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Evidence of *Coxiella burnetii* in Punjab province, Pakistan

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ABSTRACT

Coxiella burnetii causes query (Q) fever, an important zoonotic disease with worldwide significance. The role of environment in the ecology of *C. burnetti*, and its influence on seroconversion in animals has not been elucidated in Pakistan. We carried out a cross-sectional study in Punjab province to (1) determine the prevalence and distribution of *C. burnetii* in soil using an ISIIII gene-based real time-polymerase chain reaction (RT-PCR) assay, (2) analyze association between the occurrence of *C. burnetii in soil* and its predictors i.e. soil characteristics (macro- and micro-nutrients) and several likely risk factors including the seroconversion in small ruminants at places where its genome had or had not been detected, and (3) predict homology and genetic diversity of the identified strains using sequences originated from different hosts worldwide. A total of 2425 soil samples from nine districts of Punjab province were processed. *C. burnetii* DNA was detected in 47 samples (1.94%, 95% CI: ±0.55) originating from 35 villages of studied districts (7.22%, 95% CI: ±2.30). The highest prevalence was found in Attock (7.11%, 95% CI: ±3.36), followed by Lahore (4.83%, 95% CI: ±3.49), Sahiwal (4.70%, 95% CI: ±2.6), Dera Ghazi Khan (2.33%, 95% CI: ±2.02), Faisalabad (1.35%, 95% CI: ±1.18) and Sheikhupura (0.68%, 95% CI: ±0.94). The odds of detecting bacterial DNA in soil was increased with a unit increase in organic matter [2.511 (95% CI: 1.453–4.340), $p = 0.001$] and sodium [1.013 (95% CI: 1.005–1.022), $p = 0.001$], whereas, calcium [0.984 (95% CI: 0.975–0.994), $p = 0.002$] and potassium [0.994 (95% CI: 0.990–0.999), $p = 0.011$] had protective effect where a unit increase in each analyte decreased odds for its occurrence by 1.0% approximately. Likewise, for categorical variables (risk factors), the odds of detecting *C. burnetii* were higher at locations >500 m away from a main road [1.95 (95% CI: 1.06–3.78), $p = 0.04$]. The enzyme-linked immunosorbent assay (ELISA) revealed an increased prevalence of antibodies in sheep (17.9%, 95% CI: ±5.54) compared with goats (16.4%, 95% CI: ±4.34). When determining the association between soil DNA and *C. burnetii* antibodies in small ruminants, the odds of detecting these antibodies were significant in sheep at the livestock barns [2.81 (95% CI: 1.20–7.37), $p = 0.02$]. The IS1111 gene-based sequence analysis revealed a clustering of the DNA into two distinct groups with much genetic divergence (0.76–68.70%): the first group that contained sequences from Lahore district clustered with human and buffalo origin isolates, whereas the second group that contained the sequences from the remaining study districts clustered with goat-, rodent- and human-origin isolates. This study provides the first evidence of the presence of C. *burnetii* in the environment in Punjab province, Pakistan. Future studies are needed to ascertain the bacteria's molecular epidemiology over a wide geographical area, type the isolates, and evaluates the potential risks to human populations, particularly farmers and veterinarians.

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1. Introduction

Coxiella burnetii is classified as a category B biological agent and causes query (Q) fever, a World Organization for Animal Health

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(OIE) notifiable disease. Farm animals are considered as the major reservoirs of this bacterium; however, retrospective analyses of Q fever outbreaks in humans have revealed an association between disease occurrence and small ruminants (Georgiev et al., 2013). In the event of birth from infected livestock, numerous *C. burnetii* cells are released into the environment, and they can remain infectious for a long period of time (Kersh et al., 2013). Once in the environment, when the bacterial are not replicating, the organism can persist in the soil, dust and aerosol to form a spore-like small cell variant that is resistant to drying and ultraviolent radiation. Furthermore, it has the potential to disperse over a long distance under the influence of wind and rain (Hawker et al., 1998; Tissot-Dupont et al., 2004; Schulz et al., 2005; Kersh et al., 2010; Astobiza et al., 2011). Humans could acquire this infection through the inhalation of aerosolized particles from reproductive discharges, tissues, and environmental dust as well as through direct contact with the milk, urine, feces, and semen of infected animals (Tissot-Dupont and Raoult, 2008; Chmielewski and Tylewska-Wierzbanowska, 2012; Das et al., 2014; Bielawska-Drozd et al., 2013). Although infected humans and animals remain asymptomatic in most cases, undifferentiated febrile illness in humans and reproductive disorders in affected animals have been reported (Vanderburg et al., 2014).

