SERO-SURVEILLANCE AND RISK FACTORS OF BURKHOLDERIA MALLEI INFECTION IN INDIGENOUS HORSES OF BANGLADESH WITH A BRIEF REVIEW ON VALIDATION OF SERODIAGNOSIS

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ABSTRACT

Background: Glanders is a highly contagious and fatal zoonotic reportable antique disease of solipeds caused by the Gram-negative bacterium Burkholderia mallei. This disease has been eradicated from most of the western developed countries in the 20th century and its occurrence was reduced in endemic developing nations but recent reports on the occurrence of clinical cases and outbreaks of this disease in both the eradicated and endemic countries indicates that it has regained the status of a re-emerging disease in the world. However, the information on the occurrence of B. mallei infection is almost lacking in Bangladesh.

Objective: This study was conducted on the sero-surveillance and risk factors of B. mallei infection in indigenous working horses in Bangladesh

Materials and Methods: A cross-sectional study on the sero-surveillance and risk factors of B. mallei infection was carried out in 125 indigenous horses in the districts of Mymensingh and Tangail during January to August 2019. Individual serum samples were screened using Complement fixation test (CFT) at the OIE and National Reference Laboratory for Glanders, Germany and Enzyme linked immunosorbent assay (ELISA) at the National Research Centre on Equines, Haryana, India. Risk factors were identified using multivariable logistic regression analysis.

Results: The overall sero-prevalence of B. mallei infection in indigenous horses was found to be 10.4% (95% CI: 5.9 -17.5). None of the 13 CFT positive sera was positive with ELISA. The odds of B. mallei infection were 6.1 times (95% CI: 1.7-28.9) higher in horses with the history of skin lesion than those without skin lesion. Significantly higher odds of B. mallei infection (odds ratio: 5.8; 95% CI: 1.4-39.7) were observed in horses with the history of parasitic infestation than those without parasitic infestation.

Conclusions: The relatively higher prevalence of B. mallei infection observed in this study should be interpreted with caution as all CFT positive samples negative with ELISA indicating some false positive reactions. Further studies are needed to test the accuracy of the serological tests for the detection of B. mallei infection in horses in Bangladesh.

Keywords: Burkholderia mallei infection, Carrier and latent infections, Sero-surveillance, Indigenous horses, CFT. ELISA, Bangladesh. Review. Validation. Serotests

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INTRODUCTION

Glanders is a highly contagious and fatal zoonotic disease, primarily affecting solipeds (horses, donkeys, asses and mules), caused by *Burkholderia mallei* and characterized by nodular lesions in the lungs and ulcerative and nodular lesions of the skin and respiratory mucosa.\(^1\) It is an antique disease and Hippocrates was first described as a disease of horses in 450 BC and Aristotle grouped it under the generic term for epizootics (‘melis’ - Greek word or ‘mallus’ / ‘malleus’ in Latin word means severe disease) around 350 BC, from which the causative bacterium *Pseudomonas mallei* takes its name.\(^2,5\) Many synonyms have been given to this disease including Cutaneous droes, Farcy (skin form), Farcy pipes, Farcy buds, Malleus or Equinia.\(^4,6\) The glanders name has generally been accepted as the term of choice.\(^4\) However, it has also been suggested that this disease is associated with enlargement and induration of the lymph nodes of the lower jaw and hence the name glanders from the French ‘glandres’ means glands.\(^7\) Its bacterial etiology was established in 1882 when Friedrich Loeffler and Wilhelm Schultz in Germany isolated and identified the causal agent from a horse dying of glanders, which they named the *Bacillus mallei* (now known as *Burkholderia mallei*). It has a 95% case fatality rate in untreated septicemia infections in equine and a 50% case fatality rate in treated human individuals, even diagnosed early.\(^8\) It is regarded as a potential biological warfare or bioterrorism agent because of the high mortality rate and the small number of organisms needed to establish infection in humans and horses. Accordingly, this bacterium was one of the first biological warfare agents used in the 20th century which was used in the American Civil War and both the World Wars by infecting horses and subsequently soldiers.\(^9-11\) Most recently, the former Soviet Union Army was also accused to have used weaponized *B. mallei* against opposition forces in the Afghan war between 1982 and 1984.\(^5,6,12\) It is a notifiable disease under the Glanders and Farcy Act 1899 and currently, notification of OIE is compulsory for OIE member states. It is an important historic disease with high mortality rates in horses when large population of horses played a significant role in human transport and military campaigns and later on it is disappeared with the decreased of horse population and their uses. In addition, most of the developed nations have been eradicated this disease through large scale culling and slaughter, quarantine and pre-testing of animals for movement\(^13\) but the regions of endemicity still exist in several countries in Eastern Europe, Asia, South America, Middle East and North Africa. However, recently several sporadic cases and outbreaks of glanders in horses have been reported from Afghanistan, Bahrain, Brazil, Eritrea, Germany, India, Iran, Kuwait, Lebanon, Mongolia, Myanmar, Nepal, Pakistan, Russia and Turkey.\(^14-20\) It indicates that the glanders has regained the status of a re-emerging disease in both the endemic and previously eradicated countries.\(^5,21,22\) Glanders is a primary disease of solipeds and humans but it also occurs in carnivores, small ruminants, whereas cattle and pigs are considered resistant.\(^23\) However, the reservoirs of *B. mallei* are solipeds, particularly horses and the chronically affected horses can be asymptomatic carrier and latent but remain highly infectious.\(^16\) So far, no clinical glanders case has been reported either in horses or humans from Bangladesh\(^24\) but found seropositive horses.\(^25\) This paper compares the CFT and i-ELISA for the sero-surveillance of *B. mallei* infection in indigenous horses in Bangladesh with a brief review on validation of the sero-diagnosis.
Sero-surveillance of *Burkholderia mallei* infection in horses

