Molecular Characterization And Hemagglutination Activities of Flagellin Protein of *Salmonella typhi*

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Abstract. The purposes of this research are for molecular characterization and hemagglutination activity test of flagellin protein of Salmonella typhi. The research samples consist of 7 strains of S. typhi isolates from Central Java (5 strains from Semarang city, 1 strain from Salatiga and 1 strain from Magelang) and 2 strains of S. typhi from Yogyakarta (Doctor Sardjito Hospital and Bethesda Hospital). The undertaking procedures are: 1) PCR and sequencing of fliC genes using primer LPW 1856 and LPW 1857.2) Isolation and separation of flagellin protein using SDS-PAGE. 3) Hemagglutination Activity Test upon human erythrocytes of blood group A, B, AB and O.The results show that 8 strains of S. typhi have a fliC gene size of 1452 to 1488 bp including serovar H1-d, and 1 strain with the size of 1267 bp including serovar H1-J. Flagella protein resulted from SDS-PAGE protein consists of 1-2 major proteins and 1-3 minor proteins with a molecular weight of 16-116 kDa. The results of hemagglutination activity test of flagellin protein show that there are 3 strains of S. typhi (MG-1, SA02.2 and BET) which are able to agglutinate human erythrocytes of blood group A, B, AB and O (2-64HA), 6 other strains show various hemagglutination activities varied

INTRODUCTION

Salmonella typhi (S. typhi) is a rod shape and gram-negative bacteria causing systemic infections to humans and animals known as typhoid fever (Yang *et al.* 2012). Its Pathogenity highly depends on the number of virulence factors, such as adhesion (attaching) ability to the cell host, which facilitates bacteria to attach in the small intestine mucosa (Jindal *et al*., 2012; Alexan *et al*. 2009). Bacterial adhesion of *S. typhi* on the cell host is also performed by hemagglutinin protein, as the beginning of pathogenesis (Darmawati & Anwar, 2008). Hemagglutinin protein is a protein which is able to agglutinate erythrocytes, due to its ability to recognize receptors owned by the erythrocyte membranes. Darmawati and Anwar (2008) state that hemagglutinin proteins of some strains of *S. typhi* from Java have various hemagglutination activities upon the erythrocytes of mice.

The other virulence factor is flagella, composed of protein flagellin subunit, and in some bacteria, plays an important role in its life, serving as a means of motion and helping bacteria to get into the cell host (Darmawati, S. & Evy Prastiyanto 2014; Hatta et al. 2011). Flagellin subunits are targets which may be recognized by the natural body immune system through Toll-like Receptor (TLR) 5 (Baker *et al.* 2007). In addition, flagellin may stimulate the adaptive immune system (Alexan *et al.* 2009). Most *S. typhi* only have flagellin genes known as *fliC* which encodes flagella antigen phase 1 (Hd antigen). However, Indonesian *S. typhi* isolates express H-j antigen known as flagella antigen z66 (flagella antigen phase 2) encoded by the flj^{Bz66}, that is, *fliC* genes which experience deletion in the hypervariable region of linear plasmid (Hatta *et al*., 2011). Based on flagellin genes owned by *S. typhi* in Indonesia, it shows genetic diversity which results in the expressed protein diversity.

Thus, this research aims to perform molecular characterization and hemagglutination activity test of flagellin protein of S. typhi Isolates from Central Java and Yogyakarta. Molecular Characterization of flagellin genes covers the size and profile of flagellin protein subunit, while hemagglutination activity test is conducted upon human erythrocytes of ABO blood group system.

METHODS

Research samples

The research samples consist of seven strains of *S. typhi* isolates from Central Java (5 strains from Semarang, 1 strain from Salatiga, 1 strain from Magelang), 2 strains of *S. typhi* from Yogyakarta (1 strain from Doctor Sarjito Hospital and 1 strain from Bethesda Hospital). Those nine strains of *S. typhi* are isolated from blood cultures of patients with positive Widal (Darmawati *et al.*, 2011)

