liveexport

Evaluation of Diagnostic Assays for *Chlamydophila abortus* in Australian Export sheep

Final Report

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Abstract

The Complement Fixation Test (CFT) is the only assay used in Australian laboratories for the detection of antibodies to *Chlamydophila abortus* (the agent of ovine enzootic abortion). OIE recognises the poor specificity of the CFT due to cross-reactivity, resulting in significant numbers of non-infected sheep deemed positive and hence being rejected for export. A pilot study determined the diagnostic sensitivity and specificity of several assays, and the best commercial ELISA was selected for further investigation. A panel of Australian sheep sera was tested using this assay in three laboratories. The Institut Pourquier *Chlamydophila abortus* antibody ELISA gave repeatable and reproducible results with a sensitivity equivalent to the CFT, but with a higher specificity. Here we recommend that the ELISA should be included in export test protocols as an alternative to the CFT, which would result in less sheep being excluded unnecessarily from export. This would lower the cost to industry and increase confidence in market access.

Executive summary

Australia exports sheep for breeding purposes and export protocols require that individual animals be tested for exposure to *Chlamydophila abortus,* the agent of Enzootic Abortion of Ewes (EAE). The Chlamydia Complement Fixation Test (CFT) is one of the required components of pre-export health certification for sheep being prepared for export to one of our major trading partners, China.

To qualify for export to China, each animal must test negative, at a serum dilution of 1 in 4. Australia claims freedom from EAE (Animal Health Australia[1]) but Australian sheep sera often give positive reactions in the Complement Fixation Test (CFT), which excludes these animals from export. It is well-recognised that the CFT has poor specificity due to cross-reactions to other chlamydia and *Acinetobacter* (OIE[2]).

Using this assay for export testing will continue to unnecessarily exclude non-infected animals from export. A more specific assay would reduce the testing burden of selecting excess animals to allow for those that will not pass the stringent health certification requirements of some importing countries.

A pilot study undertaken in 2005 found that the commercially available Institut Pourquier *Chlamydophila abortus* antibody ELISA was a good candidate demonstrating equivalent sensitivity to the complement fixation test but with higher specificity.

A larger study tested a panel of 1,562 Australian sheep sera and found that the percentage of samples testing positive in the ELISA was 0.3% and 3.7% at two separate laboratories. The percentage of samples testing positive in the CFT at 1 in 4 at the two laboratories was 17% and 11% respectively. There is a statistically significant difference between the results obtained with the Institut Pourquier *Chlamydophila abortus* antibody ELISA and the complement fixation test ($P < 0.001$). The ELISA gave repeatable results within a laboratory and reproducible results between laboratories.

Adoption of the ELISA would result in an immediate reduction in the number of sheep being excluded from export: down from 17% (applying current CFT protocol requirements for major trading partners) to <4%. Fewer animals would have to be tested with cost savings to the exporter and farmer because the rejection rate would be lower. This would have a significant impact on Australia's export market, with improved access for Australian producers.

Contents

1 Background

Australia claims freedom from Enzootic Abortion of Ewes (EAE) (Animal Health Australia[1]) but Australian sheep sera often give positive reactions in the Complement Fixation Test (CFT), which excludes these animals from export. The CFT is the only assay available in Australian laboratories for the detection of antibodies to *Chlamydophila abortus* (the agent of enzootic abortion). It is well recognised that the CFT has poor specificity with cross-reactions to other chlamydia and *Acinetobacter* (OIE[2]).

The Chlamydia Complement Fixation Test is one of the required components of pre-export health certification for animals being exported to China, and with China's very stringent test requirements, significant numbers of sheep are rejected for live export. Using this assay for export testing will continue to exclude animals from export. The CFT is listed in both the Australian Standard Diagnostic Techniques[3] and the OIE Manual of Standards for Diagnostic Tests and Vaccines[2]. ELISA is discussed in both references, but there has been no ELISA offered as a diagnostic test in Australia for Chlamydia antibody detection. Given the nature of the CFT, ELISA would be a valuable tool, if suitable reagents could be found. In the laboratory, ELISAs are generally preferred to CFTs because they are less demanding in terms of labor and technical expertise. For these reasons, an ELISA may be chosen ahead of a CFT of equivalent sensitivity.

1.1 Pilot Study

In 2005 a pilot study was conducted by DPI PIRVic to identify suitable diagnostic assays (ELISA) to replace the CFT. An extensive review was undertaken which led to the selection of four ELISAs for evaluation. Each of the ELISAs satisfied the main criterion of using *Chlamydophila abortus* antigen and not *Chlamydophila psittaci* antigen which is known to be highly cross reactive between Chlamydophila species.

A panel of antisera were sourced for use in the pilot study and included:

- 55 positive ovine sera from Moredun Research Institute, Scotland
- 50 ovine sera from NCDI (MAF) New Zealand
- 93 Australian field sera were selected (ovine), from four states, and multiple properties

The 93 Australian field sera included in the first study were selected to represent a range of results in the CFT, from negative to high positive. Thus these samples represented a skewed population. However, results obtained showed that there was no difference in chlamydial seroprevalence between Australia and New Zealand, consistent with the lack of any strong evidence of the presence of *Chlamydophila abortus* in Australian sheep.

The positive and negative panels of sera were used to estimate the diagnostic sensitivity and specificity for each of the ELISAs. Two of these ELISAs were provided by Moredun Research Institute, Scotland and used different antigens. While these two ELISAs produced acceptable sensitivities and specificities, they were supplied only for research purposes and are not commercially available. A third ELISA (Panclabort) was found to have quite low sensitivity (70%) which may be acceptable as an indicator of exposure within a flock, but would have little value where individual animals are assessed as is the requirement for live sheep exports.

The fourth ELISA, produced by Institut Pourquier and marketed commercially, was calculated to have a specificity of 90%, compared to the specificity of the Complement Fixation Test of 76% (determined in the pilot study), when the assay is used as per export protocol requirements. The recombinant antigen used to coat the plates is designed to allow no cross-reaction with *Chlamdydophila pecorum*. As a result of the pilot study, the Institut Pourquier ELISA was identified as a candidate for further study on a large population of Australian ovine serum samples as representative of the animals that are selected for live export.

1.2 Extension of the pilot study

A fresh review was undertaken to ensure the selection of the best currently-available ELISA for evaluation, with consideration given to the availability and appropriateness/relevance of the assays. This review identified references to two products that were not being marketed when the original pilot study was undertaken:

- a Chlamydophila ELISA manufactured by Guildhay was followed up but found to be misleading as this was not a commercial product for Australian laboratories, and
- an IDEXX/Bommeli ELISA, using antigen purified from *Chlamydophila psittaci*, strain 6BC, which is based on lipopolysaccharide, a component common to many Chlamydophila and therefore not specific to *Chlamydophila abortus*. The Chlamydia Complement Fixation Test commonly uses lipopolysaccharide as its antigen, which contributes to the poor specificity of this test. There would be little to be gained in evaluating an ELISA that utilises the same antigen as the Complement Fixation Test.

Based on these results, the Institut Pourquier *Chlamydophila abortus* antibody ELISA was chosen for further study as originally planned, testing a 'blind panel' of samples to confirm the preliminary findings and ensure the robustness of the assay has been reviewed and comparisons of the findings can be made with the diagnostic sensitivity and specificity of the assay, as determined in 2005[4].

2 Project objectives

2.1 Evaluation of Diagnostic Assays for *Chlamydophila abortus* in Australian Export Sheep

2.1.1 To compare the performance of ELISA and CFT for quantification of Chlamydophila abortus antibodies in sheep.

2.1.2 To prepare a submission to the Sub-Committee of Animal Health Laboratory Standards (SCAHLS) to recognise ELISA as an appropriate diagnostic method.

