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Detection of brucella organism in crossbred cattle based on serological and molecular assay

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Abstract
A total of 110 sera sample and whole blood samples were collected separately from unorganized dairy herds of Jersey and Holstein-Friesian (HF) crossbred cattle to evaluate the sero-prevalence of brucellosis by Rose Bengal Plate Test (RBPT), indirect ELISA and its PCR assay. The prevalence rate was recorded 13.63% by RBPT and 20.90% by ELISA respectively. Milk samples (82) were screened by Milk Ring Test (MRT) with a sero-positivity of 17.07%. The disease predisposition with respect to breeds revealed that the prevalence was significantly higher in Holstein Friesian (23.07%) than Jersey (17.77%) crossbred. According to age group the prevalence of brucellosis was higher in animals of age >4years (27.27%) followed by animals in the age groups of 2–4 years (22.05%) and least in animals of 1-<2 years of age (10%). With regard to history of reproductive failure, the highest prevalence of Brucellosis was found among the animals having previous history of abortion (61.90%) followed by retention of placenta (44.44%) and repeat breeding (18%). Screening of whole blood sample and milk sample by genus specific (*bcsp-31* gene) as well as species specific *IS711* gene based PCR revealed that 21(19.09%) blood sample and 9 (10.97%) milk samples were found positive.

Keywords: Brucellosis, cattle, prevalence, PCR.

1. Introduction
Brucellosis is an infectious zoonotic disease having an impact on both human and animal health and remains endemic in India [1]. It causes considerable economic loss by causing abortion and decrease in milk efficiency. It is one of the causes of infertility in animals and poses a great risk to public health if transmitted to humans [2].
For effective control of brucellosis in animals and its transmission to human, precise diagnosis is the prime requirement. Presumptive diagnosis in livestock on the basis of its history of reproductive failures in livestock must be confirmed by laboratory methods [3]. Although isolation of *Brucella spp* is the 'gold standard' for diagnosis, they are time consuming and represent a risk for laboratory personnel [4]. However at the farm level, mass screening of animals are done by various serological test like Milk Ring Test (MRT), Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (SAT) and Enzyme-Linked Immunosorbent Assay (ELISA). But the major drawback of these test are cross reaction with other Gram-negative bacteria, such as Yersinia enterocolitica O:9, *Francisella tularensis* etc [5]. In this regard molecular techniques like Polymerase chain reaction (PCR) is being employed for rapid and confirmatory diagnosis of Bucellosis [6].
The present study aimed at to know the prevalence of brucellosis in unorganized dairy herds of bordering areas of Assam and Meghalaya by serological tests (MRT, RBPT and ELISA) and PCR assay from blood and milk. This will help in the control of brucellosis among the dairy herds and will reduce the heavy economic losses.

2. Material and methods
2.1 Sample collection: The sera sample and whole blood samples (110) were collected separately from unorganized dairy herds of Jersey and Holstein-Friesian (HF) crossbred cattle. Herds were located in the bordering areas of Assam and Meghalaya and having the history of abortion. The sera sample were collected in vacutainers without anticoagulant and whole blood were collected in heparin coated vacutainers. Milk samples (82) were collected from all the

lactating animals. All the samples were transported to laboratory on ice. Host factors studied were breed, age and association of brucellosis according to history of reproductive failure.

Serological tests: Sera sample were first screened by RBPT and subsequently by ELISA. ELISA was performed as per the manufacturer's protocol of IDEXX Brucellosis Serum X2 kit (IDEXX, Netherland) for bovine brucellosis. The absorbance was read at 450 nm with an ELISA reader (Lab systems Multiskan Plus, Thermo Fisher Scientific, USA). Samples having percent positivity value 80 or above (%P ≥ 80) were categorized as positive and below 80 as negative. Milk samples were screened by Milk ring test. RBPT and MRT antigens were procured from IVRI, Izatnagar.

