Original Research

Apparent Prevalence and Associated Risk Factor Assessment in Occurrence of Bovine Brucellosis – A Cross Sectional Epidemiological Study

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Abstract

Brucellosis is one of the highly contagious diseases with zoonotic potential affecting livestock and humans worldwide. A total of 1391 samples (821 serum samples, 483 milk samples and 87 aborted materials) were collected from eleven districts of Tamil Nadu to assess the current epidemiological status of Brucella infection in the study area. On serological analysis (n=821) the overall seroprevalence of bovine brucellosis was 33 (4.02%) by RBT, 36 (4.38%) by STAT and 55 (6.70%) by i-ELISA. In milk, prevalence rate was 4.35 per cent (21/483) by MRT and 5.80 per cent (28/483) by m-ELISA. For bacteriological isolation, 87 aborted materials were screened and 3 isolates were recovered and classified by biochemical tests as B. abortus biovar 2. The isolates were confirmed by PCR targeting bcsp 31 and IS711 genes. To identify the potential risk factors in bovine brucellosis, risk analysis was performed by using structured questionnaire. In risk analysis, aborted history animals, late aborted history animals, husbandry practices – rearing, farm sector, grazing, source of purchasing animals and improper disposal of animal wastes were found to be significant risk factors which may play a role in the prevalence and transmission of the disease.

Key words: Brucellosis, Seroprevalence, Strain Identification, Molecular, Risk Factors

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Introduction

Brucellosis is one of major abortion causing bacterial diseases of livestock and it was highly prevalent in all over the world except Denmark, UK, Netherlands and Romania where the disease is eradicated (Mukasa-Mugerwa, 1989). *Brucella* as a zoonotic pathogen causes orchitis, high fever and reduced conception in humans, whereas in livestock, brucellosis is characterized by abortion, orchitis, retained fetal membrane,

repeat breeding, reduced milk yield, pyometra, endometritis, vaginitis and metritis along with other infections (Radostits *et al.*, 2010).

Brucellosis should be ruled out whenever a cow aborts unexpectedly due to its severe economic loss, monitoring and surveillance of farm with abortion histories is warranted, except in a *Brucella*-free herd. The gold standard technique for *Brucella* identification is culture and other screening test also useful in control of brucellosis (OIE, 2009). However, due to its fastidious nature (Romero *et al.*, 1995) and the risk of acquiring the disease from laboratory condition, isolation should be processed under BSL III laboratory (Bhat *et al.*, 2012). Serological tests are most efficient tools in epidemiological surveillance by which the prevalence can be easily studied (Alton *et al.*, 1988; Kumar *et al.*, 2018a). Moreover, each serological test was having demerit over one with other test. Therefore, two or more tests are required before arriving at confirmatory diagnosis. The most widely used serological tests for diagnosis of brucellosis in animals are Rose Bengal Plate Agglutination Test (RBT), Standard Tube Agglutination Test (STAT), Enzyme Linked Immunosorbent Assay (ELISA), Complement Fixation Test (CFT) and Fluorescent Polarization Assay (FPA) (Al-Majali *et al.*, 2009; OIE, 2009). Other than serum, *Brucella* antibodies are also excreted in milk. Thus, milk can be utilized as a non-invasive sample for assessing the prevalence of brucellosis (Kumar *et al.*, 2018). Nowadays, polymerase chain reaction (PCR) for *Brucella* identification and typing targeting various genes were utilized for rapid diagnosis (Yu and Nielsen *et al.*, 2010). PCR gene target for brucellosis, such as 16S - 23S rRNA operon, *bcsp* 31 or IS711 genes are commonly utilised for laboratory diagnosis (Baddour *et al.*, 2008; Godfroid *et al.*, 2010). The overall sensitivity of the PCR was higher than culture method, (Leal-Klevezas *et al.*, 1995; Romero *et al.*, 1995; Hamdy and Amin, 2002) which has the potential to detect even low number of *Brucella* organisms from field samples.

