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Comparison of serological tests for detection of *Brucella* antibodies in cattle of an organized dairy farm

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ABSTRACT

Brucellosis has long been recognized with high seroprevalence in Indian dairy herds. Serological surveillance followed regularly to monitor the status of the disease. Four serological methods viz., ELISA, RBPT, STAT and 2-ME tests were used to determine the seroprevalence of brucellosis in an organized dairy farm. Overall, 33.85%, 32.61% and 30.90% animals were diagnosed serologically positive, respectively by ELISA, RBPT and STAT. However, only 13.66% animals were diagnosed positive by 2-ME. Maximum numbers of seropositive cases were found in female animals, indigenous breed and in 6 to <9 yrs age group, respectively by all serological tests. ELISA diagnosed highest number of seropositive cases in different categories viz., females (36.41%), indigenous breed (21.13%) and 6 to <9 yrs (52.15%), respectively. Sex, breed and age of animals were found to influence the antibody titer (χ^2 , $p < 0.05$) of animals. Therefore, ELISA could be recommended as a screening test for cattle. However, at field level RBPT (κ , 0.8597) and STAT (κ , 0.8608) may be used for initial screening of the herd. In addition, 2-ME test must be used in parallel to other serological test to rule out the infection.

Key words: Brucellosis, Cattle, ELISA, RBPT, Seroprevalence, STAT and 2-ME test.

INTRODUCTION

Brucellosis is a contagious disease, caused by a number of host adapted species of Gram-negative intracellular bacteria of the genus *Brucella* (Mantur *et al.*, 2007). It has wide socio-economic impact, especially in countries in which rural income relies largely on animal husbandry (Wadood *et al.*, 2009; Maadi *et al.*, 2011). In India, it causes approximately Rs. 350 million economic losses (PD-ADMAS, 2012). Human population is at a greater risk of acquiring brucellosis owing to its occupation. Several studies have confirmed widespread prevalence (17% to 22.18%) in different states of India (Chahota *et al.*, 2003; Trangadia *et al.*, 2009). Long-term serological studies at national level indicated, 5% cattle infected with brucellosis (Renukaradhya *et al.*, 2002). However, prevalence is variable in cattle but is generally higher among dairy cattle (Radostits *et al.*, 2007).

Reproductive system is the site of predilection for its multiplication. In addition, in lactating animals, it localizes in mammary tissues with frequent excretion in milk. Abortion in last trimester, birth of unthrifty newborn, orchitis, epididymitis, frequent sterility, etc. are the common clinical signs in domestic animals (Radostits *et al.*, 2007; OIE, 2012). Clinical diagnosis on the basis of abortion is, however, equivocal since many pathogens can induce abortion. Therefore, laboratory testing is essential (Godfroid *et al.*, 2010). Serological tests such as Rose Bengal Plate Test (RBPT), Standard Tube

Agglutination Test (STAT) and Enzyme Linked Immunosorbent Assay (ELISA) are commonly used for screening of animal population. These are inexpensive, fast and sensitive but not highly specific. Isolation of the causative agent is most accepted tool for confirmatory diagnosis, has the advantage of detecting the viable organisms, but it is time consuming, reduced sensitivity in chronic stage of infection. Furthermore, handling of suspected clinical samples requires containment level for group 3 pathogens (OIE, 2012).

Looking to the present status of high prevalence in dairy herd, the present study was aimed to determine the seroprevalence of brucellosis in cattle of an organized dairy farm using ELISA, RBPT, STAT and 2-mercaptoethanol (2-ME) test.

MATERIALS AND METHODS

Serological tests: A total of 644 serum samples (Table 1) of cattle were collected from an organized Dairy Farm (Pantnagar, India). Reference antigens and serum for RBPT, STAT and 2-ME tests were procured from the Indian Veterinary Research Institute, Izatnagar (India). AniGen *B. Brucella* Ab ELISA kit (Cat. No. EB43-01) was procured from Bionote (Korea).