2.1.3 To communicate, via a report, the findings to AQIS and Biosecurity Australia to assist in the review and update of export testing protocols for sheep, with the inclusion of ELISA as a relevant test option.

3 Methodology

3.1 Sample Panel

A panel of 1,562 Australian ovine serum samples was sourced from five Australian states and more than 150 properties (Table 1). A range of age, breed and both male and female sera were included. The exposure of these animals to *Chlamydophila abortus* was not known. Samples were selected as representative of the animals that are selected for live export with no test history for Chlamydophila, a population that would be considered negative based on the Animal Disease Status from Animal Health in Australia. The sera were contributed from several sources: stored serum that had been collected for other studies, samples collected when sheep were being bled for ovine brucellosis accreditation and samples from abattoirs.

Table 1 Distribution of samples collected by State

Source State	Number of Samples Tested
Victoria	467
Western Australia	300
New South Wales	247
Tasmania	193
South Australia	331
Mixed origin (SA/NSW)	24
Total:	**1562**

The samples were aliquotted into duplicate vials and labeled sequentially. The identity of the samples was not known to the testing laboratories.

3.2 Testing Laboratories

The samples were distributed for duplicate testing in two laboratories experienced with complement fixation test and ELISA and their use as diagnostic tools: Primary Industries Research Victoria, Attwood (Victoria) and the Animal Health Laboratories, Agriculture Western Australia (Western Australia).

3.3 Reagents

The ELISA and complement fixation test reagents were imported through the Australian supplier of these reagents, received at the Attwood laboratories of Primary Industries Research Victoria and distributed to each of the two testing laboratories. Sufficient of each of the supplied reagents was purchased to ensure that both of the testing laboratories would use the same batch of reagents throughout the testing.

3.3.1 ELISA

Chlamydophila abortus antibody ELISA manufactured by Institut Pourquier. All of the required reagents were provided in kit form.

3.3.2 Complement Fixation Test

Chlamydia Complement Fixing Antigen, positive control serum and negative control serum from Slovakia were provided to both testing laboratories. These laboratories were individually responsible for providing the remaining CFT reagents – sheep red blood cells, complement, haemolysin, buffers.

3.4 Test Protocols

Two laboratories tested all 1,562 samples by ELISA and Complement Fixation Test.

3.4.1 ELISA

The ELISA was run as per the protocol provided with the kit.

3.4.2 Complement Fixation Test

Each laboratory tested the samples using their documented procedure for Chlamydia Complement Fixation but using the same batch of antigen and positive and negative control serum.

3.5 Results Evaluation

3.5.1 Recording results

Results for the 1,562 samples tested were returned to PIRVic for analysis. For the purpose of this study samples recorded as Positive or Doubtful/Inconclusive were classified as Positive, ie. non-negative. The Complement Fixation Test was read at two serum dilutions, 1:4 and 1:32 as per China and OIE protocols respectively.

3.5.2 Statistical analysis of results

The percentage of samples giving positive results was determined for ELISA and CFT for each of the two testing laboratories using 95% Confidence Intervals.

McNemar's test was used to compare results from the different tests. McNemar's test is appropriate when the assays are carried out on the same population.

4 Results and discussion

4.1 Number of samples giving a positive result

Determinations of % positivity were established for ELISA and CFT for each of the two testing laboratories (Victoria and Western Australia) using 95% Confidence Intervals.

4.1.1 ELISA

The percentage of the 1,562 samples testing positive in the ELISA are presented in Table 2.

Table 2 Positive results reported in the Chlamydophila abortus antibody ELISA

Laboratory	% Positive (CI)a
Victoria	3.7 (2.8 - 4.8)
Western Australia	0.3 (0.1 - 0.7)

[a]One-sample binomial test with exact 95% Confidence Intervals

This result is much lower than would have been expected and compares favourably with the diagnostic specificity of 90.0%, which was established in the 2005 pilot study[4] using a New Zealand population, a country that claims freedom from *Chlamydophila abortus* and ovine enzootic abortion and has never diagnosed sporadic ovine chlamydial abortion. Thus while we would have expected 1 in 10 animals to be excluded from export, the current study of an Australian population showed that less than 1 in 25 animals would be excluded using the Institut Pourquier *Chlamydophila abortus* antibody ELISA.

4.1.2 Complement Fixation Test

The complement fixation test (CFT) was read and analysed at two serum dilutions: 1:4 and 1:32. The Australian Standard Diagnostic Technique (1993) sets the reporting dilution at 1:4 for the Chlamydia CFT. This is also the reportable end-point for China, one of Australia's major trading partners, requiring individual animals to test negative at 1:4 to be eligible for export. However, OIE states that titres less than 1:32 in individual animals would be considered to be non-specific for *Chlamydophila abortus*. The percentage of the 1,562 samples testing positive have been presented for each of the two cut-offs: 1:4 as per China export testing protocols and 1:32 as per the OIE recommendation (Table 3).

Table 3 Positive results reported in the CFT at both 1:4 and 1:32 cut-off

Laboratory	% Positive @ 1:4 (CI)[a]	% Positive @ 1:32 (CI)[a]
Victoria	10.7 (9.2 - 12.3)	2.3 (1.6 - 3.2)
Western Australia	16.8 (15.0 - 18.7)	2.6 (1.8 - 3.5)

[a]One-sample binomial test with exact 95% Confidence Intervals

It is noted that the percentage of samples in the panel of 1,562 sera tested falls from 10.7% to 2.3% and 16.8% to 2.6% (Victoria and Western Australia respectively) when the cut-off is moved to 1:32 as per OIE recommendations.

4.2 Results Comparison – ELISA and Complement Fixation Test

Comparisons were made of the results obtained between CFT and ELISA within each laboratory (Victoria and Western Australia) using McNemar's test for paired samples (Table 4).

Table 4 Comparison of results obtained using the Chlamydophila abortus antibody ELISA and the CFT (1:4 cut-off)

Value	Sample Result Same	Sample Result Different	P value[b]
ELISA and CFT Victoria	1262	300	<0.001
ELISA and CFT Western Australia	1207	355	<0.001

[b]Using two-sided exact McNemar's Test

These results show that the two assays, Institute Pourquier *Chlamydophila abortus* antibody ELISA and Chlamydia Complement Fixation, are not comparable. There is a statistically significant difference between the results obtained with the Institut Pourquier *Chlamydophila abortus* antibody ELISA and the complement fixation test ($P < 0.001$) within each of the two laboratories.

4.3 Repeatability

A subset of 45 samples from the large panel of 1562 samples was selected to be tested in a repeatability trial which was undertaken at the Attwood laboratories of Primary Industries Research Victoria.

4.3.1 Within-operator variation

These forty-five samples were tested three times by three operators (Table 5).

Table 5 Within Operator Variation (P values)

	Run 1 / Run 2	Run 2 / Run 3	Run 1 / Run 3
Operator 1	0.750	0.016	0.016
Operator 2	0.250	0.250	1.00
Operator 3	0.124	1.00	1.00

[b]Using two-sided exact McNemar's Test

The positive control serum is added to each plate twice, as per the manufacturer's instructions. A mean of the Corrected OD of the Positive Control is calculated for run validation.

All nine plates satisfied the validation criteria of the ELISA. The difference between the Corrected OD of the Positive Control was calculated for each plate. Run 3 for Operator 1 had a difference of 17% between the Corrected OD of the two Positive Controls. In the testing laboratory, a plate with a difference of 17% would be rejected and all samples retested. For the purpose of this study, to establish the repeatability of the ELISA, all plates were accepted. Run 3 for Operator 1 is therefore considered to be an outlier. P values determined for all pairs of plates were >0.05, except where this plate was included. The P value calculated for Operator 1 (Run 2 and Run 3) and (Run 1 and Run 3) may be explained by this poor replicate.

