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Distribution Flagellin Gene Variants of Salmonella Typhi in Patients with Typhoid Fever in West Kutai, East Kalimantan, Indonesia

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Abstract: *Background:* Virulence of *S. typhi* possessed an important factor for occurrence of typhoid fever in humans. Penetration of *S. typhi* in the intestinal mucosa is an important step in the establishment of infection because it allows microorganisms to pass through the epithelial barrier. This penetration is mostly determined by the motility of bacteria. Flagella are composed of a protein called flagellin that associated with the first stage of invasion which allows the bacteria to make direct contact with host cells. *Objectives:* To explore distributions of Salmonella typhi flagellin gene in effort to explain pathogenesis of typhoid fever in patients with typhoid fever in West Kutai, East Kalimantan. Indonesia. Method: This study was an observational study with cross sectional design. Blood samples collected in January 2011 to December 2012 in Damai District and Barong Tongkok District, West Kutai. Blood cultures performed in patients with suspected typhoid fever, based on clinical features determined by the medical personnel. All positive culture isolate were examine for Hd, Hj, z66 and *z66Ind* of flagellin genes by Polymerase Chain Reaction (PCR). *Results:* A total of 62 *S. typhi* isolates obtained from 425 patients with clinically suspected typhoid fever. All 62 (100%) samples found *fliC d*, *fljBz66* gene was found by 47 (75.81%) *z66Ind* 8 (12.9%) respectively and there was no samples had *fliC j*. This study shows that significant differences between flagellin gene variants in relation to the incidence of gastrointestinal bleeding (p = 0.034). *Conclusion:* We found three types of flagellin gene of *S. typhi* in West Kutai, they are *FliC d*, *FljBz66* and *z66Ind*. *S. typhi* containing *fliC d* genes provides the possibility 9 times more likely to cause gastrointestinal bleeding in patients with typhoid fever when compared with *S. typhi* containing *fljBz66* genes, and 17.5 times when compared with *z66Ind* gene.

Keyword: Salmonella *typhi*, Flagellin Gene, FliCd, *FljBz66*, *z66Ind*

1. Background

Typhoid fever is caused by *Salmonella enterica serovar typhi* (*S. typhi*), is a major public health problem, especially in developing countries [1, 2], including Indonesia. The amount of cases of typhoid fever in the world is very difficult to determine, because the disease is known to have clinical symptoms with a very broad spectrum [3]. World Health Organization estimates there are 17 million cases of typhoid fever worldwide with an incidence of 600,000 cases of deaths each year [4]. Incidence of typhoid fever in Indonesia is still high bringing Indonesia to country with fourth highest typhoid fever burden in the world. The disease is found throughout the year with annual morbidity rate reached 157/100,000 population in semi-rural areas and 810/100,000 population in urban areas and increasing every year [5, 6].

Virulence of *S. typhi* possessed an important factor the occurrence of typhoid fever in humans. Several virulence factors such as fimbria or villi found on the cell surface of *S. typhi* which playing role in the process of adhesion and colonization to the host cells [7]. Penetration of *S. typhi* in the intestinal mucosa is an important step in the establishment of infection because it allows microorganisms to pass through the

epithelial barrier. This penetration is mostly determined by the motility of bacteria. Flagella are composed of a protein called flagellin that also serves as an antigen. Flagella associated with the first stage invasion which allows the bacteria to make direct contact with host cells [8, 9]. In most of Salmonella, there are two genes encode the flagella antigen, they are FliC and fljB. FliC gene can express two types of flagellin namely flagellin Hd and Hj [10].

Very limited studies have been conducted to determine the population of the gene encoding the flagelllin. In this study we explore the distribution of flagellin gene variants of *S. typhi* spreading in the population, particularly in the West Kutai District, East Kalimantan.

