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RESEARCH ARTICLE

First Molecular Evidence of *Coxiella burnetii* in Ticks Collected from Dromedary Camels in Punjab, Pakistan

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ABSTRACT

This study surveyed the existence of the *Coxiella burnetii* in ticks removed from camels in three different regions of the Punjab, Pakistan. A total of 325 ixodid ticks were analyzed for the occurrence of *C. burnetii* DNA by real-time polymerase chain reaction. Before analyses, all ticks were pooled into 30, 20 and 15 pools from Southern, Northern and Central Punjab, correspondingly. Each pool was comprised of five engorged or semi-engorged ticks according to species. Pooled based prevalence in Southern Punjab was eighteen from 30 pools (60%), leading to maximum-likelihood of true prevalence of 0.56% (95% CI 0.23-1.31), while pooled prevalence in Northern Punjab was eight from 20 pools (40%), leading to maximum-likelihood of the true prevalence of 0.44% (95% CI 0.31-0.89) and none of the pooled samples from Central Punjab were found positive for *C. burnetii*. During classification of tick pools based on species, infection was diagnosed in 75% (09/12) of *H. dromedary*, 42.8% (03/07) of *H. anatolicum*, 50% (01/02) of *H. scupense*, 60% (03/05) of *R. microplus*, and 50% (02/04) of *R. annulatus* in Southern Punjab. In comparison, infection was detected in 37.5% (03/08) of *H. dromedary*, 23% (01/04) of *H. anatolicum*, 0% of *H. scupense*, 66.7% (02/03) of *R. microplus* and 66.7% (02/03) of *R. annulatus* in Northern region. The detection of *C. burnetii* implies important role of ticks in the dissemination of this bacterium in Punjab, Pakistan and reveals that ticks have a major contribution to the epidemiology of coxiellosis in this ecological system. These judgments warrant further molecular investigations for better perception about *C. burnetii* epidemiology and its contributions to humans and animal disease in Pakistan.

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INTRODUCTION

Tick-borne diseases are common health problems diagnosed in both animals and humans and their spectrum has been recently increased (Dantas-Torres *et al*., 2012). Ticks serve as reservoirs and vectors for numerous zoonotic pathogens (Guatteo *et al.,* 2011; Dantas-Torres *et al*., 2012). Q fever, a tick-borne zoonotic disease, is caused by *Coxiella burnetii* (Cooper *et al*., 2011; Greene 2012; Norris *et al*., 2013). *Coxiella burnetii*, is a short (0.3-1 µm) pleomorphic strict intracellular Gram-negative coccobacillary bacterium (Maurin and Raoult, 1999; Angelakis and Raoult, 2010), and it primarily affects macrophages (Maurin and Raoult, 1999). It is capable of developing highly infective forms impervious to environmental conditions, for instance, high temperature, ultraviolet light, disinfectants and osmotic pressure (Angelakis and Raoult 2010). Coxiellosis in animals is mainly linked with various reproductive disorders, for example, infertility, metritis, stillbirths and delivery of weak calves (Porter *et al*., 2011). Q fever in humans may be acute and characterized by unspecific signs such as headache, fever and pneumonia, or a chronic form characterized by osteomyelitis, hepatitis, and endocarditis (Marrie 2009). Abattoir workers, veterinarians, farmers and laboratory staff are at great risk of contracting the infection as an occupational zoonosis (Groten *et al.,* 2020).

Soft and hard ticks are one of the main arthropods recognized to be naturally infected by *C. burnetii* (Maurin and Raoult, 1999; Cutler *et al*., 2007; Angelakis and Raoult