In India, brucellosis is highly prevalent in most parts of our country. Seroprevalence studies have shown a prevalence of brucellosis of 8.8 per cent in India and 9.3 per cent in bovines of Tamil Nadu (Renukaradhya *et al.*, 2002). Purchase of animals without prior diagnosis and lack of awareness among the farmers were found as other potential risk factors for transmission of brucellosis (Shome *et al.*, 2014). The economic losses due to brucellosis were 3.4 billion US dollar in India (Singh *et al.*, 2015). There is no single test by which brucellosis can be identified. Hence, a battery of diagnostic tools like culture, serological testing and molecular identification were usually needed for confirmatory diagnosis (OIE, 2009). The choice of a particular testing strategy depends on type of epidemiological survey and its various purposes (livestock and wildlife) in a country or a region.

Considering the above facts in mind the present cross-sectional study was designed to: (i) Assess the seroprevalence of bovine brucellosis in Tamil Nadu, (ii) Identify the various risk factors associated with prevalence and transmission of bovine brucellosis in Tamil Nadu.

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Materials and Methods

Study Area

Livestock sector is a booming enterprise which solely depends on productive and reproductive performance of the healthy animals. Reproductive disorders in animals due to brucellosis cause severe economic losses to the farmers as well as to the country. In India due to under reporting system and unawareness on brucellosis, aborted and various reproductive ailed animals were sold to other farmers without proper diagnosis which plays a pivotal role in disease transmission. Hence, the present cross-sectional study aimed to determine the seroprevalence of bovine brucellosis and to identify various epidemiological risk factors from eleven districts (Erode, Salem, Kancheepuram, Villupuram, Tiruvannamalai, Tiruvallur, Tirunelveli, Pudhukkottai, Thiruvarur, Virudhunagar and Chennai) of Tamil Nadu, India (Fig. 1).

