Development of 316v antibody enzyme-linked immunosorbent assay for detection of paratuberculosis in sheep

This paper (No. 30112015-00074-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in December 2015 in issue 34 (3) of the *Scientific and Technical Review*

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Summary

An enzyme-linked immunosorbent assay (ELISA) was developed and optimised using a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) antigen prepared from a C strain (316v) passed through a French press. The optimised assay was evaluated with a panel of sera from MAP infected ($n = 66$) and uninfected ($n = 1,092$) sheep. Animals in the MAP infected category were positive on either tissue culture or histopathology but were of unknown serum antibody status. The diagnostic performance and cost of the assay were compared with those of a commercial ELISA (IDEXX). At 99.8% diagnostic specificity the assay showed a diagnostic sensitivity of 23% (95% CI:

15.1–35.8) compared with 36.4% (95% CI: 25.8–48.4) for the commercial ELISA (McNemar's test: chi-square 5.82, $p < 0.05$). The sensitivities were 5.9% (95% CI: 1–26.9), 27.9% (95% CI: 14.7–45.7) and 35% (95% CI: 18.1–56.7), for low grade, paucibacillary and multibacillary lesion grades, respectively. The cost of the commercial assay kit was 2.7 to 5.2 times greater than that of the 316v ELISA for an equivalent number of tests, the multiple depending on the number of plates processed per run. For flock-level surveillance, to account for the lower sensitivity of the 316v ELISA compared with the commercial ELISA, sample sizes would be increased but the test cost would still be lower. The 316v assay will be useful for diagnosis of Johne's disease in sheep flocks, particularly in developing countries where labour costs are low relative to the cost of consumables.

Keywords

Antibody – Diagnosis – Paratuberculosis – Sensitivity – Serum – Sheep – Specificity.

Introduction

Johne's disease (JD) is a chronic granulomatous enteritis of ruminants, commonly seen in cattle and sheep. The disease also affects other domestic and wild ruminants. It is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Clinically infected animals can show progressive wasting, and intermittent diarrhoea, depending on the species. It is a production-limiting disease that results in economic losses, especially in infected dairy herds (1, 2) and the sheep industry (3). Diagnosis of JD in ruminants can be made by detecting the causative agent in infected hosts or by measuring the immune response to infection. Detection of MAP by laboratory culture followed by molecular probe-based methods requires a long turnaround time and is expensive. Detection of a host immune response, such as antibody in serum, is more suitable for large-scale testing at low cost per animal. A commonly employed method to detect antibody mediated immune responses is the enzyme-linked immunosorbent assay (ELISA).

The sensitivities of currently available ELISAs are low but specificity is generally high when tested on uninfected populations (4, 5, 6, 7). Sensitivity varies with the stage of disease; it is low in the early or subclinical stages and increases as the disease progresses to the clinical stage (8, 9, 10). The sensitivity increases with the age of the infected host, but the specificity decreases (11). Information on the accuracy of ELISA for subclinical infection as well as for different histopathological lesion types is not available. In most studies, the estimated diagnostic sensitivity of ELISA is 34% to 41%, with a specificity of 94.8% to 99.5%, in comparison to the reference standards of histopathological lesion detection or faecal culture of MAP (6, 12, 13).

Estimates of diagnostic accuracy for ELISA often vary substantially for tests of the same type and make (7). Furthermore, most of the commercial ELISAs are expensive, and they are not always affordable in developing countries. This discourages the implementation of disease control programmes. There is a need to develop an inexpensive ELISA with high sensitivity and specificity for large-scale surveys, particularly in developing countries where ruminants play an important role in the subsistence agriculture economy. The aim of this study was to develop a relatively low-cost assay for the diagnosis of paratuberculosis that could be applied in developing countries.

Materials and methods

Optimisation

Optimisation reagents

French-pressed antigen prepared from a purified culture of the C strain 316v of MAP (Elizabeth Macarthur Agricultural Institute, New South Wales [NSW], Australia) was used for assay development and evaluation. The protein content of the antigen was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12% precast Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories Inc., USA). The protein concentration was estimated

using a spectrophotometer (NanoDrop 1000; Thermo Scientific, Rockford, USA) at 280 nm. Three plate-blocking reagents were tested: 1% v/v fetal calf serum (FCS) (GIBCO®, Life Technologies, Victoria, Australia) in phosphate-buffered saline (PBS) Tween (0.05% v/v) (PBST); 5% w/v skim milk (SM) (Diploma, NSW, Australia) in PBST; and PBST alone. Four different types of diluent were also tested: 1% w/v SM in PBST; 1% w/v ova albumin (OA) (Sigma, St. Louis, USA) in PBST; 1% v/v FCS in PBST; and PBST alone. The two conjugates tested were horseradish peroxidase (HRP)-labelled rabbit anti-sheep polyclonal antibody (PAb) (KPL Laboratories Inc., USA) and HRP-labelled mouse anti-sheep monoclonal antibody (MAb) (Sigma, St. Louis, USA), clone GT-34. Three types of microplate were tested for efficient antigen binding: Nunc MaxiSorp (Roskilde, Denmark); Immulon (Thermo Scientific, Rockford, USA); and Costar (Corning Inc., Corning, New York, USA).