**MATERIALS AND METHODS**

A cross-sectional study using 125 randomly selected horses from six upazilas of two districts-Mymensingh (n = 83) and Jamalpur (n = 42) was carried out for a period of eight months from January to August 2019. Venous blood (5 to 7 ml / animal) was collected from each of the 125 randomly selected horses by using sterile disposable syringe. Samples were placed on a tray undisturbed for one hour at room temperature in a slightly inclined position to facilitate clotting and separation of serum. The clotted blood samples were then stored overnight at 4 °C. Sera were transferred to a fresh test tube and centrifuged at 2,500 rpm for 10 minutes. The clear supernatants were transferred to fresh tubes and sent equal volume to laboratory of Germany and India with adequate measures.

Primary data was collected through personal face-to-face interviews with the horse owners. For each sample, the following data were collected: 1) age group [< 6 years, ≥ 6 years], 2) gender [male / female], 3) history of respiratory signs (nasal discharge and / cough) in last 3 months [yes / no], 4) parasitic infestation [yes / no], 5) history of skin lesions (nodules and / ulcers and / abrasions) in last 3 months [yes / no], 6) lameness and arthritis [yes / no] suggestive of glanders. Those risk factors were selected after informal discussion with equine owners.

**Complement fixation test (CFT)**

The CFT was performed at the OIE and National Reference Laboratory for Glanders, Friedrich-Loeffler-Institute, Germany (CFT) as described in the OIE manual. Briefly, serum samples were diluted 1 : 5 in CFT buffer (Institute Virion / Serion GmbH), inactivated and two fold dilutions of them were mixed with Malleus CFT antigen (Ccpro GmbH) and five complement hemolytic units-50% of guinea pig complement (Institute Virion / Serion GmbH). Sera, complement and antigen were mixed in the plates and incubated overnight at 4 °C. A 2% suspension of sensitized (amboceptor from Institute Virion / Serion GmbH) sheep red blood cells (Labor Dr. Merk & Kollegen) were added and plates were incubated for 45 minutes at 37 °C and then centrifuged for 5 minutes at 600g. Samples with 100% hemolysis in a dilution of 1 : 5 were categorized as negative, samples showing 100% inhibition of hemolysis in a dilution of 1 : 5 were classified as positive. All suspicious test results (25-75% hemolysis) were classified as positive.

