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Investigation of Reproductive Failure in Buffaloes at a Farm of Itarsi Tehsil in Madhya Pradesh in India for Detection of Antibodies to *Brucella* Species Organisms by Rose Bengal Test and Infectious Bovine Rhinotracheitis Virus by Indirect Enzyme-linked Immunosorbent Assay

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ABSTRACT

Background: Reproductive failures due to abortion in buffaloes are a major cause of concern to livestock owners that cause economic losses. Brucellosis (bacterial disease) and infectious bovine rhinotracheitis (viral disease), both infectious diseases in buffaloes are implicated in abortions in India.

Methods: The investigated buffalo farm experienced a few cases of abortions in pregnant animals in the late trimester. Blood samples were collected aseptically from individual animals without anticoagulants. After clotting of the blood, extracted serum samples were clarified and tested in Rose Bengal test and indirect enzyme-linked immunosorbent assay for the serological diagnosis of brucellosis and infectious bovine rhinotracheitis.

Result: Out of 147 buffalo serum samples tested by using the Rose Bengal test, a total of 27 buffalo serum samples (18.36%) tested positive for the presence of agglutinating antibodies against *Brucella* species. None of the tested serum samples had detectable levels of antibodies against the infectious bovine rhinotracheitis virus in an indirect enzyme-linked immunosorbent assay.

Key words: Brucellosis, Buffalo, Infectious bovine rhinotracheitis.

INTRODUCTION

Brucellosis is a contagious disease of animals and characterized by abortion in females and to a lesser extent orchitis and infection of accessory sex glands in males and infertility in both sexes. It has zoonotic importance in terms of its transmissibility to human beings (Manish *et al*., 2013). The disease attracts the attention of the animal owner through abortion in pregnant animals in the herd mainly in the late trimester and usually those in first pregnancy. Brucellosis is confirmed further by laboratory diagnosis adopting various methods such as culture and isolation, molecular (Polymerase chain reaction (PCR), Real-time PCR, Multiplex PCR), Brucellin skin test, serological tests (Rose Bengal test, Serum tube agglutination test, Complement fixation test, Enzyme-linked immunosorbent assays, Fluorescence polarisation assay) and other miscellaneous tests. Infectious bovine rhinotracheitis (IBR) is an important viral disease of large ruminants that causes abortions, retention of placenta, moderate reduction in milk production and even death in calves. IBR-affected animals exhibit salivation, red nose and necrosis of mucous membranes in the case of respiratory form and pustules on the vulva known as infectious pustular vulvovaginitis in females (https://www.nddb.coop/farmer/animal-health/disease/viral/ibr; Accessed on 17th July 2023) and infectious pustular balanoposthitis in males in the case of the reproductive form (Pandey *et al*., 2014). Milk productivity in the state of Madhya Pradesh is lower than in some other states of the country (Gulati *et al*., 2021). Investigations on

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both of these infectious diseases, brucellosis and infectious bovine rhinotracheitis, that cause reproductive failures in buffaloes in the state of Madhya Pradesh were reported previously. The present study indicated presence of agglutinating antibodies against *Brucella* species organisms in buffaloes at a farm with a history of abortions from Madhya Pradesh state using the Rose Bengal test (slide agglutination test) and the absence of antibodies against IBR virus infection using indirect ELISA.

MATERIALS AND METHODS

Location

Kiratpur village is located in Itarsi tehsil of Hoshangabad district in Madhya Pradesh state of India. The village is situated 12 kilometers away from sub-district headquarters Itarsi (tehsildar office) and 30 kilometers away from district headquarters Hoshangabad (https://villageinfo.in/madhya-pradesh/hoshangabad/itarsi/kiratpur.html; Accessed on 01 June 2023).

Livestock farm

Adult Murrah, Bhadavari and other non-descript buffalo populations were reared at a farm at Kiratpur village in two closely located but separate sheds. These buffaloes were experiencing an outbreak of brucellosis. In some pregnant animals, there was an abortion during the first term. The majority of the buffalo population at the farm included females.

