Journal of Animal Research: v.7 n.3, p. 495-499. June 2017

DOI: 10.5958/2277-940X.2017.00073.0

# Sero-prevalence and Molecular Detection of *Brucella* species in Slaughter Pigs (*Sus scrofa*) of Punjab, India

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**Received:** 04 January 2017 **Revised**: 25 April, 2017 **Accepted**: 26 April, 2017

**ABSTRACT**

Huge losses have been reported due to brucellosis in livestock populations of the country. However, limited studies have been carried out on porcine brucellosis in India. As far as we are aware, this is the first epidemiologic study carried out to determine *Brucella* species circulating in naturally infected pigs (*Sus scrofa*) in Punjab, India. The blood samples were collected from 330 pigs slaughtered in small slaughter shops located in 5 districts of Punjab state of India. The samples were screened using Rose Bengal Plate test (RBPT), Standard tube agglutination test (STAT) and ELISA. For molecular identification, conventional PCR was employed on all the seropositive and 30 negative samples. Nine (2.72%), eight (2.42%) and ten (3.03%) samples were found positive using RBPT, STAT and ELISA, respectively. Out of 10 seropositive samples, 4 were found positive using conventional PCR. The results indicate that pigs are infected with *Brucella* species and policies must be developed for prevention and control of brucellosis in the country.

**Keywords**: Brucellosis, PCR, Porcine Brucellosis, Prevalence, Punjab.

Brucellosis occurs due to gram negative facultative intracellular bacteria belonging to genus *Brucella* (family Brucellaceae). The six classical species involved are *B. abortus, B. melitensis, B. suis, B. ovis, B. canis and B. neotomae* (Alton *et al*., 1988; Corbel *et al*., 2005). They are non-motile, non-spore forming, intracellular pathogens (Quinn *et al*., 1994; Alton *et al*., 1975). The disease affects several domestic animals primarily mammalian livestock in India (Renukaradhya *et al*., 2002), broad range of wild animals and all ages of humans. In humans, the disease occurs due to ingestion of raw milk or meat from infected animals or people in close contact with their secretions such as veterinarians, farmers or slaughterhouse workers. Brucellosis in pigs is primarily caused by *B. suis*. This species consists of five biovars, but infection in pigs mainly occurs due to *B. suis* biovars 1, 2 or 3. Porcine brucellosis is of widespread occurrence; however prevalence is low, with the exception of South America and Southeast Asia where high prevalence has been reported (OIE, 2009).

Pigs usually become infected when they ingest feed contaminated by birth or abortion products, or contact aborted foetuses and membranes. Piglets can be infected during nursing, but most seem to reach weaning age without becoming infected (CFSPH, 2007). The disease in sows causes infertility and abortion at any stage of gestation resulting in birth of dead or weak piglets. In boars orchitis is common. Pigs are susceptible to infection with *B. abortus* from cattle and *B. melitensis* from sheep and goat, but reports of natural infection in pigs caused by either of these organisms are rare (OIE, 2009).

The disease was first recognized in India in 1918 (Anonymous, 1918) and is now endemic in most of the livestock populations in the country (Renukardhya *et al*., 2002). In India, approximately 80% of the human population live in 575,000 villages and thousands of small towns; have close contact to domestic and wild animal populations (Mantur and Amarnath, 2008). The human population stands at a greater risk of acquiring zoonotic

diseases including brucellosis (Mantur and Amarnath, 2008). People who consume raw milk, meat or their products are also at higher risk of contracting brucellosis. *Brucella abortus* and *B. melitensis* have been isolated from suspected human blood samples from South India (Madhukar *et al*., 2014).

From all the *Brucella* species prevalent in the Indian livestock, *B. abortus* associated bovine brucellosis is the most important concern in Punjab, India. Bovine brucellosis is widespread in India and appears to be on the increase in recent times, perhaps due to increased trade and rapid movement of livestock (Renukaradhya *et al.,* 2002). Huge losses amounting US $3.4 billion (5th-95th percentile 2.8 – 4.2 billion) has been reported due to brucellosis in Indian livestock populations (Singh *et al*., 2015). Nationally, bovine brucellosis accounted for 95.6% of the total losses occurring due to brucellosis in livestock populations (Singh *et al*., 2015).

Pig is an important livestock animal in India. There are 11.13 million pigs consisting of 2.38 million crossbred and 8.74 million indigenous pigs (DAHD, 2014). However, the people involved in pork production belong to lower and thus pork production occurs under unhygienic conditions with low input costs.

Porcine brucellosis has been associated with production losses in pregnant sows (Singh *et al*., 2015; Rahman *et al*., 2012). Seroprevalence levels of porcine brucellosis ranging from 3.2 – 11.3% have been reported from Central and South India (Soni and Pathak, 1969; Kumar and Rao, 1980; Krishnappa *et al*., 1981; Thoppil, 2000). The process of slaughtering pigs, particularly illegal slaughter, presents a risk of infection for persons involved in pig slaughter as well as pork consumers. However, extensive studies on porcine brucellosis have not been carried out in North India. Therefore, the current study was planned to estimate seroprevalence and to detect *Brucella* species circulating in pigs in North India.

