



Seroepidemiological survey of brucellosis and isolation of *Brucella suis* from swine herds of Meghalaya, North-East India

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ABSTRACT

Brucellosis is a well-known and wide spread zoonotic disease. It is endemic in several parts of Asia, including India. In this study, seroprevalence of porcine brucellosis was studied among apparently healthy pigs in Meghalaya where pig keeping plays a significant function in socio-economic development. Serum samples (3,597) from pigs were screened using Rose Bengal Plate Test (RBPT) and indirect ELISA. Isolation of *Brucella* was attempted in clinical samples. A total of 13 (0.36%) were positive by RBPT and 72(2%) by Indirect ELISA. *Brucella suis* isolate was recovered from placenta of an aborted pig. Risk factors involved in the transmission of brucellosis amongst swine herd were studied. It was observed that age (OR=0.590; P=0.04) and sex (OR=0.557; P=0.04) were significant intrinsic risk factors for transmission of porcine brucellosis. Although the seroprevalence is low, isolation of *B. suis* from an aborted pig indicated that disease is actively circulating among swine herds of Meghalaya.

Keywords: Bruce ladder PCR, *Brucella*, *B. suis*, Porcine brucellosis, Risk factors, Seroprevalence

Brucellosis is a zoonotic and economically important disease reported worldwide affecting all domestic animals including pigs and man (Radostits *et al.* 2007). Brucellosis in pigs is chiefly caused by *Brucella suis*, a facultative Gram-negative intracellular organism. Within the *B. suis* species, there are five biovars. *B. suis* biovars 1 and 3 are known to infect Suidae only, whereas biovar 2 is reported to infect Suidae and hare. Reports are available for biovar 4 infecting caribou and reindeer. Biovar 5 has been isolated from rodents in Russia (Godfroid *et al.* 2010). The characterization of porcine brucellosis is abortion, weak piglets and infertility in sows; orchitis and accessory organs infection in boars. Paralysis and lameness are reported from both the sexes. Other frequently noted extragenital lesions are lymphadenitis, arthritis, subcutaneous abscesses and spondylitis (Aparicio *et al.* 2013; OIE, 2011). Systemic infections with reproductive ailments in pigs are caused by *B. suis* only, however other *Brucella* species can cause a self limiting infection in pigs (Deyoe *et al.* 1986). It is important from public health perspective, that *B. suis* has the capability to colonize the udder of bovine and consequent shedding in milk. High risks are encountered by humans and laboratory workers who are exposed to pigs and handling samples, respectively (OIE, 2009). Modes of transmission among animals are by inhalation of aerosols, ingestion of contaminated feed/ fodder, copulation and

licking of aborted fetuses/ placenta (Shimshony *et al.* 2009). Entry of infection to an organized farm mainly occurs through addition of newly purchased pigs to the herd without screening or quarantine (Shome *et al.* 2016). Brucellas are expelled from the secretions and excretions of the infected pigs like urine, semen, vaginal discharge, milk, lochial secretion, aborted contents, and pus of subcutaneous abscess (Kebeta *et al.* 2015).

Laboratory diagnosis of porcine brucellosis involves serological testing and isolation from the clinical material. Diagnostic assays approved by OIE for porcine brucellosis are, Rose Bengal plate test (RBPT), complement fixation test, ELISA and fluorescence polarization assay (FPA) (OIE, 2011). Although the reliability of serological tests is good, it suffers the false positivity issues which decrease its specificity. This is mainly due to cross-reacting antibodies against *Yersinia enterocolitica*, *Salmonella*, *Francisella* and some other zoonotic pathogens (See *et al.* 2012). *B. suis* has 5 documented biovars. Tests like phage typing, sensitivity to dye, CO₂ requirement, H₂S production etc, are performed to distinguish different species and biovars (Alton *et al.* 1988). Recently, multiplex PCR like Bruce ladder (Lopez-Goni *et al.* 2008) and AMOS PCR (Bricker and Halling, 1994) are deployed to identify species and biotypes within *Brucella*.