PCR and sequencing of *fliC* genes of *S. Typhi*

DNA Isolation of bacterial genomes is conducted using *DNeasy Blood and Tissue Kits* (Qiagen catalog number 69504). Primary LPW 1856 **(**5'ATGGCACAAGTCATTAATACAAAC-3') and LPW 1857 **(**5'-TTAACGCAGTAAAGAGAGGACGTT-3') are used for amplification of *fliC* genes (Lau *et al.*, 2005). The reagent used for amplification of *fliC* genes with a method of *Polymerase Chain Reaction* (PCR) is Maxima Hot Start Green PCR Master Mix (2X) (Thermo Scientific, K1061) while the size used is 12.5 µl of master Mix, 1 µl of each primary LPW 1856 and LPW 1857, 1 µl of DNA template, 7.5 µl of sterile dH_2O, 25 µl of each tube volume, and the device used is *Applied Biosystems GeneAmp PCR System 2400*.

The amplification of *fliC* genes is conducted in a total of 30 cycles with conditions: a temperature of 95 ^{0}C for 30 seconds to perform DNA denaturation, a temperature of 46 ^{0}C for 30 seconds to perform DNA template annealing process (primary LPW 1856 and LPW 1857), for extension at 72 ˚C for 2 minutes with a *final extension* at a temperature of 72˚C for 10 minutes to perform DNA polymerization process. The results of DNA fragment amplification are separated by 1% of *Agarose Gel Electrophoresis* based on single band appearance at 1500bp. The amplicon visualization is conducted using *Major Science UV transluminator*. DNA sequencing is performed on a sequencer device of *ABI Prism™ 310*.

Isolation and SDS-PAGE of flagellin protein

Flagellin protein isolation is conducted using a modified method of Alexan *et al*. (2009). Flagellin protein is obtained by growing a bacterial colony of Mac Conkey media in 50ml BHI liquid medium, incubated at 37 ^{0}C for 48 hours with agitation used as a starter. The starter is then put into 500 mL of BHI media, incubated at 37 ^{0}C for 48 hours with agitation. The bacterial culture is then centrifuged at a temperature of 4 ^{0}C, with a speed of 3000 rpm for 20 minutes. Pellet is suspended with 5 mL of physiologic solution until it turns to be a thick suspension, then the suspension acidity is set into pH level of 2 by adding 1M of HCl plus, stirred for 30 minutes at room temperature, and centrifuged at 3000 rpm for 30 minutes. Supernatant containing flagellin protein is then added with 1M of NaOH that the pH level turns to be 7.2.

The isolates of Flagellin protein from Central Java and Yogyakarta are then separated using SDS-PAGE (12%) to see the protein profiles, by staining of *0.1% Coomassie Brilliant Blue R-250*. Flagellin protein is then dried-frozen and used for hemagglutination test.

Haemagglutination test

Human erythrocyte hemagglutination test is conducted using a method of Hanne and Finkeltein (Booth *et al.*, 1983). Samples are multiply diluted with PBS on micro agglutination plate with a volume of 50 µl (50 µg/ml), and then each well is added by 50 µl of 1.0% human erythrocytes in PBS. The micro agglutination plate is then shaken for 1 minute, incubated at a room temperature for 1 hour and the occurrence of hemagglutination is observed. Hemagglutination titer (HA) is shown by the opposite of the highest dilution number that still showing the occurrence of hemagglutination.

RESULTS AND DISCUSSIONS

fliC Genes of S. typhi

The PCR results of *fliC* flagellin genes using primer LPW 1856 and LPW 1857, which is then electrophoresed using 1% agarose is shown in Figure 1, while the gene size based on the sequencing results is shown in Table 1. The results show that the tape size on PCR results of *fliC* genes of a strain of *S. typhi* SLT.1

isolates from Salatiga is equal to 1260 bp, looking different with the tapes of the other eight strains of *S. typhi* with a size equal to 1500bp. This is similar to the results of research conducted by (Frankel *et al.* 1989; Lau *et al.*, 2005; Baker *et al.* 2007) that the *fliC* flagellin gene with a size equal to 1267 bp encoding flagellin protein H1-J of *S. typhi* serovar H1-j, these bacteria are less motile and less invasive when compared with *S. typhi* serovar H1-d.

