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Full Length Article

Phylogenetic Analysis of Soil Borne *Brucella* Species by Targeting Insertion Sequence 711 Element in Punjab, Pakistan

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Abstract

A metagenomics study was conducted to detect DNA of *Brucella* species from soil samples (n=1280) in nine districts of Punjab. Out of five *Brucella* species, only two (*B. abortus* and *B. melitensis*) were detected through conventional multiplex Polymerase Chain Reaction (mPCR). Out of nine, three (33.33%) districts that is Sheikhupura, Faisalabad and Sargodha were positive for *B. abortus* while two (22.22%) districts (Sheikhupura and Sargodha) were positive for *B. melitensis*. All districts were significantly positive for *Brucella* species DNA (χ^2=54.505, df=8, $p<0.05$, 95% CI). The *IS711* gene was amplified from soil DNA samples through mPCR and PCR products were sequenced to obtain the *IS711* nucleotide data. Homology level and phylogenetic relationship of nucleotide sequence of *IS711* gene fragment of soil borne *Brucella* isolates was compared to the isolates from foreign countries that were accessed in GenBank. The results showed a high homology level (up to 99%). Multiple sequence alignment was done and Phylogenetic tree was constructed through using Neighbor-joining method. The analysis showed that *Brucella* isolates from USA, Iran, India, Korea and China were closely related to the soil borne *Brucella* isolates from Pakistan. © 2017 Friends Science Publishers

Keywords: *Brucella*; DNA; Nucleotide sequence; Metagenomics; PCR; Soil

Introduction

Brucellosis is a wide spread zoonosis throughout the world and is caused by pathogenic species of *Brucella*. It is the second most common zoonotic disease after rabies (Boschiroli *et al.*, 2001). The organism is categorized as Risk group III pathogen by World Health Organization (WHO) and placed in category B pathogen by Centre of Disease Control and Prevention (CDC). *Brucella* species are intracellular facultative pathogens and mostly affect the reticulo-endothelial and reproductive system (Jarvis *et al.*, 2002). It grows slowly on nutrient media and their growth can be enhanced by the addition of serum or blood. *Brucella* species replicate inside cells and evade the innate and adaptive immunity thereby inducing disease (Ficht, 2003).

Soil provides the complex habitat for the microorganisms but their number is very high in surface soil around macropores (Bundt *et al.*, 2001). The macropores are the channels in the soil which are formed by the activities of earthworms, roots of the plants and other soil biota which is lined with organic matter mostly in top soil (Fierer *et al.*, 2007). The bacterial growth and diversity is correlated with organic matter. The microbial diversity and number is very high in top 10 cm of the soil and decreases with the depth (Eilers *et al.*, 2012). It is estimated that number of species of bacteria in the soil ranges from 2000 to 18,000 per gram (Henry *et al.*, 2004).

Brucella species are sensitive to heat and can survive for several weeks in water and can transfer to relevant hosts (Mawdsley *et al.*, 1995). *Brucella* species can survive in soil and dust for many weeks (Memish and Balkhy, 2004). *B. abortus* can survive on vegetation, soil and fetal tissues for several weeks depending on moisture, temperature and sunlight (Aune *et al.*, 2012). *B. abortus* survives up to 135 dyas in aborted fetuses and more than 60 days in cool environment and up to six months in a shaded fetus (Aune *et al.*, 2012). Sunlight and temperature has impact on survival of *B. abortus* in the environment (Jones *et al.*, 2010). *B. abortus* can survive up to 66 days in wet soil; at 90% humidity, it can survive for 48 to 73 days. *B. abortus* survives less than 4 days in dry soil and *B. suis* may survive up to 28 days between 5^0C and 22^0C (Lovell *et al.*, 1944).

Brucella species contain Insertion Sequence (IS) which is also called as *IS6501*. Different species of the genus *Brucella* contain different copy number of IS, e.g. *B. abortus*, *B. melitensis* and *B. suis* mostly contain 7 copies of

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IS whereas there are over 30 in case of *B. ovis*. At present, there is no evidence for the transposition of IS, but as it is present in many pathogenic *Brucella* species. In high copy numbers, IS likely has the capacity to transpose (Ocampo-Sosa and García-Lobo, 2008).