2.2 Molecular Work

DNA from the blood and milk was extracted by using QIAamp DNA mini kit (Qiagen, GmbH, Hilden, Germany). Confirmation of the isolates was done by Genus specific PCR targeting *bcsp-31* gene, and species specific PCR targeting IS711 gene. The list of primers is given in Table 1

The PCR reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) in 20 µL volume containing 2µl DNA sample (100 ng/ µl), 1 µl (10 pmol) of each forward and reverse primer, 10 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific™, USA) and 7 µL of nuclease free water (Thermo Scientific™, USA). The *bcsp*31 gene based genus specific PCR was optimized with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation (95 °C for 45 sec) annealing (60 °C for 1 min) and extension (72 °C for 2 min). Final extension was carried out at 72 °C for 5 min. Similarly, *IS711* gene based specific primer was optimized with the same conditions used for *bcsp*31 PCR, except for the annealing condition which was kept at 55.5 °C for 45 sec.

3. Result and Discussion

In the present study, results of sero-positivity by different serological tests and by PCR assay were presented in Table 1. All total of 110 sera sample were collected from unorganized dairy herds and out of which the prevalence of brucellosis was recorded 15 (13.63%) by RBPT and 23 (20.90%) by ELISA respectively. These findings were in close proximity with the findings of other researcher [7]. Prevalence rate of bovine brucellosis was recorded 12.69% by RBPT and 17.5 - 34.92% by ELISA in Guwahati and nearby areas by the earlier workers [8, 9]. The variation of prevalence rate is might be due to the sample size and procurement of new animals without prior testing.

Breed wise prevalence of brucellosis was significantly higher in Holstein Friesian (23.07%) than Jersey (17.77%) by ELISA which was in agreement with the earlier worker [9] ($p<0.01$).

According to age group the prevalence of brucellosis was higher in animals of age >4years (27.27%) followed by animals in the age groups of 2-4 years (22.05%) and least in animals of 1-<2 years of age (10%) by ELISA (Fig1). The differences in the prevalence of the disease among these three age groups were statistically significant ($p<0.1$), with animals in the age group of >4years being the most susceptible. Similar finding were also observed by other workers (10). Although bovines of all age groups are affected by brucellosis but advancement of age with decreased immune status makes the higher age group more susceptible.

With regard to history of reproductive failure, the highest prevalence of *Brucellosis was* found among the animals having previous history of abortion (61.90%) followed by retention of placenta (44.44%) and repeat breeding (18%) in case of cattle (Fig. 2). These finding were in close conformity of earlier worker [8]. The higher incidence of abortion in third trimester may be due to the fact that the production of erythritol makes uterine environment conducive for growth of *Brucellae*, which in turn causes abortion and as a result of sequel retention of placenta occurs [10].

Screening of 82 milk samples by MRT revealed the presence of Brucella antibody in 14 (17.07%) milk samples. Mohamand *et al*. [11] recorded 18.35% positivity in cattle by MRT. This might be due to localization of the organism in the supramammary lymph nodes and in the udder when the disease becomes chronic. Such animals may shed *Brucella* organism in their milk with a titre of diagnostic threshold [12].

Screening of whole blood sample and milk sample by genus specific (*bcsp-31* gene) as well as species specific *IS711* gene based PCR revealed that 21(19.09%) blood sample and 9 (10.97%) milk samples were found positive. All the PCR positive blood and milk samples were showing expected amplification of 223bp for genus specific and 498bp for species specific *IS711* gene based PCR. During acute phage of infection organisms are circulated in the blood and excreted heavily in milk. However, chronic carrier animals also shed *Brucella* organism in their milk. Therefore good quantity of DNA can be extracted from blood and milk of such animals. Similar observations that DNA can be extracted from blood and milk were made by previous workers [13, 14].

In conclusion, it is very difficult to detect all infected cases of bovine brucellosis using a single test. Hence, combination of both serological testing and PCR assay can be useful diagnostic tools to detect the status of the disease in the farms. As brucellosis is an economically important zoonotic disease among farmers, so it is essential to have an accurate diagnosis and awareness generation program to facilitate the prevention and control strategies against the disease pathogen.