**Enzyme-linked Immuno-sorbant Assay (ELISA)**

The indirect ELISA was performed at the National Research Centre on Equines, Haryana, India as described. Briefly, the optimal concentration of ELISA reagents (TssB protein, serum, and secondary antibody dilution) was determined by checkerboard titration method using known glanders positive and negative equine serum. The optimal ELISA reagents concentration was assumed to be for those showing the highest discrimination between positive and negative serum. After optimization of the assay, 125 equine serum samples were assayed by ELISA. Pool of four positive and four negative serum samples were included in each ELISA plate along with field serum samples for monitoring the accuracy of the assay. The 96-well microtiter plates (Greiner Bio One, USA) were coated with TssB protein for overnight at 4 °C followed by washing with PBS-T and blocking with 6% skimmed milk in PBS-T for 1 hour at
37 °C. Serum samples were diluted in dilution buffer (2% (w/v) skimmed milk in PBS-T) and 100 μl of diluted serum was added in each well. The plates were incubated for 1 hour at 37 °C and washed five times with PBS-T. The diluted anti-horse conjugate (100 μl) was added in each well and incubated for 1 hour at 37 °C. Following washing steps, 100 μl of substrate solution containing 200 μmol of orthophenylene-diamine in citrate perborate buffer (Sigma-Aldrich, USA) was added in each well and plate was kept in dark at 37 °C for 10 minutes. Finally, the reaction was stopped by addition of 50 μl of 1M H₂SO₄ and the absorbance was read at 492 nm in a microtiter plate reader (Titertek Multiskan, Finland).

**Statistical analysis**

Data were processed and recorded by MS office excel (Office 10) and analyzed by Epi Info™ (Epi Info 7). Pearson chi-square test and Fisher Exact test (where expected count was less than 5) were used to evaluate the univariable association between seropositivity for *B. mallei* and its potential categorical predictors mentioned earlier. Multiple logistic regression analysis was used to identify risk factor for glanders. Variables with p ≤ 0.05 in multivariable analysis were recorded as risk factor. Categories with lowest prevalence were used as reference.

**RESULTS**

Out of 125 horse serum samples tested, of which 13 (10.4%, 95% CI: 5.9 –17.5) were found positive by using CFT and none of the samples was positive with ELISA (Table 1 & Photo 1 & Photo 2). The CFT positive animals were found in both of the Mymensingh (10.98%) and Jamalpur (9.30%) districts but no significant difference (OR: 1.20, CI: 0.34–4.15, p > 0.05) was

<table>
<thead>
<tr>
<th>SN</th>
<th>Districts with Upazila</th>
<th>Complement fixation test (CFT)</th>
<th>Immunoblot</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Earlier report 25</td>
<td>Current study</td>
<td>Earlier report 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. tested</td>
<td>Positive No. (%)</td>
<td>No. tested</td>
</tr>
<tr>
<td>1</td>
<td>Fulpur</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Muktagachha</td>
<td>-</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Mymensingh Sador</td>
<td>-</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>Trishal</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>Total: Mymensingh</td>
<td>115</td>
<td>043 (37.4)</td>
<td>083</td>
<td>09 (10.98)</td>
</tr>
<tr>
<td>5</td>
<td>Jamalpur Sadar</td>
<td>-</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Melandah</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Total: Jamalpur</td>
<td>110</td>
<td>042 (38.2)</td>
<td>042</td>
<td>04 (09.52)</td>
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<td>Total: Tangail</td>
<td>076</td>
<td>020 (26.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td>301</td>
<td>105 (34.9)</td>
<td>125</td>
<td>13 (10.40)</td>
</tr>
</tbody>
</table>
Sero-surveillance of *Burkholderia mallei* infection in horses

found between the two districts (Table 1). Table 1 also shows that the Fulpur upazila had the highest sero-prevalence of 14.29%, followed by Muktagacha (13.64%), Melandah (10%), Trishal (9.09%), Jamalpur Sadar (8.69%) and Mymensingh Sadar (8.57%).

The univariate analysis reveals significant (p < 0.05) association of sero-positive *B. mallei* with history of respiratory signs, history of parasitic infestation and history of skin lesions (Table 2). Respiratory signs within last 3 months were included in the study and coughing, sneezing, dyspnea, nasal discharge, epistaxis, abnormal lung sound were considered as respiratory illness. Presence of nodules, ulceration, superficial or deep abscess or any kind of skin injury was recorded as skin lesions in the study. It was also evident from the analysis that animals with endo-parasitic and ecto-parasitic infestation were more likely to be seropositive. The odds of *B. mallei* infection were 6.1 times (95% CI: 1.7-28.9) higher in horses with the history of skin lesions than those without skin lesions (Table 2). Significantly higher odds of *B. mallei* infection (Odds ratio: 5.8; 95% CI: 1.4-39.7) were observed in horses with the history of parasitic infestation than those without parasitic infestation (Table 2).

