

Titer IgG anti-flagellum Antibody and Flagellin Gene Variants of *Salmonella enterica* Serovar Typhi as Risk Factor for Typhoid Fever Carriers

Ressy Dwiyanti^{1,6}, Yadi Yasir², Muhammad Sabir^{1,6}, Rosdiana Natzir³, Sutji Pratiwi Rahardjo⁴,
Nur Indah Purnamasari^{5,6}, Juhri Saning^{1,6}, Mochammad Hatta^{6,*}

¹Department of Microbiology, Faculty of Medicine, Tadulako University, Palu, Indonesia

²Department of Microbiology and Immunology, Faculty of Medicine, Mulawarman University, Samarinda, Indonesia

³Department of Biochemistry, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

⁴Department of Otholaringology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

⁵Medical Education Unit, Faculty of Medicine, Haluhuleo University, Kendari, Indonesia

⁶Molecular Biology and Immunology Laboratory for Infectious Diseases, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

*Corresponding author: hattaram@indosat.net.id

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Abstract Background: *Salmonella enterica* serovar Typhi (S. Typhi) is a human-specific pathogen that causes typhoid fever. Typhoid fever remains a global health problem especially in developing countries. Pathogenesis of typhoid fever is complex and host response is poorly understood. There is an urgent need for adequate and efficient detection methods for the establishment of carrier state of typhoid fever as a source of transmission. We compared IgG anti-flagellar antibody and flagellin gene variants of S. Typhi to explore risk factors of typhoid fever carriers. **Method:** Serum and fecal swab samples obtained from 379 suspected typhoid carriers. Typhoid carriers were identified when home visits of patients who have recovered from typhoid fever at least 1 year. In-house indirect sandwich ELISA were established to detect anti-flagellum IgG. DNA samples obtained directly from fecal swab were confirmed to be serovar Typhi by nested PCR. All specimens were examined for their Hd, Hj, z66 and z66 Ind flagellin genes by Polymerase Chain Reaction (PCR). **Results:** A total of 379 suspected patients, where examined by nested PCR to detect specific flagellin gene for S. typhi, and found 21 (5.28%) samples were positive. Serum samples from all suspected typhoid carriers were examined by ELISA to detect titer of anti-flagellum IgG. Of 21 typhoid carrier patients, there were 2 (9.5%) patients had Hd+ variant; one (4.8%) patient had Hj+ variant; 6 (28.6%) patients had Hd+Z66+ variant; one (4.8%) patient had Hj+ Z66+ variant and 11 (52.3%) patients had Hd+Z66IND+ variant. There were 34 patients positive for anti-flagellum IgG antibody after examined by ELISA. Among PCR positive patients there were 14 patients had high titer and 7 patients had low titer of anti-flagellum IgG antibody. Within PCR negative we found 13 patients with low titer of anti-flagellum IgG. **Conclusions:** We conclude that patient harboring Hd+Z66IND+ gene of S. Typhi and High titer of IgG antibody anti-flagellum S. Typhi considered to be risk factor for typhoid carriers development.

Keywords: IgG anti-flagellum, flagellin gene, *Salmonella enterica* serovar Typhi, typhoid fever carrier

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1. Background

Typhoid fever is a systemic infection in human that is caused by *Salmonella enterica* subsp. *enterica* serovar Typhi (S. Typhi) [1]. It is a febrile, systemic illness commonly affected human in areas where sanitation is poor [2]. S. Typhi is responsible for 21.7 million infections and cause death for approximately 217,000 peoples worldwide annually [3]. In Sulawesi, Indonesia, typhoid fever is one of the most frequent infectious

diseases [4]. Three to five percent of enteric fever become chronic carriers and harbour S. Typhi throughout their lifetime [5]. Typhoid fever is spread primarily through fecal contamination of food and drinking water from patients or carriers [6].

