Evaluation of Recombinant Proteins of *Burkholderia mallei* for Serodiagnosis of Glanders

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Glanders is a contagious disease caused by the Gram-negative bacillus *Burkholderia mallei*. The number of equine glanders outbreaks has increased steadily during the last decade. The disease must be reported to the Office International des Epizooties, Paris, France. Glanders serodiagnosis is hampered by the considerable number of false positives and negatives of the internationally prescribed tests. The major problem leading to the low sensitivity and specificity of the complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) has been linked to the test antigens currently used, i.e., crude preparations of whole cells. False-positive results obtained from other diagnostic tests utilizing crude antigens lead to financial losses to animal owners, and false-negative results can turn a risk into a possible threat. In this study, we report on the identification of diagnostic targets using bioinformatics tools for serodiagnosis of glanders. The identified gene sequences were cloned and expressed as recombinant proteins. The purified recombinant proteins of *B. mallei* were used in an indirect ELISA format for serodiagnosis of glanders. Two recombinant proteins, 0375H and 0375TH, exhibited 100% sensitivity and specificity for glanders diagnosis. The proteins also did not cross-react with sera from patients with the closely related disease melioidosis. The results of this investigation highlight the potential of recombinant 0375H and 0375TH proteins in specific and sensitive diagnosis of glanders.
The potential of purified recombinant proteins was investigated in an effort to develop diagnostic tools for glanders. Recombinant proteins were purified, and the serodiagnostic potential was assessed using BLASTn and BLASTp searches. The selected gene sequences were cloned, and 3,000 gene sequences of *B. mallei* were analyzed using online BLASTn and BLASTp searches offered by the National Center for Biotechnology Information (NCBI) and a BLAST search was performed using a database of *B. mallei* genes.

Recently, we described a recombinant protein of the *BimA* gene, which lost its pathogenicity in glanders specifically. Recombinant proteins have not been effectively tested (22). Other serological tests, e.g., agglutination, precipitation, indirect hemagglutination, immunodiffusion, counterimmunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA), have also been described (6, 13, 26, 33), but they have limitations. A Western blot technique using lipopolysaccharide antigen has been shown to have higher sensitivity and specificity than the currently used CFT (10).

Glanders is particularly difficult to differentiate from melioidosis, because *B. mallei* is closely related to *B. pseudomallei* and the two species cross-react in serological and hypersensitivity tests (3, 28). One consequence is that the two diseases might be confused during diagnosis; melioidosis in horses, which can resemble glanders, has occasionally been described (27, 28). Cross-reactivity can also cause complications in countries where *B. mallei* is not endemic. Horses that have been sensitized to *B. pseudomallei* (21) or related nonvirulent organisms such as *Burkholderia thailandensis* could have false-positive results in glanders screening tests (7). Diagnostic tests involving crude antigens are unable to diagnose glanders specifically. Recombinant proteins have not been exploited much in the serological diagnosis of glanders. Identification of *B. mallei*-specific diagnostic antigens still remains a challenging task, as *B. mallei* is a deletion clone of *B. pseudomallei* (11) which lost greater than 1,000 genes. Nearly all genes retained by *B. mallei* share approximately 99.5% DNA-DNA sequence identity with their *B. pseudomallei* orthologues. Recently, we described a recombinant BimA protein of *B. mallei* for serodiagnosis of glanders (15). In the present study, new diagnostic targets were identified by analyzing approximately 3,000 gene sequences of *B. mallei* NTCC 10229 using BLASTn and BLASTp searches. The selected gene sequences were cloned, recombinant proteins were purified, and the serodiagnostic potential of purified recombinant proteins was investigated in an indirect ELISA format.

### MATERIALS AND METHODS

**Bacterial strains.** *Burkholderia mallei* NTCC 10230 and *B. pseudomallei* NTCC 4845 were obtained from the Central Public Health Laboratory, Colindale, London, England. *B. mallei* and *B. pseudomallei* were grown in glucose dextrose broth and Ashdown medium, respectively, at 37°C for 48 h. The genomic DNA was prepared by using a QIAamp minikit (Qiagen). For the preparation of whole-cell sonicated (WCS) antigen, the bacteria were harvested and sonicated on ice, followed by centrifugation at 8,000 × g for 20 min. The supernatant was collected and used as a WCS antigen for ELISA. The live cultures were handled in a biosafety level 3 (BSL3) laboratory.

