

Original Research Article

Antibodies against *Shigella flexneri* adhesion molecule outer membrane protein (OMP) can cross-react with OMPs of some *Shigella* species

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Abstract

Purpose: To identify a *Shigella flexneri* hemagglutinin outer membrane protein (OMP) as an adhesion factor and examine its ability to cross-react with the OMPs of other *Shigella* species.

Methods: OMP was isolated from the bacterium *S. flexneri* after shaving the pili using a pili bacterial cutter in a solution of 0.5 % *n*-octyl- β -D-glucopyranoside. Hemagglutination was evaluated using mice erythrocytes. The molecular weight (MW) of the OMPs was profiled by SDS-PAGE. Adhesion molecules were identified by calculating the index of adhesion. Thereafter, antigen cross-reaction with antibodies was examined by Western blotting.

Results: The *S. flexneri* adhesion molecule OMP has a MW of 28 kDa. An antibody against the 28 kDa *S. flexneri* OMP adhesion molecule cross-reacted with different MW OMPs from other *Shigella* species.

Conclusion: This study showed that the 28 kDa *S. flexneri* OMP is a hemagglutinin adhesion protein, and antibodies against it can cross-react with the OMPs of other *Shigella* species.

Keywords: *Shigella flexneri* OMP, Outer membrane protein, Protein adhesion, Antibody, Cross-reaction

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INTRODUCTION

Diarrhea is the fourth leading cause of morbidity and mortality worldwide, and *Shigella* species cause an estimated 164.7 million cases of diarrhea annually. In developing countries, the 163.2 million cases resulted in 1.2 million deaths [1]. In South Jakarta, the predominant isolate was *S. flexneri*, which is often isolated in other developing regions, such as Bangladesh, Pakistan, and

Africa [2]. *S. flexneri* is endemic in developing countries [1,3].

Shigella is a highly infectious pathogen, because only 10 – 100 ingested bacteria are sufficient to cause disease [4]. The success of a pathogenic bacterial infection requires attachment to host cells, tissue colonization, and cell invasion [5].

The initial stage of infection is adhesion between the bacteria and host cells.

Outer membrane proteins (OMPs) include proteins that mediate the attachment of bacteria to the host cell more closely than do pili [6,7]. A 49.8 kDa *Shigella dysenteriae* hemagglutinin pili protein that serves as an adhesion protein was found previously [8]. Oral immunization with this 49.8 kDa *S. dysenteriae* protein can increase intestinal secretory IgA (s-IgA) levels, inhibit bacterial colonization in the colon, and prevent damage to colon epithelial cells [9]. There are two *S. dysenteriae* pili protein subunits, of 7.9 and 49.8 kDa. The *S. dysenteriae* protein prevented diarrhea in a mouse ligated ileal loop (MLIL) model [10].

The World Health Organization (WHO) has prioritized the development of safe, effective vaccines for shigellosis, and several candidate vaccines are being developed currently [1,11]. OMPs have been used successfully as vaccine antigens to prevent Lyme disease and pertussis based on their roles as adhesion molecules [11-13]. The ideal *Shigella* vaccine should be able to induce an immune response against all *Shigella* spp. [11].

EXPERIMENTAL

Sample preparation

The bacteria *Shigella flexneri* 4a, *Shigella dysenteriae* 2a, *Shigella boydii* serotype 10, and *Shigella sonnei* were obtained from the Yogyakarta Regional Health Laboratory. Culture isolates of the *Shigella* species were grown on MacConkey medium at 37 °C for 24 h and then transferred to Petri dishes containing Salmonella-Shigella Agar (SSA) medium at 37 °C for 24 h. The resulting bacterial cultures were dissolved in 10 mL phosphate-buffered saline (PBS) (pH 7.4), added to bottles containing 500 mL Brain Heart Infusion

(BHI), and shaken in a water bath for 30 min at 37 °C.

Isolation of OMP

The *Shigella* OMP was isolated in two stages. The first stage involved cutting the bacterial cell pili, and the second involved isolating the OMP from the bacterial cell after pili cutting, as described in our previous study [14]. The OMP was isolated according to the method of Evans and Evans [15]. The pellet from the last centrifugation step was resuspended in PBS (pH 7.4) until the volume reached 15-fold, and then *n*-octyl- β -D-glucopyranoside was added at concentrations up to 0.5 %. The resulting solution was homogenized using a vortex and centrifuged at 12,000 rpm at 4 °C for 15 min. This treatment was repeated three times to give a supernatant rich in OMP.

