL alpacas from each herd (plus the *index* case *M. bovis* spoligotype for each breakdown) were 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), 1 (11:A). Gender information was available for 42 of the VL alpacas: 28 VL were female and 14 VL were male. No age data was available.

(b) TB-free alpacas

Of the 257 TB-free alpacas initially tested with IFNγ and Chembio STAT-PAK rapid test, 19 were removed as test-positives for further investigation (post mortem and mycobacterial culture). There were 8 VL and 11 NVL. Of the 8 VL: 2 cases of *M. microti* infection were identified (one spoligotype 34, one spoligotype 19) following 14 weeks incubation.

Acid-fast bacilli were identified in both *M. microti* cases. In one of these 2 cases mineralized liver and mandibular lymph node lesions were also noted, while the other cases had more extensive lymph node lesions (hepatic, mesenteric, ileocaecal and pre-scapular) with a single lung lesion.

Of the other VL alpacas, tiny calcified liver lesions appeared common, one case with similar lesions also present in the mandibular and mesenteric lymph nodes. There was one case on an "unclassified mycobacterium" isolated from an alpaca that contained a single liver abscess. All were classified by histopathology as non-TB and were acid-fast-negative.

2. IFNγ test results

55 VL and 257 TB-free alpacas were available for IFNγ testing and entry into ROC analysis. Test-positivity was initially determined using cattle IFNγ test cut-offs (see Methods above, IFNγ ELISA, test positivity), which the ROC analysis subsequently showed to be an accurate reflection of sensitivity and specificity at this cut-off for alpaca responses. The test data were then re-expressed using ROC to provide higher specificity, with new cut-offs.

(a) <u>IFNγ test positivity using cattle IFNγ cut-offs</u>

Based upon cattle IFNγ test positive cut-off criteria (i.e. **PPDB-PPDA>0.1**; **EC-nil>0.1**), the number of positive responders in the 55 VL and 257 TB-free alpacas are shown in Table 2 with corresponding sensitivity and specificity values [and 95% confidence intervals, CI].

TABLE 2: IFNγ test sensitivity and specificity using cattle test cut-offs

	n/55	% Sensitivity	[95%CI]	n/257	% Specificity	[95%CI]
PPD+	**35/55**	**63.6%**	[50.9-76.3]	**28/257**	**89.1%**	[85.3-92.9]
EC+	**27/55**	**49.1%**	[35.9-62.3]	**26/257**	**89.9%**	[86.2-93.6]
EC+ PPD-	**8/55**	**14.5%**	[5.2-23.8]	**21/257**	**91.8%**	[88.4-95.2]
PPD+ *and* EC+	**19/55**	**34.6%**	[22-47.2]	**5/257**	**98.1%**	[96.4-99.8]
PPD+ *or* EC+	**44/55**	**80.0%**	[69.4-90.6]	**49/257**	**80.9%**	[76.1-85.7]

(N.B. PPD: PPDB minus PPDA)

The sensitivity and specificity values were lower for both PPD and EC as single tests compared to cattle data (test performance of BOVIGAM IFNγ test in cattle; PPD sensitivity 90.9%, specificity 96.5% [Schiller *et al*., 2009]; EC sensitivity 72.7%, specificity 98.8% [Vordermeier *et al*., 2001]).

Our study results suggest the lower specificity in SAC is 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 *M. bovis* in camelids (Oevermann *et al*., 2004; Zanolari *et al*., 2009), and as a member of the closely related TB Complex cluster of mycobacterial species, will induce positive responses to PPD (recently described for *M. microti* infection in cats, Rhodes *et al*., 2011).