4.3.2 Between-operator variation

The results obtained between the three operators were also analysed looking for differences between operators (Table 6)

Table 6 Between Operator Variation (P values)

	P value[b]
Operator 1 / 2	0.750
Operator 1 / 3	0.750
Operator 2 / 3	1.00

[b]Using two-sided exact McNemar's Test

No difference was seen between operators (P >0.05).

4.3.3 Within-plate variation

A subset of the 45 samples used to measure repeatability was tested to examine within-plate variation. Nine samples were tested 5 times on a single ELISA plate. Eight of the nine samples gave a consistent result each time (based on interpretation), ie. Positive (3 samples) or Negative (5 samples). Thus there was no difference in result/interpretation for 8 of the 9 samples. The ninth sample gave two Doubtful and three Positive results and is clearly a sample falling close to the threshold between Doubtful and Positive.

4.4 Reproducibility

The reproducibility of the assay was measured by comparing the results obtained in the two testing laboratories, analysed using McNemar's Test for paired samples (Table 7).

Table 7 Comparison of results – ELISA/ELISA and CFT/CFT between laboratories

Value	Sample Result Same	Sample Result Different	P value[b]
ELISA Victoria and ELISA Western Australia	1507	55	<0.001
CFT (1:4) Victoria and CFT (1:4) Western Australia	1315	247	<0.001

[b]Using two-sided exact McNemar's Test

The reproducibility of the Institut Pourquier *Chlamydophila abortus* antibody ELISA measured between two testing laboratories was found to be poorer than expected when compared to the performance of this ELISA in the 2005 pilot study where duplicate testing was done in a single laboratory, PIRVic Attwood, and the degree of matched results was higher than the current study suggests. The sample panels were smaller in the original study (55 positive serum samples and 50 negative serum samples) compared to the current trial (1562 blind samples) and if there are differences to be found, they will be detected in a larger study group. Thus with one laboratory reporting 5 positive samples and the other laboratory reporting 58 positive samples – the P value of <0.001 seems extreme. However, the specificity of the ELISA has been calculated at 90.0%, suggesting that 1 in 10 sheep would be reported as (false) positive. The results of the current study show a % positivity of 0.3 – 3.7% (Western Australia and Victoria respectively) which is much lower than would have been expected.

A second interlaboratory comparison was undertaken to revisit the performance of the ELISA. This additional reproducibility testing was planned and conducted using a smaller panel of samples. A third testing laboratory was introduced to assist with identification of possible concerns with the assay. A panel of 45 samples was prepared - aliquotted, labelled blind and distributed to three testing laboratories: Primary Industries Research Victoria (PIRVic Attwood), Agriculture Western Australia, Animal Health Laboratories and the Queensland Department of Primary Industries and Fisheries, Biosecurity Science Laboratory. All three laboratories were provided with the same batch of reagents. This panel of 45 samples was made up mainly of fresh sheep blood samples collected for this purpose. However, since the status of these new samples was unknown, a small number of samples (8) from the main study panel of 1562 were selected for inclusion. (Table 8).

Table 8 Comparison of results obtained in three laboratories using the Chlamydophila abortus antibody ELISA

Laboratory	Sample Result Same	Sample Result Different	P value[b]
ELISA Victoria and ELISA Western Australia	44	1	1.000
ELISA Victoria and ELISA Queensland	43	2	0.500
ELISA Western Australia ELISA Queensland	45	0	1.000

[b]Using two-sided exact McNemar's Test

The P values (>0.05) indicate good reproducibility between all of the laboratories. It is not possible to determine the cause of the earlier poor reproducibility, however, the problems seen in the original inter-laboratory comparison are no longer apparent.

5 Success in achieving objectives

5.1 To compare the performance of ELISA and CFT for quantification of *Chlamydophila abortus* antibodies in sheep

5.1.1 Testing completed using ELISA and CFT

The (Institut Pourquier) *Chlamydophila abortus* antibody ELISA was identified as the best candidate ELISA currently commercially available to Australian laboratories. Two state government laboratories provided duplicate testing using the Institut Pourquier *Chlamydophila abortus* antibody ELISA and the complement fixation test on a panel of 1.562 Australian ovine serum samples sourced for the project.

5.1.2 Comparison of the performance of ELISA and CFT

The results show that the two assays, Institute Pourquier *Chlamydophila abortus* antibody ELISA and Chlamydia Complement Fixation, are not comparable ($P < 0.001$) with the ELISA demonstrating higher specificity than the complement fixation test.

5.1.3 Performance of the *Chlamydophila abortus* antibody ELISA

Results of replicate testing between laboratories suggests the ELISA is reproducible and fit-for-purpose. The repeatability of the ELISA was also measured and found to be acceptable.

5.2 To prepare a submission to the Sub-Committee on Animal Health Laboratory Standards (SCAHLS) to recognise ELISA as an appropriate diagnostic method

A report was submitted to SCAHLS 23rd January 2007 using the Validation Template for Serological Assays. Following initial feedback from SCAHLS New Test Evaluation Working Group, the additional work on reproducibility was undertaken in the three laboratories. The final submission to SCAHLS was sent 22nd May 2007 and is currently with two independent reviewers. A response is expected early July. The SCAHLS Validation file has been attached to this report as Appendix 1.

5.3 To communicate via a report, the findings to AQIS and Biosecurity Australia to assist in the review and update of export testing protocols for sheep, with the inclusion of ELISA as a relevant test option

A report has been submitted to AQIS and Biosecurity Australia incorporating the results of the project and the SCAHLS Validation Template for Serological Assays. AQIS and Biosecurity Australia have indicated their support for the project and await the technical response from SCAHLS as to the endorsement of this ELISA for use in export testing protocols for sheep.

6 Impact on meat and livestock industry – now and in five years time

The results of this study support Australia's claim to freedom from the causative agent of Enzootic Abortion of Ewes[1].

If the (Institut Pourquier) *Chlamydophila abortus* antibody ELISA is accepted by SCAHLS, AQIS and Biosecurity Australia, a review of the current export testing protocols for sheep should incorporate ELISA as a relevant option. If Australia's trading partners endorse this it is expected that there will be a reduction in the number of sheep being excluded from export: down from 17% (applying current CFT protocol requirements for major trading partners) to <4%.

Thus smaller numbers of sheep would need to be selected for pre-export testing as more animals would meet health certification requirements, resulting in less animals being rejected. This would have a significant impact on Australia's export market, with improved access for Australian producers.

7 Conclusions and recommendations

7.1 Conclusions

The Institut Pourquier *Chlamydophila abortus* antibody ELISA gives repeatable and reproducible results of equivalent sensitivity to the CFT but with higher specificity. Replacing the CFT with the ELISA will result in fewer sheep being unnecessarily excluded from export.

7.2 Recommendations

The Institut Pourquier *Chlamydophila abortus* antibody ELISA is included as an alternative to the complement fixation test in export testing protocols.

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9 Appendix – SCAHLS Validation template

The following is a summary of the essential information required to assess the validation status of a serological assay. Validation is the process through which a test method is determined to be fit for purpose. It is recognized that validation is an incremental process. The four levels of validation described in this document - Development and feasibility, Diagnostic performance and implementation, and Technology transfer and reproducibility - represent a progression from development through to application. It is proposed that these guidelines be used to assemble the essential data used for the evaluation of a new serological assay, serological assay kit or essential serological assay reagent(s).

Proposals will be reviewed by the New Test Development (NTD) working group of SCAHLS according to SCAHLS Policy.