Pathogenesis of typhoid fever is complex and host response is poorly understood [7]. Some early studies have revealed relationship between flagellar function as determined by motility, and invasiveness in *Salmonella* species [8]. Flagella of *Salmonella* are expressed on the cell surface, they consist of a basal body embedded in the cell membrane, a central rod attached to a hook which in

turn attaches to a helical filament made up of polymerised units of flagellin protein [9,10]. Currently, we identified a new flagellin gene that encoded the z66 Ind gene. The z66 gene was present in 15.4% of isolates and this “foreign” flagellin gene was associated with increasing risk of severe clinical symptoms [11].

There is an urgent need for adequate and efficient detection methods for the establishment of carrier state of typhoid fever, perhaps based on genomic markers such as flagellin gene and immunological response of host such as titer of IgG anti-flagellum. In this study, we compared IgG anti-flagellar antibody and flagellin gene variants of *S. Typhi* to explore risks factor of typhoid fever carriers.

2. Method

2.1. Clinical Specimens

Serum and fecal swab samples obtained from 379 suspected for typhoid carrier. Typhoid carriers were identified when home visits of patients who have recovered from typhoid fever at least 1 year. DNA Samples obtained directly from fecal swab were confirmed to be serovar *Typhi* by nested PCR. This study was approved by the ethical boards of the participating institutes and informed consents were obtained from all participants or from their parents/guardians.

2.2. Serologic Analysis

In-house indirect sandwich ELISA were established to detect anti-flagellum IgG. Flat-bottom 96-well microtiterplates were coated overnight at 4°C with 100 µl of either 1 µg of antigen/ml in coating buffer (0.1 M carbonate buffer [pH 9.4], antigen positive) or coating buffer alone (antigen negative). Purified flagellum antigen of *S. Typhi* provided from Wellcome Trust Clinical Research Unit, United Kingdom. The plates were blocked for 1 hat 37°C with 200 µl of phosphate buffered saline containing 1% bovine serum albumin (BSA). Sera were either assayed in serially diluted (starting at adilution of 1/50). Sera were diluted in phosphate-buffered saline containing 0.1% BSA and 0.05% Tween 20, 100 µl was applied to the appropriate wells, and the plates were incubated for 4 hat room temperature. Bound antibody (IgG) were detected using heavy-chain-specific goat antibodies directly conjugated to alkaline phosphatase. One hundred microliters was added to each well, and the plates were incubated overnight at 4°C. One hundred microliters of p-nitrophenyl phosphate (1 mg/ml) was added to each well, and the plates were incubated at ambient temperature in the dark for 30 to 40 min. The absorbance at 405 nm (reference filter, 450 nm) was determined using an automated ELISA reader. For sera assayed at a single dilution, antibody levels were expressed in optical density (OD) units. These were taken as the mean OD of three wells with antigen minus the OD of a single well without antigen. For the titration assays, sera were assayed in triplicate (two wells antigen positive and one well antigen negative), and the titer was taken as the highest dilution giving a net OD (mean OD of antigen-positive wells minus OD of antigen-negative well) of ≥ 0.2 . Six standards were included on each plate, and the OD or titer of the samples was adjusted accordingly. Blank wells

with nosera were included to monitor background [12]. We estimated the high titer of IgG anti-flagellum in endemic area of typhoid fever were $\geq 1/200$ and Low titer $< 1/200$ according to House *et al.* [12].

2.3. Preparation of DNA

DNA was extracted from fecal swab samples according to the diatom-guanidinium isothiocyanate (GuSCN) method. For the preparation of DNA from feces, a stool sample with a volume of approximately 100 uL was attached to a cotton swab, suspended in 1 mL of sterile water, vortexed vigorously, and centrifuged at 1,000 rpm for 5 min and added in 900 uL of lysis buffer (50 mM Tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X-100) and centrifuged at $12,000 \times g$ for 10 minutes. To obtain the DNA, samples were lysed by incubation for 15 minutes at 18°C and 20 uL of diatom suspension was added. The diatom containing the bound DNA was sedimented by centrifugation at $12,000 \times g$ for 15 seconds. The diatom pellet was washed with washing buffer (5.25 M GuSCN in 0.1 M Tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 minutes. The pellet was mixed with 60 uL of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer and the DNA was eluted by incubation at 56°C for 10 minutes. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until PCR was performed [13].

