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Comparative Efficacy of Serological Tests and Molecular Analysis of Brucellosis in Dairy Cattle of Mathura District

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ABSTRACT

Background: Brucellosis is one of the threads to the global world and also one of the major zoonotic problem for both humans and animals worldwide. *Brucella* is the gram negative bacteria which produce Brucellosis and having clinical characteristics like mastitis, repeat breeding, retention of placenta, abortion in the last trimester and also there will be reduction in milk in dairy cattle. Male also shows some clinical characteristics like epididymitis, orchitis and sterility. The disease can affect Humans and can also exhibit few symptoms like undulant fever, arthritis and orchitis *etc*.

Methods: A total of 405 serum samples were collected comprising dairy Cattle (female-372 and male-33) from various blocks of Mathura district. Samples were process from detection of Brucellosis by RBPT, STAT, ELISA, MRT and 5 tissue samples were subjected to molecular analysis by PCR for the disease.

Result: Based on the analysis, out of total 405 animal samples, Dairy cattle animals showed 49 (13.17%) positivity with RBPT. STAT showed 43 (11.55%) in dairy cattle. Similarly, I-ELISA revealed 55 (14.78%). Further, C-ELISA revealed 42 (93.34%) in dairy cattle. PCR detected amplicons of 223 bp in tissue samples (n=3), employed whereas only one were detected with *B. melitensis* and *B. ovis,* where as 2 were detected for *B. abortus* and *B. suis* by AMOS PCR at 498 bp, 285 bp, 976 bp and 731 bp respectively.

Key words: Brucellosis, I- ELISA C- ELISA, PCR, STAT, RBPT.

INTRODUCTION

Brucellosis is a zoonotic problem of great animal welfare and economic implications worldwide. Brucellosis poses a major public health threat by the consumption of non-pasteurized milk and milk products produced by unhygienic dairy farms in endemic areas. It is of high endemicity in the regions of Africa, Mediterranean, Middle East, parts of Asia and Latin America (Refai, 2002). Although developed countries like Europe, Australia, Canada, Israel, Japan and New Zealand have already eradicated the disease (Geering *et al*., 1995). In India, brucellosis causes an average loss of US $18.2 per buffalo followed by 6.8 per cattle, 0.7 per sheep, 0.6 per pig and 0.5 per goat (Sing *et al.,* 2015). Brucellosis is caused by members of genus *Brucella*. The *Brucella* organisms are small, non-motile, non-spore forming, aerobic, facultative intracellular, Gram-negative coccobacilli which are arranged singly or in pairs or short chains. Also, are catalase, oxidase and urea positive. The capacity of causing persistent disease and circumventing innate and adaptive immunity are key secrets of replication and persistence in the host by *Brucella*. Depending upon the specific natural host affinity, there are six classical *Brucella* species, *viz*., *B. abortus* (cattle and buffaloes), *B. melitensis* (goats), *B. suis* (pigs), *B. ovis* (sheep), *B. neotomae* (desert wood rats) and *B. canis* (dogs) (Osterman and Moriyon, 2006). It is stated that more than 80% of dairy cows will abort if they are exposed during the time of gestation. In males orchitis and epididymitis with sterility are prominent (Radostits *et al.,* 2000). Humans become infected with *Brucella* species by ingesting organisms, getting contaminated with mucous membranes or through abraded skin. Animal abortion products, unpasteurized dairy products, uncooked or undercooked meat or meat products, unpasteurized milk and cheese prepared from infected unpasteurized milk (Pappas *et al*., 2005) play a crucial role in its transmission. Symptoms in human brucellosis can be highly variable, ranging from non–specific, flu-like symptoms (acute form) to undulant fever, arthritis, intermittent or irregular fever, orchitis and epididymitis (Smith and Kadri, 2005).

