

SEROEPIDEMIOLOGY OF MELIOIDOSIS AND ITS ASSOCIATED RISK FACTORS OF RURAL HORSES IN BANGLADESH

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ABSTRACT

Background: The disease melioidosis was named by Stanton and Fletcher in 1932 from the Greek ‘melis’ (distemper of asses) and ‘eidos’ (resemble). During the last century, this Gram-negative environmental bacterium has been variously known as *Bacillus pseudomallei*, *Bacillus whitmorii*, *Malleomyces pseudomallei*, *Pseudomonas pseudomallei*, and, since 1992, *Burkholderia pseudomallei*. In the latter half of the 20th century, melioidosis emerged as a significant public health concern in Southeast Asia and northern Australia. Severe melioidosis is associated with a case fatality rate of 50% in Thailand and 20% in Australia among all patients. Melioidosis affects both humans and animals elsewhere but is only reported in humans in Bangladesh. Clinical melioidosis has been reported in humans in Bangladesh as sporadic cases, with around 100 cases diagnosed over 60 years as of October 2023. There seems to be no inland reports on the prevalence of melioidosis in animals, even seroprevalence in Bangladesh.

Objective: This study aimed to determine the seroprevalence of melioidosis in rural horses in Bangladesh using serological and molecular diagnostic techniques.

Materials and Methods: From 2023 to 2024, a cross-sectional study was conducted in the Mymensingh, Jamalpur, and Kurigram districts of Bangladesh to determine the seroprevalence of melioidosis in local horses and associated risk factors. A total of 118 horse serum samples were collected and screened for melioidosis using the commercial kits of Antibody Rapid Detection Kit (ARDK), Enzyme-linked immunosorbent assay (ELISA), and Polymerase Chain reaction (PCR).

Results: The overall seroprevalence was 21.53%, 15.25%, and 1.69% in the ARDK, ELISA, and PCR, respectively. Multiple epidemiological risk factors, including environmental conditions, husbandry practices, and geographical distribution, were evaluated and discussed.

Conclusion: This study's findings may help us develop effective prevention and control strategies. There is a risk of zoonotic transmission of this disease to humans in contact with it. Further studies are needed to elucidate the epidemiology, pathogenesis, and clinical importance of melioidosis in animals and humans in Bangladesh, including its economic impact.

Keywords: Melioidosis, ARDK, ELISA, PCR, Sero-prevalence, Risk factors, Rural horses.

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INTRODUCTION

Glanders and melioidosis are zoonotic bacterial diseases caused by closely related Gram-negative bacteria, *Burkholderia mallei* and *B. pseudomallei*, respectively. Glanders is an infectious disease caused by *B. mallei*, a gram-negative aerobic, non-motile bacterium. It primarily infects equines but can also be transmitted to humans, making it a true zoonosis. Melioidosis is an infectious disease caused by *B. pseudomallei*, a bacterium genetically similar to *B. mallei* but an environmental saprophyte. It is a Gram-negative, aerobic, motile bacterium. It can be distinguished from *B. mallei* by its motility and usually its resistance to aminoglycosides. Both diseases are globally distributed but are predominantly encountered in tropical and sub-tropical regions, with endemicity of melioidosis in Australia and Southeast Asia, including Bangladesh.¹⁻⁷ The increasing recognition of melioidosis in humans and recent outbreaks of glanders in animals have led to their classification as emerging or re-emerging diseases, and to melioidosis being described as a neglected tropical disease.⁸ The etiological agent for melioidosis is *B. pseudomallei*, an oval, motile, gram-negative, facultative anaerobic bacillus with bipolar staining. This widespread saprophyte can adapt to various environmental conditions, isolated from multiple soil types and surface water of varying depths, and infects humans and many animal hosts. Host species infected include terrestrial and aquatic mammals, birds, and fish. Goats, sheep, pigs, and camels appear particularly susceptible. In contrast, dogs, cats, and cattle appear more resistant. Still, these may develop disease if they become immunocompromised.⁹ Sporadic cases or small outbreaks have been reported in various primates, marsupials, deer, buffalo, camels, llamas, zebra, horses, mules, rabbits, rodents, iguanas, parrots, crocodiles, dolphins, and seals.^{8,10} Although glanders has been eradicated from most countries, it has regained the status of a re-emerging disease, particularly in Asia, the Middle East, and South America, because of the numerous recent outbreaks.¹¹ Laboratory-associated infections with both bacteria have also occurred, categorizing them as Hazard Group 3 pathogens. Although rare in Western countries, both bacteria have recently attracted significant interest due to their potential use as bioterrorism agents and their expanding geographic footprint.⁸ A review indicates that there is a single report on equine glanders in Bangladesh,¹ which reports an overall 34.9% seroprevalence of glanders in horses based on the complement fixation test (CFT) and 24.8% based on immunoblot in the districts of Mymensingh, Jamalpur, and Tangail. In contrast, the higher prevalence in Jamalpur (11.81%) makes CFT a suitable screening test. There are no specific studies on the prevalence of melioidosis in horses in Bangladesh, but the clinical human disease caused by *B. pseudomallei* has been reported in Bangladesh.¹⁻⁷ While glanders (*B. mallei*) is a specific equine disease, *B. pseudomallei* can infect not only horses but also various mammals, birds, and reptiles. This bacterium is widespread in Bangladesh. Due to a lack of surveillance, many cases in humans and potentially animals are likely underdiagnosed and unreported. This paper describes the seroprevalence of melioidosis in rural horses in Bangladesh using serological and molecular diagnostic techniques.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Mymensingh (15 horses), Kurigram (20 horses), and Jamalpur (83 horses) districts of Bangladesh from January to October 2024, with laboratory work performed at the Zoonotic Disease Diagnostic Laboratory, Bangladesh Agricultural University, Mymensingh.

