Vol 18 No 1, p 47-52

EVALUATION OF 5 INDIRECT ELISA FOR THE DETECTION OF ANTIBODIES TO PARATUBERCULOSIS IN DROMEDARIES

U. Wernery[1], A. Abraham[1], S. Joseph[1], R. Thomas[1], G. Syriac[1], R. Raghavan[1] and T. Baker[2]

[1]Central Veterinary Research Laboratory, P.O. Box 597, Dubai, UAE

[2]UCB 216 Bath Road, Slough, Berkshire, SL1 3WE, UK

ABSTRACT

The four commercial indirect MAP ELISAs were only able to detect paratuberculosis positive camel sera when the kit conjugate was replaced with either Protein A or the goat anti-camel IgG conjugates from Bio-X or CVRL. The Triple J conjugate did not perform well in contrast to the findings of Kramsky and co-workers who used this in a similar protocol to detect anti-MAP antibodies in llama and alpaca sera. With the former combinations, the Checkit and ID Screen MAP antigen coated plates showed considerable non-specific cross- reactivity with paratuberculosis negative camel sera, viz. %S/P values ≥ 25% in 4 and 2 out of 4 negative sera, respectively. The Paratub MAP antigen coated plate/ Protein A conjugate combination showed better non-specificity, although one camel sample E2A which had no history of paratuberculosis showed a % S/P of 27% and also a reduced response in camel 6BI after the second vaccination dose.

Parachek, and in-house MAP antigen coated plates worked well in combination with Protein A conjugate showing acceptable non-specific cross reactivity (%S/P ≤ 12%), and a good colorimetric signal that provided an excellent anti-MAP immune response in the two vaccinated camels, with a response range of greater than 2 and 1.2 absorbance units in camels 47B and 6BI, respectively, after the second vaccine dose compared to the pre-treatment level. Similar profiles were obtained for the in-house ELISA protocol that employed OPD substrate, a format that was in common to other ELISAs in our laboratory. It was therefore concluded, that the in-house MAP ELISA was the method of choice for future studies on *M. paratuberculosis* infection in camels.

Key words: Dromedary, iELISA, paratuberculosis

Paratuberculosis (Johne's Disease) is characterised by persistent and progressive diarrhoea, weight loss, debilitation and eventually death. It affects cattle, sheep, goats, farmed deer and other domestic and wild ruminants. It also occurs in Old World Camels (OWC) (Wernery and Kaaden, 2002) and in New World Camels (NWC) (Appleby and Head, 1954; Schwarte, 1956; Belknap *et al,* 1994; Ridge *et al,* 1995). Lesions have been observed in the ileum, caecum and colon with severe intestinal thickening and enlargement of the intestinal lymph nodes. But also generalised paratuberculosis with inflammation of the spleen, liver and lung has been described (Kinne *et al,* 2010). No satisfactory treatment is known. Vaccination can be effective in reducing disease incidence, but does not eliminate infection. Camels that have been vaccinated may develop severe granulomas at the inoculation site causing camel owners to dislike the vaccine (Eckersly *et al,* 2011). All infected camels should be culled and carcasses properly disposed.

For the serological diagnosis, to the knowledge of the authors, only indirect ELISAs are currently available. These ELISAs use anti-ruminant, anti-bovine or protein G conjugates. Before these assays are used for serological surveys or diagnosis in camelids, it is essential to evaluate these tests. We describe here the evaluation of commercially available indirect ELISAs for MAP using different conjugates and the comparison with an in-house version.

Materials and Methods

The evaluation employed 5 different indirect antibody MAP ELISA kits and 3 different commercial conjugates using a panel of negative and positive dromedary sera.

