

Prevalence of *Burkholderia mallei* in Equids of Remount Depot, Sargodha, Pakistan

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Abstract.- Glanders is a highly contagious disease of solipeds caused by *Burkholderia mallei*. Nodules and ulcers are either seen in the upper respiratory tract and lungs (*i.e.* glanders) or the skin (*i.e.* farcy). Infection always results in persistence of the agent and intermittent shedding. Progressive loss of efficiency and fatal outcome resulting in massive economical losses forced veterinary authorities worldwide to start disease control including mass testing using complement fixation test and/or malleinisation, and culling of positives. In the last decade, the number of outbreaks in Asia and South America has been steadily increasing and glanders proved to be a re-emerging transboundary disease again. Pakistan has demonstrably been an endemic country for the last 120 years. Actual data however on the presence of disease among Pakistani army equid are absent. A seroprevalence study of equids rearing establishment, Remount Depot, Sargodha Pakistan having the densest working equine population from Sargodha district was made in the year 2009. A total of 920 (horses: 75; mules: 844; donkey: 01) serum samples were collected from apparently healthy paddock equids. The complement fixation test (CFT) and the highly specific and newly validated immunoblot (IB) technique were used for serodiagnosis. No positive animal (horse, mule and donkey) was found. Glanders seems to be restricted to remote, sporadic pockets of endemicity and may cause outbreaks after being introduced in native populations by asymptomatic shedders. The diagnostic specificity of the ccPro antigen based CFT was 68.39%, and of the CIDC antigen based CFT 65.87%.

Key words: Seroprevalence, Complement fixation tests, Equidae, glanders.

INTRODUCTION

Glanders is caused by the bacterium *Burkholderia (B.) mallei* a Gram-negative, non-motile, obligate intracellular mammalian zoonotic pathogen. Nodules and ulcers are either seen in the upper respiratory tract and lungs (*i.e.*, glanders) or the skin (*i.e.* farcy). Infection always results in persistence of the agent and intermittent shedding. The disease appears mostly in chronic form in horses and as an acute form in donkeys and mules. Most cases of glanders remain nonclinical or latent (Dvorak *et al.*, 2008; Khan *et al.*, 2013; OIE, 2008). Progressive loss of efficiency and fatal outcome resulting in massive economical losses forced

veterinary authorities worldwide to start disease control including mass testing using complement fixation test or malleinisation, and culling of positives. Following this, glanders was eradicated from Western Europe and North America in the 1950s. However, the number of outbreaks in Asia and South America has been steadily increasing in the last decade and glanders again proved to be a re-emerging transboundary disease. Pakistan is a good example for a country which has demonstrably been endemic at least for the last 120 years. The glanders and Farcy Act of the British government in 1899 made *B. mallei* infection a notifiable disease involving culling of positive equidae and camels. In Pakistan, low indemnity paid to the owner – it is the same amount as in 1899 - and lack of stringent implementation of this act have limited the owners' preparedness to destroy their diseased animals (Khan *et al.*, 2013; Muhammad *et al.*, 1998; Wahid, 2011) and hinder eradication of the disease. A

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cascade of outbreaks in civilian equids have been reported from 1999 to 2007 in the Pakistani Punjab and various strains of *B. mallei* have been isolated. Fifteen of these were characterised by variable number tandem repeat (VNTR) typing (Hornstra *et al.*, 2009). It appears that there exists specific *B. mallei* lineages (genotypes) of the Pakistani Punjab and the disease is spread via communal stables and water troughs. Glanders outbreaks have been reported from Indian army equids during the past 30 years (Verma, 1981). An outbreak of glanders was also noticed in the equid breeding establishment Mona Remount Depot (RD), Sargodha, Pakistan in 2006-2007. A preliminary study was carried out in equids rearing establishment RD, Sargodha to assess the clinical manifestations of glanders and anti-*B. mallei* antibodies using complement fixation test (CFT) (OIE, 2008; Elschner *et al.*, 2011) and immunoblot (IB) technique (Elschner *et al.*, 2011; Khan *et al.*, 2011). No seroprevalence study has been conducted so far to examine anti-*B. mallei* antibodies in apparently healthy Pakistani army equids. The main objectives of this study was to determine the seroprevalence of glanders in apparently healthy equid of Pakistan Army and to comparatively evaluate CFT (using two different antigens: c.c.pro, Germany and CIDC, The Netherlands) and IB for the diagnosis of glanders.