2. Method

This study was an observational study with cross sectional design. Blood samples collected in January 2011 to December 2012 in the Damai District and Barong Tongkok District, West Kutai. Blood cultures performed in patients with suspected typhoid fever, based on clinical features determined by the medical personnel who treated the patient. The patient's blood cultures performed in Department of Microbiology Medical Faculty Mulawarman University and Provincial Laboratory Ministry of Health East Kalimantan. For bacterial motility and molecular biology examination conducted at the Laboratory of Microbiology and Molecular Biology Medical Faculty Hasanuddin University Makassar, Indonesia. Ethical clearance was approved by the review boards of the participating institutes and informed consent was obtained from all participants or their parents/guardians.

2.1. Blood Culture

Five milliliters of freshly collected blood was was taken aseptically and then inserted into the BACTEC transport medium and incubated at 37°C for 24-48 hours. One milliliter of this culture was then plated on Salmonella Shigella (SS) agar (Oxoid, Basingstoke, United Kingdom), incubated for 24 hours at 37°C, and examined for growth. If growth was present, individual colonies were examined by Gram staining and identification of the bacteria was performed after subculturing on SS agar by biochemical testing with the triple sugar iron test, sulfide indole motility, methyl red Voges' Proskauer reactivity, citrate consumption, urease and decarboxylase activity, and carbohydrate fermentation of glucose, lactose, mannitol, sucrose, and arabinose.

2.2. Preparation of DNA

DNA was extracted from freshly collected culture of *S. typhi* according to the diatom guanidinium isothiocyanate (GuSCN) method. For the extraction of DNA from culture, a freshly single colony of *S. typhi* sample was mixed with 900uL of lysis buffer (50mM Tris-HCl, 5.25 M GuSCN, 20mM EDTA, 0.1% Triton X-100) and centrifuged at 12,000 × g for 10 minutes. To obtain the DNA, samples were lysed by incubation for 15 minutes at 18°C and 20uL of diatom suspension was added. The diatom containing the bound DNA was sedimented by centrifugation at 2,000× 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 60uL of 10mM Tris-HCl, pH 8.0, 1mM 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 [14]. All these PCRs should work clearly on genomic DNA template from the diatom-guanidinium isothiocyanate (GuSCN) extraction method.

2.3. Amplification of Flagellin Gene

The fliC primers set for the fliC genes amplification, which will give a result of around 1500bp for the H:d antigen or about 200bp smaller for the Hj 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-. Amplification of fliC gene was performed using primers: fliC F: TTAACGCAGTAAAGAGAG and fliC_R: ATGGCACAAGTCATTAATAC and produce a 1521bp product for the d allele and a 1273bp product for the j allele. Amplification of the *fljBz66* was performed as previously described using z66Flag_F: ATGGCACAAGTCATCAATAC and z66Flag_R: TTAACGCAGCAGAGACAGTAC. Control PCR amplicons from the aroC gene were produced using primers aroC for: CCTGGCACCTCGCGCTATAC and aroC rev: 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 is 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, flowed by an extension step of 72°C for 2 min [5]. 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 597bp 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, 12]. The Ind-specific PCR was performed with after an initial denaturation at 94°C for 2 min, for 35 cycles at 94°C for 30s, 55°C for 30s, and 72°C for 1 min, followed by an extension step of 72°C for 5 min [11].

3. Results

3.1. Distribution of S. typhi Positive Culture

A total of 62 *S. typhi* isolates obtained from 425 patients with clinically suspected typhoid fever. In the District of

Damai we found 191 patients with suspected to typhoid fever, and as many as 27 (14:14 %) were confirmed with positive cultures. while at the District Barong Tongko there are 234 patients with suspected typhoid fever and found 35 (14.96 %) positive (Table 1). In Table 1 shows that the positive blood culture only 14:59 % of 425 suspect cases examined.

Table 1. *Distribution of patients with suspected fever typhoid.*

Districts	No. Suspects	Positive Culture
Damai Districts	191	27(14.14%)
Barong Tongkok Districts	234	35(14.96%)
Total	425	62(14.59%)

3.2. Distribution of Flagellin Gene Variants in West Kutai

Detection of flagellin gene performed by PCR using primers fliC_F5'-TTAACGCAGTAAAGAGAG-3 'fliC_R 5'-ATG GCA ATT CAA AATAC GTC-3' which will produce all the 1,521 bp fragment for the gene encoding Hd and 1,273 bp for the Hj. Detection of flagellin gene encoding Z66 gene using a primer z66_F 5'ATG CAA GCA GTC AATAC ATC-3 'and z66_R 5'TTAACGCAGCAGAGACAGTAC-3' which will generate 1500 bp fragment. Detection of *z66Ind* using Ind-F: 5 'ATG TCG GAA ATC AAC CGT ATC T 3' and Ind-R: 5 'CAG GCC GTC AAC CTG AGA C 3' that will produce fragments 597 bp [11, 13]. Results of detection of *S. typhi* flagellin gene showed in figure 1 and 2 below.

