Development and validation of an ELISA to detect antibodies to Corynebacterium pseudotuberculosis in ovine sera

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Abstract

Several enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of antibodies to Corynebacterium pseudotuberculosis, the causative agent of caseous lymphadenitis (CLA). However, none are commercially available in the UK. It was therefore necessary to develop a new, economic ELISA for use in a research project studying the epidemiology of CLA in UK sheep.

The ELISA with its diagnostic qualities is presented. The ELISA was developed using sonicated C. pseudotuberculosis and optimised to detect total antibody or IgG class antibody in serum. Receiver Operating Characteristic (ROC) curves were obtained and the area under the ROC curve was used to compare the sensitivity and specificity of the two ELISAs.

Both versions of the ELISA were evaluated on a panel of 150 positive reference sera and 103 negative reference sera. Using the test at 100% specificity, the sensitivity of detection of total antibody was 71% (95% confidence interval 63-78%), and the

sensitivity of detection of IgG antibody to C. pseudotuberculosis was 83% (76-89%), which compares favourably with other reported ELISA tests for CLA in sheep. The sensitivity of the IgG antibody assay may be higher because of the greater affinity of IgG class antibodies compared with the IgM antibodies also detected by the total antibody ELISA.

The results of ROC analysis indicated that the IgG isotype ELISA was more accurate than the total antibody ELISA. The efficiency of the test was greatest when serum samples were run in a dilution series than when any single serum dilution was used. The ELISA is considered to be suitable for application in field studies of CLA in UK sheep.

Keywords:
Corynebacterium pseudotuberculosis; Sheep-bacteria; ELISA; Diagnosis; Sensitivity; Specificity

1. Introduction

Corynebacterium pseudotuberculosis (formerly C. ovis) is the causative agent of caseous lymphadenitis (CLA) in sheep and goats. The disease is characterised by abscess formation in lymph nodes and/or viscera. In the UK, affected sheep typically have abscesses in the parotid or retropharyngeal lymph nodes, and the disease can be diagnosed by bacteriological culture of pus from such abscesses. However, a proportion of infected sheep may have only internal abscesses, often in the lungs or mediastinal lymph nodes, and show no overt clinical signs of infection. Identification of sheep with internal abscesses requires alternative diagnostic methods, and therefore researchers have developed serological tests for the diagnosis of CLA.

CLA was first diagnosed in the UK in 1990, in imported Boer goats, and was first reported in sheep in this country in 1991. It has since spread widely within the UK sheep flock (Binns et al. 2002). CLA can cause economic losses for pedigree sheep breeders and concern has been raised that the disease may spread to commercial flocks and lead to an increased condemnation of lambs at slaughter. The true prevalence of infection in UK sheep has not been estimated, partly due to the lack of an adequate and available diagnostic test for infection in live sheep.

Although there are currently several serodiagnostic tests for the detection of antibodies to C. pseudotuberculosis in sheep, including haemolysis inhibition (Burrell 1980), indirect haemagglutination, anti-haemolysin inhibition, complement fixation tests (Shigidi 1979), immunodiffusion (Burrell 1980) and enzyme-linked immunosorbent assays (ELISA), none are without problems (Sutherland et al. 1987). Various antigen preparations have been used in the ELISA tests, including cell wall antigens (Sutherland et al. 1987), crude exotoxin (phospholipase D) (ter Laak et al. 1992; Dercksen et al. 2000), cell supernatant (Maki et al. 1985), and recombinant exotoxin (Menzies et al. 1994).

Typically the tests perform adequately in goats (Dercksen et al. 2000; Kaba et al. 2001), but with reduced sensitivity in sheep, especially in subclinically infected sheep with only internal abscesses. Tests may also have differing specificity when compared with bacteriological culture, possibly due to cross-reactions with related bacterial species or infected but recovered sheep (culture negative) or because of presence of maternal antibodies. Finally, the rate of seropositivity in culture-positive sheep varies with age and immune status and with the route and extent of exposure to C. pseudotuberculosis and the interval between exposure and diagnosis (Sutherland et al. 1987).

