Vet.Med.J.,Giza. Vol.55, No.4. (2007):1067-1075.

POLYMERASE CHAIN REACTION AND ADAPTED ENZYME LINKED IMMUNOSORBENT ASSAY FOR DIAGNOSIS OF CAMEL BRUCELLOSIS

M. A. AL-DUBAIB

Dept. of Veterinary Medicine, College of Agriculture and Veterinary Medicine
Qassim University, P. O. Box 6622, Buraydah 51452, Qassim, KSA

Received: 8. 10. 2007
Accepted: 22. 10. 2007

SUMMARY

In the present study, polymerase chain reaction was carried out on 10 brucella isolates recovered from camels affected with brucellosis central region of Saudi Arabia. Brucella abortus as well as Brucella melitensis specific primers were employed for the assay. All isolates were identified as B. melitensis. This was in agreement with the results of the traditional bacteriological identification. Moreover, antibodies against camel IgG was raised in rabbits and purified with polystyrene affinity chromatography. The purified anti-camel IgG was conjugated with horseradish peroxidase (HRPO) using the sodium periodate method. The anticamel-HRPO conjugate prepared in this study was tested in an indirect ELISA adapted in the same study on camel sera positive and negative for brucellosis as indicated by the Rose Bengal plate test. The conjugate was found efficient and was able to elucidate positive and negative samples at a dilution of 1/40.

INTRODUCTION

Camel husbandry is a highly viable economic and social activity in the Gulf region, particularly in Saudi Arabia (Abdel-Rahim et al., 1994). Camel brucellosis has been reviewed recently (Abbas and Agab, 2003; Abbas and Omer, 2006). The disease has been documented in all camel keeping countries, and considered by FAO/WHO as the most important animal zoonosis in camel keeping countries (FAO/WHO, 1986). Several workers have reported on camel brucellosis in Saudi Arabia (Abbas and Agab, 2003; Ramadan et al., 1998). Although most of the brucella infection in

camels is chronic or silent, i.e without overt clinical signs, some animals develop acute diseases, such as arthritis, endometritis, vaginitis and abortion (Ramadan et al., 1998; Agab, 1993; Wernery and Kaaden, 1995). There is, however, very little record of clinical signs in the literature on camel brucellosis. Zaki (1948) speculated that Brucella abortus was the main cause of brucellosis in camels while Rutter and Mack (1963) considered Brucella melitensis as the agent of camel brucellosis. Both authors based their assumptions on the results of comparative serological tests. Many ELISA kits for diagnosis of animal brucellosis are commercially available but unfortunately those kits are for livestock other than camels. The present study was planned to adapt an indirect ELISA specifically for camel brucellosis. Also, polymerase chain reaction was employed to identify brucella isolates recovered from infected camels in Saudi Arabia.

MATERIALS AND METHODS

Production of camel IgG antibodies

Serum, separated from camel jugular blood, was diluted 1:4 with phosphate buffered saline (PBS, pH 7.2). Immunoglobulin G (IgG) was extracted from the diluted serum using saturated ammonium sulfate solution (Hudson and Hay, 1990). The IgG was mixed with Freund's complete adjuvant and injected in rabbits. Each rabbit received 500 _g camel IgG in Freund's complete adjuvant intramuscularly into the thigh muscle and subcutaneously at the two sides of the back. The inoculation was repeated after 2 weeks. Serum was separated from rabbit blood to be monitored for immune response using the agar gel immunodiffusion test. When required, the immunized rabbits were bled-euthanasized and blood was collected in plain tubes to get serum. Camel IgG antibodies were purified from rabbit serum IgG using the polystyrene affinity chromatography (Staak et al., 1996).