MATERIALS AND METHODS

A total of 920 (horses: 75; mules: 844; donkey: 01) equine blood samples were collected from the Pakistani Army equine rearing establishment, RD, Sargodha. Pink spray (Komi Phrama, Korea) was used for marking the animal after blood sampling and as antiseptic at the site of blood collection. All blood samples were collected during the hot summer season *i.e.* from April to July, 2009. The temperature ranged from 40–49°C. During field conditions, blood samples were stored at 4°C and immediately transferred to local laboratory for sera isolation. The samples were centrifuged at 1300 rpm for 15 minutes at RT (800D, Jiangsu Zhengji Instruments, China). For long-term storage, the serum samples were kept at -20°C and shipped to the Friedrich-Loeffler-Institut, Jena, Germany on dry ice. For animals' ethical

issues, guidelines were followed made for the welfare of livestock from which blood is being harvested for commercial and research purposes by the Animal Advisory Committee, Ministry of Agriculture, Wellington (1996), New Zealand (NAEAC, 2009). Basic data on animals were gathered with a questionnaire (open and closed questions) for each animal. All sera were tested with the OIE prescribed CFT method (OIE, 2008) using two different antigens: ccpro (ccpro GmbH, Oberdola, Germany) and CIDC (Central Veterinary Institute of Wageningen UR, The Netherlands). Both these antigen were extracted from *B. mallei* cultures in phosphate buffer saline and the compound contained 0.5% phenol as a preservative. Working dilution in Veronal buffer (VB) was 1:40. For CIDC *B. mallei* strains were heat killed for 3 h in a Koch's steamer at 100 °C and the working dilution in VB is 1:600.

CFT procedure was adopted as described by Khan *et al.* (2011). All CFT positive/suspicious and anti-complementary sera were retested with the confirmatory IB (Elschner *et al.*, 2011). Antigen preparation and test procedures were performed as described by Elschner *et al.* (2011) with little modification (Khan *et al.*, 2011).

RESULTS

Table I shows the information retrieved through questionnaires of 920 study animals. The equines were divided into North, East and West charges and each charge was several kilometres away from each other. Each charge was further divided into different paddocks. According to the questionnaires, 920 blood samples belonged to equines of different age, sex and breeds. All animals were non-vaccinated. Two hundred and fifty four female animals (horses: 01 mules: 253) were non-pregnant by history but no pregnancy test was performed onsite. Six hundred and sixty six (horse: 74; mules: 591; donkey: 01) male equids were castrated. All animals were well managed with their pedigree records, were well fed and showed good health status. Animals used common feeding and water troughs. Mules were separated from horses into different paddocks. The age of mules was from 3-16 years while the age of horses was 3-5 years.

No equid was tested positive in the IB but depending upon the use of antigen (ccPro/CIDC/ both antigens) 5/9/10 horse sera and 88/112/139 mules sera were diagnosed false positive by CFT. The sera of fifty five equids (horse: 10; mules: 45) showed anti-complementary effect with both antigens in CFT. CFT and IB results of 920 animals have been summarized Table II. Excluding 55 anti-complementary sera, the diagnostic specificity of the ccPro antigen based CFT was 68.39%, and of the CIDC antigen based CFT was 65.87%.

Table I.- Main information retrieved through questionnaires of 920 study animals.