The most specific diagnostic test reported for C. pseudotuberculosis is an ELISA based on recombinant phospholipase D (PLD) expressed in E. coli (Menzies et al. 1994). Perhaps the best current ELISA test is that developed for use in the Dutch CLA elimination and control programme (Dercksen et al. 2000). This modified double antibody sandwich ELISA has a sensitivity of 79 ± 5% and specificity of 99 ± 1% for sheep. Neither of these ELISA tests are commercially available in the UK at present, and all those developed to date are relatively expensive. This paper describes the development of an alternative test used to for epidemiological research studies of ovine CLA in the UK.

2. Materials and Methods

2.1 Sample collection and management

Blood samples were collected from sheep by jugular venepuncture into 10 ml vacutainer tubes without anticoagulant (Becton-Dickinson). Serum was separated from clotted blood in vacutainers by centrifugation and decanting. Serum samples were aliquoted and stored at 4^{o}C until processed; long-term storage was undertaken at –20^{o}C.

To ascertain the true infection status of abscessed sheep in the positive reference sample, pus samples were taken from superficial abscesses of live sheep, or at post mortem examination from dead sheep, and transported in charcoal Amies' transport medium to the laboratory. Bacteriological culture was carried out on blood agar under conditions of 5% CO_2 for approximately 48 hours. Colonies morphologically resembling those of C. pseudotuberculosis were Gram-stained, and Gram-positive rods were tested for urease and catalase. Isolates that were urease and catalase positive were identified as C. pseudotuberculosis. A representative selection of

isolates was confirmed as C. pseudotuberculosis using the API-Coryne biochemical test system (Bio-Mérieux).

2.2 Reference serum samples

The ELISA was evaluated on a panel of 150 positive reference sera from sheep in 22 flocks, each sheep had at least one abscess positive for C. pseudotuberculosis at bacteriological culture at the time of sampling and 103 negative reference sera from sheep in four flocks which had never had a case of CLA. Many of the negative reference sera were obtained by one of the authors (LG) in 1993, before CLA had reached a high prevalence in the UK.

A pooled positive control standard serum was prepared from all test sera with a relative antibody concentration higher than 0.8 times the highest positive serum sample in the positive reference collection (n = 28). Negative control standard sera (n = 3) were obtained from three one-year-old, barn-reared experimental Dorset sheep, which were determined to be free from lesions suggestive of CLA at post mortem examination. Bacteriological cultures of parotid and mediastinal lymph nodes from these sheep were also negative.

2.3 Antigen preparation

The antigen used to coat the ELISA plates was obtained by growing up one bead from frozen stocks of an isolate of C. pseudotuberculosis obtained from an infected sheep (isolate 1620) in brain-heart infusion broth at 37^{o}C with agitation for 48 hours. The culture was then centrifuged for 10 minutes at 2000 rpm; the cells were washed twice in 10 ml phosphate-buffered saline (PBS), and resuspended in 1.5 ml PBS. The cells were pulse sonicated (Soniprobe Type 1130A, Dawe Instrumental Ltd., London)

for six 30-second pulses to disrupt the cell walls. The antigen preparation was quantified using the Coomassie Blue method (Bradford 1976). Prepared antigen was stored in 1.5 ml aliquots at –20^{o}C. Alternative antigen preparations that were initially tested included culture supernatant and non-sonicated cells, all antigens in either of these preparations were present in the sonicated cell preparation in preliminary experiments (unpublished data) and so the cell sonicate was used for further analysis.

2.4 ELISA procedure and development

Ninety-six well PVC microtitration plates (Greiner Bio-One Ltd., Stonehouse, Glos.) were coated with the sonicated bacteria at 4 μg/ml in 100 μl carbonate/bicarbonate buffer (15mM sodium carbonate, 35mM sodium bicarbonate) at a pH of 9.6, and incubated overnight at 4^{o}C. The plates were washed between each incubation step three times in PBS-Tween (phosphate-buffered saline plus 0.1% v/v Tween-20) using a commercial plate washer (Titertek M384 Atlas Microplate Washer, Biological Instrumentation Services, Ltd.). Serum was added to the wells and the plates were again incubated overnight at 4^{o}C.

