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RESEARCH ARTICLE

Seroprevalence and Risk Factors of Equine Glanders in Selected Districts of Khyber Pakhtunkhwa (KPK)

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ABSTRACT

Glanders is highly contagious and zoonotic bacterial disease of equines caused by *Burkholderia mallei*. Since many decades, glanders is endemic in eastern parts (Punjab) of Pakistan and has no information of disease in the north-western region (Khyber Pakhtunkhwa – KPK). Therefore, present study was aimed to determine the seroprevalence and associated risk factors in two draft equine populated districts (Charsada and Peshawar) of KPK. A total of 393 equine samples originating from different localities of study area were investigated for anti-*B. mallei* antibodies (glanders) using indirect enzyme linked immunosorbent assay (ID Vet, Grabels France). The overall seroprevalence was found to be 3.1% (95% CI: 1.6-5.3) which remained insignificantly (χ^2 0.421, p=0.516) between the two districts. In Charsada, prevalence estimates varied significantly (χ^2 11.986, p=0.017) among 5 localities and found insignificant (χ^2 1.287, p=0.732) in different sampling areas of Peshawar district. Donkeys were more seropositive (4.1%; 4/195) than horses (2.1%; 8/194) which did not differ statistically (χ^2 1.397, p=0.237) and all mules were tested negative. Prevalence slight increased with age of animals (>10 years) and with poor body conditions. Donkeys were more test positive (OR: 2.05, 95% CI: 0.61-6.94) than horses and similarly animals managed on communal feeding (OR: 1.84, 95% CI: 0.23-14.5) and water troughs (OR: 1.65, 95% CI: 0.21-13.04) had more chances of contracting glanders. Multivariable model indicated donkeys (OR: 4.58, 95% CI: 1.0, 20.9) and equines above 10 years of age (OR: 3.10, 95% CI: 0.7-13.3) were more likely to test positive for glanders. In conclusion, present study provides prevalence estimates of equine glanders in KPK regions, which appears to be an area of endemicity. Future large-scale investigations are seriously warranted in other equine populated districts before implementing an effective control plan for glanders.

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INTRODUCTION

Glanders is reportable equine bacterial disease of zoonotic importance which is caused by *Burkholderia* (*B.*) *mallei, a* strictly host adaptive, Gram negative bacterium that cannot live longer outside its equine host. Although, equine glanders has been eradicated from the most part of the world, the disease still occurs and consider endemic in developing countries including Afghanistan, Bangladesh, Brazil, India, Iran and Pakistan (Sellon and Long, 2014; Rahman *et al.,* 2018). Recent outbreaks in China (in 2018), Kuwait and Turkey (in 2019) that have been notified to World Organization for Animal Health – OIE (www.oie.int/wahis), highlight the re-emergence of glanders in disease free regions. Similarly, glanders caught serious attention of surveillance in Europe after its re-introduction into Germany in the wake of import of an apparently healthy horse from Brazil (Sellon and Long, 2014). Spontaneous spillover of glanders from horse to camel and a recent case of glanders in a man in Thailand (Tuan *et al.*, 2019) seriously raises a specter of transmission of the disease from glanderous equids to domestic animal species and to human in endemic region.

Equine glanders manifests three clinical forms viz., pulmonary, nasal and cutaneous, and the course of disease

may be sub-acute, acute or chronic. Clinically, the disease is characterized by fever, blood-tinged nasal discharge, epistaxis, enlargement of sub-mandibular lymph nodes, hind-quarter edema, orchitis, cutaneous lymphadenitis and lymphangitis (Constable *et al*., 2017). Sub-clinical/latent disease develops in 90% of natural infection in horses (Neubauer *et al.*, 2005), which are considered dangerous sources of infection for healthy cohorts. In horses, glanders mostly runs sub-acute to chronic course, which are thought to be the natural reservoir of *B. mallei*. While, the donkeys and mules quickly succumb to natural infection. Different risk factors including overcrowding, communal watering and feeding troughs, old age, extreme weather condition, poor nutrition, draught stress have been reported in transmission of glanders from disease to healthy equids (Dvorak and Spickler, 2008).

