New Flagellin Gene for *Salmonella enterica* serovar Typhi from the East Indonesian Archipelago

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Abstract. Phase variation is a property unique of some *Salmonella enterica* serovar Typhi strains from Indonesia. *Salmonella* Typhi isolates from Indonesia have been described that in addition to the phase 1 Hd flagellin gene contain a second flagellin gene named z66. *S. Typhi* isolates from Indonesia with a mutant Hd gene named Hj have also been described. Here, we have identified another flagellin gene of *S. Typhi*, named Ind, showing a closest homology with the flagellin gene of *Serratia marcescens*. The Ind gene was detected in 21.8% of the *S. Typhi* isolates from the East Indonesian archipelago, all of which contained the Hd gene. The Hj gene was not detected. The z66 gene was present in 15.4% of the isolates. The presence of these “foreign” flagellin genes could be associated with an increased risk for developing severe disease.

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Typhi is the causative agent of typhoid fever. The Gram-negative rod-shaped bacterium is pathogenic only in humans.¹ Infection occurs when water or food contaminated with the pathogen is consumed. *Salmonella* Typhi invasion occurs through the terminal ileum and in analogy with *Salmonella enterica* serovar Typhimurium, likely through M cells on Peyer’s patches. Subsequently, *S. Typhi* is transferred to monocytes and then traffics to cells of the reticulo-endothelial system where it may stay in a semi-dormant state. Possibly after reactivation by the immune system the bacterium re-enters the blood stream and disseminates into the liver, the gall bladder, and the bone marrow. Flagella provide the *S. Typhi* with motility and may play a role in cell entry, are down regulated inside cells, and it has been suggested that they are used for escape from an intracellular site.^{2,3} Flagella also are an important target of the innate immunity. The majority of *Salmonella enterica* serovars are biphasic, alternating between the expression of two flagellar antigens, encoded by the phase 1 and the phase 2 flagellin genes *fliC* and *fliB*.⁴ The genome of *S. Typhi* possesses only the phase 1 *fliC* gene, which encodes the Hd antigen. However, some *S. Typhi* isolates from Indonesia express an alternative H antigen, known as Hj, and or a second flagellin, called z66.⁵ The Hj antigen arises through a deletion in the hypervariable core region of the *fliC* gene, and the z66 flagellin antigen is encoded by a gene called *fliB^{z66}* located on a linear plasmid.⁶ The z66 flagellum is considered the equivalent of the phase 2 flagellum as it was found that *S. Typhi* isolates from Indonesia that are Hd and Hj antigen negative but are motile revert to Hd or Hj positivity when treated with an anti z66 antiserum.⁷ This study was initiated to determine the presence of the different flagellin genes in *S. Typhi* isolates from three of the major islands in the east Indonesian archipelago.

MATERIALS AND METHODS

Clinical specimens and patient population. Between January and June 2010, *S. Typhi* isolates were obtained from blood cultures from 136 acute febrile patients with clinical suspicion of typhoid fever presented at different hospitals and clinics in and around Makassar in South Sulawesi, in Jayapura in Papua, and in Samarinda in East Kalimantan, and 26 isolates from each of these islands were selected without knowledge of the clinical information. All isolates were confirmed to be serovar Typhi by biochemical testing and nested polymerase chain reaction (PCR) using *S. Typhi*-specific primers.^{8,9} The mean age of the patients was 31.3 years (range, 17–20) and the male to female ratio was 0.95. The duration of fever at hospital admission was 5.8 days (range, 4–9) and the average body temperature was 38.4°C (range, 37.7–39.6). The general disease condition was considered severe in five patients (6.4%) with apathy in three patients and stupor in two patients. One patient with a moderate disease condition also presented with stupor. None of the patients had respiratory involvement and the main symptoms and signs were coated tongue (96.3%), malaise (93.8%), headache (83.8%), nausea (27.5%), constipation (12.5%), abdominal distention (12.5%), diarrhea (11.3%), and melena (8.8%). The clinical information had been collected prospectively using a structured questionnaire that was completed by the responsible physician or nurse.

Ethical considerations. The project was approved by the review boards of the participating institutes and informed consent was obtained from all participants or their parents/guardians.

