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Sero-prevalence and Molecular Detection of *Brucella* Species in Pig Producers of Punjab, India

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ABSTRACT

The current study was carried out to determine the sero-prevalence and molecular detection of *Brucella* species in persons involved in pork production in Punjab state of India. The sample size was selected using survey toolbox and a total of 123 blood samples were collected from pig farmers and slaughter house workers. The serum samples were tested using Rose Bengal Plate Test (RBPT), Standard Tube Agglutination test (STAT) and indirect ELISA. Polymerase chain reaction (PCR) on blood samples using B4 and B5 primers was carried out on all sero-positive and 20 randomly selected sero-negative samples. The Bruce ladder Multiplex PCR technique was further used for confirming the species of *Brucella* positive samples. The results observed that 1 (0.81%) and 4 (3.25%) subjects were positive in RBPT/STAT and ELISA, respectively. Polymerase Chain Reaction confirmed the presence *Brucella* species in blood of one of the sero-positive samples and Bruce ladder multiplex PCR confirmed the species to be *B. abortus*. Chi-square test was applied on the results to determine the significant difference among various groups. A significant difference was found in the prevalence rates in different districts in Punjab. The results indicate that *B. abortus* is circulating in the persons involved in pork production in Punjab state of India. The preventive and control measures need to be enforced to prevent the occupational exposure of brucellosis to pig producers in Punjab (India).

Keywords: *B. abortus*, PCR, prevalence, pig producers

Brucellosis is an important occupational zoonosis prevalent worldwide (Seleem *et al*., 2008). The important transmission routes for humans include ingestion of raw milk or meat from infected animals or close contact with their secretions (Elsberg, 1981; Godfroid *et al*., 2005). The occupational groups at risk include slaughter house workers, farmers and veterinarians (FAO, 2003).The source of human infection always resides in domestic or wild animal reservoirs. The disease has been reported in all the age groups, both sexes and even congenital cases have been reported (Corbel, 2006). The disease affects various domestic animals species (Renukaradhya *et al*., 2002) and wild animals. The important clinical symptoms include joint pain, weakness, low back pain and gastrointestinal symptoms (Kose *et al*., 2014).

The disease occurs due to Gram negative facultative intracellular bacteria belonging to Genus *Brucella* (family *Brucellaceae)*. The species in *Brucella* Genus are *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B.ceti and B.pinnipediae, B. microti* and *B. inopinata* (Sung *et al*., 2011). Brucellosis in pigs primarily occurs due to *Brucella suis*. The species *B. suis* consists of five biovars. biovars 1 and 3 are highly pathogenic causing severe disease while Biovar 2 is rarely pathogenic in humans. In humans, *B. melitensis* is the most pathogenic species followed by *B. suis*, whereas *B. abortus* is considered to cause the mild type of brucellosis (Galinska and Zagorski, 2013).

The diagnosis of human brucellosis remains a clinical challenge especially in the developing countries. The *Brucella* specific tests include culture, slide or tube agglutination, indirect Coombs, Enzyme linked Immunosorbent assay (ELISA), Indirect fluorescent antibody (IFA), and use of molecular techniques such as

Polymerase chain reaction (PCR) (Araj, 2003). The most widely used serological tests are Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and ELISA. Numerous PCR assays have also been developed for the rapid identification of *Brucella* species (Fekete *et al*., 1992; Klevezas *et al*., 1995; Amin *et al*., 2001).

In India, approximately 80% of the human population live in 575,000 villages and thousands of small towns (Raghunatha *et al*., 2014), have close contact with domestic/wild animal populations (Mantur and Amarnath, 2008). Pigs are very important species reared for meat purposes in various parts of country by diverse human segment groups. In India, there are 11.13 million pigs consisting of 2.38 million crossbred and 8.74 million indigenous pigs (DAHD, 2014). Generally, people involved in pork production belong to low socio economic groups and thus pork production is being carried out under unhygienic conditions with low input costs providing the circumstances for transmission of diseases from pigs to humans.