Reports on porcine brucellosis in India are sparse. A single isolation report of *B. suis* (biovar 2) is available in the literature (Mathur, 1885). The seroprevalence of

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Brucella in pigs has been reported from some states of India like Tamil Nadu (Kumar and Rao, 1980), Karnataka, Andhra Pradesh, Madhya Pradesh, Punjab (Shome *et al.* 2016) and Uttar Pradesh, Assam (Nath *et al.* 2009). Highest seroprevalence in pigs with abortion history was reported from Assam (adjoining state of Meghalaya). A solitary report of pig brucellosis in Meghalaya carries 0% seroprevalence (Shome *et al.* 2016). In India, swine brucellosis vaccination is not in practice. North-eastern India (our study area) is the main pig rearing part in the country and pig rearing is socio-culturally blended with the livelihood of the people of this region. Owing to the paucity of reports on porcine brucellosis in Meghalaya, this study was envisaged with an objective to establish the prevalence of brucellosis among swine herds by using serological, microbiological and molecular methods.

MATERIALS AND METHODS

The study was carried out during the period from October 2012 to September 2017 in Meghalaya state, North-eastern India. Meghalaya is a state of India located on the north-east between the coordinates 20°1' and 26°5' North latitude and 85°49' and 92°52' East longitude with a geographical area of 22,429 km². In India, Meghalaya with an average annual rainfall of 1,200 cm is the wettest state. Meghalaya shares its border with Assam (another state of India) on the north-east and with Bangladesh on the south-west. It is a mountainous state with highland plateaus and valleys. Elevation ranges are from 150 m to 1961 m. Meghalaya comprises of three hills namely, Khasi (central part with highest elevations), Jaintia (eastern part) and Garo (western part and nearly plain). The climate of the state differs with the altitude. As per livestock census of India (2012), the total population of the pig in the state is 5,43,381 (GOI, 2014).

A total of 3,597 pig sera samples were collected from three hilly regions (Khasi, Jaintia and Garo) of Meghalaya. All the swine herds were owned by the tribal farmers for local meat consumption. Serum samples were collected from apparently healthy pigs. The pigs were reared under the traditional production system mostly up to 12 months (slaughter/market age). The test group consisted of 1146 male (31.85%) and 2451 female (68.15%). A total of 1350 (37.53%) animals were from less than 6 months age group, and 2247 (62.47%) animals were from more than 6 months age group. In the test population, 1,414 animals were of indigenous, 1,797 were of Hampshire and 386 were of crossbreds. Following proper restraining, blood was collected by venipuncture of ear vein and the site was disinfected with alcohol. Sera were collected following centrifugation at 2,500 rpm for 15 min and transferred to the Eppendorf tube and stored at -40°C until tested.

A total of 28 clinical samples (5 placenta, 5 stomach contents, 9 spleens and 9 uterus) from aborted sows, still-birth fetus suspected for brucellosis were included for *Brucella* isolation.

Rose bengal plate test (RBPT) was initially done to spot

Brucella antibodies as per Alton *et al.* (1988). The *B. abortus* coloured antigens for RBPT were acquired from the Institute of Animal Health and Veterinary Biologicals, Bengaluru, India. Samples exhibiting clumping of antigen (any degree) in RBPT was measured as positive (Al Dahouk *et al.* 2003). Serum samples were analysed by means of commercial indirect ELISA kit (INGEZIM BRUCELLA PORCINO 11.BP.K.1, Spain) which uses a monoclonal antibody specific for porcine IgG immunoglobulins and Lipopolisaccharide from smooth *Brucella*. Manufacturer's instructions were followed to perform ELISA. The absorbance was read at 450 nm with an ELISA reader (Lab systems MultiskanGO, Thermo Fisher Scientific, USA). Samples having percent positivity value 0.25 or above (%P ≥ 0.25) were sorted as positive and below 0.25 (%P < 0.25) as negative.

Clinical materials were inoculated in *Brucella* enrichment broth (Himedia, Mumbai, India) with *Brucella* selective supplement (Himedia, Mumbai, India) holding Polymyxin B sulphate (2500 IU), Nystatin (500000 IU), Bacitracin (12,500 IU), Nalidixic acid (2,500 mg), Cycloheximide (50 mg), and Vancomycin (10 mg). Inoculated broth was then incubated for 3 days at 37°C for enrichment. Further, the enriched broth was plated on to the *Brucella* agar with selective supplements (Himedia Laboratories) and incubated under 5% CO₂ at 37°C till the growth appeared. Plates showing circular, elevated, honey-colored colonies with an complete margin after incubation of 3 days are suspected for *Brucella* and included in the further confirmation studies.