The field diagnosis of glanders mainly rely on clinical history and results of mallein test. However, mallein gives inconclusive reactions in clinically advanced/anergic patients and subsequent serological assays cannot be considered. A number of serological tests are available for glanders (Verma *et al*., 2014), however, complement fixation test (CFT), immunoblotting (IB), enzyme linked immunosorbent assay (ELISA) have comparatively been evaluated (Elschner *et al.*, 2011; Khan *et al*., 2014) and are recommended for diagnosis and surveillance (OIE, 2018). The CF test is the most sensitive and specific for glanders in horses but has questionable diagnostic efficiency on anti-complementary sera (Khan *et al.*, 2014). Therefore, IB and ELISA are preferred alternative to CFT while testing samples from mixed equine population.

In view of epidemiologic studies (Hussain, 2011; Ghori *et al*., 2017) and retrospective record of Veterinary Medical Teaching Hospital, University of Agriculture, Faisalabad, glanders seems endemic in Punjab since many decades. As far as could be ascertained, information on the occurrence, distribution and risk factors of glanders is limited to Punjab Province. As we are expanding surveillance of glanders in other areas of Pakistan, the present study was delineated to determine the sero-prevalence and associated risk factors of the disease in draught equids populated prefectures (Peshawar and Charsada) of Khyber Pakhtunkhwa (KPK) province of Pakistan.

MATERIALS AND METHODS

Description of study area, equine population and husbandry practices: The study was conducted in draught equine populated areas of district Peshawar (34°01′N 71°35′E) and Charsada (34°09′N 71°44′E) of KPK (Fig. 1) under surveillance of OIE listed equine diseases from 2013-2017. Both prefectures are semi-arid, respectively, host 21,147 (D=18,358, H=2,368 horses, M 421) and 41,938 (D=35,262, H=6,205, M=473) equines. The animals of both regions have poor body condition owing excessive work and under feeding by economically compressed having low socioeconomic status of the owners. The animals are fed on Alfalfa supplemented with chickpea and drink water from communal-water troughs available in equine communities. No immunization and mineral–vitamins supplementation program are in place. The selection of the study areas was based on the record of draft equine communities and number of animals (Anonymous, 2006).

Samples size and sampling: The sample size was calculated by considering the unknown status of glanders (i.e., estimated prevalence of 50%) in KPK with 95% confidence interval limits and an absolute target precision of 5% as described elsewhere (Afridi *et al.*, 2017). Using the foregoing estimates, 393 sera originating from horses (n=195), donkeys (n=194) and mules (n=4) were randomly selected from different equine communities of Charsada and Peshawar districts (Table 1). In each draft equine community, allocation of samples was reached according to population in that community and simple random sampling method was followed on-site.

Using blood collection system (BD Vacutainer, USA), the samples were directly drawn into gel-activated clot tube from jugular vein. The sera were harvested upon centrifugation (4000 rpm for 10 minutes) and preserved at -20ºC until tested. Animal and owner level data, management, problem history, treatment response was registered on previously tested proforma (Hussain, 2011).

Serological examination (indirect ELISA): All samples were examined by a recently developed indirect ELISA for glanders (Laroucau *et al.*, 2017) following the manufacturer instructions (ID Vet, Grabels, France). This assay detects IgG against semi-purified *B. mallei* antigen with multispecies conjugate and carries good diagnostic accuracy. Optical density was measured at 450 nm by ELISA plate reader (Thermo Fisher, USA) and sample to positive (S/P) ratios were calculated using following formula: S/P% = (net OD sample) / (net OD positive control) x 100. The samples with S/P% ≥50% were regarded as positive, between 40-50% as suspicious and at ≤ 40 considered negative (Laroucau *et al.*, 2017).

