Acta Tropica 176 (2017) 134–139

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Seroprevalence and risk factors of glanders in working equines – Findings of a cross-sectional study in Punjab province of Pakistan

Muhammad Taslim Ghori[a,*], Muhammad Sarwar Khan[a], Jawaria Ali Khan[a], Masood Rabbani[a], Muhammad Zubair Shabbir[a], Haroon Rashid Chaudhry[a], Muhammad Asad Ali[a], Javed Muhammad[a], Mandy Carolina Elschner[b], Bhushan M. Jayarao[c]

[a] University of Veterinary and Animal Sciences, P.O. Box 54000, Lahore, Pakistan
[b] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96a 07743, Jena, Germany
[c] The Pennsylvania State University, University Park, 16802 PA, USA

ARTICLE INFO

Keywords:
Burkholderia mallei
Risk factors
Seroprevalence
Equines
Pakistan

ABSTRACT

Glanders is an infectious and contagious bacterial disease of equines. A little is known about its seroprevalence and risk factors in working equines in countries where the disease is endemic. Also, there are no reports on prevalence of the disease in areas where there is a prior evidence of *Burkholderia (B.) mallei* detection in soil. A cross-sectional study was conducted in selected districts (n = 09) of Punjab province of Pakistan during 2014–2015. A total of 1008 serum samples were screened for detection of antibodies to *B. mallei* with complement fixation test followed by western blot. The overall seroprevalence was found to be 3.17% (95% CI: 2.25–4.44). The seropositivity was significantly higher from the sampling sites where *B. mallei* was detected in soil [OR: 10.66 (95% CI: 4.42–31.66), $p = 0.00$]. Other risk factors significantly associated with animal seropositivity were: age group [OR: 1.78 (95% CI: 4.58–15.56), $p = 0.00$], location in urban area [OR: 2.99 (95% CI: 1.46–6.51), $p = 0.00$],body condition [OR: 3.47 (95% CI: 1.64–7.99), $p = 0.00$], presence of farcy lesion [OR: 7.71 (95% CI: 3.47–19.50), $p = 0.00$], proximity to water bodies [OR: 7.71 (95% CI: 3.47–19.50), $p = 0.00$]; domestic animal population [OR: 3.20 (95% CI: 1.24–10.87), $p = 0.03$] and number of households in sampling area [OR: 4.18 (95%CI: 1.82–11.30), $p = 0.00$]. The study provides an estimate of prevalence of glanders and a potential link between animal seropositivity and presence of *B. mallei* in soil. The risk factors identified in this study can be used in surveillance and disease awareness. The high prevalence of disease in draught horses and contact of infected animals with their care-takers in developing countries signify need to initiate progressive control of the disease using one health approach.

1. Introduction

Glanders, caused by *Burkholderia (B.) mallei,* is an infectious and contagious disease of equines which can negatively impact international trade, equine sportsand has potential for zoonotic transmission (Khan et al., 2013a). Clinically, the disease is characterized by the formation of nodules and ulcers on skin and respiratory tract.The disease has been reported from different regions of the world that include Southern America, Middle-East countries and Asia (Khan et al., 2012; Malik et al., 2015). Recently, the disease has regained global attention as confirmed cases have been reported from Bahrain and Germany (Elschner et al., 2016; Scholz et al., 2014).

Working horses, mules and donkeys are particularly important for livelihood and food security in rural and peri-urban areas of those developing countries that has a typical agriculture-based economy. Therefore, the individuals living either in close proximity or in close contact with the infected animals may get exposure to the infection (Van Zandt et al., 2013). Low compensation costs, poor hygienic conditions, stress, sharing of grooming equipment and communal drinking/grazing areas have been considered as risk factors for transmission and persistence of infection. In addition, movement of asymptomatic carriers without proper screening further propagate infection (Khan et al., 2013b).

Pakistan has an agriculture based economy with livestock an integral part of it. According to a latest available estimate, there are about 0.4 million horses, 0.2 million mules and 5.2 million donkeys in the country (Economic Survey of Pakistan 2016–17). Clinical evidence of glanders is not uncommon across various regions in the country;

* Corresponding author.
E-mail address: tasleemghauri@yahoo.com (M.T. Ghori).

http://dx.doi.org/10.1016/j.actatropica.2017.07.031
Received 28 April 2017; Received in revised form 25 July 2017; Accepted 27 July 2017
Available online 29 July 2017

nevertheless, a limited literature is available about serological evidence of glanders in Pakistan. The existing knowledge is patchy and inconclusive because the earlier studies were limited by the choice of study population, sampling design, sampling method and serological test employed (Naureen et al., 2007; Khan et al., 2012; Saqib et al., 2012) that may have poor detection ability and may produce non-specific results (Hatcher et al., 2015). Furthermore, the epidemiology of glanders in working horses, particularly in context to presence of *B. mallei* in soil, has not been fully understood before. The risk factors for glanders in equines used for draught and in contact with cart owner's etc. have not been yet investigated. With this background, we estimated the seroprevalence of glanders in working equines using well-accepted serological assays (combination of Complement Fixation test and Western Blot), and determined a relationship between seroconversion and potential risk factors.

