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Predominant Prevalence of *Brucella abortus* biovar-4 in Small Ruminants in Tamilnadu

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Publication Date: 15 October 2016

Article Link: <http://scientific.cloud-journals.com/index.php/IJAVST/article/view/Sci-491>



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Abstract Brucellosis being a global zoonotic disease and animal health problem most of the studies has been oriented towards bovine than small ruminants. Hence the present study is conducted to identify the biotype of predominant *Brucella spp.* in small ruminants for defined control and eradication strategies. The study was carried out by random collection of the vaginal swab, milk, blood samples from the 315 sheep and goat. The samples were subjected for isolation and identification by biochemical tests followed by bio-typing with mono-specific A and M anti-sera and confirmed by multiplex AMOS PCR. Out of 195 goats and 120 sheep, 20 (10.25%) goats and 22 (18.33%) sheep were positive by culture with overall prevalence 13.33%. Fifteen isolates from goats and 20 isolates from sheep were typed as *B. abortus biovars-4*, 3 isolates from goats were typed as *B. melitensis biovars-2*, and 2 isolates from goats were positive for *B. ovis* and also 2 isolates from sheep were un-typed biovars. The confirmation was carried out by using multiplex AMOS-PCR. The study revealed that the *B. abortus* biovar-4 was predominant in small ruminants. Since most of the control strategies are directed towards bovine brucellosis this study indicates that small ruminant brucellosis control strategy must be redefined since it is the potential source of zoonosis.

Keywords *Brucellosis; Zoonotic; AMOS PCR; Brucella abortus biovar-4*

1. Introduction

Brucellosis is an infectious disease of domestic and wild animals with serious zoonotic implication in humans. Animal brucellosis is mainly characterized by reproductive involvement resulting in abortion and infertility whereas human brucellosis leads to chronic debilitating complications. Currently, the genus consist of 10 species classified based on their host preference and phenotypic differences. *B. abortus*, *B. melitensis* and *B. suis* are divided into 7, 3 and 5 biovars. Ovine and caprine brucellosis caused by *Brucella melitensis*, sporadic infection in sheep and goats can also be caused by *Brucella abortus*, *Brucella ovis* or *Brucella suis* (OIE, 2009). Clinical disease is common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and Caribbean. In India, brucellosis was first recognized in 1942 and is now endemic throughout the country. *B. abortus*

biotype-1 in cattle and buffaloes and *B. melitensis* biotype-1 in sheep, goats and man are the predominant infective biotypes in India (Renukaradhya et al., 2002). The disease is mainly manifested as reproductive failure which includes abortion in pregnant female, stillbirth, placentitis, epididymitis and orchitis in male. Localization may also occur in mammary tissue with excretion in the milk (Pal, 2007).

The present study has been undertaken to estimate the prevalence of brucellosis in sheep and goats by culture and biovar identification by biochemical tests and multiplex AMOS-PCR assay. This study of biovar identification will be helpful for effective vaccine preparation for the control of brucellosis in small ruminants.

2. Materials and Methods

The samples were collected from 120 sheep and 195 goats in Tamilnadu. A total of 80 milk samples (38 from sheep and 42 from goats), 299 vaginal swabs (120 from sheep and 179 from goats), 210 whole blood (120 from sheep and 90 from goats) and 16 aborted materials samples from goats were collected from organised and unorganised sheep and goat units.

2.1. Isolation and Identification

Isolation from single animal is a sufficient evidence to establish the infection status of a herd and is considered to be the Gold standard test (OIE, 2009; Kaltungo et al., 2014). The milk, vaginal swab and aborted materials were cultured in *Brucella* selective media (Himedia) containing *Brucella* selective supplement (Himedia). The organism were isolated from whole blood by lysis centrifugation technique as per mentioned by Mantur (2004). Identification of *Brucella* organism were carried out by Grams staining, modified acid fast staining followed by bio-typing with biochemical tests viz., oxidase, catalase, urease, H₂S production, growth in the presence of thionin and basic fuchsin dye and agglutination with mono-specific A and M antisera as described by Alton et al. (1988).

2.2. Polymerase Chain Reaction

The isolates were grown on *Brucella* selective media and incubated for 48 hours at 37°C. The DNA were isolated from pure culture by using one step medox DNA extraction reagent. Few colonies from culture were suspended in 100 µl of medox reagent in ependorf tubes and incubated at 100°C for 15 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatant containing crude DNA were collected into sterile ependorf tubes. The DNA was stored at -20°C until use.

