Indirect Enzyme Linked Immunosorbent Assay for Diagnosis of Brucellosis in Buffaloes

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Abstract

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Brucellosis is an important zoonotic disease causing significant economic losses worldwide. Early detection of this disease is essential for its control and eradication. Presently, an Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) was developed using lipopolysaccharide (LPS) as antigen and compared with the commercial kit using one hundred negative and positive sera each from buffaloes. The agreement for the positive result between the developed and commercial I-ELISA was 78% and for the negative it was 100%. At 52.49%, 53.09%, 53.26%, 53.86% and 53.94% cut off the sensitivity was 100%, 100%, 97.53%, 88.93% and 86.42%, while the specificity was 84.03%, 84.87%, 85.71%, 87.39% and 87.39%, respectively, for developed I-ELISA. This developed test can be used for the screening of herds as the relative sensitivity is higher.

Brucella abortus, I-ELISA, lipopolysaccharides (LPS)

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of animals worldwide (OIE 2000). The disease is caused by various species of the genus *Brucella*, which are facultative, intracellular bacteria capable of surviving and multiplying inside the cells of mononuclear phagocytic system (Jarvis et al. 2002). The disease causes significant economic losses including abortion, loss in milk production, low fertility rates and cost of replacement of animals (McDermott and Arimi 2002). Timely diagnosis of the disease in sexually mature animals is very important and necessary for the control of this malady; also the transmission of the disease to humans is an important justification to undertake measures for its control and eradication.

Early detection, control and elimination of reactors are important considerations for the control of brucellosis. Brucellosis is diagnosed by classical serological techniques i.e., agglutination, precipitation, complement fixation but these techniques have several drawbacks such as poor performance and lack of standardisation (OIE 2000). At present, application of the ELISA technique is considered a better test in early detection of infection than complement fixation test (Rojax and Alonso 1995). Indirect enzyme linked immunosorbent assays (I-ELISAs) have been developed and used in various countries for sero-diagnosis of brucellosis in cattle and other animals (Omer et al. 2001; Dajer et al. 1998; Molnar et al. 1998; Romero et al. 1995), however, such kind of work in buffaloes is limited (Guarino et al. 2001).

The incidence of brucellosis in Pakistan is increasing particularly in large dairy herds. Earlier studies indicated low prevalence, i.e., 0.33 to 0.65% (Sheikh et al. 1967), whereas much higher prevalence is reported in recent studies, i.e., 21.05 to 26.1% (Sarwar 2000; Ramzan 1996; Akhtar et al. 1990). The incidence is higher in animals kept at organized farms rather than small holdings (Ahmad and Munir 1995; Lodhi et al. 1995; Ahmad et al. 1990, 1994). In spite of such a high incidence, none of the diagnostic tests has been

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standardised in buffaloes, therefore, in the present study an I-ELISA was developed using lipopolysaccharide (LPS) as antigen and compared with a commercial kit for the diagnosis of brucellosis in buffaloes.

Materials and Methods

Blood samples

One hundred negative serum samples were collected from buffaloes kept at a Livestock Research Station (LRS), National Agricultural Research Centre (NARC), having no previous history of brucellosis. These animals were tested twice a year regularly against brucellosis by RBPT. Another 100 serum samples were collected from buffaloes at a farm with clinical and serological evidence of the disease and history of abortions. The disease was confirmed by isolating *Brucella abortus* from the vaginal secretions of aborting animals, from the placenta and from the abomasum of the foetuses.

