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RESEARCH ARTICLE
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A study on bovine brucellosis in an organized dairy farm

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Abstract

Aim: To assess the status of bovine brucellosis in an organized dairy with a past history of *Brucella* abortions and where *Brucella* control measures including test and removal, calf-hood vaccination (already present adult animals were not vaccinated), use of semen obtained from a screened bull and general hygienic measures helps in the control of brucellosis in the farm have been implemented for the past four years.

Materials and Methods: A total of 195 samples including 89 blood samples, 89 serum samples and 17 milk samples were collected and analysed by isolation and identification, Polymerase chain Reaction (PCR), Milk Ring Test (MRT), Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Enzyme Linked Immnuno-Sorbent assay (ELISA).

Results: On analysis, all the 89 blood and 17 milk samples turned out to be negative for culture and PCR. MRT and ELISA tests on all the 17 milk samples and STAT on all the 89 serum samples were also negative. The percent positives for *Brucella* antibodies in serum samples were 4.5 and 6.7 by RBPT and ELISA, respectively. Of the 17 vaccinated animals, 14 were negative by all the *Brucella* antigen and antibody diagnostic tests employed. Amongst the three vaccinated animals, one animal was positive by RBPT and I-ELISA and, two animals were positive by I-ELISA alone. On the other hand, of the 72 non-vaccinated animals, 65 were negative by all the diagnostic tests employed, three animals were positive by RBPT and 4 animals were positive by I-ELISA.

Conclusion: The results of our study indicated that a combination of RBPT and I-ELISA can be successfully used for screening for brucellosis when the prevalence is low. Implementation of control measures including test and removal of the affected, calf-hood vaccination, use of semen obtained from a screened bull and general hygienic measures help in the control of brucellosis in the farm.

Keywords: bovine brucellosis, control programmes, diagnostic tests, ELISA, organized dairy farm, RBPT

Introduction

Brucellosis is a disease with worldwide distribution that affects both animals and humans. In particular, the disease in cattle is caused by *Brucella abortus* and is mainly characterized by abortions, infertility and reduced milk yield. Although incidences of brucellosis in livestock and human populations significantly declined following effective vaccination-based control and preventive programmes, it still remains a persistent problem in the endemic areas [1, 2]. A definitive diagnosis of brucellosis is challenging, in most cases, because culture of the organism (the "gold standard" test) shows low sensitivity and furthermore, the serological tests employed for screening are often inconclusive. Thus, a complete eradication of brucellosis from a farm becomes a difficult task. Techniques such as serological testing of animals and the subsequent culling of those that are seropositive for antibodies to *Brucella* spp. are commonly employed in many instances. Thus, the specificity of the serological tests employed is of paramount importance and tests with the highest possible specificity are required [3, 4]. The use of at least 2 tests applied in succession is usually recommended for accurate diagnosis and maximal specificity.

Therefore, we conducted this study to identify the status of bovine brucellosis using various antigen and antibody-based diagnostic tests in an organized dairy farm with a past history of *Brucella* abortions but has subsequently implemented *Brucella* control measures.

Materials and Methods

The farm chosen for this study was an organized dairy cattle farm (University Research Farm, Chennai, Tamil Nadu, India) with past history of *Brucella* abortions. *Brucella* vaccination was introduced four years ago as per the recommended vaccination schedule [5] and intensive farming system was followed on the farm with zero grazing, hand milking, immediate culling of *Brucella* infected animals and other healthy animal husbandry practices. The farm consisted of 89 cross bred cattle of which 17 animals were vaccinated against brucellosis using *Brucella abortus* (strain 19) live vaccine. Implementation of control measures including test and removal, calf-hood vaccination (hence already present adult animals were not vaccinated) use of semen obtained from a screened bull and general hygienic measures helps in the control of brucellosis in

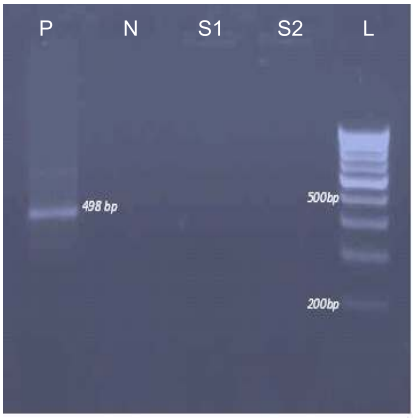

Figure-1. Agarose gel showing PCR amplified product (498 bp) for IS 711 gene in Brucella abortus. P: Positive control, N: Negative control, S_1 and S_2: Is711 gene-Negative samples, L: DNA Ladder.

