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Recombinant Truncated Flagellin of *Burkholderia pseudomallei* as a Molecular Probe for Diagnosis of Melioidosis

Yao-Shen Chen,[1,2] David Shiuan,[3] Ssu-Ching Chen,[4] Soi-Moi Chye,[5] and Ya-Lei Chen[5]*

Section of Infectious Disease, Kaohsiung Veterans General Hospital,[1] Institute of Biomedical Science, National Sun Yat-Sen University,[2] and Department of Medical Technology, Fooyin Unversity,[5] Kaohsiung, Department of Life Science and Institute of Biotechnology, National Dong-Hwa University, Hualien,[3] and Department of Medicine, China Medical Collage, Taichung,[4] Taiwan, Republic of China

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Current serological tests for melioidosis, using impure or uncharacterized cell antigens from *Burkholderia pseudomallei*, have problems in detection sensitivity and specificity. Therefore, we designed and expressed the recombinant flagellin (truncated at both the N- and C-terminal ends), and used the antigen to develop an indirect enzyme-linked immunosorbent assay (ELISA) to diagnose melioidosis. Comparison of the immunoreactivities of the full-length and truncated flagellins reveals that the truncated flagellin performed much better in detection specificity and sensitivity. Only the full-length flagellin was recognized by other bacterial causing septicemia and gave a false-positive result in Western analysis, indicating that the cross-reactive epitopes were located on the more highly conserved N- and C-terminal regions of flagellin. The indirect ELISA using recombinant truncated flagellin as the antigen achieved 93.8% sensitivity and 96.3% specificity and offered a more efficient serodiagnosis of melioidosis.

Melioidosis, caused by *Burkholderia pseudomallei*, is endemic in Southeast Asia and northern Australia in particular (8, 12). The clinical manifestations of meliodosis are protean, and the disease usually leads to death from acute septicemia (8, 13). Thus, the efficient diagnostic methods are very imperative. Several serological methods including the indirect hemagglutination test (11), immunofluorescence assay (2, 3, 15), and enzyme-linked immunosorbent assay (ELISA) (1, 4, 7) had been developed to diagnose the early *B. pseudomallei*-specific antibodies. However, the use of living bacteria as antigens in these assays creates a risk of laboratory-acquired infections (16). In addition, impure or uncharacterized cell antigens of *B. pseudomallei* usually cross-react with other bacterial in the sera, making the methods less specific. To avoid the above problems, we developed a very safe, rapid, and efficient method to identify *B. pseudomallei*-specific antibodies by using the recombinant truncated flagellin as the antigen.

Flagellin (flagellar protein) elicits early specific antibodies against *B. pseudomallei* infection in mice. It is therefore an ideal antigen for detecting melioidosis (5, 6). The N- and C-terminal regions of flagellin in *B. pseudomallei* have higher homology to those of other bacteria and may carry cross-reactive epitopes (9). Therefore, we designed a recombinant flagellin stripped of the original ends and developed an indirect ELISA to detect the *B. pseudomallei*-specific immunoglobulin G (IgG) by using the truncated flagellin as the antigen.

MATERIALS AND METHODS

Bacterial strains. *B. pseudomallei* VGH07 was isolated from a patient admitted to the Kaohsiung Veterans General Hospital in southern Taiwan. The isolate was confirmed by standard biochemical tests and the ID 32 GN profile (API system; BioMérieux, Mercy l'Etoile, France) and kept in Luria-Bertani (LB) broth at 37°C.