Serological tests

All the sera were tested for the presence of *Brucella abortus* antibodies by a commercial kit (CHEKIT®, Germany) and a home-made kit. I-ELISA performed by the commercial kit is designated as I-ELISA$_{com}$ and home-made as I-ELISA$_{dev.}$

Indirect ELISA (I-ELISA$_{dev}$)

Bacterial strain cultivation and antigen extraction

Brucella abortus strain 99 obtained from Germany was mass cultivated on tryptone soy agar (Oxoid Ltd, Basingstoke, Hampshire, England) slants and incubated at 37 °C for 24 to 48 h. After confirmation by Gram staining the bacterial growth was re-suspended in phosphate buffered saline (PBS, pH 6.4) and inoculated in Roux flasks containing tryptone soy agar and incubated for 48 h at 37 °C. Again, purity was checked by Gram staining and purified growth of the flasks were washed with 50 to 60 ml of phenolic saline and centrifuged at 10,000 *g* for 30 min to obtain the bacterial mass.

Smooth lipopolysaccharide (LPS) from *Brucella abortus* was extracted by hot phenol method (OIE 2004; Biancifiori et al. 1996). Briefly, 50 g packed wet cells were re-suspended in distilled water at 66 °C, followed by addition of 90% phenol with continuous stirring for 20 min in a shaking water bath with the same temperature. This mixture was centrifuged at 10,000 *g* and the bottom brownish phenol layer was removed and filtered (Wattman filter No.1). The LPS were precipitated by cold methanol, saturated with sodium acetate and centrifuged at 10,000 *g* for 10 min. The precipitates were separated and stirred with distilled water for 18 h followed by centrifugation at 10,000 *g*. The supernatant was kept at 4 °C. The precipitates were re-suspended in distilled water and stirred for additional 2 h at 4 °C. The supernatant was recovered by centrifugation as above and pooled with the previously recovered supernatant. Eight ml of trichloroacetic acid were added to the crude LPS (supernatant). After stirring for 10 min, the precipitates were removed by centrifugation and the translucent supernatant solution was dialyzed against distilled water. The quantity of smooth LPS in the antigen was determined by thiobarbituric assay (Waravdekar and Saslaw 1959) using purified 2-ket-3deoxyoctonate standard (Sigma, KDO catalog No. K7000).

I-ELISA$_{dev}$ procedure

Indirect ELISA was standardised by the checkerboard titration method as described by Wright et al. (1993). *Brucella* LPS antigen was diluted in carbonate coating buffer to a concentration as determined by checkerboard titration, i.e. 1.25 µg/ml. For coating, 100 µl of diluted antigen was added to each well of flat bottom micro-titration plates (Titertek, Flow Laboratories, Catalog No. 76-204-05) and incubated for 18 h at 4 °C. The plates were washed five times with phosphate buffer saline tween-20 (PBST). All the sera were diluted 1 : 50 in PBST and 100 µl of the diluted sera was dispensed in each well of the micro-titration plate and incubated for one hour at 37 °C.

After another washing cycle with PBST, 10 µl of diluted rabbit anti-bovine IgG whole molecule conjugated to horseradish peroxidase (Cappel, USA) diluted 1 : 1000 was added to all the wells of micro-titration plate followed by another 60 min of incubation at 37 °C. After the washing cycle, 100 µl of substrate (2.2′ azino diethyl benzothiazoline sulfonic acid / ABTS) solution was added into each well of ELISA plate and incubated with shaking for 15 min at 37 °C. The reaction was stopped by the addition of 100 µl of 4% SDS solution. Development of colour was assessed by a spectrophotometer (BDSL, Immunoskan MS) at 405 nm. Standard positive and negative serum controls were also included in each plate.

Data analysis

Indirect ELISA

Provided the controls were within acceptable limits, the results were expressed in percent positivity (PP) which was calculated as follows (FAO/IAEA 1991):

PP: Mean OD of duplicate tests × 100 /
Mean OD of C++ control

Sensitivity and specificity

Relative sensitivity and specificity was calculated as (Thrusfield 1986):

Relative sensitivity (%): Total number of Positive results × 100 /
Total number of positive animals sampled
Relative specificity (%): Total number of negative results × 100 /
Total number of negative animals sampled

Scatter plot was used for the descriptive analysis of PI values. Kappa statistics applied for the measurement of agreement between both I-ELISA$_{kit}$ and I-ELISA$_{dev,}$ while Receiver Operating Characteristics (ROC) graph was plotted for cut off selection at its best accuracy. All analyses were conducted using the statistical software SPSS.