Rose Bengal Plate Agglutination Test: The test was performed according to the method prescribed by OIE (2012). Equal volume of both antigen and serum sample were

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TABLE 1: Overall, sex, breed and age wise seroprevalence of brucellosis in cattle by different serological tests. Figures in parentheses indicate percentage; Figures having same superscripts are significant at 5% level of significance for respective test in respective group of animals by respective serological tests.

Animal groups	No. of serum samples tested	Positive samples			
		RBPT	STAT	ELISA	2-ME Test
Sex					
Female	596	208 (34.89) ^a	198 (33.22) ^a	217 (36.41) ^a	87 (14.60) ^a
Male	48	2 (4.17) ^a	1 (2.08) ^a	1 (2.08) ^a	1 (2.08) ^a
Breed					
Crossbred	327	148 (45.26) ^a	139 (42.51) ^a	151 (46.18) ^a	58 (17.74) ^a
Sahiwal	317	62 (19.56) ^a	60 (18.93) ^a	67 (21.13) ^a	30 (9.46) ^a
Age					
0- <3 yrs	67	15 (22.39) ^a	16 (23.88) ^a	19 (28.36) ^a	10 (14.92)
3- < 6 yrs	122	27 (22.13) ^b	26 (21.31) ^b	27 (22.13) ^b	17 (13.93)
6- < 9 yrs	209	104 (49.76) ^{abcd}	101 (48.33) ^{abcd}	109 (52.15) ^{abcd}	34 (16.26)
9- <12 yrs	143	34 (23.78) ^c	33 (23.08) ^c	34 (23.78) ^c	14 (9.79)
≥12 yrs	103	30 (19.13) ^d	23 (22.33) ^d	29 (28.16) ^d	13 (12.62)
Total					
	644	210 (32.61)	199 (30.90)	218 (33.85)	88 (13.66)

mixed, definite agglutination was taken as positive reaction, where as no agglutination as negative.

Standard Tube Agglutination Test : The test was performed according to the method described by Alton *et al.* (1975). Briefly, two fold serial dilution of serum in phenol saline (0.5 %, v/v) were prepared in agglutination tubes. Then, 0.5 ml of standardized *Brucella* SAT antigen was added to each tube with proper mixing. All serum samples were tested up to minimum of five dilutions. For high titre sera, more dilutions were prepared in order to achieve end point titre. Considering the special significance of 50 % end point, a control tube was set up to simulate 50% clearing by mixing 0.5 ml antigen with 1.5 ml of phenol saline (0.5 %, v/v) in an agglutination tube. All tubes were incubated at 37°C for 20 hrs before result was observed. The highest serum dilution showing 50 % or more agglutination was considered as the titre of the serum. The titre so obtained was expressed in unit system by doubling the serum titre as International Unit (IU) per ml of serum.

2-Mercaptoethanol test: The test was performed according to the method described by Buchanan and Faber (1980). Briefly, two fold serial dilution of serum in normal saline (containing 0.1 M 2-ME) were prepared in agglutination tubes. Equal volume (0.5 ml) of standardized antigen was added to each tube with proper mixing and incubated at 37°C for 48 hrs. All serum samples were tested up to minimum of nine dilutions. For high titre sera, more dilutions were prepared in order to achieve end point titre. Two controls were prepared with each batch of test *viz.* an antigen control (0.5 ml normal saline containing 2-ME and 0.5 ml standard antigen) and reading standard (0.75 ml normal saline containing 2-ME and 0.25 ml standard antigen). The reading standard tube was used to simulate 50% clearing of the antigen suspension after the agglutination reaction.

Test was read as; 4+ if all organisms in the *Brucella* antigen suspension are agglutinated and hence clear supernatant; 3+ reading equal to 75% agglutination of the organisms with slightly cloudy supernatant; 2+ reading indicate 50% organisms are agglutinated and the supernatant has equal density to the reading standard tube; 1+ reading indicate 25% agglutination of the organisms and supernatant is slightly less dense than that of antigen control tube; 0 reading indicate no agglutination and supernatant density equal to the standard tube. The endpoint was the highest dilution of serum producing a 2+ reading. The serum titer is the reciprocal of the serum dilution of end point titre.