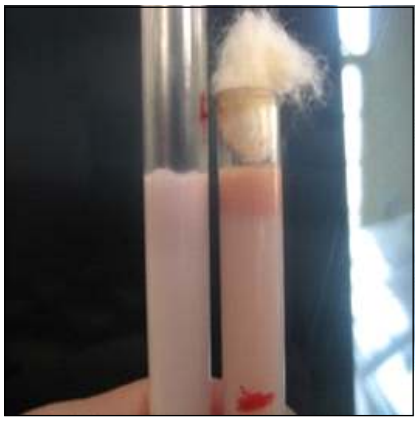

Figure-2. Milk ring test

Figure-3. Rapid plate agglutination test

the farm. A total of 89 blood, 89 serum and 17 milk samples were collected from cattle using aseptic methods.

Isolation and identification of *Brucella* was attempted from 89 blood samples using the lysis centrifugation blood culture technique [6, 7]. Isolation of *Brucella* organisms from 17 milk samples was done according to the procedure detailed in OIE [5]. The samples were inoculated onto a *Brucella* selective agar that contained selective supplements and were incubated at 37℃ for 4-7 days.

Genomic DNA was extracted from 89 blood and 17 milk samples using QIAGEN DNA kit as per the manufacturer's protocol. The isolated DNA was used for amplification of IS711 gene [8] using IS711 forward and reverse primers (IS711FP-5' GACCAAC GGAATTTTTCCAATCCC3' and IS711RP-5'TGCCGATCACTTAAGGGCCTTC At3'). PCR was performed in a reaction volume of 25 µl in a thermal cycler [8] with slight modifications [7]. The PCR conditions included 95℃ for 5 minutes for initial denaturation, with 35 cycles of 95℃ for 30 seconds for denaturation, 48℃ for 45 seconds for annealing and 72℃ for 45 seconds for extension. This was followed by 72℃ for 7 minutes for final extension. The PCR-amplified products were analysed by agarose gel electrophoresis and viewed under a UV-transilluminator. The amplification product of 498 base pairs, specific to *Brucella abortus* was visualized.

Brucella abortus bang ring antigen was obtained from Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu. All the 17 milk samples were subjected to the Milk Ring test (MRT) as described previously [5] and were examined for the formation of pink ring above the white-coloured milk column.

Rose Bengal antigen was obtained from the Indian Veterinary Research Institute (IVRI), Izatnagar. All the 89 serum samples were subjected to Rose Bengal Plate test (RBPT) as described earlier [5] and were examined for the agglutination reaction. Equal volumes of antigen (25 µl) and test serum samples were mixed thoroughly on the glass plate. The agglutination was observed after 4 minutes.

All the 89 Serum samples were serially diluted as 1:10, 1:20 and up to 1:320 in the agglutination tubes for Standard Tube Agglutination test (STAT) and *Brucella* plain antigen that was obtained from IVPM was added as described before [5]. Agglutination readings were noted after incubation at 37℃ for 18-24 hours, and 80 IU or above were considered as positives.

All the 89 sera and 17 milk samples were subjected to antibody detection by *Brucella*-Ab I-ELISA test kit (SVANOVIR, Sweden) as per the manufacturer's protocol. The optical density was measured at 450 nm and percent positivity values were calculated.