Results

The data frequency distribution of percent positive values in home-developed I-ELISA for detecting anti-*Brucella* antibodies in bovines is shown in Fig. 1.

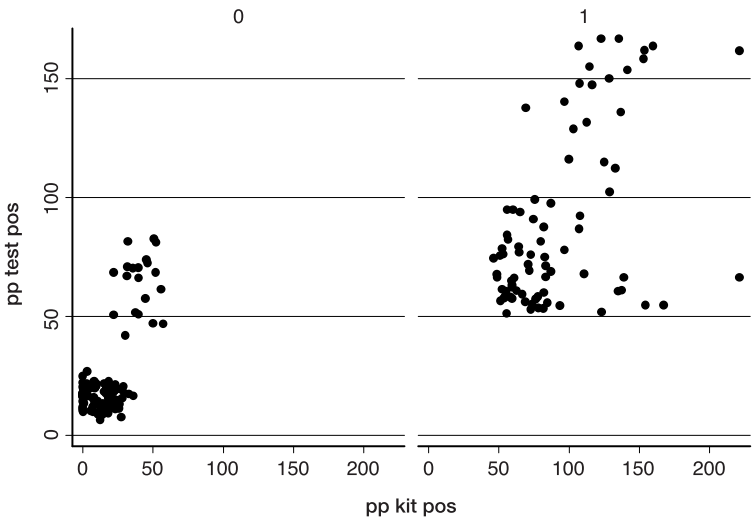

Fig. 1. Scatter graph showing the data distribution of percent positive values in home developed I-ELISA for detecting anti-Brucella antibodies in bovines: 0, negative and 1, positive by commercial I-ELISA

The distribution of percent positive values of home-developed I-ELISA for detecting anti-*Brucella* antibodies in bovines is shown in Fig. 2.

Receiver Operating Characteristics (ROC) graph for the comparison of home developed and commercial I-ELISA for detection of *Brucella* antibodies in bovines is shown in Fig. 3.

The agreement for the positive result between the I-ELISA$_{dev}$ and I-ELISA$_{kit}$ was 78% and for the negative it was 100%. Remaining 22% sera were positive by the I-ELISA$_{dev}$ and negative by the I-ELISA$_{kit.}$ Results of home-developed and commercial kit I-ELISA for detection of antibodies against bovine brucellosis are shown in contingency Table 1.

At 52.49%, 53.09%, 53.26%, 53.86% and 53.94% cut off the sensitivity was 100%, 100%, 97.53%, 88.93% and 86.42%, while the specificity was 84.03%, 84.87%, 85.71%, 87.39% and 87.39%, respectively, for I-ELISA$_{dev.}$ The relative sensitivity and specificity of I-ELISA$_{dev}$ for detection of antibodies against bovine brucellosis at various cut offs is shown in Table 2.

Discussion

In the present study, the LPS were used for the development of I-ELISA and this test was compared with commercial ELISA kit. The LPS were used because *Brucella* LPS has