**DISCUSSION**

The world’s horse population is likely to be around 60 million\(^{28}\) but it seems to be quite low in Bangladesh in comparison to other livestock animal species and it might be the reason that the horse population does not appear in both the national and international livestock statistics.\(^ {28-32}\) However, some year-wise horse population of Bangladesh has been reported to be varied from 43 to 46 thousands during the period from 1969 to 1990.\(^ {33,34}\)

Horses are very sparsely distributed throughout Bangladesh with a noticeable concentration in greater Sylhet, Tangail, Kishorgonj and greater Dhaka districts. The socio-economic status of horse owner in the districts of Mymensingh, Tangail, Sherpur and Jamalpur showed that they
are mainly used for pulling cart, transportation, land tillage and sport purposes for their livelihood. Horses are also used as horse-drawn carriages, horse rent for wedding/marriage ceremony, horseback riding and racing in Bangladesh. In addition, there are some horse establishments like President’s Guard Regiment, National Police Head Quarter, Remount Veterinary and Farm Core (RVFC), Sarda Police Academy, Bangladesh Military Academy where small number of exotic horses are maintained for various purposes. Recently, six trained horses have been imported from India for the Bangladesh Police through Benapole Port on February 4, 2020.

The horses of Bangladesh cannot be defined under any specific breed and these indigenous and non-descriptive horses have been influenced genetically by Arabian and Persian horses which have been immigrated through India from the west but their numbers are not known. However, Bangladeshi horses are observed to be close to those of the Thai native horse which are direct descendants of the Chinese South Western mountain ponies. Thus, it can be said that the native horses of Bangladesh have belonged to the lineage of the South East Asian ponies rather than that of Arabian and Persian horses. However, it claims that Bangladesh has only two breeds of horses: the Bangladesh native horse and the Rajshahi pony. The Bangladesh native is of the South-East Asian pony type and is said to have been influenced by Arabian and Persian horses migrating from the west through India. The Rajshahi pony is of unknown origin which is described as dwarf, standing 109-113 cm tall and is found mainly in Rajshahi, Jessore, Tangail, Mymensingh, Sylhet and Dhaka districts.

RS = Respiratory signs  PI = Parasitic infestation

<table>
<thead>
<tr>
<th>SN</th>
<th>Variables</th>
<th>Sub-variables</th>
<th>Total No. tested</th>
<th>CFT +ve No. (%)</th>
<th>CFT -ve No. (%)</th>
<th>Odds ratio</th>
<th>95% CI of OR</th>
<th>p value</th>
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<tbody>
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<td>Age (years)</td>
<td>&lt; 6</td>
<td>42</td>
<td>07 (16.7)</td>
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<td>2.6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>≥ 6</td>
<td>83</td>
<td>06 (07.2)</td>
<td>77 (92.8)</td>
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<tr>
<td>2</td>
<td>Gender</td>
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<td>69</td>
<td>08 (11.6)</td>
<td>61 (88.4)</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
<td>56</td>
<td>05 (08.9)</td>
<td>51 (91.1)</td>
<td>Reference</td>
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<td>3</td>
<td>History of RS</td>
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<td>26</td>
<td>06 (23.1)</td>
<td>20 (76.9)</td>
<td>3.9</td>
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<td>99</td>
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<td>92 (92.9)</td>
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<td>4</td>
<td>History of PI</td>
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<td>72</td>
<td>11 (16.4)</td>
<td>56 (83.6)</td>
<td>5.5</td>
<td>1.4 - 36.5</td>
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<td>53</td>
<td>02 (03.4)</td>
<td>56 (96.6)</td>
<td>Reference</td>
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<tr>
<td>5</td>
<td>History skin lesions</td>
<td>Yes</td>
<td>51</td>
<td>10 (19.6)</td>
<td>41 (80.4)</td>
<td>5.8</td>
<td>1.7 - 26.8</td>
<td>0.01</td>
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<td></td>
<td></td>
<td>No</td>
<td>74</td>
<td>03 (04.1)</td>
<td>71 (95.9)</td>
<td>Reference</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6</td>
<td>Lameness &amp; arthritis</td>
<td>Yes</td>
<td>03</td>
<td>01 (33.3)</td>
<td>02 (66.7)</td>
<td>4.58</td>
<td>0.39 - 54.37</td>
<td>0.28</td>
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<td></td>
<td>No</td>
<td>122</td>
<td>12 (09.8)</td>
<td>11 (90.2)</td>
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</table>
Glanders is caused by the bacterium *Burkholderia mallei* and has been variously classified in the past as *Bacillus mallei*, *Loefflerella mallei* and then *Pfeifferella mallei*, *Malleomyces*, *Pseudomonas* and *Actinobacillus*. The new name *Burkholderia mallei* was based on the 16s rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition and phenotypic characteristics. The characteristics of 14 equine isolates of *B. mallei* have been reported.