Sr. No.	Characteristics of 920 study animals	Numeric / comments
1.	Animal code	To identify the animal
2.	Species Age	Horse/mule/donkey Varies from 3-16 years
3.	Sex (male/female)	(666/254)
4.	Breed	Thorough breed/Arabian/Sufflok
5.	In case of female (pregnant or non-pregnant)	All female animals (254) were non-pregnant
6.	Vaccination (if any)	All animals were non-vaccinated
7.	Previous history of Glanders	No
8.	Any case report of Strangels	53 horses
9.	Any case report of Melioidosis	unknown
10.	Feeding and drinking behavior	Use common water troughs for drinking Use common mengers for feeding
11.	Serodiagnosis of glanders	No serodiagnosis was performed
12.	Malleinisation	All animals were non-malleinised

DISCUSSION

Recently, a glanders outbreak in Kabul, Afghanistan was attributed to illegally imported horses from Pakistan. In Pakistan *B. mallei* strains were frequently isolated from glanderous equids from the Punjab districts of Faisalabad, Lahore and Sargodha during 1999-2007 (Wahid, 2011)

demonstrating the permanent presence of *B. mallei* in the equid population of this country. In disease free periods common stables and their surroundings seem to be active reservoirs for glanders. *B. mallei* is cleared from wet surfaces or slurry within 100 days in temperate climates (Loeffler and Schutz, 1882). We assume that *B. mallei* is inactivated with a few days or even hours by the dry heat and harsh sunlight during Pakistani summer. Thus, the role of public water troughs and stables for the spread of *B. mallei* may only be of seasonal importance. Based on a literature study we also assume that especially 'healthy' carriers *i.e.* sub-clinically infected animals play the most important role for persistence and propagation of disease (Manzoor *et al.*, 2008; Pritchard, 1995). In disease free periods *i.e.* when no outbreak is obvious these animals should at least be detected by serology. Astonishingly no clinical signs of chronic infection *i.e.* scars as marks of local infection or acute glanders or anti *B. mallei* antibodies were registered. Our current study shows that glanders was not detectable in equids, RD, Sargodha having outbreaks in the past. It can be supposed that glanders only re-emerged in the form of local epidemics when glanderous equids were introduced in native populations. The authors suppose that only small local foci of glanders exist in remote areas of the Punjab from which glanders spilled over to other equine population only occasionally. This assumption is supported by the finding that a glanders outbreak happened in a Lahore Polo Club (2005) after the introduction of two glanderous horses brought from a Sargodha district farm to participate in competitions (Wahid, 2011). Another explanation for the absence of glanderous animals in our investigation is that most diseased animals may succumb to glanders during the stressful summer season. The army population of equines tested were well monitored and obviously glanders free army animals. This group should serve as a local negative control group for test evaluation as done before by Pakistani workers (Naureen *et al.*, 2007). So it was not astonishing that no positive cases were found. However, the high number of CFT positives in this group was surprising. A total of 363 (39.5%) sera tested positive/suspicious with both CFT procedures. It is well known that CFT may produce a considerable

Table II.- Complement fixation tests and immunoblot results summary of 920 animals.

Species (No of samples)	A/M/F/B ⁺	CFT ⁻ (c.c.pro [§])	CFT (CIDC ⁺⁺)	Both CFTs	IB ⁺⁺⁺	Comment
Horse (5)	3-5/4/1/A,S	Positive/suspicious	Negative	-	Negative	False positives
Horse (9)	3-5/9/0/A,S	Negative	Positive/suspicious	-	Negative	False positives
Horse (10)	3-5/10/0/A,S	-	-	Positive/suspicious	Negative	False positives
Mule (88)	3-16/56/32/H	Positive/suspicious	Negative	-	Negative	False positives
Mule (112)	3-16/69/43/H	Negative	Positive/suspicious	-	Negative	False positives
Mule (139)	3-16/117/22/H	-	-	Positive/suspicious	Negative	False positives
Horse (10)	3-5/10/0/A,S	ACA ^{§§}	ACA	-	Negative	Negative
Mule (45)	3-16/33/12/H	ACA	ACA	-	Negative	Negative
Horse (41)	3-5/41/0/A,S,T	Negative	Negative	-	Not tested	True negatives
Mule (460)	3-16/316/144/H	Negative	Negative	-	Not tested	True negatives
Donkey (1)	6/1/0/ND	Negative	Negative	-	Not tested	True negatives