Both versions of the ELISA used a two-step detection system; the primary detection antibody (mouse anti-sheep monoclonal) varied between the tests (see below), but the secondary detection was carried out using 100 μl of alkaline-phosphatase conjugated donkey anti-mouse IgG monoclonal antibody (Jackson Immunoresearch Ltd.) diluted to 1/1000 in PBS-Tween in all cases. Both detection antibodies were incubated on the plates at 37^{o}C for one hour and then 50 μl per well alkaline phosphatase substrate p-nitrophenol phosphate, disodium (1mg/ml) (Sigma) in carbonate/bicarbonate buffer was added. The plates were then left at room temperature to develop. The plates were read at 405nm against 492nm (non-specific absorption) when the highest standard

optical density (OD) reached 1.5-2.0 units on a Multiscan EX (Thermo Labsystems, Vantaa, Finland). The ELISA was optimised with respect to incubation times, and the concentrations of coating antigen and detection antibodies were optimised using chequerboard titrations.

2.5 Pan-light-chain ELISA

For this version of the ELISA, test sera were assayed using serial dilutions in 100 µl PBS-T against the positive standard, which was also run in serial dilutions. For each plate, 12 wells were filled with 100 µl PBS-T only, to serve as a background control. The primary detection antibody was the cell culture supernatant from cell line K67.3G2 supplied by Karin Haverson, School of Clinical Veterinary Science, University of Bristol. This monoclonal antibody detects ovine light chains, and therefore all antibody classes, and was diluted in PBS-Tween to 1/100 and added at 100µl per well.

2.6 Anti-IgG ELISA

For this version of the ELISA, test sera were tested at serial dilutions in 100 µl PBS-T starting at 1/100 against the positive standard. On each plate, 12 wells were filled with PBS only, to serve as a background control. The primary detection antibody used in this test was a monoclonal anti-ovine IgG (IgG1 plus IgG2) (clone VPM6, Serotec), diluted in PBS-Tween to 1/1000 (100µl per well).

2.7 ELISA analysis

Each of the sera were tested at a range of one in three dilutions; two negative standard control sera and the pooled positive standard (in duplicate) were also run in serial dilutions on each plate to provide positive and negative reference curves (serum dilution against OD). The process for calculating antibody concentrations relative to a standard has been previously described in several species (Finerty et al, 2000; Bailey et al, 2004;). Briefly, the mean background OD for completely negative wells was first subtracted from each OD reading. The concentration of antibody in the reference serum was arbitrarily assigned as 1. A graph was plotted of log[reference serum] against a transformed function for the OD. In this case, a square-root transformation of the OD produced the best straight line region within the curve. The intercept and gradient of this straight line region was calculated for each plate, and the values obtained used to calculate the amount of antibody in each well relative to the standard (equation 1).

[1] Concentration of antibody = gradient * $OD^{0.5}$ + intercept

Since each sample well contained a dilution of the original sample, the calculated relative[Ab] for each well was multiplied by the dilution to obtain a value for rel[Ab] in the original sample. Where rel[Ab] was calculated from multiple dilutions of a particular sample, only those dilutions whose OD values were within the linear part of the curve were used to obtain the mean and standard deviation (SD) of the rel[Ab]. This method required the assumption that the gradients of the line of log(dilution) against $OD^{0.5}$ (usually a reflection of affinity) were the same for all samples: in fact, the gradients of some of the samples in the negative reference population were shallower, indicating low affinity