Amplification of flagellin genes. The chromosomal DNA of *B. pseudomallei* was isolated by using a modified proteinase K digestion technique (10). Primers 5′-TTTTGGATCCATGCTCGGAATCAACAGCAACATTAAC-3′ (forward primer) and 5′-TTTTGCGGCCGCTTATTGCAGGAGCTTCAGCACTTGC-3 (reverse primer) (9) were designed to amplify the full-length flagellin gene. The nucleotide sequence of the PCR-amplified DNA fragment had been confirmed to be the flagellin gene sequence (GenBank accession number U73848). The amino acid sequence of the flagellin protein has 33 to 46% identity to that of *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Proteus mirabilis*, and *Escherichia coli*. However, the N- and C-terminal amino acid sequences (amino acids 1 to 40 and 300 to 387) of flagellin from the above bacteria have approximately 67 and 50% homology, respectively. Thus, primers 5′-AAAAGGATCCGCGTCGGCGCTGCAACAGGAACTCG-3′ and 5′-AAAAGCGGCCGCTTACATCGCCTGGTACGCGCCCGTCTGC-3′ were designed to amplify the truncated flagellin gene (encoding amino acids 41 to 299 of the flagellin). The PCR mixtures contained 20 μl of DNA extract, 2 μl of each primer, 5 μl of 10× amplification buffer (Promega, Madison, Wis.), a 10 μM concentration of each deoxyribonucleotide, and 1 U of *Taq* polymerase (Promega) in a final volume of 50 μl. The PCR amplification was conducted for 25 cycles: denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min.

Expression of flagellin. The amplicons of the flagellin gene with the full-length and truncated forms (central region) were cloned in frame into the pGEX4T-2 plasmid (Amersham Pharmarcia Biotech, Aslington Heights, Ill.) at the *Bam*HI-*Not*I site behind the junction with glutathionone *S*-transferase. Approximately 10^6 CFU of *E. coli* BL21 [*hsd*S *gal* (λ cI *ts*857 *ind*-1 Sam7 *nin*-5 *lacUV5-T7* gene 1)] per ml, carrying the full-length or truncated form of the flagellin gene, was grown in LB broth containing 50 μg of ampicillin per ml and 500 μM isopropyl-β-D-thiogalactoside (IPTG; Fluka, Buchs, Switzerland) until the optical density at 600 nm (OD_{660}) reached 0.8. After a 2 h-induction with 500 μM IPTG, the recombinant protein was extracted with 200 μl of B-PRE solution (Pierce, Rockford, Ill.). The lysate was loaded onto as immobilized glutathione column (Clontech), and the fusion proteins were eluted with 50 mM reduced gluthathione by centrifugation. The purified flagellin was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacylamide gel) and confirmed by Western blot analysis (with the rabbit anti-flagellin antiserum, produced by Genesis Biotech Inc.).

Serum specimens. A total of 32 serum samples from 16 patients with septicemia due to melioidosis, admitted to Kaohsiung Veterans General Hospital, Chang Gang Memorial Hospital—Kaohsiung, and Jiarren Hospital, Kaoshiung,

* Corresponding author. Mailing address: Department of Medical Technology, Fooyin University, 151 Chin-Hsuen Rd., Ta-Liao, Kaohsiung 83101, Taiwan, Republic of China, Phone: 886-07-7811151, ext. 627. Fax: 886-07-7827162. E-mail: dan1001@ms31.hinet.net.

TABLE 1. Antiflagellin antibody levels in sera from patients with melioidosis at different collection times after the onset of melioidosis

Patient	Antibody titer in serum collected on day[a]:						
	1	3	7	30	90	180	>360
1	1,024 (512)			1,024 (256)	512 (256)	512 (256)	
2	1,024 (512)				1,024 (256)	128 (256)	
3	1,024 (512)				1,024 (256)		256 (256)
4			1,024 (256)	1,024 (256)	256 (256)	128 (256)	
5	1,024 (512)						
6	1,024 (512)						
7	2,048 (1,024)						128 (128)
8		1,024 (512)	1,024 (512)				
9	1,024 (512)	1,024 (512)	1,024 (256)				
10	1,024 (512)	1,024 (512)					
11	128 (128)						
12	1,024 (512)						
13		2,048 (1,024)					
14	1,024 (512)						
15	4,096 (1,024)	4,096 (1,024)					
16	1,024 (512)						

[a] The highest positive dilution of sera from melioidosis patients, as determined with the full-length flagellin antigen in an indirect ELISA. The values in parentheses give the highest positive dilution with truncated flagellin antigen.