**Serum samples.** This study included a total of 123 equine serum samples collected at the National Research Centre on Equines, Hisar, India, between 2007 and 2010 for routine serological investigation of the disease. The negative or positive status of the samples was determined by use of a combination of clinical symptoms, culture, and the CFT. Group I consisted of 21 true-positive serum samples collected from outbreaks in four states, viz., Maharashtra, Andhra Pradesh, Chhattisgarh, and Himachal Pradesh, of India (Table 1). The CFT titers of positive samples ranged from 1:16 to 1:256. CFT reagent was purchased from Bioveta, S.C. in Ivanovice, Hane, Czech Republic, and testing was carried out according to the OIE-recommended procedure (22). Group II consisted of 102 serum samples, and these were determined to be negative by the CFT. These animals were healthy and did not present any clinical symptom of glanders. Sera from 10 culture-confirmed melioidosis patients (group III) were kindly provided by Chiranjay Mukhopadhyay of Kasturba Medical College, Manipal, India. Serum samples from apparently healthy humans (*n = 10; group IV*) with no history of fever in the past 3 months were also collected and used as negative controls.

**Identification of diagnostic targets.** About 3,000 gene sequences of *B. mallei* NTCC 10229 were retrieved from the Pathema-Burkholderia Bioinformatics Resource Center (http://pathema.jcvi.org/cgi-bin/Burkholderia/PathemaHomePage.cgi), and a BLAST search was performed using online BLASTn and BLASTp searches offered by the Na-

### TABLE 1 Characteristics of glanders-positive equine serum samples

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Animal</th>
<th>CFT titer</th>
<th>Clinical symptoms</th>
<th>Culture result</th>
<th>Sample origin</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>1:32</td>
<td>E</td>
<td>+</td>
<td>Panchgani (Maharashtra)</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>1:64</td>
<td>B</td>
<td></td>
<td>Panchgani (Maharashtra)</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>1:16</td>
<td>D</td>
<td></td>
<td>Panchgani (Maharashtra)</td>
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<tr>
<td>4</td>
<td>H</td>
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<td>A</td>
<td></td>
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<tr>
<td>5</td>
<td>H</td>
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<td>F</td>
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<td>Pune (Maharashtra)</td>
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<td>6</td>
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<td></td>
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</tr>
<tr>
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<td>C</td>
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<td>H</td>
<td>1:64</td>
<td>A</td>
<td></td>
<td>Raipur (Chhattisgarh)</td>
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</tbody>
</table>

**Note:**

- H, horse/pony; M, mule.
- A, mucopurulent nasal discharge; B, thick, mucopurulent nasal discharge with ulcers on nasal septum; C, seeping nodules (having thick yellowish brown exudate mainly on inside hind limb); D, apparently healthy, showing slightly nasal discharge; in direct contact with animals showing clinical symptoms of glanders; E, mucopurulent nasal discharge (seeping, having thick yellowish brown exudate mainly on inside hind limb), nodules (skin); F, thick, mucopurulent nasal discharge with ulcers on nasal septum, seeping nodules (having thick yellowish brown exudate mainly on different parts).
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Expression and purification of recombinant proteins. Transformants were screened for expression in a small culture (10 ml), and one clone for each gene exhibiting substantial expression after 4 h of induction by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was selected for further work. Ten milliliters of overnight-grown culture was inoculated into 200 ml of LB broth containing kanamycin (30 μg/ml) and grown at 37°C with shaking (200 rpm). Gene expression was induced by 1 mM IPTG after the absorbance at 600 nm (A600) reached 0.5. After 4 h of induction, the cells were harvested by centrifugation at 4,000 × g for 20 min. Expression of the recombinant proteins was checked by Coomassie blue staining following SDS-PAGE. The integrity of the cloned sequence was confirmed by restriction digestion and DNA sequencing. All the 4 expressed proteins (with a C-terminal His tag) were obtained in the insoluble fraction (inclusion bodies) and hence purified under denaturing conditions (8 M urea) by immobilized metal affinity chromatography (IMAC) using a Ni2+–nitrilotriacetic acid (NTA) fast-flow, chelating Sepharose column. The purity of the proteins was assessed by Coomassie blue staining following SDS-PAGE. The purified proteins were dialyzed against 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing decreasing concentrations of urea. The protein concentration of purified protein was determined with reference to standard bovine serum albumin (BSA) by using a bicinchoninic acid assay kit (Sigma).