We investigated the potential "masking" of PPDB IFNγ responses by high PPDA responses caused by exposure to other, non-TB Complex mycobacteria. We looked at alpacas that were "test-negative" but had both positive PPDB and positive 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 in this study originated) 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. Further, 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. 8 VL alpacas were positive to EC only, suggesting that ***not* to incorporate EC within any testing regime 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%), there is an associated loss of specificity (drops to 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+ alpacas (~85% of them) were negative to PPD, which could be due to exposure to an environmental mycobacterium, such as *M. kansasii* (which expresses these antigens and is known to elicit immune responses cross-reactive with *M. bovis* [Vordermeier *et al*., 2007]), or of course, undiagnosed *M. bovis* infection.

Next, the IFNγ data from 55VL and 257 TB-free alpacas were analysed using the ROC method for defined sensitivity, specificity and associated test cut-offs. ROC analysis of the observed test data (from Table 2) supported our initial choice of test cut-offs above in that, for the same specificities for PPD and EC shown in our alpaca cohorts, the cut-off readouts from the analysis were 0.1003 for PPD and 0.1008 for EC (as opposed to the 0.1 for both PPD and EC tests used above).

(b) Increasing the specificity of the IFNγ test

Using the ROC analysis data of the same 55 VL and 257 TB-free alpacas to increase the IFNγ test specificity to values closer those of cattle IFNγ testing (96.7%, GB Cattle Specificity Trial) provided the modified test values shown in Table 3.

TABLE 3: ROC analysis of IFNγ test for increased specificity

	n/55 % Sensitivity [95%CI]	**n/257 % Specificity [95%CI]**
PPD+	**16/55 29.1%** [17.1-41.1]	**8/257 96.9%** [94.8-99]
EC+	**16/55 29.1%** [17.1-41.1]	**8/257 96.9%** [94.8-99]
EC+ PPD-	**7/55 12.7%** [3.9-21.5]	**8/257 96.9%** [94.8-99]
PPD+ *and* EC+	**9/55 16.4%** [6.6-26.1]	**0/257 100%** [98.6-100]
PPD+ *or* EC+	**23/55 41.8%** [28.8-54.8]	**16/257 93.8%** [90.9-96.7]

(N.B. PPD: PPDB minus PPDA)

To achieve the higher 96.9% specificity shown for PPD and EC required an increased test cut-off for both PPD and EC as follows**; PPDB-PPDA>0.7272**, and **EC-nil>0.3797**. Application of these higher cut-offs for PPD and EC antigens reduced their sensitivity values by ~54% and ~40% respectively (Table 3 compared to Table 2). These cut-off values (and resulting lower sensitivities) may be deemed unrealistic, since, particularly in the case of PPD responses, the analysis offers cut-offs to mitigate for PPD "false-positive" responses in the TB-free cohort, where these responses may be real, may be relatively high, and may be driven by undiagnosed infection (e.g. *M. microti*). Such responses therefore cannot be simply removed by a simple change in cut-off value.

Interestingly, of those remaining EC-positive alpacas (i.e. positive using the higher test cut-offs) in the TB-free group, none were now positive to PPD, and **using a test criteria of positive to "PPD *and* EC" now provided 100% specificity**. But the corresponding sensitivity was poor, at 16.4% and unlikely to be adequate to identify infected herds. On the other hand, considering that all of the VL alpacas in this study were skin-test-negative, the IFNγ test, even at these stringent levels, could present a distinct improvement.

The advantage of IFNγ testing lies in its flexibility, and these data collectively may offer insight as to its potential application for camelids. For example, as a *High*

Specificity (low sensitivity) test for surveillance purposes using the "PPD *and* EC" positivity criteria (cut-offs PPDB-PPDA>0.7272; EC-nil>0.3797), and a *High Sensitivity* (low specificity) test for TB-confirmed herd clean-up situations, using the "PPD *or* EC" positivity criteria (cut-offs PPDB-PPDA>0.1003; EC-nil>0.1008).

As the IFNγ test also identifies *M. microti* infection, it would be important for mycobacterial culture to capture the infecting organism, to differentiate between notifiable *M. bovis* and non-notifiable *M. microti*. *M. microti* also causes clinical TB in SAC and therefore also requires disease-control in SAC, and consideration over zoonotic spread to humans (Niemann *et al*., 2000).

