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Comparative Efficacy of Serological Tests and Molecular Analysis of Bovine Brucellosis in Western Uttar Pradesh

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ABSTRACT

Background: Brucellosis is a major zoonotic disease that affects people all over the world. Metritis, mastitis, repeat breeding, abortion in the last trimester of pregnancy, retention of placenta and reduced milk production are all symptoms of Brucellosis in the female bovines, whereas epididymitis, orchitis and sterility are symptoms in the male animals. In humans flu-like to undulant fever, arthritis, orchitis and epididymitis can be detected.

Methods: A total of 470 bovine samples (serum, whole blood, milk samples) were taken from four districts of western Uttar Pradesh. The 262 sera samples were processed and subjected to RBPT, STAT and ELISA for the determination of seroprevalence of brucellosis and 108 whole blood samples were subjected to molecular analysis by PCR for the disease.

Result: Out of 262 (203 cattle and 59 buffalo) serum samples, the seroprevalence of brucellosis in 203 cattle serum sample was found to be 11.82%, 16.25% and 8.37%. Whereas, in 59 buffalo serum samples, the seroprevalence was 3.38%, 15.25% and 0.00% by RBPT, STAT and I-ELISA respectively. Out of 108 whole blood samples, 5 were positive in molecular detection by PCR giving a prevalence rate of 1.62%.

Key words: Bovine Serum, I- ELISA, PCR, RBPT, STAT.

INTRODUCTION

Brucellosis is one of the most important contagious and communicable bacterial diseases, with a worldwide distribution. It is reported from almost 86 countries and is classified as re-emerging though neglected zoonosis with high rates of morbidity and lifetime sterility stated by Trujillo *et al.* (1994). Though it has been eradicated in many developed countries of Europe, Australia, Canada, Israel, Japan and New Zealand Geering *et al.* (1995) yet remains highly endemic in Africa, Mediterranean, Middle East, parts of Asia and Latin America (Refai, 2002) due to poor management, limited resources (Khan and Zahoor, 2018), increased trade and frequent movement of livestock Renukaradhya *et al.* (2002). Schwabe, a WHO consultant estimated an annual loss in India to be Rs 240 million in livestock, Rs 120 millions in human beings and brucellosis costs Rs 350 million in the form of food animals. There is a several routes for Brucellosis transmission which includes mother to offspring before or at the birth, through milk, by sexual contact, direct physical contact, from polluted environments and from eating spoiled raw meat mainly placenta and birth products. Brucellosis is caused by members of genus *Brucella* which are small, non-motile, facultative aerobic, intracellular, Gram-negative coccobacilli. The ability of *Brucella* to replicate and persist in host cells is directly associated with its capacity to cause persistent disease and to circumvent innate and adaptive immunity (Fichi, 2003). The classical species of *Brucella* and their major hosts are *Br. abortus* (cattle), *Br. Melitensis* (goats), *Br. suis* (swine) and *Br. ovis* (sheep). In bovines, the disease is clinically characterized by metritis, mastitis, repeat breeding, abortion in the last trimester of pregnancy, retention of placenta and reduced milk production in the females and epididymitis, orchitis and sterility in males Radostits *et al.* (2000). In humans, brucellosis is considered to be an occupational disease that mainly affects slaughter house workers, butchers and veterinarians. The presence of brucellosis in India was first established early in the previous century and since then it has been endemic in almost all the states (Sehgal and Bhatia, 1990;

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Renukaradhya *et al*., 2002). Livestock production is a vital source of providing dietary protein for the rapidly growing human population and it is therefore, important to define clear and effective strategies for controlling infectious diseases like brucellosis that are undermining the livestock industry and are a cause of major economic loss to our country.

MATERIALS AND METHODS

Source and place of samples collection

The present study was conducted in four districts *viz*., Aligarh, Firozabad, Hathras and Mathura located in western part of Uttar Pradesh, India, from October 2017 to July 2018. These districts were chosen because of the presence of large number of smallholder dairy producers, gaushalas and good animal husbandry techniques. During the present study, a total of 470 samples (serum, whole blood, milk) were aseptically collected from the selected animals (cattle and buffaloes) from the above mentioned areas and processing of sample performed at the department of Epidemiology and Preventive Medicine, DUVASU, Mathura, U.P. Total 262 serum samples were collected for serological diagnosis and 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. Care was taken to avoid shaking the samples during transportation to prevent the destruction of the RBCs. Serum was collected from the clotted blood after centrifugation at 4000 rpm for 10 min. A total of 108 whole blood samples were collected for PCR assay in EDTA vaccutainers (BD, USA). 100 milk samples were collected in sterile polypropelene tubes 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, blood sample and milk sample were stored at -20°C till tested.

Prevalence based on different serological tests

RBPT and STAT antigens were procured from BP Division, Indian Veterinary Research Institute, (IVRI), Izatnagar, India. RBPT antigen was 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 described by Alton *et al.* (1988). To perform STAT in a serum samples, heat killed phenolised suspension of *Brucella abortus* strain 99 plain antigen was taken. The ELISA test kit was purchased from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) (Formerly PD-ADMAS) Hebbal Bengaluru, Karnataka, India and the contents were stored at 4-8°C until use. The test was performed as per the manufacturer's protocol.