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MATERIALS AND METHODS

A total of 405 serum samples were collected from cattle in and around various blocks of Mathura district. These consist of female (372) and male (33) serum samples were collected from various blocks of dairy farms and gaushalas of Mathura district, within the time period of February, 2023 to middle of November, 2023 and processing of samples performed at the department of veterinary public health and epidemiology. Total 405 serum samples were collected for serological diagnosis and 5 stomach content samples of aborted calf for PCR assay. About 5-10 ml of blood was collected by puncturing the jugular vein in sterile disposable syringes (Dispovan) or plain vaccutainers (BD, USA) for serum collection. Basic precautions was taken to avoid shaking the samples during transportation to prevent the destruction of the RBCs. Serum were collected from the clotted blood after centrifugation at 4000 rpm for 10 min. A total of 5 stomach content samples were collected for PCR assay. The samples were transported immediately to the laboratory on ice pack. The individual animals in the present study were identified by their respective identification numbers or names. None of the animals was vaccinated against brucellosis. All the serum samples were stored at -20°C till tested.

Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT) antigen were procured from Indian Veterinary Research Institute, (IVRI), Izatnagar, Bareilly, India. For serum I-ELISA, kit for cattle procured from ICAR National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bangalore, Karnataka. RBPT antigen is an 8% suspension of pure smooth killed cells of *Brucella abortus* strain 99 phenolised and stained with rose Bengal dye. Rose Bengal Plate Test is a single dilution serum agglutination test. It was performed on glass slides according to the method presented by (Alton *et al.,*1988). To detection of STAT in a serum samples, *Brucella abortus* plain antigen were taken which was heat killed phenolised suspension of *Brucella abortus* strain 99 and it show 50% agglutination at 1/500 final dilution of serum with Indian standard. In cattle serum samples antibody was detected by indirect ELISA (I-ELISA) kit procured from Svanova (Biotech-AB), Uppasala, Sweden. Briefly, each of the kit contained 96 flat bottom polystyrene with *Brucella abortus* precoated antigen wells. Competitive ELISA (C-ELISA) was performed using separate kits based on IgM for large ruminants procured from SVANOVIR (Sweden).

Bacterial DNA from stomach content was extracted by Phenol Chloroform Method (Sambrook and Russel, s2001) and from milk sample as per Pokorska *et al.* (2016) method. PCR analysis for *Brucella* genus specific bcsp31 gene was carried out according to the method described by Baily *et al.* (1992) (Table 1). The final reaction volume of 25 µl was obtained by adding 12.5 µl of Master mix (Genie, Banglore), 3 µl of DNA template, 1µl of each of the primers (forward and reverse) made up by adding nuclease free water. For bcsp31 gene amplification, the initial denaturation step was carried out at 95°C for 3 min followed by denaturation at 95°C for 30 sec., annealing at 63°C for 45 sec., extension 72°C for 45 second and a final extension step at 72°C for 10 min. *Brucella* species specific IS711 gene amplification, the PCR conditions were an initial denaturation step at 95°C for 10 min, denaturation at 94°C for 1 min., annealing 58°C for 1 minute, extension 72°C for 1 min. and followed by a final extension step at 72°C for 7 minute. For each gene 35 amplification cycles were performed. After the amplification, amplicons were separated in 1.5% gel in tris-acetate EDTA (TAE) buffer at 60 volt for 80 min, stained with 0.5% ethidium bromide solution and visualized under ultraviolet light. For species differentiation of positive samples, the method (AMOS-PCR) described by Bricker and Halling (1994) was adopted. The five primers cocktail poised to differentiate *Brucella* species DNA into *B. abortus, B. melitensis, B. ovis* and *B. suis* (Table 2).

RESULTS AND DISCUSSION

Screening of cattle (n=405) in which female (372) and male (33) serum samples were detected by RBPT, STAT and I-ELISA, different outcomes were recorded for the diagnosis of antibodies against *Brucella* species. On analysis total

Table 1: Primer sequences of genus *Brucella.*

Primers	Gene	Sequence (5′-3′)	Size of amplified product	Reference
B4 (F)	*bcsp31*	TGGCTCGGTTGCCAATATCAA	223 bp	Baily *et al.* (1992)
B5 (R)		CGCGCTTGCCTTTCAGGTCTG		

Table 2: AMOS multiplex PCR for simultaneous detection of four *Brucella* species.

