The Journal of Animal & Plant Sciences, 28(6): 2018, Page: 1717-1724
ISSN: 1018-7081

MOLECULAR DETECTION OF BURKHOLDERIA MALLEI IN NASAL SWABS FROM DRAUGHT HORSES WITH SIGNS OF RESPIRATORY TRACT INFECTION

M. T. Ghori[1*], M. S. Khan[1], J. A. Khan[1], M. Rabbani[1], M. H. Chaudhary[2] ,M. Z. Shabbir[1], R. Ahmed[1], H. R. Chaudhry[1] and J. Muhammad[3]

[1]University of Veterinary and Animal Sciences, P.O. Box.54600 Lahore, Pakistan, [2]Punjab University Lahore, Pakistan.[3]University of Swabi, Anber, Pakistan.

*Corresponding Author's Email: tasleemghauri@yahoo.com

ABSTRACT

Glanders is a highly contagious and fatal zoonotic bacterial infection of equines. The disease is endemic in South Asia. The nasal shedding of bacteria from infected carrier animals may spread disease in susceptible hosts. The purpose of this study was to determine mallein reactivity in glanders suspected draught horses and estimate prevalence of active nasal shedders. A total of n=122 animals were purposively selected based on a case definition. Initially, the horses were screened through mallein test and later their nasal swabs were tested by real time PCR for the detection of B. mallei DNA. Mallein test was positive in 35.24 % (95% CI: 26.8%-43.7) animals. *B. mallei* was detected in only two horses. To explore pattern of clinical symptoms, Principal component analysis (PCA) was carried out. PCA clustered correlated symptoms into principal components. PCA confirmed co-occurrence of nasal and skin forms of disease in most horses included in the study. The study found high prevalence of the disease and existence of active nasal shedders in draught horses. PCA grouped symptoms that were more correlated together and provides a mechanism to overcome collinearity for regression models. The findings implicate importance of targeted surveillance in glanders suspected horses.

Key words:*Burkholderia mallei*, draught horses, mallein test, real time PCR, PCA analysis.

INTRODUCTION

Burkholderia mallei (B. mallei) is an obligate, intracellular, bacterial pathogen. It is highly susceptible to environmental conditions such as temperature, exposure to direct sunlight, humidity and pH of soil and commonly used disinfectants (Neubauer *et al.*, 2005; Dvorak and Spickler, 2008). It causes glanders; a highly contagious and fatal disease of equines that can also affect humans. The disease in equines appear as formation of multiple nodules and ulcers in the respiratory tract and skin which later discharge honey like pus. Clinically, the disease appears in three forms that are nasal, pulmonary and skin / farcy form (Malik *et al.*, 2015). The course of the disease is short in mules and donkeys with acute pulmonary signs whereas in horses, the disease runs a chronic course (Wittig *et al.*, 2006; Mota *et al.*, 2010). In many cases, infected horses may remain asymptomatic (Muhammad *et al*., 1998). The disease is considered endemic in many developing countries of Asia, Africa and South America. The disease has socio-economic implications such as trade restrictions and public health hazard. Low indemnity /compensation according to Farcy act 1899 (Indo-Pakistan) exert serious negative effect on livelihood of poor families dependent on working equines (Muhammad *et al.*, 1998). The pathogen is also a potent warfare agent due to its biological features such as low infective dose, high rate of mortality, the lack of effective treatment; and unavailability of an effective vaccine (Gilad *et al.*, 2007).

In Pakistan, the disease is primarily diagnosed by clinical signs and suspected animals are subsequently subjected to mallein test for screening. Crude preparation of the mallein has limited sensitivity and specificity of the test (Naureen *et al.*, 2007). Although identification of the pathogen by culturingis considered as the 'gold' standard for its diagnosis but due to a lack of proper bio-safety laboratory facilities, low number of pathogen in secretions and body fluids, and high risk of contamination, it is not commonly used in the Pakistan (Saqib *et al.*, 2012; Khan *et al.*, 2013). Because *B. mallei* shares many antigenic properties with *B. pseudomallei*, therefore serological differentiation between melioidosis and glanders through reference complement fixation test (CFT) is not possible particularly in the regions endemic with melioidosis (Naureen *et al.*, 2007; Kumar *et al.*, 2011; Singha *et al.*, 2014; Ghori *et al.*, 2017). As *B. mallei* is a category B microorganism, therefore, it is necessary to detect it early and specifically in the diseased animals. Conventional techniques for screening of the disease are slow, cumbersome, unreliable and time-consuming; thus, molecular techniques have been introduced to detect B. mallei in clinical samples. Recently, a real-time PCR is used to detect the agent directly from clinically suspected animals with higher detection ability and specificity (Tomaso *et al.*, 2006).