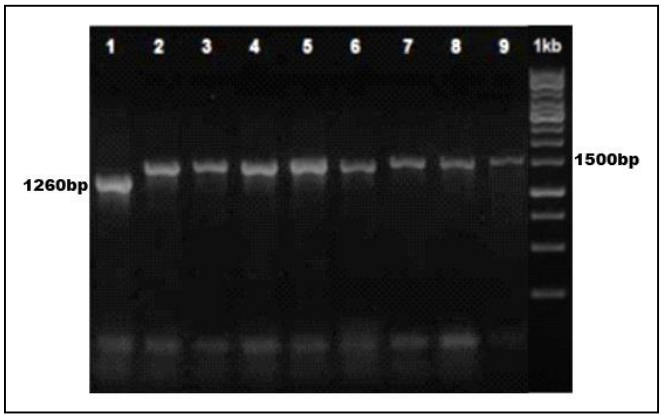

Figure 1. The PCR results of *fliC* genes of 9 strains of S. typhi, respectively: 1) SLT-1, 2) BA07.4, 3) MG-1, 4) SA02.2, 5) EM-3, 6) KD30.3, 7) KD 27.2, 8) BET, 9) SRJ, Marker (Darmawati & Prasetiyanto. 2014)

Flagellin protein of*S. typhi* Serovar H1-j is expressed by *fliC* genes experiences deletion of 260 bp that the size is only approximately 1267 bp (Table 1). *Salmonella typhi* serovar H1-j is only found in Indonesia. Thus, from those 9 strains of *S. typhi* strains, there is one strain of H1-J isolates from Salatiga and 8 strains of H1-d.

Table 1. The Size of flagellin *fliC* genes of 9 isolates of *S. typhi* from Central Java and Yogyakarta based on results of sequencing

Strain	Origin	*fliC* gene (bp)
S. typhi SLT-1	Salatiga	1267 bp
S. typhi BA 07.4	Semarang	1458 bp
S. typhi MG-1	Semarang	1454 bp
S. typhi SA 02.2	Semarang	1464 bp
S. typhi EM 3	Semarang	1456 bp
S. typhi KD 30.3	Semarang	1452 bp
S. typhi KD 27.2	Semarang	1456 bp
S. typhi BET	Yogyakarta	1454 bp
S. typhi SRJ	Yogyakarta	1488 bp

Flagellin protein profile

Flagellin protein from 9 strains of *S. typhi* after separated using SDS-PAGE 12% shows that there are 1-2 major proteins and 1-3 minor proteins (Figure 2). The molecular weight of protein subunits arranging flagellin starts from 16-116kDa.