As the extensive studies on brucellosis in persons involved in pig production have not been carried out in North India, the current study was planned to estimate sero-prevalence and to detect *Brucella* species circulating in persons involved in pig production in Punjab (India).

MATERIALS AND METHODS

Place of work

The present study was carried out in the School of Public Health and Zoonoses, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

Selection of human subjects and study area

The cross-sectional study was conducted in Punjab state of Northern India during the year 2014-2015. The samples were collected from persons involved in pork production viz., farmers and slaughter workers. The sample size was determined using survey toolbox by taking into consideration the number of pig farmers in the state and the available disease prevalence data. As per 18th livestock census, there are 26000 pigs in Punjab, India; however, there is no direct data for number of persons engaged in pig production in Punjab (India). Based on published data, we assumed that each pig farmer owns 5 pigs (Nath *et al*., 2013), indicating that there could be about 5200 pig farmers in Punjab, India. The objective information on prevalence of infection in pig farmers was not available in published scientific literature. For estimating the sample size, we assumed that prevalence of infection in pig farmers will be half the prevalence as in cattle and buffalo farmers as quoted in a Nigerian study (Baba *et al*., 2001). (The prevalence of disease in cattle and buffalo farmers was taken as 10.5% based on Bedi *et al*. (2007) study. At the minimum expected prevalence of 5.25%, a sample size of 123 pig farmers was required to demonstrate freedom from disease. As most of the pig slaughter shop owners also keep pigs, they were also included in the current study. The selected subjects represented 90% of the total districts in Punjab (India).

The study was ethically approved by the Institutional Ethical Committee, Dayanand Medical College and Hospital, Ludhiana (Ref No. DMCH/R and D/2015/256, Dated 05/06/2015). The participants were informed about the objectives of this study and a written consent was sought before being enrolled in the study.

Sampling

A total of 123 blood samples were collected from pig farmers and slaughter workers. For collection of samples, 140 persons involved in pig production were contacted. Out of these, 123 agreed to be involved in current study with an overall response rate of 87.85%. The demographic data such as age, gender and primary occupation of the participants were recorded. One Hundred twenty one participants were male and two were females. The primary occupation of 91 participants was pig farming, and the remaining 32 were pig slaughter house workers.

The 10 ml of blood sample was aseptically collected from each subject. Five ml blood was then transferred to plain tubes for serum separation and serum was separated from clotted blood by centrifuging for 10 minutes at 1200 rpm. Separated serum was collected in screw-caped sterilized vials and stored at -20^{o}C till used. Five ml of whole blood was transferred in screw capped sterilized vials containing anticoagulant EDTA for PCR and stored at -20^{o}C till used.

Table 1: Multiplex PCR (Bruce ladder) primer sequences (Garcia Yoldi *et al.*, 2006) used for differentiation of Brucella species

Primer	Target gene	Sequence(5'-3')	Size of the amplified product (bp)
BMEI0998f	*wboA*	ATC CTA TTG CCC CGA TAA GG	1682
BMEI0997r		GCT TCG CAT TTT CAC TGT AGC	
BMEI0535f	*bp26*	GCG CAT TCT TCG GTT ATG AA	450
BMEI0536r		CGC AGG CGA AAA CAG CTA TAA	
BMEII0843f	*omp31*	TTT ACA CAG GCA ATC CAG CA	1071
BMEII0844r		GCG TCC AGT TGT TGT TGA TG	
BMEII436f	*Polysaccharide Deacetylase*	ACG CAG ACG ACC TTC GGT AT	794
BMEII435r		TTT ATC CAT CGC CCT GTC AC	
BMEII0428f	*eryC*	GCC GCT ATT ATG TGG ACT GG	587
BMEII0428r		AAT GAC TTC ACG GTC GTT CG	
BR0953f	*ABC transporter binding protein*	GGA ACA CTA CGC CAC CTT GT	272
BR0953r		GAT GGA GCA AAC GCT GAA G	
BMEI0752f	*Ribosomal protein S12, gene rpsL*	CAG GCA AAC CCT CAG AAG C	218
BMEI0752r		GAT GTG GTA ACG CAC ACC AA	
BMEII0987f	*Transcriptional regulator, CRP family*	CGC AGA CAG TGA CCA TCA AA	152
BMEII0987r		GTA TTC AGC CCC CGT TAC CT	