## MATERIALS AND METHODS

### Place of work

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

### Study area and livestock systems

Punjab is situated in the northwest India with mean annual minimum and maximum temperature of 29.8 C and 16.5 C, respectively (Kaur *et al*., 2006). The state experiences hot, winter and rainy seasons with mean annual rainfall of 750 mm (Kaur *et al*., 2006). Cattle and buffalo livestock systems are most dominating in Punjab state of India (Chandel and Malhotra, 2006). In Punjab, pig farming is common in economically deprived communities (such as sweepers). The official National sample survey office data reveals that there is no dedicated agricultural land for pig farming in Punjab, India (NSSO, 2013). Therefore, pigs are usually left free ranging and are caught and slaughtered by their owners at maturity or when needed (Kaur *et al*., 2016).

### Selection of animals

For this cross-sectional study, 5 districts (approximately 20% of total area) were randomly selected from 22 districts of Punjab state of India. Further, these districts are sub-divided into 5-7 tehsils each. We randomly selected two tehsils from each district for collection of samples. As per the 18$^{th}$ livestock census of India, there are 12846 pigs living in these 5 districts of Punjab. The sample size was estimated using survey toolbox (Cameron, 1999). A sample size of 298 pigs was required at minimum expected prevalence of 3.0% (p=0.0496) given the ELISA sensitivity and specificity of 99% and 99%, respectively. The samples were collected from pigs slaughtered in small pig slaughter shops located in these areas. For collection of samples, 40 slaughter shops located in 10 tehsils in 5 districts were contacted. Out of these, 25 shop owners agreed to be involved in present study with an overall response rate of 62.5%. Depending on the pork demand, 5-6 pigs are usually slaughtered every day in these slaughter shops. Blood samples were collected randomly from a single pig every week on different weekdays.

We collected 330 blood samples from 60 farm and 270 stray pigs from 5 districts in Punjab (Table 1). There were 310 adult (> 6 months of age) and 20 young pigs (< 6 months of age) indicating that adult pigs are commonly

**Table 1:** Details of swine samples collected for detection of porcine brucellosis from different districts in Punjab, India

| District | Total number of pigs in district | Tehsil | Number of slaughter shops agreed to give samples (Contacted) | Number of samples collected |
|---|---|---|---|---|
| Ludhiana | 4822 | Ludhiana East | 4 (5) | 44 |
| | | Samrala | 3 (3) | 51 |
| Patiala | 4131 | Nabha | 1 (3) | 25 |
| | | Rajpura | 4 (6) | 30 |
| Sangrur | 2699 | Dhuri | 3 (4) | 27 |
| | | Sunam | 2 (3) | 31 |
| Jalandhar | 728 | Phillaur | 2 (3) | 33 |
| | | Nakodar | 1 (4) | 28 |
| Muktsar | 466 | Malout | 3 (5) | 30 |
| | | Muktsar | 2 (4) | 31 |
| **Total** | 12846 | | 25 (40) | 330 |

slaughtered in Punjab (India). The epidemiological data related to each pig was also recorded. Chi-square test was applied to determine any significant difference in prevalence estimates associated with age, area or management practices.

**Sampling**

About 10 ml of blood sample was collected from each pig. For serum samples, 5 ml blood was transferred to plain tubes and serum was separated from clotted blood by centrifuging at 1200 rpm for 10 minutes. Separated serum was collected in screw-caped sterilized vials and stored at -20C until use. For isolation and PCR, 5 ml of whole blood was transferred in screw capped sterilized vials containing anticoagulant EDTA and stored at -20C until use.

**Serological techniques**

The conventional serological tests such as Rose Bengal Plate Test and Standard Tube Agglutination test (Alton *et al*., 1975; OIE Manual, 2004) were used for screening of the serum samples. The RBPT and plain *Brucella* antigen were procured from the Punjab Veterinary Vaccine Institute, Ludhiana (Punjab) and stored at 4 °C until use. We considered a titre of 80 IU to be positive whereas a titre of 50IU has also been considered positive in other countries (Lord *et al*., 1997). The commercially available ELISA kits (Ingensa Pvt. Ltd.) were used and the ELISA was performed as per manufacturer's instructions (Ingensa Pvt. Ltd.).

**Conventional PCR**

The PCR was carried out on all seropositive samples and 30 randomly selected seronegative samples. 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. *Brucella melitensis* and *B. suis* standards were obtained from Indian Veterinary Research Institute, Bareilly. The DNA was extracted from blood and standard strains *B. abortus* strain 19, *B. melitensis and B. suis* culture using commercially available Himedia DNA extraction kits as per manufacturer's instructions. The previously designed B4 (5'- TGG CTC GGT TGC CAA TAT CAA – 3') and B5 (5'- CGC GCT TGC CTT TCA GGT CTG - 3') primers (Baily *et al*., 1992; Navarro *et al*., 2002) for the bcsp31 gene encoding an immunogenic 31 kDa OMP of *Brucella* species were used in the conventional PCR.