Western blot analysis. The purified recombinant proteins were resolved on SDS-polyacrylamide gels and transferred electrophotoreically onto a nitrocellulose membrane (32). Blocking of the membrane was done with 5% defatted milk powder in 0.01 M PBS overnight at 4°C. After washing the membrane with 0.01 M PBS containing 0.05% Tween 20 (PBS-T), the blot was probed with a 1:2,000 dilution of anti-His antibody- horseshad peroxidase (HRP) conjugate for 1 h at 37°C. The membrane was washed with PBS-T, and the reaction was developed using 3,3’–diaminobenzidine tetrabydrochloride (DAB)–H2O2 as a substrate. Development was carried out for 2 to 3 min until bands of the desired intensity appeared, and thereafter, the membrane was washed under tap water and dried.

Indirect ELISA. Indirect ELISA was used to detect the antibodies against four recombinant proteins of B. mallei in equine sera. To optimize the concentration of recombinant proteins and serum dilution to be used in the indirect ELISA, checkerboard titration analysis was performed using known glanders-positive and -negative horse sera (pools of 10 serum samples each). After optimization, indirect ELISA was carried out by standard methods. Briefly, an ELISA plate (Nunc-Immuno Plate MaxiSorp surface; Nunc, Denmark) was coated with 100 μl of recombinant proteins (2 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Following overnight coating, plates were washed twice with 0.01 M PBS-T (pH 7.2) and blocked with 200 μl of 1% BSA in PBS for 2 h at 37°C. Test sera diluted 1:200 in PBS containing 0.1% BSA were appended in duplicate wells (100 μl/well) for 1 h at 37°C. The wells were washed five times with PBS-T. Rabbit anti-horse (IgG) antibodies conjugated to HRP (Sigma) diluted 1:5,000 were added to the wells, followed by incubation for 1 h at 37°C. After five washes, the plate was developed in the dark for 5 min with ortho-phenylenediamine (0.4 mg/ml) and H2O2 (6%, 0.4 μl/ml) as a substrate.
Determination of cutoff for indirect ELISA. For determination of the cutoff, 20 randomly selected glanders-negative serum samples (group II) were tested by indirect ELISA essentially by the above-described method. The cutoff optical density at 492 nm (OD492) was determined by the addition of 2 standard deviations (SDs) to the mean OD492 of these samples, and test samples with an OD492 greater than the cutoff were treated as positive.

Cross-reactivities of recombinant proteins. The cross-reactivities of the recombinant proteins with melioidosis-positive human serum samples (group III) were screened by indirect ELISA. Healthy control human sera (group IV) were tested as negative controls. The procedure for indirect ELISA was the same as that described above, except that recombinant proteins were coated in the ELISA wells at a higher concentration (10 μg/ml). The WCS antigen of B. mallei or B. pseudomallei (10 μg/ml) was also used for coating as a control. Anti-human IgG-HRP conjugate (Dako) was used at a 1:6,000 dilution.

RESULTS
Cloning and expression of recombinant proteins. Full/truncated gene sequences of 444 bp (0375H), 222 bp (0375TH), 423 bp (0376TH), and 564 bp (A3050H) were amplified by PCR using B. mallei NCTC 10230 genomic DNA as the template. The purified amplicons were cloned into the pET28a expression vector. E. coli host strain BL21 (DE3) was transformed with plasmids pET0375H, pET0375TH, pET0376TH, and pET03750H harboring the corresponding full/truncated gene sequences. Four clones, viz., B. mallei Bm0375H, Bm0375TH, Bm0376TH, and BmA3050H, expressed recombinant proteins of 18, 10, 17, and 22 kDa, respectively. Study of the time course of expression of the recombinant proteins revealed maximum expression after 4 h of induction with 1 mM IPTG. Plasmids (pET0375H, pET0375TH, pET0376TH, and pET03750H) were isolated for sequencing of cloned genes/gene fragments. The nucleotide sequences of all four cloned genes/gene fragments showed 100% identity with the sequences of their respective homologues of B. mallei NCTC 10229.

Purification and Western blotting of recombinant proteins. All the four recombinant proteins with a C-terminal His tag were localized in inclusion bodies, as revealed by their association with the pellet fraction on cell lysis under native conditions. These insoluble proteins were solubilized in buffer containing 8 M urea and purified by Ni-NTA matrix IMAC. The expressed proteins were eluted using a pH 4.0 elution buffer, and various fractions were analyzed by SDS-PAGE. All the recombinant proteins were purified to almost homogeneity, as revealed by the presence of a single band on SDS-PAGE analysis (Fig. 1). The sizes of the purified proteins on Coomassie-stained SDS-polyacrylamide gels were consistent with the predicted molecular masses of 18, 10, 17, and 22 kDa (Fig. 1). The yields of purified 0375H, 0375TH, 0376TH, and A3050H isolated from a 1-liter culture were 24, 25, 22, and 28 mg, respectively. The authenticity of the recombinant proteins was verified by Western blot analysis using monoclonal antipolyhistidine antibodies directed toward the polyhistidine tags in their proteins (data not shown).