201

202 2.8 Statistical analysis

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204 Statistical analysis was carried out using Stata version v.7 (Statacorp). The variables
205 analysed were the antibody concentration relative to the positive standard over the
206 linear portion of the dilution series (rel[Ab]) at each individual dilution for the anti-
207 IgG ELISA. Within- and between-assay repeatability was assessed by calculating the
208 coefficient of variation for rel[Ab]. The agreement between test results was assessed
209 by the calculation of the kappa statistic for the dichotomous value (positive or
210 negative) using an appropriate cut-off, and Spearman rank correlation coefficients for
211 the continuous variable (rel[Ab]) (Altman, 1991). The analysis of agreement was
212 carried out for pairs of results using the same assay on different occasions, and using
213 the two different ELISA tests.
214 Receiver Operating Characteristic (ROC) curves (plots of sensitivity against [1-
215 specificity]) were plotted and used to estimate the optimal cut-off for various values
216 of sensitivity (Se) and specificity (Sp); the area under the ROC curve (AUC) was
217 estimated by non-parametric integration (Greiner et al. 1995). Exact binomial 95%
218 confidence intervals (or one-sided 97.5% CI for an estimate of 100% Se or Sp) for
219 sensitivity and specificity were calculated. The mean and geometric mean relative
220 antibody concentration were obtained for the positive and negative reference
221 populations and tested using t-tests. Further measures of diagnostic test accuracy
222 (Greiner et al. 1995) were estimated in an Excel spreadsheet. Odds ratios were
223 corrected by adding 0.5 to each cell when Se or Sp was estimated to be 100%. The
224 above analysis was carried out for each variable at cut-offs of 100% sensitivity, 100%

specificity, the maximum specificity with a sensitivity of ≥ 80%, the maximum sensitivity with a specificity of ≥ 96%, and roughly equal sensitivity and specificity.

3. Results

3.1 Distributions of the ELISA responses of reference sera

The geometric mean relative antibody concentrations, calculated for each dilution of sample independently, were significantly different between the positive and negative reference populations except for the two highest single dilutions using the anti-IgG ELISA system (Table 1). This reflects the fact that at high dilutions, OD readings for positive and negative samples fall below the linear part of the curve and become unreliable, demonstrated by the increasing 95% confidence intervals relative to the mean.

The distributions of the relative antibody concentrations in each sample were negatively skewed, so logarithmic transformations were used in the analysis. Histograms of the logarithm of Rel[Ab] for both versions of the ELISA overlapped (boxed in Fig. 1 and 2) where the results of the positive and negative reference populations overlapped. This area was substantially narrower and involved fewer sheep for the anti-IgG ELISA (Fig. 1) than for the K67 ELISA (Fig. 2), indicating the greater efficiency of the former in discriminating between the two reference populations.

3.2 Assessment of repeatability and inter-test and within-test agreement

The within-assay (duplicate samples run at the same time, although rarely on the same plate) coefficient of variation (CV) was slightly lower than the between-assay (duplicate samples run on different occasions) CV (Table 2). The agreement for repeated assays within each version of the ELISA was acceptable (Table 3). However, the agreement between the K67 and anti-IgG versions of the test was lower, particularly in the positive reference population, indicating that the latter had increased sensitivity at 100% specificity compared with the former.

3.3 Cut-off determination and determination of sensitivity and specificity

The sensitivity of the K67 test to detect total antibody and the anti–IgG test to detect IgG antibody to C. pseudotuberculosis was 71% (95% confidence interval 63-78%) and 83% (76-89%) respectively when the specificity was set at 100% (Table 4). The specificity of the K67 test was 23% (95% confidence interval 14-34%), and that of the anti–IgG test was 64% (53-74%) when the sensitivity was set at 100% (Table 4).

3.4 ROC analysis

The results of ROC analysis indicated that the IgG isotype ELISA (AUC 0.9887) was more accurate than the total antibody ELISA (AUC 0.9494), P = 0.003. Using a 1/100 dilution in the anti-IgG ELISA, the overall accuracy was not significantly different from that obtained using the whole series (AUC 0.9741, P = 0.2), but it was impossible to obtain 100% specificity using this dilution or the 1/900 dilution; the maximum specificity obtained in both cases was 98.9% (95%CI: 94.2-99.9%).

