Indian Journal of Biotechnology
Vol. 18, January 2019, pp 22-25

Serum based screening and molecular detection of brucellosis in ruminants

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Received 27 May 2018; revised 15 December 2018; accepted 18 December 2018

Brucellosis is a highly infectious bacterial disease that mainly affects cattle, sheep, pigs, goats, dogs, horses and wild animals primarily caused by *Brucella abortus*, *B. ovis*, *B. suis*, *B. melitensis*, and *B. canis*. It has a significant threat to the livestock and human community mainly in developing countries and requires accurate diagnosis, characterization and management. This study was undertaken in 238 samples (112 serum samples from the sheep, 82 serum samples from the goat and 44 serum samples from the cattle) suspected of brucellosis collected from the southern districts of Tamil Nadu. All the 238 samples were screened for the brucellosis by carrying out rose bengal plate agglutination test (RBPT). The seropositive serum samples were further subjected to *Brucella* cell surface salt extractable protein 31 (BCSP 31) gene-based PCR for *Brucella* genus confirmation. The BCSP 31-PCR positive samples were further subjected to *Brucella*-AMOS (*avis-melitensis-ovis-suis*) multiplex PCR for identification of *B. abortus, B. ovis, B. melitensis* and *B. suis* species. The study revealed that 8.92% (n = 10) serum samples from sheep, 9.75% (n = 8) serum samples from goat and 6.81% (n = 3) serum samples from cattle were seropositive for brucellosis by RBPT. All the twenty one seropositive samples produced specific amplicon of 223 bp by BCSP 31-PCR confirms brucellosis. Further molecular typing of BCSP 31-PCR positive samples by *Brucella* –AMOS PCR revealed specific amplicon of 498 bp indicating the involvement of *B. abortus* in 19 serum (10 from sheep, 6 from goat and 3 from cattle) samples. One serum sample from goat revealed specific amplicons of 498 bp and 731 bp indicating the involvement of both *B. abortus* and *B. meletensis*. Another serum sample from goat yielded specific amplicons of 498 bp, 731 bp and 285 bp suggesting the mixed infection of *B. abortus, B. meletensis and B. suis*, respectively. The *B. abortus is* the common species involved in cattle, sheep and goat infections. Two caprine samples showed mixed infection which involves *B. abortus, B. meletensis* and *B. suis* species. The study concludes that the serum can be used as an alternate specimen for the fast and reliable molecular diagnosis of brucellosis.

Keywords: Brucellosis, ruminants, molecular detection, serum

Introduction

Brucellosis is a zoonotic and most important highly infectious bacterial disease of domestic and wild animals. Multiple animal species infection with zoonotic potential of this disease gives an alarming sign to the nation. *Brucella* species are Gram-negative, facultative intracellular bacteria, which lack capsule, flagellae, endospore and native plasmids[1]. In sexually mature animals the infection localizes in the reproductive system, udder, uterus, testes and accessory glands, and typically produces placentitis followed by abortion in the pregnant animals, usually during the last third of pregnancy, and epididymitis and orchitis in the male. A healthy animal gets infection by contact with the placenta, fetus, fetal fluids, and vaginal discharges from an aborted/infected animal. Most or all *Brucella* species are also found in semen. Males can shed these bacteria for long periods or lifelong[2]. Some *Brucella* species have also been detected in other secretions and excretions including urine, faeces, hygroma fluid, saliva, milk, nasal and ocular secretions[3]. In ruminants the disease is predominantly caused by the *Brucella abortus*, *B. melitensis*, and *B. suis.* Abortion in the pregnant animals, reduced milk production and infertility accounts for greater economic losses in the ruminant production.

The geographical distribution of brucellosis is constantly changed with new foci of re-emergence. Urbanization, free grazing and movement with frequent mixing of flocks all contribute to the wide distribution of brucellosis in animals. Despite the advance made in the diagnosis and therapy, brucellosis is still wide spread and its prevalence in India is increasing. The representative samples from different regions of our country reveal that the prevalence rate has increased from 34.15% of the samples in 2006-07 to 67.28% of the year 2010-11.

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Although *B. abortus* infect cattle and *B. melitensis* infect sheep and goats but this can occur vice versa also. The situation in pigs is different, as pigs can act as maintenance hosts for *B. abortus*. Therefore, the *Brucella* species infecting animals has to be identified in order to advocate sound control measures in the maintenance host[4]. Molecular based detection of *Brucella* spp. rapidly replaces conventional serodiagnostic approach in clinical laboratories in past few decades. Blood and tissues serves as a good source of nucleic acid for the diagnosis of *Brucella* infection. Clinical specimens like serum, urine, cerebrospinal fluid, synovial or pleural fluid and pus can also be used alternatively for *Brucella* detection[5]. Serum is the preferred in molecular diagnostic assays, serves as a good source of DNA due to its anticoagulant and hemoglobin-free nature[6]. Serum based detection and subsequent typing can also be considered as an important epidemiological tool to study the prevalence of *Brucella* species in ruminants hence this study is designed.