Control sera

Positive and negative control sheep sera were obtained from an experimental trial at the University of Sydney (10). The negative control (NC) serum was obtained from sheep that were unexposed to MAP and had tested negative on faecal culture, culture of biopsied tissue, histopathology and Institut Pourquier ELISA (Montpellier, France). The positive control (PC) serum with a known MAP-specific antibody response was obtained from sheep immunised with the Gudair® vaccine (Gudair®, Pfizer Australia Pty Ltd, NSW, Australia).

Serum adsorption

To reduce cross reactions due to the exposure of hosts to environmental mycobacteria, all serum samples were adsorbed with heat-killed *Mycobacterium phlei* (University of Sydney, NSW, Australia). The optimum concentration of *M. phlei* was determined using a panel of archival sera from uninfected sheep ($n = 23$) that tested negative in the Institut Pourquier ELISA and sheep positive on MAP faecal culture ($n = 23$) that tested positive in this ELISA. The panel of sera contained archival sera stored for several years at the

Faculty of Veterinary Science, University of Sydney. These sera were tested previously and categorised accordingly using what was then the Institut Pourquier ELISA, which is now the IDEXX ELISA. The serum was diluted in 0.1% v/v FCS in PBST containing heat-killed *M. phlei* (0.8 and 1.3 mg/ml w/v were tested) and adsorbed overnight at 4°C with constant end-to-end shaking. The protein concentration was determined from the weight of cells per unit volume of diluent. The adsorbed serum was centrifuged at 2,500 × *g* for 10 min at room temperature (RT) to separate the supernatant from the particulate *M. phlei* and then used for ELISA.

General reagents

Carbonate buffer (0.1 M sodium carbonate, pH 9.6) was used as the coating antigen; substrate 3′, 3′, 5′, 5′ tetra-methyl-benzidine (TMB) (Thermo Scientific, Rockford, USA) was used for development of the chromogenic reaction; 2 M sulphuric acid was used to stop the chromogenic reaction; and FCS (0.1% v/v) in PBST was used as the conjugate diluent. Reverse osmosis purified water with Tween 20 (RO Tween water) (0.05% v/v) was used as the wash buffer.

Assay evaluation

The optimised 316v ELISA was evaluated using a set of serum samples from MAP infected and uninfected sheep. The PC and NC sera were used for plate controls.

Sera for estimation of diagnostic specificity

Specificity was defined as the proportion of uninfected animals yielding a negative result in the test. A total of 1,092 sera used for estimation of diagnostic specificity were obtained from: sheep from Western Australia ($n = 1{,}045$) that were putatively free of MAP infection at the time of sample collection (14, 15); sheep ($n = 10$) from among the negative controls in an experimental infection trial based at the University of Sydney (10); and sheep ($n = 37$) from farms in NSW, Australia, that had been monitored and tested negative for more

than three consecutive years (MN3) under the Market Assurance Programme for sheep (Animal Health Australia).

Sera for estimation of diagnostic sensitivity

Diagnostic sensitivity was defined as the proportion of infected sheep determined to be truly positive by tissue culture or histopathology that were positive in the test. A total of 66 sera from infected sheep were used for estimating diagnostic sensitivity (10). Infected sheep were categorised into four groups according to previously described histopathological lesion grades (16, 17, 18): no lesion or low grade (type 1 or 2) ($n = 17$); early paucibacillary (type 3a) ($n = 18$); multibacillary (type 3b) ($n = 20$); and paucibacillary (type 3c) ($n = 11$). Diagnostic sensitivities for paucibacillary (3a and 3c) and multibacillary (3b) lesion types were analysed.

Decision-limit quality control

Individual sera with a coefficient of variation (CV) for paired replicates of > 15% were re-tested. All the results from re-tests with a CV of < 20% were used in the analyses.

Between-plate variation

Repeatability was expressed as the CV of the NC and PC. In addition, a total of 90 sera (negative [$n = 45$], weak positive [$n = 10$], medium positive [$n = 25$], and strong positive [$n = 10$]) were tested in duplicate on three different days using independently prepared dilutions.