Organism	Name of gene	Primer sequence 5′-3′	Product size (bp)	Reference
B. abortus	IS711(F)	GACGAACGGAATTTTTCCAATCCC	498 bp	Bricker and
B. melentis	IS711(F)	AAATCGCGTCCTTGCTGGTCTGA	731 bp	Halling (1994)
B. ovis	IS711(F)	CGGGTTCTGGCACCATCGTCG	976 bp	
B. suis	ISI711(F)	GCGCGGTTTTCTGAAGGTGGTTCAGG	285 bp	
Common reverse primer	ISI711(R)	TGCCGATCACTTAAGGGCCTTCAT	-	

of 405 serum samples, 49 (13.17%) were found positive by RBPT (Fig 1), whereas 43 (11.55%) by STAT (Fig 2) in dairy cattle. Similarly, I-ELISA revealed 55 (14.78%). The overall seroprevalence of bovine brucellosis was found to be 9.92%, 16.03% and 6.48% by RBPT, STAT and I-ELISA respectively, Bohrey *et al.* (2022). Out of 405 samples, on analysis of dairy cattle (n=45) in which 40 sera samples were from female and 5 sera samples were from male cattle from various blocks of Mathura district, having history of vaccination for Brucellosis. C-ELISA test was performed and the result showed that 42 (93.34%) sample came positive in C-ELISA and 3 sera samples came negative. Out of 42 positive sample 6 sera samples were weak positive and 36 sera sample were strong positive. There have been reports of higher prevalence rates in cattle (10.74%). Ahmad *et al.* (2009) (25.7%) in Jordan, Mishra *et al.* (2022) in India and Junaidu *et al.* (2011) in Nigeria in agreement with the results of this study. Studies by Krishnamoorthy *et al.* (2015) and Tragandi *et al.* (2015) estimated the prevalence of I-ELISA to be 11.63 per cent in Southern India, 6.8 per cent in Andhra Pradesh, 8.2 per cent in Gujarat and 2.3 per cent in Odisha. Kassahun (2004) observed somewhat lower prevalence rates for intensive (2.5%) and extensive farms (1.7%) in Southern Ethiopia, whereas Berhe *et al.* (2007) reported slightly higher prevalence rates (3.19%) for extensive agricultural systems in Algeria region there is more gaushalas where culled cattle are kept with healthy cattle without proper testing of brucellosis and these gaushalas are running very unhygienic conditions so that it may be the higher finding of brucellosis.

Fig 1: RBPT Test (Positive cattle serum) for Brucellosis.

Molecular detection

The DNA was extracted directly from the 4 tissue samples (T= stomach content of aborted fetus) collected from the 4 different dairy cattle which were detected anti-*Brucella* antibodies by I-ELISA test. These were then subjected to PCR using *Brucella* genus specific primer pair B4/B5 targeting bcsp31 gene (Baily *et al.,* 1992). Amplicon size of 223 bp was obtained only in 1 tissue samples (T=1), out of 4 tissue samples were found carrying *Brucella* organisms. (Fig 3). None of the other samples yielded DNA which implied that *Brucella* organism was not present in the tissues of those animals even though the antibody titre was quite high leading to positive results in serology Singh *et al.* (2015). Various PCR procedures have been developed for the detection of *Brucella* (Probert *et al.,* 2004; Tanmay, 2007; Zamainian *et al.,* 2015). *B. abortus* is an intracellular bacteria and this poses a problem for selection of a suitable sample (Wattam *et al.,* 2009).