NOTE: Please save completed documents as 'client name_ test name_ SCAHLS_NTDWG_Serology review_date.doc' and forward for review to the Executive Officer SCAHLS for lodgement and forwarding to the Chair SCAHLS New Test Development working group

	[*Chlamydophila abortus* ANTIBODY ELISA] SEROLOGICAL ASSAY	This is a commercial kit manufactured by Institut Pourquier. It is imported into Australia and distributed by Laboratory Diagnostics Pty Ltd. The product is sold as an all inclusive kit, with antigen coated plates, positive and negative control sera and all other necessary reagents. This reagent is intended to detect antibodies specifically directed to the *Chlamydophila abortus*. This test is an indirect ELISA test based on the use of a recombinant 80-90 kDa protein, that is specific for *Chlamydophila abortus* and *Chlamydophila psittaci* and that does not cross react with *Chlamydophila pecorum.*
1	BACKGROUND	
1.1	APPLICANT *(Name and full contact details)* *(Job title within organization)* *(Type of organization i.e. commercial, institutional or governmental)*	Lisette McCauley Primary Industries Research Victoria, 475 Mickelham Road, Attwood, Victoria. 3049 Scientist Government
1.2	COMMERCIAL INFORMATION	☐ Yes 'commercial in confidence' ☐ Not commercially sensitive *If yes, then it is up to the submitter to arrange appropriate formal agreements if considered necessary*
1.3	INTENDED PURPOSE OF THE ASSAY *(Should fit one of the 6 OIE 'Statement of Purpose' - check at least one)* *A test method may have more than one application. The intended application will determine the required performance characteristics of the method and the*	☐ Population freedom (declaration) ☐ Animal Disease freedom (trade) ☐ Eradication/control ☐ Investigation of clinical signs ☐ Prevalence estimate (risk analysis) ☐ Immune status Chlamydophila abortus is the agent of Enzootic Abortion of Ewes (EAE, ovine chlamydiosis). Australia exports sheep to a number of countries for breeding and export protocols require that individual animals be tested for exposure to *C. abortus*. To qualify for export, every individual animal must test negative. Testing in Australia is only done by CFT and the protocol varies according to the importing country. One major trading partner demands a reporting dilution of 1:4. However,

	criteria for validation *If a test has more than one application, submitters must provide evidence of fitness for purpose for each application within the template or in separate templates* *Provide a brief description of microorganism/disease, the species the test will be used on, with background on what is being measured and why the new technology has been developed* *In what other laboratories is the test being used or intended to be used, what national or state programmes will it support.*	according to the OIE, an animal would not be considered infected if it tested positive at a CFT dilution of less than 1:32. Australia claims freedom from EAE but Australian sheep sera often give positive reactions in the CFT, which excludes these animals from export. However, it is well-recognised that the CFT has poor specificity with cross-reactions to other chlamydia and *Acinetobacter*. Chlamydiae have been isolated from Australian sheep faeces and have been detected in the arthritic joints of Australian lambs. The organism in both instances is likely to be *Chlamydophila pecorum*, a gut commensal that may enter the bloodstream to cause arthritis sporadically. Sheep infected with *C. pecorum* have been shown to give positive CFT reactions. *"Chlamydophila abortus* shares common antigens with *Chlamydophila pecorum* and some Gram-negative bacteria, so that the CF test is not wholly specific…. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion. Alternative serological tests have been developed, but none has been sufficiently appraised so far for field use." (OIE Manual of Standards for Diagnostic Tests and Vaccines, Chapter 2.4.7, last updated 24 July 2004.) The Australian Standard Diagnostic Technique for Chlamydiosis (1993) still sets the complement fixation reporting dilution at 1:4. This does not follow OIE's recommendations for the assay. Thus with the Chlamydia Complement Fixation Test being one of poor specificity, many animals may unnecessarily be lost to export. A pilot study was undertaken in 2005 to examine four ELISAs for the detection of antibody to *Chlamydophila abortus.* The study evaluated the performance of each ELISA by determining sensitivity and specificity. One commercial product was identified as a potential alternative to the Complement Fixation Test for Chlamydia: the Institut Pourquier ELISA with a diagnostic sensitivity of 90.9% and a diagnostic specificity of 90.0%. This is the only ELISA commercially available to Australian laboratories. This current study of Australian sheep sera has been undertaken in 2006-07. No other Australian laboratory has yet evaluated an ELISA for *Chlamydophila abortus*. A recent search has found no evidence of any other *Chlamydophila abortus* ELISA available (commercially). The Animal Health Laboratories of Agriculture Western Australia was asked to provide duplicate testing of the Australian sheep sera sourced for the 2006-07 study.
1.4	DESCRIPTION OF THE ASSAY *(Type of assay, whether derived from OIE prescribed or alternative test, published or in-house development)* *(Expected sample type to be tested: preservative used, age/shelf life,*	This reagent is intended to detect antibodies specifically directed to the *Chlamydophila abortus*. This test is an indirect ELISA test based on the use of a recombinant 80-90 kDa protein, that is specific for *Chlamydophila abortus* and *Chlamydophila psittaci* and that does not cross react with *Chlamydophila pecorum.*

	temperature, transport requirements)	The assay is a commercial ELISA, in kit form, manufactured by Institut Pourquier. All required reagents are provided in the kit, including positive and negative control sera. The Pourquier Chlamydophila abortus ELISA can be used on bovine, ovine and caprine sera. Samples would normally be submitted to the laboratory as blood or serum. Serum to be taken off the clot and stored at 4ºC until tested.
1.5	REFERENCES *(Provide relevant references including any that the test was based on; list publications resulting from this work if already published or submitted)* *Provide a statement about degree of acceptability in scientific, regulatory and client community*	Comparison of ELISA and CFT assays for *Chlamydophila abortus* antibodies in ovine sera. LME MCCAULEY, MJ LANCASTER, P YOUNG, KL BUTLER and CGV AINSWORTH. (In press) References provided with the validation file from Institut Pourquier: *Manual of Standards for Diagnostic Tests and Vaccines. Office International des Epizooties. 2000. Chap. 2-4-7. Enzootic abortion of ewes (ovine chlamydiosis).* *Buendia, A.J., Montes de Oca, R., Navarro, J.A., Sanchez, J., Cuello, F., Salinas, J., 1999. Role of polymorphonuclear neutrophils in a murine model of Chlamydophila psittaci-induced abortion. Infect. Immun. 67, 2110-2116* *Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E., Herring, A.J., 1998. Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the Chlamydophila psittaci subtype that causes abortion in sheep. Infect. Immun. 66, 1317-1324* *Souriau, A., Salinas, J., De Sa, C., Layachi, K., Rodolakis, A., 1994. Identification of subspecies-and serotype 1-specific epitopes on the 80-90 kDa protein region of Chlamydophila psittaci that may be useful for diagnosis of Chlamydophila induced abortion. Am. J. Vet. Res. 55, 510-514* *Nakane, P.D. und Kawoi, A. Peroxidase-labelled antibody. A new method of conjugation. J. Histochem. Cytochem. 1974, 22 : 1084* Paper comparing several assays, including the precursor to the Institut Pourquier *Chlamydophila abortus* ELISA: Buendía AJ, Cuello F, Del Rio L, Gallego MC, Caro MR, Salinas J. Field evaluation of a new commercially available ELISA based on a recombinant antigen for diagnosing *Chlamydophila abortus* (*Chlamydia psittaci* serotype 1) infection. *Vet Microbiol* 2001;78(3):229-239. Results from the pilot study were presented at the 2nd Annual AAVLD meeting in Sydney in October 2006. There has been considerable support for the successful search for an ELISA as an alternative assay to the Complement Fixation Test, particularly where it is used in international trade.