IDEXX ELISA methodology

The IDEXX ELISA (Montpellier, France) was performed according to the manufacturer's instructions. Briefly, serum samples, including positive and negative controls, were diluted (1:20) in a buffer containing an extract of *M. phlei*, mixed with a shaker and incubated for 1 h at RT. The adsorbed serum samples (100 µl) were dispensed to each well of an ELISA plate pre-coated with antigen, mixed with a shaker and incubated for 45 min at RT. The plates were hand-washed three times with wash buffer, and a peroxidise-labelled monoclonal

anti-ruminant IgG conjugate (100 µl) diluted in dilution buffer (1:100) was added to each well and incubated for 30 min at RT. The plates were washed as above and 100 µl of TMB substrate was added and incubated for 10 min in the dark. The chromogenic reaction was stopped by adding 100 µl of stop solution, and the optical density (OD) values were read at 450 nm using a plate reader (Multiskan Ascent, Thermo Scientific, Victoria, Australia).

ELISA result interpretation

The ELISA results from both the 316v and the IDEXX ELISA were interpreted as the signal of the test sample as a proportion of the positive control, corrected for the negative control (sample-to-positive; SP%), according to the formula: SP%= (($OD_{450\ nm}$ of test sera − $OD_{450\ nm}$ of NC) ÷ ($OD_{450\ nm}$ of PC − $OD_{450\ nm}$ of NC)) × 100

For the 316v ELISA, an SP% cut-point was chosen for optimum sensitivity while maintaining a high specificity. For the IDEXX ELISA, test sera with an SP% ≥ 55% were considered positive, according to the manufacturer's instruction. Test sera with SP% > 45% and < 55% in the IDEXX ELISA were considered as suspect and were re-tested. The re-test values were used for analysis.

Cost of 316v ELISA in comparison with IDEXX ELISA

A comparison of the cost of the 316v and the IDEXX ELISA was calculated in Australian dollars (A$). For the 316v ELISA, the retail cost of laboratory supplies, reagents, antigen and antibodies was obtained, based on testing an equivalent number of plates to those in an IDEXX ELISA kit, in Australian dollars using data for 2013. The labour differential between the in-house test and the commercial kit was determined and costed for Australian labour costs.

Statistical analyses

Statistical analysis was performed using GenStat 12.1 (VSN International Ltd., UK) and GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, USA) software. Diagnostic estimates were analysed using Epitools (Survey Tool Box, AusVet Animal Health Services,

Australia). The linear relationship of the OD values obtained from the 316v ELISA performed on three different days was analysed. McNemar's test for paired observations was used to assess the agreement between the assays with a chi-squared statistic, and $p < 0.05$ was considered to show a significant difference between the assays. The OD values were used for analysis of the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC_{ROC}). The ROC curve was analysed to examine the discriminatory power of the assay to separate the infected and uninfected groups, but not to set the cut-point.

Results

Analysis of antigen

The French-pressed crude antigen prepared from the 316v strain of MAP was analysed by SDS-PAGE, which revealed separation of three strong bands and many lesser bands. The three major bands were of molecular mass 30, 60 and 72 kDa. All the protein bands were within the range 10 to 95 kDa (Fig. 1). The presence of multiple bands suggests that the MAP 316v antigen used in this assay is a complex protein mixture.

Optimisation

Assay optimisation was performed on the basis of the signal-to-noise (SN) ratio obtained from the PC and NC serum samples. Suitable parameters for serum diluent, serum dilution, conjugate, conjugate diluent, conjugate dilution, microplate, plate-blocking reagent, antigen concentration and the amount of *M. phlei* protein required for serum adsorption were identified and optimised. The plates were blocked for 30 min at RT; antigen–antibody and antibody–conjugate reactions were incubated for 1 h at RT, and the chromogenic reaction was stopped after 20 min of incubation in the dark. The volumes of reagent used per well were 50 µl (antigen, serum, conjugate and stop solution) and 100 µl (TMB substrate and plate-blocking reagent). The plates were washed five times with RO Tween water using a plate washer (Tecan, Aim Lab, Victoria, Australia), and OD was read at 450 nm

using a plate reader (Multiskan Ascent, Thermo Scientific, Victoria, Australia). Note that hand-washing was also acceptable (data not shown).