Molecular characterization of *Brucella* by PCR

Bacterial DNA from whole blood and serum was extracted by Phenol Chloroform Method (Sambrook and Russel, 2001) 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 sec 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.

RESULTS AND DISCUSSION

In this study, a total of 470 samples from 203 cattle and 59 buffaloes were collected including 262 serum samples from 203 cattle and 59 buffaloes which were screened for seroprevalence of bovine brucellosis using RBPT, STAT and I-ELISA. The overall seroprevalence of bovine brucellosis was found to be 9.92%, 16.03%, 6.48% by RBPT, STAT and I-ELISA respectively. The distribution of antibodies against Brucella antigen varied significantly between cattle and buffalo, according to a species-wise seroprevalence investigation (Table 2). By RBPT, STAT and I-ELISA, the

Table 1: Details of primers used for PCR reaction for *Brucella* genes.

Gene target and size of amplified product (bp)	Primers	Sequences (5′-3′)	Reference
bcsp31 (223bp)	B4 (Forward)	5′-TGG-CTC-GGT-TGC-CAA-TAT-CAA-3′	Baily *et al.* (1992)
	B4 (Reverse)	5′-CGC-GCT-TGC-CTT-TCA-GGT-CTG-3′	
IS711 (498bp)	IS711 (Forward)	5′-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3′	Bricker and Halling, (1994)
	IS711 (Reverse)	5′-GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC-3′	

Table 2: Species wise seropositivity of bovine brucellosis (cattle and buffalo) detected by RBPT, STAT and I-ELISA.

Species	No. of serum sample	No. of samples positive by RBPT	Percent positivity by RBPT	No. of samples positive by STAT	Percent positivity by STAT	No. of samples positive by I-ELISA	Percent positivity by I-ELISA	p value
Cattle	203	24	11.82%	33	16.25%	17	08.37%	0.0565
Buffalo	59	02	03.38%	09	15.25%	00	00.00%	
Total	262	26	08.46%	42	09.35%	64	11.28%	

p>0.05 at 5% level of significance.

seroprevalence in cattle was 11.82 per cent, 16.25 per cent and 8.37 per cent, whereas it was 3.38 per cent, 15.25 percent and 0 per cent in buffalo serum samples. As a result, cattle had a higher prevalence of the disease than buffalo, but there was no difference that was statistically significant (p=0.0565). According to I-ELISA, Kebede *et al.* (2008) (11%), Eshetu *et al.* (2005) (10%) and Aggad and Boukra (2006) (9.7%) observed almost identical seroprevalences of bovine brucellosis. 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, Junaidu *et al.* (2011) in Nigeria and Ahmad *et al* (32.2 per cent). 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 Gujrat 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.

Molecular detection

Serum (100 samples), whole blood (108 samples) and milk (100 samples) were subjected to PCR assay and amplicons of 223 bp of *Brucella* bcsp 31 gene (genus specific Fig 1) Baily *et al.* (1992) and 498 bp of *Brucella* species specific IS711 (Fig 2) gene (Bricker and Halling, 1994) were obtained. Out of 100 serum samples, none was by PCR while out of 108 whole blood samples, 3 and from 100 milk samples, 2 were found positive by PCR (Fig 1). None of the serum samples yielded DNA which implied that *Brucella* organism was not present in the serum of those animals even though the antibody titre was quite high leading to positive results in serology Singh *et al.* (2010). Various PCR procedures have been developed for the detection of *Brucella* (Probert *et al.,* 2004; Tanmay, 2007; Zamain *et al.,* 2015). *B. abortus* is an intracellular bacteria and this poses a problem for selection of a suitable sample (Wattam *et al.,* 2009). Only during acute phase of infection, it circulates in blood, mostly inside the white blood cells and hides itself in mammary organs, genital organs and lymph nodes (Morgan and Mackinnon, 1979) hence sample should be selected according to the phase of Brucella's life cycle which is not practically possible to find out. The wide variation in the number of samples detected as positive by RBPT (26), STAT (42), ELISA (17) and PCR (5) might be due to many factors. Whole blood was found to be the better sample out of serum, whole blood and milk for DNA extraction to perform PCR assay as DNA might have been in very low or negligible quantity in serum and in milk, presence of fat globules and other proteins might have inhibited the DNA yield. Karthik *et al*. (2014) also performed bcsp 31 gene based PCR and species specific IS711 gene based PCR using whole blood samples to detect 15.13% positivity. Their results indicated that whole blood can be used for studying the molecular epidemiology of *B. abortus* in bovine and particularly detecting the active phase of infection. Further, at the time of equilibrium of host parasite interaction, the *Brucella* may persist in circulation for some time before getting localized in their preferred sites. Similar results that DNA can be extracted from whole blood and used as a sample for screening for brucellosis has been reported by Guarino *et al.* (2000) in buffaloes, Keid *et al.* (2010) in dogs and Khamesipour *et al.* (2013) in cattle and sheep. Nakkas *et al.* (2002) and Leal-Klevezas *et al.* (2000) used buffy coat instead of whole blood for DNA extraction as macrophages take up brucellae but it needs additional steps (Mitka *et al.* 2007) and hence the use of whole blood as such was tried in this study with better results. Use of commercially available kits have been said to have improved the quality as well as the quantity of extracted DNA (Queipo-Ortuno *et al.,* 2008; Keid *et al.,* 2010) as compared to conventional DNA extraction method. Alamian *et al*. (2017) described a novel PCR assay for detecting *Brucella abortus*. Daugaliyeva *et al.* (2016) developed a differential PCR assay for detection of *Brucella abortus*. Orizil *et al.* (2016) conducted a multiplex PCR technique for detection of *Brucella* spp. Hemande and Gandge (2016) showed that PCR-SSCP is more sensitive than PCR-RFLP for detection of polymorphism in bcsp31 gene. Arasoglu *et al.* (2013) detected *Brucella* genus specific bcsp31 PCR from tested milk samples. In a study by Rekha *et al.* (2013), none of the samples of blood, milk and serum were positive for brucellosis. Various factors like time of sample collection, infection status of the animal, condition of farm, number of samples collected can influence the results. The better molecular diagnostic approach for screening of field animals and for increased sensitivity and higher specificity or more than one marker-based PCR could be used.