3. Serological tests for antibody responses

Serum samples were available from a total of 52 VL and 306 TB-free alpacas for antibody test ROC analysis. The STAT-PAK rapid test, being qualitative, is shown as number and % positive for the two alpaca cohorts. Similarly the ENFERplex provided an overall test positive/negative readout. Quantitative data for the DPP rapid test and the IDEXX ELISA were both subjected to ROC analysis and the test specificities set to 96.7% and 97.4% for **DPP (cut-off >9.05)** and **IDEXX (cut-off>0.34)** respectively.

TABLE 4: Antibody test sensitivity and specificity

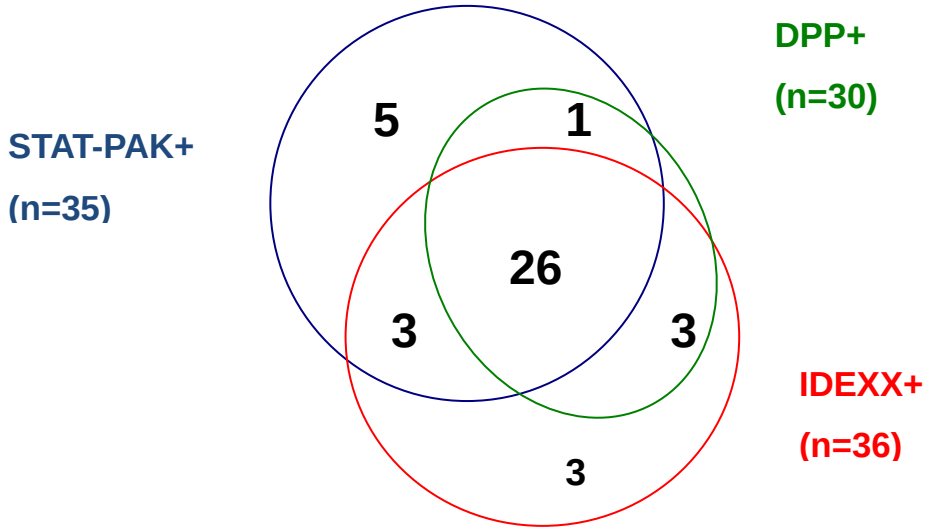
	N % Sensitivity [95%CI]	N % Specificity [95%CI]
STAT-PAK	**35/52** **67.3%** [54.5 – 80.8]	**8/306** **97.4%** [95.6 – 99.2]
DPP	**30/52** **57.7%** [44.3 – 71.1]	**10/306** **96.7%** [94.1 – 98.4]
IDEXX	**36/52** **69.2%** [56.7 – 81.7]	**8/306** **97.4**% [95.6 – 99.2]
ENFERplex	**32/48** **66.7%** [53.4 – 80.0]	**8/257** **96.9%** [94.8-99.0]

All the antibody tests showed high specificity, with STAT-PAK and IDEXX 95%CI ranging only slightly higher than the DPP and ENFERplex tests. However data from cattle has shown that antibody test sensitivity can be boosted by up to 30% in lesioned animals following the single comparative tuberculin skin test (Waters *et al*., 2011), and a boosting effect of the skin test upon STAT-PAK results in llamas was reported by Dean *et al* (2009). All of the VL alpacas in this study had been skin-tested, while none of the TB-free alpacas had received a skin test.

The data for STAT-PAK testing compare well with a recent analysis of 830 alpacas from GB breakdown herds (summarized in Alvarez *et al*., 2011) where of the 9.75% STAT-PAK-positive alpacas tested 76.3% were VL, 23.7% NVL, and 9.2% of the VL alpacas identified overall were STAT-PAK-negative.