Insertion element (IS 6501) is 836 bp in length. In genome of *B. ovis* it occurs 20 to 35 times and 5 to 15 times in all other *Brucella* species. Number of copies of IS in *B. ovis* genome is 30 and the genome of *B. melitensis* biovar 3 contains 10 copies (Ouahrani *et al.*, 1993).

Livestock sector contributes 11.9% share in GDP of Pakistan (Amjid *et al.*, 2011). Most of the population earns a living from agriculture and livestock. Many studies have been done on serological prevalence of brucellosis but no research has been performed on the prevalence of soil borne *Brucella* species in Pakistan. The persistence of *Brucella* species in soil is a great risk for animal and human health. The aim of the present metagenomics study was to establish a phylogenetic tree of pathogenic *Brucella* species/strains and to accurately survey pathogenic *Brucella* populations in the soil. The data from this study should prove instrumental in future efforts to develop recombinant vaccines from local isolates.

Materials and Methods

Sample Collection

The soil samples (n=1280) were collected from nine districts of Punjab, Pakistan, including Sargodha, Sahiwal, D.G. Khan, Chakwal, Attock, Sheikhupura, Gujranwala, Faisalabad and Lahore. The soil samples (5 samples/village) were collected three inches below the surface; four samples were collected within each surveyed village where animals and humans interact on a routine basis and one soil sample was collected outside the villages. All the geographical coordinates of the samples were recorded using a GPS receiver (Garmin, Dakota U.S.A.). Personal Protective Equipment (PPEs) were used for soil sampling. The soil samples were pooled, mixed thoroughly and divided into two aliquots, one for sample archives (~500 g) and second (~200 g) was processed for DNA extraction in University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS) Lahore.

DNA Extraction

DNA was extracted from the soil samples using PowerMax™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA) as per manufacturer's recommendations. DNA quality and quantity was determined using Nano Drop 1000 spectrophotometer (Nano Drop, USA) and Qubit flourometer (Invitrogen, USA) using DNA BR assay kit (Invitrogen, USA) as per manufacturer's instructions.

Conventional Multiplex PCR

DNA samples were subjected to conventional multiplex PCR by using species specific primers. The primers of five species such as *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis*, PCR conditions, composition of reaction mixture and agarose gel electrophoresis was performed and followed as described by Ali *et al.* (2014).

Sequencing

The nucleotide sequences of positive DNA samples were obtained using an ABI PRISM 3130 Genetic Analyzer. DNA sequencing was performed according to the methods as described previously (Pettersson *et al.*, 2009).

Statistical Analysis

The results of conventional multiplex PCR were compiled in a single Microsoft Excel spreadsheet. Data was analyzed through chi square test using 95% confidence interval and 5% level of significance. A statistical software SPSS (version 20.0; SPSS Inc., Chicago, IL) was used for statistical analysis. Normality of data was checked by using Shapiro-Wilk test. Nucleotide sequence data of local *Brucella* isolates produced by DNA sequencing were compared with those of *Brucella* isolates from foreign countries using BLAST (Basic Local Alignment Search Tool). Sequence alignment was performed through BioEdit (Version 7.0). A phylogenetic tree was constructed by Neighbor-joining method using MEGA software (version 7.0).

Results

Out of nine districts, three districts Sheikhupura, Faisalabad and Sargodha (33.33%) were positive for soil borne *B. abortus* and two districts Sheikhupura and Sargodha were positive for *B. melitensis* (22.22%) as shown in Fig. 3. Through conventional multiplex PCR (mPCR), out of five species (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis*), only two (*B. abortus and B. melitensis*) were detected. Out of 1280 soil samples, 23 soil samples (1.8%) were positive for *B. abortus*, while four soil samples (0.31%) were positive for species specific DNA of *B. melitensis*. Overall 2.1% prevalence of *Brucella* species' DNA was observed. PCR products were run on 2% agarose gel, and bands for *B. abortus* (498 bp) and *B. melitensis* (731 bp) were seen on gel as shown in Fig. 1.