2010). Until now, *C. burnetii* has been recorded in more than 40 distinct tick species in different countries (Cutler *et al*., 2007). Ticks get *C. burnetii* during blood feeding from infected animals and can spread the bacterium to other animals during the next blood-feeding or by aerosols disperse of dried tick fecal matter, so play a major role in the perpetuating *C. burnetii* in the environment (Mediannikov *et al*., 2010). Ticks can also transmit *C. burnetii* vertically to their offspring's (Walker and Fishbein 1991). *C. burnetii* proliferate in the gut cells of infected ticks, ultimately resulting in high titers of viable microbes removed with feces (Maurin and Raoult, 1999). Ticks play a substantial role in Q fever epidemiology by contaminating the environment by releasing *C. burnetii* via their feces, coxal fluids and saliva (Maurin and Raoult, 1999; Angelakis and Raoult 2010). Though, no proof is available reporting the propagation of *C. burnetii* to humans by blood-feeding ticks (Maurin and Raoult, 1999). There is no report available in Pakistan about the prospective role of camel ticks playing in *C. burnetii* epidemiology. Therefore, this investigation intended to scrutinize the existence of *C. burnetii* in camel ticks in Punjab, Pakistan.

MATERIALS AND METHODS

Description of survey region: The current survey was carried out in camel population in thirteen different districts of the Punjab, Pakistan. To make the present study worthwhile and demographically manifold, 4 Central districts (Chiniot, Jhang, Sargodha and Faisalabad), 4 Northern districts (Bhakkar, Khushab, Layyah and Mianwali) and 5 districts from Southern zone (Bahawalnagar, Bahawalpur, Lodhran, Muzaffargarh and Rahim Yar Khan) of Punjab, Pakistan were selected (Fig. 1). Punjab is the densely populated province with an approximate population of 110 million (Anonymous, 2017). Its total area is 205,344 km^2. Topographically, it is situated at 72.7097^0 E and 31.1704^0 N in the semi-arid lowlands region. The majority of the area in Punjab experiences extreme weather condition. Its temperature varies from -2°C to 45°C, but may cross 50°C during summer and may drop to -1°C over winter. Average rainfall in Punjab differs with maximum rain in northern areas as compared to middle and southern region. It has a fertile agriculture land based on widespread irrigation system (Shabbir *et al.,* 2016). Agriculture and livestock sector have major contribution in the socio-economic development particularly in rural areas of Punjab, Pakistan.

Ethics statement: The procedure for the current investigation was endorsed by the ethical research committee and board of study members of the Department of Clinical Medicine and Surgery University of Agriculture Faisalabad, Pakistan. Prior consent was obtained from the owners before tick collection from infested camels.

Collection and identification of ticks: Ticks were collected from January through June 2020 from camels raised in thirteen different camel populated districts of the Punjab province of Pakistan. These districts are situated in three zones of the Central, Southern, and Northern parts of the Punjab province with different climates, agroecology and livestock management systems. A total of 140 camels were observed for the existence of ticks on different parts of their bodies according to Abdullah *et al.* (2016). Semi or fully engorged ticks attached to the skin of each camel were cautiously detached manually using curved forceps to prevent any injury to the body and put into separate, prelabeled, 5ml cryogenic vials with 70% ethanol as preservative. Ticks from the same camel were put in the same vial. A total of 325 ticks were gathered from camels during the period mentioned above. These ticks were transferred to the molecular epidemiology laboratory, Department of Veterinary Parasitology, University of Agriculture, Faisalabad, Pakistan. Ticks were morphologically identified under a microscope using identification key described previously by Estrada-Pena *et al.* (2004) and merged them in 65 pools (each pool comprised of 5 ticks). For molecular diagnosis of pathogens, pooling of ticks is a routine exercise (Knobel *et al*., 2013). Then, these tick pools were kept at -20°C till DNA extraction.

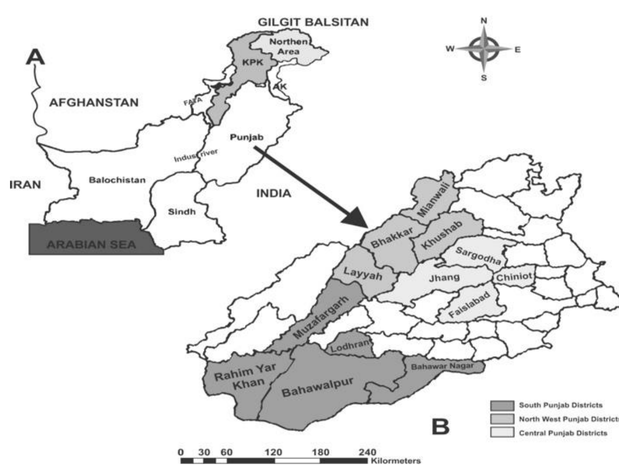

Fig. 1: A) Map of Pakistan and its neighbor countries, and B) Map of Punjab indicate the South, North and Central Districts of Punjab where samples were collected.

