

## Detection of antibodies to recombinant truncated flagellin and sonicated whole cell antigen of *Burkholderia pseudomallei* in acute melioidosis and in healthy Bangladeshi individuals

Md. Shariful Alam Jilani<sup>1</sup>, Tang Thean Hock<sup>2</sup>, Sraboni Mazumder<sup>1</sup>, Fahmida Rahman<sup>1</sup>, Md. Mohiuddin<sup>1</sup>, Chowdhury Rafiqul Ahsan<sup>3</sup>, Jalaluddin Ashraful Haq<sup>1\*</sup>

<sup>1</sup>Department of Microbiology, Ibrahim Medical College; <sup>2</sup>Advanced Medical & Dental Institute (AMDI), Universiti Sains Malaysia, 13200 Bertam, Kepala Batas, Pulau Pinang, Malaysia; <sup>3</sup>Department of Microbiology, University of Dhaka

### Abstract

**Background and objectives:** Several types of *Burkholderia pseudomallei* antigens have been used to determine the antibody response in acute and asymptomatic cases. In the present study, we have detected immunoglobulin G (IgG) antibody to recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei* in the sera of acute melioidosis cases and healthy individuals from melioidosis endemic areas of Bangladesh by indirect enzyme-linked immunosorbent assay (ELISA). In parallel, IgG antibody to sonicated whole cell antigen (SWCA) of *B. pseudomallei* was determined to compare with anti-RTFA antibody.

**Methodology:** Serum samples from culture confirmed melioidosis cases and from healthy individuals aged 21 years and above residing in melioidosis endemic rural areas were included in the study. Serum IgG antibody to RTFA and SWCA of *B. pseudomallei* was determined by indirect ELISA.

**Results:** Out of 8 culture confirmed acute melioidosis cases, 7 (87.5%) and 8 (100%) were positive for anti-*B. pseudomallei* IgG antibodies by RTFA and SWCA methods respectively. Among 361 healthy individuals, the rate of seropositivity by RTFA-ELISA was significantly less than that of SWCA-ELISA (16.1% versus 26.8%;  $p = 0.001$ ). The mean optical density (OD) of RTFA-ELISA of positive cases was significantly less than that of SWCA-ELISA in both melioidosis and healthy individuals ( $0.79 \pm 0.11$  versus  $2.4 \pm 0.08$ ,  $p = 0.0001$ ;  $0.67 \pm 0.01$  versus  $1.27 \pm 0.02$ ,  $p = 0.0001$ ). The sensitivity and specificity of RTFA-ELISA were 88.9% and 100% respectively.

**Conclusion:** Findings of the study suggest that multiple or combination of antigens should be used to study the seroprevalence of *B. pseudomallei* infection in a community. Also, prospective study is necessary to find out the duration of persistence of antibodies to different antigenic components of *B. pseudomallei* after exposure.

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### Introduction

*Burkholderia pseudomallei* is a gram negative bacillus and the causative agent of melioidosis. The organism is found in the soil and surface water of endemic areas and infects human by direct contact. Clinical disease includes localized or septicemia

infection. But asymptomatic infection is also common [1]. The bacterium is able to remain quiescent or latent in the host for decades following primary infection while maintaining the potential to relapse to cause acute and fulminating disease after many years [2,3]. So, once considered

\*Correspondence: J. Ashraful Haq, Department of Microbiology, Ibrahim Medical College, 1/A Ibrahim Sarani, Segunbagicha, Dhaka 1000, Bangladesh. Email: [jahaq54@yahoo.com](mailto:jahaq54@yahoo.com)

as obscurity, melioidosis is now recognized as an emerging disease of global importance. It is largely restricted to the Southeast Asia and Northern Australia, however, the disease has been increasingly reported in countries outside the Asia-Pacific region including Bangladesh [4,5]. It was first reported in Bangladesh in 1964 [6]. Subsequently, substantial increase in sporadic cases were reported after 1988 [4,7,8].