Working horses are used for transport and source of livelihood of many cart owners in developing countries of the world. These are used to carry bricks, as cart and transport of goods. Nomads use them for transport while migrating and in pastoral settings. Lack of medical care, absence of screening of affected horses and close link between animals and humans may facilitate disease transmission. In this situation, the affected animals particularly those shedding the bacteria in nasal secretions may serve as reservoir of infection for other animals and to the personnel who work with them. So far little is known about the epidemiological significance of these active carriers of infection in draught horses in endemic areas. The earlier studies in Pakistan and other countries of the region were focused on the sero-surveillance with little research focused on working equines. In short, the role of active carriers among draught equines in developing countries remains unclear. The objectives of this study were , i) to determine the association of clinical signs in draught horses and mallein reactivity , ii) to specifically detect B. mallei using real time PCR directly from nasal secretions of draught horses having signs of respiratory disease and could potentially be a source of infection for other animals and their care takers.

MATERIALS AND METHODS

Study design: The study was carried out in Punjab province of Pakistan during 2014-2015. Districts Lahore, Sheikhupura, Faisalabad, and Chakwal were included in the study. These districts were selected because of previous detection of *B. mallei* in the soil samples (Shabbir *et al.*, 2015; Ali *et al.*, 2017) and relatively high population of draught horses (Economic Survey of Pakistan, 2016-17). Due to poor resources and diagnostic facilities horses suffering from chronic respiratory problem are usually not subjected to mallein test and therefore may serve as reservoir of infection. A total of one - hundred- and- twenty two (n = 122) nasal swabs were collected from draught horses from Lahore (n=86), Sheikhupura (n=10), Faisalabad (n=23) and Chakwal (n=3) districts of Punjab. The animals were selected based on any of the two or more clinical signs consistent with glanders i.e. presence of nasal discharge, coughing, and enlarged lymph nodes, and farcy lesions. The animals were purposively selected from markets, farriers, brick kilns, veterinary hospitals, villages, nomads, and slum areas. All the animals were subjected to mallein test. Nasal secretions were taken by rotating the swabs gently in the nostrils. The nasal swab packs were carefully transferred to University Diagnostic Laboratory, University of Veterinary and Animal Sciences Lahore under a cold chain system for further processing.

Samples processing and testing: DNA extraction of *B. mallei* from the nasal swabs was performed as recommended by the manufacturer of the Mini DNA kit (Qiagen, UK). The DNA samples were later shifted to Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, (FLI) Jena, Germany for real time PCR. The PCR was executed according to an optimized protocol as described by (Tomaso *et al.*, 2006). The PCR mixture consisted of the followings: Master Mix (Taq Man, Thermo Inc., USA), 1X, 12.50 µL; Primer BmafliP-forward, 0.1 µM, 0.25 µL; Primer BmafliP-reverse, 0.1 µM, 0.25 µL; Bma Probe, 0.1 µM, 0.25 µL; and nuclease-free water (Thermo Inc., USA),4.45 µL. In this method, bacteriophage λ was used as an internal inhibition control. For the control reaction, the components included the following: Primer Lambda forward, 0.6 µM, 1.5 µL; Primer Lambda reverse, 0.6 µM, 1.5 µL; Lam Yak probe, 0.2 µM, 0.3 µL; to make a total volume of 21 µL. The positive control (DNA B. mallei ATCC 23344, 100pg/µl) for real-time PCR was provided by FLI Jena, Germany. Lambda DNA (0.5pg/µL, TIB MOLBIOL, Germany) and template DNA were added (2 µL each). The mixture (25 µL) was added to the PCR tubes.

Gene **Sequence**
Flip BmafliP Forward Primer 5'-CCCATTGGCCCTATCGAAG -3'
BmafliP Reverse Primer 5'-GCCCGACGAGCACCTGATT -3'
Bma Probe "6FAM CAGGTCAACGAGCTTCACGCGGATC-BHQ1"
Primer Lambda F 5'-ATGCCACGTAAGCGAAACA-3'
Primer Lambda R 5'-GCATAAACGAAGCAGTCGAGT-3'
Lam YAK YAK-ACCTTACCGAAATCGGTACGGATACCGC-DB

A real-time PCR detection machine (Step One-Plus, Applied Biosystems, USA) was used as per the following PCR conditions: Decontamination, 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 cycles of annealing of the primers at 63°C for 1 min and denaturation at 95°C for 25 Sec; and 1 cycle of cooling at 40°C for 1 minute. FAM dye was attached to the 5' end and TAMRA was attached to the 3' end for fliP detection, while YAK was attached at the 5' end and DB was attached at the 3' end for Lambda detection.

Data analyses: The data were compiled in Microsoft excel and analysed in R software version 3.4.4. A correlation matrix plot was created using corrplot package. The symptoms of disease are often correlated therefore carried out principal component analysis (PCA) to reveal pattern in data. PCA is s a multivariate data reduction technique that transforms the original variables

into a new set of uncorrelated variables called principal components. The technique has been used for exploration of symptoms data. The analysis was carried out with FactoMineR package. Bartlett test of sphericity was significant ($p \leq 0.05$) and Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.58 (p value < 0.05) indicating appropriateness of dataset for PCA.

