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Status of Infection during a Natural Outbreak of Brucellosis in Unvaccinated Buffaloes at a Livestock Farm of Madhya Pradesh in India by using Competitive Enzyme-linked Immunosorbent Assay

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ABSTRACT

Background: Brucellosis is one of India's most important bacterial diseases of large ruminants. It is of economic importance. It is caused by members of the *Brucella* species and responsible for abortion in pregnant bovines usually during the first term and generally after the fifth month of gestation. Hence, livestock farm with a history of abortion in pregnant animals has to be investigated for Brucellosis.

Methods: Whole blood samples (of 50 individual buffaloes) were collected aseptically from a herd of buffaloes experiencing a natural outbreak of Brucellosis. Extracted serum samples were tested after clarification in commercially procured competitive enzyme-linked immunosorbent assay for the serological laboratory diagnosis of Brucellosis.

Result: Out of 50 buffalo serum samples tested by using commercially procured competitive enzyme-linked immunosorbent assay, a total of 24 buffalo serum samples tested positive for the presence of Antibodies against *Brucella* species. Overall seropositivity of 48% was recorded for Brucellosis in the investigated buffalo herd experiencing a natural infection in the present study even after long-term storage of serum samples at deep freezing conditions.

Key words: Brucellosis, Buffalo, Competitive enzyme-linked immunosorbent assay.

INTRODUCTION

In India, Madhya Pradesh state milk production in 2021 was 520.66 lakh kilograms of milk per day, a whopping increase of 133% of milk production compared to the milk production in 2011 (https://timesofindia.indiatimes.com/city/ahmeabad/rajasthan-mp-outpace-guj-in-milk-output-value-growth/articleshow/100487026.cms Accessed on 01 June 2023). The contribution of the milk segment to the total value of output from livestock stands at around 83%. In Madhya Pradesh state, of the total milk production, the share of buffalo milk stands at 49% compared to cow milk (45%). However, milk productivity in the state is lower than in some other states of the country (Gulati *et al*., 2021). 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). Brucella antibodies were also detected in wild animals and avian species (Ali *et al.*, 2020). It is an important bacterial disease posing an economic threat to livestock owners (Bano and Lone, 2015). 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

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diseases/brucellosis/downloads/bruc-facts.pdf; Accessed on 1st June 2023). Few workers have reported the serological presence of the infection in the livestock population of the Madhya Pradesh state in large ruminants (Kataria and Verma, 1969; Gupta *et al*., 2017; Verma *et al*., 2019a; Verma *et al*., 2019b). However, there were very few studies reporting on Brucellosis infection at a farm experiencing the natural outbreak in the unvaccinated buffalo population in the state (Audarya *et al*., 2024). Apart from routine screening of the buffalo population, confirmatory laboratory diagnosis of Brucellosis from clinical samples of the herd is required for implementing strategies to control the infection and prevent economic losses to the animal owners. The present study reports the status of infection during a natural ongoing Brucellosis outbreak in unvaccinated buffaloes at a farm from Madhya Pradesh state, by using a commercially procured serological test; a competitive enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

Location of/about livestock farm

Kiratpur village is situated 12 kilometers away from sub-district headquarters Itarsi (tehsildar office) and 30 kilometers away from district headquarters Hoshangabad district in Madhya Pradesh state of India (https://villageinfo.in/madhya-pradesh/hoshangabad/itarsi/kiratpur.html; Accessed on 01 June 2023). Adult Murrah, Bhadavari and other non-descript buffalo populations were reared at a livestock farm in two closely located but separate sheds. These buffaloes were experiencing an outbreak of Brucellosis with abortion during the first term in some pregnant animals. The majority of the buffalo population at the farm included females.

Serum samples

Blood samples from individual buffalo were collected aseptically in a sterile vacutainers (VAKU-8® from Hindustan Syringes and Medical Devices Limited, Faridabad, India) without anticoagulant for serum and allowed to clot. Separated serum samples were clarified by centrifugation at 3000 rpm for 5 minutes to remove the traces of red blood cells if any and stored in the deep freezer at -20°C (Godrej D-Cool Horizontal Deep Freezer, India) in cryovials.