At 1 µg/ml of antigen, 1:100 serum dilution and 1:25,000 polyclonal conjugate dilution, 1% v/v FCS in PBST was identified as a suitable serum diluent as well as a plate-blocking reagent. However, the maximum SN ratio achieved was 8.9 (Figs 2a, 2b and 2c). Therefore, the polyclonal conjugate was replaced by a monoclonal conjugate. The change in conjugate increased the OD values and therefore the SN ratio (Fig. 2d). With the use of monoclonal conjugate, the amount of FCS used as serum diluent was reduced without adversely affecting the SN ratio (Fig. 3a). Among the three brands of microplate, Nunc MaxiSorp was most suitable (Fig. 3b). The maximum SN ratio was achieved at 2 µg/ml antigen and 1:100 serum dilution (Fig. 3c). The ability of the assay to discriminate uninfected from infected animals was greatest when 1.3 mg/ml of *M. phlei* protein was used (Fig. 3d).

Optimised 316v ELISA format

The optimised 316v ELISA format is summarised as follows. Briefly, 50 µl per well of 316v antigen (2 µg/ml) in carbonate buffer was coated onto flat-bottom 96-well Nunc MaxiSorp microplates and incubated overnight at 4°C. The following morning, the plate was machine-washed five times (or hand-washed five times) with RO Tween water and then blocked with 100 µl/well of a mixture of 1% v/v FCS in PBST at RT (range 22–24°C) for 30 min. The plate was washed as above.

The serum was diluted (1:100) in diluent (0.1% v/v FCS in PBST) containing 1.3 mg/ml of heat-killed *M. phlei* and adsorbed overnight at 4°C with constant end-to-end shaking. The adsorbed serum was centrifuged at 2,500 × *g* for 10 min at RT to separate the supernatant from the particulate *M. phlei*. The adsorbed serum supernatant (50 µl) was added to the required wells and incubated for 1 h at RT. The plate was washed as above prior to the addition of HRP-labelled mouse anti-sheep monoclonal conjugate (Sigma, St. Louis, USA) (50 µl; 1:40,000) in diluent (0.1% v/v FCS in PBST), and then incubated for

1 h at RT. The plate was washed as above and 100 µl of TMB substrate added. The reaction was incubated for 20 min in the dark after which the chromogenic reaction was stopped by the addition of 50 µl of 2 M sulphuric acid. The OD was measured at 450 nm using a plate reader.

Evaluation of the 316v ELISA

Diagnostic estimates of the 316v ELISA

The mean OD ± standard error of the mean (SEM) of the infected group (0.242 ± 0.021) was significantly higher ($p < 0.001$) than that of the uninfected group (0.117 ± 0.001). Two test sera from the uninfected group produced consistently high OD values after repeated testing. The AUC_{ROC} ± standard error (SE) of the 316v ELISA was 0.84 ± 0.031 (95% confidence interval [CI]: 0.774–0.896), suggesting a good ability to discriminate infected animals from uninfected. An SP% cut-point of 20 was chosen to obtain diagnostic specificity of 99.8% (95% CI: 99.3–99.9). The diagnostic sensitivity for no lesion or low-grade histopathological lesion was 5.9% (95% CI: 1–26.9). The sensitivity for the paucibacillary and multibacillary groups was 27.9% (95% CI: 14.7–45.7) and 35% (95% CI: 18.1–56.7), respectively. The overall sensitivity at the 20% SP cut-point was 23% (95% CI: 15.1–35.8).

Repeatability

The CV for the mean OD values of the positive and the negative controls was 16.8% and 9.8%, respectively. The results from 90 sera with negative, weak positive, medium positive and strong positive MAP-specific antibody responses that were tested on three different days with independent serum dilutions and reagents showed strong linear relationships between the results on days one and two ($p < 0.0001$, $r^2 = 0.983$), days one and three ($p < 0.0001$, $r^2 = 0.979$) and days two and three ($p < 0.0001$, $r^2 = 0.983$).

Diagnostic estimates of the IDEXX ELISA

The mean OD ± SEM of the infected group (0.578 ± 0.052) was significantly higher ($p < 0.001$) than that of the uninfected group (0.103 ± 0.002). The SP% values of three serum samples were in the suspect range (45–55); they were re-tested, and the re-test SP% were used for analysis. The AUC_{ROC} ± SE of the assay was 0.92 ± 0.021 (95% CI: 0.873–0.958), suggesting a very good ability to discriminate infected animals from uninfected. At the 55% SP% cut-point recommended by the manufacturer, the diagnostic specificity was 99.8% (95% CI: 99.5–100).

Among different histopathological lesion types, sensitivity was lowest in sheep with no lesion or low-grade lesions, at 5.9% (95% CI: 1–26.9). The sensitivity values for the paucibacillary and multibacillary groups of sheep were 51.7% (95% CI: 34.4–68.6) and 41% (95% CI: 21.9–61.3), respectively. The overall sensitivity at the 55% SP% cut-point was 36.4% (95% CI: 25.8–48.4).

