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Comparison of PCR with conventional techniques for the diagnosis of brucellosis in cattle

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Brucellosis is a major zoonotic disease endemic in many parts of the world. It is characterized by chronic infection in animals leading to abortion and infertility. The disease is caused by *Brucella* which is gram negative, nonspore-forming, facultative intracellular organism (Cardoso *et al.* 2006). It consist of 7 species (Ko and Splitter 2003) based on antigen variation and primary host: *B. melitensis* (sheep and goat), *B. abortus* (cattle), *B. suis* (swine), *B. ovis* (rams), *B. canis* (dogs), *B. neotomae* (wooden rats) and recently recognized *B. maris* (marine mammals). Besides, restriction in the international trade of animal and animal products, the disease can be transmitted to humans following contact with infected animals or through consumption of contaminated milk and milk products (Young *et al*. 1985). Serological assays used for the diagnosis of *Brucella* infection include Rose Bengal plate agglutination test (RBPT), serum tube agglutination test (SAT), compliment fixation test (CFT) and enzyme linked immunosorbant assay (ELISA); however, bacteriological isolation remains the definitive method (Chand *et al.* 2005). But the tedious isolation process and risk to the laboratory personnel to infection limits regular use of bacteriological isolation in diagnosis. Apart from this, brucellae could not be isolated many a times even from known positive cases (Probert *et al.* 2004). Therefore, a rapid, sensitive and specific tool like polymerase chain reaction (PCR) for precise detection and identification of such a fastidious organism(s) from a variety of samples (Amin *et al*. 2001, Leyla *et al.* 2003 and Probert *et al*. 2004) is being exploited. Several PCR based methods have been described for the detection of *Brucella* species using bacterial isolates and field samples (Leal-Klevezas *et al.* 2000). The present study compares PCR with isolation of *Brucella* from different samples *vis a vis* conventional test, viz. RBPT and SAT.

A total of 45 samples including blood (15), vaginal swabs (15) and milk (15) were collected simultaneously from cattle population from the dairy farm of the Indian Veterinary Research Institute, Izatnagar. The samples were collected aseptically and immediately transported to laboratory under cold condition. The milk samples were processed for bacteriological isolation as described by Alton *et al.* (1995) and incubated at 37°C under 5% CO_2 for several days and inspected regularly for the development of *Brucella*, like colonies. The blood containing sodium citrate was inoculated onto Ruiz Castaneda biphasic medium (Leal-Klevezas *et al.* 2000). The vaginal swabs were washed properly in 2 ml of sterilized phosphate buffer saline (PBS; pH 7.4) and were inoculated onto Ruiz Castaneda biphasic medium as for the blood.

The serum were tested by RBPT and SAT as per the standard method (Alton *et al*. 1975). Serum samples, exhibiting any degree of clumping of coloured antigen in RBPT and 50% agglutination reaction at a dilution of 1: 40 or above in SAT were considered positive. The DNA was extracted from whole blood as described by Leal- Klevezas *et al.* (2000) whereas DNA extraction kit (Biogene) was used for extracting DNA from fatty top layer of raw milk and vaginal swab washing (each 400 µl) as per the manufacturers guidelines. PCR was performed in 50 µl containing 5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris- HCl, pH 9.0), 200 µM each of the 4 deoxynucleotide triphosphates, 1U Taq DNA polymerase, 8 pmol each of the primers derived from the BCSP–31 sequence (Serpe *et al.* 1999) of *B. abortus* (Forward 5' GGG CAA GGT GGA AGA TTT 3' and Reverse 5' CGG CAA GGG TCG GTG TTT 3') and 5 µl of template DNA. The amplification was carried out in mastercycler gradient thermocycler with a preheated lid at a denaturation temperature of 94°C for 3 min followed by 35 cycles at 94°C for 45s, 53°C for 45s and 72°C for 5 min. Electrophoresis of PCR product was carried out on 1.5% (w/v) agarose gel in tris-borate EDTA (TBE; 0.5X) to visualize 443bp product in positive samples after staining with ethidium bromide.

Out of 15 blood samples, 13 were positive in RBPT and

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of these positive samples, 6 were also positive in SAT with a cut off titre of 80. The PCR was positive in 2 blood samples of animals positive in SAT and RBPT. The result of RBPT and SAT, where 7 samples positive by RBPT turned out as negative in SAT, corroborates the findings of Morgan (1974). Moreover, the RBPT recommended by WHO/FAO Expert committee as a screening test (Anonymous 1986), has been reported to be an oversensitive test (Alton *et al.* 1975a). The other 6 animals were positive to both RBPT and SAT. No isolation of *Brucella* could be made from any of the blood, milk and vaginal swabs taken from these animals. But the blood and vaginal swabs from 2 of these 6 animals, which tested positive by both serological tests, were also positive by PCR. Although cultural isolation is the gold standard in the microbial diagnosis, it may sometimes be abolished by factors inherent to the microbes besides it is tedious and not possible to isolate *Brucella* every time even from the known positive cases (Ray 1979). The observation in the present study that two culture negative but seropositive animals tested positive by PCR supports this contention. In case of vaginal swab, this could even be due to massive contamination of the samples or viability loss of the organism before culturing (Ray 1979) and in all these circumstances DNA can still be detected by PCR. However, the intracellular nature of bacteria may affect the proper release of *Brucella* from leukocyte and results in the negative isolation of *Brucella* from blood samples. Brucellae are excreted in milk intermittently which may explain the negative PCR result on milk samples from these animals. Alternatively, this could be because of the presence of the PCR inhibitors or number of organisms below the detection limit, in the milk.

The sensitivity of PCR to detect brucellae from milk was also determined by serially diluting *B. abortus* S19 in uncontaminated milk, followed by DNA isolation as above, which detected as low as 40 cells/ml of milk, which is higher than the previous report (Romero *et al.* 1995). This detection limit can be further increased by reducing sampling error, since random distribution of the cells in a sample may not have the exact number of cells or gene copy as has been calculated to contain (Wilson 1997). The sensitivity of the test in spiked blood was not performed due to the intracellular nature of the organism in leukocytes. The specificity of the PCR was confirmed by amplification of DNA extracted from different strains of *Brucella* and the lack of amplification of DNA extracted from closely related organisms, viz. *Escherichia coli, Vibrio cholerae, Campylobacter coli, Listeria monocytogenes, L. ivanovi* and *Salmonella* Typhimurium. The failure to detect amplified fragments in the DNA template of the related bacteria supported the specificity of this PCR as a genus specific assay in the detection of brucellae. The results indicated the superiority of the PCR for detecting small amount of the pathogen in various samples over isolation proving the PCR as a highly sensitive, reliable and specific method for detection of *Brucella* species from various clinical samples and can be used as an adjunct to serological tests in diagnosis of brucellosis. However, there is a need to improve the sensitivity of the PCR.

SUMMARY

Brucellosis is still a wide-spread zoonosis of international importance. In the present study, a PCR has been compared with conventional methods for detection of brucellosis, viz. RBPT, SAT and bacteriological isolation of the agent. The PCR was found highly specific for identification of *Brucella*. No isolations could be made even from the animals which were positive to RBPT, STAT and PCR. The study indicated that the PCR could be used as an adjunct in the diagnosis of brucellosis.

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