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Serosurveillance of Bovine Brucellosis Using RBPT and c-ELISA and Comparative Evaluation of Test Performance

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A B S T R A C T

The present study was conducted to evaluate the presence of *Brucella* infection amongst the randomly selected cattle and buffalo's population in and around Chennai. The Rose Bengal Plate Test (RBPT) and Bru Alert® *Brucella* Antibody c-ELISA was employed to screen 355 sera samples (295 from cattle and 60 from buffalo) for the presence of *Brucella* antibody. The overall positivity of 8.73 % (31/355), 10.14 % (36/355) in bovine population, within this 9.15 % (27/295), 11.19 % (33/295) among white cattle and 6.66% (4/60), 5 % (3/60) among buffaloes by RBPT and c-ELSA respectively. Comparatively high seropositivity was found in >5 years age groups, females and exotic cross breed animals. The agreement between the two test was excellent (Kappa=0.8847) and also, chi-square test indicated an evidence of strong (P<0.01) association between the tests. Based on this study, c-ELISA has been found to be a gold standard test for detecting *Brucella* antibodies having more sensitivity and specificity. So on, it is recommended that RBPT could be successfully used for initial screening of brucellosis in bovines and c-ELISA should be used as confirmatory test to eliminate false positive results amongst positive sera.

Introduction

Brucellosis in humans and animals is known to be a worldwide problem and still remains a major public health hazard and of great economic importance (Charisis, 1998). The

World Health Organization considers brucellosis a neglected zoonosis and classifies *Brucellae* as risk group III agents because they can be easily transmitted via aerosols (WHO, 2006). Bovine brucellosis is a highly contagious, zoonotic and economically noted

disease in India due to high prevalence and abortifacient nature in animals. The disease is caused by *Brucella abortus* majorly in bovine, *B. melitensis* in goat and rarely in sheep, *B. ovis* in sheep and *B. suis* in pigs and which is characterised by abortion, still births and reduction in milk yield in females and orchitis in males (Aparicio *et al.,* 2013).

Humans are infected either by direct contact with infected animals or by ingesting contaminated products, mainly unpasteurized milk and dairy products (Halling and Young, 1994). Brucellosis in humans is characterized by a febrile flu-like syndrome, frequent chills, headaches and general weakness (Cork and Checkley, 2010). Early diagnosis of bovine brucellosis will help us to control the transmission of disease and reduce the incidence in future.

In the diagnosis of brucellosis few difficulties are faced because of the nonspecific symptoms and signs shared with other febrile illnesses, slow growth rate of the causative agent in blood culture, and the complexity of its sero-diagnosis (Colmenero *et al.,* 1990; Memish *et al.,* 2000; Al Dahouk *et al.,* 2003). Diagnosis of brucellosis is done by battery of tests and application of the test will be based on the purpose of study.

For antigen detection, culture and PCR assay are performed and for antibody detection, Rose Bengal Plate Agglutination Test (RBT), Standard Tube Agglutination Test (STAT), Enzyme Linked Immunosorbent Assay (ELISA), Complement Fixation Test (CFT) and Fluorescent Polarization Assay (FPA) were routinely used (OIE, 2009; Al-Majali *et al.,* 2009). In brucellosis, diagnosis is quite cumbersome due to the various merits and demerits of each test. There is no single test to confirm the bovine brucellosis except the incontrovertible diagnostic approach by using cultural isolation methods (Nielsen, 1995).

Even though bacterial culture shown as a gold standard diagnostic approach, it's not so easy to retrieve the isolation from infected animals due to its less sensitivity, facultative intracellular nature of organism and the risk of laboratory acquired zoonosis (OIE, 2009). Hence, a minimum of two or more tests are needed to confirm the bovine brucellosis in antigen and or antibody detection assays (Nielsen, 2002).

On the basis of an extensive work done on serological tests, it has been reported that no individual test is perfect for diagnosis of brucellosis; however the error could be minimized using the most reliable test (Nielsen, 2002; Gall and Nielsen, 2004). It is generally considered that a positive response in the agglutination test, which detects mainly IgM, is not indicative of brucellosis if the result is not further confirmed by a positive IgG response (Bhanu Rekha *et al.,* 2013).

