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Comparison of immunogenicity of 17 *Burkholderia mallei* antigens and whole cell lysate using indirect ELISA

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ABSTRACT. Glanders is a World Organization for Animal Health (WOAH)-notifiable equine disease caused by the infection of *Burkholderia mallei*, and is endemic in Mongolia, South Asia, Africa, and South America. While the complement fixation test (CFT) has been widely used for serodiagnosis of glanders and is considered a standard serological test, it has several limitations. These limitations include poor specificity, labor intensive techniques, variability in antigen and protocol. Consequently, indirect enzyme-linked immunosorbent assays (iELISAs) based on recombinant proteins have been developed as alternative serodiagnostic assays to address some of the challenges associated with the CFT. The accuracy of iELISA relies on the *B. mallei* proteins used as an antigen. Hence, to determine the best diagnostic candidate in iELISA, in terms of sensitivity and specificity, a comparison of 17 immunogenic *B. mallei* proteins and detergent-based whole cell lysate (WCL) was performed. According to the sensitivity and specificity on the sera from glanderous and non-glanderous Mongolian native horses, iELISA using Hcp1, GroEL, and detergent-based WCL represented the highest diagnostic accuracy. These three candidates did not have cross-reactivity to horse sera with several other equine diseases. WCL, Hcp1, and GroEL showed considerable potential as antigens for iELISA in the serodiagnosis of glanders in Mongolia. Detergent-based WCL extraction offers a consistent approach for the preparation of reliable *B. mallei* antigen. WCL-iELISA should be further validated in a large-scale study to meet WOAH demands.

KEYWORDS: *Burkholderia mallei*, glanders, indirect ELISA, whole cell lysate

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INTRODUCTION

Glanders is a highly contagious and often fatal disease that mainly affects members of the *Equidae* family, such as horses, donkeys, and mules. Horses, in which the course of glanders is often chronic or even non-apparent, serve as reservoirs of the infection. It has zoonotic potential and has been reported to infect humans [31] and dromedary camels [34] after close contact with infected horses. Additionally, carnivores can be infected by the ingestion of contaminated meats [36]. The causative agent, *Burkholderia mallei* is a Gram-negative, non-motile, non-spore-forming bacterium phylogenetically close to *B. pseudomallei* and *B. thailandensis*. *B. mallei* and *B. pseudomallei* are both classified as Tier 1 select agents because they have zoonotic potential and have previously been used as bioterrorism agents [2, 35]. Glanders is endemic to Mongolia, South Asia, Africa, and South America. [8, 20–23].

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The complement fixation test (CFT) for glanders is widely used worldwide for surveillance, and is recommended for international horse transportation by the World Organization for Animal Health (WOAH) because of its high sensitivity and more than 100 years of history. However, a considerable number of false-positive results have been reported, likely due to the use of crude antigens, mainly consisting of lipopolysaccharides (LPS) and the anticomplementary activity of equine sera [36], which is a problem in horse trading and causes unnecessary financial losses for owners. In addition, owners do not want to euthanize glanders positive equines, which can lead to inadequate disease control and further propagation of the infection [1]. The CFT results are influenced by several reaction reagent components. For example, complement and sensitized sheep erythrocytes affect hemolysis efficiency. The strains used as antigens and testing protocols differ among laboratories, resulting in poor standardizability [1, 5, 15]. Thus, efforts have been made to develop alternative assays, including western blotting (WB) [7, 38], enzyme-linked immunosorbent assays (ELISAs) [4–6, 14, 17, 25, 28], microsphere-based immunoassays [18], protein microarrays, and dipstick assays [33]. Indirect ELISAs have emerged as cost-effective methods that allow for processing multiple samples simultaneously and provide results in a shorter timeframe (approximately 2–3 hr) than CFT (approximately 24 hr). Recently, several iELISAs using recombinant proteins have been developed to address the limitations of CFT, highlighting their potential for glanders diagnosis [4, 5, 15, 17, 25, 28]. Additionally, commercial iELISA using undisclosed double antigens have been reported to show high diagnostic accuracy [5, 6]. Despite their potential in the diagnosis of glanders, the diagnostic sensitivity of these iELISA is insufficient to meet WOAH demands compared to CFT, and limited to confirmatory testing of CFT-positive sera [5, 13]. Thus, there is an urgent need to develop improved iELISAs that incorporate single or multiple antigens with high serodiagnostic potential. Several studies have identified immunogenic *B. mallei* proteins using various experimental approaches, such as protein microarray [33] and mass spectrometry based on matrix-assisted laser desorption ionization (MALDI) imaging [10].