Assay agreement

At equivalent diagnostic specificity (99.8%), the 316v ELISA showed apparently lower overall sensitivity than the IDEXX ELISA. McNemar's chi-square test with Yates' correction revealed that the IDEXX ELISA was significantly more sensitive than the 316v ELISA (chi-square statistic = 5.82, $p < 0.05$). However, both assays showed similar trends of sensitivity across the range of different histopathological lesion grades.

Cost of 316v ELISA in comparison to IDEXX ELISA

The cost of reagents and disposables for the 316v ELISA to test one plate was A$35.48 (Table I). The retail cost of a five-plate IDEXX ELISA kit was A$1,125 (i.e. A$225 per plate). However, the additional cost of consumables such as pipette tips and a waste bin that are not provided with the kit resulted in a final cost of A$236 per plate. This estimate ignores the costs of laboratory overheads and labour, which may vary substantially depending on the laboratory and

the country. The 316v ELISA requires an extra step when compared with the commercial ELISA. The estimated extra labour time to perform the 316v ELISA is approximately 1 h and is not markedly different if one or five plates are tested. Using an Australian rate of A$51.58 per hour for a skilled technical officer, the total cost of the 316v ELISA is A$35.48 + A$51.58 = A$87.06 per plate when one plate is tested or [(5 × A$35.48) + A$51.58]/5 = A$45.80 per plate when five plates are tested. Thus, the cost of the IDEXX ELISA is 2.7 to 5.2 times greater than that of the 316v ELISA when differential labour inputs are considered in the Australian context. These multiples would be greater in countries with lower unit labour costs.

Discussion

A relatively inexpensive, adsorbed ELISA was developed that has comparable specificity to but lower sensitivity than that of a commercial ELISA (IDEXX). It may be useful for diagnosis and control of JD in sheep.

Published estimates of the sensitivities of MAP antibody assay are sometimes based on the testing of small numbers of sera and are limited to overt clinical cases of JD (5, 19). The evaluation process in this study involved a sufficiently large number of sera for specificity estimates and a range of sera that were representative of different stages of infection for sensitivity estimates. The evaluation process was guided by recently published consensus-based reporting check-list items in partial compliance with the Standards for Reporting of Diagnostic Accuracy (STARD) for tests under evaluation (20). There were several factors determining the diagnostic sensitivity. First, the cut-point (SP%) was chosen to achieve a very high specificity, which concurrently reduced the sensitivity. Second, unlike other studies, the panel of sera used in this study was from a spectrum of subclinical and clinical cases of ovine JD, which were characterised by the histopathological lesions: no or low-grade (1–2) lesion, early paucibacillary (3a) lesion, multibacillary (3b) lesion and paucibacillary (3c) lesion. Finally, although the majority of animals in the study were defined as infected on the basis of histopathological

lesions consistent with paratuberculosis, some animals were defined as infected on the basis of the isolation of MAP by culture from the relevant tissue, irrespective of histopathological lesions. The diagnostic sensitivity estimate of the 316v assay was similar to that of a Pourquier ELISA kit reported in a study where the performance of the Pourquier ELISA was compared with that of the agar gel immunodiffusion test (21).

In this study, the authors determined the influence of the stage of disease on diagnostic sensitivity. The highest diagnostic sensitivity estimate obtained by the 316v ELISA was from animals with multibacillary (3b) lesions, followed by early paucibacillary (3a) lesions and paucibacillary (3c) lesions. This finding is consistent with the findings from other studies which report that clinical cases are more efficiently detected by such assays (9, 22). However, the IDEXX ELISA showed a higher sensitivity for the paucibacillary group than for the multibacillary group, indicating its superior ability to detect early stage infection when compared with the 316v ELISA. The higher serum dilution (1:100) used in the 316v ELISA in comparison to the IDEXX ELISA (1:20) may also have contributed to the lower sensitivity of the former. Sheep with no or low-grade lesions had the lowest diagnostic sensitivity estimates in both assays, suggesting that these assays are less efficient for detecting subclinical than clinical infection.

The World Organisation for Animal Health (OIE) guidelines for assay validation suggest that a CV < 20% for raw OD values of positive and negative controls is indicative of adequate repeatability of an assay. The CV of the positive control (16.8%) within the assays was higher than that of the negative control (9.8%). These CVs were both within the allowable limit of 20% in the OIE standards. A plausible explanation for the higher CV for positive controls in this study may be the increased amount of analyte in positive sera, which is more likely to produce slightly different degrees of reaction each time it is tested; this situation is less likely for negative control sera without analyte. The major factors influencing ELISA repeatability are the pH, room and buffer temperatures, reagent preparation method, reagent

pipetting error (23) and the technician. The assay may show higher variability in a diagnostic laboratory where more than one staff member is involved, a situation which requires proper training and standardisation of procedures.