RESULTS

The percentage of mallein positive (n= 43)animals was 35.24 %{ 95% CI: 26.8%-43.7%}. The reactive animals belonged to Lahore, Sheikhupura and Chakwal districts. However, no infected animal was detected in Faisalabad district (Table -1). The real time PCR results revealed that *B. mallei* was positive in only 2 /122 (1.63%) nasal swabs thus confirming the active carriers among the draught horses. The locations of the mallein positive and PCR positive horses are shown in map (Figure -1). The frequency of the symptoms and their percentages were as follows: nasal discharge 76 (62.29 %), cough 91 (74.59 %), skin lesions 60 (49.18 %), enlarged sub-maxillary / sub-mandibular lymph nodes 79 (64.75 %), poor body condition 85 (69.67 %), and epistaxis 31 (25.40 %) in horses, respectively. Some of the symptoms are show in Figure -2. The correlation matrix shows weak correlations between set of symptoms: epistaxis vs body condition (r = 0.14), enlarged lymph nodes vs nasal discharge (r =0.28), farcy lesions vs epistaxis (r = 0.25), and cough vs enlarged lymph nodes (r =0.28) Figure - 3.

PCA transformed the symptoms related data into six new independent variables (eigenvectors) called principal components (PC).Table-2 shows eigenvalues, percentage variance and cumulative percentage of variance of principal components. The first two principal components had eigenvalues greater than 1 and cumulatively explained 48.57 % of total variance (inertia/information) in the dataset. The values of the loadings (weights) are given in table 3 and correspond to the Pearson correlation coefficients between variables (symptoms) and principal components. The values of the loadings vary between + 1.0 and -1.0. Larger the absolute value of the loading, greater is contribution of the variable to the principal component. For example, nasal discharge, enlarged lymph nodes, epistaxis and farcy lesion had weights of 22.80, 21.49, 20.89 and 20.81 respectively, for principal component one (PC1). To simplify, PC1 is cluster of symptoms that were occurred together in most horses. That is horses that had skin lesion also had nasal form of the disease. PC1 explained 31.04 % variance in the data. The second most predominant pattern of symptoms was concurrence of chronic cough and chronic weight loss in the principal component 2 (PC2) that explained 17.53 % variance. The most contributing symptoms in PC2 were poor body condition and presence of cough respectively. The Mallein test reaction was significantly linked to PC1 ($p \leq 0.05$). Figure 4 displays quality of representation (squared cosine, cos2) of variables (symptoms) and individuals (animals) along first two principal components (2 D summary of data). The angles show degree of correlation among variables and their lengths correspond to their relevance for the respective components. The individual animals in the periphery have better representation (cos2 values closer to 1).

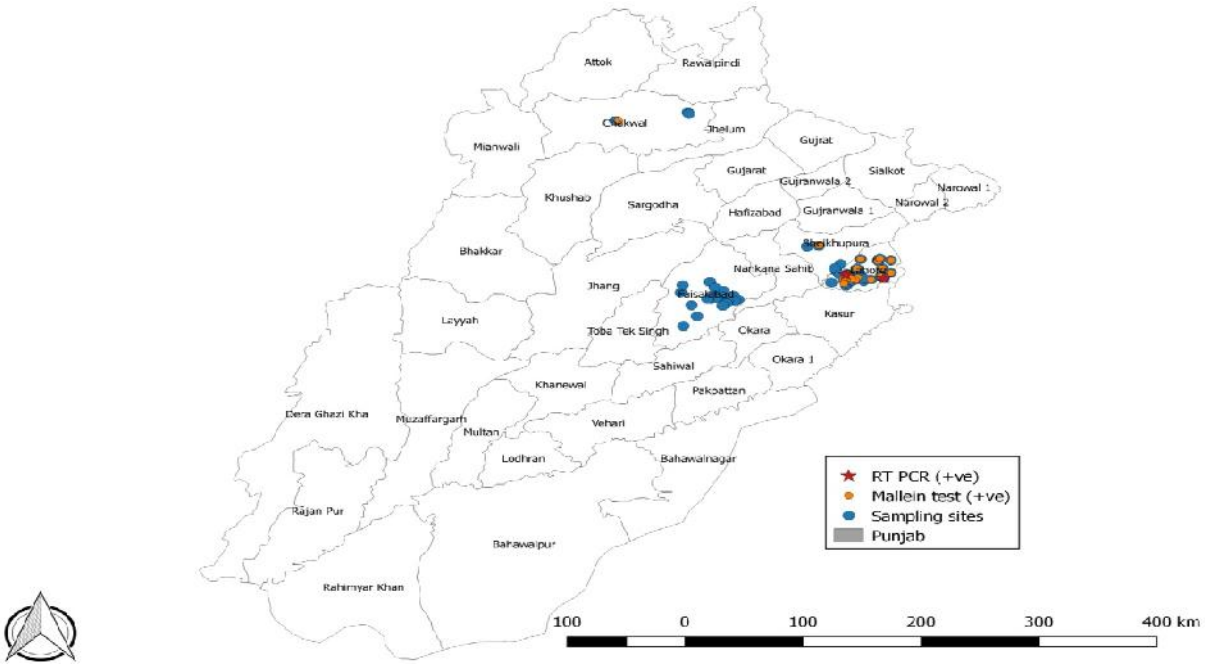
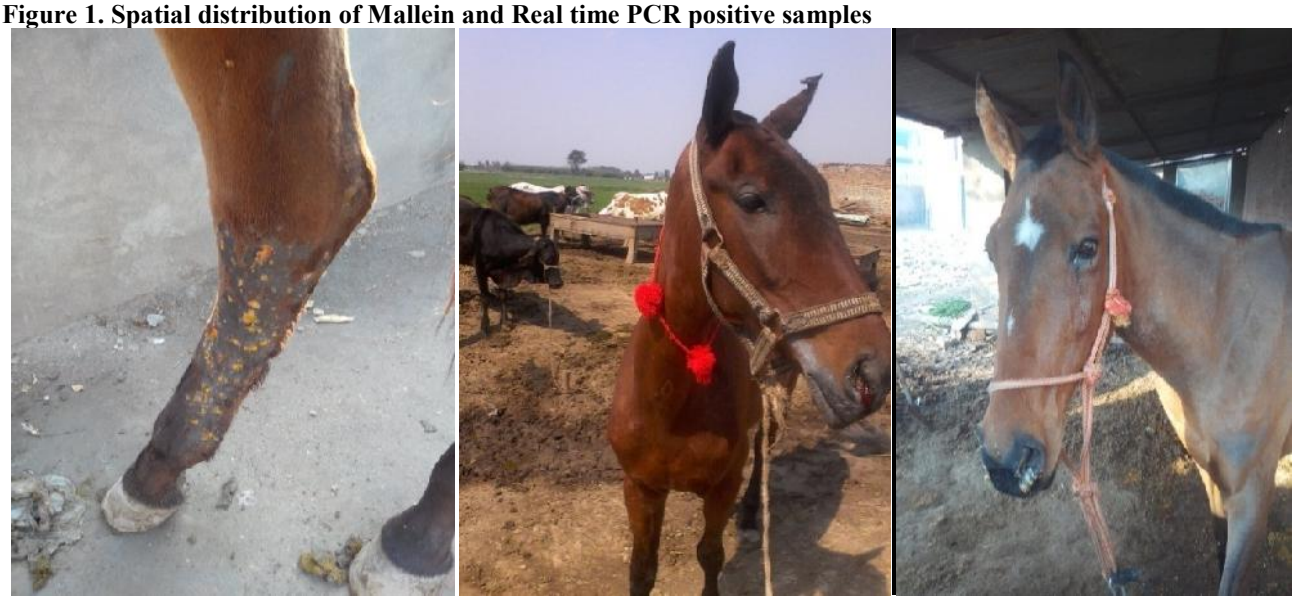