Preparation and evaluation of anticamel-IgG HRPO conjugate

Camel IgG antibodies were dialyzed against sodium carbonate buffer (0.01 M) overnight at 4°C. The IgG was conjugated to horseradish peroxidase (HRPO) using sodium periodate and sodium borohydride as described by Wilson and Nakane (1978). Camel IgG was used as an antigen in a direct ELISA for evaluation of the prepared conjugate. The anticamel conjugate was checker board tested in a direct ELISA against different dilutions of the camel IgG in a 96 well flat bottom plate (Staak et al., 1996).

The indirect ELISA test proper

To test serum samples of vaccinated animals as well as animals of flocks with history of brucellosis, ELISA was carried out using the whole cell B. abortus antigen at the proper dilution as indicated by the results of the checker-board titration (1: 46) following the methods of Alton et al. (1988).

Polymerase chain reaction

The deoxyribonucleic acid (DNA) was extracted from brucella melitenses sheep isolates recovered from infected camels and identified as Brucella melitenseis by traditional methods. Phenol: chloroform extraction was employed after disruption of the bacterial cells with SDS and lysozyme (Sambrook et al., 1989). Three primers were designed according the sequence of the IS711 (Bricker and Halling, 1994). IS711 is a unique repetitive genetic element to Brucella melitenses sheep spp. and for most species at least one copy of the element occurs at unique species- or biovar-specific chromosomal locus. The unique locations of these elements are the basis of the identification of Brucella spp. and differentiation between Brucella biovars. The primer sequences were 5'-GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC-3' (B. abortus-specific primer), 5'-AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA-3' (B. melitensis-specific primer) and 5'-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3'(IS711-specific primer). Amplification was carried out following the reaction mixture and conditions described by Bricker and Halling (1994).

RESULTS AND DISCUSSION

The magnitude of brucellosis seroprevalence in camels is almost based on serological surveys which using a variety of procedures. It is important to note that the slide agglutination test (SAT), performed at a neutral pH, has been shown repeatedly to have poor diagnostic sensitivity when compared to other conventional tests (Alton et al., 1988). The buffered plate agglutination test (BPAT), the buffered acidified plate antigen test (BAPAT) and the card or Rose Bengal test (RBT) are comparable and have greater analytical sensitivity, especially in the detection of IgG1. Although the three tests differ in diagnostic performance, it is generally agreed that they exhibit greater sensitivity and specificity than the SAT (Nielsen et al., 1984; Wright and Nielsen, 1990). Enhanced sensitivity for detection of Brucella infected animals has been reported by several workers due to the utilization of the enzyme linked immunosorbent assay (ELISA) for serodiagnosis of brucellosis (Al Dahouk et al 2003; McGiven et al., 2003; Ferreira et al. 2003). Even though, no one ELISA kit is commercially available for serodiagnosis of camel brucellosis. In the present study, anti-camel IgG, raised in rabbits, was conjugated to horseradish peroxidase (HRPO) using the sodium periodate method. When tested with checker board ELISA, the conjugate showed successful results as indicated by positive results with high OD values with antigen-coated wells and negative results with antigen-free wells of the microtitre plates. Concerning the different dilution in the checker board, the conjugate showed OD values over 0.3 up to dilution of 1/160. The results were obtained with antigen dilution up to 1/1600 (Table 1).

Table (1): Optical density values of the anticamel HRPO conjugate

Row / Column	1	2	3	4	5	6	7	8	9	10	11	12
A	1.954	1.087	0.518	0.412	0.249	0.117	0.063	0.021	0.002	0.007	0.008	0.013
B	1.877	1.103	0.582	0.392	0.241	0.118	0.052	0.024	0.003	0.007	0.015	0.016
C	1.938	1.216	0.573	0.332	0.281	0.122	0.061	0.029	0.002	0.010	0.013	0.010
D	2.091	1.265	0.527	0.331	0.268	0.126	0.047	0.023	0.008	0.008	0.020	0.019
E	1.871	1.023	0.531	0.327	0.254	0.131	0.055	0.030	0.008	0.006	0.005	0.009
F	1.712	0.911	0.465	0.322	0.197	0.102	0.048	0.031	0.008	0.009	0.013	0.011
G	1.528	0.778	0.374	0.301	0.187	0.077	0.031	0.010	0.003	0.009	0.014	0.010
H	0.032	0.003	0.003	0.010	0.003	0.006	0.017	0.020	0.019	0.016	0.017	0.000