Selection of the SP% cut-point is influenced by the intended purpose of the assay being developed. The authors chose an SP% cut-point of 20% because it conferred the optimal balance of very high diagnostic specificity with moderate sensitivity. Studies have suggested that when an assay is employed under different circumstances the cut-point may have to be reviewed, depending on the relative cost of misclassification and the distribution of ELISA OD readings for the population under investigation (24). Therefore, this assay will remain subject to continuous validation according to the requirements of the study.

Both assays showed good ability to discriminate infected from uninfected animals, and a similar trend of sensitivities among different histopathological lesion types. However, the McNemar's test showed that the IDEXX ELISA was significantly more sensitive than the 316v ELISA. The reasons are unclear but may relate to differences in the type of antigen, the type of conjugate and serum dilution rates. The commercial kit contains non-disclosed components, so direct comparisons cannot be made.

Application of ELISA to detect infection at flock level may require the testing of large numbers of samples. Sample size can be adjusted to account for test sensitivity, to achieve the desired sensitivity and confidence of detection at flock level. To account for the lower sensitivity of the 316v ELISA compared with the IDEXX ELISA, the sample size would need to be increased by a factor of 1.6 (based on the average sensitivities of the two tests), other things being equal. Given the substantially lower cost of the 316v assay (2.7 to 5.2 times lower), the total cost would be less than if the commercial ELISA were used.

Conflict of interest

We declare that there is no conflict of interest in regard to the findings from this study.

Acknowledgements

This work was supported by Meat and Livestock Australia and by the Cattle Council of Australia, Sheepmeat Council of Australia and WoolProducers Australia through Animal Health Australia. Mrs Anna Waldron, Mrs Ann-Michele Whittington and Ms Nicole Carter of the Farm Animal Health group provided assistance in accessing archival sera and ELISA reagents.

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Table I
Cost evaluation of the 316v ELISA for testing 92 samples (one plate)

Consumables	Retail cost in A$	Unit of consumables	Unit cost in A$	Quantity required for testing one plate of samples	Cost in A$ for testing one plate of samples
Plate: 60 number (Nunc MaxiSorp)	308.45	plate	5.14	1 plate	5.14
TMB, 400 ml (Thermo Scientific)	233	ml	0.58	10 ml	5.83
PBS, 200 tablets (Amresco)	76	tablet	0.38	1 tablet	0.38
Tween 20, 500 ml (Sigma)	58	ml	0.12	3 ml	0.35
FCS, 1 × 500 ml (Gibco)	280	ml	0.56	0.2 ml	0.11
MAb, clone GT 34, 1 ml (Sigma)	308.70	ml	308.70	0.005 ml	1.54
Sulphuric acid, 1,000 ml (Ajax)	139	ml	0.14	5 ml	0.70
Pipette tips, 20 boxes (Finntip)	120	box	6.00	2 boxes	12.00
Waste bin, small, 1 number	5	bin	5.00	1 bin	5.00
French pressed 316v, 4.57 mg (Elizabeth Macarthur Agricultural Institute, NSW, Australia)	500	mg	109.40	0.012 mg	1.31
M. phlei, 1,000 mg*	50	mg	0.05	62.40 mg	3.12
Total cost of testing 92 samples					35.48

A$: Australian dollars

FCS: fetal calf serum

PBS: phosphate-buffered saline

TMB: tetra-methyl-benzidine

* Takes into account the costs of producing a batch of *M. phlei*, including cultures, centrifugation, freeze drying, labour and overheads. *M. phlei* can also be purchased commercially from Allied Monitor Inc. (USA) at A$32 per 100 mg, and is available in larger volumes as required. Using a base rate of A$32/100 mg would add A$16.48 to the cost per plate, for a total cost of A$51.33. The costing of the ELISA makes some assumptions: the laboratory is already fitted with common equipment, including pipettes, a centrifuge, a plate washer (although this is not necessary because hand-washing will work), a plate shaker and a plate reader