ELISA kits

The following MAP ELISA kits were used according to the manufacturers' protocol except where stated:

1. Checkit® from IDEXX is an indirect MAP ELISA (IDEXX Laboratories/Dr. Bommeli Diagnostics, Switzerland, website www.bommeli.com)
2. ID Screen Paratuberculosis Indirect ® from ID Vet

SEND REPRINT REQUEST TO U. WERNERY email: cvrl@cvrl.ae

Innovative Diagnostics, France, email: idvet.info@id-vet.com

3. Parachek Johne's Absorbed EIA from Prionics (manufactured by Agriquality, Australia), website: www.prionics.com
4. Paratub. Serum-S and Paratub.Serum-B from Institute Pourquier, France, website: www.institut-pourquier.fr

These ELISAs were designed to detect antibodies against MAP in ruminant serum; also in milk in the case of ID Screen. In order to avoid cross-reactions, each protocol required that samples were pre-incubated in a neutralising buffer containing *Mycobacterium (M.) phlei*, before transferring them to MAP antigen coated plates. Specific antibodies would bind to the surface, if present, and then be detected by horseradish peroxidase (HRP)-conjugates in combination with tetramethyl benzidine (TMB) substrate. The ID Screen and Paratub kits employ anti-ruminant IgG conjugates, the Paracheck kit uses an anti-bovine IgG conjugate and the Checkit a Protein G conjugate. The Paratub kit offers both screening and validation protocols, whereas the latter requires measurement of the difference in signal between a test and an antigen negative control well.

Additional conjugates were obtained as follows:

Protein A - HRP (Sigma, USA, code: MFCD00132102); goat anti-camel IgG - HRP (Triple J Farms, USA, website: www.kentlabs.com/triplej.html) and guinea pig anti-camel IgG- HRP (Bio X, Belgium, code: BIO 344 website: http://www.biox.com/).

An in-house (CVRL) goat anti-camel IgG–HRP conjugate was prepared from goat F(ab)2 conjugated to HRP by the method of Hermanson (2008). The F(ab)2 resulted from a pepsin digest of affinity purified IgG, extracted from goat serum following immunisation with purified camel IgG. Conjugates were tested in the following dilutions of blocking buffer (5% skimmed milk powder, 1% bovine serum albumin and 0.05% Tween 20 in PBS): Protein A, Triple J and CVRL at 1 in 10,000 and Bio-X at 1 in 41 and substrate was prepared by dissolving one tablet of TMB 2 HCL in 10 ml sodium perborate buffer. The various antigen coated plates were compared using 1 in 10,000 diluted Protein A conjugate (1 in 8000 for the Paracheck plate) and revealed with TMB substrate.

In-house ELISA

MAP protoplasmic antigen coated plates for an in-house ELISA were prepared as follows: antigen PPA-3 from US strain 18 *M. paratuberculosis* (Allied Monitor, USA, email: info@alliedmonitor.com) was reconstituted at 10 mg/ml in de-ionised sterile distilled water, then diluted to 0.1mg/ml in 0.05 M carbonate / bicarbonate buffer, pH 9.6. Microwell plates (Nunc Maxisorp 96-well, Denmark) were coated with this solution at 100 µl per well and incubated at 4°C overnight, then washed 3 times with wash buffer (Phosphate Buffered Saline (PBS) with 0.05% Tween 20). Blocking buffer was added at 300 µl per well and plates incubated for 2 hours at room temperature.

In parallel serum samples (100 µl in tubes) were pre- incubated with 900 µl *M. phlei* suspension, (Allied Monitor, USA, reconstituted at 100 mg in 20 ml PBS) at 37°C for one hour, then overnight at room temperature. Samples were centrifuged at 13,000 rpm for 5 minutes and 500 µl of supernatant diluted with 500 µl blocking buffer.