Serological techniques

Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination test (STAT) were the conventional serological tests used for screening of the serum samples (Alton *et al*., 1975; OIE, 2004). The RBPT and plain *Brucella* antigen were procured from the Punjab Veterinary Vaccine Institute, Ludhiana (Punjab) and stored at 4^{o}C until use. A titre of 80IU was considered to be positive (Arabaci and Oldacay, 2012). The commercially available IgG and IgM ELISA kits (Demeditec Diagnostics, Germany) were also used to test serum samples. The IgG and IgM ELISA was performed as per the manufacturer's instructions.

Conventional PCR

Blood samples belonging to all seropositive samples by ELISA and 20 randomly selected seronegative samples were subjected to PCR. The reference *B. abortus*, *B. melitensis* and *B. suis* strains were used as positive controls. The standard *B. abortus* strain 19 was obtained from Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (India). *B. melitensis* and *B. suis* standard strains were obtained from Indian Veterinary Research Institute, Bareilly (India). The DNA from the whole blood and standard strains was extracted as per manufacturer's instructions using available Himedia DNA extraction kits **(**Himedia Laboratories, Mumbai, India). Primers used in the conventional PCR were previously designed B4 (5'-TGG CTC GGT TGC CAA TAT CAA – 3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG - 3') primers for the bcsp31 gene encoding an immunogenic 31 kDa OMP of *Brucella* species (Baily *et al*., 1992; Navarro *et al*., 2002).

PCR was carried out as per the protocol of Baily *et al*. (1992) with slight modifications. The reaction mixture for PCR consisted of Master mix Go Taq green (Promega Corporation, USA) 12.5 µl, 1 µl of each primer with concentration 23 picomole, 7 µl of template DNA and 3.5 µl of dH_2O for a final reaction volume of 25 µl. The thermal cycling conditions were as follows: 94°C for 5 min, 35 cycles of denaturation at 94° C for 60 sec, annealing at 65° C for 60 sec, and extension at 74° C for 60 sec with a final extension at 74° C for 3 min (Baily *et al*., 1992). A 1.5 % agarose gel stained with ethidium bromide (0.5 µg/

Table 2: District wise sero-prevalence of brucellosis in pig handlers in Punjab

Name of District	Number of samples tested	No of samples positive (%)		
		RBPT	STAT	IgM ELISA
Hoshiarpur	4	0	0	0
Jalandhar	8	0	0	0
Amritsar	9	0	0	1 (11.1)
Barnala	8	0	0	0
Bathinda	10	0	0	0
Faridkot	4	0	0	0
Fatehgarh Sahib	5	0	0	0
Fazilka	3	0	0	0
Firozpur	5	0	0	0
Gurdaspur	6	0	0	0
Ludhiana	21	0	0	1 (4.76)
Kapurthala	2	0	0	0
Mansa	6	0	0	0
Moga	5	0	0	0
Ropar	1	0	0	0
Muktsar	9	0	0	0
Sangrur	7	0	0	0
Patiala	8	1 (12.5)	1 (12.5)	2 (25.0)
Pathankot	1	0	0	0
Tarn Taran	1	0	0	0
Total	123	1(0.81%)	1(0.81%)	4(3.25%)

ml) at 70 V (60- 90 minutes) was used to analyse the PCR amplified products.