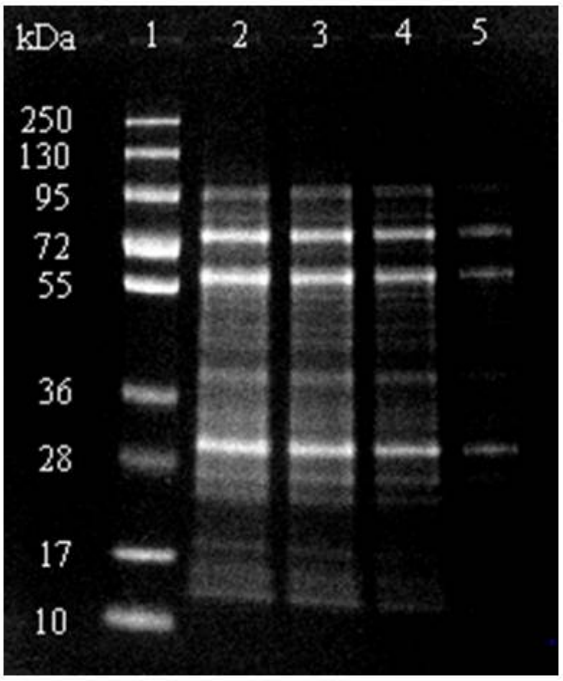
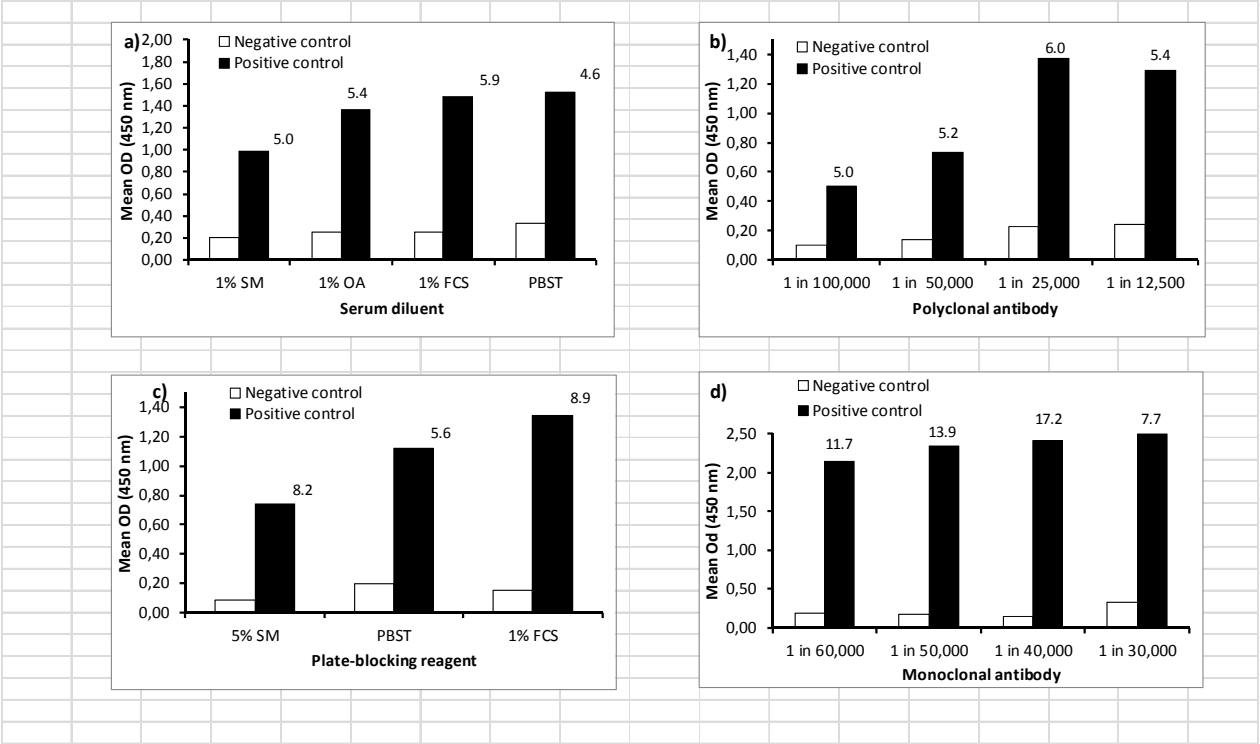


Fig. 1
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of crude *Mycobacterium avium* subspecies *paratuberculosis* (MAP) 316v antigen
Lane 1: Page Ruler™
Lane 2: 20 µl 316v (20 µg)
Lane 3: 15 µl 316v (15 µg)
Lane 4: 10 µl 316v (10 µg)
Lane 5: 5 µl 316v (5 µg)



