Comparative efficacy of conventional diagnostic methods and evaluation of polymerase chain reaction for the diagnosis of bovine brucellosis

Raheela Akhtar*,Prof. Zafar Iqbal Chaudhry ,Prof. A. R Shakoori[1],
Mansoor ud Din Ahmad and Asim Aslam

Department of Pathology,
University of Veterinary and Animal Sciences, Lahore, Pakistan
1. School of Biological Sciences, University of the Punjab, Lahore, Pakistan.
Corresponding author e-mail: teetu_meetu41@yahoo.com, Tel: 092-042-9211449

Abstract

The comparative efficacy of Rose Bengal Plate Test (RBPT) and Milk Ring test (MRT) was calculated in terms of sensitivity and specificity for the diagnosis of bovine brucellosis in cows (Group A) and buffaloes (Group B) from Lahore and Okara districts of Punjab, Pakistan. Using bacterial growth as a gold standard RBPT showed high sensitivity values of 100% in both groups. While its specificity was 96.29% (Group A) and 90.62% (Group B). On the other hands MRT showed low sensitivity (80.0% in Group A; 86.6% in Group B) while its specificity was 100% in all the animals of both groups. The calculated positive predictive and negative predictive values of both groups were in correspondence with their specificity and sensitivity values respectively. High sensitivity and low specificity of RBPT as compare to high specificity and low sensitivity of MRT in all groups suggested the poor efficacy of both tests used individually as compare to bacterial growth. In the continuation of this study polymerase chain reaction (PCR) was evaluated for its diagnostic efficacy of quick Brucella abortus isolation from same samples. PCR conducted on serum samples gave more positive results than on milk samples. Therefore, the combination of both conventional tests alongwith serum PCR can be recommended.

Key words: Diagnostic tests, PCR, Bovine brucellosis, Laboratory Tests,

Introduction

Brucellosis has been an emerging and re-emerging disease of domestic animals since the discovery of Brucella melitensis by Bruce in 1887. This peril with the synonyms of Bang disease and Malta fever is one of the five common bacterial zoonosis in the world caused by organisms belonging to the genus Brucella, a gram-negative, non-spore-forming, facultative, intracellular bacteria (WHO, 1997). This airborne pathogen is classified as a biosafety level III pathogen and is considered to be a potential bioterrorist (Greenfield et al 2002). Following penetration of the mucosal epithelium, the bacteria are transported, either free or within phagocytic cells, to the regional lymph nodes, spleen, liver, bone marrow, mammary glands, and sex organs and start multiplying there (Cutler et al. 2005). Diagnosis of brucellosis relapses is generally difficult and microbiological techniques are therefore required for confirmation. One of the major diagnostic problem results from the similarity of the O-antigenic side chain of Brucella LPS with other microbes, in particular, with Yersinia enterocolitica 0: 9 (Kittelberg et al., 1995). Blood cultures represent the "gold standard" of laboratory diagnosis on Brucella agar, Brucella selective agar, Tryptose agar, Tryptose bujon, Tryptose citrate bujon but incubation is slow (4 - 6 weeks) and the process is lengthy and labor-intensive with 60-70% yield from blood cultures. (Zerva et al 2001). A broad range of test sensitivity and low specificity in areas of endemicity, lack of usefulness in diagnosing chronic disease and relapse, presence of cross-reacting antibodies, and lack of timeliness constitute problems associated with brucellosis serology (Emmerzal et al 2002) but diagnosis still centers on isolation of the organism and serological test results, however, modern molecular techniques like polymerase chain reaction which is now replacing other methods.

Objectives

- To find a single screening test for the diagnosis of bovine brucellosis.
- To compare conventionally used methods like RBPT and MRT for their efficacy.
- To evaluate PCR as a diagnostic tool for brucellosis and find out the best specimen to be used in PCR.