In this study, a comparative assessment of various immunogenic *B. mallei* proteins and detergent-based extracted whole-cell lysates (WCL) was conducted to identify the optimal candidate in an iELISA for equine glanders with respect to diagnostic sensitivity and specificity.

MATERIALS AND METHODS

Ethics approval and consent to participate

This study was approved by the animal ethics committee of the Veterinary Science and Bio-medical Research at the Mongolian University of Life Science (VSBMR23/01/21) and carried out in accordance with the Standard Diagnosis Guide for Animal Diseases 2015 of the State Central Veterinary Laboratory, Mongolia. In accordance with the General Authority for Veterinary Services of Mongolia, no approval was needed for antemortem sampling, as it was conducted as part of standard veterinary care. Informed consent was obtained from the owners prior to the collection of the blood samples for the purposes of this study.

Protein expression and purification for recombinant B. mallei proteins

All 17 *B. mallei* proteins were expressed and purified as follows (Table 1). Genes encoding the selected proteins excluding BMA0436, BMAA0351, BMAA0749_1, and BMAA0749_2 were amplified by polymerase chain reaction (PCR) using primers with restriction site at the 5' end (Supplementary Table 1). The purified PCR products were digested with the corresponding restriction enzymes and ligated to the multiple cloning site of the linearized pET28a+ expression vector (Novagen, Madison, WI, USA), which fused a polyhistidine tag at the C-terminus of the expressed proteins. For BMA0436, BMAA0351, BMAA0749_1, and BMAA0749_2,

Table 1. Recombinant *Burkholderia mallei* proteins used in this study

Locus ID	Protein	Protein size (aa)	Signal sequence and/or transmembranal domain (aa)	rProtein coverage (aa)	Expression form	Reference
BMA0434	Putative exported protein	198	1–21	22–198	Soluble protein	[6]
BMA0436	OmpA precursor	224	1–22	23–224	Soluble protein	[13, 18, 33]
BMA1487	Antioxidant, AhpC/Tsa family	182	-	1–182	Soluble protein	[13, 18, 33]
BMA2001	GroEL	550	-	1–550	Soluble protein	[38]
BMA2002	Chaperonin, 10 kDa	97	-	1–97	Soluble protein	[13, 18, 33]
BMA2431	Chaperonin, 10 kDa	105	-	1–105	Soluble protein	[13, 18, 33]
BMA2642	50S ribosomal protein L7/L12	124	-	1–124	Soluble protein	[13, 18, 33]
BMAA0351	Oligopeptide ABC transporter	554	1–43	44–554	Soluble protein	[13, 18, 33]
BMAA0742	Type VI secretion system protein, Hcp1	169	-	1–169	Soluble protein	[5]
BMAA0743	Type VI secretion system protein, TssB	499	-	1–499	Denatured protein	[7]
BMAA0744	Type VI secretion system protein, TssA	164	-	1–164	Soluble protein	[5]
BMAA0749_1	Autotransporter, BimA, truncated	373	-	1–89	Denatured protein	[4]
BMAA0749_2	Autotransporter, BimA, truncated	373	-	146–373	Denatured protein	[4]
BMAA1523	Type III, effector BopE	261	-	1–261	Denatured protein	[13, 18, 33]
BMAA1530	Type III, target BipC	419	-	1–419	Soluble protein	[13, 18, 33]
BMAA1609	Type 4 pilus biosynthesis protein	432	-	1–432	Soluble protein	[13, 18, 33]
BMAA1751	Malate dehydrogenase	327	-	1–327	Soluble protein	[13, 18, 33]

the gene sequences were optimized for codon usage in *Escherichia coli*. Optimized sequences with restriction sites for NcoI and XhoI were synthesized and cloned into the pET28a+ expression vector. The expression vectors were transformed into chemically competent *E. coli* BL21 (DE3) cells (Novagen). Subsequently, sequencing was performed to confirm that the correct sequences were inserted into the expression vectors.

