Explor Anim Med Res.
Vol. 14, Issue 2, 2024
DOI: 10.52635/eamr/14.2.279-286

ISSN 2277-470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

MOLECULAR EVIDENCE OF *BRUCELLA ABORTUS* ASSOCIATED REPRODUCTIVE FAILURE IN DOMESTIC SWINE- AN UNEXPLORED FIELD STUDY IN SOUTHERN INDIA

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Received 30 March 2024, revised 26 October 2024

ABSTRACT: Brucellosis is an infectious bacterial zoonotic disease of domestic and wild animals frequently caused by *Brucella abortus, B. ovis, B. melitensis, B. suis,* and *B. canis* species. The present study was conducted on 100 serum samples from swine with a history of abortion and reproductive failure in the southern states of India. The samples were screened by Rose Bengal plate agglutination test (RBPT) and standard tube agglutination test (STAT) for detection and quantification of brucella antibody respectively. The samples were also subjected to 16S rRNA gene and Brucella cell surface salt-extractable (BCSP 31) based PCR assays for Brucella genome screening and confirmation. Brucella genome-positive samples were also subjected to AMOS multiplex PCR for *B. abortus, B. ovis, B. melitensis,* and *B. suis* species differential diagnosis. The serological screening revealed 3% (n=3) seropositivity for Brucellosis with antibody tires of 1: 160, 1:160, 1:320 for TL7, TL12 and KA-9 samples respectively. Molecular screening evidenced 5% (n=5) positivity for the Brucella genome including three seropositive samples yielded a specific amplicon of 800 and 223 bp amplicons by 16S rRNA and BCSP-31 PCR respectively. Further species identification by AMOS PCR yielded an amplicon of 498 bp specific to *B. abortus* genome in all three seropositive samples (TL7, TL12, and KA-9) and two PCR-positive samples (TN17 and KA-21) yielded 285bp amplicon specific to *B. suis*. Molecular screening of Brucella-positive samples for viral co-infections such as PCV2, PPV, CSFV, and PRRSV evidenced non-involvement of above screened 4 viral aetiologies in all 5 Brucella-positive samples indicating the primary infection of Brucella species.

Keywords: Swine, Reproductive failure, *B. abortus*, Seropositive.

INTRODUCTION

In recent years, Brucellosis has been re-emerging zoonotic infectious bacterial disease endemic in Asian and African continents affecting both domestic and wild animals. Shifts in geographical distribution and expanding its host range spectrum contribute to its persistence and prevalence [1]. *Brucella abortus, B. melitensis B. suis, B. canis,* and *B. ovis* - associated field infections in animals are predominant and associates with human transmission except *B. ovis* [2]. The prevalence of brucellosis in Indian livestock populations was estimated to be 12% accounting for over 500,000 cases with economic losses to the tune of 3.4 billion US $ including the loss of US$ 7.1 million attributable to brucellosis in swine [3]. Clinical outcomes of Brucella-infected swine are infertility, reproductive failure (abortion, stillbirth, with dead or weak piglets) in mature sows; orchitis in boars, and lameness and paralysis in either sexes. Both clinically affected and asymptomatic pigs disseminate Brucella

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organism in all body secretions including semen contributing to horizontal and venereal transmission [4] As per the World Organization for Animal Health (WOAH) *B. melitensis* followed by *B. abortus, B. suis* is and *B. canis* is the order of brucellosis threat to human populations. Livestock brucellosis associated with any of the above Brucella species is crucial; it must be promptly diagnosed and contained with utmost care to prevent economic loss in livestock and human transmission.