Hence, in the present study, RBPT and c-ELISA were employed as screening tests for detecting brucellosis in bovine.

Materials and Methods

In this study, bovine sera samples were collected, randomly from organized farms and unorganized farms around Chennai and Madras Veterinary College teaching hospital, Chennai, India. Most of the samples were collected, randomly from apparently healthy animals of different age, sex and breed (cattle). In a few of the animals, serum samples were collected based on history or clinical evidence of brucellosis, like abortion, from 25 cattle. Blood samples (3 ml) were collected from 355 animals (295 cattle and 60 buffaloes) by jugular vein puncture in sterile test tubes (5 ml) and were allowed to clot and then centrifuged at 2000 rpm for 15 minutes. Sera were separated and stored at − 20°C until further use.

Serological tests

Rose Bengal Plate Test (RBPT)

The coloured antigen required for RBPT was obtained from the Division of Biological products, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh and the test was performed as per the standard protocol of agglutination test (OIE, 2008). Briefly, a drop of serum (30 μl) was placed on clean grease free glass slide and an equal quantity of coloured antigen was added and mixed thoroughly with the help of inoculation loop. The mixture was observed for clumping / agglutination for one min. and the results were recorded as agglutination (+) and no agglutination (-).

Monoclonal based blocking ELISA

Monoclonal based blocking ELISA kit (Bru Alert®) for diagnosis of brucellosis in bovine was obtained from TRPVB, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India and used for testing the sera samples. All reagents were allowed to attain room temperature (22-25°C) before use.

All reagents were homogenized by inversion. The protocol given by the manufacturer was followed to perform c-ELISA. Interpretation of c-ELISA: For each sample, the Percentage Inhibition (PI) was calculated as follows using the sample and control values:

PI = 100 – {(Test sample OD/Negative control)

In order to compare different diagnostic tests and calculate percentage, Chi-squared test, kappa statistics, sensitivity and specificity were calculated as per Thrus field (2005) using MS office 2007 Excel spread sheet, coded and analyzed by SPSS version 17.

Results and Discussion

Serological tests have been used singly or in combination in detecting the prevalence of *Brucella* infection. In the present study RBPT and c- ELISA were used to screen the bovine sera samples.

The RBPT was applied to 355 bovine samples which included 295 and 60 sera samples from cattle and buffaloes respectively. The overall positivity in bovine was 8.73 % (31/355).

9.15% (27/295) positives among white cattle and 6.66% (4/60) among buffaloes (Table 1).

The LPS antigen based competitive ELISA considered very sensitive test for bovine brucellosis screening was employed in this study.

On a total of 355 total bovine sera samples screened, 10.14 % were positive (36/355). 11.19 % (33/295) positives among white cattle and 5 % (3/60) among buffaloes (Table 1).

Demographic determinants for Bovine brucellosis

Age

Majority of the antibody detection positives were found in >5 years age group (RBPT-6.5% and c-ELISA-6.8%) in cattle and 5% in bufalloes in both test. The positivity percentage was decreased from higher age group to younger age group (Table 2).

Sex

In sex wise distribution high positivity was seen in females. It was observed that 8.14% (24/295), 9.83% (29/295) among white cattle by RBPT and c-ELISA respectively, while 5% (3/60) in each of the two tests was recorded among buffaloes (Table 3).

Breed

The breed wise prevalence was only considered in cattle. The seroposivity by the RBPT and ELISA were assessed on breed wise in which Jersey cross breed showed higher level of positivity (RBPT—4.1% and c-ELISA – 4.75%) by all the tests followed by HF cross and non-descript animals (Table 1).

Aborted cases

Aborted cases were recorded only in white cattle and 4 out of 25 cases, turned to be *Brucella* sero positives 16% (4/25) of aborted cases.

Comparison of RBPT and c-ELISA – Bovines

On comparison of RBPT with c-ELISA the former had a sensitivity of 83.33 per cent and specificity of 99.69 per cent. Out of the 31 samples detected as positive by RBPT, 1 sample was negative by c-ELISA and of the 36 samples positive by c-ELISA, 6 samples were negative by RBPT.