Results

In the present study, tests pertaining to the isolation of *Brucella abortus* and nucleic acid detection of *Brucella abortus* by targeting the IS711 gene using PCR from all the blood and milk samples were negative (Figure-1). Results from MRT of 17 milk samples were also negative (Figure-2). However, the RBPT detected 5.6% positives from total of 89 serum samples tested (Figure-3). None of the serum samples were positive for *Brucella* antibody detection by STAT. Antibody detection by *Brucella* I-ELISA kit revealed 6.7% positives amongst the 89 serum samples tested (Figs. 4a and 4b), whereas none of the 17 milk samples were positive (Table-1).

Of the 17 vaccinated animals, 14 were negative by all the *Brucella* antigen and antibody diagnostic tests employed. Amongst the three vaccinated animals, one animal turned out to be positive by RBPT and I-ELISA, two animals were positive by I-ELISA. Of the 72 non-vaccinated animals, 65 were negative by all the diagnostic tests employed and three animals were positive by RBPT, while the remaining 4 animals were positive by I-ELISA (Table-2).

Discussion

Testing for brucellosis among livestock is

Table-1. Results of different tests conducted for prevalence of brucellosis

Tests	Culture		PCR		MRT	RBPT	STAT	ELISA	
	Blood	Milk	Blood	Milk	(Milk)	(Serum)	(Serum)	Serum	Milk
Total	89	17	89	17	17	89	89	89	17
Positive	0	0	0	0	0	4	0	6	0
% positive	0	0	0	0	0	4.5	0	6.7	0

Table-2. Results in vaccinated and unvaccinated animals

Animals	No. of animals	Positive		Negative by all tests
		RBPT+i-ELISA	i-ELISA	
Vaccinated	17	1	2	14
Unvaccinated	72	4	3	65

primarily conducted as a component of the disease eradication and surveillance programmes rather than as a diagnostic support [9]. In the present study we were unable to isolate the *Brucella* organism from blood and milk samples. The recovery methods used for *Brucella* from blood and milk samples are often insensitive as reported earlier [10]. This is because, majority of the *Brucella* strains are slow growing organisms during primary isolation process and some of the strains are fastidious as they require serum-enriched culture media,a fact that is highlighted by the finding that even laboratories with rich experience report only low isolation rates that range from 20-50% [11].

Yet, isolation and identification of the bacterium remains the gold standard test for brucellosis [12] However, these conventional methods are time consuming and hence there is an urgent need for development of rapid diagnostic methods with high sensitivity and specificity. One such method is identification of nucleic acid of the bacterium by PCR [11]. In this study, 89 blood and 89 serum samples were negative for antigen-based detection using *Brucella abortus* IS711 gene by PCR. Our PCR results were in accordance with the results of others who have noticed that the presence of PCR inhibitors, the intracellular localization of the pathogen [13] and a high concentration of leukocyte DNA could be some of the factors that inhibit the PCR assay [14]. Detection of *Brucella* antigen via culture and PCR was negative and this result may be attributed to either very low concentration or absence of the organism in the farm animals at the time of this study. The negative results from culture and PCR might be a result of effective control measures taken to mitigate the *Brucella* infection.

Serological results are dependent upon the variable titers of antibodies in different phases of the disease. RBPT, usually a less sensitive test [10], detected antibodies in 5.6% of the serum samples whereas STAT could not detect antibodies in any of the cases. As detection of IgM by agglutination test, is not confirmation of brucellosis, the results have to be checked by IgG response by I-ELISA within a week [15]. Hence all the three RBPT positive animals tested for IgG presence by I-ELISA and where they were proved to be negative thus confirming absence of brucella infection in those animals. 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 [15].

Although theoretically it would be best to measure IgM antibody titres because of their early onset of appearance, it is important to note that a number of other microorganisms also contain antigens with epitopes similar to *Brucella* outer polysaccharides and therefore, measurement of IgM antibody sometimes results in false positive reactions leading to low specificity of the assay [16]. Because the ideal antibody measurement for serological tests for brucellosis is IgG1 [17], in this study we utilized the IgG1-based ELISA kit. I-ELISA could detect 6.7% cases as positives in serum samples, whereas RBPT could detect only 4.5% cases as positives. In our study, except RBPT and I-ELISA, no other tests were able to detect *Brucella* antibodies in the samples. Our results were in accordance to that of earlier reports by various authors. In one study 5.5% positives by STAT, 50% by RBPT and 100% by dot ELISA among the *Brucella* suspected bovines [18] and in another study 8.5% positives by I-ELISA and only 3% by MRT [19].