Reading was at 405 nm Row H contains no antigen (conjugate control)

Table (2): ELISA screening of camels pretested with RBP test for brucellosis

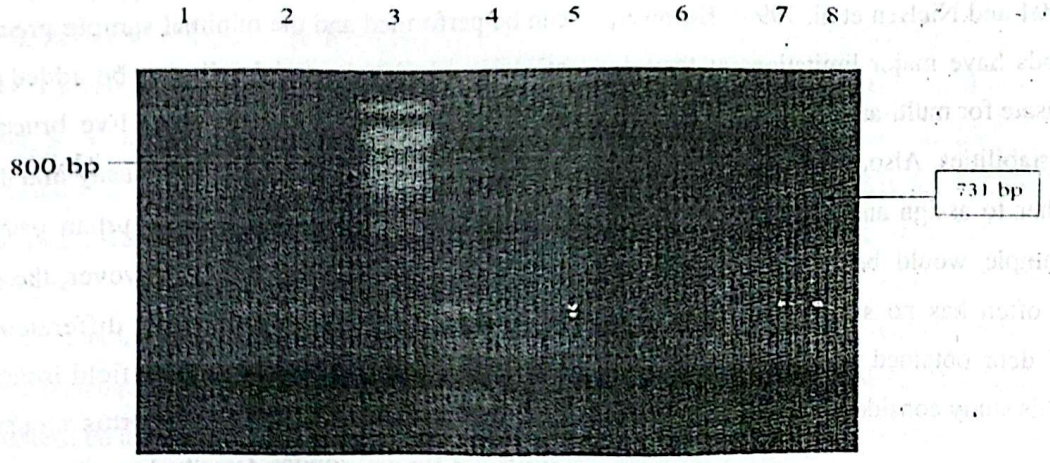
Rose Bengal test results	Number	ELISA positive		ELISA negative	
		Number	%	Number	%
Positive	55 (11.83%)	52	94.54	3	5.45
Negative	410	5	1.22	405	98.78
Total	465	57	12.26	408	87.74

Table (3): Brucella isolates recovered from clinical specimens of seropositive camels manifesting brucellosis-like symptoms

Clinical picture	Specimens *	Number	Positive samples	
			Number	%
Abortion	Foetal stomach content	6	3	50%
Mastitis	Milk	5	0	0.0%
Retained placenta	Placenta	5	2	40%
Dystocia	Uterine fluids	7	0	0.0%
Vaginitis	Vaginal swabs	4	0	0.0%
Orchitis	Semen and urethral swabs	5	2	40%
Rye-neck syndrome	Prescapular lymph node (aspirate)	4	1	25%
Hygroma	Synovial fluid	3	2	66.66%
Total		39	10	25.64%

* Specimens were collected from animals with positive results with brucella Rose Bengal, slow agglutination and complement fixation serological tests

Figure (2): Agarose gel electrophoresis of PCR products obtained with brucella genomic DNA using *B. melitensis* specific primers



Lane 1 (Rev. 1 strain), lane 2. (Brucella melitensis sheep isolate), lane 3 100 bp DNA size marker (Amersham, Germany), lanes 4-7 (camel Brucella melitenses isolates) and lane 8 (negative control i. e. PCR master mix without DNA template).

