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Serological and molecular analysis for brucellosis in selected swine herds from Southern India

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ABSTRACT

Background: Swine brucellosis is a zoonotic disease caused by *Brucella suis*. The study describes the occurrence of brucellosis in two organized piggeries in Southern India.
Methods: A total of 585 serum samples comprising 575 from pigs and 10 from animal handlers were collected and tested by serological tests and PCR. Tissue samples were collected for isolation of the pathogen.
Results: Out of 575 serum samples screened, 236 (41.04%) were positive for brucellosis by both Rose Bengal plate test (RBPT) and indirect ELISA (iELISA) and 47 (8.17) samples showed *Brucella* DNA amplification by genus specific PCR. The sows those aborted and 19 boars with orchitis were seropositive for brucellosis indicating association of clinical symptoms with brucellosis seropositivity. Two of 10 pig handlers were positive by RBPT and showed significant serum agglutination test (SAT) titres of >1:160 and 1:320. *B. suis* bvI was isolated and identified by biochemical tests and confirmed by amplification *Brucella* genus and Bruce ladder PCRs from vaginal and testicular samples.
Conclusions: The introduction of untested breeding boars in the farms might have resulted in the disease transmission and spread. The present study highlighted the diagnosis of *B. suis* bvI as a cause of abortions in the pigs and occupational exposure to farm personnel.

Introduction

Brucellosis in swine is a contagious disease with greater zoonotic potential [1] and clinically characterized by infertility, abortion and birth of dead or weak piglets in sows; orchitis and infection of secondary sex organs in boars and lameness and paralysis in both sexes [2]. The disease is generally transmitted during copulation and by consumption of feed contaminated by birth and/or abortion products and uterine discharges [3]. Transmission through the conjunctiva or via broken skin and by inhalation were also reported in pigs [4]. Both symptomatic and asymptomatic boars excrete *Brucella* in semen, hence, venereal transmission is also common [2].

There are several methods for diagnosis of *Brucell*a infection, however, isolation of the pathogen remains the most reliable method [5,6]. Isolation and identification of *Brucella* is time-consuming, hazardous, laborious and suffers with the limitation of low sensitivity and hence serological and molecular detection of antigens/antibodies and nucleic acids are employed frequently.

Swine brucellosis is enzootic and highly prevalent in South America and South East Asia. The disease is sporadically recorded in Europe, Asia, and Oceania, while, Israel recorded free status [7]. In India, annual economic median loss of US$ 7.1 million due to porcine brucellosis has been reported [8]. Though higher prevalence of porcine brucellosis with greater economic losses and zoonotic transmission cases have been reported in India, still the routes of disease transmission and associate risk factors are unknown [9]. The objective of the present study was to investigate brucellosis outbreak by multimodal diagnostic approaches in two

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Table 1
Serological and PCR analysis for serum samples collected from pigs.

Farm	Total animals in the farms	Total animals sampled	Male/female animals	RBPT & iELISA positive	χ^2	P value	PCR positive	χ^2	P value
1	800	357	Male — 36	18 (50.00)			10 (27.77)		
			female — 321	161 (50.15)	0.02	0.887	26 (8.09)	8.27	0.004*
	Total			179 (50.14)			36 (20.11)		
2	650	218	Male — 13	3 (23.07)			2(15.38)		
			female — 205	54 (26.34)	0.01	0.9203	9 (4.39)	0.95	0.329
			Total	57 (26.14)			11 (5.04)		
Total	1450	575		236 (41.04%)			47 (8.17%)		

* P value <0.05 is considered statistically significant.

organised swine farms in southern India which had experienced high incidence of abortions and orchitis.

Materials and methods

Study area and samples

The two organised pig farms were located about 50 km apart in the outskirts of Bengaluru, Karnataka, Southern India and has a steppe climate with temperature ranging from 28–33 °C and humidity 46–52% and the area receives 800–1000 mm rainfall. The farmers had basic knowledge about rearing, breeding, management, vaccination against common infectious diseases like classical swine fever, haemorrhagic septicaemia and foot and mouth disease. Swine were fed with mixture of maize, ground nut cake and mineral mixture and ground water as a source for drinking and cleaning. Natural breeding was practiced in the farms and none of the animals were vaccinated or tested for brucellosis.