The incubation was carried out in Master cycler Pro (Eppendorf, T-Gradient) thermal cycler. The PCR was carried out as per Baily *et al*. (1992) with slight modifications. For PCR, the reaction mixture consisted of Master mix (Go Taq green) 12.5 µl, 23 pmol of each primer, 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 94 C for 60 sec, 65 C for 60 sec, and 74 C for 60 sec and a final extension at 74 C for 3 min (Baily *et al*., 1992). The

**Table 2:** Comparative prevalence of porcine brucellosis in different districts in Punjab, India

| District | Tehsil | Number of samples tested | Number of positive samples (%) | | |
|---|---|---|---|---|---|
| | | | RBPT | STAT | ELISA |
| Ludhiana | Ludhiana East | 44 | 3 (6.81) | 3 (6.81) | 3 (6.81) |
| | Samrala | 51 | 1 (1.96) | 1 (1.96) | 2 (3.92) |
| Patiala | Nabha | 25 | 2 (8.00) | 2 (8.00) | 2 (8.00) |
| | Rajpura | 30 | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| Sangrur | Dhuri | 27 | 1 (3.70) | 1 (3.70) | 1 (3.70) |
| | Sunam | 31 | 1 (3.22) | 0 (0.00) | 1 (3.22) |
| Jalandhar | Phillaur | 33 | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| | Nakodar | 28 | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| Muktsar | Malout | 30 | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| | Muktsar | 31 | 1 (3.22) | 1 (3.22) | 1 (3.22) |
| **Total** | | 330 | 9 (2.72) | 8 (2.42) | 10 (3.03) |

PCR amplified products were analyzed on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) at 70 V (60-90 minutes).

## RESULTS AND DISCUSSION

Of the total 330 sera samples tested, 9 (2.72%) and 8 (2.42%) were found positive by RBPT and STAT respectively. However, 10 samples were detected positive by ELISA (Table 2). With the prevalence of 2.72% (based on RBPT), it was concluded that the study population is diseased at a confidence level of 99% (p=0.006). In conventional PCR, from 10 seropositive samples, 4 (1.21%) were found positive (Fig. 1) for *Brucella* infection using B4 and B5 primer for amplification of *bcsp31* gene (Baily *et al*., 1992). Based on STAT, prevalence ranged between 0 – 4.21% in 5 districts and no significant difference (chi-square = 3.33, p = 0.5) was recorded. Similarly, no significant difference (chi-square = 0.33, p = 0.56) was recorded for prevalence between adult (2.58%) and young (0%) pigs. The prevalence was 1.66% in farm and 2.59% in stray pigs and significant difference (chi-square = 0.18, p = 0.67) was not recorded.

The results indicate that *Brucella sp.*is prevalent in pigs in Punjab, India. High prevalence of brucellosis has been reported in cattle from Punjab (Dhand *et al*., 2005). A screening using Rose Bengal Test (RBT) and further confirmed by using Slow Agglutination Test (SAT) showed a sero-prevalence of 42.9% in aborted sows and 1.6% from non-aborted pigs in tribal areas in Bangladesh (Rahman *et al*., 2012). We reported higher disease prevalence in stray pigs. Further studies are required to determine risk factors associated with porcine brucellosis in Punjab, India.

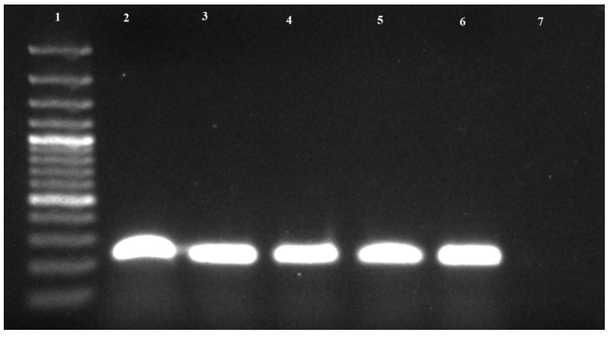


**Fig. 1:** *Brucella* genus specific PCR of gene Bcsp31 region from Pig blood sample. From left to right: Lane 1- Ladder; Lane 2- Control positive; Lane 3- Sample 1; Lane 4- Sample 2; Lane 5- Sample 3; Lane 6- sample 4; Lane 7- Negative control.

We employed battery of tests to estimate seroprevalence of brucellosis in pigs in Punjab (India). Biosafety concerns prevented us from isolating bacteria from suspected tissues such as lymph nodes, which could have improved our epidemiological insights. Case-control studies could further help determine the risk factors associated with porcine brucellosis. As far as we are aware, this is the first epidemiologic study carried out to determine *Brucella* species circulating in naturally infected pigs (*Sus scrofa*) in Punjab, India. We detected 10 sero-positive samples and only 4 samples

were confirmed using molecular detection. Further studies are required to re-confirm the identities of the *Brucella* species circulating in pigs in India.

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