For species differentiation of *Brucella* organisms, AMOS-PCR using five primers which was developed by Bricker and Halling (1994) was employed. This assay was able to detect *B.abortus, B.melitensis, B.ovis* and *B.suis*. However, it was unable to detect *B.canis, B.neotomae, B.pinnipedialis, B.ceti* and some biovars of *B.abortus* and *B.suis*. Again, 4 extracted DNA samples were subjected to this assay using four specific primers of four different species along with one IS711 primer. Amplicon of 976 bp and 731 bp was seen in 1of the featal stomach content sample which indicates presence of *B.ovis* and *B. melentensis* in the sample. An amplicon of 976 bp and 731 bp was seen in 2 of the tissue (featal stomach content) sample which indicates presence of *B. ovis* and *B. melentensis* in the sample. An amplicon of 498 bp and 285 bp was seen

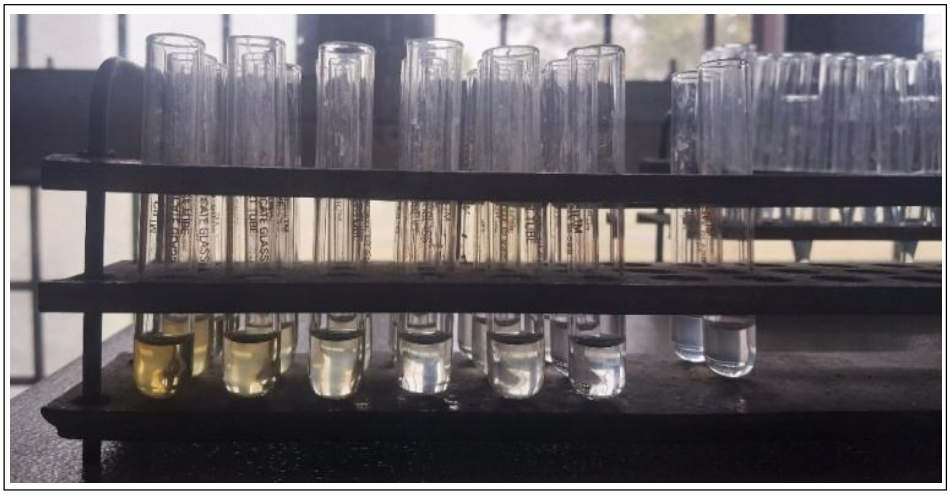
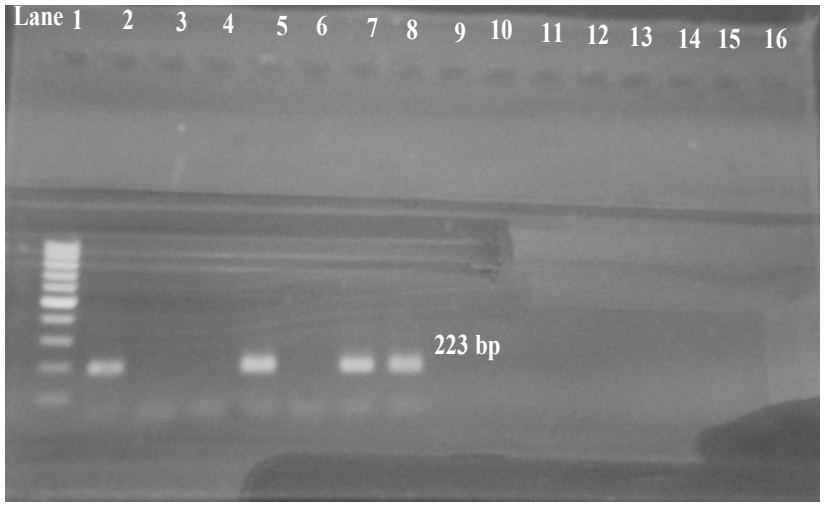
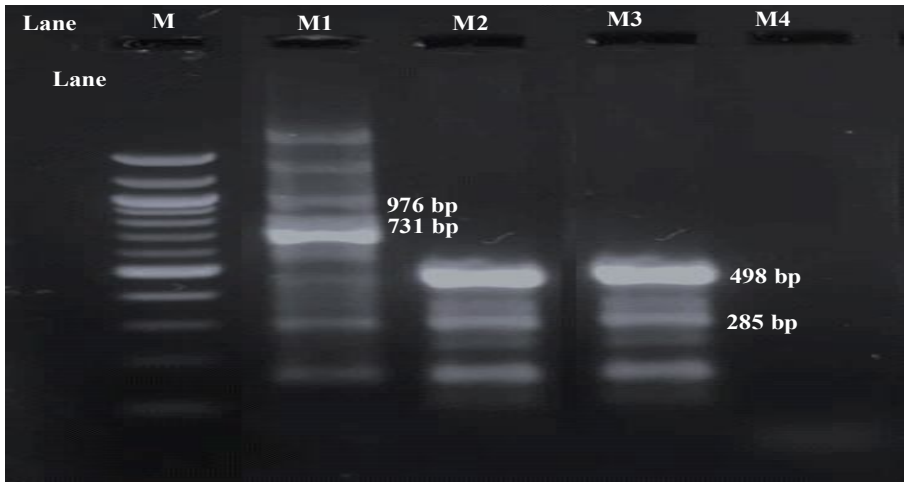