Bruce Ladder Multiplex PCR assay

Blood samples positive for *Brucella* genus were subjected to Bruce Ladder PCR for species identification. Multiplex PCR using eight pair primer cocktail (Table 1) was carried out as per recommended protocols. For PCR, the reaction mixture consisted of 1.5U Taq polymerase, PCR buffer 1X, 1.5mM $MgCl_2$, 400 µM of each dNTP, 6.25pmol of each primer, 100 ng template DNA and dH_2O for a final reaction volume of 25 µl. The thermal cycling conditions were as follows: 95°C for 7 min, 25 cycles of 95° C for 35 sec, 65° C for 45 sec, and 72° C for 3 min and a final extension at 72° C for 6 min (Garcia-Yoldi *et al.*, 2006). A 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml) at 70 V (60- 90 minutes) was used to analyse the PCR amplified products.

Statistical analysis

Chi-square test was applied on the results of the study at 5% level of significance by using Microsoft Excel software to determine the significant difference among the various groups.

RESULTS AND DISCUSSION

Out of 123 samples, one (0.81%) sample was found positive using both RBPT and STAT. Four samples were detected positive using IgM ELISA and none of the samples were found positive by IgG ELISA (Table 2). The results are adequate to reject the null hypothesis that the population is free from disease (at the expected minimum prevalence of 5.25%). Only one seropositive sample was found positive in conventional PCR (Fig. 1) for *Brucella* infection using B4 and B5 primer for amplification of *bcsp31* gene. Using Bruce ladder multiplex PCR the *Brucella* positive sample was found to be *B. abortus* species. The eight pair

primer cocktail amplified five regions in test samples of *Brucella* positive samples of 152, 450, 587, 794 and 1682 bp products. The Reference *B. abortus* strain 19 amplified four regions in 152, 450, 794 and 1682 products. In *B. suis and B. melitensis* standard strains, an additional 272 bp and 1071 bp products were seen, respectively (Figure 2). Further Chi-square test was applied on the results to determine the significant difference among the various groups. There was no significant difference in the prevalence between different age groups ($p<0.05$). There was also no significant difference in the prevalence between both sexes ($p<0.05$). However, there was a significant difference ($p<0.05$) in the prevalence rates in different districts in Punjab. There was no significant difference in the prevalence between pig farmers and pig slaughter house workers ($p<0.05$).

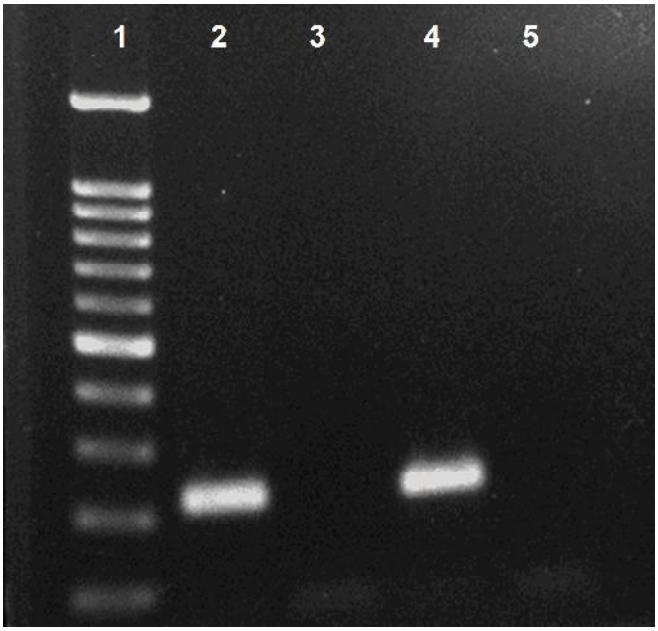

Fig. 1: *Brucella* genus specific PCR of gene bcsp31 on human blood samples. From left to right: Lane 1- Ladder; Lane 2- Control positive; Lane 3- Negative Control; Lane 4- Sample 1; Lane 5- Negative Control.

The results indicate that *B. abortus* is prevalent in persons involved in pig production in Punjab, India, possibly due to transmission of *B. abortus* from cattle to pigs as high prevalence of brucellosis has been reported in cattle from Punjab and these pigs could serve as an important source of infection for pig farmers and pig handlers. Four samples were detected positive by ELISA IgM and none of the samples were found positive by ELISA IgG. This shows that there were no chronic infections.