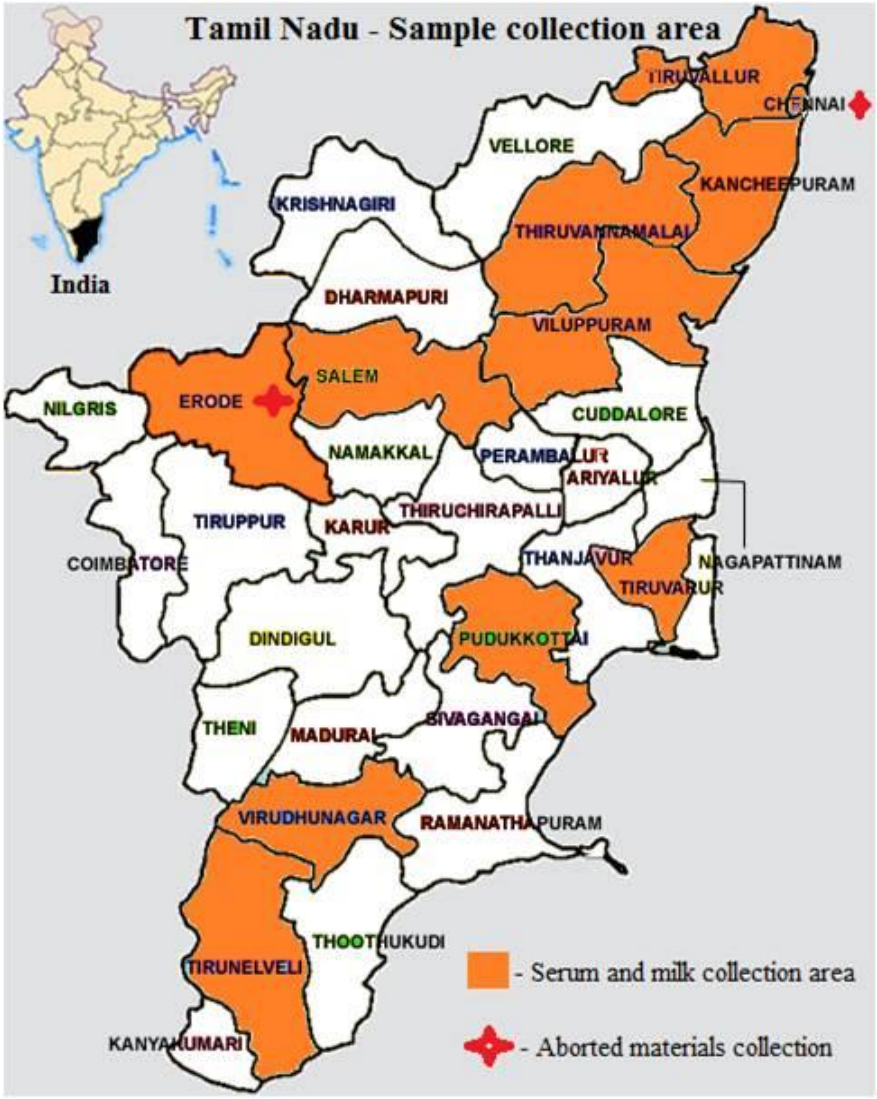

Fig. 1: Sample collection area

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Sample Size

In the present study a total of 1391 samples (821 serum samples, 483 milk samples and 87 aborted materials) were collected from eleven districts of Tamil Nadu to assess the current epidemiological status of *Brucella* infection in major part of Tamil Nadu. Sexually matured cattle were selected randomly with the history of abortion, retained fetal membrane, repeat breeding, infertility, pregnant, prepubertal anestrus heifers and unknown reproductive histories. All the clinical samples were properly transported to laboratory under chilled conditions. Aborted samples for isolation of organism were processed following biosafety protocols. Blood samples (3 ml) were collected using vacuette® with EDTA from 821 cattle by jugular vein puncture in sterile test tubes (5 ml) and stored at - 20°C until use. Sera were separated by centrifugation at 2000 rpm for 15 minutes and stored at - 20°C until further use. Milk samples were collected from 483 lactating cattle. The udder was thoroughly washed and cleaned with potassium permanganate solution (1:1000) and dried with sterile gauze. Teat orifices were disinfected with 70 per cent ethyl alcohol. After discarding the first few drops of milk, approximately 10 ml of milk from each quarter was collected in two sets of sterile screw capped plastic vials (50 ml) and transported on ice to the laboratory.

Isolation

The aborted clinical samples namely fetal tissue, placental tissues and uterine discharges were enriched in *Brucella* broth (HI media) containing *Brucella* selective supplement (HI media) for three days at 37^0 C which had the following antibiotics; Vancomycin - 10 mg, Polmyxin B sulphate - 2,500 IU, Bacitracin - 12,500 IU, Nystatin - 50,000 IU, Cyclohexidine - 50 mg and Nalidixic acid - 2.5 mg. The three days old enriched suspension were directly streaked on the *Brucella* selective medium (HI media) with *Brucella* selective supplement (HI media) followed by incubation of agar plate at 37°C with 5 per cent CO_2. The plates showing small, circular, elevated, honey coloured colonies were further subjected to various identification protocols.

Identification

For identification, the presumptive isolates were subjected to various biochemical tests viz., oxidase, catalase, H_2S production test, CO_2 requirement and dye reduction test viz., thionin at 1:25,000, 1:50,000, 1:1, 00,000 dilution and basic fuchsin dye at 1:50,000, 1: 1, 00,000 dilution were performed as per Alton *et al.* (1998) and PCR assay were utilised in this study. For molecular confirmation, genomic DNA was extracted from the isolates and aborted tissue samples by using "Ultra-Pure Genomic DNA Spin Minipreps" DNA extraction Kit obtained from Bio basic, Canada. The extracted genomic DNA was subjected to *bcsp* 31 based PCR (Baily *et al.*, 1992) and *IS*711 *B. abortus* specific (in-house designed) PCR. For PCR, the quantity and purity of extracted genomic DNA were determined by using Nano-Drop 1000