The concordance between these two tests was 98.03% per cent with a kappa value 0.8847 indicating RBPT to have almost perfect agreement with the gold standard c- ELISA. Statistical analysis using chi-square test indicated an evidence of strong (P<0.01) association between the tests (Table 4).

Brucellosis has recently been identified as one of the greatest problem in cattle and buffaloes in India and this infection is consistently found on the rise.

There are various reasons behind this problem like the unavailability of testing facilities in the field, lack of awareness and ignorance of animal owners and socio-economic and religious beliefs (Walunj *et al.,* 2019).

In India, about 80% of people live within close contact to domestic livestock animals or companion animals, a critical risk factor for zoonotic disease transmission such as brucellosis; yet, the true incidence of human brucellosis is unknown. Seroprevalence studies suggest infection may range between 0.9% – 18.1%, with higher risk in veterinarians and farm attenders.

Indeed, five of the ten countries with the highest incidence for human brucellosis are in this area, including Syria that has the highest annual incidence of brucellosis worldwide (Agasthya *et al.,* 2007).

The success of eradication program depends on diagnose of the disease precisely. Further it is necessary to have easy, robust, sensitive and specific test so as to take the appropriate control measures to prevent the further spread of infection (Walunj *et al.,* 2019). Hence, in the present study, RBPT and c-ELISA were employed as screening tests for detecting *Brucella* antibodies in bovines.

In the present study, the number of positives reactors in cattle by RBT was 9.15% (27/295) and 11.19 % (33/295) by c-ELISA. This is in agreement with the findings of (Rahman *et al.,* 2011; Kumar *et al.,* 2017) which ELISA recorded high positives over RBPT, but their prevalence was lesser compared to this study. The findings are not in agreement with the few other works (Mai *et al.,* 2012; Al-Ameen *et al.,* 2016; Gupta *et al.,* 2017), in which RBPT recorded high seropositives over c-ELISA and also the prevalence in their findings was very high compared to the present study. Manishimwe *et al.,* (2015) also recorded high seropositives in RBPT over c-ELISA, but their prevalence was less compared to our findings.

In buffalloes, the seropositivity recorded was 6.66% (4/60) and 5 % (3/60) by RBPT and c-ELISA respectively.

Table.1 Results of bovine brucellosis

Animal species	RBPT			ELISA		
	No. of sera samples	Positive	% positivity	No. of sera samples	positive	% positivity
Cattle	295	27	9.15	295	33	11.19
Jersey cross	111	12	4.07	111	14	4.75
Holstein cross	89	9	3.05	89	12	4.07
Non descripts	95	6	2.03	95	7	2.37
Buffalo	60	4	6.66	60	3	5.0

Table.2 Age wise distribution

Animals	RBPT			c-ELISA		
	1-2 years	3-4 years	≥5 years	1-2 years	3-4 years	≥5 years
Cattle	1(0.34%)	5 (1.70%)	21(7.11%)	3(1.01%)	8(2.71%)	22(7.50%)
Buffaloes	1(1.67%)	1(1.67%)	2(3.33%)	-	1(1.67%)	2(3.33%)

Table.3 Sex wise distribution

Animals	RBPT		c-ELISA	
	Male	Females	Male	Females
Cattle	3(1.02%)	24(8.14%)	4(1.36%)	29(9.83%)
Buffaloes	1(1.67%)	3(5.00%)	-	3(5.00%)

Table.4 Comparison of RBPT with c-ELISA in bovines

Test		ELISA		Total	Sensitivity	Specificity	Concordance	Kappa value	Chi – square test
		Positive	Negative		83.33	96.69	98.03	0.8847	**279.76****
RBPT	Positive	30	1	31					
	Negative	6	318	324					**(P<0.01)
Total		36	319	355					

The result was in agreement with the findings of Hussain *et al.,* (1994) and Al-Iraqi *et al.,* (2009) where they have fond higher prevalence by RBPT than c-ELISA but recorded high prevalence compares to our findings. The result disagree with the findings of Brahmabhatt *et al.,* (2009) and Rahman *et al.,* (2011) in which they found high prevalence by ELISA than RBPT but recorded high and low prevalence respectively.