PCR amplification and detection. The PCRs were performed in a 25 µL reaction mixture containing 1 µL silica-guanidinium isothiocyanate purified *S. Typhi* DNA, 5 pmol of each primer and 22.5 µL of the Platinum PCR Supermix (Invitrogen, Carlsbad, CA) with the PCR cycle conditions specified below for each of the different primer sets.¹⁰ Amplicons were sized on a 10 cm 1% agarose gel, stained with ethidium bromide and viewed under UV illumination.

PCR detection of the Hd and Hj genes. Initially, the primers I and II described by Frankel and coworkers¹¹ were used for the PCR amplification of the Hd and Hj expressing *fliC* genes. The PCR amplification using these primers produces a 1,530 bp product in case of the Hd gene and a 1,269 bp product

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in case of the H_j gene. After an initial denaturation at 94°C for 2 min, the PCR reaction was performed for 35 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 150 s, followed by an extension step of 72°C for 7 min. Because of difficulties in the amplification of these relatively long DNA stretched for some of the DNA isolates, two new PCR primers were designed and designated Hd/j-F (5' GAA ATC GAC CGT GTA TCC GG 3') and Hd/j-R (5' CAA CCT GTG CCA AGG CAG C3'), which are predicted to produce in a PCR a 927 bp product in case of the Hd gene and a 666 bp product in case of the H_j gene. After an initial denaturation at 94°C for 2 min, the PCR reaction was performed 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.

PCR amplification of the z66 and detection and characterization of a novel flagellin gene. The z66flag_F and z66flag_R primers described by Baker and coworkers⁶ were used for PCR amplification of the full-length 1.467 nucleotides (nt) long z66 gene. After an initial denaturation at 94°C for 2 min, the PCR reaction was performed for 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 150 s, followed by an extension step of 72°C for 7 min. After an initial analysis of seven *S. Typhi* isolates (isolates T503, T548, and T566 from Sulawesi and isolates T521, T531, T548, and T561 from Papua) three isolates that did not yield the expected 1.467 bp product produced a distinct product of ~1.050 bp. Bidirectional sequence analysis of the shorter product and nucleotide alignment with the z66 gene revealed that the product had a length of 1.044 nt and showed some homology with the z66 gene. This shorter gene was tentatively named Ind (GenBank accession no. HQ645960). The three isolates that amplified the 1.044 bp product were obtained from patients from Makassar, the capital of South-Sulawesi, and the isolates that amplified the 1.467 bp were from Jayapura, the capital of Papua. Sequence analysis was performed for the Ind gene of three independent *S. Typhi* isolates and all showed 100% identity. In further studies the primers PZ66-A and PZ66-B developed by Huang and coworkers¹² were used for the specific amplification of a 375 nt segment of the z66 gene and two new primers 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 nt 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. The z66-specific PCR was performed with after an initial denaturation at 94°C for 2 min, for 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by an extension step of 72°C for 7 min. 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.

PCR amplification of terminal inverted repeat sequences. Many linear plasmids are characterized by the presence of terminal inverted repeats (tir). The pBSSB1 tir-specific primer pairs a/c and b/d described by Baker and coworkers⁶ were used for the amplification of the two tirs of pBSSB1 harboring the z66 gene. After an initial denaturation at 94°C for 2 min,

the PCR reaction for the a/c primer pair was performed 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. For the b/d primer pair the PCR conditions was the same except that the primer annealing step was performed at 50°C.

RESULTS

The result of the different PCRs for the flagellin genes is illustrated in Figure 1 for a selection of seven *S. Typhi* isolates. The PCR using the z66flag_F/R primer pair amplified in three of these isolates a 1.044 bp product revealing the presence of a previously unknown flagellin gene here named Ind. Sequence analysis of the 1.044 bp PCR product showed some homology with the z66 gene with 82% homology for the first 468 nt at the 5' end of the gene and 85% homology for the last 233 nt at the 3' end of the gene. The internal portion of the Ind gene did not show homology with z66. The Ind gene could be specifically detected using the Ind-F/R primer pair.