2. Materials and methods

A cross-sectional study was carried out in Punjab province. Punjab is the second largest province of Pakistan in terms of area and has highest population density. Its economy is mainly dependent on agriculture and livestock. It has one of the largest irrigation systems in the world. The province has nine administrative divisions which are further divided into 36 districts/counties. First, we included Lahore, Sheikhupura, Faisalabad, Attock, Chakwal, Sargodha, Gujranwala, Sahiwal and Dera Ghazi Khan Districts in this study. These districts were purposively selected because they have an 1) increased population of equines, 2) prior evidence of presence of *B. mallei* DNA in soil (Ali et al., 2016; Shabbir et al., 2015) and 3) had previous history of case reports (Animal Disease Reporting and Surveillance Department, Government of Punjab, Pakistan). In the second stage, while assuming 95% confidence interval, 10% expected prevalence and 5% margin of error,a sample size of 139 villages was calculated from a total number of villages (n = 4883) in selected districts of Punjab province (WinEpi software, http://www.winepi.net/uk/sample/indice.htm). However, we increased the study villages (n = 233) for further accuracy and validity in results. At third step, as per availability of subject animal conveniently at sampling site, a total of 1008 serum samples were collected from horses (n = 257), donkeys (n = 727) and mules (n = 24). The sera were collected from those villages (n = 233) of select districts where *B. mallei* was (n = 339) and was not (n = 669) detected in soil previously (Ali et al., 2016; Shabbir et al., 2015).

Following consent of the owner, blood samples (approximately 5 mL) were collected in VACUETTE® (VWR, Radnor, PA, USA). Sera were separated by centrifugation at 4000 rpm for 10 min, labeled and stored at −20 °C. For each sample, the following data were collected i) history of respiratory signs (nasal discharge and/cough) in last 3 months [yes, no], ii) history of skin lesions (nodules and/ulcers) in last 3 months [yes, no], iii) body condition [poor, normal], iv) use of the animals [tonga, cart], v) location of sampling site [rural, urban], vi) age group [≤6 years, ≥6 years], vii) sex [male, female], vii) evidence of detection of *B. mallei* in soil samples from the sampling site [yes, no], viii) distance of sampling site from nearest water body [≤500m, ≥500m], ix) distance of sampling site from nearest animal market [≤1 km, ≥1 km], x) domestic animal population in the sampling village [≥1000, ≤1000], xi) number of households in the sampling village [≤300, ≥300]. Those risk factors were selected after informal discussions with equine practitioners.

Each sera were processed for anti-*B. mallei* antibodies through complement fixation test (CFT) followed by western blot. Since equine sera may have 1) anti-complementary or hemolytic activity, 2) certain unknown bodies that may facilitate non-specific fixation of complement and 3) an increased immunoglobin G isotype (T) (Kappmeyer et al., 1999; Robinson, 1939), we diluted each sample sera in CFT buffer (1:5) (Virion/Serion, pH 7.2) and incubated in a water bath at 60 °C for 30 min (for horses), and at 63 °C for 30 min (for donkeys and mules) as described previously (Khan et al., 2014). A CFT antigen containing mixture of three different strains of *B. mallei* (c.c.pro. GmbH, Germany), a complement and a ready-to-use hemolytic system (Virion/Serion, Germany) were used in the test. Positive control serum (commercial horse serum, c.c.pro, GmbH, Germany) and negative serum (commercial horse serum, Bio & Sell, Feucht, Germany) were also used. Any sample was considered positive if there is no hemolysis of RBCs at 1:5 dilution and, negative if there was a complete hemolysis.

All the sera that were positive or positive for anti-complementary reaction for glanders in the CFT were subjected to western blot analysis. The western blot assay was performed according to the procedure described previously (Elschner et al., 2011) with minor changes. Initially, hyper immune sera were raised in separate specific pathogen free (SPF) rabbits using a suspension (10^6 CFU/ml) of heat-inactivated *B. mallei* (Zagreb), *B. pseudomallei* (ATCC 23343), *Streptococcus equi ssp. zooepidemicus* (ATCC 700400), *Streptococcus equi ssp. Equi* (ATCC 9528), *Rhodococcus equi* (8DSM 20307 ATCC 25729), and *Streptococcus equinus* (DSM 20558 ATCC 9812). The animals were kept in SPF containment facility throughout the experiment. The experiment was authorized by the government of Thuringia, Germany (Reg.-Dr. 04-53/00 and 04-51/04).To cover a broad antigenic variation of pathogen (*B. mallei*) originating from different regions of the world, a mixture of LPS (350 µL volume) containing three strains of *B. mallei*, namely Bogor, Dubai-7, and Mukteswar, was used to separate bands on a precast 4–12% polyacrylamide gradient gel (Invitrogen, USA). Using an I-Blot Module (Invitrogen, USA), the LPS was transferred to a 0.45 µm nitrocellulose membrane (Invitrogen) for 7 min. The membrane was dipped in blocking solution (Candor Bioscience, Germany) overnight followed by 3 times washing in washing buffer (Candor Bioscience, Germany) for 20 min each on a shaker and cut into 3-mm-wide strips. The strips were then stored in a freezer at −20 °C or were processed immediately for the immunoblot assay. LPS-coated strips were labeled properly and placed in incubation trays (Bio Rad, USA). Then, the strips were covered with the equine test sera (1:50 dilution) or rabbit sera (1:400 dilution) in Low Cross buffer (Candor Bioscience, Germany) and incubated at room temperature for 1.5 h on a shaker. After incubation, the strips were subjected to three washing steps in washing buffer (Candor Bioscience, Germany) for 20 min each. Then, a 1:5000 dilution of alkaline phosphatase-conjugated rabbit anti-horse-IgG or goat anti-rabbit-IgG (Sigma, Germany) was prepared in Low Cross buffer. The strips were then incubated with the respective diluted, conjugated secondary antibodies for 1.5 h at room temperature with continuous shaking. Following incubation, the strips underwent three washing steps for 20 min each. Then, the strips were stained with NBT-BCIP® solution (Sigma, USA). The staining of the strips was stopped after 10 min by dipping them in distilled water. A sample was considered positive if the LPS banding pattern within the region of 20–60 kDa was clearly visible, was scored suspicious if weak bands were detected, and was scored negative if no bands were observed.