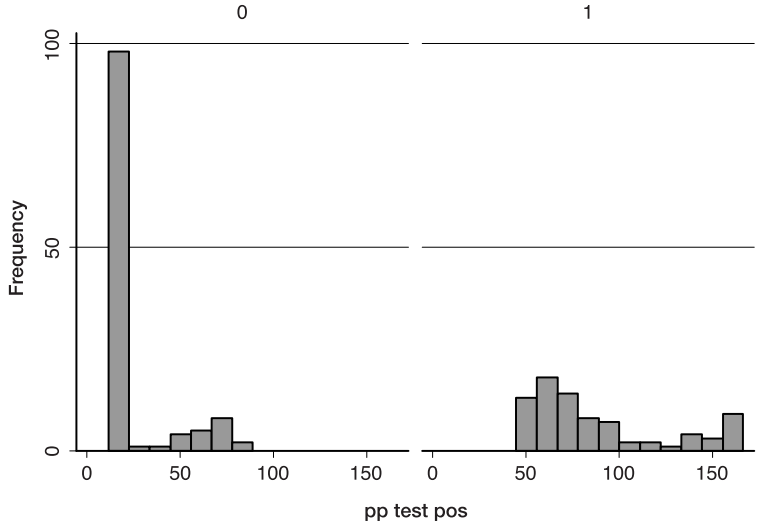

Fig. 2. Histogram showing the distribution of percent positive values of in home developed I-ELISA for detecting anti-*Brucella* antibodies in bovines: 0, negative and 1, positive by commercial I-ELISA

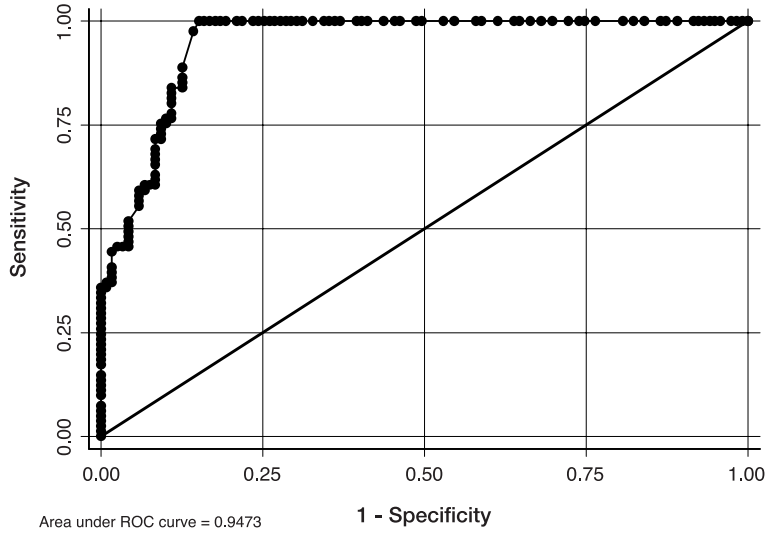

Fig. 3. Receiver Operating Characteristics (ROC) graph for the comparison of home made developed and commercial I-ELISA for detection of *Brucella* antibodies in bovines

been considered the most important antigen during immune response and are the target for many serological and immunological studies. Also LPS can be extracted easily, quantified and standardised as compared to other antigens. Moreover, it gives better sensitivity and specificity with good reproducibility. It also possesses a convenient cut off value for diagnostic purposes. Finally, it is not restricted to bovine cattle and can be adapted to different species of animals as well as to humans. In addition, a small quantity is required

Table 1. Contingency table showing results of home-developed I-ELISA and commercial kit I-ELISA for detection of antibodies against bovine brucellosis

		I-ELISA$_{dev}$		
	Status	Positive	Negative	Total
I-ELISA$_{kit}$	Positive	78	0	78
	Negative	22	100	122
	Total	100	100	200

Table 2. Relative sensitivity and specificity of developed I-ELISA for detection of antibodies against bovine brucellosis at various cut offs

Cut off (PP)	Sensitivity	Specificity
(> = 43.12)	100.00%	83.19%
(> = 52.49)	100.00%	84.03%
(> = 53.09)	100.00%	84.87%
(> = 53.26)	97.53%	85.71%
(> = 53.86)	88.89%	87.39%
(> = 53.94)	86.42%	87.39%
(> = 54.96)	85.19%	87.39%
(> = 56.07)	83.95%	87.39%
(> = 56.16)	83.95%	89.08%
(> = 57.52)	82.72%	89.08%
(> = 57.78)	81.48%	89.08%
(> = 58.71)	80.25%	89.08%
(> = 59.31)	77.78%	89.08%
(> = 60.16)	76.54%	89.08%
(> = 60.84)	76.54%	89.92%
(> = 60.93)	75.31%	89.92%
(> = 61.44)	75.31%	90.76%

PP = Per cent positivity

for the screening of a large number of samples. However, by using LPS the specificity was slightly lower than the specificity (99.2%) observed by Abalos et al. (2000) when using the same antigen. This may be due to a lower number of samples tested by I-ELISA and the use of polyclonal anti-bovine IgG in our studies.