Serum samples

Blood samples from individual buffalo were collected aseptically in sterile containers without anticoagulant for serum and allowed to clot. Separated serum samples were clarified by centrifugation at 3,000 revolutions per minute for 5 minutes to remove the traces of red blood cells if any. Initially, these serum samples were received and stored in the deep freezer at -20°C at the Department of Veterinary Microbiology College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Dr. Ambedkar Nagar-Mhow, Indore, Madhya Pradesh, India. Subsequently, another set of these serum samples was transported by following cold chain conditions in a thermocol box to Virus Laboratory at the Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Rose Bengal test antigen

The Rose Bengal test (RBT) antigen was procured from Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh.

Procedure for Rose Bengal test

This test is a simple spot agglutination test using antigen stained with Rose Bengal and buffered to a low pH. The test was performed as per the directions contained in the chapter on brucellosis published by World Organization for Animal Health, Paris (https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.01.04_ BRUCELLOSIS.pdf). Serum samples and RBT antigen were brought to room temperature. A clean grease-free glass slide was used to perform the slide agglutination test. A total volume of 25 µl of each serum sample was placed on a glass slide by using a micropipette. After uniform mixing of antigen in the bottle equal volume of antigen was placed near each serum spot and immediately with the micropipette tip serum and antigen mixed thoroughly to produce a circular or oval zone approximately 2 cm in diameter. The mixture was agitated gently for 4 minutes at room temperature and read for agglutination. Any visible colored agglutination was considered as positive reaction. This test was performed at Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Dr. Ambedkar Nagar-Mhow, Indore, Madhya Pradesh, India.

Indirect infectious bovine rhinotracheitis enzyme-linked immunosorbent assay kit (IBR ELISA)

Indirect IBR ELISA kit was obtained from Svanova, Biotech, Sweden. The kit contains the Bovine herpes virus-1 (BHV-1) coated plate, Control positive serum sample, Control negative serum sample, Conjugate (Horse radish peroxidase (HRPO) conjugated anti-bovine immunoglobulin (Ig) G monoclonal antibodies), Phosphate buffer saline (PBS) -Tween solution (20X concentrate), Substrate solution (Tetramethyl benzidine (TMB) in substrate buffer containing H_2O_2) and Stop solution (2M H_2SO_4). The 1st column was coated with IBR virus antigen whereas the 2nd column was coated with Madin Darby Bovine Kidney (MDBK) cell supernatant. Similarly, all the odd column wells were coated with virus and all even column wells were with MDBK cell supernatant. This test was conducted in the Virus Laboratory at the Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Procedure for testing of serum samples

The IBR ELISA was performed as per the method provided by the manufacturer. A volume of 90 µl of 1X Phosphate buffer saline (PBS) -Tween buffer was added to all the wells of the plate coated with IBR antigen. A volume of 10 µl each of different test serum samples was added in duplicate such as A1 and A2 up to F11 and F12. A volume of 10 µl of the negative serum sample was added to G11 and G12 wells and a volume of 10 µl of the positive serum sample was added to H11 and H12 wells, respectively. The plate was incubated overnight at 4°C followed by washing the plate 5 times in 1X washing buffer. 100 µl of ready-to-use anti-bovine IgG Horse radish peroxidase (HRPO) conjugate was added to all the wells and incubated at 37°C for 1 hour. After washing the plate 5 times, the substrate solution (TMB substrate) was added at the rate of a volume of 100 µl per well. The plate was kept in the dark at room temperature for 10 minutes for the development of color. The blue color

was developed in the positive cases. The color was stopped by adding 50 µl volume of stop solution and the color was changed from blue to yellow. The plate was read at 450 nm in an ELISA reader (Thermo Labsystems).

Interpretation

The result was interpreted by using the following formula:

Corrected optical density (OD) values

The OD values in wells coated with IBR antigen are corrected by subtracting the OD values of the corresponding wells containing the control antigen.