The ELISA was carried out on camel serum samples that were previously tested with Rose Bengal plate test (Table 2). From the table, brucellosis incidence as indicated by ELISA was 12.26% while that indicated by RBP test was 11.83%. This finding denotes that RBP test screening is worth to be applied for the serosurvey of camel brucellosis especially with the lack of commercial ELISA kits for camel brucellosis. However, we succeeded to prepare anti-camel HRPO-conjugate which was found efficient for application with a complex disease like brucellosis. If anticamel conjugate can be found, ELISA will be then applied and accepted instead of complement fixation test which is a laborious test. However, when applying ELISA for camel brucellosis, more specific antigens are to be selected to avoid false positive due to high sensitivity originating from cross reactivity of the test. Many methods for interpreting ELISA data have been described (Heck et al., 1980; Gall and Nielsen, 1994 and Nielsen et al. 1996). However, most methods have major limitations as they do not compensate for multi-animal, inter-well, or inter-plate variabilities. Also, many systems require the researcher to assign an arbitrary value above which a sample would be considered positive. This value often has no statistical validity. The analysis of data obtained with the ELISA performed in this study considered these variabilities.

In the present work, genomic DNA was extracted from 10 brucella isolates proved to be Brucella melitenses by bacteriological methods recovered from different clinical cases of camel brucellosis (table 3). The PCR assays were carried out on the extracted DNA using two sets of brucella-specific primers. This was to identify and differentiate Brucella species recovered in this study. This was based on the observation that the genetic element IS711 occurs at several species-specific or biovar-specific chromosomal loci. The assay was designed to amplify species-specific-sized products by using three primers, one of which hybridizes to the IS711element (Halling et al., 1993 and Ouaharni et al., 1993) and the other hybridizes to one of two species-specific regions adjacent to the element. The vaccinal strain Rev-1 and B. melitensis field isolates were identified by amplification of a 731-bp fragment (figure 1). The PCR assay has several advantages over the current microbiological methods used to identify Brucella species. A major advantage is the speed with which the assay can be performed and the minimal sample preparation as only 104 bacterial cells can be added directly to the reaction mixture. Also live brucella organisms are not necessary for the assay and this is significant because brucella is a human pathogen (Bricker and Halling, 1994). However, the assays performed in the study can not differentiate the B. melitensis vaccine strain from field isolates and other references strains (B. abortus strains). Ewalt and Bricker (2000) described another assay that can differentiate the vaccine strains from field isolates and other references strains. This

will be carried out in a next step of the project during which PCR assays will be performed on clinical samples instead of bacterial isolates.

Acknowledgment:This study was performed through the project No. (AT-23-60), funded by King Abdulaziz City for Science and Technology (KACST), KSA.

REFERENCES

Abbas, B. and Agab, H. (2003): Review of camel brucellosis. Preventive Veterinary Medicine 55, 47-56.

Abbas, B. and Omer, O. H. (2006): Review of infectious diseases of the camel. Veterinary Bulletin, Vol. 75, No. 8, pp. 1N-16N.

Abdel-Rahim, S. A.; Abdul Rahman, K. and El Nazier, A. E. (1994): Camel production and reproduction in Qassim, Saudi Arabia. Journal of Arid Environments 26, 53-59.

Agab, H. (1993): Epidemiology of camel diseases in Eastern Sudan with emphasis on brucellosis. M.V.Sc. Thesis. University of Khartoum.

Al Dahouk, S., Tomaso, H., Nockler, K., Neubauer, H. and Frangoulidis, D. (2003): Laboratory-based diagnosis of brucellosis. Serological tests for brucellosis. Clin. Lab. 49:577-589.

Alton, G. G., Jones, L. M., Angus, R. D. and Verger, J. M. (1988): Techniques for the Brucellosis laboratory. Institut National de la Recherch Agronomique· (INRA). Paris.

Bricker, B. J. and Halling, S. M. (1994): Differentiation of Brucella abortus bv. 1, 2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv.1 by PCR. J. Clinical Microbiology 32(11):2660-2666 .

Ewalt, D. R. and Bricker, B. J. (2000): Validation of the abbreviated Brucella AMOS PCR as a rapid screening method for differentiation of Brucella abortus field strain isolates and the vaccine strains, 19 and RB51. J Clin Microbiol. Aug; 38(8):3085-3086.