In the culture of the transformed bacteria for recombinant protein expression, when the optical density (OD) at 600 nm of exponentially growing bacterial culture reached 0.4, the expression of recombinant proteins was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 4 hr or with 0.2 mM IPTG at 18°C for 20 hr. The cells were collected by centrifugation at 9,400 × *g* for 10 min and the supernatant was removed. Cell pellets were lysed using the BugBuster Master Mix (Novagen) according to the manufacturer's protocol. The lysate was centrifuged at 13,000 × *g* for 5 min and the supernatants containing soluble recombinant proteins were collected. The pellet, containing the inclusion form of the recombinant protein, was solubilized in 8 M urea.

The recombinant proteins were purified using Ni-NTA agarose (Wako Pure Chemicals, Osaka, Japan) on a PD-10 column. Purified proteins were evaluated for size and purity using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein concentration was measured using the BCA Protein Assay Kit (Takara Bio, Inc., Kusatsu, Japan), with reference to a standard BSA solution.

Preparation of WCL using detergents

The BSL3-compliant laboratories of the Hokkaido University International Institute for Zoonosis Control (Japan) and the Institute of Veterinary Medicine (Mongolia) were used for bacterial propagation and lysis. The lysate was removed from the BSL3 laboratory after verifying the absence of viable bacteria. *B. mallei* strain ATCC 23344 was grown on an agar with Brain Heart Infusion (BHI) broth (Shimadzu Diagnostics Corp., Tokyo, Japan) and 4% glycerol at 37°C for 48 hr under aerobic condition. All bacterial colonies were resuspended in PBS. The suspension was centrifuged in 500 µL at 13,000 × *g* for 5 min and the supernatant was discarded. The harvested cells were stored at −80°C. For WCL preparation, cell pellet (wet weight of 0.1 g) was resuspended in 4 mL lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 800 µg lysozyme (Wako Pure Chemicals) and then swayed on ice for 30 min using a shaker. This lysis buffer was a modification of the conventional Radio-Immunoprecipitation Assay (RIPA) buffer, containing lysozyme and replacing SDS with CHAPS to minimize protein denaturation while maintaining efficient extraction. Aliquots of the bacterial lysates were plated onto BHI agar and incubated at 37°C for 48 hr. The absence of colony formation on the agar confirmed that the bacteria in the lysates were not viable. The lysate was added with a final concentration of 50 mM magnesium sulfate and 8 µL of DNase I (Nippon Gene, Tokyo, Japan). The suspension was again shaken on ice for 30 min using a shaker and subsequently centrifuged at 9,400 × *g* for 10 min. The supernatants were used as antigens for iELISAs.

Complement fixation test (CFT)

The CFT was performed as previously described [8]. Briefly, the serum of each horse was diluted 1:5 in 0.9% NaCl and inactivated for 30 min at 56°C. Diluted sera, 1:40 diluted guinea pig complement, and CFT Antigen (Biocombinat, Ulaanbaatar, Mongolia) were mixed in quadruplicates in the wells of 96-well-round-bottom microwell plates and incubated at 4°C for 16–18 hr. A 2% suspension of sensitized sheep red blood cells was added (100 µL/well), and the plates were incubated for 45 min at 37°C and centrifuged at 600 ×*g* for 5 min. Samples with 100% (4 wells) hemolysis were "negative", and samples showing no (0 well) hemolysis were classified as "positive".

Mallein test

The Mallein test was performed as described previously [8]. Briefly, tested horses were selected by the presence of typical clinical signs and the positivity of the CFT and injected intradermally with 0.2 mL (1.0 mg/mL) of concentrated mallein purified protein derivative (PPD) (Biocombinat, Ulaanbaatar, Mongolia) on the middle of the vertical side of the horse's neck. The reaction to PPD injection was examined in the necks of the horses at 24, 48, and 72 hr. The test was considered positive based on the presence of a marked firm painful swelling of the skin, measuring at least 6 mm in diameter, at the injection site after 24 and 48 hr.