Suspected colonies were further confirmed by various tests, i.e. Gram staining, catalase, oxidase, production of urease and H₂S, nitrate reduction, methyl red and Voges Proskauer test (Alton *et al.* 1988). The cultures were grown for 18 h by inoculating in *Brucella* selective broth at 37°C and genomic DNA was extracted employing QIAamp DNA Kit (Qiagen, Germany). The genomic DNA was subjected to PCR to detect *Brucella* genus by targeting *bcsp31* gene as described by Baily *et al.* (1992). The PCR was carried out in a Thermal Cycler (Eppendorf, Germany). The PCR reaction mixture for amplification consisted of 12.5 µl of 2× PCR master mixtures (ThermoFisher Scientific, US), 1 µl (10 pmol/µl) of each primer (ILS, Haryana, India), 2 µl of DNA template and nuclease-free water to make final volume to 25 µl. The cycling conditions for PCR consisted of 5 min initial denaturation at 95°C followed by 35 cycles each of 45 s denaturation at 95°C, 1 min annealing at 60°C and 2 min extension at 72°C and a final extension step of 5 min at 72°C. Further to confirm the species the Bruce Ladder multiplex PCR was employed as per the protocol of Lopez-Goni *et al.* (2008). PCR conditions were similar as of *bcsp31* gene except for primer annealing, which was done at 64°C. PCR products (7 µl) were subsequently analyzed by electrophoresis on a 1.5% agarose gels containing ethidium bromide (0.5 µg/mL) and observed under gel documenter.

Seropositivity was determined as the number of animals positive for ELISA, divided by total number of animals

tested. Odds ratio for all the variables (breed, herd size, sex, age) were calculated using online statistical computation tool (<http://vassarstats.net/odds2x2.html>).

RESULTS AND DISCUSSION

Brucellosis, an economically important reproductive disease of livestock is prevalent in nearly all developing nations including India. In this study, of the 3,597 serum samples tested, 13 (0.36%) were found to be positive by RBPT and 72 (2%) by Indirect ELISA. The seroprevalence in Khasi, Jaintia and Garo hills were 1.34%, 11% and 0%, respectively. In this study, we evaluated breed, herd size, age and sex and found age and sex were significant intrinsic risk factors for brucellosis in swine herds of Meghalaya (Table 1). The results of the present study showed that higher seroprevalence of brucellosis in female (2.32%) than male (1.30%). Higher seroprevalence was observed in older (≥ 6 months) (2.29%) as compared to younger (< 6 months) pigs (1.96%). Seroprevalence in herd size less than 10 and more than 10 was found to be 2.26% and 1.56%, respectively. And the seroprevalence in indigenous and crossbreeds was 2.11%, whereas in Hampshire breed, it was 1.89%.

Isolation of *Brucella* from aborted and clinical materials is a gold standard for diagnosis. In this study, on bacteriological analysis of the clinical samples, one *B. suis* isolate was obtained. Biochemically, the isolate was confirmed as *B. suis* biovar 1. The isolate exhibited amplification of the *bcs31* and the species was confirmed by Bruce ladder multiplex PCR assay.

In this study, seroprevalences based on RBPT and indirect ELISA were recorded as 0.36% and 2%, respectively. In previous studies, the seroprevalence of *Brucella* in pigs has been reported from some states of India such as Tamil Nadu 11.3% (Kumar and Rao, 1980), Karnataka 8.5%, Andhra Pradesh (28.2%), Madhya Pradesh (14.6%), Punjab (9.9%), Uttar Pradesh (16.7%) and Meghalaya (0%) (Shome *et al.* 2016). The highest prevalence (87.1%) in pigs with the history of abortion was reported from Assam (Nath *et al.* 2009). Shome *et al.* (2016) also reported very low seroprevalence in Rajasthan and Gujarat. In India, risk attributes like improper farm hygiene, rapid livestock movements; lack of awareness has been found associated to seropositivity of Brucellosis among dairy animals (Chand and Chhabra, 2013). Meghalaya is a poor tribal state in India, where farmers hardly purchase

stocks from market for their farm. The low animal movement in Meghalaya might be the reason for lower seroprevalence.