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spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For positive control *B. abortus* S19 genomic DNA was used. The primers and other reagents were procured from Eurofins Genomics, India. The primer sequence for *bcsp* 31 forward TGG CTC GGT TGC CAA TAT CAA and reverse CGC GCT TGC CTT TCA GGT CTG with 224 bp as expected product size as per Baily *et al.* (1992). In house designed *B. abortus* specific *IS*711 gene forward primer sequence was AGG CCG ATA GCA TCG ACA A and reverse sequence were AAT GGA ACC GGA TCG AAG CA with 378 bp as expected product size. The cyclical conditions for *bcsp* 31 gene-based PCR, initial denaturation was done at 94˚C for 5 min followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 55 ˚C for 45 s and extension at 72 ˚C for 1 min. Final extension was performed at 72 ˚C for 10 min. The cyclical conditions for IS711 *B. abortus* specific PCR, initial denaturation was done at 95˚C for 10 min followed by 30 cycles of denaturation at 95 ˚C for 2 min, annealing at 63 ˚C for 45 s and extension at 72 ˚C for 45 s. Final extension was performed at 72 ˚C for 10 min. The PCR products were analysed by gel electrophoresis using 1.5% agarose gel containing ethidium bromide. The gel was visualized under UV illumination (Gel Doc Mega – Bio Rad). The isolates which showed amplification with *bcsp* 31 were identified as *Brucella* spp., and amplification with IS711 were identified as *B. abortus*.

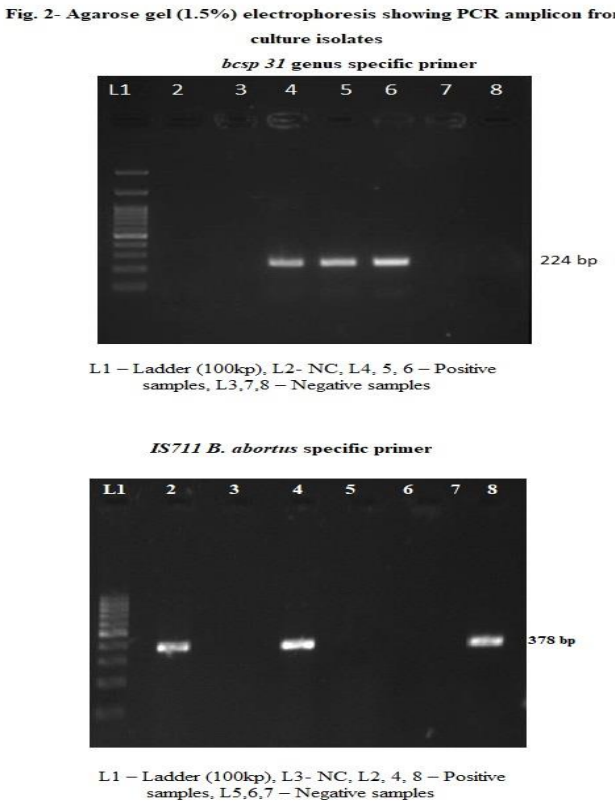

Fig. 2: Agarose gel (1.5%) electrophoresis showing PCR amplicon from culture isolates

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Apparent Prevalence

To ascertain the prevalence, all the serum samples were subjected to Rose Bengal Test (RBT), Standard Tube Agglutination Test (STAT) and milk samples were subjected to Milk Ring Test (MRT) as per Alton *et al.* (1998). RBT, STAT and MRT antigens were procured from Indian Veterinary Research Institute, Izatnagar, India. Formation of clumps, agglutination more than 40 IU and formation of clear ring in the cream layer were considered as positive for RBT, STAT and MRT respectively. For confirmatory diagnosis, all the serum and milk samples were subjected to indirect Enzyme Linked Immunosorbent Assay (i-ELISA). The *Brucella* antibody ELISA test kit was purchased from Svanova, Sweden and used for screening serum and milk samples as per manufacturer's instruction. The samples were run on Svanovir *Brucella*-Ab indirect ELISA kit and the optical densities (ODs) were determined in a microplate spectrometer (Bio rad) at 450-nm wavelength. Positive and negative control serum samples were included in each test. Interpretation of the results was based on Per cent Positivity (PP) calculations; PP is calculated by (Test sample or negative control (OD) x 100) / (Positive control (OD)) and results were interpreted as positive for PP $\geq$ 60 and negative for PP < 60 for the individual serum (10 µl) sample. For milk the results were interpreted as positive for PP $\geq$ 10 and Negative for PP < 10 for individual and pooled milk samples.