Sample collection and processing

Blood samples (8-10 ml) were collected from 118 randomly selected horses, placed in tubes with clot activator (Fig. 1), transported to the laboratory within 12 hours, and sera were separated by centrifugation at 2,500 rpm for 10 minutes and stored at -20°C until tested.

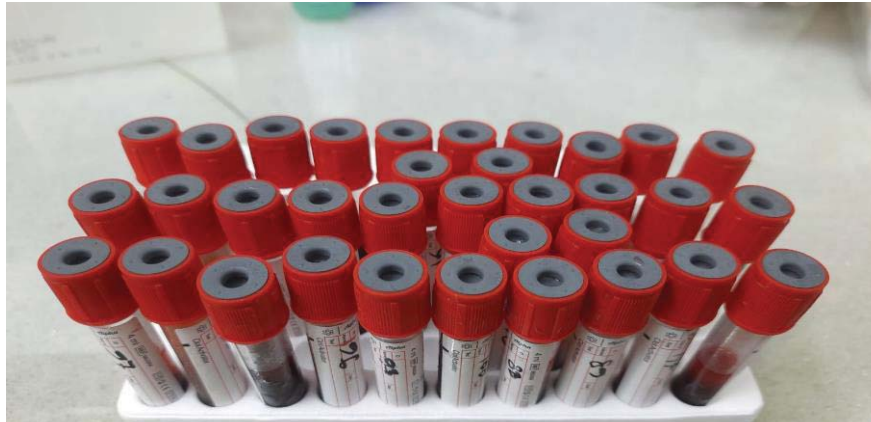


Fig. 1. Blood samples placed in blood collection tube

Epidemiological data collection

Data on potential risk factors were collected through face-to-face interviews with horse owners. For each animal, the following parameters were recorded: history of respiratory signs (nasal discharge and/or cough) in the last 3 months [yes/no], history of skin lesions (nodules, ulcers, abrasions) in the previous 3 months [yes/no], floor type [concrete/soil], use of the animals [draft/race], age, gender [male/female], color, parasitic infestation [yes/no], and stress-related history. These risk factors were selected after consultation with equine practitioners familiar with the study region.

Antibody rapid detection test

To validate the presence of the *B. pseudomallei* in the original serum samples, the Glanders Rapid Detection Test Kit (Genomix Biotech, USA). The Glanders Ab Rapid Detection Test Kit was carried out according to the manufacturer's instructions in the supplied catalogue. 10 μ L of the serum sample was added to the sample well on the test cassette using the dropper. Two drops of test buffer were placed in the identical sample well on the test cassette. After 20 minutes, a line appeared on both the control (C) and test (T) lines, indicating a positive sample. Still, a line appearing just in the control (C) line region was classified as a negative sample. (Fig. 2)