Glanders is a highly communicable disease of equids but acute in donkeys and mules whereas mostly the chronic in horses and the recovered animals remain carrier. This infection has also been reported in sheep and goats, wild animals, canids and felids. Guinea pigs and hamsters are reported to be highly susceptible and caused mortality with 24 hours following inoculation whereas pigs and cattle have reported to be resistant.

This organism can invade its host through mucous membrane, gastro-intestinal tract and the integument. Glanders is pathologically characterized by ulcerating nodular lesions of the skin and mucous membrane and clinically characterized by fever, malaise, depression, cough, anorexia and weight loss.

Glanders is primarily classified into two forms include clinical and sub-clinical but it can also be classified into four forms: cutaneous, pulmonary, nasal and asymptomatic carrier.

Cutaneous glanders may result from skin injury or may be due to a secondary manifestation of the respiratory form. It consists of nodules, pustules and ulcers that occur over any part of the body but most frequently observed on the legs. The lesions appear as nodules in chains along the lymphatic vessels, which tend to break down and form crater-like ulcers discharging thick yellowish viscid and sticky purulent material heavily laden with the organism. This is usually referred to as farcy pipes. It induces a neutrophilic leukocytosis and severe anemia probably because of depressed erythropoietic activity of the bone marrow.

The pulmonary form is most commonly associated with the formation of round, greyish, firm, encapsulated nodules embedded throughout the lung tissue and clinically characterized by acute broncho-pneumonia with cough and high fever.

The nasal form is the formation of nodules or ulcers in the upper air passages commonly on the lower parts of the turbinate and on the cartilaginous nasal septum. It is clinically characterized by bloody muco-purulent nasal discharge when those nodules rupture.

The asymptomatic carrier form of glanders develops after a period of illness of some months. The affected animal makes an apparent recovery but persists as an occult case. Mallein test is positive but no obvious skin lesions can be seen.

Equine glanders may be diagnosed based on clinical signs, the Mallein test, serological tests and bacterial isolation. The isolation and identification of *B. mallei* bacterium from cutaneous lesions, lymph nodes, and nasal and respiratory exudates are considered to be the ‘gold standard’ method for diagnosis of glanders.

The majority of the glanders cases (approximately 90%) occur as asymptomatic latent and carrier forms under field condition that make the difficulty to diagnose these sub-clinical glanders on the bacteriological method. Accordingly, various immune-diagnostic tests have been used for the diagnosis of the asymptomatic glanders which include the Agar-gel immunodiffusion (AGID) test, Counter-immuno-electro-phoresis (CIE) test, Indirect
fluorescent antibody (IFA) test, Indirect haemagglutination test (IHA) test, Rose Bengal test, Complement fixation test = CFT, Enzyme linked immunosorbent assay = ELISA, and Immunoblot.

The accuracy of the sero-diagnosis tests is challenging due to false-positive and false-negative test results. However, there is a general agreement that the CFT is superior to other serological tests. CFT can detect carriers and chronically infected animals and still the only OIE mandatory serological test for international trade of equids and also recommended for sero-surveillance. The CFT is known to have high sensitivity but it gives a considerable number of false-positive results. It may give a positive cross-reaction in horses suffering from strangles, equine influenza or petechial fever and in emaciated horses not suffering from glanders. These limitations of the CFT might be due to use of non-standardized antigen that caused different diagnostic accuracies. The CFT antigens have also been evaluated and the selection of antigens used for CFT has also to be updated and standardized.