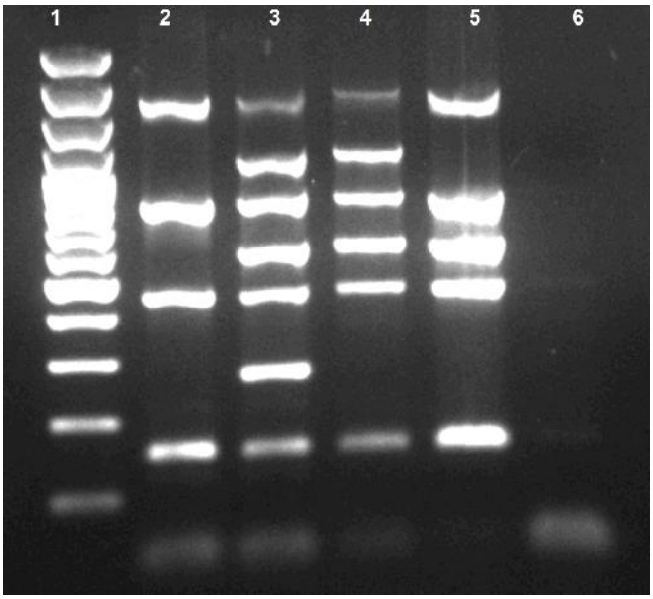

Fig. 2: *Brucella* species specific Multiplex PCR on human blood samples. From left to right: Lane 1- Ladder; Lane 2- Control positive (*B. abortus***)**; Lane 3- Control positive **(***B. suis***)**; Lane 4- Control positive (*B. melitensis*); Lane 5-Sample 1; Lane 6- Negative Control.

Pig farming is restricted to certain parts of the country. Accordingly, the seroprevalence levels of 3.2% in Madhya Pradesh (Soni and Pathak, 1969), 11.3% in Tamil Nadu (Kumar and Rao, 1980) and 6.3% in Karnataka (Krishnappa *et al.*, 1981) have been recorded. Thoppil (2000) observed 9.5% seroprevalence in 756 pigs slaughtered in Karnataka. Mathur (1985) also isolated *B. suis* biotype-2 from Yorkshire pigs in Tamil Nadu, India.

Rahman *et al.* (2012) described seroprevalence of brucellosis in swine in Bangladesh. Blood from a total of 105 pigs was collected from selected areas of Bangladesh. All samples were screened using RBPT and STAT. Out of the 105 sera analyzed, 7 (6.7%) and 5 (4.8%) were found to be positive by RBPT and STAT, respectively. High brucellosis seroprevalence rates in domestic swine herds have been reported in Wallus and Fatuna Islands (Guerrier *et al.*, 2011) and are associated with a significant burden of human infection by *Brucella suis*. The present study was an attempt to represent the existing situation of brucellosis in persons involved in pig production in

Punjab state of India. A battery of tests was employed to estimate sero-prevalence of brucellosis in persons involved in pig production in Punjab (India). However, isolation of bacteria from suspected blood samples could not be attempted which might have projected more information on brucellosis especially biovars circulating in the pig owners. Also, the prevalence of brucellosis in pigs of study area shall need to be studied to determine the risk of transmission of brucellosis from pigs to humans.

In Punjab, there are no modern abattoirs for slaughtering of pigs. Additionally, scavenging of pigs, absence of vaccination program in pigs and lack of knowledge among persons engaged in pig production are the important factors responsible for occurrence of brucellosis in pig farmers in India.

The results indicate that porcine brucellosis could be an important zoonosis in Punjab state of India and it is important that medical practitioners in Punjab consider brucellosis for differential diagnosis when investigating the cases of pyrexia especially of unknown origin. Further, the preventive and control measures need to be enforced to prevent the occupational exposure of brucellosis to pig producers in Punjab (India).

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