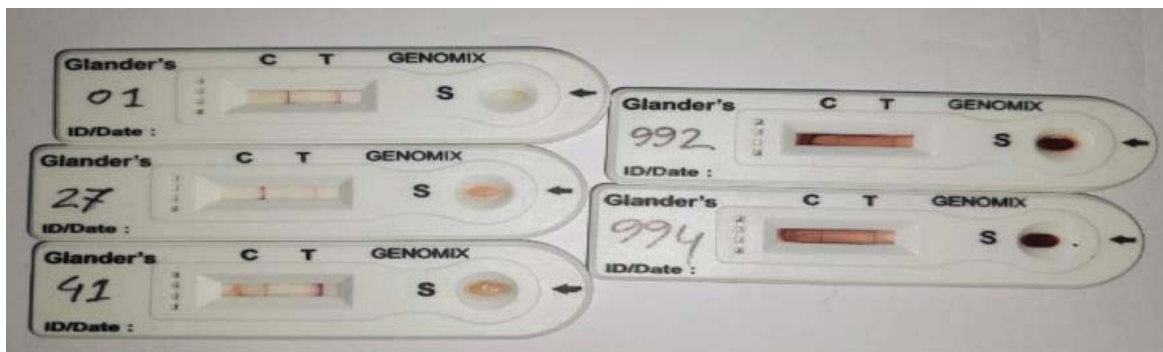


Fig.2. Antibody Rapid Detection Test results of serum collected from rural horses

ELISA Test

Hcp1-specific antibodies were detected using diluted samples (1:100) and incubation at 37°C, as per the kit manufacturer's instructions (Genomix Biotech, USA). Results were calculated as percent positivity (PP%), with values <20% considered negative, 20% equivocal, and >20% positive. (Fig. 3)

Calculations

Percent positivity (PP%) was calculated from the reference sample as per the following formula:

$$\text{Positivity (\%)} = \frac{(\text{OD450 sample serum} - \text{OD450 Negative control}) \times 100}{\text{OD450 Positive control} - \text{OD450 Negative control}}$$



Fig 3. ELISA to detect melioidosis seropositivity

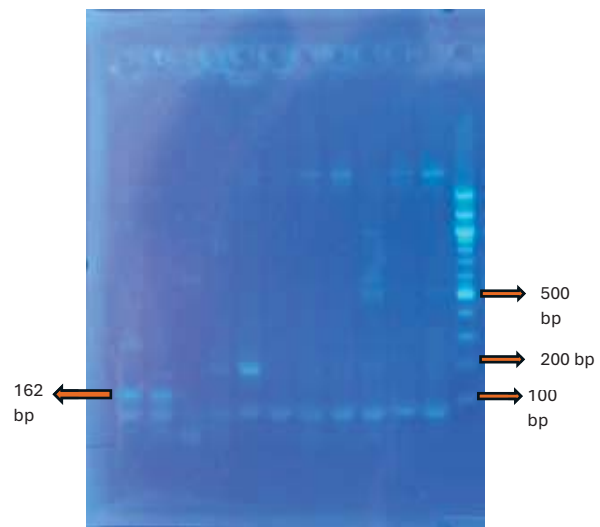


Fig.4. Gel electrophoresis image of amplicon produced from zmpA PCR Assay (162 bp)

PCR Test

The DNA was amplified using a *Burkholderia pseudomallei* genus-specific primer set, **mpr** (forward: 5'-TCT CCG ATA GCC GCC TTG-3'; reverse: 5'-CGT ATC ACA TCG CAT CGC-3').³⁰ A final volume of 50 mL was used for the PCR reactions, which included 1 PCR buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25 U of Taq DNA polymerase, 5 mL of DNA template, and distilled water. According to (27), amplification consisted of 35 cycles of denaturation at 94°C for 30s, annealing at 52°C for 30s, and extension at 72°C for 45s. The initial denaturation was carried out at 94°C for 5 min. After the last cycle, a previous extension lasting an additional two minutes was performed at 72°C. A 100 bp DNA ladder (Invitrogen™), was used to compare the band on an agarose gel. The amplified PCR products were confirmed on agarose gels (1%) stained with ethidium bromide on the gel documentation system (EZEE Clearview UV transilluminator).

Statistical Analysis

Data was analyzed using SPSS version 22. Bivariate logistic regression analysis identified relationships between variables and melioidosis at a 95% confidence level ($p \leq 0.05$). The Hosmer-Lemeshow test assessed model fit.

RESULTS

Seroprevalence of melioidosis

A total of 118 horse serum samples from rural areas of Bangladesh were screened for melioidosis using ELISA, Antibody Rapid Detection Test (ARDT), and PCR. The overall seroprevalence was 15.25% (18/118) by ELISA, 21.18% (25/118) by ARDT, and 1.69% (2/118) by PCR (Table 1).