The farmers procured (farm-1 = 10 and farm-2 = 6) landrace boars from outside the state for breeding purpose. The problems of abortions and still births erupted in the farm 4–6 months after introduction of new boars. Based on the clinical signs such as abortions in later stage of pregnancy, still births, lameness of hind legs due to swollen knee joints, orchitis and enlargement of lymph nodes, veterinarian attending the farms advised for brucellosis testing. Demographic data, history in structured questionnaire and clinical samples were collected.

A total of 166 abortions (farm 1—158; farm 2—08) and 70 still births (farm 1—48; farm 2—22) were reported. The animals showed symptoms of lamness due to swollen knee joints especially hind legs (71), orchitis (19) and enlargement of lymph nodes (5). Blood samples (about 3 ml each) were collected from 357 of 800 animals from farm-1 and 218 of 650 animals from farm-2 from the ear vein of animals above 6 months of age in vacutainers without anticoagulant (Becton Dickson, UK). Serum samples were stored at −20 °C until tested. Vaginal swabs from 4 aborted sows and testicular tissue material from 2 boars showing enlarged testicles were collected aseptically into the *Brucella* selective broth containing antibiotic supplements (Pronadisa-Conda, Spain) and transported to laboratory on ice within 2 h of collection for isolation. Ten blood samples (farm 1 = 6 and farm 2 = 4) of 5 ml each were drawn from animal handlers in vaccutainers without anticoagulants after obtaining the verbal and written consents. The age, sex, duration of association with pigs, clinical symptoms and treatment, if taken, were recorded.

Bacterial cultures

The reference cultures *Brucella melitensis* 16 M, *Brucella abortus* S99, *Brucella suis* 1330 cultures were procured from National *Brucella* Culture Repository, Indian Veterinary Research Institute, Izatnagar, India and *E. coli* culture from ATCC (ATCC 25922).

Serological screening of serum samples

The serum samples (n = 575) from pigs and animal handlers (n = 10) were tested by Rose bengal plate test (RBPT) and results were rated as negative when agglutination was absent and 1+ to 3+ as positive, according to the strength of the agglutination observed within 1–3 min [5]. The RBPT positive human serum samples were further tested by serum agglutination test (SAT) by preparing two-fold serial dilutions of the serum starting at 1:10–1280 dilution according to Weybridge technique [5]. The highest dilution of the serum which showed 50 percent agglutination was considered end point titre and titres of 1:160 (320 IU/ml) and above were declared as positive for human brucellosis [10]. *B. abortus* S99 coloured and plain antigens for RBPT and SAT, respectively were procured from Institute of Animal Health and Veterinary Biologicals, Hebbal, Bengaluru-560024, India. Indirect ELISA (iELISA) to detect antibodies against *Brucella* was standardized and evaluated in our laboratory using rabbit anti-swine IgG-HRP conjugate (Sigma, USA) [1,9].

PCR

DNA was extracted from serum samples using QIAamp DNA Mini kit (Qiagen, Germany) as per the instructions of the manufacturer. The purity and quantity of the DNA were assessed using NanoDrop 2000 (Thermo Scientific, USA) and stored at −20 °C until used. Primers targeted to amplify the 223 bp fragment of *Brucella* cell surface 31 kDa (*bscp31*) and PCR conditons as described earlier [11] were used in the present study. The primer sequences were: Forward 5-TGGCTCGGTTGCCAATATCAA-3′ and Reverse 5-CGCGCTTGCCTTTCAGGTCTG-3′. The amplified PCR products were analysed on 1.5% agarose gel electrophoresis and stained with ethidium bromide (1.0 μg/ml). DNA extracted from serum sample spiked with *B. abortus* S99, *B.suis* 1330, *B. melitensis* 16 M and *E. coli* were used for PCR positive and negative controls, respectively.

Isolation of Brucella *from clinical samples*

The isolation of the pathogen was attempted in biosafety level-II plus laboratory facility. The clinical samples collected in *Brucella* selective broth were incubated with and without 10% CO_2 at 37 °C for 72 h. A loopfull of broth culture from both the sets were streaked onto *Brucella* selective agar containing supplements (Pronadisa-Conda, Spain) and incubated with and without 10% CO_2 at 37 °C till the appearance of growth or up to one week. The suspected colonies were purified, stained by Gram's and modified Ziehl Neelsen (ZN) staining and tested for oxidase, urease, H_2S in triple sugar iron agar and dye sensitivity tests (1:25 000, 1:50 000 and 1:100 000 dilutions of thionin and basic fuchsin, Himedia, Mumbai) [5]. Positive and negative reference cultures were simultaneously tested to interpret the biochemical test results. The cultures were tested for agglutination with monospecific anti-A serum procured from Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15

3NB, United Kingdom. DNA extracted from purified colonies (Qiagen, Germany) were amplified by genus specific primers [11] and multiplex Bruce ladder PCR [12].