4. Discussion

The sensitivity and specificity of the anti-IgG ELISA compared favourably with other reported ELISA tests for CLA in sheep (Dercksen et al. 2000). The current ELISA was simpler and cheaper to run than the Dutch test which is an indirect double-sandwich antibody ELISA.

For a disease such as CLA, deciding a gold standard with high discriminatory efficacy is challenging. In the current study the gold standard used to define the positive reference population was culture of C. pseudotuberculosis from typical lesions. This is a reasonable positive standard. However, the assumption that negative culture is a negative control may not be valid. Many C. pseudotuberculosis-infected sheep do not display clinical signs, and lesions may be non-culturable rather than negative for C. pseudotuberculosis. It is impossible to be certain that the negative reference population is truly uninfected and has never been in contact with the bacterium (Menzies et al. 1994). We aimed to maximise our confidence of the status of our negative reference population by using a combination of sheep from clinically-negative flocks, specific-pathogen-free sheep and experimental sheep that had never been in contact with CLA. If any of the negative reference population were infected, this would have resulted in misclassification, which in turn would bias the estimates of sensitivity and specificity (Staquet et al. 1981).

The representativeness of the negative reference population used for establishing the cut-off value is of major importance (Greiner and Böhning 1994). Negative samples ideally need to come from the same population as positive samples so that other biological factors within the target population can be ignored. In our case, the negative reference population were younger than the target population, and many of the samples were obtained in 1993, several years earlier than the current study field samples for which the ELISA was developed. This was done to ensure that the

300 negative control sheep were truly uninfected, but these factors are a potential
301 limitation to the ELISA qualities.
302 The ELISA presented in this paper was based on crude bacterial antigen, containing
303 all the antigens in the cell supernatant and cell wall, to detect antibodies to as many
304 antigens of C. pseudotuberculosis as possible, thus combining both cell-wall and
305 toxin-based assays (Sutherland et al. 1987). Other authors have reported that a cell
306 supernatant antigen performed better than sonicated cells (Maki et al. 1985) but this
307 was not our experience. Muckle et al. (1992) reported that the specificity of crude
308 antigen preparations was low, we addressed this by using a double antibody detection
309 system and the specificity of our ELISA was reasonable. However, as demonstrated
310 here the specificity of an IgG antibody test is higher than that of the total antibody
311 ELISA for a given sensitivity, because the latter also detects IgM which may result in
312 much greater cross-reactivity than with IgG alone.
313 The ROC analysis was used to detect the best trade-off between sensitivity and
314 specificity, to compare the accuracy of the two ELISAs and to investigate the use of
315 single dilutions of test sera in the anti-IgG assay. The use of a series of dilutions of
316 test sera was necessary initially to establish a cut-off point for the ELISA, to detect
317 any prozone effect, and to identify a single serum dilution that could be routinely
318 employed for test samples. The dilution series in fact proved more accurate than any
319 single dilution of test sera in the anti-IgG assay. Most other reported ELISA tests for
320 CLA use only a single serum dilution for each sample (Sutherland et al. 1987;
321 Sutherland et al. 1987; ter Laak et al. 1992; Dercksen et al. 2000). The validation of a
322 choice of one dilution against a series of dilutions is not described in these papers.
323 However, such assays may have serious inaccuracies due to prozone effects at low
324 dilutions and increasing errors at high dilutions (Figure 4). For the IgG ELISA

325 presented here the 1/100 dilution gave a similar AUC ROC to the dilution series, but
326 it was not possible to obtain 100% specificity with this dilution. If this were not an
327 important consideration, the test could be run using single 1/100 dilutions of test sera,
328 resulting in a decrease in the cost of running the assay.