Materials and Methods

Screening of Serum Samples for Brucellosis by Rose Bengal Plate Agglutination Test (RBPT)

A total of 238 samples (112 serum samples from the sheep, 82 serum samples from the goat and 44 serum samples from the cattle) suspected of brucellosis collected from the southern districts of Tamil Nadu were screened. Serum samples were screened by carrying out RBPT using *B. abortus* colored antigen procured from IVPM, Ranipet as per the standard procedure[7].

BCSP 31 Gene Based Genus Specific PCR

DNA was extracted from the positive serum samples by DNeasy blood and tissue kit (Qiagen, USA) and was subjected to PCR by using standard primer pair B4 F (5 TGG CTC GGT TGC CAA TAT CAA 3) and B5 R (5 CGC GCT TGC CTT TCA GGT CTG 3) which amplified 223 bp fragment of the gene encoding 31 kDa *Brucella* cell surface salt extractable protein (BCSP 31) of *Brucella* genus. The PCR reaction was carried out at 93°C for 5 minute followed by 35 cycles of denaturation at 90°C for 1 minute, annealing at 64°C for 30 seconds and extention at 72°C for 1 minute, with final extension at 72°C for 10 minutes[8]. The PCR products were analyzed by electrophoresis in 1.5% agarose gel at 100 V for 45 minutes and documented. Amplicon of size 223 bp is specific for *Brucella* genus.

Bru-AMOS Multiplex PCR for *Brucella* Species Identification

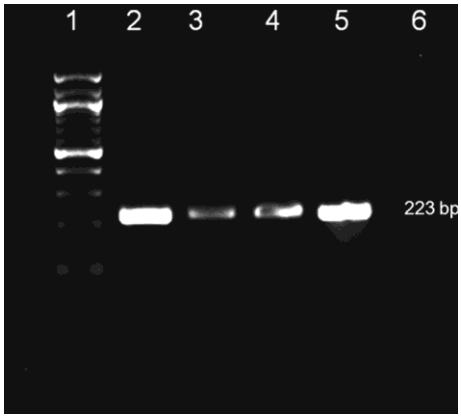
The PCR (BCSP 31 gene) positive samples were further subjected to Bru-AMOS PCR for *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* species identification and it was carried out as described by Bricker and Halling (1994)[9]. A total of 25 µl PCR reaction mixture consisted of 2X PCR mastermix, cocktail of 5 primers sets (20 pmol each) including *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis* and IS711 specific primer (Table 1) and 5 µl DNA template. The PCR reaction was carried out at 93°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 1.15 minute, annealing at 55.5°C for 2 minutes and extention at 72°C for 2 minutes, with final extension at 72°C for 10 minutes. PCR products were analyzed by electrophoresis in 1.5% agarose gel at 100 V for 45 minutes and documented. Specific amplicon of 498 bp is specific for *B. abortus*, 731 bp is specific for *B. melitensis*, 976 bp is specific for *B. ovis* and 285 bp is specific for B. *suis*.

Results

The study revealed that 8.92% (n = 10) serum samples from sheep, 9.75% (n = 8) serum samples from goat and 6.81% (n = 3) serum samples from cattle were seropositive by RBPT. All the twenty one seropositive samples produced specific amplicon of 223 bp by BCSP 31 PCR confirms *Brucellosis* (Fig. 1). Further molecular typing of BCSP 31-PCR positive samples by *Brucella* -AMOS PCR revealed specific amplicon of 498 bp indicating the involvement of *B. abortus* in 19 serum (10 from sheep, 6 from goat and 3 from cattle) samples. One serum sample from caprine revealed specific amplicons of 498 bp and 731 bp indicating the involvement of both *B. abortus* and *B. meletensis*. Another serum sample from goat yielded specific amplicons of 498 bp, 731 bp and 285 bp suggesting the mixed infection of B. *abortus*, *B. meletensis* and *B. suis*, respectively (Fig. 2).

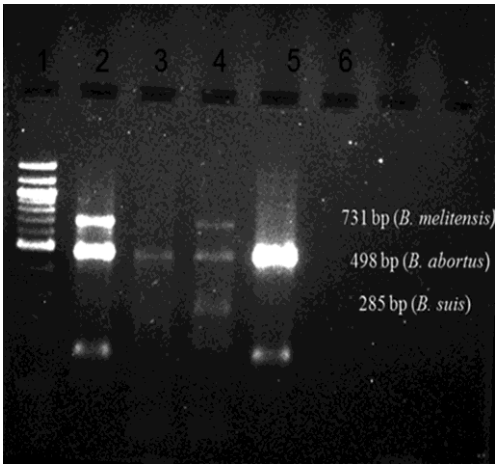
Table 1 — Primer sequences (5´-3´) for AMOS PCR (Bricker and Halling, 1994)

B. abortus specific primer;
GACGAACGGAATTTTTCCAATCCC
B. melitensis specific primer;
AAATCGCGTCCTTGCTGGTCTGA
B. ovis specific primer;
CGGGTTCTGGCACCATCGTCG
B. suis specific primer;
GCGCGGTTTTCTGAAGGTTCAGG
IS711 specific primer;
TGCCGATCACTTAAGGGCCTTCAT



Lane 1:100 bp ladder
Lane 2: Caprine serum
Lane 3: Cattle serum
Lane 4: Ovine serum
Lane 5: Positive control
Lane 6: Non template control

Fig 1. — BCSP 31 gene based PCR.