Figure 1. Spatial distribution of Mallein and Real time PCR positive samples



(a)Skin lesions(b) Epistaxis(c) Mucopurulent nasal discharge

Figure 2. Distribution of clinical symptoms

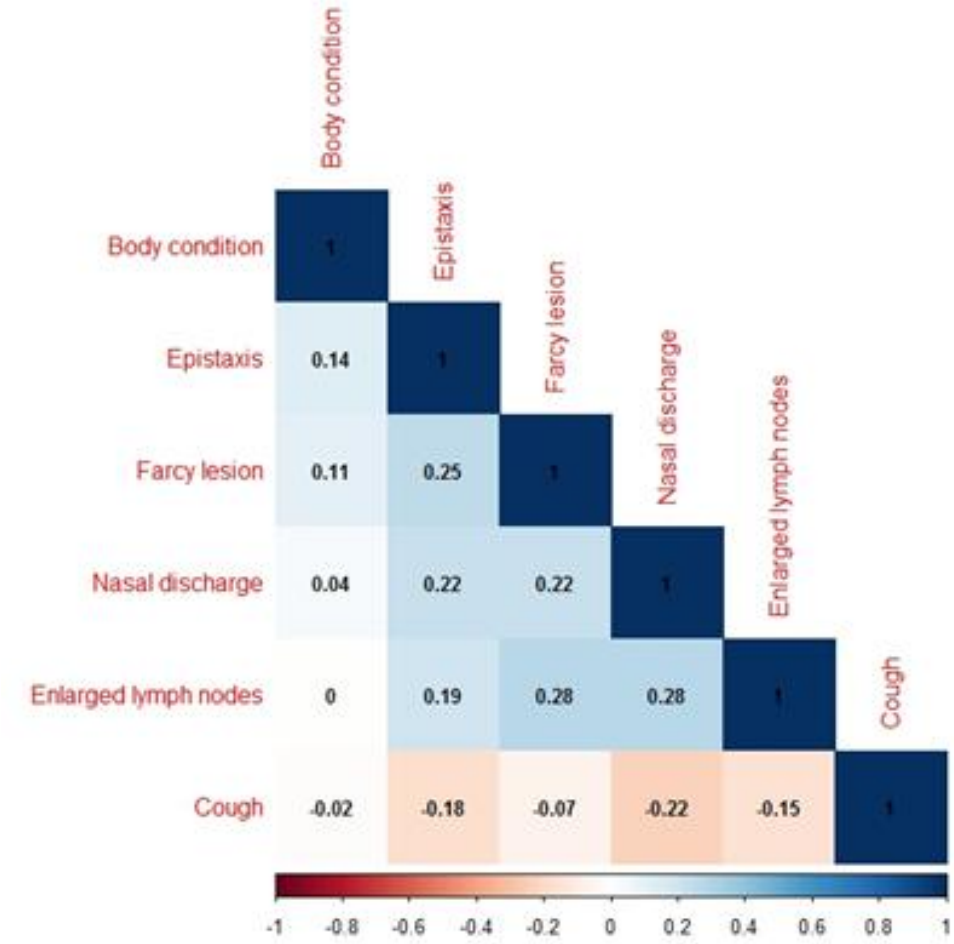


Figure 3. Correlation matrix analysis of six symptoms

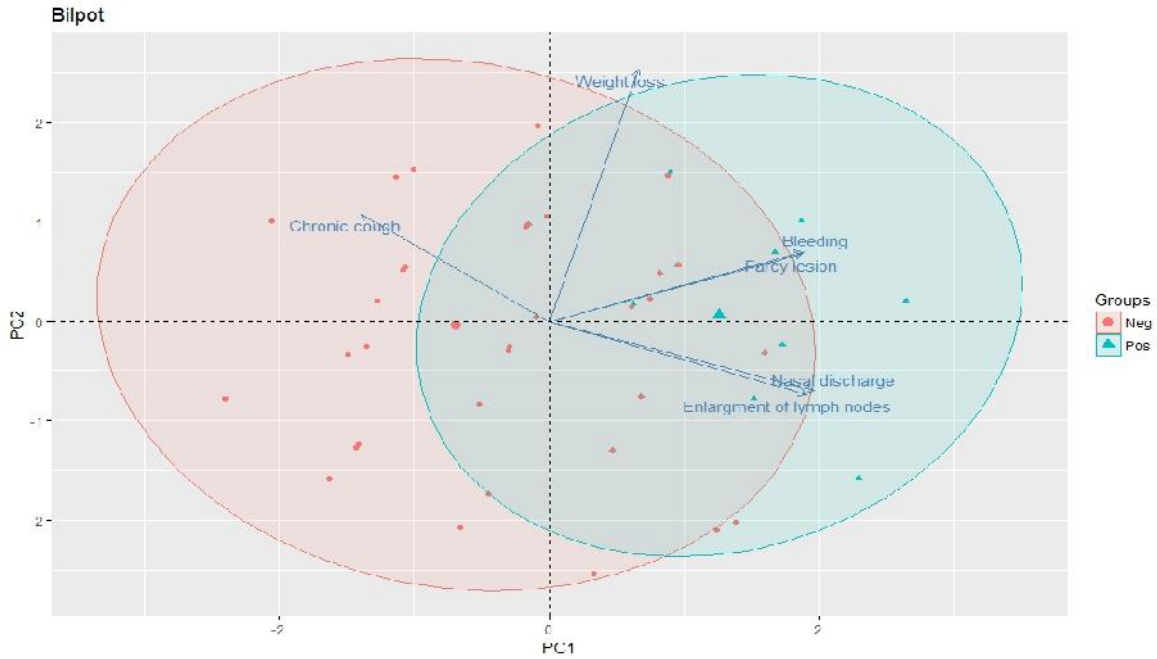


Figure 4. Squared cosine presentation of principal components

Table 1. Prevalence of *B. mallei* based on Mallein test and Real Time PCR in draught horses.

	Mallein test		Real Time PCR			
District	**Pos**	**Neg**	**Pos**	**Neg**	**Total**	**Percentage prevalence**
Lahore	41	45	2	84	86	2.38
Sheikhupura	1	9	0	10	10	0
Faisalabad	0	23	0	23	23	0
Chakwal	1	2	0	3	3	0
Total:	**43**	**79**	**2**	**120**	**122**	**1.63**

Table 2. Eigenvalue distribution and percentage of variance explained by principal components.

Principal Components	**Eigenvalue**	**Percentage of Variance**	**Cumulative percentage of variance**
PC1	1.86	31.04	31.04
PC2	1.05	17.53	48.57
PC3	0.94	15.64	64.21
PC4	0.76	12.74	76.95
PC5	0.71	11.86	88.81
PC6	0.67	11.19	100.00

Table 3. Results of the principal components analysis showing the weights for each variable.