329 A proportion of C. pseudotuberculosis culture-positive sheep were negative to our
330 ELISA. This has been reported for all ELISAs developed to date. This is unlikely to
331 be due to the particular infecting strain of C. pseudotuberculosis, because sheep
332 experimentally infected with either sheep or goat strains of the bacterium showed
333 similar responses on Western Blot analysis, and differed from the responses of
334 identically-treated goats (Kamp et al. 2001). It is possible that some 'false negative'
335 sheep did not produce antibody to the particular antigen(s) used, but this is less likely
336 with crude antigen. It is also unlikely that infected sheep tested negative to the
337 antibody-ELISA because they have lost antibodies because, although the half-life of
338 experimentally-transferred antibody is only about three weeks, antibody can usually
339 be detected for at least a year in natural infections. Even in sheep without gross
340 lesions, it is likely that small quantities of antigen are sequestered by the follicular
341 dendritic cells in lymph nodes, and that these periodically stimulate memory cells
342 (KoscoVilbois and Scheidegger, 1995).

343 Although the immune response to CLA in most sheep has a strong humoral
344 component (Pépin et al. 1993), it is possible that the low sensitivity of ELISA tests
345 designed to detect antibodies to C. pseudotuberculosis may result from some sheep, or
346 sheep during certain stages of infection, expressing a predominately cell-mediated
347 immune response to the pathogen. In general, T_H1 cells promote cell-mediated
348 immunity (CMI) while T_H2 cells stimulate a humoral immune response (Infante-
349 Duarte and Kamradt, 1999). Genetic predisposition to express Th1 or Th2 responses

does occur in sheep and contributes to differences in the ability to control infectious agents (Gill et al, 2000). Some pathogens, such as Mycobacterium avium subsp. paratuberculosis stimulate different arms of the immune response at different stages of pathogenesis (Burrells et al. 1998). Alternative techniques to identify diseased but Ab negative sheep include an ELISA to IFN-γ produced by stimulated leucocytes (Prescott et al. 2002) and polymerase chain reaction (Çetinka et al. 2002), but the feasibility of these techniques in diagnosing infection in live sheep on farms has not yet been demonstrated.

The anti-IgG ELISA test reported here has since been used in epidemiology studies in the UK. The lack of sensitivity in individual sheep was overcome by increasing the sample size per flock, to ensure that if infection was present then it would be detected. An alternative would have been to reduce the cut-off value, resulting in an increased sensitivity at the expense of reduced specificity.

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Table 1: Mean and geometric mean relative [Ab] (rel[Ab]) in positive and negative reference populations

Relative [Ab] calculated using:	**Positive reference population geometric mean rel[Ab] (95% CI)**	**Negative reference population geometric mean rel[Ab] (95% CI)**	**P-value (t-test)**
Total Ab*, all dilutions	0.19 (0.16-0.23)	0.022 (0.017-0.027)	<0.001
IgG[#], all dilutions	0.14 (0.11-0.18)	0.007 (0.006-0.008)	<0.001
IgG, serum diluted 1/100	0.025 (0.024-0.027)	0.006 (0.005-0.006)	<0.001
IgG, serum diluted 1/300	0.057 (0.051-0.064)	0.009 (0.008-0.01)	<0.001
IgG, serum diluted 1/900	0.102 (0.087-0.12)	0.020 (0.019-0.022)	<0.001
IgG, serum diluted 1/2700	0.16 (0.13-0.19)	0.051 (0.048-0.054)	<0.001
IgG, serum diluted 1/8100	0.26 (0.22-0.30)	0.14 (0.14-0.15)	<0.001
IgG, serum diluted 1/24300	0.49 (0.43-0.56)	0.43 (0.40-0.45)	0.11
IgG, serum diluted 1/72900	1.12 (1.03-1.21)	1.27 (1.17-1.38)	0.03

*Total Ab: Total Antibody ELISA (K67)

[#] IgG: anti-IgG ELISA

Table 2. Within- and between-assay coefficients of variation (CV):

	K67 ELISA		Anti-IgG ELISA	
Sample:	**Median within-assay CV[#] (%)**	**Median between-assay CV (%)**	**Median within-assay CV (%)**	**Median between-assay CV (%)**
Whole reference population	25.2	27.6	19.6	30.2
	IQ*: 12.7-34.3	IQ: 10.7-55.0	6.7-39.0	IQ:15.2-47.8
Positive reference samples	19.9	22.1	Insufficient data	31.5
	IQ: 8.5-34.3	IQ: 9.2-42.9		IQ:15.2-49.3
Negative reference samples	17.1	50.4	19.6	30.1
	IQ: 13.3-25.5	IQ: 26.8-95.6	6.7-39.0	IQ: 11.1-47.8