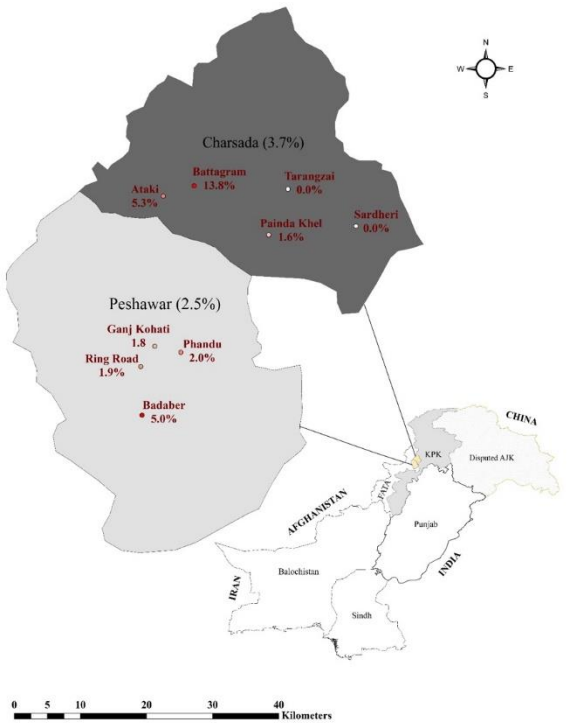

Fig. 1: Choropleth map showing seroprevalence of equine glanders in different localities of 2 prefectures of Khyber Pakhtunkhwa Province, Pakistan.

Data analysis: Epidemiological data generated was analyzed to investigate different epidemiological attributes of glanders. Prevalence along with 95% confidence interval was calculated by using the binomial exact method. Chi-square testing was performed to find out significant difference among sex, age and locations based prevalence of glanders. Univariate analysis was performed to calculate Odds ratio (OR) for different determinants of disease. Based on the univariable analysis, all the variables with p value <0.25 were included in the multivariable analysis. A backward stepwise removal method was used to remove the variables with P>0.05 at each step until only significant variables were left in the final model. The values of Nagelkerke R-Square (NR^2) and Hosmer Lemeshow Test (HLT) were used to assess the fit of the final model.

RESULTS

Overall prevalence of antibodies to *B. mallei* (glanders) was 3.1% (12 out of 393; 95% CI: 1.6-5.3) which differed insignificantly (χ^2 0.421, p=0.516) between the districts of Peshawar and Charsada. However, the prevalence varied from 1.8 to 5% among 4 localities of Peshawar and 1.6 to 13.8% among 5 localities of Charsada. In Charsada, seroprevalence of glanders varied significantly (χ^2 11.986, p=0.017) between the communities whilst remains insignificant (χ^2 1.287, p=0.732) between the communities of Peshawar district (Table 1; Figure). Species related seroprevalence was found to be highest in donkeys (4.1%; 4/195) followed by horses (2.1%; 8/194) which differed non-significantly (χ^2 1.397, p=0.237) between the species. None of the mules tested positive for glanders and were not included in analysis of data owing to insignificance (Table 2). Other statistics showed higher seroprevalence of glanders in equine less than 10 years old (3.0%; 5/168) than above 10 years (3.2%; 7/221) and in animal with poor body conditions (3.5%) than normal (2.5%). Analysis of occurrence with watering and feeding methods showed higher seroprevalence in animals fed on communal feeding (3.3%; 11/334) than separate feeding (1.8%; 1/55) and drinking on communal watering trough (3.2; 11/339) than bucket watering (2%; 1/50). However, prevalence remained statistically non-significant. Anemia was noted in 3.4 % cases which did not differed significantly (p=0.405) between glanders positive and negative equids (Table-2). Univariable analysis of the data showed that donkeys were more likely to test positive (OR:2.05; 95% CI: 0.61-6.94) for glanders than horses. Other variable indicated that animals fed on communal feeding (OR: 1.84; 95% CI: 0.23-14.53) water troughs (OR:1.65; 95% CI: 0.21-13.04) were more likely to be test positive for glanders (Table 2).