Per cent positivity values (PP)

All corrected OD values for the test samples as well as the negative control are related to the corrected OD values of the positive control as follows.

$$PP = \frac{OD^{450} \text{ of the sample (corrected)}}{OD^{450} \text{ of the positive control (corrected)}} \times 100$$

As per the PP values sample having PP <12 was considered negative and PP >12 was considered positive respectively.

RESULTS AND DISCUSSION

The buffalo population of Madhya Pradesh state as per the 20th livestock census is 10.30 million. Brucellosis in different host populations is caused by one of the several members of the bacterial agent of *Brucella (B)* species (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, *B. ceti* and *B. Pinnipedalis*) (Quinn *et al*., 2011). It is an important bacterial disease posing an economic threat to livestock owners in Asia (Bamaiyi *et al*., 2014). The disease is contagious in nature, spread fast and affects ruminants, wildlife and also human beings. The losses due to the disease can be counted as a) decreased milk production, b) weight loss, c) loss of young, d) infertility and e) lameness (https://www.aphis.usda.gov/animal_health/animal_diseases/brucellosis/downloads/bruc-facts.pdf; Accessed on 1st June 2023). Detailed reviews about the disease in animal populations had been published by several researchers emphasizing etiology, epidemiology, pathogenesis, immune response, diagnosis, vaccination and its control (Smits and Kadri, 2005; Gul and Khan, 2007; Boral *et al*., 2009; Manish *et al*., 2013; Shoukat *et al*., 2017; Khurana *et al*., 2021). Several researchers have reported the presence of *Brucella* species infection (Kataria and Verma, 1969; Isloor *et al*., 1998; Mehra *et al*., 2000; Gogoi *et al*., 2017; Gupta *et al*., 2017; Verma *et al*., 2019; Islam *et al*., 2021) and IBR infection (Nandi *et al*., 2004, Nandi *et al.,* 2007; Audarya *et al*., 2017; Patel *et al*., 2018) in the livestock population. Some of the researchers have also used molecular methods for the identification and characterization of the organisms (Ghodasara *et al*., 2010; Pandey *et al*., 2014a).

The results of the present investigation are presented in Table 1, 2 and Fig 1, 2. A total of 147 unvaccinated buffalo serum samples collected from a livestock farm were tested by an agglutination test, RBT. Some of these buffaloes at a farm experienced abortion and also a drop in milk production during the year, mid-2015. This history of the animals pointed towards possible infection due to *Brucella* species organisms and hence in that year in the late-2015 collected serum samples from these buffaloes were received for initial screening by agglutination test. On finding positive samples (27 positive samples out of 147 tested), it was advised to farm authorities to send and get tested those samples at Indian Veterinary Research Institute,

Table 1: Presence of agglutinating antibodies against *Brucella* species in serum samples from buffaloes of a livestock farm with a history of abortion.

Tag number	RBT outcome
5	Positive
8	Positive
17	Positive
49	Positive
116	Positive
134	Positive
141	Positive
145	Positive
146	Positive
164	Positive
167	Positive
174	Positive
176	Positive
181	Positive
183	Positive
185	Positive
190	Positive
229	Positive
254	Positive
259	Positive
1511	Positive
1513	Positive
1516	Positive
1518	Positive
1519	Positive
2501	Positive
2505	Positive

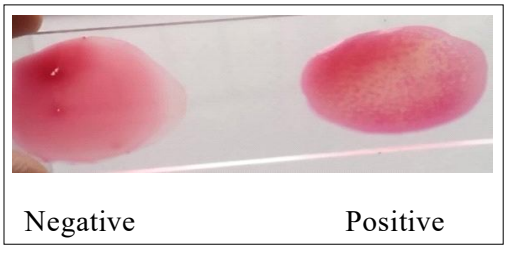

Fig 1: Rose Bengal test revealing absence and presence of agglutinating antibodies on mixing of antigen and serum sample for *Brucella* species.