Lane 1:100 bp ladder
Lane 2: Caprine serum 1
Lane 3: Cattle serum
Lane 4: Caprine serum 2
Lane 5: Ovine serum
Lane 6: Non template control

Fig. 2 — Bru-AMOS multiplex PCR.

Discussion

This study reported 8.92% of serum samples from sheep, 9.75% of serum samples from goat and 6.81% of serum samples from cattle were seropositive for brucellosis by rose bengal plate agglutination test (RBPT). Singh *et al* (2010)[10] reported that the prevalence rate of brucellosis is 8.58% in cattle, 8.85% in goat and 7.08% in sheep from the states of Rajasthan and Bihar by RBPT. The B4/B5 primer pair was used in many parts of world to amplify BCSP 31 gene that confirms *Brucella* genus[8]. Garshasbi *et al* (2014)[11] compared the sensitivity and specificity of two target genes viz., BCSP31 and IS711 to detect *Brucella* DNA in human blood or serum samples and showed that both the targets were 96% sensitive in *Brucella* detection. The first multiplex PCR, called AMOS PCR[9] for *B. abortus, B. melitensis, B. ovis,* and *B. suis*, was published in 1994. This method could detect biovar 1, 2 and 4 of *B. abortus*, biovars 1, 2 and 3 of *B. melitensis* and biovar 1 of *B. suis* and *B. ovis*. This assay could not differentiate individual biovars within a species.

In this study serum samples were used for the extraction of DNA for molecular detection of brucellosis. There are only very few studies used serum samples for amplifying *Brucella* DNA. However, the use of serum instead of whole blood samples offers several advantages for nucleic acid amplification methods. The quality and quantity of *Brucella* DNA extracted from whole blood may be hampered by anticoagulants, hemoglobin and host cell DNA present in it, but not in serum. Red blood cell lysis, washings by centrifugation and adjustment of isolated DNA concentrations are not required when serum is used as a sample for nucleic acid extraction. The origin of pathogen nucleic acids in serum samples, most probably they are released in the circulation as breakdown products during bacteremia. Several studies have documented the presence of circulating pathogen DNA in serum samples[12]. Serum samples collected from aborted cows, buffaloes, ewes and goats were positive with the genus specific BCSP31 real-time PCR assays.

The present study also revealed out of 21 serum samples screened 19 serum (10 from sheep, 6 from goat and 3 from cattle) samples showed *B. abortus* indicating as this is the predominant species identified with the clinical infections of cattle, goat and sheep. Similar results were reported by Wareth *et al* (2015)[13] and showed *B. abortus* was the common species in all the serum samples collected from cows, buffaloes, ewes and goats. Darshana *et al* (2016)[14] also revealed that 15 isolates from goats and 20 isolates from sheeps were positive for *B. abortus*, 3 isolates from goats were positive for *B. melitensis* and 2 isolates from goats were positive for *B. ovis* indicating involvement of *Brucella* species to non-preferred hosts. This study revealed mixed infection of *B. abortus, B. meletensis* and *B. suis* species in two

caprine samples. Aparicio (2013)[15] reported both *B. abortus* and *B. melitensis* infection in one animal and demonstrated that one host can be infected with two different species of *Brucella* at the same time. Small ruminants are raised with cattle in close contact in the same pasture; transmission of host specific *Brucella* species to non-preferred hosts may occur. Brucellosis in ruminants may involve any of the *Brucella* species but *B. abortus* is the predominant species in many instances it is associated with clinical infections in cattle, goat and sheep[6,13]. Accidental *B. abortus* infections in small ruminants may even play a role for the persistence of brucellosis in cattle. The possible reason for mixed *Brucella* infection could be sheep and goats are reared alongside dairy units and transmission could be through person, vehicle, fodder, drainage etc. Expansion of animal industries, urbanization, lack of hygienic measures in animal husbandry and in food handling partly account for the spread brucellosis in humans. Future studies are necessary to investigate the ability of *Brucella* isolates to be transmitted to and replicate outside its preferred host species in field conditions. Detection and characterization animal brucellosis in wider geographical region is highly essential for prevention and control of brucellosis in southern districts of Tamil Nadu.

Acknowledgements

The authors are thankful to The Dean, Veterinary College and Research Institute, Tirunelveli and Tamil Nadu Veterinary and Animal Sciences University for the facility and support.

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