Table 1: Homology level of soil borne *Brucella* isolates (*B. abortus* & *B. melitensis*) with the isolates from foreign countries accessed in GenBank

GenBank Accession numbers of	Country	*B. abortus*	*B. abortus*	*B. abortus*	*B. abortus*	*B. melitensis*
ACCESSION CP013964	China	97%	94%	96%	98%	99%
ACCESSION CP010851.1	USA	97%	94%	96%	98%	99%
ACCESSION CP006962.1	China	97%	94%	96%	98%	99%
ACCESSION CP003129.1	USA	97%	94%	96%	98%	99%
ACCESSION AE008917	USA	96%	92%	95%	96%	98%
ACCESSION AM040265	USA	94%	95%	95%	96%	99%
ACCESSION GU433108	India	94%	95%	95%	96%	99%
ACCESSION DQ845342	Iran	98%	93%	98%	98%	99%
ACCESSION CP003177.1	Korea	94%	95%	95%	96%	99%
ACCESSION AJ314586	Italy	95%	96%	96%	97%	99%
B. abortus Pak/UVAS-1 isolate	Pakistan	100%	91%	90%	92%	93%
B. abortus Pak/UVAS-2 isolate	Pakistan	91%	100%	93%	93%	95%
B. abortus Pak/UVAS-3 isolate	Pakistan	90%	93%	100%	93%	94%
B. abortus Pak/UVAS-4 isolate	Pakistan	92%	93%	93%	100%	96%
B. melitensis Pak/UVAS isolate	Pakistan	93%	95%	94%	96%	100%

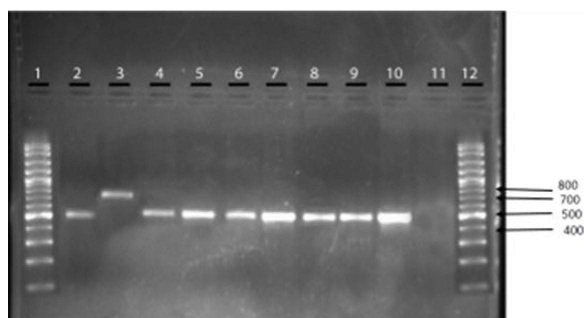

Fig. 1: Conventional Multiplex PCR for *Brucella* species. Lane 1 and 12 shows Marker (100 bp)
Lane 10 shows Positive control for *B. abortus* and Lane 11 shows negative control. Lane 2, 4 to 9 shows Bands for *B. abortus* and Lane 3 shows band for *B. melitensis* DNA

In district Sheikhupura, out of 145 soil samples collected from 29 villages, when processed through mPCR, five samples (3.44%) were positive for *B. abortus* while two samples (1.38%) were positive for *B. melitensis*. In district Faisalabad, out of 155 soil samples collected from 31 villages, when processed through mPCR, nine samples (5.80%) were positive for *B. abortus*. None of the samples was found positive for *B. melitensis* in this district. In district Sargodha, out of the 145 soil samples collected from 29 villages when processed through mPCR, nine samples (6.20%) were positive for *B. abortus* while two samples (1.38%) were positive for *B. melitensis*. *Brucella* species were detected significantly in all districts ($\chi 2=54.505$, df=8, $p<0.05$ and 95% CI).

Soil samples collected from Gujranwala, Lahore, Sahiwal, D.G. Khan, Chakwal and Attock districts, when processed through mPCR none of the samples was found positive for *Brucella* species. The nucleotide sequences of IS711 gene of soil borne *B. abortus* and *B. melitensis* species were deposited in GenBank under the accession numbers KX764595, KX764596, KX764597, KX764598 and KX764599, respectively. The sequences were BLAST in NCBI and aligned through Clustal W alignment by MEGA. Multiple sequence alignment was done through BioEdit (version 7.1.9) software. Phylogenetic tree was constructed through MEGA (7.0) software by using Neighbor-Joining method as shown in Fig. 2. The analysis showed that *Brucella* isolates from USA, Iran, India, Korea and China are closely related to the soil borne *Brucella* isolates from Pakistan.

Discussion

In the present study, we found that soil of districts Sargodha, Faisalabad and Sheikhupura is positive for *Brucella* species. District Sargodha shows positive soil samples for *Brucella* species as the soil of this district is of sandy loam type near rivers. Chances of DNA survival and stability are very high in this type of soil as described (Mallmann and Litsky, 1951).

In Faisalabad districts, the positive soil of tehsils Chak Jhumra, Tandlianwala and Samandari is a silty, clay and sandy type and this soil supports DNA stability up to months as described by Romanowski *et al.* (1993) and the soil of district Sheikhupura is also a loamy sand type and according to Recorbet *et al.* (1993) this type of soil is also conducive to DNA stability.