PCR based molecular detection assays are a reliable approach for identification and typing of *Brucella* species directly from animal field samples with high specificity and sensitivity which may be used as a rapid alternate approach on par with stringent gold standard culture-based identification and characterization [5]. Serological investigation of swine brucellosis all over India in 5431 stratified random serum samples collected during 2018-2019 recorded an apparent prevalence of 4.33 percent [6]. Meta-analysis on the prevalence of *Brucella* spp across Indian pigs during 2010-2023 with 22,846 events through common effect and random effects models estimated overall prevalence of nine percent and six percent respectively evidencing its significant prevalence in Indian pigs [7]. Though a higher prevalence of brucellosis is documented in swine populations in India, detailed data on different *Brucella* species associated with field infections and their possible routes and sources are scarce [8]. As of date, there is no serum-based molecular detection study on brucellosis documented from swine populations of Tamil Nadu. This paper documents the foremost report of *B. abortus* genome-associated reproductive infection in swine and its containment in southern India.

MATERIALS AND METHODS

Sample collection and preliminary examination

This study is undertaken in 100 serum samples from domestic swine belonging to Large White Yorkshire and its crossbreeds in organized farms collected from suspected animals with a history of stillbirth, abortion, and reproductive failure in January to December 2021 from Andhra Pradesh, Karnataka, Tamil Nadu, and Telangana states in India. Sample details along with vaccination history are presented in Table 1.

Ethical requirements

This research work was done in only suspected field samples, which don't have any specific experimental procedures and invasive techniques hence institutional animal ethics committee approval was not obtained for this study. Oral and written consent from swine farm authorities was obtained before the collection of the blood samples and survey questions. All the pig samples in this study are sampled by a qualified veterinarian following all applicable guidelines and care.

Serological screening for Brucella antibodies

All the 100 samples in this study were initially screened for Brucella-specific antibodies by Rose Bengal plate agglutination test (RBPT) and standard tube agglutination test (STAT) for detection and quantification respectively according to the standard procedure described in WOAH, 2022. *B. abortus* S99 colored antigen plain antigens for serological screening by RBPT and STAT respectively were procured from the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu, India.

Molecular screening for Brucella genome by PCR assay

DNA was extracted from all the serum samples using commercial DNeasy Blood and Tissue Kit (Qiagen, USA) and subjected to molecular screening targeting 16S rRNA gene and Brucella cell surface salt-extractable (BCSP31) protein-based PCR assays for Brucella genome detection and confirmation. An 800 bp fragment of 16S rRNA was amplified with primers and cycling conditions as reported by Bricker *et al*. [9]. Simultaneously PCR targeting 223 bp portion of the 31kDa Brucella cell surface salt extractable protein (bcsp31) gene of *Brucella* was amplified with primers and cycling conditions as reported by Baily *et al*. [10].

Identification and differentiation of *Brucella* species by multiplex AMOS-PCR

All the PCR-based Brucella genus positive samples were further subjected to AMOS multiplex PCR for *B. abortus, B. ovis, B. melitensis,* and *B. suis* identification with four specific forward primers for *B. abortus, B. melitensis, B. ovis*, and *B. suis* with 20 picomoles concentration of each and one common universal reverse primer IS711 with 100 picomoles concentration (Table 2). PCR cycle conditions include initial denaturation of 93°C for 5 min followed by 35 cycles starting with denaturation at 95°C for 1.15 min, annealing at 55.5°C for 2 min. and extension at 72°C for 2 min with a final extension at 72°C for 10 min. Specific amplicons 976

bp, 731 bp, 498 bp, and 285 bp specific for *B. ovis, B. melitensis, B. abortus,* and *B. suis* respectively confirm its molecular positivity [11].

Molecular screening of Brucella-positive samples for other viral co-infections

All five brucella-positive samples were further subjected to DNA viruses which results in reproductive failure such as porcine circovirus 2 (PCV2) [12] and porcine parvovirus genome (PPV) [13]. Further from brucella-positive samples RNA was extracted by using TRIZOL® LS reagent (Invitrogen, USA Cat. No. 10001 96-010), and cDNA synthesis was carried out using PrimeScript™ RT reagent kit, (Takara, Japan, Cat.No.RR037A) following manufacturer instructions and screened for RNA viral aetiologies such as Classical swine fever virus (CSFV) [14] and Porcine reproductive and respiratory syndrome virus (PRRSV) [15] which may also contributes in reproductive failure. Primers used in the PCR screening for all these four pathogens are presented in Table 2.