We compared the capture of the 52 VL alpacas tested with 3 of the antibody tests (Figure 1 below) and found that (i) 41 out of 52 VL alpacas (78.8%) were captured using a combination of STAT-PAK and IDEXX antibody tests, (ii) the DPP test did not add extra value in this cohort since alpacas positive to DPP were positive to one or other antibody test, (iii) for the STAT-PAK and IDEXX tests there were 6 antibody-positive VL alpacas (15% of antibody-positives) that were positive to one test but not the other, therefore using both antibody tests together gave the best outcome in terms of VL capture by antibody tests in this study.

FIGURE 1: Capture of 52 VL alpacas using antibody tests



In a direct comparison between the IDEXX and ENFERplex ELISA tests, all but 3 ENFERplex-positive VLs were detected using the IDEXX test. All three ENFERplex+/IDEXX- alpacas were detected using other tests (two alpacas using the STAT-PAK, one using the IFNγ test).

Of the **11/52 antibody-negative VL alpacas** in this cohort there was IFNγ test data for 9 alpacas; of these 9, a further 5 were captured by a positive IFNγ test using *High Specificity* cut-offs (1 PPD+EC- alpaca, 4 EC+PPD- alpacas), while all 9 were identified by a positive IFNγ test using *High Sensitivity* cut-offs (3 PPD+EC- alpacas, 2 PPD+EC+ alpacas, 4 EC+PPD-alpacas).

Not performing the STAT-PAK test would have resulted in 6 VL alpacas escaping detection, only one of which would be identified using the IFNγ test (at *High Sensitivity*), while removal of the IDEXX test would have resulted in 6 VL alpacas escaping detection, but 5 of which would be detected using the IFNγ test (at *High Sensitivity*). This suggests that in terms of detecting diseased (VL) animals, the application of both the cell-based IFNγ and an antibody test would give the highest overall detection rate, and this data suggests that the combination of IFNγ and

STAT-PAK, might be better than combining IFNγ and IDEXX. However it must be remembered that VL animals were originally identified using IFNγ and STAT-PAK, and so by default this may appear the better combination of tests.

4. IFNγ and antibody combination testing for breakdown herds

ROC analysis cut-offs for the IFNγ and antibody tests were applied to the **48 VL and 257 TB-free alpacas for which both IFNγ and antibody test data were available** in order to compare test performance as single tests and in test combinations to illustrate potential options for their use in culture-confirmed TB breakdown situations. While we used as many animals as possible to generate IFNγ and antibody cut-offs in the ROC analyses above, between test comparisons could only be carried out on animals on which all tests had been conducted. Therefore single tests are shown again, as applied to these smaller groups of animals (i.e. 48 out of the 55 VL used for IFNγ, and 257 out of the 306 used for antibody test analysis) and then shown in various test combinations.

IFNγ results are shown using the *High Sensitivity* IFNγ cut-offs only (PPDB-PPDA>0.1003; EC-nil>0.1008), since in a breakdown situation the priority would be to detect as many infected animals as possible. ROC cut-offs for DPP and IDEXX tests were 9.05 and 0.34 respectively, as previously.

(a) <u>Antibody test combinations</u>

Table 5 shows the relative sensitivity and specificity values for single antibody tests and for antibody test combinations as applied to 48 VL and 257 TB-free alpacas for which IFNγ and antibody test data were available.

As in Table 4 above these antibody test data as stand-alone tests suggested high specificity and good sensitivity. Combining more than one antibody test suggested a significant increase in sensitivity at the expense of a small drop in specificity, e.g. sensitivity of STAT-PAK alone could be boosted from 68.8% to 81.3% by combining it with the IDEXX test, with a corresponding 1.9% drop in specificity. However even in combination, antibody tests alone did not capture all diseased alpacas; Table 5 shows that even if all four antibody tests were employed, **16.7% of the diseased alpacas (8/48 VL) animals would have been missed using antibody tests alone.**