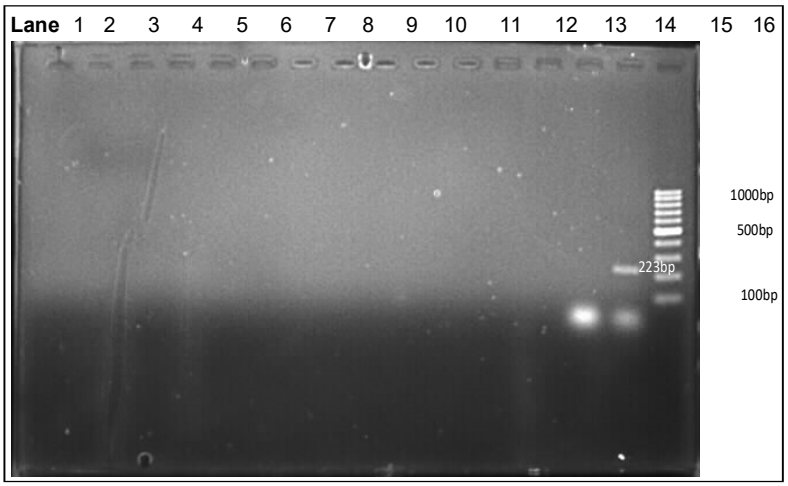

Fig 1: Agarose gel showing PCR amplified product for *Brucella* genus specific gene.
Lane: 16: 100bp DNA Ladder; **Lane:** 15: bcsp31 gene.

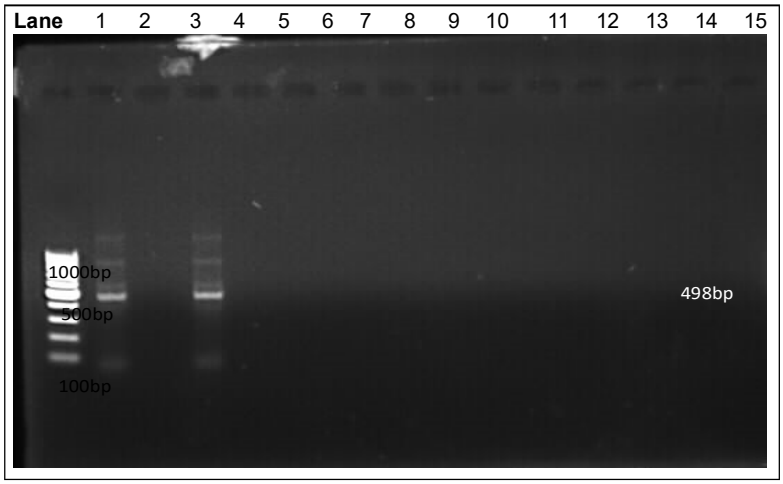

Fig 2: Agarose gel showing PCR amplified product for *Brucella* species specific genes.
Lane 1: 100 bp DNA Ladder; **Lane 6 and 9:** IS711 gene.

CONCLUSION

Among the suitability of different samples *viz*. whole blood, serum and milk for PCR assay, whole blood was found to be better than the other two. As the percent positivity by whole blood was found to be 2.8% in comparison to that of milk (2%) and none by serum. So combination of RBPT, STAT and I-ELISA and confirmation by PCR assay using whole blood was found to be the most suitable combination for the confirmatory diagnosis of bovine brucellosis in absence of isolation of the organism. 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: None.

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