Purification of *Shigella* spp. OMP

The molecular weight of the OMP was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [16]. The OMP was purified by electroelution [12]. The elution product was dialyzed for 24 h in a glass beaker with sterile PBS and stirred at 4 °C for 24 h. The obtained protein was precipitated by centrifugation at 12,000 rpm for 15 min at 4 °C.

Hemagglutination test

The hemagglutination test was performed according to the method of Hanne and Finkelstein with modifications [14]. First, 50 μ L PBS were placed in the wells of a Microplate V, and 50 μ L serial dilutions of the sample were added. Then, 50 μ L 0.5 % mice erythrocyte suspension were added to each well, and the plate was shaken gently for 1 min on a plate rotator. The hemagglutination titer was the lowest

dilution causing agglutination of the red blood cells.

Isolation of mouse enterocytes

Mice enterocytes were isolated using the Weisser method [17]. The mice were anesthetized using chloroform, their stomachs opened, and the small intestines removed. The small intestine was cut into small pieces and washed several times until clean using a solution of PBS containing 1 mM dithiothreitol (DTT) at 4 °C. Then, the intestinal tissue was added to a solution containing 1.5 mM KCl, 9.6 mM NaCl, 27 mM Na-citrate, 8 mM KH₂PO₄, and 5.6 mM Na₂HPO₄ at pH 7.4. The solution was incubated on a shaking incubator at 37 °C for 15 min. The supernatant was discarded and the tissue was transferred to a solution containing 1.5 mM EDTA and 0.5 mM DTT. The liquid was shaken vigorously for 15 min at 37 °C, and the supernatant was discarded. The tissue was washed with PBS and then centrifuged at 1500 rpm at 4 °C for 5 min; this was repeated at least three times until the supernatant was clear. Enterocytes were obtained after centrifugation at 1500 rpm for 5 min and resuspended in PBS. For the adhesion test, 10⁶ enterocytes/mL were used.

Adhesion test

The adhesion test was performed according to the method of Nagayama *et al.* with modifications [14]. *S. flexneri* was cultured in BHI liquid for 24 h at 37 °C and then centrifuged at 6000 rpm at 4 °C for 10 min. The pellet was resuspended in PBS containing 1 % bovine serum albumin. The bacterial count was set at 10⁸/mL using a spectrophotometer at a wavelength of 600 nm. Then, 0, 6.25, 12.5, 25, 50, or 100 µg of OMP was added to 300 µg of the enterocyte suspension and rocked

gently on a shaking water bath at 37 °C for 30 min.

Finally, 300 µg of the bacterial suspension (10⁸/mL) were added to each mixture, incubated on a shaking incubator for 30 min at 37 °C, and then centrifuged at 1500 rpm at 4 °C for 3 min. The pellet was washed twice with PBS. A sample was smeared on a glass slide and subjected to Gram staining.

Immunization of mice with *S. flexneri* OMP

The study used 6–8-week-old male BALB/C mice. The antigen used was the purified 28 kDa *S. flexneri* hemagglutinin adhesion OMP. Mice were acclimatized for 7 days before immunization. After acclimatization, the mice were injected intraperitoneally with 100 µg OMP that had been emulsified with 0.1 mL complete Freund's adjuvant [18].

Isolation of polyclonal antibodies

Blood was collected from the hearts of the mice, placed in an Eppendorf tube, which was left tilted for 30 min, and then centrifuged at 10,000 rpm for 5 min. The supernatant serum was stored at –20 °C [8].

Antigen/antibody cross-reactions

The cross-reaction of *Shigella* spp. OMP antigen with antibody against the *S. flexneri* 28 kDa OMP was examined by Western blotting. Before performing the antigen antibody response test, the checkerboard test was necessary to determine the effectiveness of the antigen and antibody reactions to be evaluated. The checkerboard test uses a dot blot method and reacts the *S. flexneri* 28 kDa OMP antigen with serially diluted antibodies. The antigen was diluted 1/5 to 1/10240, and the antibody was diluted 1/100 to 1/2800. The results reveal a color gradation that can be analyzed using Corel Photo-Paint

to determine the mean value [19]. Western blotting was performed using the method of Towbin *et al* [20].