Several serological methods have been developed to diagnose the infection by *B. pseudomallei* in acute cases as well as to determine the status and magnitude of exposure to the organism in healthy individuals. The most commonly used serological method – the indirect hemagglutination test (IHA) has limited clinical value in regions of endemicity due to the high background antibody titers in healthy individuals, most likely the result of repeated environmental exposure to *B. pseudomallei* [9]. A critical limitation of this assay is the lack of standardization between laboratories with respect to the antigens used; the antigens remain poorly characterized and are likely to be variable between isolates [10]. The indirect immunofluorescence antibody test (IFAT) using whole *B. pseudomallei* cells as antigen was found to be sensitive and superior to IHA and requires only a day to obtain the results [11]. The only drawback is that IFAT requires a fluorescence microscope and skilled personnel which might not be readily available in rural endemic regions of South and Southeast Asia. ELISA is being considered more favorably as a rapid and reliable tool for detection of *B. pseudomallei* infection [12]. Various antigen preparations such as crude and purified exopolysaccharide (EPS) and lipopolysaccharide (LPS) outer membrane proteins (Omps) and Bip components of *B. pseudomallei* type III secretion system (TTSS-3) have been reported as potential candidate antigens to detect antibodies in infection by *B. pseudomallei* in an ELISA format [12,13]. However, crude or uncharacterized antigens of *B. pseudomallei* cross-react with antibodies induced by other bacterial infection, making the methods less specific. To avoid the cross reactivity, a recombinant truncated flagellin antigen was developed to identify *B. pseudomallei*-specific antibodies [14]. The truncated flagellin protein of *B. pseudomallei* was supposed to be devoid of cross

reactive epitopes and would elicit specific antibodies.

The present study was undertaken to detect IgG antibody to recombinant truncated flagellin antigen (RTFA) and sonicated whole cell antigen (SWCA) of *B. pseudomallei* in the sera of acute melioidosis cases and healthy individuals from melioidosis endemic areas of Bangladesh by ELISA. The presence of IgG antibody to RTFA and SWCA in healthy individuals would provide evidence for extent of exposure of individuals to *B. pseudomallei*.

### Materials and methods

The present study was carried out to determine the presence of anti-*B. pseudomallei* IgG antibody in acute melioidosis cases and in endemic healthy individuals. Serum IgG antibody to *B. pseudomallei* was determined by ELISA using RTFA and SWCA. Details of the methods are described below.

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) approved the study. Informed written consent was obtained from all participants prior to collection of blood samples.

**Serum samples:** Serum samples in this study were collected from 361 healthy individuals aged 21 years and above residing in rural areas of two melioidosis endemic districts of Bangladesh. Eight serum samples from culture confirmed acute melioidosis cases admitted at BIRDEM General Hospital were included. Sera from 35 healthy newborn babies of Dhaka city who were presumed not to be exposed to *B. pseudomallei* were enrolled as negative control. About 2 ml of venous blood was collected from each individual with proper aseptic technique.

**Expression and preparation of recombinant flagellin:** Plasmid pGEX4Y-2 containing recombinant flagellin protein of *B. pseudomallei* used for the study was kindly provided by Ya-Lei Chen, Department of Medical Technology, Fooyin University, Kaohsiung 83101, Taiwan, Republic of China [14].

Flagellin protein was over expressed from the plasmid pGEX4T-2 cloned with *flagellin* gene of *B. pseudomallei* and transformed into *E. coli* BL21 strain. For protein purification, positive clone

chosen was inoculated into 100 ml Luria Broth (LB) cultures and allowed to grow at 37°C until the OD<sub>600</sub> reached about 0.5 to 0.6, induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for about 4 hours. Cell pellet was resuspended in 10 ml of ice cool lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 1% Triton X-100) and subjected to sonication on the ice bath. The cell lysate was then centrifuged at 4000g for 20 minutes at 4°C and supernatant was discarded. 400 µL of Ni<sup>2+</sup>-NTA resin (Qiagen, GmbH) was added to the supernatant and mixed at 4°C for 2 hours on a rotator. Bound recombinant protein was collected from the resin by adding 10 mM reduced glutathione. The purified antigen was reconstituted with sterile distilled water to make up to a concentration of 1 µg/µl and aliquoted for further use.

**Preparation of SWCA:** To prepare SWCA, 50 ml of Trypticase Soya Broth (TSB) was inoculated with pure colonies of *B. pseudomallei* USM strain and incubated overnight at 37°C. Organisms were harvested by centrifugation for 30 minutes at 4000g at 10°C. Pellets were suspended with 3 ml of 25 mM Tris-HCl (pH 7.4) and washed three times with Tris-HCl for 30 minutes at 4000g at 10°C. Deposited pellet, suspended in 5 ml of ice-cold Tris-HCl, was sonicated at 40W for 8 minutes in each pulse inside the assigned biosafety cabinet. Sonicated bacterial suspension was then centrifuged at 5000xg at 10°C for 30 minutes. After centrifugation, the supernatant containing the bacterial proteins was collected and its protein concentration was determined.