Indirect Enzyme Linked Immunosorbent Assay : Indirect ELISA was performed by using AniGen *B. Brucella* Ab ELISA kit. Manufacturer instructions were followed to perform the test. Briefly, concentrated enzyme conjugate, washing solution and sample diluents were diluted to make as working solutions with respective diluents. Each of diluted serum samples, undiluted strong positive controls, undiluted weak positive controls and undiluted negative controls, were taken in pre-coated ELISA plate respectively, predetermined wells. An incubation of 1 hr at 37°C was given, thereafter, plate was washed five times. The bound serum antibodies were traced with conjugate for 30 min at 37°C. Substrate solution was added and kept for 15 min at room temperature to make reaction visible, thereafter reaction was stopped by stopping solution. OD of ELISA plate was taken at 450 nm to calculate the percent positivity (% P) of serum. Serum which has % P value ≥25 was taken as positive whereas, samples having % P value < 25 were negative. % P of serum was calculated from OD as follow.

$$\% P = \frac{\text{OD of sample}}{\text{Average of OD of standard strong positive control}} \times 100$$

Statistical analysis: Chi square (χ^2) test was used to know the differences in different category of animals with respect to their antibody titre were either significant or non-significant at 5% level of significance. For concern test. Kappa (κ) statistics was used to know the level of agreement between different serological tests for diagnosis of brucellosis.

RESULTS AND DISCUSSION

Serological tests have been used singly or in combination in detecting the prevalence of *Brucella* infection. In the present study RBPT, STAT, 2-ME test and ELISA were used to screen the cattle sera from an organized dairy farm. Overall seroprevalence was 33.85%, 32.60% and 30.90%, respectively by ELISA, RBPT and STAT. The higher seroprevalence in the farm indicates presence of carrier animals (Sethi *et al.*, 1971), common feeding and watering points, relatively poor management. This may enhance transmission (Omer *et al.*, 2010) among the susceptible animals.

ELISA detected comparatively higher proportion of animals possessing *Brucella* antibody. It detects all antibody isotypes (Nielsen *et al.*, 1988) and hence makes it more sensitive and specific. Similar conclusion has also been given by different workers (Patel, 2007; Ghodasara *et al.*, 2010; Trangadia *et al.*, 2012). ELISA is also considered a better test in early detection of infection than classical diagnostic tests like complement fixation, agglutination and precipitation tests.

RBPT is rapid, simple and sensitive test but has low specificity (Flad, 1983). However, in present study, it (32.61%) diagnosed more positive animals than STAT (30.90%). Serum samples having low titre (40 IU/ml) tested negative in STAT but may be positive in RBPT (Morgan *et al.*, 1969). Therefore, RBPT is considered to be suitable for primary screening of individual animals even if the antibody levels are less. However, some cross-reacting antibodies have been detected by this test and hence false negative reaction may notice (OIE, 2012). Earlier workers also concluded with similar finding (Genc *et al.*, 2005; Dinka and Chala, 2009; Kungu *et al.*, 2010).

Sex-wise seroprevalence : Most of cases of brucellosis were in females in comparison to male animals by all serological test employed. ELISA detected higher numbers of seropositive animals in females (36.41%) than in males (2.08%), followed by RBPT (34.89% and 4.17%), STAT (33.22% and 2.08%) and 2-ME test (14.60% and 2.08%), respectively (Table 1). Difference in seropositive cases among sex was statistically significant ($p < 0.05$) for respective serological tests.

Erythritol content of the placenta facilitates the multiplication of *Brucella* in gravid uterus hence, makes female more susceptible to the brucellosis. Earlier studies also indicated higher infection level in female than male

animals (Patel, 2007; Upadhyay *et al.*, 2007; Junaidi *et al.*, 2011). However, this was not always the case; some worker detected no difference 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 or showed low antibody titers (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, they 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).

Breed-wise seroprevalence: Proportion of indigenous cattle found to serologically positive was low. It was 21.13%, 19.56%, 18.93% and 9.46%, respectively, by ELISA, RBPT, STAT and 2-ME test. However, in crossbred group, comparatively higher proportion of animals found positive by different serological tests. It was 46.18%, 45.26%, 42.51% and 17.74% respectively, by ELISA, RBPT, STAT and 2-ME test. These differences among the breed were found to be statistically significant ($p < 0.05$) for all serological test used. Exotic germplasm of the crossbred animals make them more susceptible under stress conditions (Aulakh *et al.*, 2008). It has also been found, level of brucellosis infection tends to be relatively high in intensive farms (FAO-WHO, 1989).

