

A COMPARISON FOR THE DETECTION OF *Corynebacterium pseudotuberculosis* IN SHEEP AND GOATS BETWEEN ELISA TEST AND THE AGAR GEL IMMUNODIFFUSION

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ABSTRACT Several enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of antibodies against *Corynebacterium pseudotuberculosis*, the causative agent of caseous lymphadenitis (CLA). However, none is commercially available. The ELISA using sonicated *C. pseudotuberculosis* to detect total antibody or IgG class antibody in serum was developed in Veterinary Research Institute, Ipoh (VRI). Our objective was to estimate the sensitivity and specificity of the developed ELISA and the agar gel immunodiffusion test (AGID) which is the diagnostic test for the detection of CLA. AGID test takes three days and ELISA only takes six to eight hours for testing. Using ELISA test, more samples can be tested in a shorter time. A total number of 500 sheep and goat sera were collected for the AGID and ELISA testing. The correlation between the two serological tests was analyzed. The estimated specificity was 95.1% for the AGID; 93.0% for the ELISA using an optical density measured cutoff point of 0.30; 90.0% using a cutoff of 0.25. The estimated sensitivities were 83.6% for the AGID; 88.0% and 93.0% for the ELISA using the cutoff of

0.30 and 0.25 respectively. Using the cutoff of 0.30 OD, the specificity is higher, 93.0% compared to 89.9% for the cutoff of 0.25 OD. The correlation between both serologic tests was 91.6%. The AGID and ELISA were specific but lack sensitivity and consistently identified the infected animals with moderate overlap between the positive test samples.

Keywords: *Corynebacterium pseudotuberculosis*, goats and sheep, CLA, AGID, ELISA

INTRODUCTION

Over the past ten years, according to the census by the Department of Veterinary Services, Malaysia, the small ruminant population in Malaysia has been steadily increasing. In 2008, the total population of sheep and goats had achieved 127,749 head and 437,805 head respectively (<http://agrolink.moa.my/jph>).

Corynebacterium pseudotuberculosis, CP (formerly *C. ovis*) is the causative agent of caseous lymphadenitis (CLA), a common disease in small ruminants (Brown and Oleander, 1987). This disease is characterized

by abscess formation in lymph nodes and viscera which can cause significant economic losses (Williamson, 2001). The affected animals typically have abscesses in parotid or retropharyngeal lymph nodes and can be diagnosed by bacteriological culture from the abscess. However, some animals are infected internally often in the lungs or mediastinal lymph nodes, and show no clinical sign of infection. To identify the infected animals with internal abscesses require alternative diagnostic methods and therefore researchers have developed serological tests for the diagnosis of CLA.

Several serological tests have been described to be applicable for the diagnosis of CLA such as microagglutination assay (Menzies and Muckle, 1989), an immunodiffusion test (Burrell, 1980), haemolysis inhibition test (Knight, 1977), a dot-blot (Prodhan *et al.*, 1993), Western blotting (Ter Laak *et al.*, 1992), complement fixation tests (Shigidi, 1979) and a variety of enzyme linked immunosorbent assays (ELISA). None is without problems (Sutherland *et al.*, 1987). The most specific ELISA based on recombinant phospholipase D (pLD) has been reported (Menzies *et al.*, 1994) but these ELISA are not commercially available at present.

The two most common serological tests used are AGID and ELISA. Both serological tests detected the same biological product, total antibody or IgG class antibody in serum. Currently in VRI, AGID is the routine diagnostic test to detect CLA and it takes three days for testing.

This problem had been a concern and a rapid serological test, ELISA, which is as reliable and efficient as AGID had been developed. The ELISA was developed using sonicated cell walls of locally isolated *C. pseudotuberculosis* (DI 3660/07) as antigen to optimize the detection to antibodies and it only takes six to eight hours to complete the testing. Several studies have evaluated the sensitivity and specificity of the AGID and ELISA in sheep. The AGID usually has slightly higher specificity (99-100%) than ELISA (95-100%) but this depends on the gold standard used and the ELISA cut-off chosen (Clarke, 1996; Hope, 2000; Sergeant, 2003).

The optical density measurement cutoff value for the ELISA was determined by parallel testing of 500 sheep and goat sera collected from various states in Malaysia, using the AGID as reference test. Receiver operating characteristic (ROC) curve was plotted for the ELISA and the sensitivity and specificity were calculated for six different cut-off values. Based on these values, two cutoff values had been chosen, 0.25 and 0.30. The specificity for the cut-off point 0.30 is slightly higher than the cutoff point 0.25 but less sensitive. It is recommended the cut-off point 0.30 is suitable for routine diagnosis and lower cutoff point 0.25 is useful when test is used for eradication scheme or control scheme during disease outbreak.