Since its first identification in Australian abattoir workers, Q fever has been considered endemic, and it has emerged and re-emerged worldwide (Das et al., 2014; Maurin and Raoult, 1999; Vaidya et al., 2010; Akbarian et al., 2015; Magouras et al., 2015). This disease received international attention following a recent large outbreak in the Netherlands and other European countries that involved humans and farm animals (Georgiev et al., 2013). However, because of the lack of proper diagnostic support in developing countries such as Pakistan, most cases of Q fever remain undiagnosed and tend to be grouped with other diseases such as fevers or abortions of unknown origin. Given the public and animal health perspectives to control the spread of *C. burnetii* among animals and humans from natural environment (soil) as well as from animals to humans, a consolidated prevalence data is essential for policy makers and concerned authorities for implementing prevention and control practices. Nevertheless, we lack baseline data relevant to the occurrence of Q fever in Pakistan, its epidemiology and distribution in the environment (particularly in the soil and potential disease reservoirs) despite (1) a large population of small ruminants (98 million head), (2) associated reproductive disorders such as fevers or abortions that remain undiagnosed because of the lack of appropriate diagnostics, and (3) the serological evidence of *C. burnetii* in humans and animals obtained in the 1980s (Ahmed, 1987). In this study, we report the prevalence of *C. burnetii* in soil from locations with and without human and animal interactions based on the IS1111 gene (Transposase)-based molecular detection assay. A possible relationship in survival and occurrence of *C. burnetii* with soil characteristics (macro- and micro-nutrients) and several risk factors has been assessed. A causal relationship between the presence/absence of *C. burnetii* in soil and the seroconversion in small ruminants originating from the same ecological area was determined. Furthermore, the IS1111 gene-based genetic diversity in the prevalent strains was predicted and evaluated using sequences that originated from different hosts worldwide.

2. Methods

2.1. Study area and sampling design

A cross-sectional study was carried out in Punjab province (31.1704°N and 72.7097°E) from 2011 to 2014. The province is the second largest of Pakistan with area of 205,344 square kilometers. It is bordered by Kashmir to the North-East, the Indian states of Punjab and Rajasthan to the East, the province of Sindh to the South, the province of Baluchistan to the Southwest, the province of Khyber Pakhtunkhwa to the West, and the Islamabad Capital Territory to the North. It has 9 administrative divisions and 36 districts. It is homeland of five rivers ('*Panj*', meaning five and '*Aab*' meaning water) which traverse from north to south that provides one of the world's largest irrigation system to fertile lands across the province. Agriculture and livestock are main sources of income particularly in rural areas. As per available census (2006), the Punjab dominates the Pakistan's livestock sector. The province claimed to have 49% of Pakistan's cattle, 65% of the buffaloes, 24% of the sheep, and 37% of the goats.

For our study, we used three-stage sampling design. In first stage, we purposively selected nine districts (Table 1). Because no previous reports of *C. burnetii* have been made from any of the districts so far that might help us to choose a particular area/region to be studied, we selected these districts as these represent the main livestock production areas in the province and there exist an increased annual incidence of human and animal diseases (Directorates of Animal and Human Health, Punjab). Moreover, husbandry practices in these districts represent most of the province. In second stage, assuming 50% prevalence, 95% confidence interval, and 5% margin of error, a sample size of 357 villages was calculated for total number of villages in study inclusive districts (n = 4883). Nevertheless, for more valid results, we randomly selected ten percent villages (n = 485) representing each of the study district. In third stage, five sites within each village were conveniently selected for soil sampling. Four soil samples were collected from livestock barns where animals and humans were living in close proximity to one another, and the fifth sample was collected from an agriculture land where no apparent interactions between humans and animals were observed (Fig. 1).

2.2. Soil sample collection

From each location, after removing up to 3–5 in. of the top surface soil, three samples approximately one meter apart from one another were collected and pooled together in triplicate; the first pooled sample (500–800 g) was used for the soil chemistry analysis, the second (150–200 g) was used for a real time-polymerase chain reaction (RT-PCR) analysis and the third was kept as archived sample (500–800 g). A brief history of the sample collection sites, including the geographical coordinates of location, the presence or absence of domestic animals in the farm, animal density, the presence or absence of vegetation and human dwellings, and the distances from the animal market, main road and water sources (e.g., rivers, canals, streams, and drains) were obtained for the risk factor analysis.

2.3. Molecular identification and sequence analysis

Bacterial genomic DNA was extracted from the soil samples (0.25 g) using the PowerSoil® DNA Isolation Kit (MoBio, West Carlsbad, CA, USA) following the manufacturer's guidelines. DNA quality was measured ($A_{260/280}$ and $A_{260/230}$) using a spectrophotometer (NanoDrop, USA), and the DNA quantity (ng/L) was determined using a DNA BR Assay kit with a Qubit fluorometer (Invitrogen, USA). The prevalence of *C. burnetii* was determined by a real time PCR assay (CFX96, BioRad, USA) using highly specific and sensitive primers (5′-GTCTTAAGGTGGGCTGCGTG-3′ and 5′-CCCCGAATCTCATTGATCAGC-3′) and probe (5′-FAM-AGCGAACCATTGGTATCGGACGTTTATGG-TAMRA-3′) targeting the IS1111 gene (Transposase gene) (Tozer et al., 2014). Prior to the soil sample analysis, the assay was optimized and validated using positive controls and the proficiency testing samples that were provided by Pennsylvania State University, USA. Soil samples that

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