Blocked MAP antigen coated plates were rinsed 3 times with wash buffer, then 100µl per well of diluted samples added in duplicate and incubated at room temperature for one hour. Plates were washed 3 times and 100 µl per well of Protein A-HRP conjugate, diluted at 1 in 4000 in blocking buffer, was added and incubated for 30 minutes at room temperature. Again the plates were washed 3 times and 100 µl per well of OPD substrate (o-phenylene-diamine : 1 tablet, 5 µl 30% Hydrogen peroxide in 10 ml distilled water) was added. Plates were incubated at room temperature in the dark for approximately 7 minutes, when the reaction was stopped by the addition of 50 µl per well 1 M sulphuric acid and absorbance read at 492 nm.

Sera

Positive sera originated from 2 vaccinated dromedaries (47B and 6BI) kept at CVRL, as well as from a clinical case (camel 845) from which a faecal sample revealed acid fast rods in Ziehl-Neelsen stain and a positive culture. The former were twice subcutaneuosly vaccinated within 4 weeks with 4 ml of the Gudair(R) paratuberculosis vaccine purchased from Pfizer, Australia. The blood was taken several times after vaccination to follow the antibody development. In total four positive sera were obtained from this trial.

Five negative paratuberculosis sera were taken from camels kept at CVRL with no history of paratuberculosis (973, DB0, E2A, 47B and 6BI) of which the latter two sera originated from dromedaries before vaccination. Furthermore, 1119 serum samples from the Dubai Camel Dairy Farm (EICMP) were tested with the in house indirect ELISA for antibodies

against paratuberculosis. This farm had no history of paratuberculosis.

Results

Initially the four commercial indirect MAP ELISAs were evaluated for their ability to detect camel IgG using the kit conjugate according to the manufacturer's instructions, i.e. using each kit antigen coated ELISA plate in combination with the respective kit conjugate. Results obtained from testing a clinically positive serum (camel 845) and three negative paratuberculosis sera are compared in Fig 1 together with the respective kit positive and negative control samples as supplied. The Paratub, ID Screen, Parachek and Checkit kits produced a 24-fold,17-fold, 9-fold and 3-fold greater signal for the positive kit control over the negative kit control, respectively, indicating that the kit reagents were functioning as expected. However, all four kits resulted in very poor or no discrimination between the camel positive and negative sera. This was not unexpected for the anti-ruminant (ID Screen and Paratub) and anti-bovine (Parachek) detection reagents, since there was no guarantee that these would cross react with camel IgG. But it was surprising that also the Protein G conjugate of the Checkit kit produced no discrimination of the camel sera, as Protein G has been reported to bind camel IgG (Hamers-Casterman *et al,* 1993).

Since selection of conjugate was critical for reliable detection of anti-MAP camel antibodies, a further 4 conjugates were evaluated using antigen coated plates from the ID Screen kit in combination with TMB substrate. Protein A-HRP conjugate was compared to commercial goat anti-camel IgG and guinea pig anti-camel IgG HRP conjugates and also against an in-house goat F(ab)2 anti-camel IgG-HRP conjugate (CVRL). Again the camel positive serum was tested alongside three paratuberculosis negative sera and the results for each conjugate are shown in Fig 2.

The Protein A and CVRL conjugates performed best, producing a similar range of positive to negative sample ratio ranging from 2.8 to 22 and 2.5 to 21, respectively. In contrast, the Triple J goat anti-camel conjugate performed poorly with a positive to negative sample ratio range of 1.5 to 3.5, whilst the Bio-X guinea pig conjugate resulted in an intermediate ratio range of 3 to 12. Owing to the good performance and commercial availability of the Protein A-HRP conjugate further work focused on using this detection reagent.