The data were analyzed in SPSS version 21 (SPSS Inc., Chicago, IL, USA). Chi-square test and, Fisher Exact test (where expected count was less than 5) were used to evaluate association between seropositivity for *B. mallei* and its potential categorical predictors mentioned earlier. Binary logistic regression analysis was carried out to quantify the risk factors. Seropositivity was kept as dependent variable and independent variables with p value less than 0.05 in univariate analysis were included in the model through backward selection (LR method). Categories with lowest prevalence were used as reference. For Wald test, p value < 0.05 were considered as significant.

3. Results

A total of 1008 sera representing 233 villages across the nine select districts were examined for the presence of *B. mallei* antibodies. Based on the soil-positivity to genome of *B. mallei* in each district, the number of sampled villages varied from 10 in Lahore to 34 in both Sheikhupura

Table 1
Seroprevalence of B. mallei in equines in selected districts of Punjab, Pakistan.

District (no. of equine samples)	Samples screened with CFT			Samples confirmed with Western Blot		Seroprevalence
	Negative	Positive	Suspected	Negative	Positive	
Lahore (n = 95)	46	25	24	69	26	27.36%
Sheikhupura (n = 175)	152	7	16	175	0	0.00%
Gujranwala (n = 191)	131	24	36	190	1	0.52%
Faisalabad (n = 110)	98	9	3	109	1	0.90%
Chakwal (n = 69)	57	2	10	68	1	1.44%
Attock (n = 184)	177	2	5	184	0	0.00%
Sargodha (n = 56)	41	3	12	53	3	5.35%
Sahiwal (n = 78)	76	1	1	78	0	0.00%
Dera Ghazi Khan (n = 50)	44	0	6	50	0	0.00%
Total	822	73	113	976	32	3.17%

All samples were initially screened with CFT. Only those samples, found positive and anti-complementary in CFT were examined through Western blot. The prevalence was calculated on the basis of number of samples positive in western blot (numerator) and total number of samples collected in each district (denominator). There was significant difference in seroprevalence across the districts ($p < 0.05$).

and Chakwal, while 189 villages were sampled from the districts where genome to *B. mallei* was not detected (Shabbir et al., 2015; Ali et al., 2016). These were 45 villages from Gujranwala, 41 from Faisalabad, 61 from Attock, 15 from Sargodha, 17 from Sahiwal and 10 from Dera Ghazi Khan. A complete description of number of villages representing each district and number of equine sera collected has been indicated in Table 1.

Seropositivity to *B. mallei* was detected in only 35 (3.17%, 95% CI: 2.25–4.44) of the total equine samples (Table 1). Fig. 1 shows the distribution of sampling sites and locations where animals were found positive. Interestingly, prevalence of *B. mallei* was significantly higher in horses (12.45%) while donkeys and mules were found seronegative. The prevalence of antibodies was significantly higher from the districts where there was an earlier evidence of *B. mallei* DNA in the soil (Table 2). The seroprevalence in soil-positive districts was 7.96% compared to 0.74% in soil negative districts. We also found significant differences among the study districts ($p < 0.05$). Seropositive animals belonged to Lahore, Gujranwala, Faisalabad and Chakwal districts. Lahore had the highest prevalence of 27.36%, followed by Sargodha (5.35%), Chakwal (1.44%), Faisalabad (0.90%) and Gujranwala (0.52%). None of the horses sampled in Sheikhupura, Attock, Sahiwal and Dera Ghazi Khan were seropositive.

The univariate analysis revealed significant association of glanders with i) history of skin lesions ii) body condition, iii) location of sampling site, vi) age group, v) evidence of detection of *B. mallei* in soil samples, vi) distance of sampling site from nearest water body, vii) animal population in the sampling village, viii) number of households in the sampling village ($p < 0.05$) (Table 3). Table 4 shows odds ratio (OR) and 95% CI of the risk factors. Animals with history of skin lesions and poor body condition were more likely to be seropositive [OR: 7.71 (95% CI: 3.47–19.50), $p = 0.00$] and [OR: 3.47 (95% CI: 1.64–7.99), $p = 0.00$], respectively. The risk of the disease was high in equines in urban areas [OR: 2.99 (95% CI: 1.46–6.51), $p = 0.00$]. Another animal related risk factor was the age of the animals. We found more seropositivity in animals less than six years of age [OR: 1.78 (95% CI: 4.58–15.56), $p = 0.00$]. It was evident from the data that the animals that were in close proximity to water bodies were more likely to give a seropositive response than those located away from water bodies [OR: 7.71 (95% CI: 3.47–19.50), $p = 0.00$]. Domestic animal population and number of households in the area were also significantly associated with the disease [OR: 3.20 (95% CI: 1.24–10.87), $p = 0.03$] and [OR: 4.18 (95% CI: 1.82–11.30), $p = 0.00$], respectively.