1.6	Test METHOD PROTOCOL *(Sufficient detail needs to be provided so that the test can be repeated by someone with experience in the basic technique)*	*To be provided in Appendix B-1.5, as it would be appear in the ANZSDP*
2	DEVELOPMENT AND FEASIBILITY *(Describe and reference for each parameter)*	
2.1	PRIOR TREATMENT OF SPECIMEN *(Selection of specimens, include collection, transport, method of preparation, dilution etc,)*	There is no prior treatment required for specimens to be tested in this assay, which tests clean, fresh serum.
2.2	REAGENT SELECTION *Describe the controls and reagents that are being used and why they were selected for this assay development e.g. type of conjugate etc* *Provide details of suppliers and information on the continuous availablity of supply of essential reagents*	This is a commercial ELISA. The kit contains all required materials. A full test protocol is provided with the kit. A validation file provided by the manufacturer has been included. See 2.3 Assay Optimisation. The Australian supplier of this ELISA is: Laboratory Diagnostics Pty Ltd PO Box 2011 Taren Point NSW 2229 Laboratory Diagnostics import a range of Institut Pourquier products used in Australian (and New Zealand) laboratories.
2.3	ASSAY OPTIMISATION *(Describe what optimisation and standardisation has been conducted)*	This is a commercial ELISA, manufactured for distribution by Institut Pourquier. See attached: Appendix A-1 (2.3), Validation File, Pourquier *Chlamydophila abortus* Serum ELISA
2.4	FEASIBILITY TESTING *(Samples with varying levels of antibody consistent with expected range in clinical (test) samples and an equivalent number*	As Australia claims freedom from EAE, it is not possible to test a representative panel of ovine serum samples that would cover an expected range in clinical samples. The pilot study established the diagnostic sensitivity and specificity of this assay by testing known positive ovine serum samples imported from Scotland and a panel of ovine serum samples from New Zealand, a country

	of samples containing no antibody) *In the case of an exotic disease it may be difficult or impossible to obtain positive specimens from infected animals. In these cases there may be no alternative but to use an interlaboratory comparison type of validation* *Describe any problems requiring reassessment What changes were made to the assay to resolve these?*	that claims freedom from EAE. The current study tested a panel of Australian sheep sera. The panel was selected blind as there is no evidence of enzootic abortion in Australia. Thus it was not possible to obtain positive specimens from infected animals. The same sample panel was tested in two experienced veterinary laboratories – Primary Industries Research Victoria and Agriculture Western Australia, Animal Health Laboratories – as an interlaboratory comparison. No changes were made to the commercial kit for this evaluation.
2.5	REPEATABILITY *Testing conducted with the same method on identical samples in the same laboratory by the same operator using the same equipment within short intervals of time..* *Include number of replicates (minimum of three operators, three runs each)* *Intra and inter-assay variation, and between operator variation, expressed by coefficient of variation, e.g. CV ≤ 20%*	A panel of 1562 ovine serum samples was accumulated from five Australian states (Victoria, Western Australia, Tasmania, New South Wales and South Australia), and more than 155 properties of origin. Testing was conducted at the PIRVic laboratory and in parallel by the Western Australian Department of Agriculture. The same kit batch was used by both laboratories. A subset of 45 of the 1562 samples was selected for repeatability testing in the PIRVic laboratory. Three operators each ran the assay three times on all 45 samples. The same kit batch was used throughout. Testing was completed per operator over either 1 or 2 days. The same plate reader was used. A further subset of 9 samples was tested to measure within-plate variation. Intra and inter-assay variation and between operator variation were measured. The results have also been statistically analysed using McNemar's Test. *Within-operator variation.* Of the nine plates, one showed a difference in the duplicate positive control reading, which in the PIRVic laboratory would have been rejected and all samples retested in a diagnostic situation. For the purpose of this study, the results of all nine runs were used and this one plate run was not repeated. However, it is noted that this single plate is considered an outlier. P values determined for all pairs of plates were >0.05, except where this plate was included. All nine plates satisfied the validation criteria of the ELISA. *Between-operator variation*. No difference was seen between operators, (P >0.05). *Within-plate variation.* In a separate run, nine samples were tested on a single plate, repeated five times each. The results showed no difference in result/interpretation of 8 of the 9 samples. The ninth sample gave two Doubtful and three Positive results and is

		clearly a sample falling close to the threshold between Doubtful and Positive.
2.6	ANALYTICAL SENSITIVITY *Smallest detectable amount of antibody. Including endpoint dilution analysis, earliest time after exposure and duration of antibody response, e.g. serial testing* *Compare with other methods including Gold Standard*	This is a commercial ELISA kit, manufactured by Institut Pourquier. The specificity analysis was carried out at the Institut Pourquier. Analytical sensitivity was determined on 176 sera coming from an infected herd. Experimental infections: 4 sheep with *Chlamydophila abortus* and 4 sheep with *Chlamydophila pecorum.* Clear seroconversion was demonstrated with the sheep infected with *Chlamydophila abortus*. No seroconversion was induced in any of the sheep infected with *Chlamydophila pecorum.* Detectability was evaluated by measuring the correlation between S/P obtained by ELISA and CFT titre (cut-off titre = 10). The validation reports that generally good correlation was seen, but discrepancies were observed with sera around the cut-off. The Gold Standard is the micro-immunofluorescence test, however this test has never been used in Australia. The complement fixation test has been the only serological assay offered in Australian laboratories for the detection of antibodies to *Chlamydophila abortus*. See 3.1 for discussion of the Gold Standard for *Chlamydophila abortus* antibody assays. See attached: Appendix A-1 (2.3), Validation File, Pourquier *Chlamydophila abortus* Serum ELISA
2.7	*ANALYTICAL SPECIFICITY* *(Differentiation of target analyte from that produced by other non-target but related agents* *Describe degree to which the assay does not cross-react with other analyte, e.g. number of false positives* *Include a comparison of results with a Gold Standard* *Testing should be based on a panel of sera from animals of known status. These should include animals exposed to agents found in similar environments, that produce similar symptoms and/or have similar taxonomy*	The Institut Pourquier ELISA is intended to detect antibodies specifically directed to *Chlamydophila abortus*. This test is an indirect ELISA based on the use of a recombinant 80-90 kDa protein, (POMP – Polymorphic Outer Membrane Protein) that is specific for *Chlamydophila abortus* and *Chlamydophila psittaci* and that does not cross react with *Chlamydophila pecorum.* The complement fixing antigen is Lipopolysaccharide, common to all Chlamydophila. Analytical specificity was determined by Institut Pourquier on 261 ovine sera from populations without pathological evidence of the presence of *Chlamydophila abortus*. These serum samples were provided by the French Department of Pyrenees Atlantiques. The observed specificity was recorded as 96.2%. 251 sera were reported as negative, 6 as doubtful and 4 positive. The non-negative samples were negative or doubtful by CFT (titre $\leq$ 10). The Gold Standard is the micro-immunofluorescence test, however this test has never been used in Australia. The complement fixation test has been the only serological assay offered in Australian laboratories for the detection of antibodies to *Chlamydophila abortus*. See 3.1 for discussion of the Gold Standard for *Chlamydophila abortus* antibody assays.