Symptoms	**PC1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Nasal discharge	22.80	5.09	0.29	15.26	56.13
Cough	11.42	12.20	52.28	0.04	16.54
Farcy lesion	20.81	4.79	22.50	1.14	3.79
Enlarged lymph nodes	21.49	5.61	13.58	7.91	19.10
Body condition	2.59	67.17	9.11	18.83	0.88
Epistaxis	20.89	5.14	2.24	56.81	3.56

The loadings correspond to the Pearson correlation coefficients between variables and components. The loadings of the variables most relevant for the component interpretation are bolded. One of the

variables that contributes most strongly to each component

DISCUSSION

Glanders is a notifiable disease of the equines caused by *B. mallei* (OIE, 2015). The previous published data shows endemicity of the disease in the country (Nasreen, 1977; Muhammad *et al*., 1998; Khan *et al*., 2012). A delayed-type hypersensitivity test (Mallein test) is recommended for screening of equines in remote endemic areas (OIE, 2015). The test has the potential to identify the true positive and true negative animals for glanders (Silva *et al.*, 2014). Furthermore, this test has an advantage on the reference serological test CFT that it can effectively be used in the equines whose sera are anti-complementary. Additionally, mallein test also eliminates the possibility of cross reactivity in serological testing of animals in the melioidosis endemic countries (Silva *et al.*, 2013). Therefore, in the current study, clinically suspected animals for glanders were screened by mallein test and 35.24 % (95% CI: 26.8%-43.7%) were found reactive to the purified protein derivate (PPD) mallein. Compared to our results, previous investigators showed low prevalence of the disease, ranging from 0.5% (Nasreen, 1977) to 1% in working horses of Lahore (Vaid *et al.*, 1981) using mallein PPD. The difference in these rates might be due to choice of the surveyed animal population or increased over period of time. In another epidemiological investigation 35 horses reacted positively to mallein in Turkey, however only few of them were confirmed positive for the disease at necropsy (Arun*et al.*, 1999).

Despite the advantages of mallein as being cheaper, easy to apply and clarifies the doubts of serological results, however it is unable to produce conclusive results in clinically advanced cases and in anergic animals. Moreover, it gives cross reactivity when applied in animals, infected with strangles (Muhammad *et al.*, 1998; Naureen*et al.*, 2007; Silva *et al.*, 2013). Hence in a country like Pakistan, which is endemic with strangles (Ijaz*et al*., 2010), screening of horses only through mallein test is not a reliable approach (Khan *et al.*, 2012). Furthermore, this test takes more than 48 hours to produce the results. Apart from significance of identification through culturing, low bacterial count in body secretions and in infected tissues of the diseased animal often lead to false negative results in case of asymptomatic or chronic cases (Scholz *et al.*, 2006). However, a fliP based real time PCR with lower detection ability, can minimize the chances of such false negative results on clinical samples (Tomaso *et al.*, 2006).Additionally, the PCR assays used without internal amplification control do not guarantee the true negative results in clinical samples (Pal *et al*., 2016). Therefore, in addition to mallein test, we used real time PCR technique with internal PCR control using DNA, directly extracted from nasal swabs to determine the active carriers of the disease in the draught horse population. We selected such type of animals because they are more prone to infection being kept in poor hygienic conditions and are under stress of heavy work (Malik *et al.*, 2015). Recently, nasal swabs were also successfully used for isolation of *B. mallei* in India from glanderous draught horses (Malik *et al.*, 2015). Khan *et al.*, (2013) reviewed that discharges from nasal and skin lesions are infective and can spread the infection to other animals. We confirmed the two active carriers of *B. mallei* from nasal swabs using real time PCR. Interestingly, both positive samples belonged to horses from the sites in Lahore where the *B. mallei* was previously detected in soil (Shabbir*et al*., 2015). It has been observed that certain physicochemical characteristics of soil and water increase the viability of *B. mallei* in the animal environment consequently increase the exposure risk to the animal (Coenye and Vandamme, 2003; Limmathurotsakul *et al.*, 2010; Ribolzi*et al.*, 2016).The lower number of positive results in PCR might be due to intermittent shedding of the bacteria in nasal secretions and large number of contaminating bacteria in nasal septum (Verdegaal and Oijen, 2016). The overdosing of antibiotics suppresses the signs of the disease (Malik *et al*., 2015). Similarly, the concurrent use of antimicrobials can delay the diagnosis of disease up to several months (Verdegaal and Oijen, 2016). Hence the animals become carriers of the disease and continuously spread the disease in the premises over a long period of time. Therefore, it is necessary to raise the awareness about the disease and suitable compensation should be paid to the equine owners. Strict legislation must be implemented to prevent quackery in the country.

PCA has been used for empirical evaluation of data to group most correlated variables (i.e. symptoms in our case) for better visualization and reveal latent patterns in the data. The symptoms which are highly correlated are grouped together into new summary variables called principal components. This approach not only makes interpretation easier (2 D visualization) but is known to overcome the problem of collinearity in multivariate analyses. The technique has been used in human medicine in case of several diseases (Poyhonen *et al.*, 2013). To the best of our knowledge, there has been no earlier attempt to explore the symptoms of glanders using this approach. With application of PCA, we found two principal components that represent predominant clinical profiles of the studied horses. The values of the loadings (weights) are given in table 3 correspond to the Pearson correlation coefficients between variables and principal components. For example, nasal discharge, enlarged lymph nodes, epistaxis and farcy lesion had weights of 22.80, 21.49, 20.89 and 20.81respectively, for principal component one (PC1). To simplify, PC1 is cluster of

symptoms that were occurred together in most horses. That is horses that had skin lesion also had nasal form of the disease. The second most predominant pattern of symptoms was concurrence of chronic cough and chronic weight loss. The principal component 2 explained 17.53% variance. The most contributing symptoms in PC2 were poor body condition and presence of cough respectively. Saqib *et al.*, (2012) studied distribution of symptoms in horses during investigation of Glanders outbreak in Lahore Polo club. The most common symptoms were nasal discharge, debility, enlarged lymph nodes, cough, nodules and ulcer like lesion. The co-occurrence of nasal and farcy symptoms is also evident in this study. Malik *et al.*, (2015) also described symptoms of glanders in indigenous population of equines in India. The results of this study are in agreement with our findings. The mechanism of co-occurrence of nasal and skin forms of the disease is unknown. One possible explanation could be unawareness regarding diagnosis and treatment of diseased horses.

