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Apparent seroprevalence, isolation and identification of risk factors for brucellosis among dairy cattle in Goa, India

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ABSTRACT

Brucellosis is a highly contagious zoonotic infection affecting livestock and human beings. The disease has been reported worldwide except in few countries where it has been eradicated. The prevalence of brucellosis among cattle from 11 farms having a history of abortions was studied. A total of 481 samples comprising of blood, milk, vaginal swabs, vaginal discharges, placental tissues and fetal tissues were collected from 296 animals. Clinical samples were processed for the isolation of *Brucella*. Serum samples (n = 296) were tested by Rose Bengal Plate Test (RBPT) and indirect ELISA. A total of 90 (30.40%) and 123 (41.55%) samples were positive by RBPT and indirect ELISA, respectively. Also 27.02% samples were positive by both the tests. *Brucella* isolates (n = 8) were recovered from clinical samples using *Brucella* selective media. All the isolates demonstrated PCR amplification for the *bcsp31* and *IS711* genes. Amplification of *Brucella abortus* specific primer was demonstrated by all the isolates in AMOS PCR indicating isolates to be of either *B. abortus* biotype 1, 2 or 4. Risk factors for transmission of brucellosis among cattle population were studied by field surveys. It was observed that lack of awareness about brucellosis (OR = 8.739, P = 0.138) and inadequate floor space (OR = 0.278, P = 0.128) were crucial risk factors for transmission of bovine brucellosis.

1. Introduction

Brucellosis is the most common zoonosis reported worldwide and is responsible for considerable economic losses due to abortion and culling of infected animals [1]. Bovine brucellosis is usually caused by *Brucella abortus* and rarely by *Brucella melitensis* [2]. The disease is characterized by abortions generally in last trimester of pregnancy, retention of placentae, endometritis, birth of weak or dead calves and reduced milk yield in bovines [3]. Transmission in animals occurs by contact with secretions of infected animals, licking of aborted fetuses and placenta, inhalation of aerosols and ingestion of fodder contaminated with *Brucella* [2,4]. Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection [5,6]. The most common routes of human infection are through consumption of unpasteurized milk products and close contact with infected animals [1].

Laboratory diagnosis of bovine brucellosis involves serological testing, isolation of the pathogen from clinical material and its identification. A battery of serological tests including Milk Ring Test (MRT), Fluorescence polarization assay (FPA), intradermal test, Rose Bengal plate test (RBPT), complement fixation test (CFT), Coombs test and ELISA are extensively used for diagnosis of bovine brucellosis [7,8]. Currently, there is no diagnostic test sufficiently sensitive and specific to detect all stages of infection in live animals [9,10]. Previously, Standard Tube Agglutination Test (STAT) was used for determination of titers, however, it was observed that the test might give false positive reaction due to cross-reacting antibodies. Serological tests are reliable but sometimes false positivity due to cross reacting antibodies against *Yersinia enterolitica* and some other zoonotic pathogens may reduce the sensitivity [11].

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The introduction of new animals in the herds has been identified as the main risk factor for seropositivity [12] besides, abortion in animals, age of the animal and awareness about brucellosis [13].

Isolation of *Brucella* from aborted fetuses, vaginal secretions, blood and milk of infected animal confirms the presence of disease. *B. abortus* has seven recognized biovars, the most reported are biovars 1, 2, 3, 4 and 9 [14,15]. The identification of *Brucella* species and biovars is based on a microbiological methods including testing for CO_2 requirement, H_2S production, urease activity, agglutination with monospecific sera (A and M), selective inhibition of growth on media containing dyes such as thionin or basic fuchsin, and phage typing [16,17]. Currently, AMOS PCR is used to identify species and selected biotypes within genus *Brucella* [18].

Bovine brucellosis is endemic in India [13,19,20]. The disease has been reported from almost every state with significant seropositivity in livestock. Various risk factors including lack of farm hygiene, overcrowding of animals and movement of animals for trade purpose have been recognized in India [13,21]. Singh et al. [22] reported that brucellosis in livestock was responsible for a median loss of 3.4 billion US dollars to India. Lack of awareness about calf hood vaccination is a major setback which increases severity of problem. The objective of this work was to study the prevalence of brucellosis among dairy farms in Goa using serological, bacteriological and molecular methods. The study also focused on identification of risk factors for spread of this infection among dairy cattle in Goa.