4. Discussion

Mallein test has been used commonly in Pakistan for screening of equine glanders. However, for initial screening of subject animal for being either positive or negative to glanders, we preferred CFT over Mallein due to the reasons that 1) the subject animals are non- cooperative and exhibit an aggressive behavior in field conditions, 2) the test requires a prolonged time (48–72 h) for result interpretation and one can't rely on farmers/owners to share accurate outcome of the results and, 3) the test has certain limitations including non-specific reactions with other bacteria, sero-conversion in subject animal, non-responsive in clinically advanced cases and false negative results with an increase in age or debility of subject animal (Naureen et al., 2007; Arun et al., 1999).

We used both CFT and WB assay for serological evidence of anti-*B. mallei* antibodies among equines (horse, donkey and mule) representing select districts of Punjab province of Pakistan. However, the percent prevalence obtained through WB assay was taken as conclusive. This is so because 1) CFT is not considered a reliable test for animals whose sera show anti-complementary activity or self-react with normal antigen (Hagebock et al., 1993), 2) it may not detect antibodies as late as 40 days from disease onset (Gilad et al., 2007) and 3) may cross-react with endemic Strangles-infected sera and could yield false-positive results (Sprague et al., 2009; Ijaz et al., 2010). Contrary to CFT, significantly higher specificity has been observed with WB assays (Elschner et al., 2011; Khan et al., 2012) and demonstrated to be two to four times more efficient in the serological detection of glanders (Katz et al., 1999). The same was evident in this study where *B. mallei* only cross-reacted with much genetically-related *B. pseudomallei,* while all other related bacteria such as *S. equi ssp. zooepidemicus*, *S. equi ssp. equi*, *R.equi* and *S. equinus* were negative by western blot (Fig. 2). It is important here to indicate that clinical evidence of Strangles are being reported in equines in Pakistan; however, none of cases of animal Melioidosis has been reported until today.

An overall seroprevalence of *B. mallei* was found to be 3.17% in our study. The prevalence was highest in district Lahore followed by Sargodha, Chakwal, Faisalabad, and Gujranwala districts. A number of studies have shown varying prevalence (0.5–16.7%) of glanders according to district/region, subject animals and assay employed (Nasreen, 1977; Vaid et al., 1981; Bashir, 1983; Hussain, 2011). A retrospective analysis of outbreaks data of glanders from 1999 to 2007 also identified diseased animals in district Lahore, Faisalabad, and Sargodha (Hornstra et al., 2009; Muhammad et al., 1998). Glanders cases were also observed in race horses of Polo club lahore during an outbreak in 2005 (Saqib et al., 2012). Taken together, results of this study and previously documented data, it implicates the endemicity of disease across a wide region of the province.

We found a significant difference in prevalence among the study districts, where it was much higher in district Lahore (27.36%) than others.Differences in prevalence across the study districts might be due to variation in population structure, agro-ecological conditions and