AMOS-PCR (*B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* – Polymerase Chain Reaction) was carried out as described by Bricker and Halling with minor modification (Bricker and Halling, 1994). A total of 25 µl PCR assay reaction mixture consisted of 2x PCR mastermix (Apliquon), four sets of primer (20 pmol each) *B. abortus*, *B. melitensis*, *B. ovis* and IS711 -specific primer (Table 1) and 5 µl DNA template.

Table 1: Primer sequences (Bricker and Halling, 1994)

Name of Primers	Sequences (5'-3')
<i>Brucella abortus</i> specific	GAC GAA CGG AAA TTT TCC AAT CCC
<i>Brucella melitensis</i> specific	AAA TCG CGT CCT TGC TGG TCT GA
<i>Brucella ovis</i> specific	CGG GTT CTG GCA CCA TCG TCG
IS711 specific	TGC CGA TCA CTT AAG GGC CTT CAT

The PCR was performed for 35 cycles in thermocyclar, each cycle consist of initial denaturation at 94°C for 1 minute, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extention at 72°C for 1 minute. The final extention of PCR product was carried out at 72°C for 10 minutes. The PCR products were separated by 1.5 % agarose gel electrophoresis at 100 V for 45 minute and gel documented. AMOS-PCR shows amplicon of size 498 bp for *B. abortus*, 731 bp for *B. melitensis* and 976 bp for *B. ovis*.

3. Results

A total of 45 isolates, 20 (10.25%) from goats and 22 (18.33%) from sheep were identified as gram negative coccobacilli and positive for modified acid fast staining. Biotyping was carried out by biochemical tests viz., oxidase, catalase, urease, H₂S production, growth in the presence of thionin and basic fuchsin dye and agglutination with monospecific A and M antisera. Fifteen isolates from goats and 20 isolates from sheep were typed as *B. abortus* biovar- 4, 3 isolates from goats were typed as *B. melitensis* biovars- 2, and 2 isolates from goats were positive for *B. ovis* and also 2 isolates from sheep were un-typed biovar (Table 2). All 42 isolates were detected by the AMOS-PCR and 35 isolates confirmed as *B. abortus* (498 bp), 3 isolates belong to *B. melitensis* (731 bp), 2 isolates identified as *B. ovis* (976 bp) and 2 isolates identified as un-typed *B. abortus* biovar (498 bp) and 3 isolates were showed negative to PCR. (Figure 1)

Table 2: Result of biochemical test for *Brucella* Isolates

Species	Total no. of isolates	Oxidase	Catalase	Urease	H ₂ S Production	Growth in the presence of Dye		Agglutination with Monospecific antisera		Biovar typing
						Thionin	Fuchsin	A	M	
Sheep	20	+	+	+	+	-	+	-	+	<i>Brucella abortus</i> biovars -4
Goat	15	+	+	+	+	-	+	-	+	
Goat	3	+	+	+	-	-	+	+	-	<i>Brucella melitensis</i> biovar -2
Goat	2	+	+	-	+	+	-	-	-	<i>Brucella ovis</i>
Sheep	2	+	+	+	+	+	+	+	+	Untyped biovar

Thionin : 20µg/ml (1:25,000) +: positive
 Basic fuchsin dye : 20µg/ml (1:25,000) -: negative

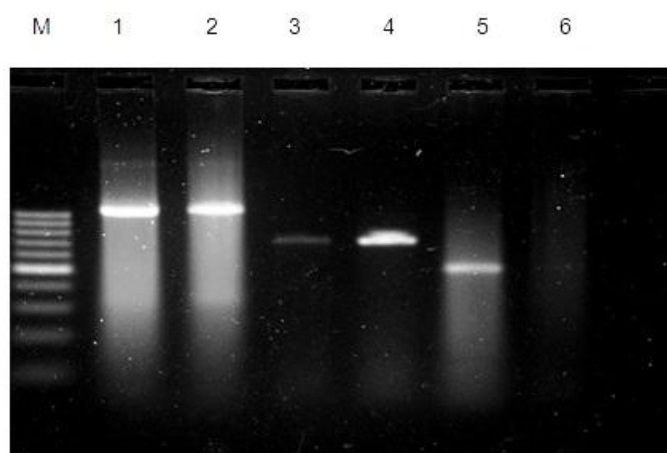


Figure 1: AMOS-PCR

Lane M: 100 bp ladder; Lane 1-2: *B. ovis* (976 bp); Lane 3-4: *B. melitensis* (731 bp); Lane 5: *B. abortus* (498 bp); Lane 6: Negative Control