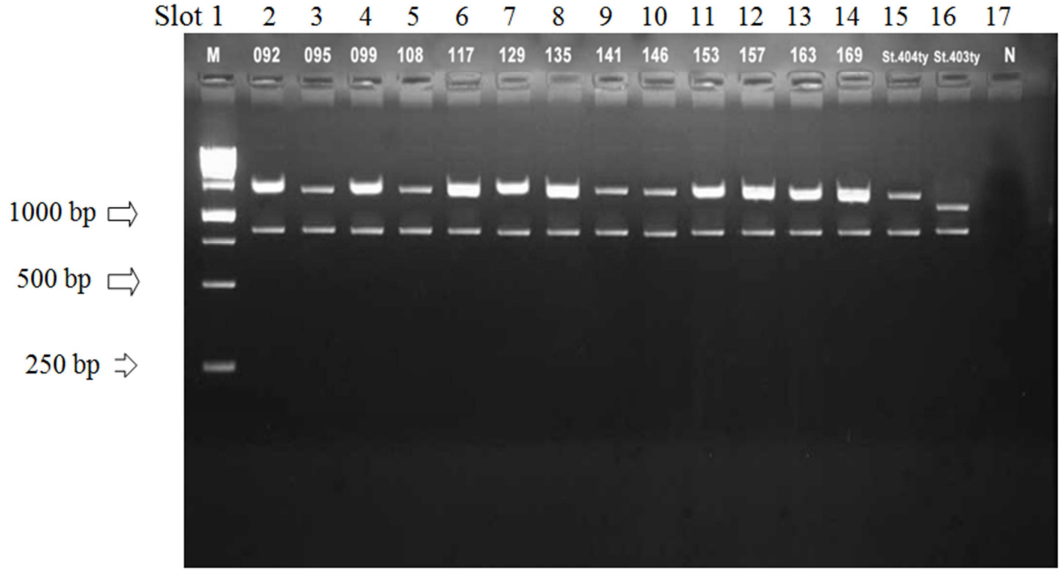

Figure 1. *Electrophoresis PCR products with AroC Primer that produce fragments of 800 bp, fliC fragments 1521 bp for the Hd, fragments 1273 to Hj, and z66 1500 bp fragments (slot 1 = Marker 1 kb, slot 2-14 = sample, slot 15 = fliC d positive control, slot 16 = fliC j positive control, slot 17 = negative control).*