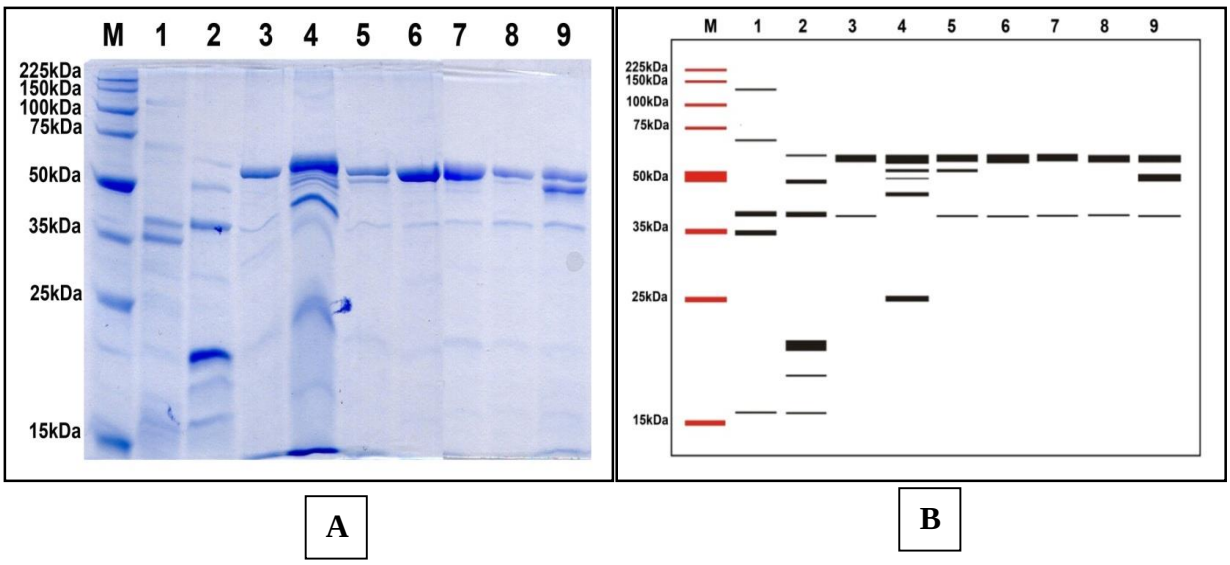

Figure 2. SDS-PAGE protein flagellin Profile in (A) and (B) of 9 strains of bacterial isolates of *S. typhi* consisting of 7 isolates from Central Java and 2 isolates from Yogyakarta respectively M) protein Marker, 1) *S. typhi* BA07.4, 2) SLT-1, 3) MG-1, 4) SA02.2, 5) EM-3, 6) KD30.3, 7) KD 27.2, 8) BET, 9) SRJ

Major protein tapes with a molecular weight of 60kDa are owned by 7 strains of isolates from Central Java and Yogyakarta, excluding *S. typhi* flagellin BA07.4 and SLT-1. This is irrelevant with a research conducted by Alexan *et al.* (2009) stating that flagellin protein of S. typhi which is isolated from chickens with diarrhea, consists of one major protein tape (54.11 kDa) and 3 minor protein tapes (41 kDa; 36.6 kDa and 25.7 kDa). The differences of flagellin protein profile from different strains show that there are genetic variations in flagellin genes owned by each strain. The differences of Flagellin protein profile probably results in virulence differences when playing its roles causing the occurrence of pathogenicity.

Table 2. Flagellin protein profile of 9 Strains of *S. typhi* from Central Java and Yogyakarta based on SDS-PAGE

No.	flagellin protein character (kDa)	*S. typhi* BA07.4	*S. typhi* SLT-1	*S. typhi* MG-1	*S. typhi* SA02.2	*S. typhi* EM-3	*S. typhi* KD30.3	*S. typhi* KD27.2	*S. typhi* BET	*S. typhi* SRJ
1	116	+	0	0	0	0	0	0	0	0
2	70	+	0	0	0	0	0	0	0	0
3	60	0	+	+	+	+	+	+	+	+
4	52	0	0	0	+	+	0	0	0	0
5	46	0	+	0	+	0	0	0	0	+
6	42	0	0	0	+	0	0	0	0	0
7	36	+	+	+	0	+	+	+	+	+
8	35	+	0	0	0	0	0	0	0	0
9	25	0	0	0	+	0	0	0	0	0
10	20	0	+	0	0	0	0	0	0	0
11	18	0	+	0	0	0	0	0	0	0
12	16	+	+	0	0	0	0	0	0	0

Note: (+) has, (0) does not have

Haemagglutination Activities

The results of hemagglutination (HA) Test of flagellin protein of 9 bacterial strains of *S. typhi* upon human erythrocytes of blood group A, B, AB and O show that the strains having hemagglutination activities upon human erythrocytes of 4 blood types are *S. typhi* MG-1, SA02.2, and BET (Figure 3 and Table 3).