The contaminated soil by *Brucella* species (which are excreted through vaginal discharge, aborted fetuses, milk and semen of infected animals) is the source of infection to animals through parenteral, air droplets, contact and through oral-fecal route (Klietmann and Ruoff, 2001). The organism is highly infectious can be aerosolized and very difficult to diagnose due to non-specific symptoms associated with its infection (Yagupsky and Baron, 2005).

DNA has been amplified from thousands of years old specimens through PCR (Pääbo *et al.*, 1989). DNA resists environmental conditions, surviving for a long time and is prevented from degradation by nucleases. The survival of

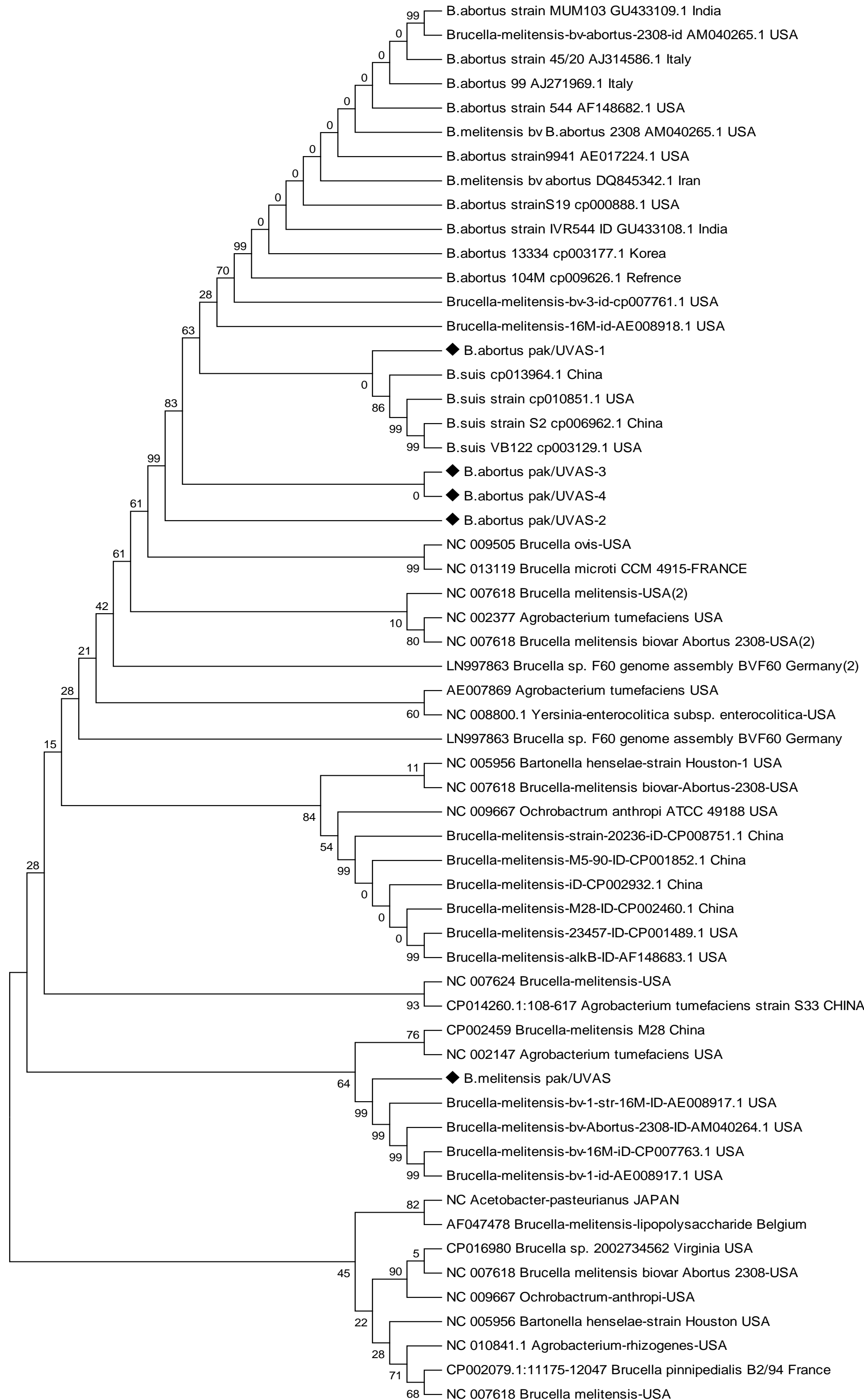

Fig. 2: Phylogenetic tree of *Brucella* isolates constructed by Neighbor-joining method in MEGA 7.0