Identification of Risk Factor

To identify risk factors, a structured questionnaire-based survey was conducted from livestock owners. All the collected samples were categorized based on demography, age, breed, husbandry practices and clinical condition of the animals. Univariate logistic regression analysis was made to identify the potential risk factors in the prevalence of bovine brucellosis. As per Thrusfield, 2018 Odds Ratio (OR) or cross product ratio was calculated to identifying the risk factors involved in the prevalence and transmission of disease by using MedCalc online software.

Results and Discussion

Serological Prevalence of Brucellosis

Brucellosis is one of the economically important diseases in India. In the present study, the overall seroprevalence of bovine brucellosis was 33 (4.02%) by RBT, 36 (4.38%) by STAT and by i-ELISA was 55 (6.70%) (Table 1). In milk, prevalence rate was 4.35 per cent (21/483) by MRT and by m-ELISA was 5.80 per cent (28/483). Comparison of different serological test was done by utilizing i-ELISA as the most sensitive test. In the evaluation, RBT had the sensitivity and specificity of 54.54 and 99.60 per cent respectively. STAT had the sensitivity and specificity of 61.81 and 99.73 per cent respectively. The MRT and m-ELISA were compared and MRT had sensitivity and specificity of 53.57 and 98.68 per cent respectively. Many researchers proved that brucellosis was highly endemic in many parts of world.

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Table 1: Summarize of diagnosis of Brucellosis by various diagnostic tests results of serological, bacteriological and molecular methods in diagnosing brucellosis

Location	Nº *	Seroprevalence			Nº *	Milk Prevalence		Nº *	Aborted Tissue Prevalence	
		RBT	STAT	i-ELISA		MRT	m-ELISA		Culture	PCR
Erode	125	6	3	5	82	3	3	8	1	4
Salem	74	4	2	10	56	2	3	-	-	-
Kancheepuram	83	2	1	3	68	4	2	-	-	-
Villupuram	70	2	3	-	45	2	3	-	-	-
Tiruvannamalai	114	9	7	19	41	2	4	-	-	-
Tiruvallur	48	-	2	1	33	1	2	-	-	-
Tirunelveli	51	2	1	1	37	1	2	-	-	-
Pudhukkottai	89	2	3	3	45	2	3	-	-	-
Thiruvarur	91	1	5	4	39	1	3	-	-	-
Virudhunagar	61	5	9	9	31	2	3	-	-	-
Chennai	15	-	-	-	6	1	-	79	2	10
Total	**821**	**33**	**36**	**55**	**483**	**21**	**28**	**87**	**3**	**14**

(*Nº – Total number of screened sample)