		See attached: Appendix A-1 (2.3), Validation File, Pourquier *Chlamydophila abortus* Serum ELISA
3	DIAGNOSTIC PERFORMANCE *(Describe and reference for each parameter)*	
3.1	REFERENCE TEST (GOLD STANDARD) *Justification for the reference method e.g. being more accurate, reference for method*	The Gold Standard is the micro-immunofluorescence test, however this test has never been used in Australia. While the Complement Fixation Test has been the only antibody assay offered in an Australian laboratory to date, it is also recognised as lacking specificity. *"Chlamydophila abortus* shares common antigens with *Chlamydophila pecorum* and some Gram-negative bacteria, so that the CF test is not wholly specific…. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion. Alternative serological tests have been developed, but none has been sufficiently appraised so far for field use. The serological responses to *Chlamydophila abortus* and *Chlamydophila pecorum* can be resolved by indirect micro-immunofluorescence, but the procedure is too time-consuming for routine diagnostic purposes. ELISAs developed independently by several research groups have not been adapted for general diagnostic work, partly because of difficulties associated with the use of particulate antigens. However, a novel ELISA that incorporates a stable, solubilised antigen has been used to test experimental and field samples, and has given results that, though lacking species specificity, have a higher sensitivity than the CF test. Of note has been the recent development of monoclonal antibody technology in a competitive ELISA and recombinant antigen technology in indirect ELISAs to discriminate between antibodies to *Chlamydophila abortus* and *Chlamydophila pecorum*." (OIE Manual of Standards for Diagnostic Tests and Vaccines, Chapter 2.4.7, last updated 24 July 2004.) The Institut Pourquier ELISA remains the only commercially available assay that is equivalent to or improves on the sensitivity and specificity of the complement fixation test in current use.
3.2	CONTROLS AND REFERENCE REAGENTS *Describe any further controls required (positive and negative) and the reasons for their use.* *Describe the availability of these controls to other laboratories*	This is a commercial ELISA. The kit contains all required materials. A single positive and a single negative control is provided. Ideally, a low positive control should be available.
3.3	SELECTION OF REFERENCE POPULATIONS	

	(The reference population should reflect the target population and include an appropriate spectrum of disease/infected animals and also animals free of the target disease/agent). *The sampling frame should be an unbiased representation of the reference population.* *Describe the criteria used for selection of reference animals e.g. time, location, animals characteristics as breed, age, gender etc.* *Describe the confirmation of the status of selected reference animals (positive or negative).*	The reference populations used to determine the diagnostic sensitivity and specificity were ovine serum samples imported from Scotland (positive panel) and New Zealand (negative panel). The positive panel constituted fifty-five positive ovine serum samples from Scotland. These samples came from sheep experimentally infected with *Chlamydophila abortus* and confirmed to have infected placentas. Fifty ovine serum samples from male sheep, (10 sera each from 5 different regions, collected in late 2004) constituting the negative group, were obtained from New Zealand. The negative population relied on the fact that New Zealand claims freedom from *Chlamydophila abortus* and ovine enzootic abortion and has never diagnosed sporadic ovine chlamydial abortion.
3.4	SAMPLING OF THE REFERENCE POPULATION *(Sample size must be stated and should reflect the degree of the required statistical certainty)* *Describe sampling procedure and any exclusion and/or inclusion criteria (n=300 from infected animals and n=1000 from uninfected animals seen as indicated by the OIE). Describe any limitations that exist relating to obtaining the appropriate number of samples.* *Random and systematic sampling are the preferred options*	Australia claims freedom from EAE (*Animal Health in Australia*. Animal Disease Status. Australian Government Department of Agriculture, Fisheries and Forestry GPO Box 858. Canberra ACT 2601. Chapter 1 2004;6) http://www.animalhealthaustralia.com.au/aahc/status/ahia.cfm Therefore it was not possible to use Australian sera to determine the sensitivity and specificity of this assay. This was done as previously described. n=55 from infected animals n= 50 from uninfected animals These numbers allowed reasonable 95% confidence limits to be calculated.
3.5	DIAGNOSTIC SENSITIVITY *(Proportion of known infected reference*	Diagnostic sensitivity was established for this assay in 2005 on a group of 55 positive sera supplied by Moredun Research

	animals that tested positive in the assay) *A reasonable number of known infected animals should be tested, the numbers will depend on the population of animals available and the disease process, relative sensitivity compared to other methods can be provided; include 2x2 calculations*	Institute. Samples were supplied freeze-dried and reconstituted before testing. Samples were tested in duplicate runs. n=55 from infected animals 50 positive, 3 negative, 2 inconclusive A diagnostic sensitivity of 90.9% was calculated for the ELISA, with a 95% Confidence Interval of 80.1 - 97.0.
3.6	DIAGNOSTIC SPECIFICITY *(Proportion of known uninfected reference animals that tested negative in the assay)* *A reasonable number of animals from known uninfected herds/flocks should be tested, the numbers will depend on the population of animals available and the disease*	Diagnostic specificity was established for this assay in 2005 on a group of 50 ovine sera provided by New Zealand, a country that claims freedom from Enzootic Abortion of Ewes. Samples were supplied frozen. Samples were tested in duplicate runs. Samples were also tested in the Complement Fixation test. 50 samples: 45 negative, 2 positive, 3 doubtful A diagnostic specificity of 90.0% was calculated, with a 95% Confidence Interval of 78.2 - 96.7.
3.7	REPRODUCIBILITY *Testing conducted by different operators in different laboratories. Describe the laboratories involved (location, previous experience in this type of testing, any special equipment used),details of the reproducibility panel (including composition and labelling, selection of samples, other data relating to sample status) administration of the trial (including distribution of panel, collection and collation of results, blinding), trial results (including variation within and between*	Testing was conducted on a panel of 1562 ovine serum samples at two laboratories: Primary Industries Research Victoria (PIRVic Attwood) and Agriculture Western Australia, Animal Health Laboratories. Both laboratories are experienced with the complement fixation test and ELISA and their use as diagnostic tools. The sera tested in this study were sourced from five Australian states and more than 150 properties, a population that would be considered negative based on the Animal Disease Status from Animal Health in Australia. The sera came from both female and male sheep. The exposure of these animals to *Chlamydophila abortus* was not known. Samples were selected as representative of the animals that are selected for live export. The sample panel was made up of serum contributed from several sources: stored serum that had been collected for other studies, samples collected when sheep were being bled for ovine brucellosis accreditation, samples from abattoirs. Sample distribution (source) is as follows: • Victoria – 467 samples

	laboratories).	• Western Australia – 300 samples • New South Wales – 247 samples • Tasmania – 193 samples • South Australia – 331 samples • Mixed origin (SA/NSW) – 24 samples Once the sample panel was compiled, samples were allocated a unique number and aliquotted into duplicate vials. One vial was held at PIRVic Attwood and the duplicate forwarded to the Animal Health Laboratories, Agriculture Western Australia. The identification of the samples was known only to the project leader. The ELISA was run as per the protocol provided by the manufacturer. Sufficient of a single kit batch was supplied to both laboratories to conduct the testing. Results were returned to PIRVic for analysis. The reproducibility of the Institut Pourquier *Chlamydophila abortus* ELISA measured between two testing laboratories was found to be poorer than expected when compared to the performance of this ELISA in the earlier study. Duplicate testing in the 2005 pilot study was done in a single laboratory, PIRVic Attwood and the degree of matched results was higher than the current study suggests. While the sample panels were smaller in the original study (55 positive serum samples and 50 negative serum samples) compared to the current trial (1562 blind samples), if there are differences to be found, they will be detected in a larger study group. Thus with one laboratory reporting 5 positive samples and the other laboratory reporting 58 positive samples – the P value (determined using two-sided exact McNemar's Test) of <0.001 seems extreme. However, the specificity of the ELISA has been calculated at 90.0%, suggesting that 1 in 10 sheep would be reported as (false) positive. The results of the current study show a % positivity of 0.3 – 3% which is much lower than would have been expected. Two interlaboratory comparisons were carried out. It was not possible to obtain known positive and known negatives. Samples were sourced from Australia, which claims freedom from ovine enzootic abortion. Trial results: a) Victoria and Western Australia, 1562 samples distributed November 2006. Statistically significant differences were seen (McNemar's Test for paired samples) between the two laboratories (P.< 0.001). b) Victoria, Western Australia and Queensland, 45 samples (36 new samples and 8 from the previous panel) distributed April 2007. There were no statistically significant differences seen between any two of the three laboratories (P.> 0.05). The second interlaboratory comparison was planned to revisit the performance of the ELISA. This additional reproducibility testing