A previous study evaluating the status of Brucellosis in organized dairy farms with a history of abortions, using ELISA and RBPT, revealed that the *Brucella* seropositive animals were 22.18% and 13.78%, respectively with these diagnostic tests [20]. Our present work also supports the claim that ELISA is a dependable screening test, especially when employed in combination with the RBPT [21]. As demonstrated by us and others, ELISA appears to be better in terms of sensitivity than the tube agglutination test. ELISA is more sensitive and also rapid than STAT and other conventional tests used for the diagnosis of brucellosis [22,23]. In this context, it is important to note that no serological test is 100% accurate and hence diagnosis is often made based on the results obtained from two or more tests. During the course of *Brucella* eradication programme, a test of adequate sensitivity but high specificity is highly desirable when the prevalence of infection is very high. On the other hand, a test with adequate specificity but high sensitivity is recommended as the prevalence dwindles. In this study, I-ELISA was found to be the most sensitive of the various tests

conducted. However, as we now show, a combination of RBPT and I-ELISA should be utilized for screening of brucellosis.

3 of the 17 vaccinated animals revealed *Brucella* antibodies in their system. The interval between vaccination and screening in one of the animal was 8 months and for the other two was 18 months. It is likely that vaccination might have contributed for the presence of *Brucella* antibodies in these animals.

Studies on the kinetics of immune response in cattle have shown that humoral IgG responses persist after the peak of the response (3-4 weeks post-infection) and remain detectable over long periods of time (up to several years) [15]. In contrast, the IgM response is rapidly induced (within 2-3 weeks) after exposure and may also quickly disappear within a few months [15]. As detection of IgM by agglutination test, is not confirmation of brucellosis, the results have to be checked by IgG response by I-ELISA within a week [15]. Hence all the three RBPT positive animals tested for IgG presence by I-ELISA and where they were proved to be negative thus confirming absence of brucella infection in those animals. Hence all the three RBPT positive animals tested for IgG presence by I-ELISA and where they were proved to be negative thus confirming absence of brucella infection in those animals. Detection of IgG antibodies in four of the unvaccinated animals by I-ELISA might be due to either a past exposure to the infection or due to chronic infection with brucellosis. None of the unvaccinated animals were positive for both RBPT and I-ELISA indicating the possibility of absence of acute brucellosis cases, because only concomitant presence of IgM (detected in agglutination test) and IgG (detected in I-ELISA) antibodies suggests acute brucellosis. Thus, various antigen and antibody detection tests in our study revealed absence of any active or acute cases of brucellosis in the farm.

Conclusion

Based on our study we recommend that a combination of RBPT and I-ELISA can be successfully used for screening of brucellosis when the prevalence is low. For successful brucellosis eradication programme, implementation of various control regimens including test and removal of the affected, calfhood vaccination, use of semen obtained from a screened bull and general hygienic measures will help in the control of brucellosis in an organized farm. A gradual decline in incidence of brucellosis in the farms can be achieved by quarantine, testing of animals for brucellosis before their integration into the farm, vaccination of newly arrived animals, culling of infected animals, preventing unrestrained grazing, use of semen obtained from a screened bull and practicing farm hygiene protocols [24,25]. All the animals have to be tested periodically for brucellosis for an effective prevention and control of the disease. Vaccination should never be terminated until the prevalence of the disease is undetected. Vaccination should be continuously maintained for at least 8–12 years so that the risk of introduction of *Brucella* infection into the farm can be avoided [12]. In detecting *Brucella* infections, accurate and rapid diagnosis is still a persistent challenge, which requires standardization and development of more advanced techniques.

Authors' contributions

VBR carried out the research work, sample analysis, drafting and revision of manuscript and statistical analysis along with GY. LG planned the study, helped in discussion and provided support in organizing the study along with AS. AS, GY and VBR contributed in sample collection. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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