Molecular diagnosis

Extraction of DNA from ticks: Before DNA isolation, all ticks were thoroughly washed by 10% ethyl alcohol and dipped in distilled water then dried off using sterile filter paper. Genomic DNA was extracted from tick specimens using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) in accordance with manufacturer's guidelines. The DNA was quantified with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and stored at -40°C under sterile environment to prevent contamination till the sample was used for real time PCR.

Detection of *C. burnetii* using real-time PCR: The extracted DNA was examined for *C. burnetii* through a commercial real-time PCR kit (Liferiver ^{TM} Bio-Tech Co., Ltd.), which depends on the fluorogenic 5' nuclease test. While PCR reaction, the DNA polymerase cleaves the probe at 5' and separates the receptor dye from the quencher dye only as the probe joins with the target DNA. For a single PCR reaction, the reaction mix included 35-µl of master mix, 0.4µl of enzyme mix, and 1µl of internal control. Finally, 4-µl of extracted DNA was added. The real-time PCR reaction was executed on CFX96^{TM} Real

Time System (BIO-RAD Laboratories, Inc. USA) with the following protocol; 37°C for 2 min. for 1cycle, 94°C for 2min. for 1cycle, succeeded by 40 cycles of 93°C for 15 seconds and 60°C for 1min. A sample was deemed as positive if the Ct (threshold cycle) value of the target gene was ≤38. Molecular grade water was served as negative control.

Data analysis: Data were analyzed by MS Excel and SAS statistical software to compute prevalence of *C. burnetii* DNA conforming to independent variable like, tick pools for every study region. Maximum-likelihood of true prevalence of infection for each tick specimen in a pool and 95% confidence intervals (CI) was calculated by EpiTools epidemiological calculator (Cowling *et al*., 1999).

RESULTS

A total of 325 engorged and semi-engorged adult camel ticks comprising 150 in Southern Punjab, 100 in Northern Punjab and 75 in Central Punjab were selected for genomic identification (Table 1). Tick taxonomical identification correspond two genera: *Hyalomma* and *Rhipicephalus*. Ticks were pooled on the basis of geographic location and species into 30, 20 and 15 pool samples in Southern, Northern and Central Punjab, correspondingly. Each pool was comprised of 5 ticks. In all study areas, pools of the genus *Hyalomma* were more prevalent (Table 2). The number of pool samples according to species is presented in (Table 3). The true prevalence was calculated for each tick specimen by the method of maximum-likelihood estimation (MLE) from pooled data. In Southern Punjab overall pooled based prevalence was eighteen out of 30 pools (60%) leading to maximum-likelihood estimate (MLE) of true prevalence of 0.56% (95% CI 0.23-1.31), while in Northern Punjab, pooled based prevalence was eight from 20 pool samples (40%) leading to maximum-likelihood of true prevalence of 0.44% (95% CI 0.31-0.89) and none of the pool sample from Central Punjab were positive for *C. burnetii* (Table 2). Pursuant to species, infection was noticed in *H. dromedary* (75%), *H. anatolicum* (42.8%), *H. scupense* (50%), *R. microplus* (60%) and *R. annulatus* (50%) in South region, while infection was detected in *H. dromedary* (37.5%), *H. anatolicum* (25%), *H. scupense* (0%), *R. microplus* (66.7%) and *R. annulatus* (66.7%) in North region. None of the pools from Central Punjab was tested positive for *C. burnetii* DNA (Table 3).