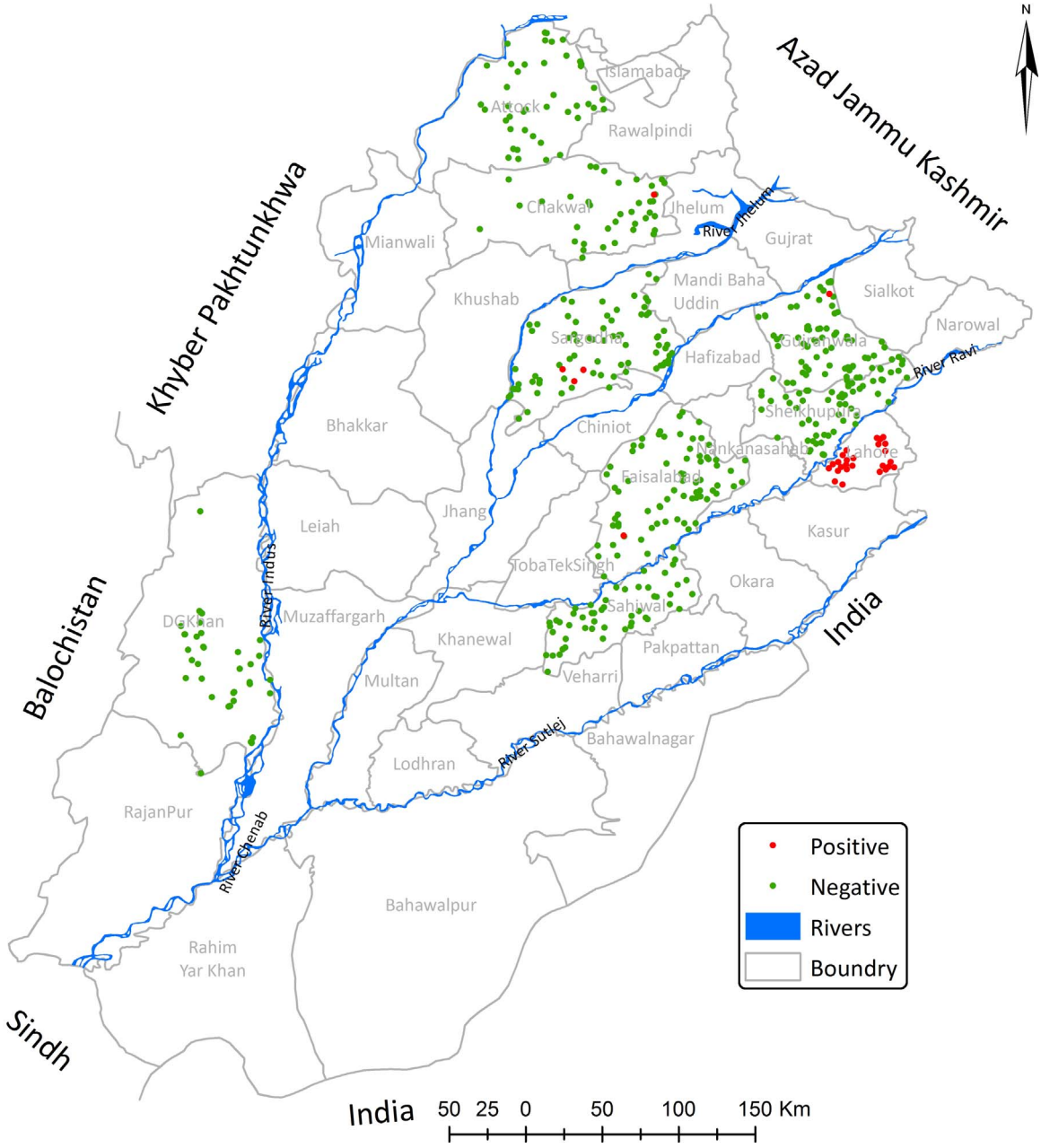

Fig. 1. Spatial distribution of sampling sites to estimate seroprevalence of B. mallei in working equines in Punjab, Pakistan. The geographical locations are red highlighted where antibodies to B. mallei were identified. The samples were taken from Lahore, Sheikhupura, Gujranwala, Faisalabad, Chakwal, Attock, Sargodha, Sahiwal and Dera Ghazi Khan Districts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other factors that may influence transmission of infection. For instance, communal water troughs in big cities and proportion of carriers may vary among the districts. An increased frequency of anti-*B. mallei* antibodies detection from Lahore district is of particular concern to public health and puts human and animal (equine) at a higher risk of exposure.

We concluded a higher proportion of seropositive animals in districts where there was a prior evidence of *B. mallei* DNA in soil (Ali et al., 2016; Shabbir et al., 2015). Similar observation has also been observed in a recent study where a higher seroprevalence was observed in small ruminants at places where genome of *Coxiella burnetii* was detected in soil as compared to the soil negative area (Shabbir et al., 2016). Although, it is an interesting finding and may require further studies but it does not confirm cause-effect relationship unless isolated from either DNA positive soil or clinical sample.Unfortunately, for us, it was not possible to culture either DNA positive soil or clinical sample from study equines because there lack BSL-3 containment facilities for culturing and propagation of pathogens of veterinary origin in the country. However, there exists a strong relationship between characteristics of the soil, turbidity of the water and the survival of the pathogen in the environment which may increase the exposure risk (Ribolzi et al., 2016). *B. mallei* has been shown to survive in the soil and water troughs in animals houses (Coenye and Vandamme, 2003), and, the physicochemical properties of soil and vegetation in the surroundings may affect the survival of the bacteria (Limmathurotsakul et al., 2010).

The history of lesions on skin, age, body condition and use of animal in urban areas were found to affect odds of seropositivity. In our study, the lowest prevalence was evident in the old-age group (older than 6 years). This could be due to the fact that CFT gives a false-negative result in older animals due to poor immune response (Sprague et al., 2009). Since animals over two years of the age are more susceptible to the disease (Al-Ani and Roberson, 2007) and young animals (below 6

Table 2
Distribution of B. mallei antibodies in equines from soil positive and negative districts.

District	Prevalence of *B. mallei* antibodies	
	Soil positive areas	Soil negative areas
Lahore	26/95 (27.36%)	–
Sheikhupura	0/175 (0%)	–
Gujranwala	–	1/191 (0.52%)
Faisalabad	–	1/110 (0.90%)
Chakwal	1/69 (1.44%)	–
Attock	–	0/184 (0%)
Sargodha	–	3/56 (5.35%)
Sahiwal	–	0/78 (0%)
Dera Ghazi Khan	–	0/50 (0%)
Total	27/339 (7.96%)	5/669 (0.74%)

Soil positive areas means sampling districts where *B. mallei* DNA was detected in soil. Soil negative areas means sampling districts where *B. mallei* was not detected in soil. The prevalence of antibodies against B. mallei in equines was significantly high in districts where *B. mallei* was detected in soil samples.

Table 3
Summary of categorical variables and their association with seropositivity against B. mallei in working equines in Punjab, Pakistan.