Age-wise seroprevalence: *Brucella* seroreactors among different age groups were estimated for which authentic age record was available. The highest proportion of seropositive were found in age group of 6 to <9 yrs (52.15%, 49.76%, and 48.33%), respectively by ELISA, RBPT and STAT. From highest to lowest number of seropositive animals after 6 to <9 yrs age group were found in 0 to <3 yrs (28.36%), followed by ≥ 12 yrs (28.16%), 9 to <12 yrs (23.78%) and 3 to <6 yrs (22.13%) respectively, by ELISA. Similarly, by RBPT, it was highest in 6 to <9 yrs, followed by 9 to <12 yrs (23.78%), 0 to <3 yrs (22.39%), 3 to <6 yrs (22.13%) and ≥ 12 yrs (19.13%), respectively. STAT diagnosed highest seropositive animals in age group of 0 to <3 yrs (23.88%), followed by 9 to <12 yrs (23.08%), ≥ 12 yrs (22.33%) and 3 to <6 yrs (21.31%), respectively. Statistically, age group of 6 to <9 yrs was significantly ($p < 0.05$) differ with all other age groups for respective serological test while, all other possible age groups combinations were non-significant ($p < 0.05$) for their respective serological test.

Seroprevalence was found to be higher in adult mature animals (6 to <9 years) by all serological tests used. This agrees with finding of other workers (Berhe *et al.*, 2007; Kebede *et al.*, 2008; Abubakar *et al.*, 2010). It has been reported that susceptibility of animal is influenced by its age (Walker, 1999) and sex (Gul and Khan, 2007).

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.

Differentiation between infection and vaccination: IgG is the indicator of active infection in host, whereas presence of IgM is associated with initial exposure of pathogen or animals vaccinated with killed vaccine (Kracker and Radbruch, 2004). Use of 2-ME in 2-ME test, split the disulfide bonds of IgM, makes it non-functional. Therefore, it only detects IgG, which is specific for active infections. Its use for differentiation between infection and vaccination has been practiced earlier (Alton *et al.*, 1975). In present study, it diagnosed 13.66% of cattle was infected with brucellosis. Similar to other serological tests, it also diagnosed maximum number of *Brucella* infected in age group of 6 to <9 yrs (16.26%), followed by 0 to <3 yrs (14.92%), 3 to <6 yrs (13.93%), ≥12 yrs (12.62%) and 9 to <12 yrs (9.79%), respectively. However, these difference among different age groups were not statistically significant ($p>0.05$).

Diagnostic efficiency : Chand and Sharma (2004) advocated the use of ELISA for assessing the situation of brucellosis in cattle. It has better results because chances of non-detection of an infected animal in ELISA are minimal. According to OIE (2012), indirect ELISA should

be considered more as a screening test rather than a confirmatory test for testing of vaccinated cattle/herds. Therefore, considering ELISA as standard test, Kappa (κ) values of RBPT and STAT were 0.8597 and 0.8608, respectively. In other words, 86% agreement between the ELISA and both RBPT/STAT in detection of antibody against *Brucella* in cattle. It further warrants RBPT and STAT could be used for primary screening of animals. More or less similar result was also estimated by Ghodasara *et al.* (2010) among these tests. Due to inherent reproducibility and high sensitivity and specificity, ELISA could replace not only the currently used confirmatory CFT, but also other two routine screening tests, namely the RBPT and STAT (Paweska *et al.*, 2002).

CONCLUSION

Sex, breed and age of the animals were found to influence the antibody titer (χ^2 , $p<0.05$) in cattle. ELISA is recommended as screening test in cattle for brucellosis. However, at field level RBPT (κ , 0.8597) and STAT (κ , 0.8608) may be used for initial screening. 2-ME test must be used in parallel to other serological test to rule out the infection at field level.

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