2.4. Amplification of Flagellin Genes

The *fliC* primers set for the *fliC* genes amplification, which will give a result of around 1500 bp for the H:d antigen or about 200 bp smaller for the H:j antigen. The final set is for the z66 antigen, which will give a product of about 1500 bp if the strains are z66+, and will give no result if they are z66- [14]. Amplification of *fliC* gene was performed using primers: *fliC_F*: TTAACGCAGTAAAGAGAG and *fliC_R*: ATGGCACAAGTCATTAATAC and produce a 1521 bp product for the d allele and a 1273 bp product for the j allele. Amplification of the *fljBz66* was performed as previously described using *z66Flag_F*: TGGCACAAGTCATCAATAC and *z66Flag_R*: TTAACGCAGCAGAGACAGTAC. Control PCR amplicons from the *aroC* gene were produced using primers *aroCfor*: CCTGGCACCTCGCGTATAC and *aroCrev*: CCACACACGGATCGTGGCG. Primers position on chromosome *fliC_F* 2011173 and *fliC_R* 2012674; *aroC_F* 2450480 and *aroC_R* 2449674. The other primers are on a plasmid. Cycles consist of an initial denaturation at 94°C for 1 min, 30 cycle at 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, followed by an extension step of 72°C for 2 min [14]. For z66Ind primer set designated Ind-F: 5' ATG TCG GAA ATC AAC CGT ATC T 3' and Ind-R: 5' CAG GCC GTC AAC CTG AGA C 3' were selected for the specific amplification of a 597 bp segment of the Ind gene. The PZ66-A and PZ66-B primers are located in the central region of the z66 gene that is largely deleted in the Ind gene and the primers Ind-F and Ind-R are located in the 5' and 3' portion of the Ind gene that shows homology with the z66 gene, but these primers are chosen such that the number of mismatches with this gene is too high to warrant efficient

amplification of the z66 gene [11,15]. The Ind-specific PCR was performed with after an initial denaturation at 94°C for 2 min, for 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by an extension step of 72°C for 5 min [11].

2.5. Statistical Analysis

All statistical calculations were performed using the computer program EPI Info Version 6.0. The statistical data between 2 groups were tested by Fischer exact test. Considered significant when p value ≤0.05.

3. Results

3.1. Flagellin Genes Variants and Anti-Flagellum IgG titer in Typhoid Fever Carrier Suspects

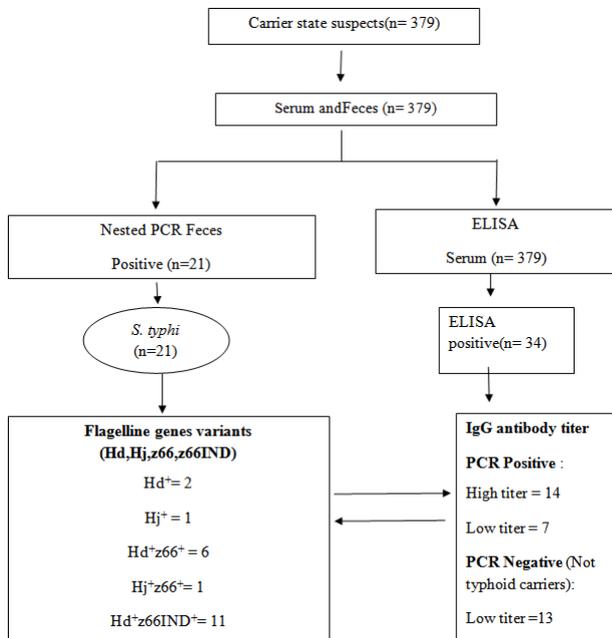


Figure 1. Flowchart of detection of carriers state, flagellin gene variants and IgG results