The variations in the positive percentage by RBPT and ELISA with different workers could be due to false positive and negative reactions, sampling size, demography and different clinical conditions of animals.

Majority of the antibody detection positives were found in >5 years age group (RBPT-6.5% and c-ELISA-6.8%) in cattle and 5 % in bufalloes in both test. The positivity percentage was decreased from higher age group to younger age group.

This finding agrees with finding of other workers (Berhe *et al.,* 2007; Kebede *et al.,* 2008; Abubakar *et al.,* 2010; Kushwaha *et al.,* 2016). It has been reported that susceptibility of animal is influenced by its age (Walker, 1999).

Younger animals tend to be more resistant to infection, although latent infections have also been reported (Radostits *et al.,* 2007). Sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in concentration with age and sexual maturity.

On sex wise distribution high positivity was seen in females. It is observed that 8.14% (24/295), 9.83% (29/295) among cattle by RBPT and c-ELISA respectively, while 5% (3/60) in each of the two tests was recorded among buffaloes.

Erythritol content of the placenta facilitates the multiplication of *Brucella* in gravid uterus, hence makes female more susceptible to the brucella infection. Other studies of this aspect also indicated higher infection level in female than male animals (Patel, 2007; Upadhyay *et al.,* 2007; Junaidu *et al.,* 2011). However, in otherwise no difference was found in infection level in male and female animals (Turkson and Boadu, 1992; Muma *et al.,* 2006). Infected male animals were usually observed to be non- reactors (Crawford *et al.,* 1990), more resistant than females (Kebede *et al.,* 2008; Tolosa *et al.,* 2008) and may be diagnosed false negative (Pati *et al.,* 2000). In addition, male animals are kept for relatively shorter period in breeding herd, thus chance of getting exposed is low (Kebede *et al.,* 2008). The possibility of venereal transmission being rare and hence limits the spread of infection, even when prevalence in females is high (McDermott *et al.,* 2002).

The breed wise prevalence was only considered in cattle. The seropositives by the RBPT and ELISA were assessed on breed wise in which Jersey cross breed showed higher level of positivity (RBPT—4.1% and c-ELISA – 4.75%) by all the tests followed by Holstein Friesian cross and non-descript animals. The result was in agreement with the findings of Kushwaha *et al.,* (2016).

Exotic germplasm of the crossbred animals make them more susceptible under stress conditions (Aulakh *et al.,* 2008).

In aborted cases 16% (4/25) *Brucella* sero positives have been found in white cattle alone. These findings were in agreement with the findings of Ibrahim and Habiballa (1975) in which the report of prevalence was 14.2% in cows that had previously aborted. Other researchers have also reported similar findings (Sandoval *et al.,* 1979; Shaw, 1986; Barman *et al.,* 1989; Sandhu *et al.,* 2001).

The agreement between the two test was excellent (Kappa=0.8847) and also, chi-square test indicated an evidence of strong (P<0.01) association between the tests. This study has given results that consistent with findings from a study conducted in Sudan, where the agreement between RBPT and C-ELISA was excellent with a Kappa of 0.86 (Adil and Hind, 2012). Also in Kigali, Manishimwe *et al.,* (2015) reports an excellent agreement with the kappa value of 0.92. In India, close results have been reported where the agreement between the two tests was very good with a Kappa value of 0.72 (Islam *et al.,* 2013). However in a study done in Iran, contradict between the two tests, with a kappa value of 0.353 had been reported (Iraqi *et al.,* 2009).

For a better assessment of the situation of brucellosis in cattle, it is recommended to use c-ELISA over RBPT (Erdenebaatar *et al.,* 2004) to eliminate false positive results amongst positive sera (Chand and Sharma, 2004).

The overall prevalence of bovine brucellosis detected by c-ELISA is 10.14 % (36/355) in bovine, 11.9 % (33/295) among white cattle and 5% (3/60) in buffaloes, showing that there is noticeable presence of Brucella antibodies in bovine population in the study area, portraying the presence of Brucella infection in population and justifying the need for continued sero-surveillance of the disease.

Based on our study, we recommend RBPT could be successfully used in initial screening of brucellosis in bovine population and c-ELISA as a confirmatory test to eliminate false positive results amongst positive sera.

Acknowledgement

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