The study has certain limitations that could affect the validity of results. We have not considered the seasonality in shedding pattern and it was a cross sectional study. Furthermore, we could not get enough number of samples. This was mainly due to non - cooperation from the animal owner in local settings even with provision of incentive. However in future, using the similar approach with involvement of government authorities a comprehensive survey should be done to identify the nasal shedders of disease. Similar surveys can also be applied in other race horses and army horses, because, in confinement nasal carriage could be even more.

Conclusion: To our knowledge, in endemic areas there is no such attempt made in the past, to find the nasal shedders of *B. mallei* in draught horse population. Although a high percentage of horses was reactive to mallein but only a small number of animals were confirmed as active carriers by molecular analysis. Based on our experience with application of PCA on our data we conclude that PCA provided insight into distribution of symptoms in horses infected with glanders. The PCA accommodates the co-linearity that can make regression analysis estimates unstable, in case the symptoms are used as covariates. Moreover, our PCA depicted a strong correlation of clinical signs with mallein positivity in draught horses.

Acknowledgements: This work was funded by the Defense Threat Reduction Agency, Basic Research Award # HDTRA1-10-1-0080 to The Pennsylvania State University, USA and University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. We are thankful to the Higher Education Commission of Pakistan for providing funds to work in Friedrich-Loeffler-Institut, Germany. We are also thankful to the University Diagnostic Laboratory (UVAS) staff for their assistance and support in collecting the field samples.

REFERENCES

Ali, M., M. Khushi, M. Rabbani, A. A. Aftab, A. Mansur-ud-Din, M. Z. Shabbir, H. Chaudhry and B. M. Jayarao (2017). Spatial distribution of Burkholderia mallei genome in Punjab, Pakistan.197-213. 10.1109/IBCAST.2017.7868056.

Anonymous.(2017).Pakistan Economic Survey (2016-17), Economic Affairs Division, Govt. Pakistan., Islamabad.

Coenye, T. and P. Vandamme (2003). Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. 5, 719–729.

Arun, S., H. Neubauer and A. Gürel (1999). Equine glanders in Turkey. Vet. Rec. 144, 255–258.

Dvorak, G. D., and A. R. Spickler (2008). Glanders. J. Am. Vet. Med. Assoc. 233: 570-577.

Ghori, M.T., M.S. Khan, J.A. Khan, M. Rabbani, M.Z. Shabbir, H.R. Chaudhry, M.A. Ali, J. Muhammad, M. C. Elschner and B. M. Jayarao(2017). Seroprevalence and risk factors of glanders in working equines–Findings of a cross-sectional study in Punjab province of Pakistan. Acta tropica. 176: 134-139.

Gilad, J., D. Schwartz and Y. Amsalem (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.

Ijaz, M., M. S. Khan, M. A. Khan, M. Avais, A. Maqbool, M. Ali and W. Shahzad (2010). Prevalence and serum protein values of strangles (Streptococcus equi) affected mules at Remount Depot, Sargodha (Pakistan). Equine Vet. Edu. 22: 196-198.

Khan, I., L.H. Wieler, F. Melzer, M.C. Elschner, G. Muhammad, S. Ali, L.D. Sprague, H. Neubauer and M. Saqib (2013). Glanders in animals: a review on epidemiology, clinical presentation, diagnosis and countermeasures. Transbound. Emerg. Dis. 60: 204-221.

Khan, I., L.H. Wieler, M. A. Butt, M. C. Elschner, A. H. Cheema, L. D. Sprague and H. Neubauer (2012). On the Current Situation of Glanders in Various Districts of the Pakistani Punjab. J. Eq .Vet .Sci. 32: 783-787.

Kumar, S., P. Malik, S. K. Verma, V. Pal, V. Gautam, C. Mukhopadhyay and G. P. Rai (2011). A recombinant Burkholderia intracellular motility A (BimA) protein for immunodiagnosis of glanders.Clin.Vaccine Immunol.18: 1456– 1461.

Limmathurotsakul, D., V. Wuthiekanun, N. Chantratita, G. Wongsuvan, P. Amornchai, N.P. Day and S. J. Peacock (2010). Burkholderia pseudomallei is spatially distributed in soil in northeast Thailand. PLoSNegl. Trop. Dis. 4: e694.

Malik, P., H.Singha, S. K. Goyal, S. K. Khurana, B. N. Tripathi, A. Dutt, D. Singh, N. Sharma and S. Jain (2015). Incidence of Burkholderia mallei infection among indigenous equines in India. Vet. Rec. Open. 2,e000129.