DISCUSSION

It is well established that ticks may serve as vectors for several viral, bacterial, and protozoan pathogens. The potential of disease transfer varies among different tick species. Most of the tick-borne diseases do not limit to

Table 1: Region wise number of camels, ticks sampled and tick pools in Southern, Northern and Central Punjab, Pakistan

Region	Number of camels	Number of ticks	Number of tick pools
Southern	64	150	30
Northern	52	100	20
Central	24	75	15
Total	140	325	65

Southern Punjab: Bahawalnagar, Muzaffargarh, Bahawalpur, Rahim Yar Khan, Lodhran, Northern Punjab: Mianwali, Bhakkar, Layyah and Khushab. Central Punjab: Faisalabad, Chiniot, Jhang and Sargodha.

Table 2: Region wise Real time PCR- based pooled prevalence of *C. burnetii* DNA in two different genera of ticks collected from camels in Southern, Northern and Central Punjab, Pakistan

Region	Tick genera	No. of pools	PCR pos. pools	No. (%) of positive pools	% positive [a]
Southern	*Hyalomma*	21	13	13/21(61.9%)	0.76
	Rhipicephalus	09	05	5/9 (55.5%)	0.68
Total		30	18	60%	0.56(0.23-1.31)
Northern	*Hyalomma*	14	05	5/14 (37.5%)	0.38
	Rhipicephalus	06	03	3/6 (50%)	0.49
Total		20	08	40%	0.44(0.31-0.89)
Central	*Hyalomma*	10	0	0	0
	Rhipicephalus	05	0	0	0
Total		15	0	0	0

a= (Maximum-likelihood estimate of true prevalence).

Table 3: Region wise Real time PCR- based pooled prevalence of *C. burnetii* DNA in different species of ticks collected from camels in Southern, Northern and Central Punjab, Pakistan

Region	Tick species	No. of pools	PCR pos. pools	No. (%) of positive pools	% positive [a]
Southern	*H. dromedary*	12	09	09/12(75%)	0.72
	H. anatolicum	07	03	3/7 (42.8%)	0.81
	H. scupense	02	01	1/2 (50%)	0.53
	R. microplus	05	03	3/5 (60%)	0.61
	R. annulatus	04	02	2/4 (50%)	0.58
Total		30	18	60%	0.59(0.41-1.03)
Northern	*H. dromedary*	08	03	03/08 (37.5%)	0.43
	H. anatolicum	04	01	1/4 (23%)	0.38
	H. scupense	02	00	0	0
	R. microplus	03	02	2/3 (66.7%)	0.82
	R. annulatus	03	02	2/3 (66.7%)	0.82
Total		20	08	40%	0.54(0.31-1.44)
Central	*H. dromedary*	05	0	0	0
	H. anatolicum	03	0	0	0
	H. scupense	04	0	0	0
	R. microplus	02	0	0	0
	R. annulatus	01	0	0	0
Total		15	0	0	0

a= (Maximum-likelihood estimate of true prevalence); *H*= *Hyalomma*, *R*= *Rhipicephalus*.

animals but pass to humans (Dantas-Torres *et al*., 2012). In particularly, bacterial diseases like *Borrelia* spp., *Rickettsia spp.*, and *C. burnetii* cause health issue for humans and animals (Eldin *et al*., 2017). *Coxiella burnetii* in nature is present mainly in cycles including ticks and vertebrates predominantly rodents. *Coxiella burnetii* spread to animals by tick bite as well exposure to infected excreta (Angelakis and Raoult 2010). Ticks are regarded as prime reservoirs of *C. burnetii* and are liable for spread of the infection to domestic and wild animals (Norlander 2000; Duron *et al.,* 2015). It is assumed that ticks acquire infection while feeding on infected animals and then serve as reservoirs of *C. burnetii* and subsequently play a vital role in preserving the bacteria in the environment, which can cause infection in domestic animals and humans (Angelakis and Raoult, 2010). *Coxiella burnetii* can multiply in the mid-gut cells of infected ticks resulting in elevated titers, stay viable during their entire life, and may be transovarially transmitted to next progeny (Maurin & Raoult, 1999).