4. Discussion

Brucellosis is a communicable disease endangering the efforts to improve productivity in sheep and goat. Determining the biovar is an important step for epidemiologic characterization of the disease and also provides preliminary data to design control strategies with regard to vaccine development. Though eradication of brucellosis in small ruminants can be achieved by depopulation of infected flocks, in developing country like India, this strategy is very difficult to adopt. Further, extensive type of rearing, nomadic system, common grazing ground and transhumance of the flock makes it difficult to design a suitable control strategy. Since brucellosis is endemic, the first step is directed to control the susceptible flock by immunization with appropriate vaccine strain. In this regard this study was carried out to identify the most prevalent brucella biovar among small ruminants.

Out of 315 samples collected from sheep and goat, 42 isolates were recovered from milk, vaginal swab, blood and aborted material. Percentage of recovery from milk, vaginal swab, aborted material and whole blood were 11.90%, 21.42%, 11.90% and 54.76% respectively. As brucella is intracellular organism percentage of recovery found to be more from whole blood samples.

Brucellosis is a worldwide zoonotic disease that is recognized as a major cause of heavy economic losses to the livestock industry and poses serious human health hazard. It is considered to be the most important cause of abortion and reduced fertility in infected sheep and goats. Determination of biovars is an important step for epidemiologic characterization of the disease and provides preliminary requirements for designing of control and eradication programs. The present study was undertaken for identification of *Brucella* biovars from small ruminants based on the biochemical tests and confirmation and differentiation of *Brucella* spp. was carried out by multiplex AMOS PCR.

Based on isolation the prevalence among sheep and goat were recorded as 18.33% (22 no.) and 10.255 (20 no.) respectively. Following isolation the isolates were conventionally characterized by biochemical tests viz, oxidase, catalase, urease, H₂S production, growth in presence of thionin and fuchsin dye and agglutination with monospecific sera A and M. Di Giannatale et al. (2008), Affi et al. (2011), Behroozikhah et al. (2012) was used these tests for characterization of brucella biovars. Isolates were further confirmed by multiplex AMOS PCR was carried out for confirmation and differentiation of *Brucella* species and the result recorded. Among 42 isolates 39 isolates were positive and 3 isolates found negative which could be due to different strain of *Brucella* organism. Ancora et al. (2005) and Matope et al. (2009) used biochemical profile and AMOS-PCR for the differentiation and typing of *B. abortus* and *B. melitensis* biovar. Similarly Mirnejad et al. (2013) recorded 45.3% *B. abortus* and 54.6% *B. melitensis* from culture positive samples by multiplex PCR.

In the present study, it was found that out of 42 isolates 35 were identified as *B. abortus* biovar-4 (83.33%), 3 isolates as *B. melitensis* biovar-2, 2 isolate identified as *B. ovis* and 2 isolates were typed as un-typed biovar. In 1975 Sen and Sharma recorded *B. abortus* biovar 4, 6, and 9 from cattle and buffalo whereas *B. melitensis* biovar 2 from sheep and goats. On contrary to this majority of the isolates were identified as *B. abortus* biovar-4 among small ruminants. The possible reason could be in organized farms sheep and goats are reared alongside dairy units and transmission could be through person, vehicle, fodder, drainage etc. In un-organized farm, since sampling has been done from small farmers they share a community pastures land which could be the source of infection. It needs to be assessed whether *B. abortus* biovar 4 has higher transmission ability than other biovars. Further work need to be carried out to find the ability of *B. abortus* biovar-4 to produce either abortion or clinical disease in small ruminants or to act as carriers in spreading the disease.

5. Conclusion

The findings of the study revealed higher distribution of *B. abortus* biovar -4 among the small ruminants which found mainly in cattle and buffalo as reported by Sen and Sharma (1975). The presence of *B. abortus* biovar -4 in sheep and goats might be due to integrated farming system comprising of infected and healthy cattle which shares a common pasture land. The untyped biovar could be due to emerging of new strain of *Brucella* in small ruminants. Vaccine developed with the prevalent strain may help to reduce the disease in both cattle and small ruminants.

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