Figure 1. showed the number of samples recorded from primary health care center and hospital. A total of 379 suspected patients, where examined by nested PCR to detect specific flagellin gene for *S. typhi*, and found 21 (5.28%) samples were positive. Serum samples from all suspected typhoid carrier were examined by ELISA to detect titer of anti-flagellum IgG. Of 21 typhoid carrier patients, there were 2 (9.5%) patients had Hd+ variant; one (4.8%) patient had Hj+ variant; 6 (28.6%) patients had Hd+z66+ variant; one (4.8%) patient had Hj+ z66+ variant and 11 (52.3%) patients had Hd+z66IND+ variant. There were 34 patients positive for anti-flagellum IgG antibody after examine by ELISA. Among PCR positive patients there were 14 patients had high titer and 7 patients had low titer of anti-flagellum IgG antibody. Within PCR negative we found 13 patients with low titer of anti-flagellum IgG. Thus, we exclude this samples from our study to focus only in typhoid carriers.

3.2. Flagellin Genes Variantsof *S. typhi*in Typhoid Carrier Patients

Flagellin gene variants *S. typhi* of typhoid carrier patients shown in Figure 2., Hd+z66+ shown as 1500 bp band (Hd+ = 1500 bp and Z66+=1500 bp) and AroC+ act as amplicon control 800 bp band (lane 2). Variation in flagellin gene Hj+z66+ shown as 1200 bp and 1500 bp band (Hj+ = 1200 bp and Z66+=1500 bp) and 800 bp of AroC+ as amplicon control (lane 3). In lane 4, AroC+ 800 bp band as control.

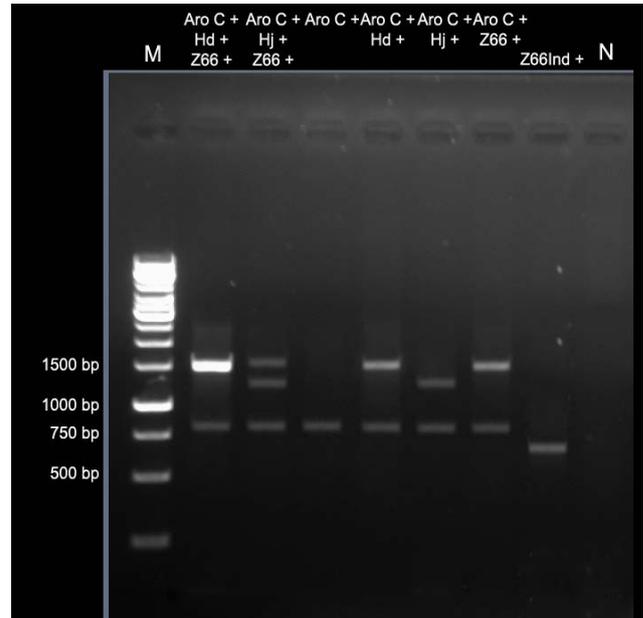


Figure 2. Electrophoresis gel results of flagellin gene variants *S.typhi*in carriers states of typhoid fever

Note:

- Lane 1: Marker 100 bp
- Lane 2: Hd+z66+; 1500 bp (Hd+), 1500 bp (z66+) and 800 bp (AroC+ for control)
- Lane 3 : Hj+z66+; 1200 bp (Hj+); 1500 bp (z66+) and 800 bp (AroC+ for control)
- Lane 4: AroC+; 800 bp (AroC+ for control)
- Lane 5: Hd+: 1500 bp (Hd+) and 800 bp (AroC+ for control)
- Lane 6: Hj+: 1200 bp (Hj+) and 800 bp (AroC+ for control)
- Lane 7: z66+: 1500 bp (z66+) and 800 bp (AroC+ for control)
- Lane 8: z66IND+: 600 bp (z66IND+)
- Lane 9: Negative control

4. Discussion

In previous study, we have been reported that the Ind gene was detected in 21.8% of the *S.typhi* isolates from East Indonesian archipelago, all of which contained the Hd. The z66 gene was present in 15.4% of the isolates. Also, in our previous study shows that variant of Hd+z66IND+ predominant in carrier state compared with acute typhoid patients (47.8% vs. 27.7%) [16].