Materials and Methods

A total of 400 suspected brucellosis samples (200 milk and 200 serum samples) from animals (cows and buffaloes) with history of abortion were collected from government and private farms of two Punjab districts, Lahore and Okara.

Animals were divided into two groups:

Group A: cows

Group B: buffaloes

Rose Bengal plate agglutination test:

The serum samples were subjected to (RBPT) Rose Bengal Plate Test for screening (Stryszak et al 1986) at Hematology Laboratory, Department of Pathology, UVAS, Lahore. The slides were read on the basis of agglutination flakes observed.

Milk Ring Test

Milk ring test was conducted on milk samples as described by Blythman et al (1977). The positive samples were differentiated on the basis of blue ring present on the top of milk after overnight reaction.

Culture and Isolation

Culture and isolation was performed as described previously. Briefly samples were streaked on tryptose soya agar with antibiotic and 1% gention violet. Cultural plates were incubated for 48 hours in 5% Co2 incubator. Resultant colonies were confirmed by biochemical tests like H2S and Polymerase Chain Reaction (PCR).

DNA extraction of serum and milk samples was performed by using Gentra DNA Isolation kit. The primer set for IS711 genomic region of B. abortus as used by Leary et al (2006) was commercially prepared and the sequences were as follows:

5-GACGAACGGAATTTTTCCAATCCC-3? (Forward)

5-TGCCGATCACTTAAGGGCCTTCAT-3? (Reverse)

The PCR was performed in 50µl reaction mixture and following conditions were applied to each assay; 1X Taq Buffer, 0.2mM dNTPs mixture, 1.5mM, MgCl2, 2.5U/µl Taq Polymerase, 4µM of each primer, 4µl of DNA extracted and 26.5 µl of DNase free deionized water. Each sample was tested in triplicate. The tubes containing the mixture were subjected to 30 cycles of amplification in a thermocycler. During each cycle the sample of DNA was denatured at 93ºC for 30 seconds, annealed at 55.5ºC for 30 seconds, and extended at 72ºC for 30 seconds. Prior to the cycling the mixture was subjected to incubation at 94ºC for a period of 4 minutes. PCR product was then analyzed at 1% of agarose gel electrophoresis. The bands of Brucella abortus DNA were detected by using gel documentation system to confirm the presence of Brucella abortus (Leary et al 2006).

Positive and negative control

For each PCR test distilled water was used as negative control and Brucella abortus strain 99 culture obtained from Veterinary Research Institute, Lahore was used as positive control.

Statistical analysis. The sensitivity and specificity of each test was determined using the formula as follows:

Sensitivity = True positive/True positive + false negative x 100

Specificity = True negative/True negative + positive x 100

The statistical analysis was made by using software win episcope 1.

Results

RBPT was found more sensitive and less specific while MRT was more specific and less sensitive in detecting Brucella specific antibodies. The sensitivity, specificity, positive predictive, negative predictive and kappa values of the RBPT and MRT are given in table 1 and 2. The antigenic detection of Brucella using PCR gave more positive results than the conventional RBPT and MRT. PCR was used to detect Brucella abortus in serum and milk samples using the primers for IS711 genetic element and gave an amplicon size of 498bp. The ladder used was 100bp. PCR gave more positive result from serum than milk samples.

Discussion

Brucellosis, a ubiquitous infection of zoonotic importance wreaks havoc on the livestock industry. As much as the disease jeopardizes the economy of the country by inflicting heavy losses, the means of definitive diagnosis are required for its effective eradication. Conventional serological tests serve as important diagnostic aid but are not fully reliable at the different stages of infection. In recent years, a number of new tests have been developed and inducted as aids for the diagnosis of brucellosis.