[#]**CV**: Coefficient of variation

*IQ: inter-quartile range

Table 3: Agreement between results of repeated applications of the same ELISA, and between results obtained with K67 and IgG ELISAs

	K67		Anti-IgG		Between K67 & IgG	
Sample:	**Kappa (P-Value)**	***Spearman (P-value)**	**Kappa (P-Value)**	**Spearman (P-value)**	**Kappa (P-Value)**	**Spearman (P-value)**
Reference population (100% specificity)	0.78 (<0.001)	0.91 (<0.001)	0.87 (<0.001)	0.93 (<0.001)	0.41 (<0.001)	0.77 (<0.001)
Reference population (100% sensitivity)	0.60 (<0.001)		0.62 (<0.001)		0.49 (<0.001)	
Positive reference (100% specificity)	0.76 (0.001)	0.86 (0.001)	0.67 (<0.001)	0.88 (<0.001)	0.22 (<0.001)	0.64 (<0.001)
Negative reference (100% sensitivity)	0.49 (0.002)	0.38 (0.006)	0.46 (<0.001)	0.78 (<0.001)	0.15 (0.14)	0.05 (0.79)

*Spearman rank correlation coefficient

Table 4: Accuracy at Specificity = 1 (97.5% lower confidence limit for specificity = 0.96; Positive predictive value = 1; Likelihood ratio of a positive test = 0)

Rel[Ab] calculated using:	Sensitivity	95% CI (sensitivity)	Efficiency[c]	NPV[d]	Youden's Index[e]	LR-[f]	Odds ratio (corrected)
Total Ab[a], all dilutions	0.708	0.627-0.781	0.804	0.625	0.708	0.292	340.06
IgG[b], all dilutions	0.833	0.764-0.889	0.897	0.790	0.833	0.167	930.18
IgG, serum diluted 1/100				Not obtained			
IgG, serum diluted 1/300	0.020	0.004-0.057	0.398	0.390	0.020	0.98	4.48
IgG, serum diluted 1/900				Not obtained			
IgG, serum diluted 1/2700	0.587	0.503-0.666	0.749	0.607	0.587	0.413	267.62
IgG, serum diluted 1/8100	0.207	0.145-0.280	0.512	0.441	0.207	0.793	49.82
IgG, serum diluted 1/24300	0.193	0.133-0.266	0.504	0.437	0.193	0.807	45.89
IgG, serum diluted 1/72900	0	0-0.024	0.385	0.385	-1	1	0.628

[a]Total Ab: Total Antibody ELISA (K67)
[b] IgG: anti-IgG ELISA
[c] Efficiency: (true positives + true negatives) / total
[d] NPV: Negative predictive value
[e]Youden's Index: Se + Sp – 1
[f]LR-: Negative likelihood ratio: [Probability of no disease/Probability of disease] given test result, divided by the odds [prevalence]
[g]Odds ratio: Positive likelihood ratio / Negative likelihood ratio

Table 5: Accuracy at Sensitivity = 1 (97.5% lower confidence limit for sensitivity = 0.98; Negative predictive value = 1; Likelihood ratio of a negative test = 0)

Rel[Ab] calculated using:	Specificity	95% CI (Specificity)	Efficiency[c]	PPV[d]	Youden's Index[e]	LR+[f]	Odds ratio (corrected)[g]
Total Ab[a], all dilutions	0.229	0.137-0.344	0.748	0.727	0.727	1.296	87.50
IgG[b], all dilutions	0.638	0.533-0.735	0.861	0.815	0.815	2.765	527.84
IgG, serum diluted 1/100	0.734	0.633-0.820	0.898	0.857	0.857	3.759	820.37
IgG, serum diluted 1/300	0.511	0.405-0.615	0.812	0.765	0.765	2.043	313.95
IgG, serum diluted 1/900	0.021	0.003-0.075	0.623	0.620	0.620	1.022	8.14
IgG, serum diluted 1/2700	0	0-0.038	0.614	0.614	0.614	1	1.59
IgG, serum diluted 1/8100	0	0-0.038	0.614	0.614	0.614	1	1.59
IgG, serum diluted 1/24300	0	0-0.038	0.614	0.614	0.614	1	1.59
IgG, serum diluted 1/72900	0.0106	0.0003-0.058	0.619	0.617	0.617	1.010	4.83