The performance of MAP antigen coated plates from four commercial kits, and an in-house MAP antigen coated plate were compared using the Protein conjugate in combination with TMB substrate. Fig 3a contrasts the resulting signal from the clinical positive camel (camel 845) serum against that from a single negative serum (camel 973) for each case. The ID Screen and in-house MAP antigen coated plate produced the best window of discrimination of 2.7 and 1.56 absorbance units, respectively, followed in rank order by the Parachek, Paratub and Checkit plates. Cross reactivity can be a problem with indirect ELISAs owing to irrelevant immunoglobulin from the sample binding non-specifically to the solid phase, therefore each of the MAP antigen coated plates were challenged with four other paratuberculosis negative sera. Cross reactivity of these were calculated as %S/P values, where:

%S/P = 100 x (sample OD – negative control OD) / (positive control OD - negative control OD)

and where the positive control was camel 845 serum, and the negative control was camel 973 serum. The data are shown in Fig 3b. The Checkit plate performed poorly with all negative sera resulting in %S/P values greater than 40%, whereas all the other plates gave values under 40%. Of these the Parachek plate exhibited the best specificity with %S/P values below 3% followed by the in-house plate with %S/P below 13%. The latter therefore provided a good compromise between a high positive sample signal and reasonable specificity.

A final evaluation of the five MAP antigen coated plates in combination with Protein A-HRP conjugate and TMB substrate involved monitoring the immune response of two dromedaries vaccinated against paratuberculosis. All five ELISAs demonstrated rising titres of anti-MAP antibody for both camels in samples collected two weeks after the first and second vaccination compared to samples collected before vaccination (Fig 4). For three of the ELISAs, samples collected after a resting interval of 17 months, showed a fall in signal almost back to pre-vaccination levels. As in the previous experiment, the ID Screen assay and in particular the Checkit assay gave higher signal for the pre-vaccination samples, suggesting a significant degree of cross-reactivity. After the second dose a reasonably similar level of signal was achieved in all five ELISAs, with the ID Screen assay giving the highest signal for both camels and the Paratub ELISA producing a somewhat lower signal for camel 6BI. Two weeks after the primary vaccination the in-house ELISA generated the greatest signal increase over the pre-vaccination sample for camel 47B, whereas the Parachek assay was more sensitive at the time point in the case of camel 6BI.

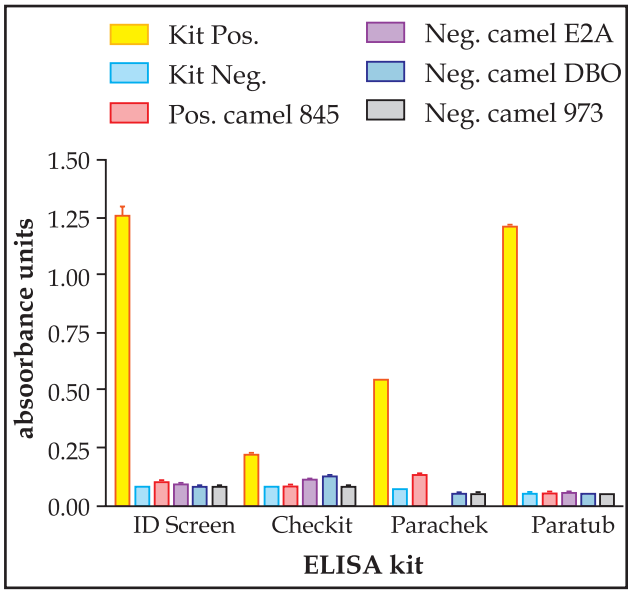


Fig 1. Evaluation of four commercial MAP ELISA kits with respect to their ability to detect and discriminate paratuberculosis positve and negative camel sera.