Fig 2: STAT test (Positive cattle serum) for Brucellosis.



Lanes-1: 100 bp DNA Ladder, Lane-2: Positive control, Lane-3: Negative control, Lane-5, 7 and 8: Calf sample positive for *Brucella genus* at (223 bp).

Fig 3: PCR amplified fragments by using B4/B5 primer pair.



Lanes: M- 100 bp DNA Ladder, M1- Positive for *B. melitensis* (731 bp) and *B. ovis* (976 bp) amplicons, M2 & M3- positive for *B. abortus* (498 bp) and *B. suis* (285 bp) amplicons.

Fig 4: PCR assay based on IS711 gene for *Brucella* spp.

in 3 of the tissue (featal stomach content) sample which indicates presence of *B.abortus* and *B.suis* in the sample (Fig 4). No other amplicons at 498 bp, 731 bp, 976 bp and 285 bp were observed in 4 tissue (featal stomach content) sample indicating absence of *B. abortus, B. melitensis, B. ovis* and *B. suis* respectively. Among the other tissue samples, none of such amplicons were detected. Similar study was done by Kurmanov *et al.* (2022) Cases are reported annually across the range of known infectious species of the genus *Brucella*. Globally, *Brucella melitensis*, primarily hosted by domestic sheep and goats, affects large proportions of livestock herdsand frequently spills over into humans. Same study was done by Gumaa *et al*. (2020), duplex recombinase polymerase amplification (Duplex RPA) assay for the specific detection of *Brucella melitensis* and *Brucella abortus* was developed in this study. Primers were designed targeting hypothetical protein genes and membrane transporter genes of *B. melitensis* and *B. abortus,* respectively. *Brucella melitensis* biovar 3 was isolated from 86 (84.31%) cows. Additionally, universal PCR showed the presence of *Brucella* DNA in all tissue extracts from which *Brucella melitensis* has been isolated as well as DNA extracts of their cultures.

CONCLUSION

Among the different samples *viz*. serum were tested by serological tests and stomach content of aborted foetus of cattle were used for confirmation of active infection of brucellosis by PCR assay. As the percent positivity by serological tests was found to be 14.78% (55/372) in female

cattle and 30.30% (10/33). So combination of RBPT, STAT and I-ELISA and confirmation by PCR assay using tissue samples was found to be the most suitable combination for the confirmatory diagnosis of brucellosis in cattle in absence of isolation of organisms. The incidence of brucellosis cases is increasing over the recent years especially in developing countries due to poor management, limited resources and increased trade and frequent movement of livestock. The disease's higher prevalence in this location raises the likelihood of zoonotic transmission, posing a substantial threat to the human population as well as a significant economic impact due to lost production and animal population.

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Conflict of interest

The authors declare that there is no conflict of interest.

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