Procedure for testing of serum samples by using competitive enzyme-linked immunosorbent assay kit (cELISA)

The Asur D×TM *Brucella* Multispecies Antibodies cELISA Test Kit (10043-02) from Biostone Animal Health LLC, Southlake, TX 76092 was commercially procured and employed in the present study. The ELISA kit is stored at 4°C. This test was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Kuthuliya - 486 001, Rewa, Madhya Pradesh, India in the year 2022. *Brucella* antigen-coated plate and all components of the cELISA kit were brought to room temperature before using it for laboratory diagnosis of Brucellosis. *Brucella* positive control (mixed well by vortexing for 20 seconds) followed by *Brucella* negative control (50 µl) were added to two wells each in the antigen-coated plate. Positive control was placed at the upper left-hand while negative control at the bottom left-hand corner of the wells of the plate so that both of them were placed well separated from each other. Now, 50 µl of diluted test serum sample was added to each well separately. 50 µl of Anti-*Brucella* Antibody horse radish peroxidase (HRP) conjugate, diluted test serum sample was added to each of the controls or samples well. The solution in the wells was mixed by gentle rocking of the plate manually for 1 minute. After proper mixing, the plate was covered with aluminum foil and incubated for an hour at room temperature in dark conditions avoiding air currents. On completion of incubation, the solution in the wells was discarded. 250 ml of 1× wash solution was added to each well of the plate. The plate was tapped on dry tissue papers after discarding the wash solution. A total of five washes were given by not allowing any time gap between washings. Thereafter,3, 3′ 5, 5′-Tetramethylbenzidine (TMB) substrate 100 ml was added to each well of the plate and after covering it with foil, it was incubated for 15 minutes at room temperature. Lastly, 100 ml of stop solution was added in each well and the plate was read immediately by using ELISA reader (LISA plus microplate reader) at 450 nm wavelength to measure optical densities (OD).

Interpretation

Percentage inhibition (PI) of samples is expressed as PI relative to the mean OD of the negative control by using the following formula:

$$PI = 1 - \frac{\text{OD450 test serum sample}}{\text{Mean OD450 negative control}} \times 100$$

As per the PI values: i) if PI was less than 50% *Brucella* Antibodies are absent in the test serum sample and the result is recorded as negative, ii) if PI was equal or greater than 50% *Brucella* Antibodies are present in the test serum sample and the result recorded as positive.

RESULTS AND DISCUSSION

The results of the present investigation are presented in Table 1 and Fig 1. A total of 50 unvaccinated buffalo serum samples collected from a livestock farm were tested by a commercially procured competitive enzyme-linked immunosorbent assay kit (cELISA) in the present study. 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 of the *Brucella* organisms and hence in that year in the late-2015 collected serum samples from these buffaloes were received for initial testing by plate agglutination tests at the

laboratory at Dr. Ambedkar Nagar-Mhow. However, one set of serum samples was stored in the deep freezer for future testing and research purposes at the laboratories located at Dr. Ambedkar Nagar-Mhow. There are several tests available for the diagnosis of Brucellosis in cows and buffaloes by using various clinical specimens namely isolation of the *Brucella* organisms, serological testing and advanced molecular detections (Brahmabhatt *et al.*, 2009; Madhukar *et al.*, 2014; Upadhyay *et al.*, 2019; Bohre *et al.*, 2021). 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 (Niranjan *et al.*, 2015). In previous studies, high as well as lower percentages of Anti-*Brucella* Antibodies (2.5%-38.5%) from randomly collected or suspected/infected cases of bovines were reported in India and abroad by using different diagnostic tests (Amin *et al.*, 2005; Islam *et al.*, 2018; Priyanka *et al.*, 2018; Priyantha *et al.*, 2021). Bovine Brucellosis in Madhya Pradesh was reported as early as 1969 (Kataria and Verma, 1969) and continued to be reported from the state (Gupta *et al.*, 2017; Verma *et al.*, 2019b; Audarya *et al.*, 2024). The cELISA test with high specificity is capable of detecting all immunoglobulin classes (Quinn *et al.*, 2011). The cELISA kit employed in the study detects Antibodies specific to *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* in bovine, ovine, caprine or swine from serum/plasma/milk samples.

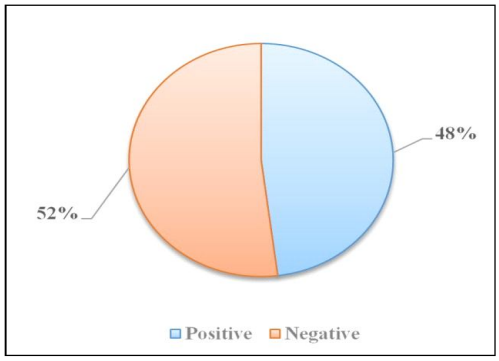

Fig 1: Infectious status of a buffalo herd at a farm during a natural outbreak of Brucellosis.

Table 1: Percentage inhibition values of buffalo serum samples tested for the laboratory diagnosis of Brucellosis by using competitive enzyme-linked immunosorbent assay and status of *Brucella* infection in individual buffalo.