Statistical analysis

The data were analyzed using SPSS 23. One-way analysis of variance with Tukey's *post hoc* test was used to determine significant differences in the mean index of adhesion among the sample groups. Pearson's correlation test was used to determine correlations between the sample doses and adhesion index. Significance level was set at $p < 0.05$.

RESULTS

OMP *Shigella* spp profiling

Figure 1 shows the molecular weights of the four *Shigella* species OMPs determined using SDS-PAGE. The profiles of the *Shigella* spp. OMPs were identical. There were various *Shigella* spp. OMPs, including OMPX, OMPA, OMPT, OMPLA, TOLC, α -hemolysin, porin OMP32, FhuA, and FEPA [5].

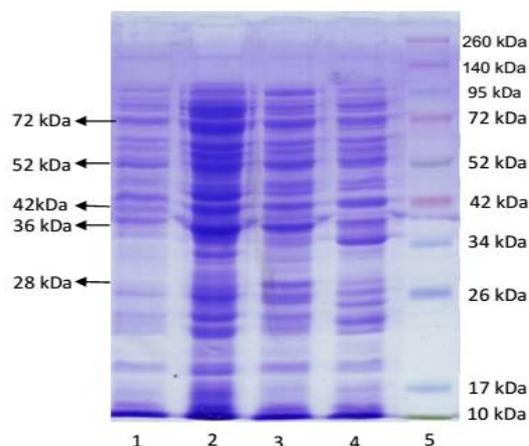


Figure 1: The results of 12.5% SDS-PAGE of *Shigella* OMPs. **Note:** Lane 1, *S. boydii* OMP; lane 2, *S. dysenteriae* OMP; lane 3, *S. flexneri* OMP; lane 4, *S. sonnei* OMP; lane 5, marker

Hemagglutination

The *S. flexneri* OMP bands with molecular weights of 28, 36, 42, 52, and 72 kDa were identified as candidate adhesion proteins. These five protein bands were excised, collected, and purified by electro elution and dialysis for the hemagglutination test. The hemagglutination test results shown in Figure 2 indicated that the 28 kDa OMP had the highest hemagglutination titer (1/32), and thus it was selected for the adhesion tests.

Adhesion

The adhesion test was conducted using 0 (control), 6.25, 12.5, 25, 50, and 100 μ g of the 28 kDa *S. flexneri* OMP (Figure 3). The adhesion index was calculated by counting the number of bacteria attached to 100 enterocytes. The result is shown in Table 1, which shows that the number of *S. flexneri* attached to mouse enterocytes decreased with increasing OMP dose. There was a significant and strong negative correlation between the dose and adhesion index ($p=0.000$). This proved that OMP inhibited the attachment of *S. flexneri* to enterocytes.

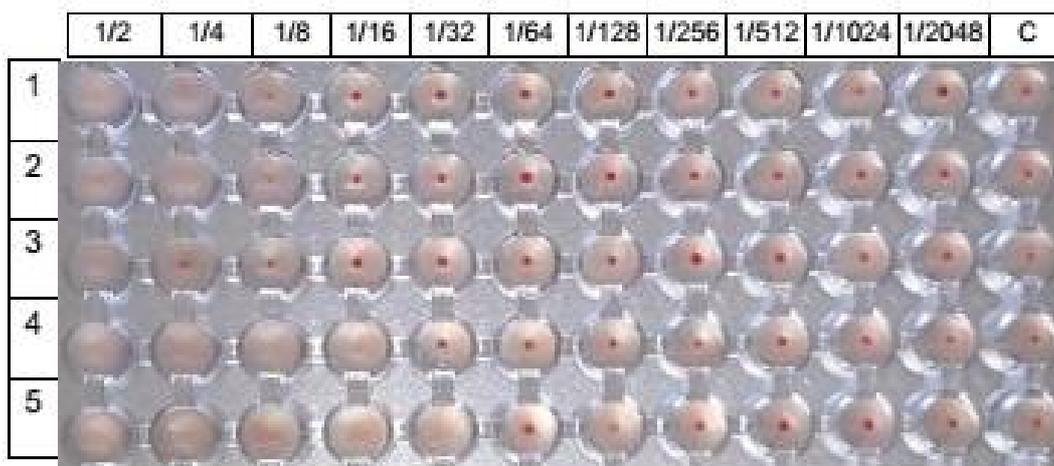


Figure 2: The results of the hemagglutination assay using mice erythrocytes and various molecular weight fractions of *S. flexneri* OMPs. **Note:** Lane C, negative control containing erythrocytes and PBS; no clumping of the erythrocytes was observed; lane 1, 72 kDa OMP; lane 2, 52 kDa OMP; lane 3, 42 kDa OMP; lane 4, 36 kDa OMP; lane 5, 28 kDa OMP