Variables	Levels	Positive	Negative	p-value
History of respiratory signs	Yes	15	378	0.45
	No	17	598	
History of skin lesions	Yes	26	545	0.007
	No	06	431	
Body condition	Poor	23	414	0.001
	Normal	09	562	
Use of animals	Cart	19	687	0.25
	Tonga	13	289	
Location of sampling site	Urban	21	380	0.004
	Rural	11	596	
Age group	≤6 years	28	590	0.00
	≥6 years	04	386	
Sex	Male	14	555	0.19
	Female	18	421	
Evidence of *B. mallei* in soil	Yes	27	312	0.00
	No	05	664	
Distance of sampling site from nearest water bodies	≤500 m	25	309	0.00
	≥500 m	07	667	
Distance of sampling site from nearest animal market	≤1 km	12	396	0.86
	≥1 km	20	580	
Animal population in sampling village	≥1000	28	670	0.03
	≤1000	04	306	
No. of households in village	≥300	26	497	0.00
	≤300	06	479	

Table 4
Estimates from the logistic regression model for association between seropositivity for glanders and its predictors in Punjab.

Variable	β-value	S.E.	p-value	OR (95% CI)
History of skin lesions	2.04	0.43	0.00	7.71 (3.47–19.50)
Body condition	1.24	0.39	0.00	3.47 (1.64–7.99)
Location of sampling site	1.09	0.37	0.00	2.99 (1.46–6.51)
Age group	1.52	0.53	0.00	1.78 (4.58–15.56)
Evidence of *B. mallei* in soil	2.36	0.49	0.00	10.66 (4.42–31.66)
Distance of sampling site from nearest water bodies	2.04	0.43	0.00	7.71 (3.47-19.50)
Animal population in sampling village	1.16	0.53	0.03	3.20 (1.24–10.87)
No. of households in village	1.42	0.45	0.00	4.18 (1.82–11.30)

years) are preferred for draught purpose in the country, this may be the reason that we noted a higher seroprevalence in this age group. Our results are also consistent with the observations during an outbreak of glanders in Lahore Polo Club horses, where the median age of affected

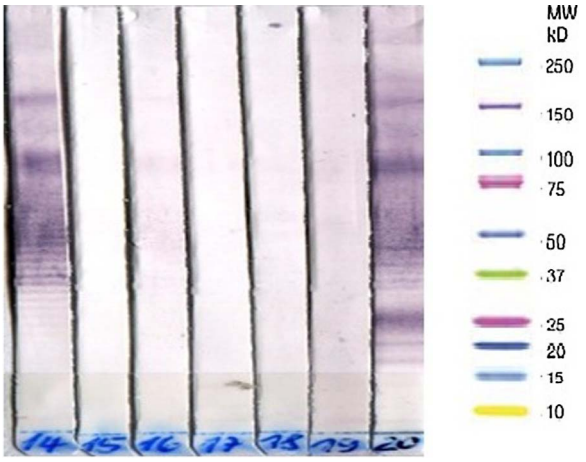

Fig. 2. Western Blot based Banding pattern of sera raised against antigen similar to *B. mallei*. *14 – *B. mallei (Zagreb)*, 15 – *Negative control*, 16 – *Str. equi ssp. zooepidemicus*, 17 – *Str. equi ssp. equi*, 18 – *Rhodococcus equi*, 19 – *Streptococcus equinus*, 20 – *B. pseudomallei*. Positive if the banding pattern of the sample falls within the region of 20–60 KDa.

horses was 3.5 years (Saqib et al., 2012).

We found statistically significant species difference with highest seropositivity in horses. This may correspond to the fact that disease is chronic in horses evidenced by the skin nodules and hematological features (Muhammad et al., 1998), while it is acute and fatal in donkeys and mules (Neubauer et al., 2005; Whitlock et al., 2007) and requires a higher infecious dose to produce disease (Altemann et al., 2012). Communal water troughs and stables serve as a reservoir of glanders (Hornstra et al., 2009). The secretions of the diseased animals may contaminate the water and transfer the infection to others in-contact animals. It was observed that draught equines mostly graze along the banks of canals, where they may shed the bacteria over the pasture, resulting in the sero-conversion of healthy animals. Also, in the present study, the animals from sampling sites located close to water bodies such as canals/streams/drains were more likely to develop a seropositive response. Another reason for the increased risk is that people mostly throw dead animals near the water bodies. In addition, farriers and hair groomers also work near the canal banks. They use the equipment for grooming/shoeing without disinfection. Thus, any type of injury during harnessing may transmit the infection to other healthy animals. This statement strengthens our findings that the seropositivity of the animals was also associated with previous history of skin lesions. Similarly, high animal population and households were also significantly associated with the seropositivity. Overcrowding has also been attributed to affect the risk of glanders transmission (Dvorak and Spickler, 2008).

This study had certain limitations that must be considered while interpreting the results. We determined test prevalence rather than true prevalence, because the agent was not isolated from the sero-positive animals. With some variables, such as number of households in village, we were dependent on owner's response which is subjective rather objective way of data collection.

5. Conclusions

This study revealed seroprevalence of glanders and its risk factors in working equines using well-known CFT and WB assay. To our knowledge, this is the first study that compared seroprevalence of glanders from areas where there was and was not earlier detection of *B. mallei* in soil. Relatively, a high prevalence of glanders was found in districts with earlier evidence of *B. mallei* detection in the soil, horses less than 6 years of age, equine population near water bodies, area with high animal population and number of households. In addition, seropositivity was dependent on three animal attributes that is age, body condition,

and history of skin lesion. The risk factors identified in this study should be given priority in surveillance, prevention and disease awareness. For future studies, an extensive surveillance should be done to monitor seroconversion in high risk human population which remains in close and unprotected contact with working equines in unhygienic conditions. Given the much better specificity and sensitivity of WB assay than CFT, it should be preferred for surveillance of glanders in a disease endemic country.