The relative sensitivity of I-ELISA$_{dev}$ was comparable with I-ELISA$_{kit}$, whereas the specificity was a little bit compromised. This developed test can be used for the screening of herds, as the relative sensitivity is higher. In developing countries like Pakistan with a need for screening herds it can be used as a tool for screening purposes. Different researchers have described various reasons for the higher sensitivity of I-ELISA. According to Guarino et al. (2001), the high percentage of positivity was due to the ability of this test to detect very low levels of antibodies present in the early stage of infection, while RBPT and SAT cannot detect it. The use of smooth LPS as antigen in the I-ELISA$_{dev.}$ might be one of the reasons for higher sensitivity as the stronger immune responses are elicited against LPS in infected animals.

In a campaign for the control of a disease like brucellosis, it is desirable that the screening test is reliable and detects almost all positive cases in a herd. In our studies, I-ELISA$_{dev}$ detected equal positive samples as I-ELISA$_{kit}$.

The main purpose of this study was the standardisation of I-ELISA to detect anti-*Brucella* antibodies in buffalo sera and its comparison with the commercial kit. In this study, the I-ELISA$_{dev}$ proved to be equally sensitive as I-ELISA$_{kit}$. This standardized I-ELISA could be a useful diagnostic test for detection of *Brucella* antibodies. Moreover, the developed technique would be particularly useful for a region where little epidemiological information is available about this disease.

Nepřímý ELISA test v diagnóze brucelózy u buvolů

Brucelóza je významná zoonóza, která celosvětově způsobuje závažné ekonomické ztráty. Včasná diagnostika onemocnění je pro její kontrolu a eradikaci zásadní. Nyní byl vyvinut nepřímý ELISA test (Indirect Enzyme Linked Immunosorbent Assay, I-ELISA) s využitím lipopolysacharidu (LPS) jako antigenu a porovnán s komerčním kitem s využitím 100 negativních a 100 pozitivních sér buvolů. Počet shodně pozitivních výsledků mezi komerčním a vyvinutým I-ELISA testem byl 78 %. Negativní výsledky se shodovaly

ve 100 %. Při 52,49 %, 53,09 %, 53,26 %, 53,86 % a 53,94 % byla pro vyvinutý I-ELISA test mezní senzitivita 100 %, 100 %, 97,53 %, 88,93 % a 86,42 %, zatímco specificita byla 84,03 %, 84,87 %, 85,71 %, 87,39 % a 87,39 %. Tento test by mohl být využit ke screeningu stád vzhledem ke své vyšší senzitivitě.

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References

ABALOS P, DAFFNER J, PINOCHET L 2000: Evaluation of three *Brucella* soluble antigens used in an indirect ELISA to discriminate S-19 vaccinated from naturally infected cattle. Vet Microbiol **71**: 161-167

AHMAD R, MUNIR MA 1995: Epidemiological investigation of brucellosis in Pakistan. Pakistan Vet J **15**: 169-172

AHMAD R, MUNIR MA, LATIF M 1994: Production systems and brucellosis in buffaloes. Pakistan J Agri Sci **31**: 341-344

AHMAD R, JAVED S, LATEEF M 1990: An investigation on the prevalence and treatment of brucellosis in buffaloes and cows. Pakistan Vet J **10**: 107-109

AKHTAR S, AFZAL M, ALI S, KHAN MI 1990: Effect of reactor retention on the spread of brucellosis in Jersey cattle and buffalo herds. Rev-Off Int Epizoot **9**: 1179-1185