A/M/F/B⁺: age/male/female/breed

c.c.pro[§]: CFT antigen from c. c. po GmbH, Germany

ACA^{§§}: anticomplementary activity

CIDC⁺⁺: CFT antigen from Central Veterinary Institute of Wageningen UR, The Netherlands

CFT⁻: Complement fixation test

IB⁺⁺⁺: Immunoblot

- Not Applicable

A, S, H, T, ND: Arabian, Suffolk, Hybrid, Thorough-breed, Non-descript

number of false-positive results. False-positive CFT results were attributed to cross-reactions to other bacteria *e.g. Streptococcus equi* (Ijaz *et al.*, 2010; Misra and Arora, 1990). A high prevalence of anti *S. equi* antibodies may be supposed as the reason for the observed cross reactions in our study populations. It is well known that strangles is endemic in Pakistani army horses (Ijaz *et al.*, 2010) and the civilian equid population of Sargodha and Lahore (Ashraf, 2000; Manzoor *et al.*, 2008). Furthermore, Ijaz *et al.* (2010) reported highest (2.6%) incidence of strangles from the end of January to the beginning of May, thus shortly before or in the beginning of the sampling period. Two other Pakistani working groups reported 32.2% strangles in mules less than 2 years of age and 35.4% in mules of more than 2 years of age in Pakistan and 54% strangles in foals in Punjab, Pakistan (Ijaz *et al.*, 2010, 2011). Melioidosis can not be ignored in differential diagnosis of glanders. No report on animals' melioidosis, (*Burkholderia pseudomallei*) which shows 98% genetic resemblance with *B. mallei* in Pakistani Punjab, is available. Fifty five (6%) serum samples predominantly from mules showed anti-complementary activity. Mule and donkey sera are more prone to anti-complementary activity (Naureen *et al.*, 2007). The reason for this high

number is not clear. Local strains can improve the sensitivity of tests for glanders detection (Sprague *et al.*, 2009). To proof our preliminary data which are biased towards sera from an army establishment, a bigger seroprevalence study among the equids of the Pakistani Punjab is highly recommended in the future using CFT and IB containing also composite mixtures of local *B. mallei* strains.

Our study showed that it may be very difficult to control glanders in a country like Pakistan. The only effective control option for glanders lacking effective vaccines is testing and culling of positives. It has to be stressed that a number of false positives will always be 'produced' by serological tests and that destruction of these few healthy animals is unavoidable. This line of action is the only way to stop the establishment of *B. mallei* in niches and also to avoid animal to human transfer (Sprague *et al.*, 2004). Testing has to be carried on even after the disease has obviously become rare and countermeasures have to be set into action to avoid reintroduction of disease by 'healthy' carriers. It has to be stressed that it is impossible to demonstrate absence of disease in equid in every case. An element of risk of spreading the disease still remains even if all precautions have been strictly followed. Public veterinary health actions have to be flanked by a wise re-imburement policy

to get full acceptance by the animal owners. However, all equines of the country have to be registered in advance and have to be made available for testing. Safe destruction of carcasses, decomposition of manure and disinfection of premises will round up the control program. It is of eminent importance to bear special social or regional particularities in mind to gain acceptance of the countermeasures at the best.

CONCLUSIONS

Glanders seems to be restricted to remote, sporadic pockets of endemicity and may cause epidemics after being introduced in naive populations by (asymptomatic) shedders. In serodiagnosis, animals tested false-positives and false-negatives should be retested with a highly sensitive and specific test like immunoblot to avoid the loss of healthy animals and introduction of glanderous animals in healthy populations, respectively.

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Conflict of interest

No conflict of interest exists.

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