Table 1: Seroprevalence of equine glanders in different areas (localities) of Peshawar and Charsada districts of KPK, Pakistan

District	Community	Horse		Donkey		Mule		Total	
		Pos. / Tested	Prev. % (95% CI)	Pos. / Tested	Prev. % (95% CI)	0	Prev. % (95% CI)	Pos. / Tested	Prev. % (95% CI)
Peshawar	Ganj Kohati	0/34	0 (0-10.3)	1/22	4.6 (0.1-22.8)	-	-	1/56	1.8 (0-9.6)
	Badabir	1/24	4.2 (0.1-21.1)	1/16	6.3 (0.2-30.2)	-	-	2/40	5 (0.6-16.9)
	Pandu	0/25	0 (0-13.7)	1/25	4 (0.1-20.4)	-	-	1/50	2 (0.1-10.6)
	Ring Road	1/40	2.5 (0.1-13.2)	0/14	0 (0-23.2)	-	-	1/54	1.9 (0-9.9)
	Total	2/123	1.6 (0.2-5.8)	3/77	3.9 (0.8-11)	-	-	5/200	2.5 (0.8-5.7)
Charsada	Ataki	0/14	0 (0-23.2)	2/24	8.3 (1-27)	-	-	2/38	5.3 (0.6-17.7)
	Batagram	1/11	9.1 (0.2-41.3)	3/18	16.7 (3.6-41.4)	-	-	4/29	13.8 (3.9-31.7)
	Penda Khel	1/22	4.6 (0.1-22.8)	0/39	0 (0-9)	0/2	0 (0-84.2)	1/63	1.6 (0-8.5)
	Sarderi	0/12	0 (0-26.5)	0/19	0 (0-17.6)	-	-	0/31	0 (0-11.2)
	Torenzai	0/13	0 (0-24.7)	0/17	0 (0-19.5)	0/2	0 (0-84.2)	0/32	0 (0-10.9)
	Total	2/72	2.8 (0.3-9.7)	5/117	4.3 (1.4-9.7)	4	0 (0-60.2)	7/193	3.6 (1.5-7.3)
Overall		4/195	2.1 (0.6-5.2)	8/194	4.1 (1.8-8)	0/4	0 (0-60.2)	12/393	3.1 (1.6-5.3)

Table 2: Univariable analysis of the seroprevalence of glanders in the equines sampled from two districts of KPK, Pakistan

Variable	Category	Pos. / Tested	Prev % (95% CI)	Odds Ratio	95% CI	P value	χ2
District	Charsada	7/189	3.70 (1.8-7.44)	1.48	0.46-4.73	0.5059	0.44
	Peshawar	5/200	2.5 (1.07-5.72)	-	-		
Equine Species	Donkey	8/194	4.12 (2.1-7.92)	2.01	0.60-6.77	0.2518	1.31
	Horse	4/195	2.06 (0.8-5.15)	-	-		
Age Groups	>10 Y	7/221	3.17 (1.54-6.4)	1.06	0.33-3.40	0.9166	0.01
	≤ 10 Y	5/168	2.98 (1.28-6.78)	-	-		
Gender	Male	4/128	3.13 (1.22-7.77)	1.02	0.30-3.44	0.9752	0.00
	Female	8/261	3.07 (1.56-5.94)	-	-		
Body conditions	Poor	9/258	3.49 (1.85-6.5)	1.52	0.41-5.70	0.5303	0.39
	Normal	3/131	2.29 (0.78-6.52)	-	-		
Kept with	Alone and other equines	7/225	3.11 (1.51-6.28)	1.02	0.32-3.26	0.9728	0.00
	Other animal species	5/164	3.05 (1.31-6.94)	-	-		
Feeding	Communal	11/334	3.29 (1.85-5.8)	1.81	0.23-14.07	0.5677	0.33
	Separate	1/55	1.82 (0.32-9.61)	-	-		
Watering	Communal	11/339	3.24 (1.82-5.71)	1.62	0.21-12.60	0.6434	0.21
	Separate	1/50	2 (0.35-10.5)	-	-		
Anemic	No	7/181	3.87 (1.89-7.77)	1.61	0.50-5.14	0.4196	0.65
	Yes	5/208	2.40 (1.03-5.5)	-	-		