**Determination of anti-*B. pseudomallei* IgG antibody by ELISA:** Serum anti-*B. pseudomallei* IgG antibody was determined by an indirect ELISA as described by Voller et al [15]. The 96 well ELISA plate (Linbro, USA) was coated with 2.5 µg/ml of RTFA or 10 µg/ml of SWCA in 0.5 M carbonate/bicarbonate buffer (pH 9.6). To each well 100 µl volume of coating buffer was added and incubated overnight at 4°C. The plate was washed three times with phosphate buffered saline-0.05% Tween 20 (PBS-T, pH 7.4) and blocked by incubating for 2 hours with PBS-T containing 2% bovine serum albumin (BSA) at 37°C. The plate was then washed three times with PBS-T. A volume of 100 µl serum

(1:400 dilution for RTFA and 1:1600 dilution for SWCA) samples was added into each well and incubated for 4 hours at 37°C. After washing with PBS-T three times, 100 µl of horseradish peroxidase conjugated anti-human IgG antibodies (MP Biomedicals, USA) (1:4000 dilution) was added and incubated at 37°C for 2 hours. After washing three times with PBS-T, 50 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 30 minutes in dark. Then 50 µl of 1 M sulfuric acid was added in each well. The colour developed was measured by ELISA plate reader (Human ELISA Reader) at 450 nm. Optimum concentration of the antigen (2.5 µg/ml for RTFA and 10 µg/ml for SWCA) and serum dilution (1:400 dilution for RTFA and 1:1600 dilution for SWCA) were predetermined by checkerboard titrations.

Cut-off OD values for anti-*B. pseudomallei* IgG antibody against RTFA and SWCA were determined to designate *B. pseudomallei* seropositivity of the study population. ELISA was performed with sera from 35 healthy newborn babies of Dhaka city who were presumed not to be exposed to *B. pseudomallei*. The mean OD+3xSD of these sera was taken as cut-off OD value to designate the case as seropositive. Table-1 shows the calculated cut-off OD values for RTFA and SWCA based ELISA. Any sample showing OD above the cut-off OD value of more than 0.4 and 0.8 by respectively RTFA and SWCA based ELISA was considered positive and referred to as exposed to *B. pseudomallei* infection.

**Table-1:** Calculated cut-off OD values for ELISA using RTFA and SWCA

Antigen	Mean OD value	SD	Cut-off value (Mean OD+3xSD)
RTFA	0.2	0.06	0.4
SWCA	0.2	0.2	0.8

Note: RTFA = recombinant truncated flagellin antigen; SWCA = sonicated whole cell antigen; OD = optical density; SD = standard deviation

## Results

Total 8 serum samples from culture confirmed melioidosis cases and 361 serum samples from

**Table-2:** Detection of anti-*B. pseudomallei* antibodies in melioidosis cases and in endemic healthy individuals by ELISA using RTFA and SWCA

Study population	Total sample number	Anti- <i>B. pseudomallei</i> IgG positive by ELISA with			Mean OD±SE of		
		RTFA N (%)	SWCA N (%)	p value by Z test	RTFA-ELISA	SWCA-ELISA	p value by t test
Culture positive melioidosis case	8	7 (87.5)	8 (100)	0.303	0.79±0.11	2.4±0.08	0.0001
Healthy individuals	361	58 (16.1)	97 (26.87)	0.001	0.67±0.01	1.27±0.02	0.0001

Note: Mean OD of negative cases for RTFA – 0.23±0.01; Mean OD of negative cases for SWCA – 0.38±0.01; RTFA = recombinant truncated flagellin antigen; SWCA = sonicated whole cell antigen; OD = optical density; SE = standard error.

healthy individuals aged 21 years and above residing in melioidosis endemic rural areas were included in the study. Out of 8 culture confirmed acute melioidosis cases, 7 (87.5%) and 8 (100%) were positive for anti-*B. pseudomallei* IgG antibodies by RTFA and SWCA methods respectively (Table-2). The mean OD of RTFA based ELISA of positive cases was significantly less than that of SWCA-ELISA (0.79±0.11 vs. 2.4±0.08;  $p = 0.0001$ ).