TABLE 5: Antibody test combinations - sensitivity and specificity

	n/48 % Sensitivity [95%CI]	n/257 % Specificity [95%CI]
SINGLE TESTS		
STAT-PAK	33/48 **68.8%** [56.6-81]	6/257 **97.7%** [95.9-99.9]
DPP	29/48 **60.4%** [47.5-73.3]	8/257 **96.9%** [94.8-99]
IDEXX	34/48 **70.8%** [58.8-82.8]	6/257 **97.7%** [95.9-99.5]
ENFERplex	32/48 **66.7%** [53.4-80.0]	8/257 **96.9%** [94.8-99.0]
TEST COMBINATIONS		
STAT-PAK/IDEXX	39/48 **81.3%** [71-91.6]	11/257 **95.8%** [93.3-98.2]
STAT-PAK/ENFERplex	37/48 **77.1%** [65.2-89.0]	14/257 **94.6%** [91.8-97.4]
STAT-PAK/DPP	36/48 **75.0%** [63.6-86.4]	14/257 **94.6%** [91.8-97.4]
IDEXX/DPP	35/48 **72.9%** [61.1-84.6]	13/257 **94.9%** [92.2-97.6]
IDEXX/ENFERplex	37/48 **77.1%** [65.2-89.0]	12/257 **95.3%** [92.7-97.9]
ENFERplex/DPP	35/48 **72.9%** [61.1-84.6]	15/257 **94.2%** [91.3-97.1]
STAT-PAK/IDEXX /DPP/ENFERplex	40/48 **83.3%** [73.4-94.2]	25/257 **90.3%** [86.7-93.9]

(b) IFNγ and antibody test combinations

Here we illustrate IFNγ and antibody test combinations using the IFNγ test option of positivity to PPD *or* EC to maximise sensitivity, and using associated *High Sensitivity* cut-offs (PPDB-PPDA>0.1 and EC-nil>0.1).

Table 6 shows that combining one antibody test with the IFNγ test option offered significant increases in the sensitivity of VL detection, with correspondingly small decreases in specificity, compared to the IFNγ test alone. Notably, this **combination of the "PPD *or* EC" IFNγ test option with two antibody tests was able to detect all VL animals, providing 100% sensitivity.** This data supports the Venn diagram shown in Figure 1 above, in that (i) different antibody tests can detect different infected animals, and (ii) a combination of antibody and IFNγ tests is required for maximum detection of diseased animals. In this study, the "PPD *or* EC" IFNγ test option, together with the STAT-PAK and IDEXX antibody tests appeared to be the best combination.

TABLE 6 : "PPD *or* EC" IFNγ and antibody test combinations - sensitivity and specificity

TEST COMBINATIONS	VL alpacas (n=48)			TB-free alpacas (n=257)		
	n/48	Se %	95%CI	n/257	Sp %	95%CI
IFNγ/STAT-PAK	45	**93.8**	87.0-100	52	**79.2**	74.2-84.2
IFNγ/IDEXX	44	**91.7**	83.9-99.5	55	**78.6**	73.6-83.6
IFNγ/DPP	44	**91.7**	83.9-99.5	58	**77.4**	72.3-82.5
IFNγ/ENFERplex	45	**93.8**	87-100	58	**77.4**	72.3-82.5
IFNγ/STAT-PAK/IDEXX	48	**100**	[92.6-100]	57	**77.8**	72.7-82.9
IFNγ/STAT-PAK/DPP	48	**100**	[92.6-100]	60	**76.7**	71.5-81.9
IFNγ/STAT-PAK/ENFERplex	47	**97.9**	93.8-100	60	**76.7**	71.5-81.9
IFNγ/IDEXX/DPP	45	**93.8**	87.0-100	62	**75.9**	70.0-81.2
IFNγ/IDEXX/ENFERplex	44	**91.7**	83.9-99.5	62	**75.9**	70.0-81.2
IFNγ/ENFERplex/DPP	47	**97.9**	93.8-100	65	**74.7**	69.4-80

Se sensitivity; Sp specificity

5. Statistical modelling of IFNγ and antibody test data

Statistical analysis was attempted using test data from TB breakdown and TB-free alpacas. For TB-free alpacas however, none of the models were satisfactory as there were too few test-positives. For the TB breakdown herds we used a total of 139 alpacas (containing VLs, test-positives and test-negatives) from herds with confirmed breakdown during the time span of this project. The statistical analysis of this data however was un-interpretable due to low sample numbers giving wide 95% confidence intervals.