RESULTS AND DISCUSSION

Seropositivity of brucellosis in swine

Serological screening of all 100 serum samples by RBPT assay revealed 3% (n=3) seropositivity for Brucella-specific antibodies (Fig. 1a). STAT-based quantification evidenced antibody tires of 1:160, 1:160, and 1:320 for TL7, TL12 (Telangana), KA-9 (Karnataka-9) samples respectively (Fig. 1b). Seropositivity of brucellosis in this study was 3% (n=3) whereas, molecular positivity was 5%(n=5), apart from three seropositive samples, two seronegative samples were also found to be positive for brucella genome by both screening and species differentiation PCR assays confirming higher sensitivity of PCR assays in comparison to both RBPT and STAT serological assays. A study in the year 2020, screened 3597 pig serum samples from Meghalaya using Rose Bengal Plate Test (RBPT) and indirect ELISA revealed 0.36% (n=13) positivity by RBPT and 2% (n=72) positivity by Indirect ELISA [16]. But, earlier studies in India from 1980-2010 on sero-prevalence of brucellosis in pigs ranged

Table 1. Details of Serum samples screened for *Brucella* genome.

Sample source (State)	Location, type of farm and Latitude & longitude	Farm size	Vaccination History	No of samples collected	No of samples positive RBPT and STAT	No of samples positive by PCR (AMOS based species typing)
Tamil Nadu	Chennai, Organized, 13.1488° N, 80.2306° E	100	Vaccinated only for CSFV, FMD; Non vaccinated for PPV & PCV2	10	0	0
Tamil Nadu	Chengalpattu, Organized, 12.8259° N, 80.0395° E	320	Vaccinated for CSFV , FMD & PCV2 Non vaccinated for PPV	20	0	1 (*B. suis*)
Andhra Pradesh	Tirupati-Organized 13.6288° N, 79.4192° E	100	Vaccinated for CSFV, FMD; Non vaccinated for PPV& PCV2	10	0	0
Karnataka	Anekal, Organized, 12.7091° N, 77.6992° E	200	Vaccinated for CSFV and FMD; Non vaccinated for PPV & PCV2	15	1	1 *(B. abortus)*
Karnataka	Chikkaballapur, Organized, 13.4355° N, 77.7315° E	200	Vaccinated for CSFV and FMD; Non vaccinated for PPV& PCV2	15	0	1 (*B. suis*)
Telangana	Hyderabad, Organized, 17.4065° N, 78.4772° E	500	Vaccinated for CSFV and FMD; Non vaccinated for PPV& PCV2	30	2	2 (*B. abortus*)
Total				100	3	5

Table 2. Details of primers used in this study.

Primer name	Primer sequence (5'-3')	Product size (bp)	Reference
Primers used for screening of Brucella genome			
16S rRNA- Bru FP	GTGCCAGCAGCCGCGGTAATAC	800	[9]
16SrRNA- Bru RP	TGGTGTGACGGGCGGTGTGTACAAG		
BCSP31- FP	TGGCTCGGTTGCCAATATCAA	223	[10]
BCSP31--RP	CGCGCTTGCCTTTCAGGTCTG		
Primer detail of AMOS PCR used in the differentiation of four Brucella species			
B. abortus FP	GACGAACGGAATTTTTCCAATCCC	498	[11]
B. melitensis FP	AAATCGCGTCCTTGCTGGTCTGA	731	
B. ovis FP	CGGGTTCTGGCACCATCGTCG	976	
B. suis FP	GCGCGGTTTTCTGAAGGTTCAGG	285	
IS711 RP	TGCCGATCACTTAAGGGCCTTCAT	-	
Primers used in screening of PCV2 genome			
PCV2-FP	TAGGTTAGGGCTGTGGCCTT	264	[12]
PCV2-RP	CCGCACCTTCGGATATACTG		
Primers used in screening of PPV, CSFV & PRRSV genomes			
PPV-FP	AGTTAGAATAGGATGCGAGGAA	265	[13]
PPV-RP	GAGTCTGTTGGTGTATTTATTGG		
CSFV-FP	GACACTAGYGCAGGCAAYAG	449	[14]
CSFV-RP	AGTGGGTTCCAGGARTACAT		
PRRSV-FP	TGACACCTGAGACCATGAGG	803	[15]
PRRSV-RP	GTGCAAAAGCCCTAGCAGTC		