Out of total 361 healthy individuals, 58 (16.1%; 95% CI: 0.125, 0.203) and 97 (26.87%; 95% CI: 0.224, 0.318) individuals were positive for anti-*B. pseudomallei* IgG antibody by RTFA and SWCA based ELISA respectively. The rate of seropositivity by RTFA-ELISA was significantly less ( $p = 0.001$ ) than that of SWCA-ELISA (Table-2). The mean OD values of RTFA and SWCA based ELISA were 0.67±0.01 and 1.27±0.02 respectively ( $p=0.0001$ ).

The sensitivity and specificity of RTFA-ELISA, when calculated, were 88.9% and 100% respectively and for SWCA-ELISA it was 100% and 97.9%.

## Discussion

Infection by *B. pseudomallei* can be detected by either culture, molecular or serological methods. These methods are usually employed to diagnose acute melioidosis cases. Apart from diagnosis of acute infection, serology is employed to assess the

extent of exposure to particular organism in a community. Several antigens of *B. pseudomallei* have been employed for serodiagnosis of acute and past infection with varying results.

A previous study evaluated 4 purified *B. pseudomallei* recombinant proteins (TssD-5, Omp3, smBpF4 and Omp85) using ELISA as potential diagnostic agents for melioidosis. TssD-5 demonstrated the highest sensitivity of 71% followed by Omp3 (59%), smBpF4 (41%) and Omp85 (19%). All 4 antigens showed equally high specificity (89-96%). A combination of four antigens provided improved sensitivity of 88.2% and good specificity (96%) [13]. Anuntagool et al. evaluated five different *B. pseudomallei* antigens including a 19.5-kDa antigen, a crude cell extract, a veronal extract, a 39.0-kDa antigen, and an immunoaffinity-purified antigen by indirect ELISA. The 19.5-kDa antigen exhibited the most satisfactory results, with 92% sensitivity and 91% specificity [16].

We determined anti-*B. pseudomallei* IgG antibody in culture confirmed melioidosis cases and in healthy population residing in melioidosis endemic rural area of Bangladesh, by ELISA using RTFA and SWCA of *B. pseudomallei* in an in-house indirect ELISA. The rate of positive cases by RTFA-ELISA was significantly less (16.1% versus 26.87%) compared to cases by SWCA-ELISA among the healthy people from melioidosis endemic area. Also, we observed lower mean OD values in both acute melioidosis

and healthy cases by RTFA-ELISA than SWCA-ELISA indicating presence of less concentration of antibodies or reactants in serum against RTFA than SWCA. Similar low OD or absorbance (mean OD ~ 0.4) was observed in culture confirmed melioidosis cases against truncated flagellin fragment [17]. We also presume that the antibody to RTFA might decline earlier overtime following exposure to *B. pseudomallei*. Also RTFA-ELISA detected antibody produced only against flagellar proteins instead of proteins from whole cell. But in case of SWCA, the antibodies detected were a mixture of antibodies against several antigenic components of the *B. pseudomallei* some of which might be long persisting against specific proteins. It appears that the rate of positivity of anti-*B. pseudomallei* antibody might vary depending on the assay method and antigen used in the serological assays. Therefore, prospective study should be undertaken to find out the duration of persistence of antibodies to *B. pseudomallei* flagellar proteins as well as to other cellular components following exposure.

High seropositivity rate in healthy population has been reported from other countries of the region. The seroprevalence rate of *B. pseudomallei* among the healthy people in Haiti was found as 9.8% by lipopolysaccharide based ELISA [18]. Another study reported 29% seropositivity among the adults in coastal areas in Southwestern India by indirect hemagglutination assay (IHA) using polysaccharide antigens of *B. pseudomallei* [19]. In a previous study conducted on more than 900 people in 2016, we found 21.5% of the study population as positive for anti-*B. pseudomallei* antibody by SWCA-ELISA [8]. The sensitivity and specificity of our RTFA-ELISA were 88.9% and 100% respectively. Similar range of sensitivity and specificity of flagellar protein based ELISA was reported by other studies [14,17].

The findings of the present study indicate that multiple or combination of antigens should be used to determine the actual presence of antibody to *B. pseudomallei* in seroprevalence study in a community.

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#### Competing interest

The authors hereby, declare that no conflict of interest exists.

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