BIANCIFIORI F, GIOVANNINI A, DI MATTEO A, URBANI G, NANNINI D 1996: Standardizazione di una tecnica ELISA per la ricerca degli anticorpi brucellari nel latte bovino. Vet Ital **32**: 42-46

DAJER AA, GUTIERREZ RE, ZAPATO VD 1998: Use of the ELISA and rivanol agglutination tests for the diagnosis of bovine brucellosis in Yucatan, Mexico. Vet Mexico **29**: 167-171

GUARINO A, FUSCO G, SERPE L, GALLO P, DI MATTEO A, URBANI G, TITTARELLI M, DI VENTURA M, CONDOLEO R 2001: Indirect ELISA for the diagnosis of brucellosis in water buffaloes (*Bubalus bubalis*) in Italy. Vet Rec **149**: 88-90

JARVIS BW, HARRIS TH, QURESHI N, SPLITTER GA 2002: Rough lipopolysaccharide from *Brucella abortus* and *Escherichia coli* differentially activates the same mitogen-activated protein kinase signaling pathways for tumor necrosis factor alpha in RAW 264.7 macrophage-like cells. Infect Immun **70**: 7165-7168

LODHI LA, JAMIL H, QURESHI ZI, AHMAD I 1995: Sero-prevalence of brucellosis in buffaloes in and around Faisalabad. Pakistan Vet J **15**: 127-128

McDERMOTT JJ, ARIMI SM 2002: Brucellosis in sub-Saharan Africa: epidemiology, control and impact. Vet Microbiol **90**: 111-134

MOLNAR E, MOLNAR L, VALE W 1998: Value of different serological tests in the diagnosis of bovine brucellosis in the Amazonian region. Acta Vet Hung **46**: 199-210

OIE 2000: Bovine brucellosis. In: Manual of standards for diagnostic tests and vaccines. 4th ed. OIE, Paris, pp. 328-345

OIE 2004: In OIE manual of the diagnostic tests and vaccines for terrestrial animals, mammals, birds and bees. 5th ed. OIE, Paris, pp. 409-436

OMER MK, SKJERVE E, MACMILLAN AP, WOLDEHIWET Z 2001: Comparison of three serological tests in the diagnosis of *Brucella* infection in unvaccinated cattle in Eritrea. Prev Vet Med **48**: 215-222

RAMZAN M 1996: Incidence of brucellosis in farm animals based on serology. M Phil Thesis Dept Biol Sci, Quaid-i-Azam Uni, Islamabad, 52 p.

ROJAS X, ALONSO O 1995: ELISAs for the diagnosis and epidemiology of *Brucella abortus* infection in cattle in Chile. Arch Med Vet **27**: 45-50

ROMERO C, PARDO M, GRILLO MJ, DIAZ R, BLASCO JM, LOPEZ GI, GONI IL 1995: Evaluation of PCR and indirect enzyme linked immunosorbent assay on milk samples for diagnosis of brucellosis in diary cattle. J Clin Microbiol **33**: 3198-3200

SARWAR H 2000: Sero-prevalence of brucellosis in domestic animals in Peshawar district of North West frontier Province (NWFP) of Pakistan. M.Sc. Thesis, Dept. Zoology, Uni. of Peshawar, 42 p.

SHEIKH SA, SHAH MA, KHAN SA 1967: Some observations on the incidence of brucellosis in West Pakistan. Pakistan J Sci **19**: 189-192

WARAVDEKAR VS, SASLAW LD 1959: A sensitive colorimetric method for the estimation of 2-deoxy sugars with the use of the malonaldehye-thiobarbituric acid reaction. J Biol Chem **234**: 1945-1950

WRIGHT PF, NILSSON E, VANROOIJ EMA, LELENTA M, JEGGO MH 1993: Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev-Off Int Epizoot **12**: 435-450

FAO/IAEA 1991: Indirect Brucellosis ELISA kit manual, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria.

THRUSFIELD M 1986: Veterinary Epidemiology. Butterworth, London, 280 p.