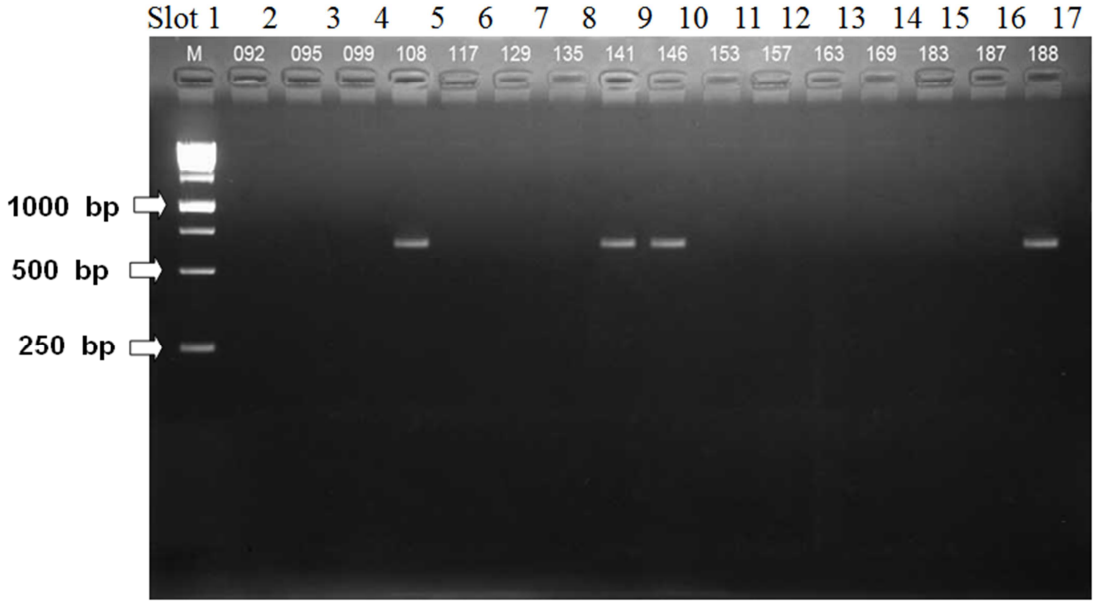

Figure 2. *Electrophoresis of PCR product of z66Ind with 597bp fragments (slot 1 = Marker 1 kb, slots 2-17 = samples).*

Table 2. *Distribution of flagellin gene S. typhi in West Kutai.*

Flagellin genes	Positive isolates		
	Damai district	Barong Tongkok district	Total
fliC d	2(28.6%)	5(71.4%)	7(11.29%) (SD=0.3)
fliC j	0	0	0 (SD=0.0)
fliC d + fljBz66	25(53.2%)	22(46.8%)	47(75.81%) (SD=0.4)
fliC d + z66Ind	0	8(100%)	8(12.9%) (SD=0.3)
Total	27(43.5%)	35(56.5%)	62(100%)

Table 2 shows that all 62 (100%) samples found *fliC d*, *fljBz66* gene was found by 47 (75.81%) *z66Ind* 8 (12.9%) and no (0%) samples had *fliC j*, This result is slightly different with previous studies in several places in Indonesia, because *fliC j* genes was not found. Sabir et al., (2014) in Palu found *FliC d*, *fliC j*, *fljBz66* and *z66Ind*; 9.9%, 22.7%, 39.7% and 27.7% respectively. Baker et al., 2008 in Jatinegara find where *fliC d*, *fliC j* and *fljBz66* by 85 (61%), 55 (40%), and 59 (42%) respectively. Research conducted by Hatta et al., 2011 on isolates originating from Eastern Indonesian Islands find *fliC d* in 100% of samples, *fljBz66* 15.4%, *z66Ind* 21.8%, but *fliC j* was not found. Study conducted by Song et al. in Korea on 375 isolates of *S. typhi* found only one isolate that has Hj but it considered Indonesian strain because the patient is showing symptoms of typhoid fever while in Indonesia.

In this study we also analyzed the relationship between flagellin gene variants between typhoid fever patients with gastrointestinal bleeding complications presented in Table 3.

Table 3. *Relationship between flagellin gene variants between typhoid fever patients with gastrointestinal bleeding complications.*

Flagellin gene variants	Gastrointestinal bleeding complications				P	OR	CI 95%
	Positif		negatif				
	n	%	n	%			
fliC d + z66Ind	1	12.5	7	87.5	0.035	17.500	(1.22; 250.36)
fliC d + fljBz66	10	21.3	37	78.7	0.014	9.250	(1.556; 54.98)
fliC d	5	71.4	2	28.6	Ref		
Total	16	25.8	46	74.2	0.034		

In Table 3 shows that significant differences between flagellin gene variants in relation to the incidence of gastrointestinal bleeding. (p = 0.034). Furthermore, no significant difference (p = 0.014) between *fliC d* with *fljBz66* with OR at 9.25, there was a significant difference (p = 0.035) between *fliC d* with *z66Ind* with OR at 17.50. These results indicate that the presence of flagellin gene *fliC d* likely to cause gastrointestinal bleeding of 9.2 times greater when compared with the presence of the gene *fljBz66* and 17.5 times greater when compared with the presence of the gene *z66Ind*. Similar results were conducted by Grossman (1995) on isolates of *S. typhi* in Jakarta, Yogyakarta, Palembang and Surabaya, showed a correlation between motility and the presence of flagellin gene variants. Hd strains show motility and invasiveness higher than Hj [14]. Likewise with the clinical picture caused by strain Hd worse than the serotype Hj. Chanh (2004) concluded that the deletions on Hj strain causing a less efficient interaction with cell surface receptors, thereby reducing the ability of invasive and virulence of this serotype [15].