Taiwan, were collected at different times as listed in Table 1. Patients in Kaoshiung Veteran General Hospital with septicemia caused by other infectious bacteria were used as disease controls. The serum specimens contained 100 samples from disease controls infected with bacteria including *Pseudomonas* group (*P. aeruginosa* [$n = 18$] and *P. putida* [$n = 4$]), *Klebsiella* group (*K. pneumoniae* [$n = 12$] and *K. oxytoca* [$n = 3$]), *E. coli* ($n = 11$), *Salmonella* group ($n = 11$), *Enterobacter cloacae* ($n = 11$), other gram-negative bacteria (*Citrobacter freundii* [$n = 3$], *Serratia marcescens* [$n = 5$], *Moraxella* spp. [$n = 5$] *Chromobacterium* sp. [$n = 1$] and *Aeromonas* spp. [$n = 4$]), and other gram-positive bacteria group (*Staphylococcus* spp. [$n = 7$] and *Streptococcus* spp. [$n = 5$]). The etiology of the septicemia in these patients was identified by biochemical and morphological characterization of clinical isolates from blood culture. The sera collected from 60 healthy adults served as the negative control group.

Evaluation of ELISA for detecting *B. pseudomallei*-specific antibody. The 96-well polystyrene microtiter plates were coated with the flagellin antigens (full-length form, 0.5 μg/ml; truncated form, 1 μg/ml) in coating buffer (50 mM carbonate/bicarbonate buffer [pH 9.6]) at 4°C overnight. The plates were blocked for 2 h using 100 μl of bovine serum albumin (1 mg/ml; GIBCO, Grand Island, N.Y.). After being washed with saline-Tween solution (0.9% [wt/vol] NaCl and 0.05% [vol/vol] Tween 20 in phosphate-buffered saline [PBS]) three times, the wells were incubated at 37°C for 1 h with twofold serial dilutions of various sera in PBS. Then the wells were washed with saline-Tween solution and incubated with diluted (1:1,000) anti-human IgG conjugated with peroxidase (Zymed, South San Francisco, Calif.) at 37°C for 1 h. The wells were washed with PBS three times, and 100-μl volumes of 1-Step Turbo TMB-ELISA substrate (Pierce) were added. The OD_{450} of the reactions products were determined using a microplate reader (Anthos 2010). The mean and standard deviations of six repeated measurements were calculated for each tested specimen. On each plate, sera from six healthy individuals were used as the negative controls. In addition, rabbit anti-flagellin antiserum and anti-rabbit IgG conjugated with peroxidase were used as positive groups for detecting the presence of flagellin antigen coating in the plate. When the average of the OD readings of the tested sample was greater than that of the negative controls plus 2 standard deviations, the tested sample was considered positive for specific antibody. The highest dilution of the tested sample which still gave a positive result was considered the endpoint titer and listed on the data sheet.

RESULTS AND DISCUSSION

The cutoff dilutions in the indirect ELISA with full-length flagellin were determined to be 1:1,024, which was greater than the mean endpoint titer plus 2 standard deviations for the healthy control group ($n = 60$). The results indicated that 24 (75%) of 32 sera from patients with acute septicemic melioidosis were diagnosed as positive (Table 2). In the same manner, 5 (23%) of 22 from the *Pseudomonas* group, 4 (27%) of 15 from the *Klebsiella* group, 3 (27%) of 11 from the *E. coli* group 3 (27%) of 11 from the *Salmonella* group, 1 (9%) of 11 from the *Enterobacter cloacae* group, 4 (22%) of 18 from the other gram-negative bacteria group, 0 (0%) of 12 from the other gram-positive bacteria group, and 8 (13%) of 60 from the healthy group were recognized as false-positive reactions to full-length flagellin. In brief, with the full-length flagellin as antigen, this assay showed a moderate sensitivity (75%; 24 of 32) and specificity (82.5%; 132 of 160) based on 192 serum specimens (melioidosis group, $n = 32$; control disease group, $n = 100$; healthy group, $n = 60$). The profile was significantly different when the truncated flagellin was used as the antigenic material, since 30 (93.8%) of 32 sera from patients with melioidosis could be distinguished from those from patients in the healthy groups at a 1:256 cutoff dilution, which was the maximum endpoint titer in sera from all the healthy individuals in the indirect ELISA using the truncated flagellin as antigen (Table 3). We then used this 1:256 cutoff dilution to evaluate