Conflict of interest

There is no conflict of interest.

Acknowledgments

This work was financially supported by the Defense Threat Reduction Agency, Basic Research Award # HDTRA1-10-1-0080 to The Pennsylvania State University, USA (Prime Awardees) and University of Veterinary and Animal Sciences, Lahore Pakistan (Subcontract Awardees). We are thankful to the Higher Education Commission of Pakistan for financial support to travel and process sera at Friedrich-Loeffler-Institut, Germany. We thank the Livestock and Dairy Development Department, Punjab, and the local veterinarians and equine owners for facilitating the serum sampling. We also thank the laboratory staff for their assistance and support in collecting the field samples at the University of Veterinary and Animal Sciences Lahore, Pakistan. The authors are thankful to Dr. U. Wernery, CVRL Dubai, for providing of the *B. mallei* strain Dubai-7.

References

Al-Ani, F.K., Roberson, J., 2007. Glanders in horses: a review of the literature. Vet. Arhiv. 77, 203–218.
Ali, M.A., Muhammad, K., Anjum, A.A., Ahmad, M-u-D., Rabbani, M., Shabbir, M.Z., Ahmad, A., Muhammad, J., Chaudhry, M.H., Chaudhry, H.R., Ghori, M.T., Jamil, T., Haisem, M., Jayarao, B.M., 2016. Spatial distribution of Burkholderia mallei in Punjab, Pakistan. J. Equine Vet. Sci. 4 (Supplement S105), 39.
Altemann, D., Bauerfeind, R., Wernery, U., 2012. Pathogenesis of glanders in experimentally infected feral donkeys using different infection routes and doses. J. EquineVet. Sci. 32, 82.
Arun, S., Neubauer, H., Gürel, A., 1999. Equine glanders in Turkey. Vet. Rec. 144, 255–258.
Bashir, A., 1983. Incidence and epidemiology of glanders in and around Faisalabad. M.Sc. Thesis. Department of Veterinary Microbiology, Univ. of Agriculture, Faisalabad, Pakistan.
Coenye, T., Vandamme, P., 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. 5, 719–729.
Dvorak, G.D., Spickler, A.R., 2008. Glanders. J. Am. Vet. Med. Assoc. 233, 570–577.
Elschner, M.C., Scholz, H.C., Melzer, F., Saqib, M., Marten, P., Rassbach, A., Dietzsch, M., Schmoock, G., de Assis Santana, V.L., de Souza, M.M., 2011. Use of a Western blot technique for the serodiagnosis of glanders. BMC Vet. Res. 7, 4.
Elschner, M.C., Liebler, T.E., Brügmann, M., Melzer, F., Neubauer, H., 2016. Re-appearance of glanders into Western Europe −clinical, laboratory and pathological findings in a German horse. OIE Sci. Technol. Rev. Bull. 1, 76–79.
Gilad, J., Schwartz, D., Amsalem, Y., 2007. Clinical features and laboratory diagnosis of infection with the potential bioterrorism agents burkholderia mallei and burkholderia pseudomallei. Int. J. Biomed. Sci. 3, 144–152.
Hagebock, J.M., Schlater, L.K., Frerichs, W.M., Olson, D.P., 1993. Serologic responses to the mallein test for glanders in solipeds. J. Vet. Diagn. Invest. 5, 97–99.
Hatcher, C.L., Muruato, L.A., Torres, A.G., 2015. Recent advances in Burkholderia mallei and B pseudomallei research. Curr. Trop. Med. Rep. 2, 62–69.
Hornstra, H., Pearson, T., Georgia, S., Liguori, A., Dale, J., Price, E., O'Neill, M., Deshazer, D., Muhammad, G., Saqib, M., 2009. Molecular epidemiology of glanders. Pak. Emerg. Infect. Dis. 15, 2036–2039.
Hussain, M.H., 2011. Serosurvey Of Equine Infectious Anemia, Glanders And Piroplasmosis In Five Draught Equine Populated Urban Areas Of Punjab. University of Agriculture, Faisalabad.
Ijaz, M., Khan, M., Khan, M., Avais, M., Maqbool, A., Ali, M., Shahzad, W., 2010. Prevalence and serum protein values of strangles (Streptococcus equi) affected mules at Remount Depot, Sargodha (Pakistan). Equine Vet. Educ. 22, 196–198.
Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to Babesiacaballi by recombinant B. caballirhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37, 2285–2290.
Katz, J.B., Chieves, L.P., Hennager, S.G., Nicholson, J.M., Fisher, T.A., Byers, P.E., 1999. Serodiagnosis of equine piroplasmosis, dourine, and glanders using an arrayed immunoblotting method. J. Vet. Diagn. Invest. 11, 292–294.
Khan, I., Wieler, L.H., Butt, M.A., Elschner, M.C., Cheema, A.H., Sprague, L.D., Neubauer, H., 2012. On the current situation of glanders in various districts of the Pakistani Punjab. J. Equine Vet. Sci. 32, 783–787.