The presence of these “foreign” flagellin genes could be associated with an increased risk for developing severe disease [11]. Furthermore, In this study we did not find a

significantly difference in percentage of positive Hj^+ , $z66^+$, Hd^+z66^+ , and Hj^+z66^+ of flagellin genes variant between the acute and the carriers state of typhoid fever. In contrast, Hd^+ , $z66Ind^+$, $Hd^+z66Ind^+$, and $Hj^+z66Ind^+$ of flagellin genes variant was found strongly associated with carriers state of typhoid fever compared with acute illness. This finding indicates the possibility that *S. typhi* containing Hd^+ and $z66Ind^+$ of flagellin genes variants are more motile and more pathogenic compared with other *S. typhi* isolates. Our results demonstrate that ELISA provides significant result that high titer on IgG anti-flagellum antibody more prevalent in patients who have $Hd^+Z66IND^+$ variant compared to other variants (10/11 or 90.9% vs 4/10 or 40%, Fisher exact test p value : <0.05). The relative predominant of certain variant of flagellin genes circulating in different endemic regions are still not been fully understood. An in-depth analysis of the host-pathogen interactions and their influence on gallbladder microorganisms maybe able to explain the adaptation and persistence mechanisms of certain strain in carrier state. Unfortunately, they are still less reliable considering the fact that in the carriers, bacteria are shed intermittently. Thus, there is an urgent need for adequate and efficient detection methods for the establishment of carrier state of typhoid fever, perhaps based on genomic markers such as flagellin gene. In this study, titers of IgG antibodies to the

flagellum antigens raised following exposure to Salmonella serotype Typhi harboring $Hd+Z66IND+$ gene among carriers state typhoid fever patients, and this fact presumably contributes as risk factor to develop typhoid fever carrier in regions of typhoid endemicity. Carrier state of typhoid fever is extremely important to prevent the transmission of these diseases. Previously many reports describe the persistence of positive *S. Typhi* among this asymptomatic state of typhoid fever by using conventional methods such as bacterial culture of faecal or urine samples. Although high titer of IgG in patient harboring $Hd+Z66IND+$ gene of *S. Typhi*, this antibody insufficient to eliminate the pathogen, it suggests that the immunity in this state to be incomplete [17]. Consistent with other study demonstrated that titers of IgG-class antibodies to the flagellum antigens can be raised following exposure to serotype Typhi [18]. We found 13 patients with positive IgG anti-flagellum in low titer (<1/200) but negative PCR. These patients considered to be non carrier subjects. Therefore, we exclude these samples from our study. It could be argued that the serum anti-flagellum antibody titers seen in the sera of 13 subjects were due to current or recent exposure with serotype Typhi, it is common in endemic areas such as in Indonesia.

Table 1. The comparison between flagellin gene variants and anti-flagellum IgG titer in carriers state of typhoid fever

Flagellin gene variants		High and low of IgG antibody titer (percentage)		p value
		High titer	Low titer	
Hd+z66IND+ (n= 11)		10 (90.9)	1 (9.1)	p <0.05*
Others (n= 10)	Hd+	4 (40)	6 (60)	
	Hj+			
	Hd+z66+			
	Hj+z66+			
Total (n=21)		14 (66.7)	7 (33.3)	

*Fisher exact test

5. Conclusions

We conclude that patient harboring $Hd+Z66IND+$ gene of *S. Typhi* and High titer of IgG antibody anti-flagellum *S. Typhi* considered to be risk factor for typhoid carriers development.

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