2. Materials and methods

2.1. Study area

Goa is a small coastal state of India located on the West coast between the co-ordinates 14°53′ to 15°47′ North latitude and 73°40′ to 74°20′ East longitude with a geographical area of 3702 sq. km. It bounded by Arabian sea in the West and the Western Ghat region runs along the eastern length of the state and consists of a wide belt of rich forest. The intermediate region that lies between the high Western Ghat region and the coastal plains, the mid land region, have distinct geographical and ecological characteristics. The climate of the region is humid tropical. The monthly maximum temperature varies between 24–33 °C (mean 27 °C). The relative humidity varies from 65 to 100% and annual rainfall varies from 2500 to 3400 mm. The state is not a traditional livestock growing area. However, the state has a sizable livestock population comprising about 76 thousand cattle mainly non-descript, 37 thousand buffaloes and 88 thousand pigs. The state does not have a recognized cattle market. So, the farmers have to purchase new stock of animals from neighbouring states. This is resulting in introduction of new animals with variety of problems. Having contracted with severe production losses, the local Animal Husbandry and Veterinary Services department requested to undertake a survey of the state to know the reproductive status of the animals. The bovine population of the state is very sparse. Herd size ranges from two to 130 animals.

2.2. Samples

A total of 481 samples comprising of blood (296), milk (60), vaginal swabs (80), vaginal discharges (20), placental tissues (10) and fetal tissues (15) were collected from 11 dairy farms located in Goa, India. The farms were selected on basis of incidences of abortions reported to the state veterinary officials. Blood samples from every adult cattle on each farm were collected irrespective of their health status. Data containing number of cattle with and without reproductive disorders related to brucellosis was recorded. The disorders included abortion, endometritis, retention of placenta, infertility, and reduced conception rate. All the clinical samples were transported to laboratory under chilled conditions. Bacteriological samples were processed for isolation of the pathogen. Serum was separated by centrifugation at 3000*g* and stored at −20 °C till further testing.

2.3. *Isolation of* Brucella

The clinical samples including vaginal swabs, vaginal discharge, milk, placental tissues and fetal tissues were processed for isolation of *Brucella*. Vaginal swabs and vaginal discharges were inoculated in *Brucella* broth (M348, HiMedia) with *Brucella* selective supplement (FD005, Himedia Laboratories, Mumbai, India) containing Polymyxin B sulphate (2500 IU), Bacitracin (12500 IU), Nystatin (500000 IU), Cycloheximide (50 mg), Nalidixic acid (2500 mg) and Vancomycin (10 mg). Samples were incubated for 72 h at 37 °C for enrichment. After enrichment vaginal swabs and discharges were spread on *Brucella* agar with selective supplements (Himedia Laboratories). Milk was centrifuged at 7000 rpm for 5 min. The upper cream and bottom deposit layers were spread on *Brucella* agar (M822, Himedia Laboratories Mumbai) with selective supplements. Placental tissues and fetal tissues were minced with sterile scalpel and directly placed on *Brucella* agar with selective supplements for isolation. The plates were incubated under 5% CO_2 at 37 °C till the growth appeared. The plates were first observed after 72 h for growth of characteristic small, circular, elevated, honey colored colonies with entire margin.

2.4. Identification

The isolates were tested by biochemical and PCR based methods for identification of *Brucella* spp. For biochemical identification, the isolates were tested by catalase, oxidase, urease production, H_2S production, CO_2 requirement, nitrate reduction, methyl red, Voges Proskeur test, Gram staining, Modified Ziehl-Neelsen staining and selective inhibition of growth on tryptic soy agar containing dyes, thionin and basic fuchsin at 10 μg/ml (1:25000) and 20 μg/ml (1:50000) concentrations as described by Alton et al. [16]. For molecular identification by PCR, genomic DNA was extracted by phenol: chloroform method and subjected to amplification of the *bcsp31* (B4/B5) and *IS711* genes [23,24]. The quantity and purity of extracted DNA were determined by using Nano-Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Genomic DNA from *Brucella abortus* S19 was used as positive control, and DNA from *E. coli* ATCC 8739 was used as negative control. The primers and other reagents were procured from Sigma Aldrich, Co., (St. Louis, MO, USA). The reaction mixture (25 μl) consisted of 12.5 μl of Ready Mix Taq buffer with $MgCl_2$ (Sigma Aldrich, Co., St. Louis, MO, USA), 0.5 μl forward primer (10 pmole/μl), 0.5 μl reverse primer (10 pmole/μl), 10 μl of nuclease free water. To this mixture 1.8 μl of template DNA was added. The PCR was performed using thermal cycler (Eppendorf Master Cycler, Germany). For detection of the bcsp31 gene initial denaturation was done at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 2 min. Final extension was performed at 72 °C for 10 min. For detection of the *IS711* gene, the PCR conditions were same as the *bcsp31* except the primer annealing was done at 55 °C. The PCR products were analyzed by gel electrophoresis using 1.5% agarose gel containing ethidium bromide. The gel was visualized under UV illumination (AlphaImager, USA). Isolates showing amplification of both the *bcsp31* and *IS711* genes were regarded as belonging to *Brucella* spp.

AMOS PCR was performed for identification of species and selected biotypes within genus *Brucella*. The reaction was performed according to Bricker and Halling [18] with suitable