SUMMARY and DISCUSSION

This project has defined the performance of the IFNγ and antibody tests in SAC. The overall summary of the main findings are as follows;

1. 'More tools in the tool box' (= tests of defined performance) which will allow informed and evidence-based policy discussions between stakeholders.

2. In a breakdown herd situation both IFNγ and antibody tests could be applied with high sensitivity; a combination of IFNγ and antibody tests will offer an improved detection rate (higher number) of infected individuals. This would be achieved by accepting lower specificities.

3. Data from the TB-free cohort suggested that the IFNγ test specificity for *M. bovis* infection may be compromised by its detection also of *M. microti* infection.

4. The IFNγ data suggest that application of this test should depend on the outcome required. Thus for TB breakdown herds *High Sensitivity* test cut-offs using the "PPD *or* EC" IFNγ test option could be employed to eliminate infected animals from the herd. The specificity of the IFNγ test could be increased but at the expense of much-reduced sensitivity. However, with a potential 100% specificity when using the "PPD+ *and* EC+" IFNγ test option, the IFNγ test could be considered as a surveillance test, but sensitivity (16.4% in this study) would very likely need to be enhanced further to identify all infected herds. The target sensitivity for such a surveillance herd test could be modelled, Herd level sensitivity will be dependent on herd size and disease prevalence in an infected herd.

5. Combinations of more than one antibody test gave significantly higher sensitivities of VL detection, compared with single antibody tests, with correspondingly small decreases in specificity. Further combination of antibody tests (STAT-PAK and IDEXX, or STAT-PAK and DPP) with IFNγ could maximise sensitivity of VL detection. **N.B. since VL alpacas were originally identified following IFNγ and STAT-PAK testing of breakdown herds, some caution on IFNγ and STAT-PAK combined test sensitivity in the VL group must be applied.**

6. 'Head line' conclusions:

Application of these blood tests as individual animal test to maximise detection of the detection of infected animals plays to their strength (e.g. to clean-up infected herds, tracing operations, trade test), whilst application of these tests for surveillance (herd level test) is not a likely and probable application scenario without significant specificity improvements (see below for potential further research).

POTENTIAL USE AND FUTURE RESEARCH

1. Potential application scenarios (subject to a full appraisal of costs and benefits and discussions between stakeholders and government):

1. The anamnestic (i.e. boosted by skin test, see 2 below) STAT-PAK or IDEXX antibody test could be employed as a follow-up to the skin test, potentially offering the best combination of sensitivity and specificity for herd clean up, pre-/post-movement TB testing, pre-export testing, and also for tracings from infected herds.

2. To resolve TB outbreaks in known infected herds one could use skin and anamnestic StatPak (or IDEXX) serology as a minimum to lift restrictions, with additional voluntary IFNγ testing to boost Se, if the herd owner so wishes.

2. Potential future research:

1. Define tuberculin skin test performances using existing data from infected herds (and obtain more data from TB-free herds – most conveniently those that were included in this study) comparing the single intradermal comparative test (using PPDA and PPDB) with the single intradermal test (using PPDB measurement only) as a potential herd surveillance test.

2. Increase sensitivity of IFNγ test when results are based on positivity to PPD AND ESAT-6/CFP-10 by adding more such defined antigens.

3. Define *M. microti*-specific antigens to allow better differential diagnosis.

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