FCS: fetal calf serum

OD: optical density

PBS: phosphate-buffered saline

PBST: PBS-Tween

Fig. 2

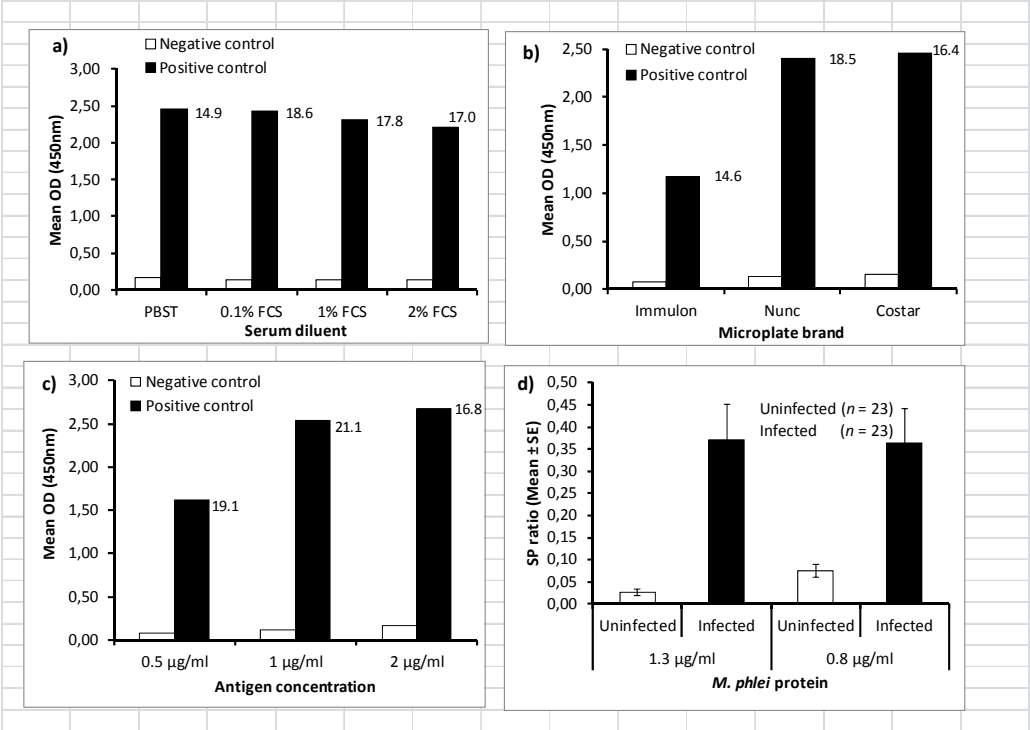
Selection of serum diluent, plate-blocking reagent and conjugate dilution

a) Selection of serum diluent (SM, skim milk; OA, ova albumin): antigen (1 µg/ml), serum (1:100), *Mycobacterium phlei* (0.8 mg/ml), polyclonal conjugate (PAb) (1:25,000) and conjugate diluent (0.1% FCS in PBS-Tween

b) Titration of PAb: antigen (1 µg/ml), serum (1:100), serum diluent (1% FCS in PBST), *M. phlei* (0.8 mg/ml), conjugate diluent (0.1% FCS in PBST)

c) Selection of plate-blocking reagent: antigen (1 µg/ml), serum (1:100), serum diluent (1% FCS in PBST), *M. phlei* (0.8 mg/ml), PAb (1:25,000) and conjugate diluent (0.1% FCS in PBST)

d) Titration of monoclonal conjugate (MAb): antigen (1 µg/ml), plate blocking (1% FCS in PBST), serum (1:100), serum diluent (1% FCS in PBST), *M. phlei* (0.8 mg/ml) and conjugate diluent (0.1% FCS in PBST). Numerals on top of bars are signal-to-noise (SN) ratios



FCS: fetal calf serum

OD: optical density

PBS: phosphate-buffered saline

PBST: PBS-Tween

Fig. 3

Selection of microplates, optimisation of serum diluent, antigen concentration and serum absorption

a) Titration of FCS as serum diluent: antigen (1 µg/ml), plate blocking (1% FCS in PBST), serum (1:100), *Mycobacterium phlei* (0.8 mg/ml), monoclonal antibody (MAb) (1:40,000) and conjugate diluent (0.1% FCS in PBST)

b) Plate selection: antigen (1 µg/ml), plate blocking (1% FCS in PBST), serum (1:100), serum diluent (0.1% FCS in PBST), *M. phlei* (0.8 mg/ml), MAb (1:40,000); conjugate diluent (0.1% FCS in PBST)

c) Antigen titration: plate blocking (1% FCS in PBST), serum (1:100), serum diluent (0.1% FCS in PBST), *M. phlei* (0.8 mg/ml), MAb (1:40,000) and conjugate diluent (0.1% FCS in PBST)

d) Serum absorption: antigen (2 µg/ml), plate blocking (1% FCS in PBST), serum (1:100), serum diluent (0.1% FCS in PBST), MAb (1:40,000) and conjugate diluent (0.1% FCS in PBST). Numerals on top of bars are signal-to-noise (SN) ratios