Brucella species in the environment is a great risk for humans and animals health. The lab workers, veterinary doctors, butchers, veterinary technicians, insemination service employees, zoo technicians, farmers working on multi-herd farms, employees of meat and milk processing enterprises are at great risk of exposure with *Brucella*

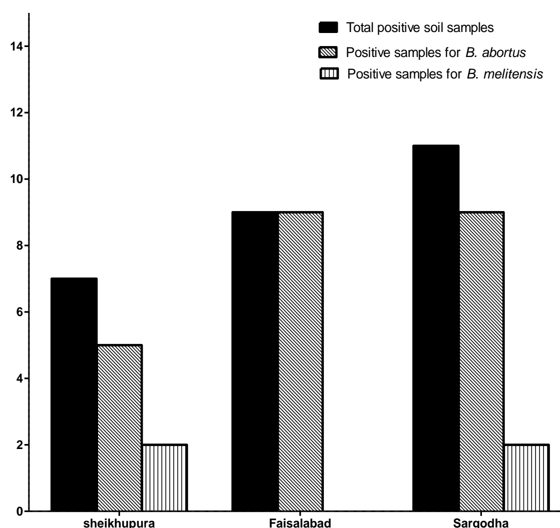

Fig. 3: Distribution of positive samples for *Brucella* species in three districts of Punjab

species (Galinska and Zagórski, 2013). Infected people show the symptoms of fever, arthralgia, myalgia, back pain, hepatomegaly, splenomegaly, endocarditis, neurobrucellosis, epididymitis and orchitis (Young, 1995).

In the present study, an overall 2.1% prevalence of *Brucella* species was observed while 12.4% was observed in Argentina (Samartino, 2002), 15.9% in Brazil (Borba *et al.*, 2013), 8.5% in Georgia (Mamisashvili *et al.*, 2013), 26.3% in Algeria (Aggad and Boukraa, 2006), 20.3% in Cameroon (Bayemi *et al.*, 2009), 4.98% in Egypt (Samaha *et al.*, 2008), 20.4% in Ethiopia (Mekonnen *et al.*, 2010), 42.2% in Libya (Gameel *et al.*, 1993), 77.5% in Nigeria (Mai *et al.*, 2012), 56.4% in Zambia (Muma *et al.*, 2007), 3.7% in Iran (Akbarmehr and Ghiyamirad, 2011), 25.8% in Jordan (Al-Majali *et al.*, 2009), 12.0% in Kyrgyzstan (Bonfoh *et al.*, 2012), 4.1% in Tajikistan (Lindahl *et al.*, 2014), 35.3% in Turkey (Şahin *et al.*, 2008) and 13.6% in India (Kumar *et al.*, 2005, Trangadia *et al.*, 2010). *Brucella* species are not detected in districts Lahore, D.G. Khan, Chakwal, Sahiwal, Gujranwala and Attock. The possible reasons for not detecting DNA may be (a) low host animal density (b) low number of households (c) high nuclease activity in the soil (d) exposure to high temperature (Bauer *et al.*, 2003).

Homology levels are considered high between isolates at 60% or more (Ma *et al.*, 2002). Soil borne *Brucella* isolates and other isolates from foreign countries accessed in Genbank were compared and homology levels determined. Soil borne *Brucella* isolates (*B. abortus* and *B. melitensis*) are>96% homologous with isolates from China [CP013964, CP006962.1], USA [CP010851.1, CP003129.1, AE008917, AM040265], Italy [AJ314586], India [GU433108], Iran [DQ845342] and Korea [CP003177.1] as shown in Table 1.

Conclusion

In conclusion, *Brucella* species such as *B. abortus* and *B. melitensis* are distributed in the soil of districts Faisalabad, Sheikhupura and Sargodha. This pattern of distribution of *Brucella* species in the districts of Punjab puts both human and animal population at a high risk of exposure. Further studies are required to explore molecular diversity (biovars) of the pathogens together with sero-conversion in animals and humans.

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