Determination of cutoff for indirect ELISA. Cutoff values for the indirect ELISAs were determined separately for four recombinant proteins. Randomly picked glanders-negative serum samples (n = 20) were tested independently by indirect ELISAs at a 1:200 dilution. The OD of the samples was read at 492 nm, and the mean OD492 was determined. Standard deviations for indirect ELISAs using 0375H, 0375TH, 0376TH, and A3050H recombinant proteins were calculated to be 0.153, 0.106, 0.09, and 0.058, respectively. The cutoff OD492 (mean OD492 + 2 SDs) for indirect ELISAs using the 0375H, 0375TH, 0376TH, and A3050H recombinant proteins are described in Table 2.

Evaluation of diagnostic potential of four purified recombinant proteins by indirect ELISA. The four purified recombinant proteins, 0375H, 0375TH, 0376TH, and A3050H, were evaluated for their diagnostic potential. Indirect ELISAs for the detection of antibodies specific to these antigens in CFT-positive equine sera were performed. All the glanders–positive serum samples (group I, n = 21) tested positive with the 0375H and 0375TH recombinant proteins in the indirect ELISA format (Table 2). For the 0375H ELISA, the OD492s ranged from 0.869 to 1.384 and the mean OD492 was 1.02, and for the 0375TH ELISA, the OD492s ranged from 0.683 to 1.02 and the mean OD492 was 0.79. The 0375H and 0375TH-based ELISAs did not give any false-positive result with 102 equine serum samples (group II; Table 2), and their OD492s ranged from 0.236 to 0.772 (mean OD, 0.514) and from 0.162 to 0.533 (mean OD, 0.366), respectively. Six serum samples (OD492 range, 0.380 to 0.488) and four serum samples (OD492 range, 0.304 to 0.436) from group I tested false negative by the 0376TH- and A3050H-based ELISAs, respectively (Table 2). The 0376TH-based ELISA gave 4 (OD492 range, 0.5 to 0.586) and the A3050H-based ELISA gave 8 (OD492 range, 0.47 to 0.546) false positives from group II serum samples (Table 2). The 0375H- and 0375TH-based ELISAs were found to be highly specific and sensitive for serodiagnosis of glanders.

Cross-reactivity of four recombinant proteins with melioidosis patient sera. The reactivity of four recombinant proteins with melioidosis patient sera was determined by indirect ELISA. The OD492 of the melioidosis patient (group III) sera were found to be <0.2. Sera from healthy individuals (group IV) also had OD492 values of <0.2, suggesting that none of the group III or group IV sera reacted with the 0375TH and 0375H proteins (Fig. 2). Recombinant proteins A3050H and 0376TH also did not cross-react with melioidosis patient sera (data not shown). This shows the
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**TABLE 2 Analysis of equine serum samples by recombinant protein-based indirect ELISAs**

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>No. of serum samples (OD range) (n = 123)</th>
<th>Cutoff value for indirect ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 21)</td>
<td>Negative (n = 102)</td>
</tr>
<tr>
<td></td>
<td>Test positive</td>
<td>Test negative</td>
</tr>
<tr>
<td>0375H</td>
<td>21 (0.869–1.384)</td>
<td>0</td>
</tr>
<tr>
<td>0375TH</td>
<td>21 (0.683–1.02)</td>
<td>0</td>
</tr>
<tr>
<td>0376TH</td>
<td>15 (0.483–0.869)</td>
<td>6 (0.380–0.488)</td>
</tr>
<tr>
<td>A3050H</td>
<td>17 (0.526–1.08)</td>
<td>4 (0.304–0.436)</td>
</tr>
</tbody>
</table>

**FIG 2** Reactivity of 0375H and 0375TH proteins with melioidosis patient (pt.) sera or healthy human control sera. The reactivity of melioidosis patient sera with the WCS antigen of *B. pseudomallei* NCTC 4845 (Bpm) or *B. mallei* NCTC 10230 (Bm) is also shown as control.