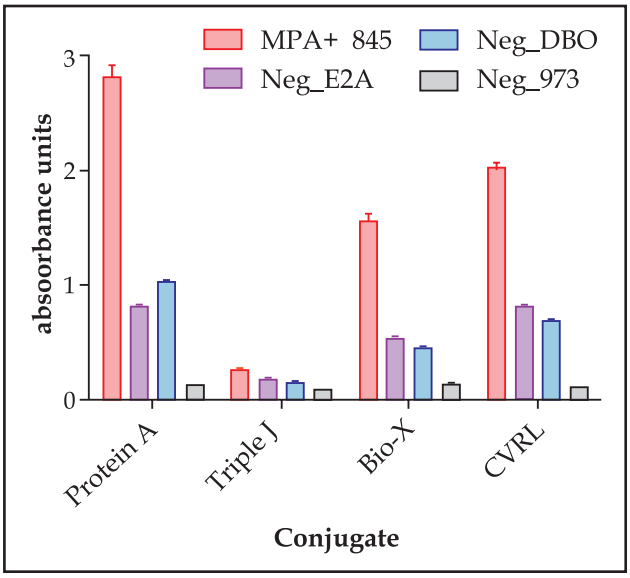
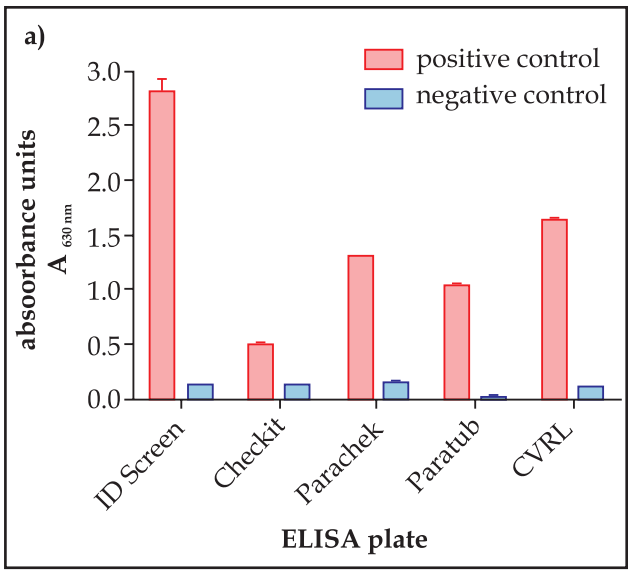


Fig 2. Evaluation of three anti-camel IgG and Protein A HRP-conjugates for detecting Paratuberculosis positive camel sera, using IDVET MAP coated ELISA plate.



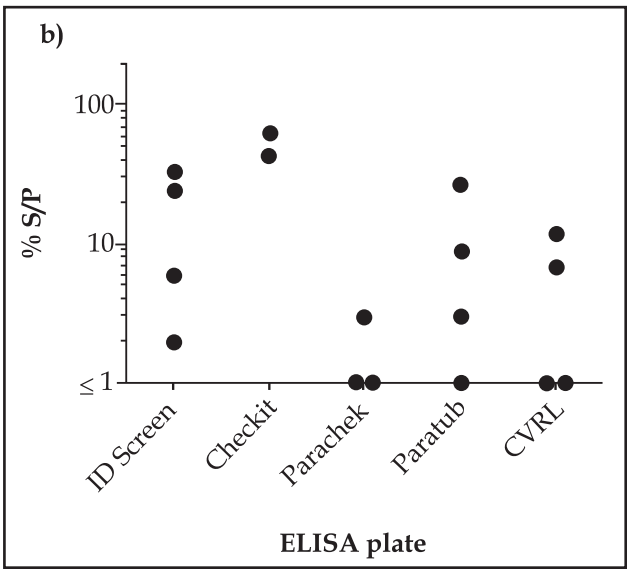


Fig 3. Comparing assay performance of five different MAP coated plates testing dromedary control sera with Protein A conjugate.
Panel a): signal / noise using clinical positve (camel 845) versus negative (camel 973).
Panel b): Cross reactivity of 4 additional Paratuberculosis negative sera expressed as % S/P values.

Overall, the in-house ELISA provided a robust assay monitoring paratuberculosis sera titres in dromedaries but since OPD substrate was common to other ELISA protocols in our laboratory, this option was run in parallel to TMB substrate on the vaccination samples. The resulting profile as shown in Fig 4 compares very favorably to that of TMB, thus confirming that the in-house protocol employing MAP antigen coated plate, Protein A-HRP conjugate and OPD substrate was suitable for future studies of paratuberculosis infection in dromedaries. All 1119 sera from the camel dairy farm were negative with the inhouse indirect ELISA.