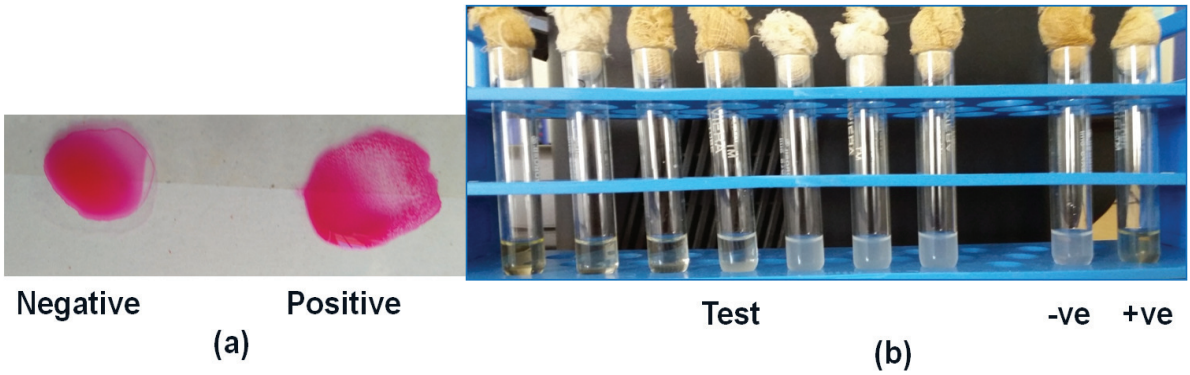
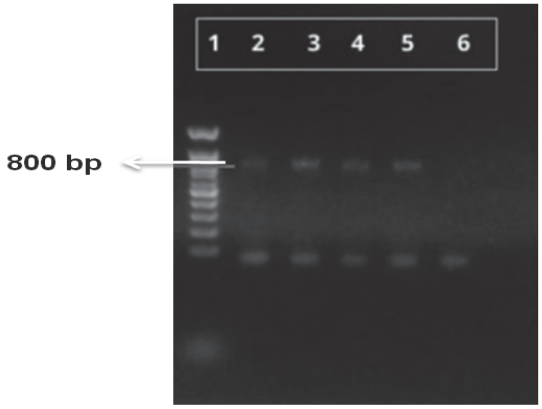


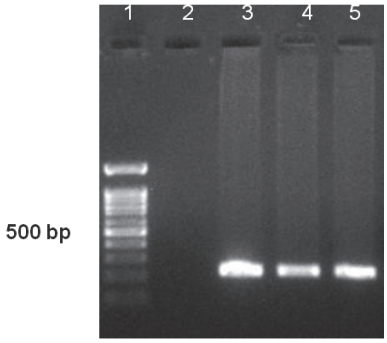
Fig. 1. Serological Screening for *Brucella* antibody. [(a): Rose Bengal agglutination test; (b): Standard tube agglutination test].

from 6.3% and 9.5% in Karnataka [17], 11.3% in Tamil Nadu [18], 16.7% in Punjab and Himachal Pradesh [19] and 87.10% in Assam [20]. A nationwide seroprevalence study in the year 2016 with 2576 serum samples from 10 states of India showed apparent prevalence of 14.2% [21]. A study in Karnataka with a serological screening of 575 swine serum samples by both RBPT and iELISA assays for brucellosis from organized farms in Karnataka with a history of abortions and stillbirth documented seropositivity of 41.04% [8] witnessing unpredictable brucellosis incidence rates across the nation. Most of the retrospective studies across India and other countries indicated varying brucellosis prevalence probably due to varying sampling strategies or at times it is very difficult to generalize the prevalence status in a particular geographical region which recommends systematic sampling and serological and molecular screening of swine at herd level and individual levels at a periodic interval.