4. Discussion

Results of this study indicate that there are genetic biodiversity on flagellin genes in Kutai Barat. There are 3 variations of flagellin gene that is *fliC d*, *fljBz66* and *z66Ind*. *fljBz66* and *z66Ind* not commonly found in other countries. The results support the previous information has also been carried out in several major cities in Indonesia including Jakarta, Yogyakarta, Palembang, Surabaya, Makassar and Palu. An interesting result of this study was Hj genes that are not found as frequently found in other regions of Indonesia. Intensive research conducted in Africa, India, Israel, Mexico, Madagascar, Nepal, Singapore, Thailand, the United States and other countries have failed to find any gene encoding Hj. Indonesia with a high incidence of typhoid fever Hd and Hj commonly found, but absence of Hj in West Kutai may relate on a mechanism of flagellin genes regulated by fljA.

According to Grossman, et al., 1995 and Baker et al., 2008, basically Hj genes are highly homologous with Hd, except for deletion of 261 bp in the central part *fliC j* genes which is responsible for the flagellin gene variation [5, 14]. This deletion occurs as a result of homologous recombination intragenic involving the repetition of 11 bp. Flagellin gene expression on Salmonella is controlled by repeated inversion of DNA segments called H segment, which contains promoter fljB. H inversion occurs through a specific part recombination between inverted repeated sequence flanking H segments. FljB and fljA gene construct operon that encodes a negative regulator for FliC expression. FljA gene inhibits the expression of FliC through post transcriptional control mechanisms [16-18].

Z66 gene encoded by extra chromosomal DNA or plasmid is called pBSSB1 [5]. This is different from Hd and Hj encoded on the chromosome. Zu, et al. reported a difference in expression z66 and FliC on osmotic pressure, bile acids and oxidative stress [19]. Flagelin gene variations found in this study indicate that there is a process of *S. typhi* adaptation on chromosomes and plasmids. Changes in the DNA are a bacterial adaptation in an effort to defend them gradually and continuously. Adaptation of living organisms including bacteria to environmental changes is a response to natural selection for survival. According to Okazaki et al., Genetic processes between flagellin genes for example in point mutation, deletion and insertion as a phenomenon that can describe the lateral transfer of genetic material that produces inter specific recombination between flagellin genes. In addition to these processes, the interaction between

genes fliC and fliB also considered one of the causes of biodiversity flagellin genes in Salmonella serovar [20].

Controversy of several studies on the correlation between the type of flagellin and the motility or the virulence properties of *S. typhi* triggers intensive research by experts to undertake a thorough study of the motility and invasiveness of *S. typhi* in vivo. Flagella mediated motility is one of the factors that play a role in the invasion of the host cell. Flagella function associated with the first stage invasion in which motility and chemotaxis power causing bacteria make direct contact with host cells. Reduced function of motility in serotypes Hj as a result of changes in the function of flagella would result in reducing invasion power.

5. Conclusion

In this study, we found three types of flagellin gene of *S. typhi* in West Kutai, namely; *fliC d*, *fljBz66* and *z66Ind*. The absence fliC j gene of S. typhi in West Kutai indicate that this event is not common in Indonesia, it would be a challenge for further research whether flagellin gene variation S. typhi is the original clone or clones that are distributed from the surrounding areas.

S. typhi with fliC d more invasive compared to others, it can be shown on these data where *fliC d* containing genes provides 9 times more likely to cause gastrointestinal bleeding in patients with typhoid fever when compared with *S. typhi* containing *fljBz66* genes, and 17.5 times when compared with *z66Ind* gene.

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