Discussion

In previous investigations (Wernery *et al*, 2007, 2008) we have shown the applicability of serological tests for the diagnosis of infections in camelids. This referred mainly to cELISAs which work well in many different animal species because the conjugate is

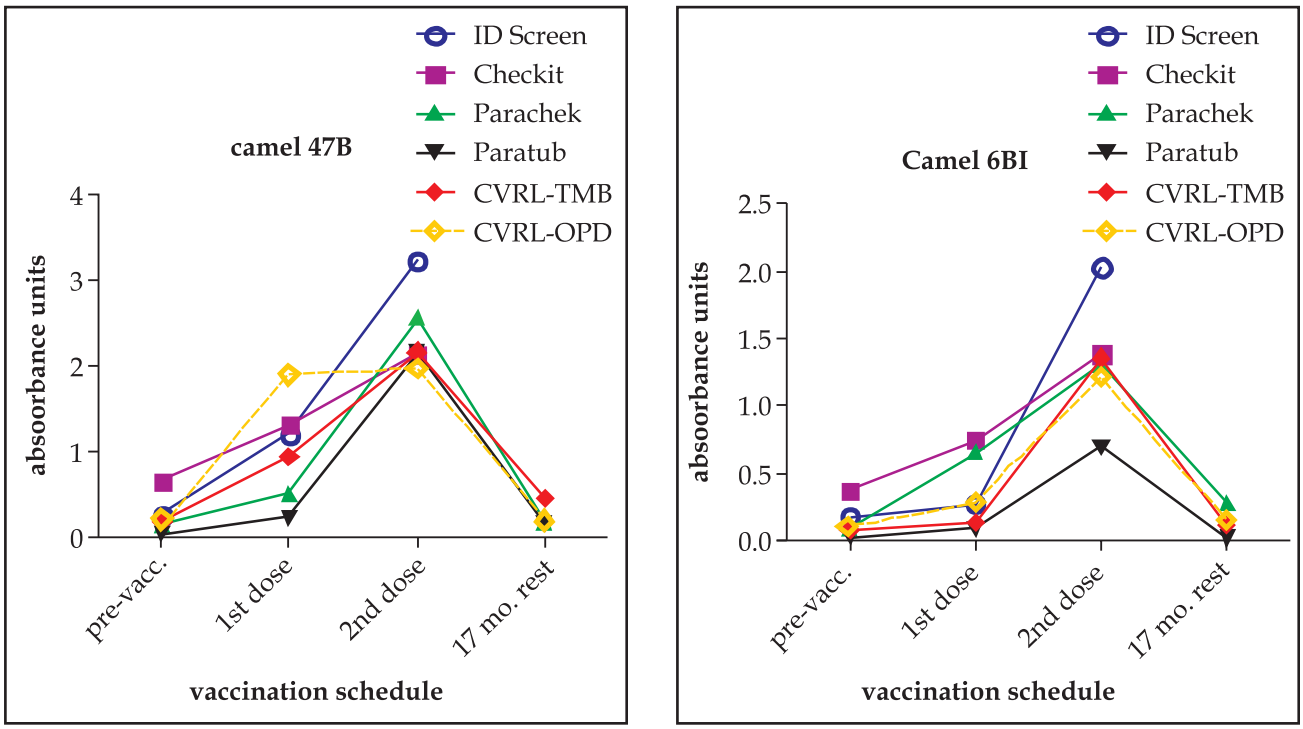


Fig 4. Monitoring anti-MAP immune response in two dromedaries vaccinated with Paratuberculosis comparing five different antigen coated plates using ProteinA conjugate.

not directed against the species investigated. With a new study we now want to concentrate our research efforts on two important camel infections for which only indirect ELISAs are currently available. The first disease is paratuberculosis.