The objective of this study is to estimate sensitivity and specificity of AGID and ELISA in the absence of a gold standard. The comparison and correlation

between the AGID and ELISA was significantly high. Therefore, the ELISA procedure based on the whole cell antigen is suitable to be used as an alternative routine diagnosis for screening large numbers of sera with high specificity and sensitivity thus allowing the identification of positive animals in shorter time.

MATERIAL AND METHODS

Bacterial strain and antigen preparation

The *C. pseudotuberculosis* strain was a local clinical isolate obtained from the abscess of an infected goat DI 3660/07. For antigen preparation, *C. pseudotuberculosis* was initially cultivated on blood agar and a single colony of the bacteria was inoculated into 50 ml of brain heart infusion, Oxoid, England (BHI) broth, incubated at 37°C with agitation for 24 hrs. From the 50 ml overnight culture, 5 ml was transferred into 100 ml BHI broth and incubated at 37°C with agitation for 24hrs. This 50 ml overnight culture was expended to 9 x 100ml of BHI broth with the total volume of 1 liter. The bacterial cell was harvested by centrifugation (8000 rpm for 10 min) and then washed twice with cool Phosphate Buffered Saline (PBS). The pellet was resuspended in 50 ml of PBS and pulse sonicated to disrupt the bacterial cell wall. The antigen protein concentration was quantified using Bradford, Coomassie Blue method (Bio Rad Protein Assay, U.S).

The prepared antigen was stored in 1.5 ml aliquots and kept at -20°C.

Sera

The positive control serum serving as the reference standard was obtained from the infected goat DI 3660/07 with clinical symptoms of CLA (abscess on the right parotid lymph node). The diagnosis was confirmed by positive pus culture. A negative control serum was from a 2 year old goat from a farm with no history of CLA. The 500 serum samples used to determine the cut-off values with reference to AGID were obtained from various farms in Malaysia. These serum samples are the routine diagnostic samples sent to Veterinary Research Institute, Ipoh (VRI) for CLA detection.

Enzyme linked immunosorbent assay (ELISA)

The ELISA was performed on MaxiSorb flat bottom plate (Nunc, Denmark). Plates were coated with the sonicated bacteria at 15 µg/ml in 100 µl carbonated buffer (15 mM sodium carbonated, 35 mM sodium bicarbonate) at pH 9.6, and incubated at 37°C with agitation for 1 hour or incubated overnight at 4°C. The plates were washed three times with PBS-T (PBS containing 0.05% Tween 20) using 500 ml squeeze wash bottle or a plate washer. The plates were blocked with 100 µl of Blocking buffer (PBS-T with 5% of skim milk), incubated at 37°C with agitation

and repeated washing. Pre-diluted serum samples 1:100 in blocking buffer was added to the wells in duplicate, incubated with agitation again. Plates were washed three times, a peroxidase Rabbit anti-goat IgG, whole molecule (Sigma Chemical) was added. The same incubation and washing was repeated. o-Phenylenediamine dihydrochloride (oPD) with H_2O_2 was used as a substrate for the colour development. The plate was incubated for 10 min and the colour development was stopped with 1 M sulfuric acid. The colour was measured (optical density, OD unit) at 490 nm using the ELISA reader (Opsys MR, Dynex). In order to validate the ELISA assay, the positive control OD reading must be more than 1.0 OD and the negative control should be lower than 0.20 OD.

Agar gel immunodiffusion test (AGID)

The AGID test involves a precipitation reaction between antigen and antibody in the semi solid agar which forms a visible line. The semi solid iron agar plates were prepared with 1% purified agar (Oxiod, England) containing 1.6 g sodium chloride, 0.5 ml phenol. Thirteen milliliters of agar were poured in the 100 mm petri dishes and allowed to cool. Five milliliters wells were punched in the cooled agar with one centre and 6 surrounding wells placed 3 mm from the centre well. Exotoxin antigen of *C. pseudotuberculosis* prepared using Zaki method (Zaki, 1968) was placed in the central well. A positive control serum was placed in every other well alternating

with the test serum samples. The plates were placed under moist temperature for 3 days. Test was considered positive if a line of precipitation was fully formed between the test well and antigen well and was continuous with the line formed by positive control wells.