Khan, I., Wieler, L.H., Melzer, F., Elschner, M.C., Muhammad, G., Ali, S., Sprague, L.D., Neubauer, H., Saqib, M., 2013a. Glanders in animals: a review on epidemiology, clinical presentation, diagnosis and countermeasures. Transbound. Emerg. Dis. 60, 204–221.
Khan, I., Ali, S., Gwida, M., Elschner, M.C., Ijaz, M., Anjum, A.A., Neubauer, H., 2013b. Prevalence of Burkholderia mallei in Equids of Remount Depot Sargodha, Pakistan. Pak. J. Zool. 45, 1751–1756.
Khan, I., Wieler, L., Saqib, M., Melzer, F., Santana, V., Neubauer, H., Elschner, M.C., 2014. Effect of incubation temperature on the diagnostic sensitivity of the glanders complement fixation test. Rev. Sci. Technol. Off. Int. Epiz. 33, 869–875.
Limmathurotsakul, D., Wuthiekanun, V., Chantratita, N., Wongsuvan, G., Amornchai, P., Day, N.P., Peacock, S.J., 2010. Burkholderia pseudomallei is spatially distributed in soil in northeast Thailand. PLoS Negl. Trop. Dis. 4, e694.
Malik, P., Singha, H., Goyal, S.K., Khurana, S.K., Tripathi, B.N., Dutt, A., Singh, D., Sharma, N., Jain, S., 2015. Incidence of Burkholderia mallei infection among indigenous equines in India. Vet. Rec. Open 2, e000129.
Muhammad, G., Khan, M., Athar, M., 1998. Clinico-microbiological and therapeutic aspects of glanders in equines. J. Equine Sci. 9, 93–96.
Nasreen, N., 1977. A study on the incidence of glanders at Lahore. M.Sc. Thesis. College of Veterinary Science, University of Agriculture, Faisalabad, Pakistan.
Naureen, A., Saqib, M., Muhammad, G., Hussain, M.H., Asi, M.N., 2007. Comparative evaluation of Rose Bengal plate agglutination test, mallein test, and some conventional serological tests for diagnosis of equine glanders. J. Vet. Diagn. Invest. 19, 362–367.
Neubauer, H., Sprague, L., Zacharia, R., Tomaso, H., Al Dahouk, S., Wernery, R., Wernery, U., Scholz, H., 2005. Serodiagnosis of Burkholderia mallei Infections in horses: State-of-the-art and Perspectives. J. Vet. Med.B, Infect. Dis. Vet. Public Health 52, 201–205.
Ribolzi, O., Rochelle-Newall, E., Dittrich, S., Auda, Y., Newton, P.N., Rattanavong, S., Knappik, M., Soulileuth, B., Sengtaheuanghoung, O., Dance, D.A., 2016. Land use and soil type determine the presence of the pathogen Burkholderia pseudomallei in tropical rivers. Environ. Sci. Pollut. Res. Int. 23, 7828–7839.
Robinson, E.M., 1939. Some observations on dourine. J. S. Afr. Vet. Assoc. 10, 44–55.
Saqib, M., Muhammad, G., Naureen, A., Hussain, M.H., Asi, M.N., Mansoor, M.K., Toufeer, M., Khan, I., Neubauer, H., Sprague, L.D., 2012. Effectiveness of an antimicrobial treatment scheme in a confined glanders outbreak. BMC Vet. Res. 8, 214.
Scholz, H.C., Pearson, T., Hornstra, H., Projahn, M., Terzioglu, R., Wernery, R., Georgi, E., Riehm, J.M., Wagner, D.M., Keim, P.S., 2014. Genotyping of Burkholderia mallei from an outbreak of glanders in Bahrain suggests multiple introduction events. PLoS Negl. Trop. Dis. 8, e3195.
Shabbir, M.Z., Jamil, T., Ali, A.A., Ahmad, A., Naeem, M., Chaudhary, M.H., Bilal, M., Ali, M.A., Muhammad, K., Yaqub, T., Bano, A., Mirza, A.I., Shabbir, A.B., McVey, W.R., Patel, K., Francesconi, S., Jayarao, B.M., Rabbani, M., 2015. Prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan. Front. Microbiol. 6, 917.
Shabbir, M.Z., Akram, S., Hassan, Z., Hanif, K., Rabbani, M., Muhammad, J., Chaudhary, M.H., Abbas, T., Ghori, M.T., Rashid, H., Jamil, T., Islam, Z., Rasool, H., Bano, A., Ahmad, A., Ali, M.A., Yaqub, T., McVey, W., Jayarao, B.M., 2016. Evidence of *Coxiella burnetii* in Punjab province, Pakistan. Acta Trop. 163, 61–69.
Sprague, L.D., Zachariah, R., Neubauer, H., Wernery, R., Joseph, M., Scholz, H.C., Wernery, U., 2009. Prevalence-dependent use of serological tests for diagnosing glanders in horses. BMC Vet. Res. 5, 32.
Vaid, M., Muneer, M., Naeem, M., 1981. Studies on the incidence of glanders at Lahore [Pakistan]. Pak. Vet. J. 1, 75.
Van Zandt, K.E., Greer, M.T., Gelhaus, H.C., 2013. Glanders: an overview of infection in humans. Orphanet J. Rare Dis. 8, 131.
Whitlock, G.C., Estes, D.M., Torres, A.G., 2007. Glanders: off to the races with Burkholderia mallei. FEMS Microbiol. Lett. 277, 115–122.