TABLE 2. IgG results with full-length flagellin antigen in an indirect ELISA

Group	No. of positive sera at antibody titer of:							% Positive (no. positive/ total no.)[a]
	<1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096	
B. pseudomallei	0	4	2	2	20	2	2	75 (24/32)
Pseudomonas spp.	3	10	3	1	5	0	0	23 (5/22)
Klebsiella spp.	4	5	1	1	4	0	0	27 (4/15)
E. coli	3	3	2	0	3	0	0	27 (3/11)
Salmonella spp.	4	3	0	1	3	0	0	27 (3/11)
Enterobacter spp.	4	3	2	1	1	0	0	9 (1/11)
Other gram-negative spp.	9	2	1	2	4	0	0	22 (4/18)
Other gram-positive spp.	9	3	0	0	0	0	0	0 (0/12)
Healthy group	27	16	6	3	8	0	0	13 (8/60)

[a] the numbers of sera with antibody titers above or equal to cutoff value/ the numbers of sera with antibody titers below cutoff value, with the cutoff value of 1:1,024.

TABLE 3. IgG results with truncated flagellin antigen in an indirect ELISA

Group	No. of positive sera at antibody titer of:					% Positive (no. positive/ total no.)[a]
	<1:64	1:128	1:256	1:512	1:1,024	
B. pseudomallei	0	2	12	14	4	93.8 (30/32)
Pseudomonas spp.	19	2	0	1	0	4.5 (1/22)
Klebsiella spp.	14	1	0	0	0	0 (0/15)
E. coli	8	2	0	1	0	9.1 (1/11)
Salmonella spp.	7	3	0	1	0	9.1 (1/11)
Enterobacter spp.	9	0	1	1	0	18.2 (2/11)
Other gram-negative spp.	16	1	0	1	0	5.6 (1/18)
Other gram-positive spp.	11	1	0	0	0	0 (0/12)
Healthy group	47	13	0	0	0	0 (0/60)

[a] The numbers of sera with antibody titers above or equal to the cutoff value/ the numbers of sera with antibody titers below cutoff value, with a cutoff value of 1:256.

the truncated flagellin and observed that six sera from the group of control diseases (*P. aeruginosa*, $n = 1$; *E. coli*, $n = 1$; *Salmonella* group D, $n = 1$; *E. cloacae*, $n = 2$; *S. marcescens*, $n = 1$) were positive for IgG (Table 3). All six sera but one cross-reacted simultaneously with both the full-length and truncated flagellin. The interactions of various sera with the full-length and truncated flagellins were also examined by Western blot analysis. The results were the same irrespective of the use of full-length or truncated flagellin in Western blot analysis or indirect ELISA. (data not shown). Taken together, the present results clearly indicate that the cross-reactive epitopes were in the N- and C-terminal regions of flagellin. Use of the truncated flagellin as the antigen in the indirect ELISA for detection of antibodies specific to *B. pseudomallei* has greatly enhanced the sensitivity to 93.8% and specificity to 96.3%.

The specificity and sensitivity of assays using various antigens and the same panel of sera for serodiagnosis of meliodosis had seldom been above 90% (14). One of the recently improved methods using partially purified culture filtrate as antigen claimed rather high sensitivity (96%) and specificity (94%) in detecting specific IgG in patients with melodosis (7); however, the possible laboratory-acquired infection of *B. pseudomallei* in the preparation of culture filtrate antigens with living materials is still a serious drawback. Attempts to use recombinant antigen have been only partially successful. One successful example which used the recombinant Bps-1 protein as antigen achieved a moderate sensitivity (69.7%) based on Western blot analysis (16).

In conclusion, we expressed the recombinant truncated flagellin encoding the immunodominant epitopes and used it as the antigen to detect *B. pseudomallei*-specific antibodies. The indirect ELISA method using the recombinant truncated flagellin achieved 93.8% sensitivity and 96.3% specificity and offered safer and more rapid and efficient means of serodiagnosis of melioidosis in areas such as Taiwan, where the disease is not endemic. However, this assay still needs to be tested while evaluating a large number of sera collected from patients with melioidosis in an area of endemic infection, such as Thailand, Australia, or Malaysia; before it can be widely used for serodiagnosis.

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