ELISA has been developed for the diagnosis of glanders in horses. The ELISA has also been compared with CFT, AGID and HA test with culture results, and the results showed that ELISA correctly identified 100% of confirmed clinical cases of horses with glanders where other tests gave only 90.0% positive reaction. Accordingly, it has been suggested that ELISA could be routinely adopted as a highly sensitive diagnostic test for glanders. However, these serological tests are not only incapable of discriminating between B. mallei and B. pseudomallei antibodies, they are also unable to differentiate between malleinized and naturally infected animals.

The Western blot (WB) and ELISA have also been evaluated to overcome the disadvantages of the CFT but these tests have not been fully validated in large scale studies. Comparative evaluation CFT, WB and five different ELISAs have been reported that the WB and all ELISAs except BimA antigen had significantly more specific than the CFT, whereas ELISAs based on TssA, TssB and BimA antigens had significantly lower sensitivity compared...
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to CFT and the sensitivities of the Hcp1-ELISA, the IDVet-ELISA and the WB did not differ significantly from that of the CFT.\(^{26}\) However, all these evaluated serological diagnostic tests are unable to differentiate between *B. mallei* and *B. pseudomallei* in the regions and country where both the close phylogenetic bacteria prevalent endemically. However, combined use of both serological and molecular detection methods increases the detection rate of glanders.\(^{43,86,87}\)

The CFT and indirect ELISA were used for the detection of antibodies against *B. mallei* infection in indigenous horse serum of Bangladesh. Of the 125 serum samples of apparently healthy indigenous horses screened with CFT and i-ELISA, of which only 10.4% animals found positive with CFT but none of the samples was positive with i-ELISA. These findings support the best test sensitivity results for the CFT followed by WB, Hcp1- and IDVet-ELISAs for the serodiagnosis of glanders in horses.\(^{26}\) The lack of specificity of the CFT especially in areas where false positive rate is very high that results in constant obstacles to trade in horses.\(^{88}\) However, the comparative sensitivities and specificities of the recommended sero-tests for *B. mallei* infection in horses have been reported as 98.0% and 96.4% with CFT, 96.8% and 99.4% with the WB, 95.3% and 99.6% with the Hcp1-ELISA and 92.5% and 99.5% with the IDVet-ELISA, respectively.\(^{26}\) Therefore, a confirmatory test for *B. mallei* infection with both the higher sensitivity and higher specificity would be developed to meet OIE requirements to avoid an introduction of infected animals into disease free populations at national and international levels.\(^{79}\)

The CFT and Mallein test subcutaneously, intracutaneously or ophthalmically and the resulting fever, swelling or reflux of pus from the eye in positive animals have historically been used as indirect diagnosis methods of equine glanders for the eradication of this disease.\(^{5}\)

Glanders which is eradicated from most of the Western Europe and Northern American countries including Australia, Japan and some other countries in the last century, it has regained the status of a re-emerging disease because of the numerous recent outbreaks throughout South American and Asian countries.\(^{4,5,85,89-91}\) However, cases of glanders still periodically occur in South America, Eastern Europe, certain parts of Asia, the Middle East, northern Africa and various Mediterranean regions and Indo-Pakistan sub-continent.\(^{92-95}\) The major outbreaks of this disease were reported between 1976 to 1982, followed by sporadic cases in 1988, 1990 and 1998 in India and remained free for eight years and re-emerging of outbreaks occurred during 2006 to 2011 in India.\(^{22,96}\) The re-emerging of glanders cases have been reported in Maharstra, Haryana and Punjab after a gap of 10 years with an overall sero-prevalence of 0.62% to 1.15% with Hcp1 indirect ELISA followed by confirmatory diagnosis by CFT during 2015 to 2018.\(^{97}\)

Lack of awareness, inadequate veterinary care and unrestricted movement of equids across state borders might have led to the introduction and establishment of the infection in these states. This base line data on glanders are suggesting for devising surveillance and control strategies.