Serum samples

In this study, horses privately owned by Mongolian nomadic owners were used. Following the collection of blood samples and the implementation of the Mallein test, all horses were safely released. In total, 147 equine serum samples were used in the present study and divided into two groups: Group A for the identification of the best candidate antigen for iELISA, and Group B to further evaluate the specificity of the selected antigens. For Group A, 87 equine serum samples were collected from the Tuv, Ulaanbaatar, Khentii, Sukhbaatar, Selenge, Dornod, and Dundgovi provinces in Mongolia between 2018 and 2020. This Group consisted of patients with positive serum (n=37) and negative serum (n=50). The positive or negative status of the samples was determined by a combination of clinical signs, the CFT, and Mallein test; Positive indicates CFT-positive, Mallein-positive, and showing typical clinical signs of glanders such as nasal discharge and ulcerating skin nodules; Negative indicates CFT-negative and no clinical signs. Group B comprising 60 serum samples collected in Japan that were officially free from glanders. This group included sera from Thoroughbred racehorses with fever (n=15), phlegmon (n=15), shipping fever (n=15), or experimental infection with *Streptococcus equi* subsp. *zooepidemicus* (n=3). Sera from horses infected with *S. equi* subsp. *zooepidemicus* were collected at five different time points (2, 4, 6, 14, and 28 days post-infection).

Indirect ELISA (iELISA)

Individual proteins were immobilized in 96 well plates using carbonate buffer (pH 9.6) and incubated at 4°C for 16–18 hr. In pre-experiments, 17 recombinant proteins and WCL were screened for reactivity to a small panel of sera from 2 glanderous horses by indirect ELISA at 3 different antigenic concentrations (1, 5, and 10 µg/mL). The final antigen concentration in this study was set at the saturation of the optical density or the highest optical density obtained with positive sera:1 µg/mL, BMA2431 (Chaperonin, 10 kDa), BMA0436 (OmpA precursor), BMA1487 (AhpC), BMA2002 (chaperonin, 10 kDa), BMAA0749_1 (truncated BimA), BMA2001 (GroEL), BMAA0742 (Hcp1), BMAA0744 (TssA), BMAA0743 (TssB), WCL; 5 µg/mL, BMA0434 (Putative exported protein), BMAA1751 (Malate dehydrogenase), BMA2642 (50S ribosomal protein L7/L12); 10 µg/mL, BMAA0351 (Oligopeptide ABC transporter), BMAA1523 (BopE), BMAA1530 (BipC), BMAA0749_2 (truncated BimA), BMAA1609 (Type 4 pilus biosynthesis protein). After immobilization, the supernatant was discarded and the wells were washed three times (300 µL/well) with washing buffer (phosphate-buffered saline pH 7.4 (PBS) with 0.05% Tween-20). The wells were then blocked for 1 hr at 20–25°C with blocking buffer (washing buffer with 5% skim milk powder; Yukijirushi, Sapporo, Japan) and washed three times. In order to consistently compare the immune reactivity of sera towards each antigen, serum dilution was fixed at 1:1,000 in blocking buffer. Diluted sera were added to duplicate wells (100 µL/well) and incubated at 20–25°C for 1 hr. Duplicate wells were arranged symmetrically with respect to the plate center. The wells were then washed three times. Horseradish peroxidase-conjugated rabbit anti-horse-IgG (Abcam, Cambridge, UK) diluted 1:50,000 in blocking buffer was added and the plates were incubated at 20–25°C for 1 hr. After washing three times, the wells were filled with 50 µL of TMB One substrate (Bethyl Laboratories, Montgomery, TX, USA). The reaction was terminated by the addition of 0.18 M H_2SO_4 (50 µL/well) and the absorbance was measured at 450 nm using an ELISA plate reader (Corona Electric Co., Ltd, Hitachinaka, Japan).

ID Screen® Glanders Double Antigen Multi-species

Horse serum samples were analyzed using the ID Screen® Glanders Double Antigen Multi-species (Innovative Diagnostics, Grabels, France), a commercially available serodiagnostic ELISA kit for glanders. Equine serum samples diluted 1:10 were added to the plate and incubated for 1 hr at 25°C. All samples were tested in duplicate, and the kit control samples were included in each run. Validation and interpretation of the results were performed according to the kit protocol. The cut-off value for the assay was set at S/P% of 70%.

Statistical analysis

Receiver operating characteristic (ROC) curve analysis was performed using the pROC package [27]. The cut-off values of the optical densities were determined by analyzing the ROC curve and calculating the mean plus 4 standard deviations in negative serum samples.