Statistical analysis

The ELISA and AGID test results were analyzed and the sensitivity and specificity were estimated. Sensitivity is calculated as the percentage of animals with CLA that had a positive test result. Specificity is calculated as the percentage of CLA negative animals that had negative tests result. The sensitivity and specificity for the ELISA at the six cut-off values for the 500 serum samples are calculated as shown in Table 1.

Receiver-operator characteristics curve (or ROC curve) is a graph of the true positive rate (sensitivity) against the false positive rate ($100 - \text{Specificity}$) for the different possible cut-off points of a diagnostic test. This graph was used to estimate the optimal cut-off for the various values of sensitivity and specificity. Six different cut-off OD values were 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and the estimated cut-off OD values were 0.25 and 0.3 (Figure 1). The comparison between two test results was analyzed using the SPSS program and the interactive box file graph was plotted (Figure 2). The correlation between AGID and ELISA was determined using the

TABLE 1. Sensitivity and specificity of the ELISA in relation to AGID result for different cut-off values.

ELISA RESULTS			AGID AND ELISA RESULTS				SENSI- TIVITY SE= A/(A+C) X 100 %	SPECI- FICITY SP= A/(B+D) X 100 %	FALSE POSI- TIVE 100%- SP
CUT OFF OD	POSI- TIVE	NEGA- TIVE	(A) AGID + ELISA +	(B) AGID - ELISA +	(C) AGID + ELISA -	(D) AGID - ELISA -			
0.10	395	105	139	256	5	100	96.50	28.10	71.90
0.15	299	201	137	162	7	194	95.10	54.50	45.50
0.20	224	276	136	88	8	268	94.40	75.30	24.70
0.25	168	332	134	34	10	322	93.10	90.40	9.60
0.30	152	348	127	25	17	331	88.20	93.00	7.00
0.35	107	393	94	13	50	343	65.30	96.30	3.70

Sensitivity = number of True Positives/ (number of True Positives + number of False negatives)
 Specificity = number of True Negatives/ (number of True Negative + number of False positives)

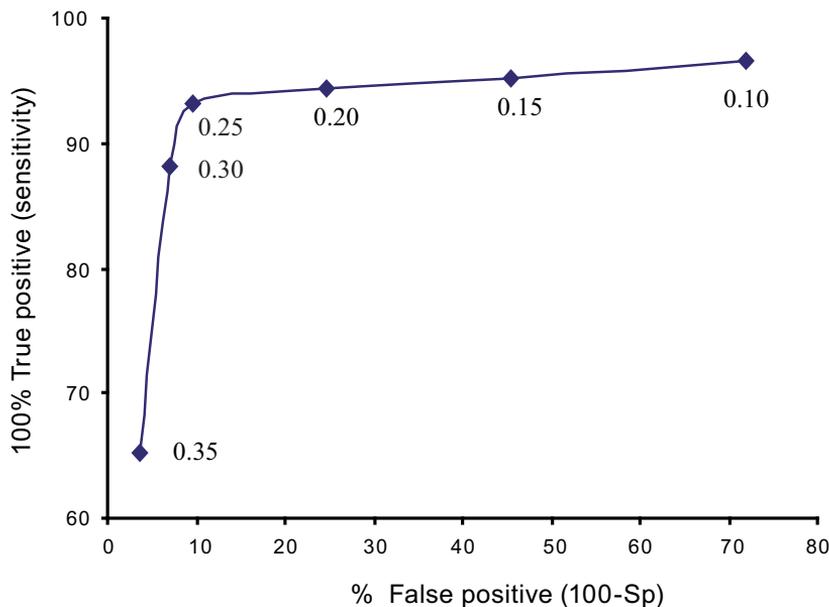


FIGURE 1. Receiver-operator characteristic (ROC) curve for ELISA. The data are based on a comparison of 500 serum samples in ELISA and AGID (Table 1).