The results of the present study showed higher seroprevalence of brucellosis in female 2.32% than male 1.30%. This finding was in agreement with the observation of earlier studies conducted in Bangladesh (Rahman *et al.* 2012) and Ethiopia (Kebeta *et al.* 2015). In contrast to this finding Ngbede *et al.* (2013) found relatively a higher prevalence of brucellosis in males than female pigs in Nigeria. With regard to the age of the animal, higher seroprevalence was observed in older (≥ 6 months) (2.29%) as compared to younger (< 6 months) pigs (1.96%). This result was in agreement with the findings of Rahman *et al.* (2012) who also found a higher prevalence of brucellosis in aged animal than young. In Meghalaya, farmers mostly keep two to four pigs for household consumption and they maintain the breeding stock. In farms, where the herd size is more than ten, purchase of animals from markets may be a practice. In north-eastern India, pigs are mostly reared under the traditional production system with less hygiene and floor space. Report on isolation of *B. suis* in pigs is very rare in India. In an earlier study, Mathur (1985) reported the isolation of *B. suis* (biovar 2) from Tamil Nadu, Southern India. Another report of PCR confirmation of *B. suis* abortion in swine is also available (Shome *et al.* 2011). To the best of our knowledge, this is the first report on the isolation of *B. suis* from pigs in north-east India.

Precise diagnostic methods are vital for the successful eradication and control of the disease, and hence the identification up to species level is of great epidemiological importance (Lopez-Goni *et al.* 2008). In the current study, Bruce ladder multiplex PCR was used to establish the species of *Brucella*. AMOS PCR can differentiate *B. abortus* (biovars 1/2/4), *B. melitensis* (biovars 1/2/3), and *B. ovis* and *B. suis* (biovar 1) (Bricker and Halling, 1994). However, other *Brucella* species (such as *B. canis*, *B. pinnipedialis*, *B. neotomae*, and *B. ceti*) and a few other biovars (*B. abortus* biovars 3/5/6/7/9 and *B. suis* biovars 2/3/4/5) cannot be identified by AMOS PCR. Notably, Bruce ladder PCR can distinguish the species of *Brucella* including vaccine strain (Lopez-Goni *et al.* 2008).

Though the seroprevalence is low, isolation of *B. suis* from an aborted pig indicated that disease is actively circulating among swine herds of Meghalaya. From in

Table 1. Risk factors related to brucellosis seropositivity among swine herds in Meghalaya

Risk Factor	Variable	No. of animals	Seropositivity (%)	Odds Ratio	95% CI	P value
Breed	Indigenous and Cross breeds	1800	38 (2.11%)	1.1183	0.70081-1.7846	0.63904
	Hampshire	1797	34 (1.89%)			
Herd size	Less than 10	2252	51 (2.26%)	1.4609	0.8749-2.4395	0.145387
	More than 10	1345	21 (1.56%)			
Age	< 6 months	1350	19 (1.40%)	0.5909	0.3483-1.0024	0.048574
	> 6 months	2247	53 (2.35%)			
Sex	Female	2451	57 (2.32%)	0.557	0.314-0.9881	0.04263
	Male	1146	15 (1.30%)			

zoonoses point of view, *B. suis* is extremely rare in human. However, Naha *et al.* (2012) reported a rare case of *B. suis* in a 27-year-old man admitted for pyrexia of unknown origin in Manipal Hospital, India. Strikingly it was a seronegative but culture proven case. This case markedly demonstrates the significance of isolation of the organism in culture, despite the higher specificity and sensitivity of serological tests, especially in areas where brucellosis is known to be prevalent. Another study from India also documented the seroprevalence (3.25%) of *Brucella* in pig farmers and pig slaughterhouse workers in Punjab (Jindal *F* 2016). Therefore the zoonotic potential of *B. suis* should not be neglected because the occupational risk among pig farmers and handlers are high. According to the data of 19th Livestock census, India is largest in livestock sector holding 11.6% of livestock population of world which consists of 1.2% pigs (GOI, 2014). In India, pig contributes 8% of total meat production (GOI, 2014). The consumption of pork is much higher (68.75%) in the north-eastern region which reflects the significance of pigs in this region (Kadirvel *et al.* 2018). But local production of pigs in this region is significantly lower than the consumption. Unscreened infected pigs might be ingrained to uncontaminated herds from other parts of country which could be a prospective peril in spreading the infection to a healthy swine herd and it may turn into a public health concern. Owing to the geographical location of this state, it shares the border with Bangladesh, which bears a regular threat to the country's livestock for invasion of exotic as well as transboundary diseases. So the low seroprevalence in this region must not be overlooked because ingrain of live animals for meat purpose from other parts may facilitate transmission of the brucellosis within no time.

This study reported the seroprevalence among the swine herds in Meghalaya. The clinical samples revealed isolation of *B. suis* biovar 1. The isolate was confirmed by Bruce ladder PCR. The essence of the present study suggests that continued surveillance and removal of infected pigs should be strictly followed to control and eradicate the disease in swine herds, as vaccination against swine brucellosis is not in practice in India.

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