RESULTS

Selection and expression of B. mallei proteins for serodiagnosis

In this study, 17 proteins were selected as potential antigens for ELISA (Table 1). BMAA0743 (TssB), BMA2001 (GroEL), BMAA0749 (BimA), BMAA0744 (TssA), and BMAA0742 (Hcp1) were selected based on their high immunogenicity, as reported in previous iELISA studies on glanders [4, 5, 15, 17, 25, 28]. Because the full-length BimA protein could not be expressed using the *E. coli* expression system, two truncated forms of BimA (BMAA0749_1 and BMAA0749_2) were generated. The remaining proteins were selected based on previous investigations of *B. pseudomallei* protein microarray, which reported that their orthologs elicited robust IgG responses in sera from humans or horses infected with *B. mallei* (glanders) [32, 33], or in sera from humans infected with *B. pseudomallei* (melioidosis) [9, 16, 29]. To assess the diagnostic potential of the selected 17 *B. mallei* proteins, recombinant polyhistidine-tagged proteins were expressed in *E. coli* expression system. Signal sequences and transmembrane domains of secretory and/or membrane proteins were excluded from the expression constructs (Table 1), and recombinant soluble proteins were harvested and purified from the 13 *B. mallei* proteins. However, for BMAA0743 (TssB), BMAA0749_1 (BimA), BMAA0749_2 (BimA), and BMAA1523 (BopE), recombinant proteins were expressed in the inclusion bodies as insoluble forms even at low temperature (18°C) during the induction time. These proteins were purified in denatured form from the inclusion bodies. The SDS-PAGE images of the purified recombinant proteins are shown in Supplementary Fig. 1.

Diagnostic sensitivity and specificity of B. mallei individual proteins and WCL

iELISAs using the 17 recombinant *B. mallei* proteins as antigens were performed on serum samples from Group A (Supplementary Table 2). The dot plot distribution of each serum sample at an optical density at 450 nm for each antigen is shown in Fig. 1. Based on ROC curve analysis, cut-off values were calculated using the mean plus 4 standard deviations of optical densities in negative serum samples. The diagnostic sensitivities and specificities are shown in Table 2. Among the tested antigens, WCL exhibited the highest diagnostic accuracy (sensitivity, 100%; specificity, 100%; cut-off value, 0.243) (Table 2). Followed by WCL, BMAA0742 (Hcp1) demonstrated a high sensitivity (97.3%), and specificity (100%) with a cut-off value of 0.096, while BMA2001 (GroEL) displayed a sensitivity of 91.9% and specificity of 98.0% with a cut-off value of 0.264 (Table 2). These three antigens were selected for further evaluation of their cross-reactivity with serum samples from horses with other infectious diseases. The remaining 12 proteins showed insufficient diagnostic sensitivity, although high optical densities were obtained from several positive serum samples (Fig. 1 and Table 2). Three proteins, BMAA1523, BMA2642, and BMAA0351, showed positive reactions in some negative sera (Fig. 1).

To compare the results of the iELISA with those of a commercially available kit, serum samples from Group A were tested using ID Screen® Glanders Double Antigen Multi-species, resulting in a sensitivity and a specificity of 100% (Supplementary Fig. 2).