Sero-prevalence varied by district. In Mymensingh, positivity rates were 20% by ELISA, 33.33% by ARDT, and 6.67% by PCR. Kurigram had the highest seroprevalence: 35% by ELISA, 40% by ARDT, and 5% by PCR. Jamalpur Sadar had the lowest rates, with 10.63% positive by ELISA, 14.8% by ARDT, and 0% by PCR (Table 1)

S/N	District	Upazila	Total No. of samples tested	ELISA Positive No. (%)	RAK Positive No. (%)	PCR Positive No. (%)
1	Mymensingh	Mymensingh Sadar	15	3 (20.0)	5 (33.33)	1 (6.67)
2	Jamalpur	Jamalpur Sadar	47	5(10.6)	7(14.8)	0
		Dewangonj	36	3(8.3)	5(13.8)	0
3	Kurigram	Nagessory	20	7(35)	8(40.0)	1(5.0)
	Overall	-	118	18(15.25)	25(21.18)	2(1.69)

Epidemiological Risk Factors

Several risk factors were evaluated for their association with melioidosis seropositivity. Age-related analysis showed that adult horses (≥ 4 years) had slightly higher seropositivity (16.33% by ELISA, 22.45% by ARDT, and 2.04% by PCR) than younger horses (14.49%, 20.28%, and 1.45%, respectively). Gender appeared to have minimal impact on seroprevalence, with females showing marginally higher rates (15.78% by ELISA, 22.81% by ARDT) than males (14.75%, 19.67% respectively).

Clinical history was significantly associated with seropositivity. Horses with respiratory signs showed markedly higher seroprevalence (30% by ELISA, 40% by ARDT, and 10% by PCR), making this the most substantial risk factor identified. The history of lameness also showed a higher association (22.22% by ELISA, 33.33% by ARDT) compared to the overall population. Conversely, horses with no visible clinical signs had the lowest sero-positivity (3.85% by ELISA, 7.69% by ARDT).

S/N	Variable	Categories	Total No. of samples tested	ELISA positive No. (%)	RAK positive No. (%)	PCR positive No. (%)
①	Age	Young (≤ 4 years)	69	10 (14.49)	14 (20.28)	1 (1.45)
		Adult (≥ 4 years)	49	08 (16.33)	11 (22.45)	1 (2.04)
②	Gender	Male	61	09 (14.75)	12 (19.67)	1 (1.64)
		Female	57	09 (15.78)	13 (22.81)	1 (1.75)
③	History of clinical findings					
a.	Respiratory signs		20	06 (30.00)	08 (40.00)	2(10.00)
b.	Skin lesions		29	04 (13.79)	06 (20.67)	0
c.	Lameness		09	02 (22.22)	03 (33.33)	0
d.	Fever		34	05 (14.71)	06 (17.65)	0
e.	No visible signs		26	01 (03.85)	02 (07.69)	0

DISCUSSION

The disease melioidosis was named by Stanton and Fletcher in 1932 from the Greek ‘melis’ (distemper of asses) and ‘eidos’ (resemble). During the last century, this Gram-negative environmental bacterium has been variously known as *Bacillus pseudomallei*, *Bacillus whitmorii*, *Malleomyces pseudomallei*, *Pseudomonas pseudomallei*, and, since 1992, *Burkholderi pseudomallei*.¹² In the latter half of the 20th century, melioidosis emerged as a significant public health concern in Southeast Asia and northern Australia.¹³ Severe melioidosis is associated with a case fatality rate of 50% in Thailand and 20% in Australia among all patients.^{14,15} Melioidosis affects both humans and animals elsewhere, but only clinical cases are reported in humans in Bangladesh. Clinical melioidosis has been reported in humans in Bangladesh as sporadic cases, with around 100 cases diagnosed over 60 years as of October 2023.⁵ There seems to be no inland reports on the prevalence of melioidosis in animals, even seroprevalence in Bangladesh.