Table 3: Multivariable analysis of the seroprevalence of glanders in the equines sampled from two districts of KPK, Pakistan

Variable	Exposure	Comparison	Wald	df	Sig.	OR	95% C.I.	
							Lower	Upper
Equine Species	Donkey	Horse	3.871	1	0.049	4.58	1.00	20.87
Age	>10 Y	<10 Y	2.317	1	0.128	3.09	0.72	13.27

In Multivariable model, initially following variables including district, equine species, age, gender, BCS, housing, feeding, watering and presence of anemia, were kept considered in analysis. However, all variables were knocked out during the logistic regression analysis (P>0.05). But when equine species and age were kept together, the model indicated that donkeys (OR: 4.58; 95% CI: 1.0-20.9) and equines above 10 years of age (OR 3.10, 95% CI: 0.7-13.3) were more likely to test positive for glanders (Table 3). The values of Nagelkerke R^2 (0.038) and Hosmer Lemeshow Test (χ^2 0.058, p=0.971) indicated that it is a weak model to predict glanders in equines of the selected districts.

DISCUSSION

Glanders and Farcy Act (1899) is in enforce in Pakistan since decades, however, due to low indemnity, it doesn't not lure the owners to let their animals be destroyed (Muhammad *et al*., 1998). Field diagnosis of glanders depends merely on mallein (purified protein derivative of *B. mallei*) test, (type IV hypersensitive reaction) which has limitations in advanced/anergic cases and in some fractious equids (Naureen *et al.*, 2007). Serology of glanders relies upon complement fixation test, however, the test is complex, difficult to standardized (Khan *et al*., 2011; OIE, 2018) and has constraint in employing on donkey serum due to anti-complementary activity (Rahman *et al*., 2018). In order to overcome these problems, alternative serologic tests including WB and ELISAs have been developed and validated (Elschner *et al*., 2011; Singha *et al*., 2014). Therefore, in this study indirect enzyme linked immunosorbent assay (iELISA) was used to determine the seroprevalence of glanders in draft equines (horses, donkeys and mules) populated localities of Charsada and Peshawar district of KPK, Pakistan. This iELISA has been evaluated in comparison to other serological tests (CFT and WB) recommended for the diagnosis of glanders (Laroucau *et al*., 2017).

The overall seroprevalence of glanders was found to be 3.1% in the studied equine population and indicated towards the possible endemic nature of disease among the draught equines in KPK. This finding concord with past reports on prevalence of glanders in draft/working equids from different parts Pakistan (Hammad, 2011; Ghori *et al*., 2017). As far as could be ascertained, first record to glanders in KPK (Peshawar) was published in 1877 during Bengal Presidency, and it is considered that glanders has quite old history this area.

Statistically indifferent seroprevalence of glanders between the 2 districts can be ascribed to similar husbandry practices and socioeconomic status of the owners. Consistent rate of seroprevalence between studied prefectures can be attributed to analogous climate, nutrition, and stressful management. Similar factors have been reported in the occurrence of glanders (Neubauer *et al.*, 2005). Significantly high prevalence of glanders in localities of Charsada can be due to almost double equine population and animals are put to work in close grouping under stressful environmental conditions (Afridi *et al.*, 2017). Similarly, Charsada has large equine market, where animals with unknown disease status are brought for sale from distant areas which may resulting in more risk of spread of infection among susceptible equids.