Results

Out of 1450 pigs housed in two piggeries, 357 (44.62%) were sampled from farm-1 and 218 (33.53%) from farm-2 including pigs having a history of abortion, stillbirths, symptoms of orchitis and apparently healthy pigs. Overall, out of 575 serum samples screened, 236 (41.04%) were positive by both RBPT and iELISA (Table 1). Farm-1 had higher seropositive pigs (50.14%) than farm-2 (26.14%).

Higher seroprevalence was recorded in males from farm-1 (50.0%) than the farm-2 (23.07%). Prevalence recorded in males was significantly higher (27.77%) than the females (8.09%) from farm-1 by PCR with P value 0.004. From farm-1, all sows having history of abortion and boars with orchitis were seropositive. Seropositivity was 45.23% and 34.78% in pigs suffering from infertility and lameness. Similarly, high seropositivity was observed in pigs with infertility (84.61%) and lameness (100%) at farm-2. Among the breeding boars, 10 out of 36 from farm −1 and 6 out of 13 from farm-2 were tested seropositive for brucellosis. Pigs with no clinical signs showed 6.19% and 10.50% seropositivity from farm-1 and 2, respectively (Table 2).

Brucella DNA was amplified by PCR in 20.11% samples from farm-1 and 5.04% samples from farm-2 with an overall detection rate of 8.17%. Three seronegative samples were positive by PCR. Out of 16 boars tested by PCR, 9 turned out to be positive (Fig. 1). Out of 6 clinical samples processed for isolation, one each of *Brucella* suspected colonies from vaginal and testicular samples from farm-1 appeared on the plates. The cultures were confirmed biochemically as *B. suis* bv I and amplification of the *bcsp* 31 genus and species specific Bruce ladder PCR (Fig. 2).

Of the 10 animal handlers tested, 2 of 6 animal handlers from farm-1 were positive by RBPT. The SAT titres of >1:160 and 1:320 in female and male handler, respectively were recorded. In PCR, one seropositive human serum sample showed positive amplification for the *bcsp* 31 genus specific PCR. Intermittent fever, backache, joint pains and redness in the eyes were the common symptoms with pain in testicle and orchitis in male animal handler.

Discussion

Swine brucellosis is widely prevalent in countries having pig population. India has 13.51 million pig population and piggery provides employment opportunities to rural and tribal farmers. Though Government of India had initiated a program for the control of brucellosis during 2011, it was limited to bovines and swine was not included. The lack of knowledge about swine diseases and veterinary supervision of swine herds are resulting in various diseases including brucellosis and causing huge economic losses to the piggery sector.

Investigations carried out in two pig farms having a history of abortions and stillbirths revealed overall seropositivity of 41.04%. In the pig farms investigated, 50% and 23% boars from farm-1 and 2, respectively and boars with orchitis were all seropositive. The farms had a history of introduction of new boars without brucellosis testing. The infected boars had been implicated for brucellosis spread in the farms through natural breeding [2]. Strict import controls and limited international movement of swine have prevented the introduction of swine brucellosis into Canada and now the country is free of brucellosis [13]. Compulsory screening of pigs before introducing into the farm, periodical brucellosis screening within the farms and removal of infected animals to prevent the spread

Table 2
Correlation of clinical symptoms with seropositivity for brucellosis.