Omer *et al.* (2000) reported that the prevalence of brucellosis in cattle was 8.20 per cent in Eritria. Silva *et al.* (2000) observed that prevalence of brucellosis in Sri Lanka was 4.7 in cattle. In Pakistan, Nasir *et al.* (2004) found 14.7 per cent prevalence in cattle. Otlu *et al.* (2008) stated that 34.64 per cent of cattle were positive for brucellosis in Turkey. Samaha *et al.* (2008) in Egypt found prevalence of brucellosis in cattle with 3.52 per cent. State-wise prevalence of bovine brucellosis in India was studied by Renukaradhya *et al.* (2002) and the highest prevalence was recorded in Punjab (23 per cent) followed by Gujarat (16 per cent), Tamil Nadu (9.3 Per cent), Goa (6.3 per cent), Southern union territory of Pondicherry (3.4 per cent), Maharashtra (2.4 per cent) and Andhra Pradesh (1.7 per cent). Similarly, Islam *et al.*, 2018 documneted 15.12 % positivity in Buffaloes of Punjab. Earlier prevalence studies in Tamil Nadu were recorded by many researchers. Jai Anandh (2005) recorded the overall seroprevalence of brucellosis in Tamil Nadu which varied from 9.14 per cent in selected districts of Tamil Nadu and concluded that prevalence was more in Vellore (25.71%), followed by Madurai (15.38%), Coimbatore (13.88%), Theni (11.76%) and Chennai (9.52%). Chandramohan *et al*. (1992) reported that 18.26 per cent of cows showed the seropositivity for *Brucella* infection in Tirunelveli District. Seroepidemiological studies to monitor bovine brucellosis in Tamil Nadu was conducted by Isloor *et al.* (1998) and showed 2.5 per cent positivity for brucellosis. Anuradha (2004) reported 8.8 per cent seroprevalence of brucellosis in bovine in Tamil Nadu. The variation of seroprevalence might be due to methods in sampling, test applied, endemicity of infection in an area, inclusion of selected districts for seroprevalence in the present study, husbandry practices and hygiene measures of farms.

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Cultural and Molecular Isolation of Isolates

In this study 87 aborted materials from cattle were screened by conventional culture methods. A total of 3 isolates were isolated and identified by utilising a battery of biochemical test and the isolates showed characteristics of *Brucella abortus* bio var 2 with a positivity of 3.44 per cent (3/87) (Table 1). All the three isolates showed a specific product size of 224 bp for *bcsp* 31 gene and 378 bp for IS711 gene (Fig. 2). In *Brucella* diagnosis, culture is considered as the gold standard technique. However, due to fastidious and zoonotic nature of the pathogen cultural identification is a laborious process. In India, cultural isolates studies have identified *Brucella* abortus bio var 1 and 3 as the most common bio var (Pathak *et al.,* 2016). Results of our study showed *Brucella abortus* bio var 2 as the predominant bio var from our study area which needs continuous isolation studies strategy to identify the common circulating bio var in the study area and to elucidate its zoonotic potential and to facilitate control strategy. Cultural isolation was attempted by many researchers all over the world with varying success. Leyla *et al.* (2003) screened *Brucella* organism from 126 aborted fetuses and showed 13 per cent (39) of the samples were positive. Kaur *et al.* (2006) showed 27.86 per cent (17/61) positivity with cattle and buffalo aborted contents viz., 37 foetal stomach contents, 9 vaginal mucus and 15 foetal membranes. Priyantha *et al.* (2008) isolated *B. abortus* from eight aborted animals and all eight isolates were identified as bio var 3 by various biochemical tests. Variation in results may also be attributed due to source of samples, sampling methods, laboratory protocols, media used and microbial adoption in various environments (Bhat *et al.*, 2012).

***Brucella sp.*, DNA Identification from Aborted Tissue**

Upon direct DNA extraction from 87 aborted materials, 14 (16.09%) samples were positive for both *bcsp* 31 and IS711 PCR (Table 1). In a similar fashion various workers attempted to identify the *Brucella* by PCR assay with various samples viz., aborted materials and to prove its sensitivity (Fekete *et al.*, 1992; Scarcelli *et al.*, 2004; O'leary *et al.*, 2006), uterus, udder, spleen, lymph nodes, kidney and liver (Gallien *et al.*, 1998) and stomach content of aborted foetuses (Cetinkaya *et al.*, 1999). Our results are in agreement with the early workers who have also reported that PCR is a sensitive antigen-based detection method which can be utilised directly by extracting DNA from aborted contents.

Risk Analysis

Risk factor assessment by risk analysis is an important tool to identify risk factors which facilitates the disease transmission. In this study, aborted history animals (OR = 8.4562, P < 0.0001**), late aborted history animals (OR = 4.6118, P=0.0115*), husbandry practices - rearing (OR = 2.1741, P = 0.0298*), farm sector (OR = 0.0424, P=0.0306*), grazing (OR = 0.3302, P=0.0001**), source of purchasing animals (OR = 3.2517, P=0.0501*) and improper disposal of animal wastes (OR = 0.1990, P< 0.0001**) were found to

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be important and identified risk factors which aid in the prevalence and transmission of disease in the study area (Table 2).