FAO/WHO (1986): Joint FAO/WHO Expert Committee on Brucellosis. 6th report. Technical Report Series No. 740. World Health Organization, Geneva.

Ferreira, A.C., Cardoso, R., Travassos, D. I. and Mariano, I. (2003). Evaluation of a modified Rose Bengal test and an indirect ELISA for the diagnosis of Brucella melitensis infection in sheep. Vet. Res. 34: 297-305.

Gall, D. and Nielsen. K. (1994): Improvements to the competitive ELISA for detection of antibody to Brucella abortus in cattle. Journal of Immunoassay 15: 277–291.

Halling, S. M., Tatum, F. M., and Bricker, B. J. (1993): Sequence and characterization of an insertion sequence IS711 from Brucella ovis Gene 133:123-127.

Heck, F. C., Williams, J.D., Pruett, J., Sanders, R., and Zink, D.L. (1980): Enzyme-linked immunosorbent assay for detecting antibodies to Brucella abortus in bovine milk and serum. Am. J. Vet. Res. 41: 2082– 2083.

Hudson, L. and Hay, F. C. (1991): Practical Immunology, Blackwell Scientific Publications, Oxford.

McGiven, J. A., Tucker, J. D., Perret, L. L., Stack, J. A., Brew, S. D. and MacMillan, A. P. (2003). Validation of FPA and cELISA for the detection of antibodies to Brucella abortus in cattle sera and comparison to SAT, CFT, and iELISA. J Immunol. Methods 278: 171-178.

Nielsen, K.; Heck, F. C.; Wagner, G. G.; Stiller, S.; Rosenbaum, B.; Pugh, R. and Flores, E. (1984): Comparative

assessment of antibody isotypes to Brucella abortus by primary and secondary binding assays. Prev. Vet. Med. 2: 197 – 205.

Nielsen, K. , Smith, P. , Gall, D. , Perez, B. , Cosma, C. , Mueller, P. , Trottier, J., Cote, G. , Boag, L. and Bosse, J. (1996): Development and validation of an indirect enzyme immunoassay for detection of antibody to Brucella abortus in milk. Vet. Microbiol. Sep;52(1-2):165-173.

Ouahrani, S., Michaux, S., Widada, J. S., Bourg, G., Tournebize, R., Ramuz, M. and Liautard, J. P. (1993): Identification and sequence analysis of IS6501, an insertion sequence in Brucella spp.: relationship between genomic structure and the number of IS6501 copies. J. Gen. Microbiol. 139:3265-3273.

Ramadan, O. R.; Hatem, M. E. and Abdin Bey, M. R. (1998): Isolation of Br. melitensis from carpal hygroma in camels. J. of Camel Practice and Research. 5 : 239 – 241.

Rutter , T. E. G. and Mack, R. (1963): Diseases of camels. Bacterial and fungal diseases. Vet. Bull. 33 : 119 – 124.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989): Molecular cloning: A laboratory manual, second edition., Cold Spring Harbor Laboratory Press.

Staak, C., Sahchow, F., Clausen, P. H. and Luge, E. (1996): Polystyrene as an affinity chromatography matrix for the purification of antibodies., J. Immunol. Meth., 194, 141-146.

Wernery, U. and Kaaden, O. R. (1995): Infectious Diseases of Camelids. Blackwell Wissenscafts Verlag, Berlin.

Wilson, M. B. and Nakane, P. K. (1978): Recent developments in the periodate method conjugating horse radish peroxidase (HRPO) to antibodies. Immunofluorescence and related techniques., W. Knapp, K. Holubar, G. Wick eds., 215.

Wright, P. F. and Nielsen, K. (1990): Current and Future Serological Methods. International Symposium on Advances in Brucellosis. Adams, L. G., (ed). College Station, Texas A and M University Press.

Zaki, R. (1948): Brucella infection among ewes, camels and pigs in Egypt. J. Comp. Path. 58: 145 – 151.