Farm no.	Farm 1				Farm 2				Total (Farm 1 + Farm 2)			
Clinical signs	No. of animals tested	Sero positives	PCR positive	Sero negatives	PCR positive	No. of animals tested	Sero positive	PCR positive	Sero negative	PCR positive	No. of animals tested	Seropositivs	PCR positive	Sero negative	PCR positive
Abortion	111	111 (100.0)	18 (16.22)	0 (0.0)	0 (0.0)	07	07 (100.0)	4 (57.14)	0 (0.0)	0 (0.0)	118	118 (100.0)	22 (18.64)	0 (0.0)	0 (0.0)
Infertility	42	19 (45.23)	4 (21.05)	23 (54.76)	1 (4.35)	26	22 (84.61)	2 (9.09)	4 (15.38)	0 (0.0)	68	41 (60.2)	6 (14.63)	27 (39.70)	1 (3.84)
Orchitis	18	18 (100.0)	9 (50.0)	0 (0.0)	0 (0.0)	01	01 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	19	19 (100.0)	10 (52.63)	0 (0.0)	0 (0.0)
Lameness	69	24 (34.78)	1 (4.17)	45 (65.22)	1 (2.22)	02	02 (100.0)	1 (50.0)	0 (0.0)	0 (0.0)	71	26 (36.6)	2 (7.69)	45 (63.38)	1 (2.22)
Enlargement of lymph nodes	04	00	0 (0.0)	4 (100.0)	1 (25.0)	01	01 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	05	01 (20.0)	1 (100.0)	4 (80.0)	1 (25.0)
No clinical signs	113	07 (6.19)	1 (14.28)	106 (93.80)	0 (0.0)	181	24 (13.25)	2 (8.33)	157 (86.74)	0 (0.0)	294	31 (10.5)	3 (9.67)	263 (89.45)	0 (0.0)
Total	357	179 (50.1)	33 (18.43)	178 (49.86)	3 (1.68)	218	57 (26.14)	11 (19.29)	161 (73.85)	0 (0.0)	575	236 (41.0)	44 (18.64)	339 (58.95)	3 (0.88)

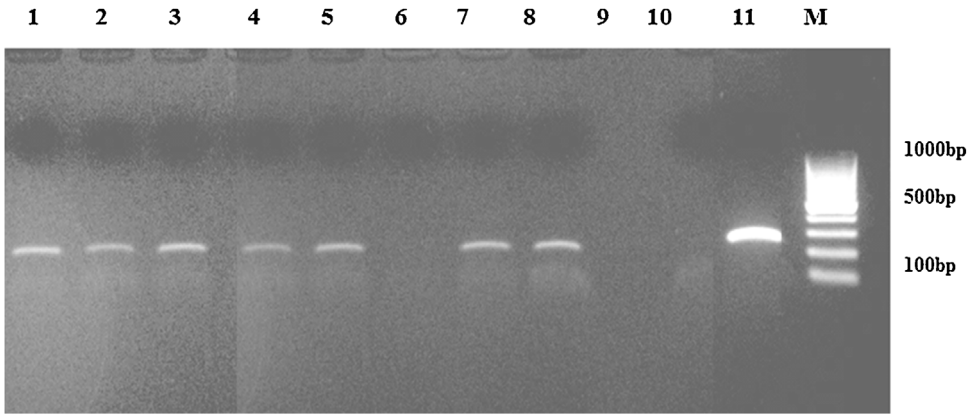

Fig. 1. *Brucella* genus specific PCR for swine serum samples: Lane 1–5 & 7–8: positive serum samples; Lane 6 & 9: negative serum samples; Lane 10: negative control; Lane 11: *B. suis* 1330; Lane M:100 bp DNA ladder.

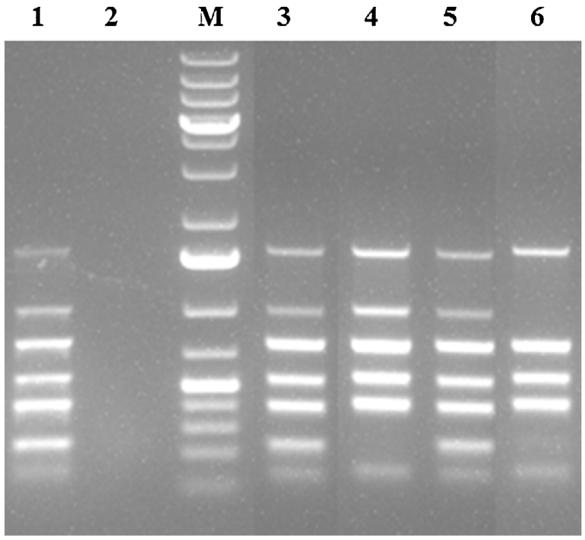

Fig. 2. Bruce ladder PCR for swine field isolates: Lane 1: *B. suis* field isolate (DBT BE1- S1); Lane 2: negative control; Lane 3: *B. suis* field isolate (DBT BE1- S2); Lane 4: *B. melitensis* 16 M; Lane 5: *B. suis* 1330; Lane 6: *B. abortus* S99; Lane M:100 bp DNA ladder.

of the disease are essential to curtail outbreaks. Swine brucellosis is basically a herd problem rather than individual pig and regular screening at the event of reproductive failure or outbreak is important to prevent further losses in the farm [3].