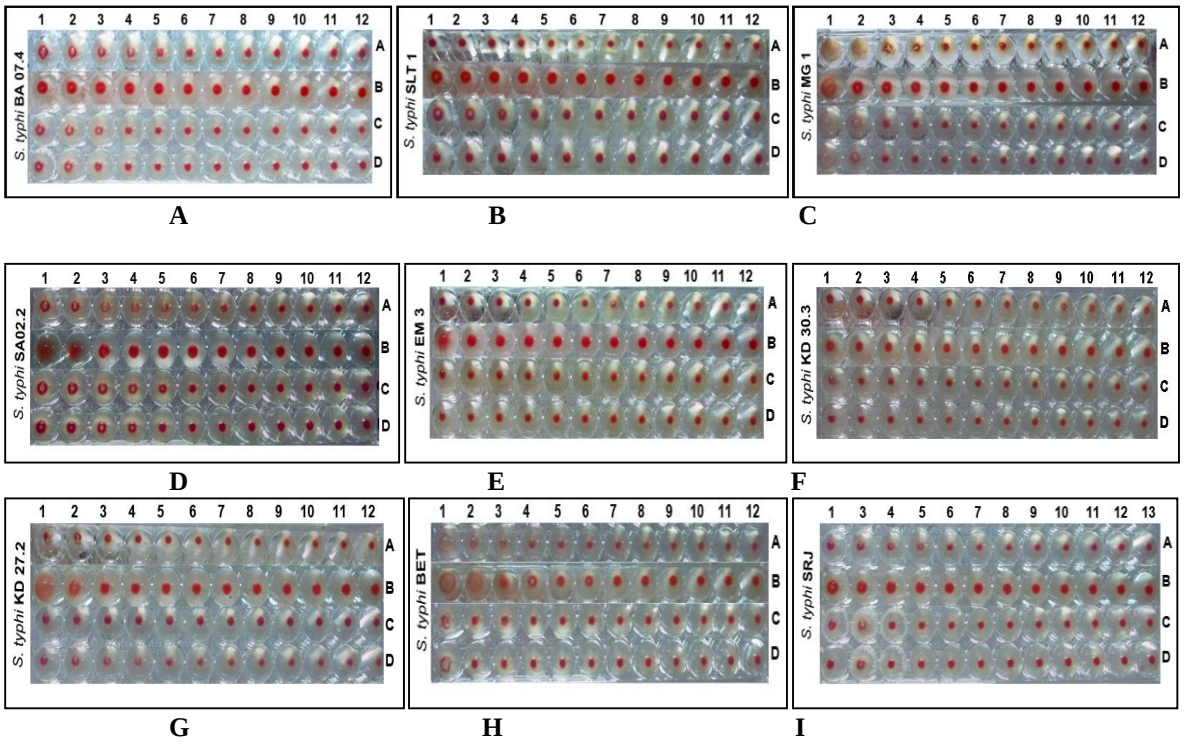

Figure 3. Flagellin protein hemagglutination activities upon human red blood cells of blood group A, B, AB and O of 9 strains of *S. typhi* isolates from Central Java and Yogyakarta respectively A) BA07.4, B) SLT-1, C) MG-1, D) SA02.2, E) EM-3, F) KD30.3, G) KD 27.2, H) BET, I) SRJ

Flagellin protein ability to agglutinate erythrocytes is due to its ability to recognize receptors on the surface of erythrocytes owned.

Table 3. Flagellin protein hemagglutination activities upon human red blood cells of blood group A, B, AB and O of 9 strains of S. typhi isolates from Central Java and Yogyakarta.

No.	Golongan Darah	*S. typhi* BA07.4	*S. typhi* SLT-1	*S. typhi* MG-1	*S. typhi* SA02.2	*S. typhi* EM-3	*S. typhi* KD30.3	*S. typhi* KD27.2	*S. typhi* BET	*S. typhi* SRJ
		Titer hemaglutinasi (HA)								
1	A	16	–	16	64	–	–	4	8	–
2	B	–	2	2	4	2	2	4	16	2
3	AB	8	4	4	16	–	2	–	2	4
4	O	2	–	4	16	–	–	–	2	4

CONCLUSION

the research results on Molecular characterization and hemagglutination activities of flagellin protein of *Salmonella typhi* Isolates from Central Java and Yogyakarta show that: There are 8 strains of *S. typhi* which have a *fliC* gene size of 1452-1488 bp including serovar H1-d, and 1 strain with the size of 1267 bp including serovar H1-J. Flagella protein resulted from SDS-PAGE consists of 1-2 major proteins and 1-3 minor proteins with a molecular weight of 16-116 kDa. The results of hemagglutination activity test of flagellin protein show that there are three strains of *S. typhi* (MG-1, SA02.2 and BET) which are able to agglutinate erythrocytes of blood group A, B, AB and O (2-64HA), while six other strains show various hemagglutination activities. Thus, showing flagellin protein variations expressed by *S. typhi* from different strains indicate the presence of genetic variations.

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