Table 2: Risk factors associated with seroprevalence of brucellosis in Tamil Nadu

Risk Factors	Variable	Nº *	No. of Positive	OR	95% CI	P value
Breed	Local	64	2	0.4285	0.1020 – 1.8004	= 0.2472^{NS}
	Cross	757	53			
Abortion history	Present	64	19	8.4562	4.4941 - 15.9114	< 0.0001**
	Absent	757	36			
Abortion time	Last	31	14	4.6118	1.4090 – 15.0943	= 0.0115*
	Early & Mid	33	5			
Husbandry practice	Farm	90	11	2.1741	1.0790 – 4.3804	= 0.0298*
	Single cow herds	731	44			
Farm sector	Organised	40	0	0.0424	0.0024 – 0.7443	= 0.0306*
	Unorganised	50	11			
Purchase of screened animals	Yes	28	2	1.074	0.2482 – 4.6479	= 0.9239^{NS}
	No	793	53			
Awareness on brucellosis	Yes	48	3	0.9224	0.2778 – 3.0755	= 0.8980^{NS}
	No	773	52			
Proper screening	Yes	17	2	1.8893	0.4209 – 8.4800	= 0.4063^{NS}
	No	804	53			
Separation of pregnant animals	Yes	31	1	0.4543	0.0608 – 3.3957	= 0.4420^{NS}
	No	790	54			
Grazing	Present	609	28	0.3302	0.1898 – 0.5746	= 0.0001**
	Absent	212	27			
Breeding	AI	763	48	0.4891	0.2107 – 1.1356	= 0.0961^{NS}
	NS (Bull)	58	7			
Source of animal purchase	Sandy	697	52	3.2517	0.9993 – 10.5809	= 0.0501*
	Own farm	124	3			
Disposal of animal waste	Proper	398	14	0.199	0.1066 – 0.3714	< 0.0001**
	Improper	323	41			

*(*Nº – Total number of screened sample)*

Various reasons to prove the role of identified risk factors are discussed below. Due to inherent abortive nature of *Brucella* organisms aborted animals and late history aborted animals (Islam *et al.*, 2018) were found to be significant risk factor. Husbandry practices as a risk factor was compared with organised and unorganized farm sector. Due to close contact between animals in farm and unhygienic practices at unorganised sector plays a role in disease transmission. Grazing pattern, movement of animals were favouring transmission of *Brucella* among animals. Because of poor disease reporting and diagnostic system in Tamil Nadu, the aborted animal is usually sold to other farmer in local sandy, this might be a significant factor which influence the high prevalence and improper disposal of animal waste in farm level may plays a pivotal role in disease transmission. Chand and Chhabra, (2013) observed that intra-herd spreading of *Brucella* infected animals plays a major risk factor in farms of Punjab. Al-Majali *et al.* (2009)

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reported that large size herd and mixed farming showed highest seropositivity for brucellosis in Jordan**.** Patel *et al.* (2014) and Bakhtullah *et al.* (2014) concluded that native breeds were resistant to brucellosis than other breeds. Risk factor identification varies with aim of study design. Strategic management of the identified risk factor in the study area will help in devising effective control at both farm and single cow herd's practices.

Conclusion

The present study concluded that, the prevalence of bovine brucellosis in the study area were 6.70 per cent in serum and 5.80 per cent in milk. Bacteriological analysis of aborted materials identified all the three isolates were to *B. abortus* biovars 2. PCR targeting *bcsp* 31 and *IS*711 gene can be effectively utilised for direct screening of *Brucella* organisms from aborted materials owing to its high sensitivity. Proper management of identified risk factors and routine screening of animals for brucellosis will help in combating the disease with zoonotic potential.

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