Lane 1:100 bp ladder
Lane 2-4: Serum samples
Lane 5: Positive control
Lane 6: Non Template control

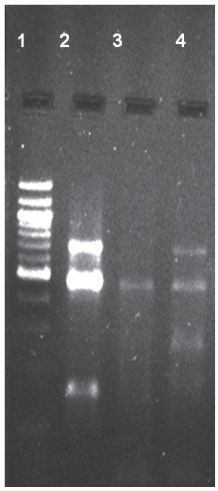
(a)



Lane 1:100 bp ladder
Lane 2: Non Template control
Lane 3: Positive control
Lane 4: Swine Serum with history of abortion
Lane 5: Swine Serum with history of abortion

(b)

Fig. 2. Molecular screening and confirmation of *Brucella* spp. [(a): *Brucella* genome screening by 16SrRNA gene; (b): *Brucella* genome screening by BCSP-31 gene].



Lane 1:100 bp ladder
Lane 2: ***B. abortus and B. meletensis*** **Positive control**
Lane 3: Test Porcine serum
Lane 4: ***B. abortus, B. meletensis & B.suis*** **Positive control**

Fig. 3. *Brucella* species differentiation by AMOS-PCR.

Detection of brucellosis genome by genus-specific PCR

Molecular screening of all 100 serum samples in this study evidenced 5% (n=5) positivity for the Brucella genome including three seropositive samples TL7, TL12, KA-9 and in addition, two TN17 (Tamil Nadu-17) and KA-21(Karnataka-21) samples which are negative in serological screening also yielded a specific amplicon of 800 bp (Fig. 2a) and 223 bp (Fig. 2b) in both 16S rRNA and BCSP31 gene PCR respectively confirming the presence of Brucella genome in all 5 samples. All five brucellosis-positive animals belong to LWY and none were found positive from crossbreeds in this study.

Handling genetic material from field samples especially serum in the laboratory greatly reduces the chances of occupational exposure to Brucellosis in humans when compared to the isolation of *Brucella* from aborted contents [22]. Mukherjee *et al*. [23] analyzed csp31, omp2, and 16S rRNA gene-based specific PCR assays for Brucella diagnosis in Indian bovine populations witnessed bcsp31gene-based PCR as most sensitive in comparison to other gene-based detection methods. Whereas, this study revealed equal sensitivity of BCSP31 and 16SrRNA genes based *Brucella* genome detection in swine samples.

Identification of *Brucella* species in field infections by multiplex AMOS-PCR

Brucella species identification in all five (TL7, TL12, KA-9, KA-21, and TN17) samples by Brucella-AMOS PCR revealed a specific amplicon of 498 bp corresponding to *B. abortus* genome in all three seropositive TL7, TL12, KA-9 samples and remaining two PCR positive samples TN17 and KA-21 yielded 285 bp amplicon corresponding to *B. suis* (Fig. 3). No involvement of *B. meletensis* and *B. ovis* genomes could be detected in this study.

Nagalingam *et al*. [24] typed *Brucella* isolates from Indian livestock through Bruce-Ladder and AMOS-

PCR assays and detected the *B. abortus* genome from the *Brucella* isolates of the pig but its association with field infection is not completely explored whereas, the present study evidences *B. abortus* associated field reproductive failure in domestic Large White Yorkshire. Outside India, Venezuela recorded varying seroprevalence rates of brucellosis in swine ranging from 5 to 89% [25]. Whereas, a study in Kenyan pigs revealed 0.57% (4/700) prevalence of brucellosis by RBPT, and all these sera were also positive by PCR, while two sero-negative samples also tested positive on qPCR. *B. abortus* was detected in four out of the six PCR-positive samples [26] as evidenced in the present study.