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Table 1: List of primers for Genus specific and *IS711* gene based PCR

Name	Sequence (5/-3/)	Product Size (bp)	Reference
Genus specific PCR primers (*bcsp*31) B4 B5	TGG CTC GGT TGC CAA TAT CAA CGC GCT TGC CTT TCA GGT CTG	223	Baily *et al.*, 1992 [15]
***IS711* gene based PCR Primers** IS711FP IS711RP	GACCAACGGAATTTTTCCAATCCC TGCCGATCACTTAAGGGCCTTC	498	Bricker *et al.*, 1994 [16]

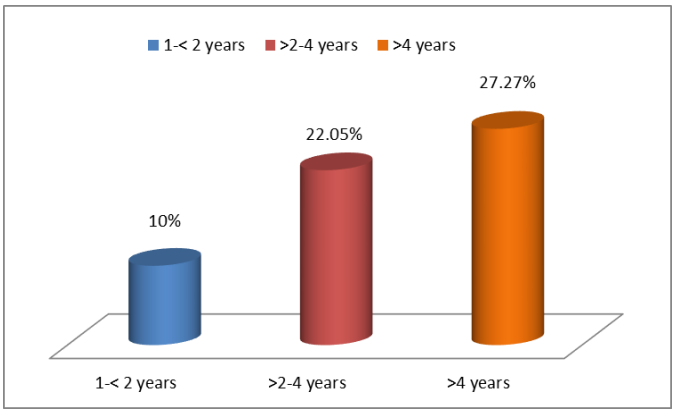

Fig 1: Age group wise sero-prevalence of bovine brucellosis

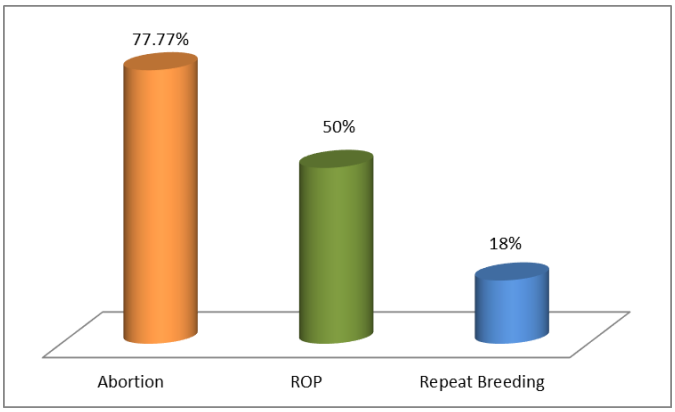

Fig 2: Association of brucellosis according to history of reproductive failure

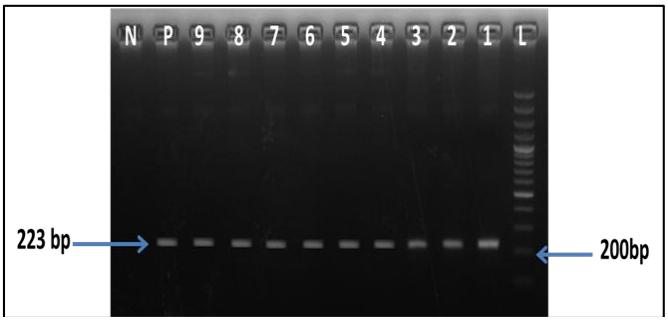

Fig 3: *bcsp* gene detection by PCR for Brucella. L: 100 bp plus ladder; 1to 9: Isolates P: Positive control; and N: Negative control

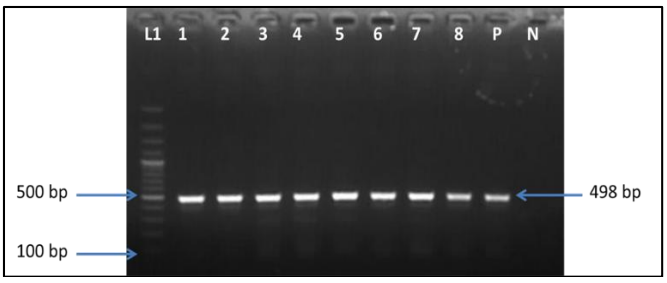

Fig 4: *IS711* gene based PCR for Brucella. L: 100 bp plus ladder; 1to 8: Isolates P: Positive control; and N: Negative contro

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