In this study the high sensitivity and low specificity of Rose Bengal Plate Test (RBPT) as compare to high specificity and low sensitivity of milk ring test (MRT) is highly suggestive of the fact that none of the test is reliable individually. However the increase no. of positive cases in RBPT may be due to many factors like antigen purity, storage temperature, contamination, vaccinal status of animals and infection with other phylogenetically related bacteria. This difference may be attributed to the non-specific agglutinating material for Brucella which has been demonstrated in the serum of animals with no history of brucellosis (Falade et al 1978) and which was latter confirmed to have characteristic similar to those for the IgM class of antibodies (Stemshorn et al 1985). The possible involvement of this non-specific agglutinating material has further been substantiated by Stryszak et al (1986) and Mathia et al (1983). The results of this study were consistent with the findings of Falade et al (1978), Mathia et al (1983) and Stryszak et al (1986)

Table 1: The percentage values for positive samples by RBPT and MRT in grpoup A and B.

	Group A (Cows)		Group B (Buffaloes)	
	RBPT	**MRT**	**RBPT**	**MRT**
Total	100	100	100	100
+ve	26	05	43	16
-ve	74	95	57	84
%	26%	5%	43%	16%

who also reported a large number of false positive reactions of brucellosis by the Rose Bengal plate test. The less number of positive cases by bacterial culture as compare to RBPT may be due to the reasons given by Baily et al (1992) who explained that the most crucial point associated with the isolation of Brucella is the stage of infection. We can predict that it may be possible that the animals under study were in later stage of infection and the production of antibodies was at peak while the numbers of organisms excreted were towards decline.

The milk ring test established the sensitivities of 80% (Group A) 86.6% (Group B) while its specificity was 100% in both groups that shows less chances of false positive reactions as compare to RBPT but as this test is less sensitive than RBPT this flaw inhibits the individual usage of this test for the diagnosis of bovine brucellosis. These findings are in agreement with findings of Hamdy et al (2002) and Leal-Klevezas et al. (1995) who detected low sensitivity of MRT. Fresh milk samples before refrigeration were preferred for this study as described by Blythman et al (1977) who demonstrated that fresh milk samples had fewer chances of false positive reactions. On contrary, Rolf et al (1987) and Erasmus et al (1988) had suggested the false positive reactions up to 3.4% by MRT that might be due to vaccination with strain 19 or sampling in very early or late lactation. Emmerzal et al (2002) narrated that MRT detected the non-specific antibodies produced by the other phyllogenetically related bacteria such as Yersinia, E.coli, Pasturella etc. The low sensitivity of MRT in this study was in accordance with the studies of Huber et al (1986).

This study also emphasizes the diagnostic usage of the PCR as explained by Sifuentes et al (1997) and Darla et al (2000) As antigenic detection is always more reliable than the antibody detection. Serum samples were found to be more appropriate for Brucella isolation that may be because of the fact that milk protein hamper the organism isolation.

Conclusion

The invariability of the sensitivity and specificity of RBPT and MRT in cows and buffaloes suggested that no single test was recommended for the accurate diagnosis of brucellosis however the combination of both is recommended for initial screening and afterwards the confirmation by PCR is needed for culling. Moreover, in our circumstances PCR cannot be used as initial screening tests for large herds because of high cost as compared to other two tests, unchecked quality control measures, sample contamination and time consumption. Because antigenic detection by PCR is always more reliable and as the serum PCR detected more cases rather than milk PCR therefore serum PCR is highly recommended as a reliable tool along with initial screening by RBPT and MRT for the diagnosis of bovine brucellosis.

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Table 2: The calculated sensitivity and specificity values of RBPT and MRT in Group A and B.

	Group A (Cows)		Group B (Buffaloes)	
	RBPT	**MRT**	**RBPT**	**MRT**
Sn	100	45.45	100	94.11
SP	83.78	100	89.47	100
PPV	68.42	100	87.75	100
NPV	100	93.68	100	98.81
Kappa	0.83 (0.753-0.921)	0.454 (0.160-0.748)	0.894 (0.815-0.974)	0.941 (0.829-1.00)

Sn: sensitivity, Sp: specificity, PPV: positive predictive values, NPV: Negative predictive values
(Bacterial culture was used as a gold standard)

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