Melioidosis is endemic in many tropical regions, and the highest isolation rates have been reported in rice paddies, rubber plantations, and other cleared and cultivated areas, but high rates have also been recorded in urban areas, fields, and animal grazing sites. Factors that may influence environmental distribution include temperature, humidity, rainfall, ultraviolet exposure, soil composition, vegetation, fertilizers, and soil disturbance such as excavation or ploughing. Recent modelling and epidemiological studies highlighted the underdiagnosis and underreporting of melioidosis, which was estimated to have infected 165,000 people and caused 89,000 deaths worldwide in 2015.¹⁶ This equates to 4.6 million disability adjusted life-years, a greater burden than those for dengue and leptospirosis, and suggests that melioidosis should be formally categorized as a re-emerging neglected tropical disease.^{16,17} India, Bangladesh, Vietnam, Thailand, Nigeria, and Indonesia are predicted to account for almost three-quarters of the global disease burden.¹⁷

Fifty-one melioidosis cases in humans with a significant male (80%) dominance between 1961 to 2017 have been reported from 16 out of 64 districts in Bangladesh. The occupations of the selected 39 patients were: farmers (36.0%), housewives (21.0%), workers/day laborers (10.0%), unemployed (10.0%), carpenters (5.0%), and others (18.0%). Over 88% (23/26) of cases in Bangladesh had a definitive history of soil exposure. Of the 50 clinical cases, the highest percentage of cases were affected with diabetes mellitus (83%), followed by chronic kidney disease (4.0%), hypertension (4.0%), smoking (6.0%), and others (6.05%).² The most affected age group was 46 to 60 years, and most of them (88.2%) had diabetes mellitus. Septicemia (51.5%) was the most common presentation followed by skin and SC tissue abscess (29.4%), septic arthritis (16.2%), UTI (13.2%), pneumonia (10.3%), visceral abscess in lung (5.95%), liver (5.9%), spleen (7.3%) and death was reported among 32.5% cases. The organism was isolated most frequently from blood (40.7%) and pus/wound swab (30.2%).⁷

Melioidosis is a fatal infectious disease caused by the aerobic, Gram-negative, non-fermenting, and non-spore-forming bacillus, *B. pseudomallei*, which is widely distributed in the rhizosphere, soil, and water. Bangladesh is considered a definite country for melioidosis owing to the isolation of the organism both from clinical samples and soil. Only around 100 cases of melioidosis have been diagnosed sporadically in the country for the past 60 years till October 2023. Organism is acquired by inoculation, inhalation, and ingestion, which is why agricultural workers and the rural population are considered high-risk groups in endemic regions.⁵

Melioidosis should be considered in any person or animal who has visited or migrated from an endemic area presenting with septicemia and/or abscesses, especially if they have a predisposing condition such as diabetes in humans. Confirmation of the diagnosis relies on culturing the organism from blood, sputum, pus, or other body fluids indicated by the clinical presentation. Recently, ELISA employing better characterized antigens such as purified O-polysaccharide (OPS) and hemolysin co-regulated protein

(Hcp-1) has been shown to have better performance than the IHA for serodiagnosis of melioidosis in endemic areas. A rapid immunochromatographic test (ICT) using Hcp-1 was evaluated in cohorts of melioidosis patients, with an overall sensitivity of 88.3%.

Culture is considered the current gold standard for diagnosis, and culture confirmation should always be sought in patients with suspected disease. Any Gram-negative bacilli that are oxidase-positive, typically resistant to aminoglycosides (e.g., gentamicin), colistin, and polymyxin but sensitive to amoxicillin/clavulanic acid should be considered as *B. pseudomallei*. This bacterium is inherently resistant to penicillin, ampicillin, first and second-generation cephalosporins, gentamicin, tobramycin, streptomycin, and polymyxin. For the intensive phase (10-14 days), ceftazidime or carbapenem is the drug of choice.³

B. pseudomallei possesses a diverse set of genes that encode a vast array of biological functions reflecting its clinical, ecological, and phenotypic diversity. This soil-dwelling Gram-negative pathogen causes melioidosis, a tropical disease endemic in northern Australia and Southeast Asian regions, including Bangladesh. Phylogeographic analyses of *B. pseudomallei* isolates using molecular typing techniques could be used to examine the diversity of this organism and track melioidosis epidemics. *B. pseudomallei* was isolated for the first time in the Indian subcontinent from soil samples in the Gazipur district of Bangladesh. Since then, Bangladesh has been considered a definitive country for melioidosis due to the presence of *B. pseudomallei* in both clinical and soil samples.⁶ This study used 20 *B. pseudomallei* isolates from clinical samples and two from soil samples. Multilocus sequence typing (MLST) was conducted with all 22 isolates, and four novel sequence types (STs), of the 12 different STs determined, STs 1005, 1007, and 56 were the most common sequence types identified in Bangladesh. ST 1005 was found in both clinical and soil samples. ST 56 was isolated from five septicemic patients.