Sample number	Value; Tag number (Outcome)	Sample number	Value; Tag number (Outcome)	Sample number	Value; Tag number (Outcome)	Sample number	Value; Tag number (Outcome)
1	10.8; 30 (Negative)	14	22; 130 (Negative)	27	19.3; 236 (Negative)	40	78.4, 174 (Positive)
2	17; 177 (Negative)	15	66.1; 48 (Positive)*	28	82.1; 259 (Positive)	41	24; 161 (Negative)
3	30; 1315 (Negative)	16	71.6; 183 (Positive)	29	82; 141 (Positive)	42	81.9; 254 (Positive)
4	73.8; 49 (Positive)	17	60; 1516 (Positive)	30	28.1; 267 (Negative)	43	14.8; 170 (Negative)
5	14.2; 286 (Negative)	18	74.9; 185 (Positive)	31	81.18; 2515 (Positive)	44	46; 180 (Negative)
6	50.8; 118 (Positive)*	19	33; 2516 (Negative)	32	62; 2507 (Positive)	45	66.8; 119 (Positive)*
7	23.6; 290 (Negative)	20	71; 192 (Positive)*	33	21.9; 226) (Negative	46	38; 169 (Negative)
8	78; 178 (Positive)*	21	7.5; 10 (Negative)	34	41.79; 292 (Negative)	47	78.2; 1518 (Positive)
9	21.3; 287 (Negative)	22	0; 41 (Negative)	35	82; 2502 (Positive)	48	83; 100 (Positive)*
10	74.83; 2519 (Positive)*	23	66; 188 (Positive)*	36	5.6; 1562 (Negative)	49	46.6; 224 (Negative)
11	73; 39 (Positive)*	24	15.75; 225 (Negative)	37	40; 240 (Negative)	50	78; 2501 (Positive)
12	11; 6 (Negative)	25	75; 1511 (Positive)	38	13; 173 (Negative)		
13	63; 150 (Positive)*	26	14, 189 (Negative)	39	17.1; 2503 (Negative)		

*- Negative in plate agglutination test (10); Negative in both (26); Positive in both (14)

The present study reported a higher seroconversion rate (48%) by cELISA 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. Further, samples from the same area were not tested due to vaccination in the herd and no report of an outbreak. However, Brucella infection is being detected in swine, small and large ruminant populations of the state (Gupta *et al*., 2017; Yadav, 2022; Audarya *et al.*, 2024). An overall seropositivity of 18.36% was reported when samples from the outbreak were tested by rose Bengal test (Audarya *et al.*, 2024). So, cELISA is more sensitive in detecting seropositivity to *Brucella* infection than the rose Bengal test. Brucellosis is responsible for more than double the economic loss in an infected buffalo compared to an infected cow (Jadav *et al.*, 2022). Hence, it is important to assess the status of infection at a farm especially when unvaccinated buffaloes are experiencing abortion. All the positive samples in the plate agglutination test (10) were also evaluated as positive in cELISA (Table 1). A total of 24 serum samples tested positive out of 50 buffalo serum samples in cELISA even when these serum samples were stored at deep freezing conditions for the long-term for more than 6 years indicating the robustness of both the test and Anti-*Brucella* Antibodies. On finding positive samples in initial studies, it was advised to farm authorities to send and test those samples at the Indian Veterinary Research Institute, Izatnagar-243 122, Bareilly, Uttar Pradesh for further confirmation of individual animals by ELISA due to the unavailability of an ELISA kit at the laboratory at that time. Results of which were not made available to the authors hence studies regarding deterioration of antibody titers/ positivity and negativity in corresponding samples could not be made in respect to ELISA studies. However, those samples that tested positive in the plate agglutination test were also evaluated as positive in the present investigation. Hence, stored serum samples were of diagnostic value even after storage at long cold conditions. So, in the present investigation, 48% of buffalo serum samples tested positive in a cELISA at a livestock farm indicating an outbreak of Brucellosis in the buffaloes of the farm. At least one male buffalo was positive for the infection in the study. Some animals even after abortion may act as carriers and transmit the infection to othe in-contact healthy animals. Hence, further bacterial isolation and molecular studies will be planned (Gokmen *et al.*, 2019). 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.*, 2013) to minimize economic losses to livestock owners. Brucellosis is also a zoonotic disease hence screening manpower at farms and being involved with related activities and treatment of affected individuals if any is necessary (Madhukar *et al.*, 2014; Nagappa *et al.*, 2018).

The study findings also highlight the importance of vaccinations to the country's buffalo and other susceptible livestock populations as envisioned in the Government of India policies to contain the losses incurred due to Brucellosis.

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 outbreak standard preventive measures are recommended. The study's findings also highlight the importance of regular screening of livestock population at farms for the disease and vaccinations to buffalo and other livestock populations of the country to contain the losses incurred due to Brucellosis.

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Conflict of interest

All author declare that they have no conflict of interest.

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