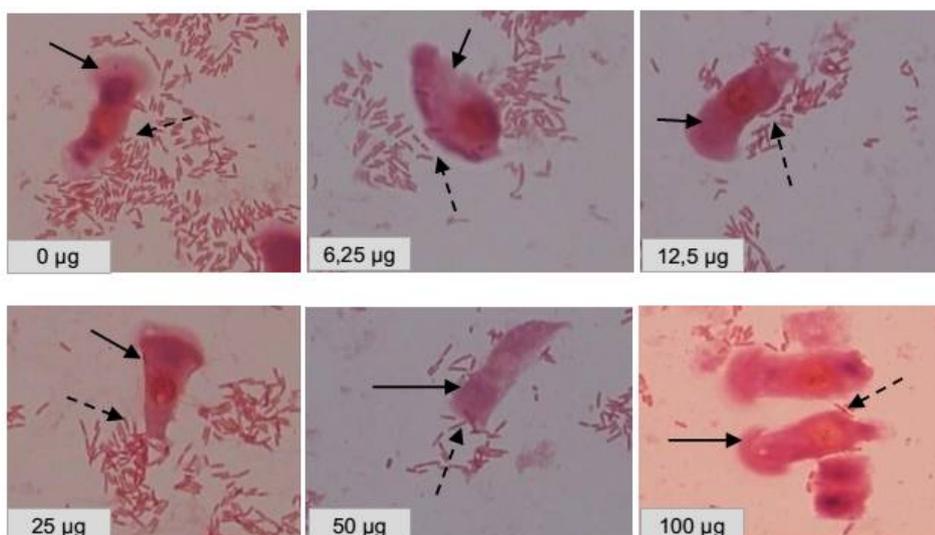


Figure 3: Adhesion test using the 28 kDa *S. flexneri* OMP and mouse enterocytes. Black arrow (→), enterocytes; dotted arrow (···), *S. flexneri* cells. Gram staining, 1000x magnification

Table 1: Correlation of various doses of OMP *S. flexneri* with adhesion index (n = 4)

Doses (µg)	Mean ± SD	P-value	Pearson correlation
0 (control)	1427.8±265.8		
6.25	1244±69.4		
12.5	785.5±148.1	0.000α	-0.786
25	596.0±20.2		
50	437.8±28.2		
100	332.3±31.8		

Antigen/antibody cross-reaction

In the checkerboard test, the *S. flexneri* OMP antigen diluted 1/320 and antibodies against the 28 kDa *S. flexneri* OMP diluted 1/400 showed the lowest density (data not shown). In this zone,

there is a balanced antigen to antibody ratio. In Western blotting, antibodies against the 28 kDa *S. flexneri* OMP diluted 1/400 reacted with *Shigella* spp. OMP.

In Figure 4, lane 1 represents the 28 kDa *S. flexneri* OMP reacting with the antibody against the 28 kDa *S. flexneri* OMP, demonstrating that the 28 kDa *S. flexneri* OMP is an antigen capable of stimulating an antibody response. OMPs from other *Shigella* spp. (Figure 4, lanes 2 to 4) also reacted with the 28 kDa *S. flexneri* OMP antibody.

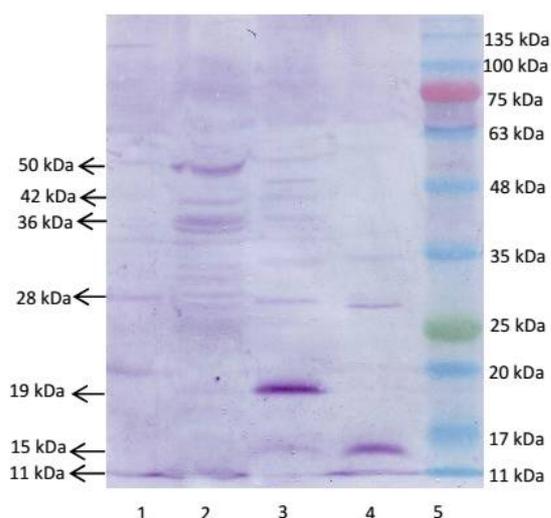


Figure 4: Western blotting of the *Shigella* spp. OMPs using an antibody against the 28 kDa *S. flexneri* OMP. **Note:** Lane 1, *S. flexneri* OMP; lane 2, *S. dysenteriae* OMP; lane 3, *S. sonnei* OMP; lane 4, *S. boydii* OMP; lane 5, marker