**DISCUSSION**

In recent years, large numbers of equine serum samples for import/export testing were found to have CFT-discrepant glanders results. This is because the internationally mandated test makes use of crude whole-cell preparations and therefore is prone to false-positive reactions with cross-reacting antigens. The accuracy of the CFT has been reported to be 90 to 95%, with serum samples being positive within 1 week of infection and remaining positive in the case of exacerbation of the chronic process (31). Identification of new protein antigens can solve the problems still existing in the serological diagnosis of glanders (21). These highly specific and sensitive antigens can be applied in development of diagnostic assays for *B. mallei* infections in a time of ever increasing animal transport.

Here, we describe the identification and generation of four recombinant proteins of *B. mallei* for glanders diagnosis. Purified recombinant proteins were used in an indirect ELISA format to evaluate their serodiagnostic potential. Recombinant proteins 0375H and 0375TH showed 100% sensitivity and specificity. The Rose Bengal plate agglutination test and counterimmunoelectrophoresis technique reported earlier (13, 19) either have technical shortcomings or have low specificity and/or sensitivity. Two competitive ELISAs with comparable sensitivity (98.9 and 98.6%) and specificity (100%) were described for glanders diagnosis (14, 29). Due to the sophisticated techniques and high costs of mallein and monoclonal antibodies, these competitive ELISAs will not be available in routine laboratories. Approaches using polysaccharide-based microarray technology (23) are expensive and will not be suitable for routine mass testing of serum samples. Recently, an LPS-based Western blot technique with 100% sensitivity and specificity has been described for serodiagnosis of glanders (10). Preparation of LPS is sophisticated and each time of preparation requires culture handling in a BSL3 laboratory. Apart from BimA (15), purified recombinant proteins have not been reported for serodiagnosis of glanders.

One of the major drawbacks of serological tests for glanders has been that they are unable to differentiate between *B. mallei* and *B. pseudomallei* infections (22). *B. mallei* and *B. pseudomallei* are very close at the genome level; therefore, it has always been difficult to develop a specific immunodiagnostic test for glanders. An orthologue of recombinant protein 0375H is absent in all but two strains of *B. pseudomallei*, viz., 7894 and BCC215, exhibiting 80 and 61% sequence identities, respectively. *B. pseudomallei* strain 7894 is a human isolate obtained from Ecuador in 1962, while strain BCC215 was isolated from a fatal case of septicemic pseudomallei strains NCTC 10229 (unnamed protein product; GenBank accession no. YP_105718), SAVP1 (hypothetical protein BMASAVP1_0039; GenBank accession no. YP_989656), and NCTC 10247 (hypothetical protein BMA10247_A1209; GenBank accession no. YP_001024199), ATCC 23344 (unnamed protein product; GenBank accession no. YP_001078395).

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positive results in equines infected with these strains of \textit{B. pseudomallei}. However, these two strains do not phylogenetically belong to Asian or Australian populations of \textit{B. pseudomallei} (24); hence, 0375H- and 0375TH-based ELISAs can be used for specific detection and differentiation of glanders from melioidosis in Asia, Australia, and other regions where \textit{B. pseudomallei} strains 7894 and BCC215 are not endemic. Earlier, we described the use of recombinant BimA antigen in a plate ELISA format for immunodiagnosis of glanders (15). A BimA-like protein has also been reported from \textit{B. pseudomallei} strain 668 and may thus give false-positive results in animals infected with \textit{B. pseudomallei} strain 668 (15). 0375H- and 0375TH-based ELISAs may prove useful in distinguishing glanders from melioidosis in geographic areas where \textit{B. pseudomallei} strain 668 is endemic.

The cross-reactivity of 0375H- and 0375TH-based ELISAs was evaluated with sera from melioidosis patients. The ELISA ODs obtained with melioidosis patient and healthy human sera were similar and close to 0.2, indicating that no antibody against 0375H and 0375TH is present in these sera. This further demonstrates the high specificity of the 0375H and 0375TH proteins.

In conclusion, the present study involved the expression and purification of four recombinant proteins. The purified recombinant proteins were used as antigen to detect \textit{B. mallei}-specific antibodies in equine sera. Of the four recombinant proteins investigated, the indirect ELISAs using recombinant proteins 0375H and 0375TH were far superior to those using A3050H and 0376TH for glanders diagnosis. The results for the 0375TH- and 0375TH-based ELISAs are very encouraging, and further evaluation with a large sample number is warranted.

**ACKNOWLEDGMENTS**

We thank the director of the Defense Research and Development Establishment, Gwalior, India, for providing necessary facilities and encouragement, and we thank Chiranjay Mukhopadhyay of Kasturba Medical College, Manipal, India, for providing melioidosis serum samples.

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