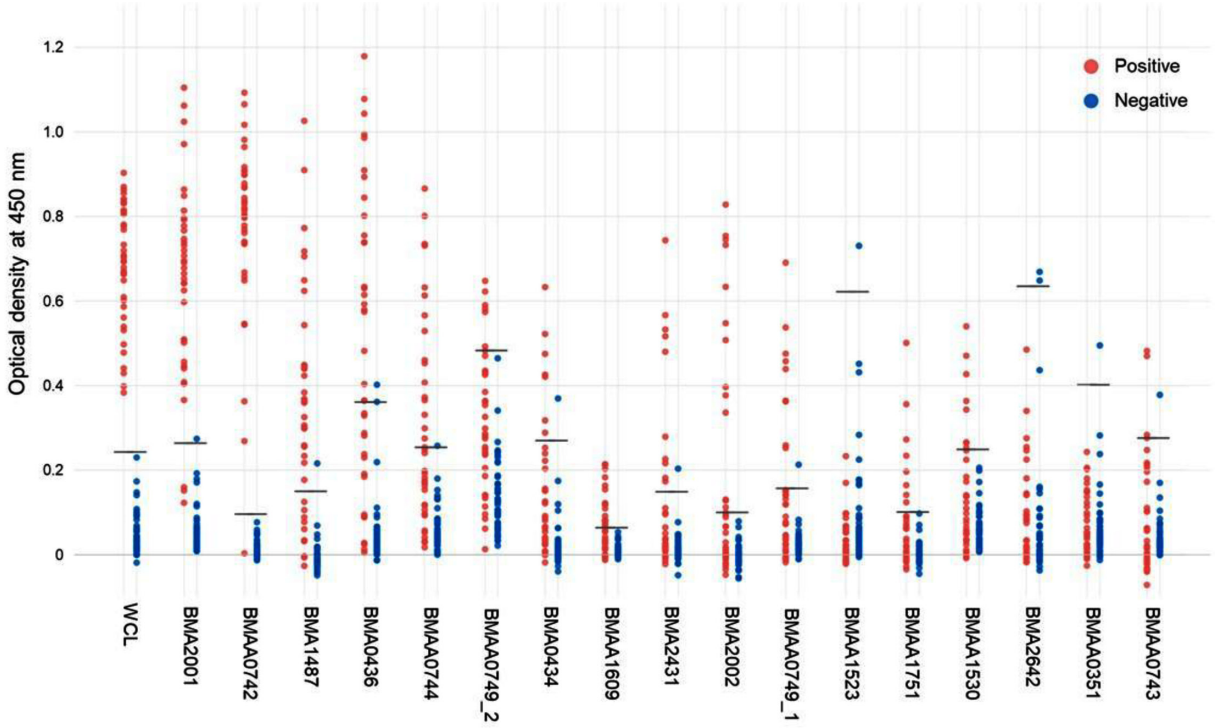

Fig. 1. Identification of the best candidate antigen for indirect enzyme-linked immunosorbent assay using serum samples of Group A horses. Positive serum (Red) and Negative serum (Blue) of Group A were used. Each gray bar indicates cut-off value of each antigen. The actual optical density at 450 nm for each sample is shown.

Table 2. Cut-off values, area under the curve (AUC), sensitivity, and specificity of indirect enzyme-linked immunosorbent assay using individual recombinant *Burkholderia mallei* proteins and whole cell lysate (WCL)

Antigen	Cut-off value	AUC (95% CI)	% Sensitivity (No. of positive)	% Specificity (No. of negative)
WCL	0.243	1.00 (1.00–1.00)	100 (37)	100 (50)
BMA2001	0.264	0.994 (0.984–1.00)	91.9 (34)	98.0 (49)
BMAA0742	0.096	0.984 (0.951–1.00)	97.3 (36)	100 (50)
BMA1487	0.150	0.951 (0.900–1.00)	73.0 (27)	98.0 (49)
BMA0436	0.361	0.903 (0.827–0.980)	59.5 (22)	96.0 (48)
BMAA0744	0.254	0.872 (0.787–0.958)	46.0 (17)	98.0 (49)
BMAA0749_2	0.483	0.838 (0.746–0.930)	16.2 (6)	100 (50)
BMA0434	0.270	0.827 (0.733–0.920)	18.9 (7)	98.0 (49)
BMAA1609	0.064	0.807 (0.694–0.919)	48.7 (18)	100 (50)
BMA2431	0.149	0.763 (0.657–0.870)	27.0 (10)	98.0 (49)
BMA2002	0.100	0.728 (0.611–0.845)	37.8 (14)	100 (50)
BMAA0749_1	0.157	0.724 (0.596–0.851)	24.3 (9)	98.0 (49)
BMAA1523	0.622	0.709 (0.595–0.823)	0 (0)	98.0 (49)
BMAA1751	0.101	0.678 (0.555–0.802)	21.6 (8)	100 (50)
BMAA1530	0.249	0.657 (0.531–0.784)	24.3 (9)	100 (50)
BMA2642	0.635	0.601 (0.477–0.724)	0 (0)	96.0 (48)
BMAA0351	0.402	0.534 (0.403–0.664)	0 (0)	98.0 (49)
BMAA0743	0.276	0.530 (0.381–0.679)	8.1 (3)	98.0 (49)

Cross-reactivity of WCL, BMAA0742, and BMA2001 in serum samples of horses infected with other pathogens

The potential cross-reactivity of WCL, BMAA0742 (Hcp1), and BMA2001 (GroEL) with serum samples obtained from horses infected with other pathogens was tested using the sera from Group B (Supplementary Table 3). All three antigens showed low optical density values when tested against all serum samples in Group B (Fig. 2 and Supplementary Table 3).