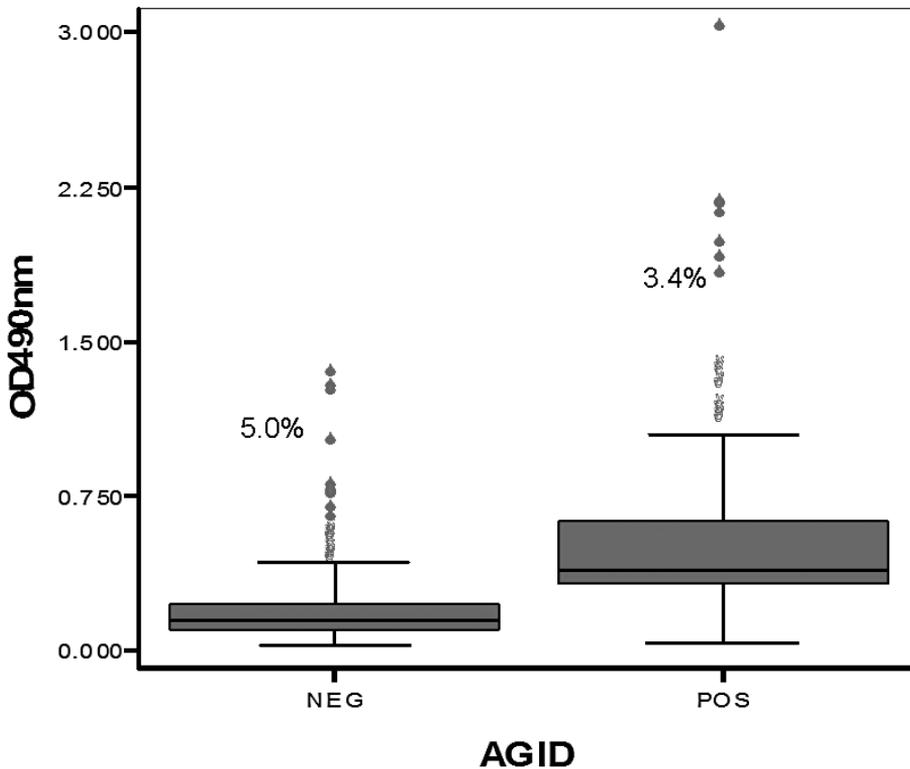


FIGURE 2. The interactive box file plot using the SPSS program. The scatterplot indicate the relationship of the AGID positive and negative results with the optical density reading at 490 nm for the ELISA assay.

TABLE 2. Correlation of ELISA and AGID for the detection of CLA antibodies

CORRELATION BETWEEN TESTS		FRACTION FROM TOTAL	PERCENTAGE
Agreement	127 positives, 331 negatives	458/500	91.6%
Disagreement	25 positives by ELISA and negatives by AGID	25/500	5.0%
	17 positives by AGID and negative by ELISA	17/500	3.4%

T. Blanino procedure (<http://www.iaea.org/programme>) (Table 2).

RESULTS

Establishment of the ELISA

The preparation of ELISA whole cell *C. pseudotuberculosis* antigen protein yielded approximately 3000 µg/ml. For optimal ELISA assay, the concentration of antigen coated into the ELISA plate were from 5 µg/ml to 30 µg/ml and the Rabbit anti-goat peroxidase conjugate was serially diluted from 500 to 256,000 times with blocking buffer. The positive and negative reference standard sera were constant at 100 times dilution. The 490 optical density absorbent results were plotted and analysis to optimize the concentration of antigen used for coating the ELISA plate and the dilution factor for the anti-goat peroxidase conjugate. The optimal antigen concentration for coating ELISA plates was determined to be 15 µg/ml per well and the dilution for peroxidase conjugate concentration was at 5000 times. Assay to assay variation was assessed based on repeated testing (5 times) of 40 serum samples with the AGID as reference test to determine the correlation of both tests. The average correlation between both tests was 92.0%.

Determination of cut-off value with reference to AGID test

To determine the cut-off values, a total of 500 serum samples were obtained from the routine diagnostic for CLA in VRI were parallel-tested in the ELISA and AGID as reference test in the absence of gold standard method. The sensitivity and specificity of the test were calculated for the six different cut-off values ranging from 0.10 to 0.35 optical densities (OD). At the lowest cut-off value, 0.10 OD, the sensitivity and specificity was 96.5% and 28.1% using the highest cut-off value, 0.35 OD, the sensitivity and specificity was 65.3% and 96.3%. This result indicates that when the cut-off value is low the sensitivity is high but lack of specificity and when the cut-off value is high the sensitivity is low and specificity is high (Table 1). To estimate the cut-off value all the six different possible OD values were plotted on a receiver-operator characteristic curve (ROC curve). Based on this curve, two cut-off values were chosen 0.25 OD and 0.30 OD (Figure 1). For these cut-off values, the calculated sensitivity and specificity were 93.1%, 90.4% and 88.2%, 93.0% respectively.