Horse, donkeys and mules are the only known natural reservoir of *B. mallei*. Asymptomatic or carrier animals are the potential source of reintroduction of the infection into glanders free areas and countries that caused trade restrictions with animals and products from regions or outbreak areas or countries.\(^{88}\) Recently it has been reported that *B. mallei* can persist as latent infection similar to *B. pseudomallei* which may not be detected by the currently used diagnostic tests.\(^{88}\) The CFT has been recommended by the OIE for international trade in equids but this
test has been reported to have varying sensitivities and specificity depending on the antigen and methodology used. False positives are problematic for the horse owners and veterinary authority whereas false negatives may allow the reintroduction of *B. mallei* into free areas and countries. The limitations of the currently available diagnostic tests and epidemiological knowledge on glanders indicate that infection with *B. mallei* remains a major risk in the context of international movement of equids.

The persistence of *B. mallei* infection in horse population poses a potential threat to occupational exposed human especially equine handlers and Veterinarians. The positive sera with positive isolation of *B. mallei* are very difficult to collect from field cases because to pose a zoonotic risk and spread of infection to equine population and accordingly, the clinically glanders positive cases are usually destroyed immediately before samples can be collected. However, the WB has been used to exclude false-positive CFT results as a confirmatory test on CFT-positive sera and use of a combination of tests can enhance the accuracy for sero-diagnosis. However, the replacement of CFT with the WB or any of the ELISAs cannot be recommended for testing equids for national and international trade purposes. Under these situations, the importation of horses from glanders endemic region of India for the Bangladesh Police might be dangerous like importation of horses from Brazil to Germany.

The present study also represents identification of various risk factors associated with sero-positivity of *B. mallei* infection of horse in Bangladesh. The results of this study suggest that the presence of *B. mallei* infection related symptoms, history of lesions on skin, age, presence of parasitic infection and history of respiratory signs are highly significantly associated with *B. mallei* infection sero-positive horses of Mymensingh and Jamalpur districts in Bangladesh.

Of the 125 serum samples tested with CFT and i-ELISA, of which only 13 (10.4%) horses showed positive to *B. mallei* infection with CFT but none with ELISA. The CFT was performed at the OIE and National Reference Laboratory for Glanders, Germany and i-ELISA at the National Research Centre on Equines, India. This difference of results of these two tests conducted in two different countries might be due to different antigens used, sample was preserved for longer period of time, longer transportation period of the samples and also presence of different antigenic variance of the *B. mallei* organisms in the country where the sample were tested or other epidemiological factors. However, comparatively higher sero-positivity of *B. mallei* infection in horses in Bangladesh has been reported in our earlier report with CFT (34.9%) and immunoblot (24.8%) techniques. The presence parasitic infection and history of skin lesions were found to be significantly (p < 0.05) associated with *B. mallei* infection. The lower sero-prevalence was recorded in the adult age (≥ 6 years) group (6.02%) than the young (< 6 years) age group (19.05%) of horses. This could be due to the fact that CFT gives a false-negative result in older animals due to poor immune response and more susceptible to the infection. Animals with history of lesions on skin were found at 5.77 times higher risk than the animals with no skin lesions in the conducted study. This may correspond to the fact that disease is chronic in horses evidenced by the skin nodules and hematological features. Also horse with parasitic infestation were found apparently about four times more likely to be *B. mallei* seropositive as compared to horse without parasitic infestation. This may be due to most the farmers do not treat their horses with anthelmintic regularly and hardly use
any disinfectants to clean the water troughs and stables. Communal water troughs and stables serve as a reservoir of *B. mallei* infection. The secretions of the affected animals may contaminate the water and transfer the infection to others in-contact animals.

**CONCLUSIONS AND RECOMMENDATION**

The risk factors associated with *B. mallei* infection in horses can be strengthened by increasing the number of samples and also ensuring a more representative sample including mules and donkeys. The relatively higher sero-prevalence of *B. mallei* infection recorded in indigenous horses should be interpreted with caution as all the CFT positive samples were negative with ELISA indicates some CFT positive samples might be false positive. Further studies are needed with highly sensitive and specific test to detect the accurate sero-prevalence status of *B. mallei* infection in indigenous horses in Bangladesh. The risk factors associated with *B. mallei* infection in indigenous horses should be emphasis on further studies on sero-surveillance, disease awareness and control. However, counter-measures against *B. mallei* infection include early detection of disease in susceptible animals, stringent quarantine measures, testing and safe destruction of infected carcasses, adequate compensation to the animal owners, disinfection of infected premises and awareness about glanders and the zoonotic implications through veterinary extension services.

**CONFLICT OF INTEREST**

No conflict of interest to declare

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