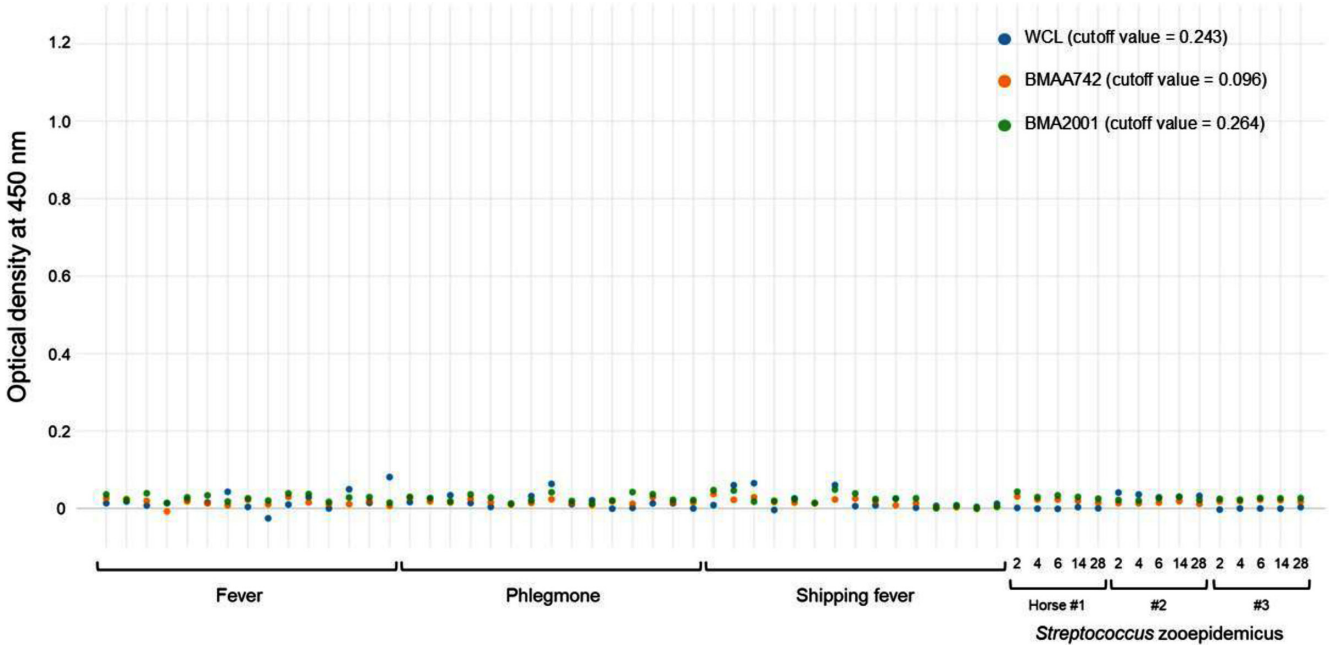

Fig. 2. Further evaluation of specificity of BMAA0742 (Hcp1), BMA2001 (GroEL), and whole cell lysate (WCL) using serum samples of Group B horses. WCL (Blue), BMAA0741 (Hcp1, Orange), and BMA2001 (GroEL, Green) were used as an antigen of indirect enzyme-linked immunosorbent assay. The actual optical density at 450 nm for each sample is shown.

DISCUSSION

This is the first report on the comparison of serodiagnostic performance of *B. mallei* antigens in iELISA using sera from Mongolian native horses. Based on the analysis of these sera, Hcp1 and GroEL were identified as recombinant proteins with the highest potential for glanders diagnosis in Mongolia. Recent studies have demonstrated the reliability of Hcp1 as an antigen for ELISA (sensitivity=95.3%, specificity=99.6%) [5] and microsphere-based immunoassays (sensitivity=100%, specificity=99.5%) [18] using equine sera collected in Pakistan and India, respectively. The present results suggest the diagnostic potential of Hcp1 and GroEL is not influenced by strain variation between countries and horse breeds. Furthermore, the present study provided additional data regarding the absence of cross-reactivity of Hcp1 and GroEL with sera from horses affected by other racehorse diseases (Fig. 2). This finding further supports the potential of Hcp1 and GroEL as specific diagnostic antigens for the serodiagnosis of equine glanders.