Mota, R.A., A.A. da Fonseca Oliveira, A.M. da Silva, J.W. Junior, L.B. da Silva, M. de Farias Brito and S.S. Rabelo (2010). Glanders in donkeys (Equus Asinus) in the state of pernambuco, Brazil: A case report. Braz. J. Microbiol. 41: 146-149.

Muhammad, G., M. Khan and M. Athar (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 ofVeterinary Science, Uni. Agri., Faisalabad, Pakistan.

Naureen, A., M. Saqib, G. Muhammad, M. H. Hussain and M. N. Asi (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., L. Sprague, R. Zacharia, H. Tomaso, S. Al Dahouk, R. Wernery, U. Wernery and H. Scholz (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.

OIE (World Organization for Animal Health), (2015). Available at :http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/

Pal, V., S. Singh, A.K. Tiwari, Y.K. Jaiswal and G. P. Rai (2016). Development of a Polymerase Chain Reaction Assay for Detection of Burkholderia mallei, a Potent Biological Warfare Agent. Defence Sci. J. 66: 458-463.

Poyhonen, A., J. T. Hakkinen, J. Koskimaki, M. Hakama, T.L. Tammela and A. Auvinen (2013).Empirical evaluation of grouping of lower urinary tract symptoms: principal component analysis of Tampere Ageing Male Urological Study data.B. J. U. Int. 111: 467-473.

Ribolzi, O., E. Rochelle-Newall, S. Dittrich, Y. Auda,P. N. Newton, S. Rattanavong, M. Knappik, B. Soulileuth, O. Sengtaheuanghoung and D. A. Dance (2016). Land use andsoil type determine the presence of the pathogen Burkholderia pseudomallei in tropical rivers. Environ. Sci. Pollut. Res. Int. 23; 7828–7839.

Saqib, M., G. Muhammad, A. Naureen, M. H. Hussain, M. N. Asi, M. K. Mansoor, M. Toufeer, I. Khan, H. Neubauer and L. D. Sprague (2012). Effectiveness of an antimicrobial treatment scheme in a confined glanders outbreak. BMC Vet. Res.8: 214.

Scholz, H.C., M. Joseph, H. Tomaso, S. Al Dahouk, A.Witte, J. Kinne, R.M. Hagen, R. Wernery, U. Wernery and H. Neubauer (2006). Detection of the reemerging agent Burkholderia mallei in a recent outbreak of glanders in the United Arab Emirates by a newly developed fliP-based polymerase chain reaction assay. Diagn. Microbiol. Infect. Dis. 54: 241-247.

Shabbir, M. Z., T. Jamil, A. A. Ali, A. Ahmad, M. Naeem, M. H. Chaudhary, M. Bilal, M. A. Ali, K. Muhammad, T. Yaqub, A. Bano, A. I. Mirza, A. B. Shabbir, W. R. McVey, K. Patel, S. Francesconi, B. M. Jayarao and M. Rabbani (2015). Prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan. Front. Microbiol. 6: 917.

Silva, K. P. C. D., G.M.C. Takaki , L. B. G. D. Silva, T. N. Saukas, A. S. Santos, and R. A. Mota (2013). Assessment of the effectiveness of the PPD-mallein produced in Brazil for diagnosing glanders in mules. Braz. J. Microbiol. 44: 179-188.

Silva K.P.C., , J.A.A. Teles, A.F.M. Dantas, M.M. Costa, W.P. Felix and R.A. Mota (2014). Partially purified malleo-proteins production for glanders diagnosis in equidae. Pesquisa Veterinária Brasileira. 34: 57-61.

Singha, H., P. Malik, S. K. Goyal, S. K. Khurana, C. Mukhopadhyay, V. K. Eshwara and R. K. Singh (2014). Optimization and validation of indirect ELISA using truncated TssB protein for the serodiagnosis of glanders amongst equines. Sci. World J. 469407.

Tomaso, H., H.C. Scholz, S. Al Dahouk, M. Eickhoff, T.M. Treu, R. Wernery, U. Wernery and H. Neubauer (2006). Development of a 5'-nuclease real-time PCR assay targeting fliP for the rapid identification of Burkholderia mallei in clinical samples. Clin Chem. 52: 307-310.

Vaid, M., M. Muneer and M.Naeem(1981). Studies on the incidence of glanders at Lahore (Pakistan). P. V. J. 1: 75.

Verdegaal, E.J.M.M. and L.A.A.M. van Oijen (2016). Atypical cases of equine Glanders could form a risk for re-emerging Glanders disease worldwide.J. Equine Vet. Sci. 4 (S105): 39.

Wittig, M. B., P.Wohlsein, R.M. Hagen, S. A. L. Dahouk, H. Tomaso, H. C. Scholz, K. Nikolaou, R. Wernery, U. Wernery, Kinne, M. Elschner and H. Neubauer (2006).Glanders-a comprehensive review.Dtsch. Tierärztl.Wochenschr. 3; 323–330.