Sero-prevalence of melioidosis

Specific seroprevalence data for melioidosis in animals, especially horses, are limited. However, an overall 3.2-71.4% seroprevalence for human melioidosis, as determined by ELISA, IHA, and IFA, has been reported in Southeast Asia.¹⁸ This study investigated melioidosis seroprevalence and risk factors of rural horses in Bangladesh, a region where the pathogen's persistence is favored.¹⁹ Of 118 horses, seropositivity was 21.18% (n=25) by Rapid kit and 15.25% (n=18) by ELISA, while PCR confirmed 1.69% (n=2). Such discrepancies between serological and molecular results may be due to sample size, preservation, or antigenic variance,²⁰ a diagnostic complexity noted in other animal studies.²¹ The 1.69% active infection rate is a significant finding, as equine melioidosis has not been previously documented in Bangladesh, where prior detection was limited to soil at 1.11% prevalence.⁶ Regional prevalence was higher in Mymensingh (6.67%) and Kurigram (5.0%) than in Jamalpur (0.00%), likely reflecting agro-ecological differences that influence transmission from environmental sources.^{16,22}

Sero-prevalence for melioidosis in animals and humans is highly variable and depends on geographical location, with endemic areas having higher rates. Studies in specific regions have shown varying results; for example, a study in India (Odisha) found a human seroprevalence of 20.9%,²³ 6.2% in animals, including livestock in Peninsular Malaysia²⁴ while a survey in Laos found cattle had the highest seroprevalence (22.8%), followed by buffalo (19.7%) and swine (4.0%).²⁵ Sero-prevalence data can be influenced by diagnostic capabilities and reporting in the different areas.

Epidemiological risk factors

Epidemiological risk factors for melioidosis in animals include environmental factors, such as living in an endemic area and contact with contaminated soil and water, especially after heavy rainfall or flooding. Other factors include animal-specific risks, such as age, breed, and immune status, as well as potential transmission via importation of animals from endemic regions.

a. Environmental and geographical risk factors

Animals in regions where melioidosis is endemic, such as Southeast Asia and Northern Australia, are at higher risk. The bacteria are often found in soil and water, and cases often increase after heavy rainfall or floods, which bring the bacteria to the surface. Typhoons and other severe weather events are strongly linked to increased transmission. Animals can be exposed through direct contact with contaminated soil, water, or aerosols created during severe weather. Specific activities on farms (farm practices), such as soil excavation, bush cleaning, and land development, can increase exposure risk.

b. Animal-specific risk factors

Some species and breeds may be more susceptible than others. For example, seroprevalence has been shown to vary among deer and rabbit breeds. Young animals (age) and those with compromised immune systems may be more vulnerable. A compromised immune system or co-existing chronic conditions in animals can increase their risk of developing melioidosis if exposed.

c. Management and other risk factors

Animal management practices on the farm, such as the type of feed, water source, and general herd health management, can play a role. Animal movement, especially the importation of animals from endemic areas, can introduce the disease to new regions.

This study has investigated minimal epidemiological risk factors for melioidosis in horses, including only age, gender, and a history of clinical findings. Adults had a higher seroprevalence (2.04%) than younger horses (1.45%) in this study, consistent with older animals' greater susceptibility.¹⁰ Respiratory signs were a strong risk factor (30% ELISA, 40% Rapid kit, 10% PCR positivity), aligning with the known respiratory pathogenesis of *B. pseudomallei*.²⁶ The high serological prevalence suggests widespread exposure, highlighting risks to animal health and potential zoonotic transmission to humans.²⁷ These findings underscore the need for enhanced surveillance and preventive measures through integrated 'One Health' approaches.^{28,29}

CONCLUSION

This study provides the first evidence of *B. pseudomallei* exposure in rural horses of Bangladesh, with serological tests (15.25% by ELISA, 21.18% by Antibody Rapid Detection Kit) indicating higher prevalence than PCR confirmation (1.69%). The identification of risk factors associated with melioidosis could be strengthened by increasing sample size and ensuring more representative sampling. The discrepancy between serological and molecular results necessitates cautious interpretation and highlights the importance of multiple diagnostic approaches. Further studies with highly sensitive tests are needed to determine accurate seroprevalence throughout Bangladesh. Future research should emphasize expanded surveillance, investigation of additional epidemiological risk factors, and development of control strategies. Countermeasures should include early detection, appropriate management practices, proper disposal of infected materials, environmental disinfection, and raising awareness about melioidosis and its zoonotic implications through veterinary extension services.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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