Paratuberculosis is a chronic infection caused by MAP. It is often referred to as Johne's Disease, which may be misleading because MAP does not always cause diseases. MAP infection can be present in a bovine herd at high prevalence with significant production losses but without actually resulting in any cases of diarrhoea (Nielsen, 2009). The situation in camel dairy herds is unknown, one reason for this may lie in the uncertainity how to diagnose MAP serologically.

The disease has been causing considerable concern to the dairy industry world wide due to the unresolved issue of its potential role in the inflammatory bowel condition in humans known as Crohn's disease, and that this organism may not be completely inactivated by milk pasteurisation. Research on MAP has clearly revealed that this organism is capable of surviving commercial milk pasteurisation (high temperature short time = HTST=72°C for 15 seconds; Grant, 2003). Consequently, a public health issue exists if a link between MAP and Crohn's disease is ever firmly established. Viable MAPs have recently been isolated in peripheral blood of individuals with Crohn's disease which contributes to the evidence that MAP might be a cause of Crohn's disease (Naser *et al,* 2004).

Only few scientists used serological tests for the diagnosis of paratuberculosis in camelids, and even less researchers have evaluated these tests for use in camelids. Alluwaimi (2008) for example used the ID Vet indirect ELISA without evaluating it for dromedaries. Miller *et al* (2000) who investigated the specificity of 4 serological assays for MAP in llamas and alpacas clearly observed that the highest specificity of 98% was achieved when an anti-llama conjugate was applied in their indirect ELISAs from IDEXX, USA. Also Kramsky *et al* (2000) modified the bovine indirect MAP ELISA (CSL Parachek, Australia) for the detection of paratuberculosis antibodies. They achieved a 99% specificity. In general, cross reactivities between antispecies IgG polyclonal antisera exist. It has been shown that dromedary IgG has 74.3% sequence identity to porcine, and 73.1% to both equine and bovine, whereas anti-goat IgG has a much lower sequence identity of only 61.6%. Some researchers do not use anti-camel conjugates, but HRP labeled A or G proteins instead. These proteins derived from the staphylococcal cell wall do react with the FcH- chain structures of gamma- globulins of

most of the mammalians but not with fish, amphibia, reptiles or avians. Phylogenetic studies clearly show that the binding regions of IgGs with which the proteins A and G strongly react, are highly conserved (Kronvall *et al*, 1970). But since IgG 1 totals only 25% of camelid IgGs and 75% are IgG 2 and IgG 3 (heavy chains), the binding capacity of protein A and G may not be sufficient.

The aformentioned particularities may explain that it is essential to screen any potential antispecies IgG for camelid diagnostic use using a panel of positive and negative sera in order to minimise false positive and negatives. Several publications have compared ELISAs with different conjugates with inconsistent results .

Soliman *et al* (1992) tested camel sera for Q – Fever using an in house cELISA and a HRP protein A ELISA. Their data showed a 50% less sensitivity for the Protein A ELISA compared to the cELISA. Zweygarth *et al* (1986), however, found no difference for the detection of *Trypanosoma evansi* antibodies between an anti-camel and a protein A conjugate. From these investigations it is obvious that more studies on indirect ELISAs of camelids are necessary especially if non-species-specific conjugates are used. In this study, we therefore evaluated several indirect ELISAs from different countries in combination with different conjugates. Our results showed that Protein A conjugate worked well with all three commercial kits (Parachek, Institut Pourquier Screening and verification) and with the in house ELISA. Camel samples did not work with the anti-ruminant or anti-bovine conjugates. We conclude that among the five different indirect ELISAs, the in house ELISA using OPD substrate and Protein A conjugate was the test of choice for camels.

References

Alluwaimi AM (2008). The efficiency of bovine ELISA in detection of the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in camel (*Camelus dromedarius*) at different age. Journal of Camel Practice and Research 15(2):163-165

Appleby EC and Head KW (1954). A case of suspected Johne's Disease in a llama (*L. glama*). Journal of Comparative Pathology 64:52-53.

