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