		was planned and conducted using a smaller panel of samples. A third testing laboratory was introduced to assist with identification of possible concerns with the assay. A panel of 45 samples was prepared - aliquotted, labelled blind and distributed to three testing laboratories: Primary Industries Research Victoria (PIRVic Attwood), Agriculture Western Australia, Animal Health Laboratories and the Queensland Department of Primary Industries and Fisheries, Biosecurity Science Laboratory. The same batch of reagents was provided to all three laboratories. This panel of 45 samples was made up mainly of fresh sheep blood samples collected for this purpose. However, since the status of these samples was unknown, a small number of samples (8) from the main study panel of 1562 were selected for inclusion. All plates met the validation criteria set by the manufacturer. It is not possible to determine the cause of the earlier poor reproducibility, however, the problems seen in the original inter-laboratory comparison are no longer apparent.
3.8	PERFORMANCE OF TEST AGAINST REFERENCE TEST (GOLD STANDARD) *Results of new test and reference test are evaluated independently (blinded)*	There is no 'gold standard' for diagnostic laboratories in the true sense of the word. The Complement Fixation Test is the only assay offered by Australian laboratories for the health certification of sheep (live sheep, embryos) for export, although ELISA does appear on some export protocols. However, the CFT for Chlamydia is recognised internationally as being deficient. Thus the ELISA was not tested against the gold standard (micro-immunofluorescence), a test which has never been offered in Australian veterinary diagnostic laboratories. It was tested against the Complement Fixation Test. The % positivity of the ELISA was 3.7% and 0.3% (Victoria and Western Australia respectively). The % positivity of the CFT when read at a serum dilution of 1:4 was recorded as 10.7% and 16.8% (Victoria and Western Australia respectively). There are statistically significant differences between ELISA and CFT ($P < 0.001$). The two assays, ELISA (Institute Pourquier *Chlamydophila abortus*) and Chlamydia Complement Fixation, are not comparable.
3.9	ASSAY INTERPRETATION *Describe interpretation including expected results from low positive, high positive and negative samples*	The 1562 Australian samples tested in the 2006-07 study were considered a negative population, based on AHC's status report for Chlamydophila abortus. The pilot study undertaken in 2005 determined a diagnostic sensitivity and specificity for the Institut Pourquier ELISA using the sera from Scotland and New Zealand. The sample size was considered large enough based on the

	Calculate precision and accuracy *Establishment of cut-off(s)*	Confidence Limits. The results for the Australian population were unknown in that these animals are considered to represent a negative population. An analysis of the results obtained within and between laboratories was made. Thus there was no expected result for these samples. The diagnostic sensitivity and specificity of the Institut Pourquier ELISA was established in 2005. Results for the Australian population tested in the 2006-07 study were examined against the specificity. The false positive rate was lower than that suggested by the diagnostic specificity of the ELISA of 10.0% (3.7% and 0.3% for Victoria and Western Australia respectively). Establishment of cut-offs was not determined as this evaluation was on the performance of a commercial kit as per the manufacturer's protocol.
3.10	PRESENTATIONS OF RESULTS *Se and Sp estimates are presented together with sample sizes and confidence intervals* *2x2 table* *Receiver operator characteristic ROC analysis* *Uninterpretable and intermediate results should be explained.*	Diagnostic sensitivity and specificity have been determined and are presented with sample sizes and confidence intervals. ROC analysis has not been undertaken as it is more applicable when cut-offs are being established or altered. To undertake such an analysis, we need a positive and negative population and would then need to assume that the Australian population is a mix of positive and negative animals. We cannot obtain estimates of Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for *Chlamydophila abortus* in Australian sheep as there is no evidence that this disease is present in the population therefore there is no way of knowing if a sample should be positive or negative. This study was the evaluation of the fitness-for-purpose of a commercial ELISA kit. Manipulating the cut-off for a commercial ELISA is not usually done and is considered to be outside of the scope of this evaluation. Manufacturers may withdraw their support for a product when a laboratory chooses to stray from the protocol. In the first instance, we are reviewing the suitability or otherwise of this assay for Australian veterinary diagnostic laboratories.
3.11	DISCUSSION OF RESULTS *Test performance parameters should be discussed in relation to the study design and the intended or current use of the test* *If the gold standard is imperfect, this should be discussed in relation to the effect on the study results*	*Complement Fixation Test (CFT)* "*Chlamydophila abortus* shares common antigens with *Chlamydophila pecorum* and some Gram-negative bacteria, so that the CF test is not wholly specific…. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion. Alternative serological tests have been developed, but none has been sufficiently appraised so far for field use." (OIE Manual of Standards for Diagnostic Tests and Vaccines, Chapter 2.4.7, last updated 24 July 2004.)

		According to the OIE, an animal would not be considered infected if it tested positive at a CFT dilution of less than 1:32. Australia claims freedom from EAE but Australian sheep sera often give positive reactions in the CFT, which excludes these animals from export. The Australian Standard Diagnostic Technique for Chlamydiosis (1993) still sets the complement fixation reporting dilution at 1:4. This does not follow OIE's recommendations for the assay. Thus with the Chlamydia Complement Fixation Test being one of poor specificity, many animals may unnecessarily be lost to export. The current study examined the results of complement fixation test at a serum dilution of 1:4 and 1:32. The % positive rate recorded for the panel of 1562 samples tested in this study for the Complement Fixation Test falls from 10.7% to 2.3% and 16.8% to 2.6% (Victoria and Western Australia respectively) when the CFT cut-off is moved to 1/32 as per OIE recommendations. While the sensitivity of the assay shows no statistically significant difference when read at a serum dilution of 1/32 ($P > 0.05$), the specificity is substantially reduced ($P < 0.05$). *ELISA* Sufficient of the same batch of kits was purchased such that both testing laboratories could run all test samples. Western Australian reported a faint colour to the substrate which was not seen in the Victorian laboratory. The kits were received by PIRVic and half of them shipped to Western Australia, received by the laboratory without any obvious delay. The 5 positive results reported by Agriculture Western Australia, Animal Health Laboratories were a subset of the 58 positive results reported by the PIRVic laboratory. The reproducibility of the ELISA when tested in two laboratories, showed a statistically significant difference in the false positive rate (3.7% cf 0.3%). However, these values are still fall well short of the false positive rate determined in the 2005 pilot study (10.0%). Either the observed difference is due to the way in which the assay was set up and read between the two laboratories, or to a variable within the product. The only explanation offered is the colour of the substrate (Western Australia reported a slight colour in the substrate, while Victoria reported a clear solution.) The same kit batch was supplied to both testing laboratories. Every test plate (in both laboratories) met the validation criteria, however there were differences in the recorded OD of the Positive Control.In the PIRVic laboratory, the mean of the Corrected OD of the Positive Control (>2.0) was higher than the raw data values reported by Western Australia. Eleven of 36 plates had an OD close to 1.5, which resulted in a lower cut-off for these plates. However, no obvious correlation could be made with the samples tested on these plates between the two laboratories. The repeatability study indicates the performance of the ELISA is comparable, despite one of nine runs showing poor duplicate OD for the Positive Control. This plate is identified as an outlier, which is supported by the statistical analysis. Based on these results, a recommendation was made for a small set of samples, to be tested in several laboratories with fresh ELISA kit(s) to review the reproducibility. A panel of 45 samples was made up mainly of fresh sheep blood samples collected for this purpose. However, since the status of these samples was unknown, a small number of samples (8) from the main study panel