Ticks and vertebrates like rodents are important component of the enzootic cycle (Aitken, 1987). Infected ticks evict a massive number of *C. burnetii* through their feces on skin of the animal during feeding. Proper tick control tactics and better hygienic measures can reduce contamination of the environment (Angelakis and Raoult 2010).

Serological investigation indicates that humans and animals in Pakistan are exposed to *C. burnetii* (Ahmad, 1987; Shabbir *et al*., 2016; Zahid *et al*., 2016; Qudrat Ullah *et al*., 2019) and no information is available about camels and camel ticks. Thus, the present study was carried out to investigate the occurrence of *C. burnetii*, etiological agent of Q fever, in camel ticks in Punjab, Pakistan as a probable route of infection in Q fever epidemics. For the best of author's information, this is the maiden epidemiological investigation about the prevalence of *C. burnetii* in camel ticks. In the current study, for molecular diagnosis of *C. burnetii* in pooled tick specimen, qPCR assay was utilized.

Pooled samples offer the possibility of testing a massive number of ticks gathered from the field. *Coxiella burnetii* DNA was noticed in 20 and 2.5% of ticks harvested from dogs and cattle, respectively (Knobel *et al*., 2013). *Coxiella burnetii* DNA was detected in a significant number (25%) in questing ticks collected from farm animals in Ethiopia (Kumsa *et al*., 2015). In contrast in the Netherlands, merely 0.2% *C. burnetii* DNA was detected in *Ixodes ricinus* ticks in farm animals by applying a multi plex qPCR (Sprong *et al*., 2012). Although molecular techniques are more specific and sensitive for the diagnosis of the Q fever, few researchers used these methods in the screening of Q fever in either camels or ticks.

Our observation of *C. burnetii* in *H. dromedary*, *H. anatolicum, H. scupense*, *R. microplus*, *R. annulatus* first-ever in Pakistan is additional information regarding the species of camel ticks that host *C. burnetii* in this province. Among the various species of camel ticks, *H. dromedary* is one of the most frequently found ticks throughout all study areas. In line with our observation, *C. burnetii* had been reported in 31.0 and 7.7% of tick pools collected from sheep and goats, respectively in Punjab, Pakistan (Qudrat Ullah *et al*., 2019).

The overall frequency of *C. burnetii* (40%; 26/65) in our study is higher, contrary to the overall prevalence of 20% in ticks collected from small ruminants in Punjab, Pakistan. This difference in prevalence is most probably attributed to factors such as different host species, number of ticks examined, geographical point, seasons and variances in the sensitivity of the genes applied in PCR by the two studies. We have tested a total of 325 ticks comprising of 5 different species from 13 districts in Punjab; though, in preceding investigation 163 ticks (78 from goats and 85 from sheep) were gathered from seven different livestock experimental farms in Punjab, Pakistan.

During current investigation, the overall frequency of *C. burnetii* in ticks from the Southern region was higher than Northern region. This disparity is most likely attributable to factors for instance the presence of greater population of cattle, goats, sheep, and camel that are frequently managed by a pastoral type of management system attributed to unobstructed movement of animals in quest of water and grazing in deserts in the Southern region. Absence of *C. burnetii* in central region ticks might be due to discrepancies in geographic sites, restricted movement of animals and use of acaricide treatments can affect *C. burnetii* prevalence in ticks.

Conclusions: The current investigation confirms the occurrence of *C. burnetii* in different tick species collected from camels in Punjab, Pakistan. This observation suggests that *C. burnetii* is fairly distributed within this ecosystem and role of ticks as possible reservoirs for the microorganism, imply the need for better surveillance measures to safeguard humans and livestock from the health risks of Q fever. Generally, the results of the current study vindicate further molecular studies to have an improved insight into the epidemiology of *C. burnetii* infections and its contribution in human and animal diseases in Pakistan.

Authors contribution: SH and MS conceived the study. SH, MS and KA collected and analyzed the samples. SH, ZS compiled data set for manuscript. SH wrote the manuscript and all authors have read, edited and approved the final manuscript.

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