Comparison between Hcp1 and GroEL revealed that GroEL exhibited low serum specificity. Because GroEL is a heat shock protein that is highly conserved among different bacterial species, the results from GroEL-iELISA suggest possible antigenic cross-reactivity between *B. mallei* GroEL and other bacterial GroELs. A previous study on *B. pseudomallei* GroEL, which shares 96% similarity in amino acid sequence with *B. mallei* GroEL, showed cross-reactivity of *B. pseudomallei* GroEL with sera from human patients with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* bacteremia [37]. Therefore, further studies are required to confirm the specificity of *B. mallei* GroEL in Mongolia.

Taking advantage of the heterogeneous antibody response to different antigens, the utilization of multiple antigens in iELISAs has been employed to increase the sensitivity in the diagnosis of tuberculosis [39], melioidosis [11], african swine fever [19], and contagious bovine pleuropneumonia [12]. ID Screen® Glanders Double Antigen Multi-species using the unrevealed double antigens has been reported to exhibit sensitivity (98.1%) comparable to that of CFT for diagnosing glanders in India and Pakistan [25]. Among the 37 positive serum samples, one did not react with any of the recombinant proteins tested in this study (Supplementary Table 2). However, this serum displayed considerable reactivity (optical density=0.441) towards WCL, a complex mixture of various *B. mallei* proteins. This result suggests that, similar to the other infectious diseases mentioned above, the combination of *B. mallei* antigens has the potential to improve the sensitivity of iELISA, although a specific protein combination that would yield optimal results was not identified in the current study. Additionally, WCL does not contain Hcp1 [3, 30]. Thus, an increased sensitivity may be expected when Hcp1 and WCL are combined.

In a previous report, the use of crude whole-cell antigens for the serodiagnosis of glanders was associated with false-positive results in the CFT [24]. However, this study demonstrates that detergent-based WCL are the most sensitive and specific candidates for diagnostic antigens. Following the current WOAH standard, the crude antigen for CFT is generated by heat-sterilizing *B. mallei* liquid culture and subsequently precipitating proteins from the culture supernatant [36]. This process results in the fragmentation and aggregation of proteins in the liquid medium upon exposure to live steam, resulting in a crude antigen that consists of capsular polysaccharides (CPS), mainly LPS. LPS is a cross-reactive antigen with common structural epitopes in various Gram-negative bacteria [26]. In contrast, detergent-based preparation is a gentler extraction process that relatively leaves soluble proteins intact.

A recent report highlighted the reliability of WB using *B. mallei* whole-cell proteome extracted by sonication for the serodiagnosis of glanders in Iran [38]. However, ensuring operator safety is a crucial consideration when preparing WCL because of the zoonotic properties of *B. mallei*. Compared to sonication extraction, detergent-based extraction reduces the risk of infection to operators and enables individuals without specialized skills and equipment to consistently produce high-quality proteins. The strain used for WCL production is *B. mallei* ATCC 23344, a reference strain available worldwide. Further large-scale validation is required to confirm the diagnostic potential of WCL because this study was based on a small sample size.

Although a relatively small number of serum samples from a limited number of countries were analyzed in this study, the iELISA using WCL showed high sensitivity and specificity comparable to the commercially available ELISA kit ID Screen® Glanders Double Antigen Multi-species, and therefore may be a practical and effective alternative for serological testing of glanders in Mongolia.

On the other hand, it should be noted that preparation of WCL requires working with live bacteria and therefore must be performed in a BSL-3 facility. The use of recombinant *B. mallei* proteins (Hcp1 and GroEL) provides a significant advantage in terms of biosafety by eliminating the need to handle live bacteria under BSL-3 conditions.

In conclusion, this study highlights Hcp1 and GroEL as highly specific recombinant antigens for serological diagnosis of equine glanders in Mongolia native horse same as horses in other endemic countries. Furthermore, detergent-based WCL, which can be prepared with a consistent quality, is a promising candidate for iELISA of glanders.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that there is no conflict of interest.

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