Table 2: Absence of agglutinating antibodies against *Brucella* species in serum samples from buffaloes of a livestock farm with a history of abortion.

Tag number	RBT outcome
3	Negative
4	Negative
6	Negative
9	Negative
10	Negative
18	Negative
21	Negative
22	Negative
30	Negative
36	Negative
37	Negative
38	Negative
39	Negative
41	Negative
44	Negative
45	Negative
48	Negative
50	Negative
51	Negative
54	Negative
56	Negative
57	Negative
62	Negative
85	Negative
86	Negative
87	Negative
88	Negative
89	Negative
91	Negative
98	Negative
99	Negative
100	Negative
102	Negative
117	Negative
118	Negative
119	Negative
120	Negative
121	Negative
123	Negative
124	Negative
127	Negative
128	Negative
130	Negative
132	Negative
132*2	Negative
133	Negative
136	Negative
138	Negative
140	Negative
142	Negative
143	Negative
144	Negative
147	Negative
148	Negative
149	Negative
150	Negative
162	Negative
163	Negative
169	Negative
170	Negative
171	Negative
172	Negative
173	Negative
175	Negative
176	Negative
177	Negative
178	Negative
179	Negative
180	Negative
182	Negative
184	Negative
186	Negative
187	Negative
188	Negative
189	Negative
191	Negative
192	Negative
193	Negative
194	Negative
197	Negative
198	Negative
223	Negative
224	Negative
225	Negative
226	Negative
236	Negative
240	Negative
242	Negative
267	Negative
268	Negative
269	Negative
283	Negative
285	Negative
286	Negative
287	Negative
288	Negative
289	Negative
290	Negative
292	Negative
300	Negative

Table 2: Continue....

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Tag less	Negative
1315	Negative
1512	Negative
1520	Negative
1559	Negative
1562	Negative
1581	Negative
1582	Negative
2502	Negative
2503	Negative
2504	Negative
2506	Negative
2507	Negative
2508	Negative
2511	Negative
2512	Negative
2513	Negative
2515	Negative
2517	Negative
2519	Negative

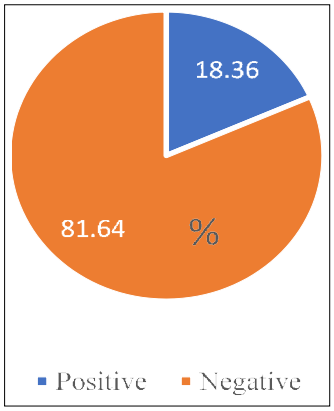

Fig 2: Per cent positivity for *Brucella* species infection of buffaloes from a livestock farm with a history of abortion in Rose Bengal test.

Izatnagar, Bareilly, Uttar Pradesh for further confirmation of individual animals by ELISA due to the unavailability of an ELISA kit at the laboratory. However, one of the sets of serum samples from these buffaloes was stored in the deep freezer and sent to Virus Laboratory at the Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India. These serum samples were tested during the year 2018-2019 for the presence or absence of antibodies to the IBR virus since this virus is also implicated in reproductive failures including abortion. There are several tests available for the diagnosis of brucellosis in cows and buffaloes by using various clinical specimens namely isolation of the *Brucella* species organisms, serological testing and advanced molecular detections (Rahman *et al.* 2011; Sharma and Bist, 2012; Jadav *et al.*, 2022). Isolation of the agent is considered the gold standard for the diagnosis of brucellosis but this can be a time-consuming and laborious process besides posing a risk of infection to laboratory personnel. That's why serological tests are generally preferred for their simplicity and low cost. In previous studies, high as well as lower percentages of anti-*Brucella* antibodies from randomly collected or suspected/infected cases of bovines were reported in India and abroad by using different diagnostic tests (Rahman *et al.*, 2011; Shome *et al.*, 2014; Priyanka *et al.*, 2018). Bovine brucellosis in Madhya Pradesh was reported as early as 1969 (Kataria and Verma, 1969) and continued to be reported in the state in large ruminants (Gupta *et al.*, 2017; Verma *et al.*, 2019). Mehra *et al.* (2000) reported an overall prevalence of 6.3% in cattle and buffaloes irrespective of sex and age and higher seroprevalence rates in buffaloes (11.4%) than in cows (9.6%) in Madhya Pradesh state. The RBT is very sensitive. However, like all other serological tests, it could sometimes give a positive result because of vaccination, however, the buffaloes in the present study were unvaccinated against both of these diseases, brucellosis and IBR. Therefore, positive reactions in RBT can be reliable. Still, it can be further investigated using suitable confirmatory or complementary strategies (including epidemiological investigation). Since few of the pregnant buffaloes in the present investigation suffered from abortion in the late trimester of their first pregnancy, the RBT results could be supported by the history. False-negative reactions occur rarely in the case of RBT. RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds or flocks (https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf).