Predominantly, brucellosis in cattle is associated with *B. abortus*, but, involvement of *B. melitensis* and B. suis-associated infections were also documented indicating infections of any *Brucella* species in non-preferred hosts [27, 28]. Cvetnic *et al*. [29] isolated *B. suis* from horses in Croatia evidencing cross-species infection of *B. suis.* Higgins *et al.* [30] isolated B. abortus from swine herds in the close vicinity of ruminants farms in Greater Yellowstone Area (GYA) and Texas from 1998 to 2011 and De Massis *et al*. [31] isolated *B. melitensis* from swine herds associated with ruminants in Italy evidencing adaptability and infectious ability of different *Brucella* species in different species of livestock. A study in southern India serum-based *Brucella* species typing through AMOS PCR evidenced *B. abortus* as a common species involved in cattle, sheep, and goat infections in addition *B. abortus, B. meletensis, B. suis* mixed infections were detected in goats and documented that serum can be an alternate specimen for the rapid and reliable molecular diagnosis of Brucellosis in ruminants [32].

Molecular screening of *Brucella*-positive field samples for other viral co-infections

Screening of all five Burcella positive (TL7, TL12, KA-9, KA-21, and TN17) samples for DNA viruses such as PCV2, PPV, and RNA viruses such as CSFV and PRRSV evidenced non involvement of all above four viral aetiologies in the *Brucella* positive samples indicating the primary infection and reproductive failure of *Brucella* species. An extensive review of the literature evidenced no mixed infection of brucellosis with other viral pathogens in domestic pigs witnessed which aligns with this study.

The source of infection in the study locations could not be identified in this study. Brucellosis-infected animals can shed organisms in semen, uterine/vaginal discharges, placenta, and tissues from abortions/dead piglets, as also in urine and milk, and can be transmitted through both direct contact with mucous membranes and ingestion [8, 33]. Feed, water, and vegetation that have been contaminated with *Brucella* spp. can also possible transmission routes for in pigs [34]. All five brucellosis-confirmed individual pigs were isolated retested and culled from the rest of the swine population in all the study farms to prevent further spread of this infection and subsequent screening of pigs in farms for brucellosis 3 months later evidenced complete seronegativity. Stringent biosecurity followed in all the study farms. Complete screening of farms, products, and associated human personnel associated with them is crucial in containing brucellosis transmission. Even though our study findings witnessed *B. abortus* infections in swine, its origin could not be found specifically which needs to be explored further by screening surrounding livestock farms and human handlers.

CONCLUSION

B. suis is the most commonly documented species in brucellosis of swine. However, the present study documents the foremost field incidence of *B. abortus* mediated primary reproductive infection in addition to *B. suis* infection in domestic pigs especially in the LWY breed when compared to its cross breeds in southern India. The possible source and route of *B. abortus, B. suis* infection in the present study locations are not explored. Feeding pigs on waste from the market could also contribute to the cross-transmission of *B. abortus* from ruminants to pigs. Screening of human personals associated with swine farm activities and other livestock populations surrounding the farm along with isolation and molecular characterization of brucellosis positive infections may through light on its possible source and transmission. The future study focusing on whole genome analysis of *B. abortus* and *B. suis* isolates associated with reproductive infection in swine may help in the identification of its biovar and serovar details which is essential for the development of suitable vaccine candidates to control brucellosis in swine.

ACKNOWLEDGMENT

The authors express sincere thanks to Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai -51, for all the necessary support. The authors express sincere gratitude. The authors extend sincere thanks Institute of Animal Health and Veterinary Biologicals, Bangalore-24.

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Cite this article as: S. Parthiban, Naidu S, Raja P, Parthiban M, Senthilkumar K, Muthuramalingam T, Balasubramanyam D, Ranganatha S, Sumanth Kumar R. Molecular evidence of *Brucella abortus* associated reproductive failure in domestic swine - an unexplored field study in Southern India. Explor Anim Med Res. 2024; 14(2), DOI:10.52635/eamr/14.2.279-286.