[a]Total Ab: Total Antibody ELISA (K67)
[b] IgG: anti-IgG ELISA
[c] Efficiency: (true positives + true negatives) / total
[d] PPV: Positive predictive value
[e]Youden's Index: Se + Sp – 1
[f]LR+: Positive likelihood ratio: [Probability of disease/Probability of no disease] given test result, divided by the odds [prevalence]
[g]Odds ratio: Positive likelihood ratio / Negative likelihood ratio

Table 6: Results of ROC analysis

Rel[Ab] calculated using:	**AUC ROC**	**Max Se at ≥0.96 Sp (95% CI)**	**PPV**	**NPV**	**Max Sp at ≥80% Se (95% CI)**	**PPV**	**NPV**	**Equal Se/Sp (95% CI Se)**	**PPV**	**NPV**
Total Ab[a], all dilutions	0.9494**	0.771 (.69-.84)	0.98	0.67	0.943 (.86-.98)	0.97	0.70	0.861 (.79-.91)	0.93	0.75
IgG[b], all dilutions	0.9887	0.933 (.88-.97)	0.98	0.90	1.0 (.96-1.0)	1	0.79	0.947 (.90-.95)	0.97	0.92
IgG, serum diluted 1/100	0.9741	0.860 (.79-.91)	0.98	0.81	.968 (.91-.99)	0.98	0.81	0.940 (.89-.97)	0.97	0.91
IgG, serum diluted 1/300	0.9677	0.887 (.83-.93)	0.98	0.84	0.98 (.93-.99)	0.98	0.75	0.920 (.86-.96)	0.95	0.88
IgG, serum diluted 1/900	0.9372**	0.827 (.76-.88)	0.98	0.78	.989 (.94-.99)	0.93	0.41	0.887 (.82-.93)	0.92	0.83
IgG, serum diluted 1/2700	0.8351**	0.673 (.59-.75)	0.97	0.66	0.585 (.48-.69)	0.76	0.68	0.747 (.67-.81)	0.83	0.66
IgG, serum diluted 1/8100	0.6408**	0.387 (.31-.47)	0.95	0.50	0.287 (.20-.39)	0.65	0.49	0.573 (.49-.65)	0.69	0.47
IgG, serum diluted 1/24300	0.4532**	0.193 (.13-.27)	0.94	0.43	0.850 (.037-.16)	0.59	0.22	0.400 (.30-.51)	0.52	0.30
IgG, serum diluted 1/72900	0.3429**	0.093 (.052-.15)	0.82	0.40	0.351 (.26-.46)	0.67	0.53	0.633 (.55-.71)	0.74	0.52

**Significantly different ($P < 0.05$) from AUC ROC for αIgG series.

[a]Total Ab: Total Antibody ELISA (K67)

[b] IgG: anti-IgG ELISA

Figure captions:

Fig. 1: Histogram of Relative [Ab] for positive and negative reference serum samples: Anti-IgG ELISA. Box represents overlap between positive and negative reference populations

Fig. 2: Histogram of Relative [Ab] for positive and negative reference serum samples: K67 ELISA. Box represents overlap between positive and negative reference populations

Fig. 3: Receiver Operating Characteristic (ROC) curves for αIgG and K67 ELISAs

Fig. 4: Sample data comparing sqrt(OD) against log(dilution) for standard (plus regression line) and two samples. Estimation of the concentration of sample 1 would be inaccurate from a single dilution of 1/100, while estimation of sample 2 would be inaccurate at a dilution of 1/1000.