Correlation of ELISA and AGID test

The comparison between the ELISA and AGID was summarized in Table 2 and Figure 2. A 91.6% correlation was obtained between the tests. Of 42 serum samples that did not agree between the tests, 5%

were positive by ELISA but negative using AGID whereas 3.4% were AGID positive but ELISA negative. The relationship of the AGID positive and negative results with the OD for the ELISA assay was also shown in the interactive graph plot using the SPSS program.

CONCLUSION

In this study, we estimated the sensitivity-specificity and correlation between ELISA and AGID in order to validate and establish the developed ELISA for serodiagnosis of CLA-infectious animals. Currently AGID is the routine diagnostic test for CLA and this test had been fully established. Therefore, the development and evaluation of the ELISA was done by parallel testing using AGID as reference test. A whole cell lysate was used as solid phase antigen, and the serum from culture positive animal served as internal reference standard. The ELISA is evaluated by comparison with AGID results and shown to have a good assay to assay reproducibility.

For diagnosis of CLA, several ELISA tests had been developed. In an early study Maki *et al.* (1985) compared crude exotoxin and different forms of cell lysates with respect to their suitability as ELISA solid-phase antigens. The exotoxin was more sensitive solid-phase antigen than the whole cell sonicate. Shutherland *et al.* (1987) reported a comparison of crude toxin and purified cell walls as solid-phase antigens showing that the latter had higher sensitivity but lower

specificity. The most specific diagnostic test for *C. pseudotuberculosis* is an ELISA based on recombinant phospholipase D (pLD) expressed in *E-coli* (Menzies *et al.*, 1994). Neither of these ELISA tests is commercially available at present.

The ROC analysis was used to detect the best tradeoff between sensitivity and specificity and the accuracy of the both tests. Six different possible cutoff values of the ELISA were plotted on the ROC curve, (Greiner *et al.*, 1995). Based on the curve, two cutoff values were chosen 0.25 and 0.30 OD. The sensitivity for cutoff 0.25 is higher than cutoff 0.30 OD but less specific. Cutoff values should be chosen carefully because they can alter the results (Menzies and Noorhuizen, 1997). This 0.25 OD cut-off value is useful when the test is used in both as an eradication scheme with truly infected animals and in a disease control scheme. Using cutoff 0.30 OD for ELISA, the sensitivity and specificity were 88.2% and 93.0% and these combination values were suitable used for the CLA routine diagnosis. When using cut-off values 0.25 and 0.30 OD ELISA as reference test, the sensitivity for AGID was 79.8, 83.0% and specificity was 97.0, 95.1% (Table 3). The AGID specificity is slightly higher but less sensitive compared to ELISA, possibly because of the antigen used in the test. Both serological tests detected the same biological product, which are CLA antibodies. The ELISA was coated with whole cell antigen and AGID used crude exotoxin as antigen. A possible explanation is AGID used crude exotoxin as captured

TABLE 3. Sensitivity and specificity of the AGID in relation to ELISA assay with cut-off 0.25 and 0.30 OD.

ELISA	AGID RESULTS		AGID AND ELISA RESULTS				SENSI- TIVITY SE= A/(A+C) X 100 %	SPECI- FICITY SP= A/(B+D) X 100 %	FALSE POSI- TIVE 100%- SP
	POSITIVE	NEGATIVE	(A) ELISA + AGID +	(B) ELISA - AGID +	(C) ELISA + AGID -	(D) ELISA - AGID -			
0.25	144	356	134	10	34	322	79.8	97.0	3.0
0.30	144	356	127	17	25	331	83.0	95.1	4.9

CLA antibodies and had no cross-reacting antibody as in ELISA test. The correlation between the two serological tests was significantly high at 91.6% thus confirming the developed ELISA is the alternative test for the detection of CLA.

ELISA is a sensitive, fast and inexpensive method for determining the infectious animals and widely used for prevalence and disease control program. This test is usually sensitive and subsequently identified more positive animals. The AGID assay was however less sensitive than ELISA, therefore it is advisable to retest the positive CLA samples with AGID for confirmation. From the result, the agreement between ELISA and AGID is good thus proving that ELISA can be used as a diagnostic tool for detecting CLA. More research work must continue on further refinement of this ELISA test. The modifications based on the use of different types of solid-phase antigens instead of the whole cell antigen for detecting the CLA antibodies might improve the ELISA specificity.

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