It has been observed that all the sows those aborted were seropositive for brucellosis indicating abortions due to brucellosis. Pigs with no clinical signs showed 6.19% and 10.50% seropositivity from farm-1 and 2, respectively suggesting brucellosis transmission in the sheds either through close contact with infected animals or aerosol or ingestion of contaminated feed. In India, varying seroprevalences in pigs have been reported from different states such as 3.2% from Madhya Pradesh [14]; 6.3% and 9.5% from Karnataka [15]; 11.3% from Tamil Nadu [16]; 16.7% from Punjab and Himachal Pradesh [17] and highest prevalence of 87.10% in pigs with history of abortion from Assam [18]. Recently, a nation wide seroprevalence study with 2576 serum samples from 10 states of India showed apparent prevalence of 14.2 (95% CI 12.9–15.6) and true prevalence of 7.2 (95% CI 5.6–8.7) [9]. Elsewhere, serological testing of 2,228 swine sera from six states of Venezuela revealed 5 to 89% prevalence [19]. These retrospective studies clearly indicated varying brucellosis prevalence probably due to different sampling strategies or at times it is very difficult to generalise the prevalence status in a particular geographical region.

Two serological tests are taken into consideration to declare brucellosis status [1] and samples positive by both RBPT and iELISA tests were interpreted positive for brucellosis in the study. The relative diagnostic sensitivity (Se) and specificity (Sp) of iELISA were 94.0% and 92.0%, respectively and kappa agreement with commercial indirect iELISA kit was 0.86 (95% CI 0.78–0.93) [20]. During test interpretations, a low number of samples were RBPT negative and iELISA positive (2.9%) or iELISA negative and RBPT positive (0.6%).

In this study, serum samples from two male animal handlers were RBPT positive with significant SAT titres and PCR amplification of *Brucella* DNA in one sample. It is well established fact that brucellosis infected animals act as a source of infection to humans through contact and food. Presence of *Brucella* antibody titers in farm workers in a brucellosis outbreak infected farm in Brazil [21] and *B. suis* infection in feral swine populations posing serious threat to livestock and humans [19] have been reported. In the present study, role of feral swine and other livestock near to the vicinity of the farms was ruled out. The pig handlers were exclusively associated with piggery activities and probably acquired infection while handling abortions/stillbirth piglets or while cleaning and feeding the pigs.

A number of genus specific PCR primers such as 16S rRNA [22], 16S-23S intergenic spacer region [23], *omp* 2[24] and *bcsp* 31[11] have been established. These assays were adopted for detection of genus and not the species of *Brucella* in different clinical specimens [25–27]. The *bcsp* 31 PCR carried out on all the samples collected for serology could detect *Brucella* DNA in 8.17% of the samples. Significantly higher sensitivity was reported for PCR due to its ability to detect DNA from bacteria that were stressed and nonviable than isolation by conventional culture methods [28–31]. In the PCR, three seronegative samples showed *Brucella* DNA amplification and this might be attributed to variability in detectable limits of antibody titres in different stages of the infection [31].The results indicated PCR as an important method to detect *Brucella* DNA directly in the clinical samples and has been suggested in diagnostic practices because of minimum risk, improved sensitivity and specificity and speed of performance [32]. The DNA isolated from serum samples was used for PCR in earlier studies by others [33] and also in our laboratory and compared with other methods [34,9].

Attempts to isolate the pathogen from clinical samples collected from farm-1 yielded one each isolate from vaginal and testicular samples. The isolates were biochemically identified as *B. suis* bv I and confirmed by Bruce ladder PCR. Earlier, isolation of *B. suis* bvII from a cow [16] and *B. suis* bvI and II to V from aborted sows have been reported [35,36,9].

Conclusions

The results of the present study confirmed *B. suis* infection in pigs farms and zoonotic transmission to pig handlers. Vaccination against swine brucellosis is not practiced in India and hence compulsory screening of pigs before introducing into the farm, continued farm surveillance and sensitising farmers on brucellosis in swine need to be strictly adopted to control the disease in swine herds and humans.

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Competing interests

None declared.

Ethical approval

The study was approved by Institutional Animal Ethics Committee, ICAR — National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India. Blood samples from animal handlers were collected as per the guidelines of Indian Council of Medical Research (ICMR), Government of India with verbal and written consents.

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