DISCUSSION

Shigellosis is caused by four *Shigella* species: *S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei* [4]. *Shigella* spp. OMP was isolated after shaving the bacteria pili using a pili bacterial cutter. The profiles of the *Shigella* spp. OMPs on SDS-PAGE gels were identical, and their calculated molecular weights were similar. The number of OMP bands observed on SDS-PAGE gels indicated that *Shigella* species expressed many OMPs of various molecular weights, including OMPX, OMPA, OMPT, OMPLA, TOLC, α -hemolysin, porin OMP32, FhuA, and FEPA [5].

The *S. flexneri* 28 kDa OMP had the highest hemagglutination titer (1/32). Hemagglutination is an indicator of the ability of bacteria to attach to animal cells, because the erythrocyte membrane receptor is homologous to the mucosal host cell surface receptors [21]. In order to confirm that the *S.*

flexneri 28 kDa OMP is an adhesion protein, we conducted adhesion tests. The results showed that the adhesion of *S. flexneri* to mouse enterocytes was inhibited by the *S. flexneri* 28 kDa OMP, as shown in Table 1 and Figure 3. There was a significant ($p=0.000$) correlation between the dose and adhesion index. Therefore, OMP was able to inhibit the attachment of *S. flexneri* to enterocytes. We conclude that the 28 kDa *S. flexneri* OMP was an adhesin protein and a bacterial virulence factor [10]. Histopathological studies have demonstrated that inflammatory lesions in shigellosis are caused by the entry of bacteria into the upper rectum and distal colon. [4]. Immunization of mice with the 49.8 kDa *S. dysenteriae* pili adhesion protein resulted in minimal colonic epithelial cell damage with elevated s-IgA levels. s-IgA binds to intracellular enterocyte antigens, minimizing the destruction of epithelial cells in the intestinal mucosa [9]. The 49.8 kDa *S. dysenteriae* pili adhesion protein also protected against the movement of solution from the intestine to lumen in the MLIL model [14].

The antigen–antibody cross-reaction examined by Western blotting showed that many *Shigella* spp. OMPs reacted with the antibody against the *S. flexneri* 28 kDa OMP (Figure 4). Therefore, the OMP antibody would be capable of recognizing epitopes present in the OMPs of all *Shigella* species. Antibodies produced against one antigen may bind to a different structurally related antigen, referred to as a cross-reaction [22]. These results are consistent with another research who detected cross-reaction between a 34 kDa OMP and intact cells of *Shigella* species. An antibody against the 34 kDa *S. flexneri* OMP recognized an epitope on the surface of *S. flexneri* [23]. After identifying the antigen–antibody response to *Shigella* OMPs, we

postulated that the antibody would also prevent the adhesion of *Shigella* to the host cell, and hence could serve as a candidate shigellosis vaccine against all *Shigella* species.

The 34 kDa *S. flexneri* OMP has already been purified with a promising subunit vaccine candidate for shigellosis [11, 23]. Our hemagglutinin assay also showed a positive result for a 36 kDa *S. flexneri* OMP, which may also be an adhesion molecule (Figure 2). Mitra *et al* found that the colonization ability of *Shigella* spp. was directly correlated with the hemagglutination activity in a suckling mouse model [24]. A recent *in vivo* study by Hamong *et al.* showed that a 49.6 kDa *Helicobacter pylori* hemagglutinin protein prevented damage to the stomach caused by homologous bacteria in mice [25].

We postulated that adhesion molecule epitopes are involved in protein hemagglutinin due to various OMPs [14]. This finding should be clarified before developing peptide vaccines based on molecule adhesion epitopes.

CONCLUSION

A 28 kDa *S. flexneri* OMP is identified here in this study as a hemagglutinin protein that serves as an adhesion protein. Antibodies against this protein cross-react with the OMPs of other *Shigella* species. Further research is needed to determine whether the 28 kDa *S. flexneri* OMP hemagglutinin adhesion protein can protect against colon epithelial cell damage and the movement of solution into the intestinal lumen in MLIL model [10].

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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