Belknap EB, Getzy DM, Johnson LW, Ellis RP, Thompson GL and Schenlaw WP (1994). *Mycobacterium paratuberculosis* infection in two llamas. Journal of American Veterinary Medical Association 204:1805-1808.

Eckersley AM, Petrovski N, Kinne J, Wernery R and Wernery U (2011). Improving the dromedary antibody response: The hunt for the perfect adjuvant vaccine. Journal of Camel Practice and Research 18:35-46.

Grant IR (2003). *Mycobacterium paratuberculosis* and milk. Acta Veterinaria Scandinavica 44:261-266.

Hamers-Casterman *et al* (1993) Naturally occurring antibodies devoid of light chains. Nature 363:446-448.

Hermanson GT (2008) In Bioconjugate Techniques, 2nd ed. Academic Press, London. Preparation of Antibody – Enzyme conjugates 20:783-823

Kinne J, Johnson B, Joseph M and Wernery U (2010). Camel paratuberculosis (Johne's Disease) – A case report. Proceeding of International Camel Symposium in Garissa, Kenya.

Kramsky JA, Miller DS, Hope A and Collins MT (2000). Modification of a bovine ELISA to detect camelids antibodies to *Mycobacterium paratuberculosis*. Veterinary Microbiology 77:333-337.

Kronvall G, Seal US, Finstad J and Williams Jr RC (1970). Phylogenetic insight into evolution of mammalian Fc fragment of γ Globulin using staphylococcal protein A. The Journal of Immunology 104(1):140-147.

Miller DS, Collins MT, Smith BB, Anderson PR, Kramsky J, Wilder G and Hope A (2000). Specificity of 4 serological assays for *Mycobacterium avium* subspecies *paratuberculosis* in llamas and alpacas: a single herd study. Journal of Veterinary Diagnostic Investigation 12:345-353.

Naser SA, Ghobrial G, Romero C and Valentine JF (2004). Culture of *Mycobacterium avium* subspecies paratuberculosis from the blood of patients with Crohn's disease. Lancet 364:1039-1044.

Nielsen SS (2009). Use of diagnostics for risk – based control of paratuberculosis in dairy herds. In Practice 31:150-154.

Ridge SE, Harkin JT, Badman RT, Mllor AM and Larsen JWA (1995). Johne's Disease in alpacas (*Lama pacos*) in Australia. Australian Veterinary Journal 72(4):150-153.

Schwarte LH (1956). Johne's Disease in a llama. Veterinary Bulletin Cited in JAVMA, 354.

Soliman AK, Boutros BAM and Watts DM (1992). Evaluation of a competitive enzyme immunoassay for detection of *Coxiella burnetii* antibody in animal sera. Journal of Clinical Microbiology 30(6):1595-1597.

Wernery U and Kaaden O-R (2002). Infectious Diseases in Camelids. Blackwell Science, Berlin, Vienna. pp 83-91.

Wernery U, Thomas R, Syriac G, Raghavan R and Kletzka S (2007). Seroepidemiological studies for the detection of antibodies against nine infectious diseases in dairy dromedaries (Part-I). Journal of Camel Practice and Research 14(2):85-90.

Wernery U, Thomas R, Raghavan R, Syriac G, Joseph S and Georgy N (2008). Seroepidemiological studies for the detection of antibodies against eight infectious diseases in dairy dromedaries of the U.A.E. using modern laboratory techniques(Part-II). Journal of Camel Practice and Research 15(2):139-145

Zweygarth E, Sabwa C and Roettdor R (1986). An enzyme – linked immunosorbent assay for the detection of antibodies to *Trypanosoma* (T.) *evansi* in camels (*Camelus dromedarius*) using peroxidase – conjugated protein A. Tropical Medicine Parasitology 37:105-106.