		of 1562 was selected for inclusion. The results of this additional testing have been discussed in 3.7 Reproducibility. There were differences in the Corrected OD of the Positive Control between the three laboratories testing a panel of 45 samples with Victoria>Western Australia>Queensland. However once again, all plates met the validation criteria set by the manufacturer. There were 4, 5 and 6 positive results (Victoria, WA and Queensland respectively). All 4 of the positive results reported by Victoria were reported positive by the other two laboratories. One positive result reported by WA and Queensland was a very high negative in the Victorian laboratory. The 6th positive result reported by Queensland was reported as a high negative and a doubtful by Victoria and WA respectively. The results obtained by the three laboratories in the additional study showed no statistically significant differences in any two of the three laboratories (P >0.05). It is not possible to determine the cause of the earlier poor reproducibility, however, the problems seen in the original inter-laboratory comparison are no longer apparent. CFT vs ELISA In the absence of a gold standard assay in use for diagnostic purposes in this country, the ELISA was measured against the complement fixation test, the only test offered in Australia for antibody to *Chlamydophila abortus*. The % positivity of the ELISA was 3.7% and 0.3% (Victoria and Western Australia respectively). The % positivity of the CFT when read at a serum dilution of 1:4 was recorded as 10.7% and 16.8% (Victoria and Western Australia respectively). There are statistically significant differences between ELISA and CFT (P < 0.001). The results of this evaluation show that the two assays, ELISA (Institute Pourquier *Chlamydophila abortus*) and Chlamydia Complement Fixation, are not comparable when the CFT is reported at a serum dilution of 1:4.
4	TECHNOLOGY TRANSFER *(Describe and reference for each parameter if appropriate)*	
4.1	TECHNOLOGY TRANSFER *(Evidence and method of technology transfer)* *Has the new method been implemented in any other laboratories (beyond reproducibility testing set out above).*	No. This ELISA is in commercial kit form. Details of the samples used in the evaluation has been provided within the template. The assay has not been implemented in any other laboratories at this stage.

	How was the technology transferred, to which laboratories, and over what period of time? *Details of the number and type of samples and their results.* *How did the test perform in other laboratories in comparison to the originating laboratory?*	
4.2	MONITORING VALIDITY OF ASSAY PERFORMANCE *(Discrepant analysis, i.e. use of a different test to confirm findings or retesting the sample or animal for example)* *Interpret with knowledge of the positive and negative predictive values* *Validity of the assay can also be monitored by:* *- Assessment of categorical (positive/negative) test data as it becomes available e.g. after 12 month* *- Estimation of prevalence in target populations* *- Calculation of positive and negative predictive values (PPV, NPV) e.g. for a number of relevant and or anticipated prevalences e.g. 0.1, 1 and 5 % prevalence* *- Assessment of precision and accuracy (repeatability, intra- and interassay, basic statistics and Levey-Jennings charts)* *- Assessment of reproducibility estimates through participation in external QAPs*	Based on the results of the pilot study where sensitivity and specificity were established, using duplicate testing of samples in two assays, complement fixation test and ELISA, there were differences noted in the serum samples returning a positive result. Samples were tested twice initially. If the second result confirmed the first, no further testing was undertaken. If the second result differed from the first in interpretation, a third test was done on the sample. Where the three tests were unable to produce the same interpretation, the sample was classified as Inconclusive/Doubtful. However, the samples that gave a positive result in the complement fixation test did not match in every instance, the samples that gave a positive result in the ELISA. This is presumed to be due to the assays detecting different antibodies - to the shared lipopolysaccharide or to the more specific recombinant antigen used in the ELISA. In the current study, testing 1562 ovine serum samples, it was again noted that there is no clear match between samples giving a positive CFT result and samples giving a positive ELISA result. The results of this study indicate that there is some systematic difference between the two laboratories. No statistical analysis is going to identify why this is the case. Positive and negative predictive values cannot be calculated for *Chlamydophila abortus* as there is no strong evidence of its presence in the Australian sheep population. This is supported by Australia's claim of freedom from EAE (*Animal Health in Australia*. Animal Disease Status). Until and unless an ELISA for the detection of *Chlamydophila abortus* antibodies is accepted for use in Australian laboratories, an assessment of reproducibility estimates through participation in external QAPs cannot be made. The ELISA being evaluated is a commercial kit. The upper and lower control limits have been established by the manufacturer, and test validation is provided as part of the protocol. See attached: Appendix A-1 (2.3 Assay Optimisation) Validation File, Pourquier *Chlamydophila abortus* Serum ELISA

	e.g. ANQAP z-score, Youden Plots, estimates for random vs systematic errors, basic statistics e.g. Coefficient of variation (CV), mean, etc *- Establishing lower and upper control limits (LCL, UCL) for internal controls (C++, C+ and C- e.g. using mean value of control plus three standard deviations from at least 20 runs)*	
4.4	MAINTENANCE AND ENHANCEMENT OF VALIDATION CRITERIA *Description of a range of appropriate test controls, including plans for continuous validation assessment.* *Continuous validation assessment may include:* *- Additional results from samples tested from target population as they become available* *- Extending validation criteria into other populations or adjusting assumptions for original population e.g. as prevalence may drop during an eradication campaign (when both true positive and false positive animals are removed)* *- Replacement of reagents (new reagents are standardised by comparison with reagents currently in use and participation in external QA programmes)*	Should this ELISA be taken up by Australian laboratories, it would be expected that there would be ongoing assessment. Acceptance of the assay by laboratories offering a health certification service for international trade would result in an expectation that interlaboratory proficiency testing would be offered by ANQAP. This would provide a source on continuous monitoring of the performance of the assay. There may be some further development of this assay by the manufacturer. Other ELISAs may become available and an assessment of their fitness for purpose and comparison with the Institut Pourquier product would be expected and encouraged.
4.5	QUALITY ASSURANCE *(Will the test will be included in Quality Assurance programs? - ANQAP, other)*	Yes, if the performance of the assay is judged to be worthy of introduction into Australian veterinary laboratories.

	Describe progress, plan, communications	
5	APPROVAL PROCESS RECORD *(To be completed by the Chair of the SCAHLS New Test Development Group or E/O SCAHLS)*	
5.1	*Dates (dd/mm/yyyy)*	Received by SCAHLS NTD Group: ___/___/____ Referred for peer review: ___/___/____ Peer review completed: ___/___/____ Decision to submit to SCAHLS or not, communicated to submitter: ___/___/____ SCAHLS decision: ___/___/____ SCAHLS decision communicated to submitter: ___/___/____
5.2	*Description of the review process undertaken (e-mail consultation, teleconference, face to face meetings etc)*	
5.3	*Review result (check one and provide reason)*	☐ Recommended to SCAHLS and AHC ☐ Recommended for review and resubmission ☐ Not recommended Initial and date: Reason:
5.4	*Process for Reporting the Recommendation*	☐ From Chair NTD working group through EO and Chair SCAHLS ☐ From Chair NTD working group directly to client

APPENDIX B-1.5

TEST METHOD PROTOCOL FOR [NAME] [SEROLOGICAL ASSAY]
FOR INCLUSION IN THE ANZSDP FOR [NAME] DISEASE

(Sufficient detail needs to be provided so that the test can be repeated by someone with experience in the basic technique). This detailed method is normally included in the ANZSDP Appendix.

Include such things as:

Reagents (including any preferred suppliers) – The ELISA is manufactured by Institut Pourquier and supplied in kit form. Bi-well coated microplates, positive and negative control sera, dilution buffers, conjugate, substrate and stop solution.

Test procedures – The method is supplied by the manufacturer and attached. This includes the validation criteria for the assay and the interpretation of results.

Specimen collection and handling and treatments – Clean fresh serum is required. The test can be used on bovine, ovine and caprine serum.

Throughput and turn around time expectations – The Institut Pourquier Chlamydophila abortus serum ELISA is an indirect ELISA. High volume throughput can be established within each testing laborator, but will be similar to that achieved with other commercial ELISA kits. Automation can be applied but the volume of reagents supplied may be insufficient. Additional reagents can be supplied free of charge. Under manual conditions, this ELISA could be set up and read within a day, including the preparation of samples.