The present study reported a higher seroconversion rate (18.36%) by RBT testing of buffaloes with a history of abortion at the livestock farm due to the *Brucella* species infection. Higher seroconversion could be due to the presence of infected aborted buffaloes and shedding of bacteria in large quantities and the transmission of the organisms to susceptible buffalo populations that were in close contact and unhygienic practices. Brucellosis is responsible for more than double the economic loss in an infected buffalo compared to an infected cow (Jadav *et al.*, 2022). Shome *et al.* (2014) reported a higher percentage of prevalence of brucellosis in buffaloes (39.32%) than in cows (4.33%) and also concluded that the disease was more prevalent in farms with a history of abortion and repeat breeding problems. Indian breeds of cattle (Hallikar, Ongole) and buffalo (Surti) were found negative except for 50% of Murrah buffaloes that tested positive in this study. Hence, it is important to assess the status of infection at a farm especially when unvaccinated buffaloes are experiencing abortion. Trangadia *et al.* (2009) investigated organized dairy farms in India and concluded that *Brucella* species organisms as well as the IBR virus were implicated in cases of abortions. However, in the present investigation,

18.36% (27 positive out of 147 tested) of buffalo serum samples tested positive in RBT and no detection of antibodies against the IBR virus at a livestock farm confirmed an outbreak of brucellosis in buffaloes of the farm. These findings and the history of buffaloes also revealed the *Brucella* species organisms as the sole member that caused abortions in buffaloes at the farm under the study. Prevention and control of brucellosis is done by following standard biological practices related to the quarantine of infected/diseased animals from healthy animals and vaccinations of the remainder. Eradication and control of the disease are achieved elsewhere by following testing and slaughter of infected animals and vaccinations to susceptible livestock populations including buffaloes (Puran *et al*., 2015) for minimizing economic losses to livestock owners. Brucellosis is also a zoonotic disease (Yohannes and Gill, 2011). In pregnancy, the disease is associated with adverse outcomes such as spontaneous abortion, preterm delivery, chorioamnionitis and fetal death (Vilchez *et al*., 2015). Hence screening manpower at farms and being involved with related activities and treatment of affected individuals if any is necessary. The study findings also highlight the importance of vaccinations to buffalo and other susceptible livestock populations of the country as envisioned in the Government of India policies to contain the losses incurred due to brucellosis. Newer strategies of control are also being investigated (Saxena, 2021) elsewhere.

CONCLUSION

The results of the study indicate the presence of *Brucella* infection in the buffalo population at the farm under investigation. Test and slaughter policy followed elsewhere in the world is not practical in India so to contain the infection standard preventive measures are recommended. The study findings also highlight the importance of regular screening of livestock populations at farms for the disease and vaccinations to buffalo and other livestock populations of the country to contain the losses incurred due to brucellosis. It highlights the importance of the implementation of the control programme under the aegis of the National Animal Disease Control Programme (NADCP) for foot-and-mouth disease and brucellosis envisioned by the Government of India.

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Conflict of interest

All authors declared that there is no conflict of interest.

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