Relatively high but non-significant (p=0.251) seroprevalence was noted in donkeys than horses which contradict to earlier studies where high prevalence was seen in horses owing to chronic nature of disease in this species (Kettle and Wernery, 2016). Notwithstanding, chronic course of glanders has been reported in ass and its crosses that ascribed to involvement of less pathogenic strain of *B. mallei* and immune status of the animals. It has been observed that occult *B. pseudomallei (*ancestor of *B. mallei)* infections are seen when host-immune system not fully responding to bacterium either due to its cytosolic translocation or only partially counteracted (Gan 2005; Kettle and Wernery, 2016). In our study, detection of more seropositive may be due to higher number of donkeys enrolled from Charsada districts where disease prevalence was found to be higher than Peshawar. Furthermore, donkeys might have phase of early incubation period which was not detectable at the time of sample collection. It is noteworthy that seroconversion starts from day 6 after entry of *B. mallei* whereas length of incubation periods varies from 1-2 weeks depending upon dose the infectious agent ingested and involvement of strain type (Altemann *et al.*, 2012).

Occurrence of glanders in relation to age of animals remained non-significant. However, relatively higher prevalence was noted in older animals (>10years old) which could be ascribed to more chances of exposure to glanders with the increase of age. Our results contradict to finding of earlier studies (Ghori *et al.*, 2017) that could be due to use different serologic test (i.e. CFT) which generate false positive reactions in aged equids (Naureen *et al*., 2007). It was observed that older animals met in this study is likely due to financially compressed status of draft equine owners in Charsada and Peshawar which cannot buy young animals owing to high monetary value.

Non-significant association of seroprevalence of glanders with sex (P=0.97) is similar to earlier findings (Neubauer *et al.* 2005; Ghori *et al.*, 2017) where in the authors found insignificance of sex with seropositivity of glanders. Similarly, glanders was unlikely (OR: 1.02; 95% CI: 0.32-3.2) to associate with housing pattern or cohorts which support the findings reported by Hammad (2011). However, comparatively higher numerical values of glanders in animals kept alone or with equid cohorts may be attributed to overcrowded, communal housing system which are considered an important transmission factors for glanders (Dvorak and Spickler, 2008; Pawaiya and Chauhan 2008; Constable *et al*., 2017)

Univariable analysis of data has shown that the communal feeding (OR: 1.81; 95% CI: 0.23-14.07), watering (OR: 1.62; 95% CI: .21-12.6), anemia (OR: 1.61; 95% CI: 0.50-5.11) and body condition (OR: 1.52, 95% CI: 0,41-5.7), affect odds of seropositivity for glanders in working quines. However, these differences were statistically non-significant (P>0.05). In Pakistan, communal watering and feeding troughs have been reported as important reservoirs for the transmission of glanders (Hornstra *et al*., 2009). Although, *B. mallei* is fragile to the environment, it can survive up to 4 weeks in water (Miller *et al*., 1948). Normally water troughs are shared by many equines being used for transportation in cities. Moreover, sharing of feeding and watering buckets is fairly common practice by equine owners which

augmenting the risk of transmission of glanders from disease to healthy equids. The role of contaminated utensil in the transmission of glanders is well documented in the literature (Ghori *et al.*, 2017). *Burkholderia mallei* is an intracellular pathogen that causes chronic wasting and bone marrow depression during occult infection (Constable *et al.*, 2017) which may be ascribed to findings of anemia and poor body conditions met in seropositive equids of this study.

Conclusions: The present study is the first ever report on seroprevalence and associated risk factors of glanders in Charsada and Peshawar which indicated an endemic nature of the disease in these districts. Area, equid species, sex and housing of animals had no bearing on the burden of disease. Before devising a control plan, future studies are seriously warranted on epidemiology of glanders both in working and game horses in KPK.

Authors contribution: AS, MSS, GM and MS conceived and designed the study. AS, MSS and MS executed the experiments and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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