A comparison of the amino acid sequence of Ind with those of Hd and z66 revealed several amino acid substitutions in the conserved amino- and carboxy-terminal regions of the proteins (Figure 2). A three amino acid deletion at the junction between the variable central region and the conserved carboxy-terminal region was also noted. The central heterogeneous part of Ind is 86 amino acids long compared with 224 amino acids for z66 and 158 amino acids for Hd. The highest sequence homology for the Ind gene was found for the nucleotide sequence of the *fliC* gene of *Serratia marcescens*. A 95% identity was observed with 72% of this gene and an 89% identity was observed with 72% of the *Edwardsiella tarda fliC* gene. The homology was observed for the conserved 5' and 3' portions of the genes. Furthermore, a stretch of 102 nt in the 5' portion of the heterogeneous central part of the Ind gene showed 72% identity with the *S. marcescens fliC* gene and a nucleotide stretch of 47 nt in the 5' portion of the heterogeneous central part of the gene showed 85% homology with the *fliC* gene of *Escherichia coli*.

The PCR amplification using the *fliC*-specific Hd/j-F/R primer set detected a 927 bp product indicating the presence of the Hd *fliC* gene in all (100%) 78 *S. Typhi* isolates from the eastern Indonesian archipelago (Table 1). The 666 bp product was not detected in any of the PCR reactions indicating the absence of the H_j gene in these *S. Typhi* isolates. The 375 bp PCR product of the PZ66-A/B primer pair detecting the z66 gene was observed in 12 (15.4%) isolates and the 597 bp PCR product of the Ind-F/R primer pair detecting the Ind gene was observed in 17 (21.8%) isolates. These PCRs were somewhat more sensitive than the PZ66-A/B PCR that produces the 1.467 bp or 1.050 bp and several of the isolates that tested negative for the longer products tested positive for either the PZ66A/B PCR or the Ind-F/R PCR. Just two isolates both from Papua contained three, the Hd, the z66, and the Ind flagellin genes. Whereas the z66 gene was detected equally often in isolates from South-Sulawesi, Kalimantan and Papua, the Ind gene was detected most frequently ($P = 0.1$) in isolates from Papua (Table 1). The PCR amplification of tirs using both the a/c and b/d primer sets was positive for isolates containing the z66 gene and tested negative for isolates containing the Ind gene and not the z66 gene (data not shown).

Five (6.4%) patients were hospitalized with severe mental conditions including three from Papua, one from Kalimantan,

FIGURE 1. Polymerase chain reaction (PCR) mediated detection of flagellin genes in *Salmonella enterica* serovar Typhi isolates from East Indonesia. (A) PCR using primer pair z66flag-F/R 1/2 (Baker and others⁶) for the amplification of full-length z66 (1.467 nt). (B) PCR using primer pair Ind-F/R for the specific amplification of a 597 bp fragment of Ind. (C) PCR using primer pair PZ66-A/B for the specific amplification of a 375 bp segment of z66. (D) PCR using primer pair Hd/j-F/R for the amplification of a 927 bp product of the Hd gene and a 666 bp product of the H:j gene (Huang and others¹²). PCRs were applied on 7 *S. Typhi* isolates from Indonesia and the products were run on a 1% ethidium bromide stained agarose gel and viewed under UV illumination. Isolates in lanes 2, 5, and 8 (isolates T503, T548, and T566, respectively) were from South-Sulawesi and isolates in lanes 3, 4, 6, and 7 (isolates T521, T531, T548 and T561, respectively) from Papua. Lane 9 is a negative control (no DNA) and lanes 1 and 10 are ladder markers.

and one from Sulawesi. Three of these patients presented with apathy and two with stupor. A *S. Typhi* isolate harboring a second flagellin gene was cultured from three (60%) patients with a severe mental condition. A *S. Typhi* isolate containing the Ind gene in addition to the Hd gene was obtained from two patients, one from Papua and one from Kalimantan, with apathy and a *S. Typhi* isolate containing the z66 gene and the Hd gene was cultured from the blood of one patient with stupor from South-Sulawesi. Three out of 28 patients infected with a *S. Typhi* strain harboring either Ind or z66 had severe mental disease compared with three out of 50 patients infected with a *S. Typhi* strain harboring Hd only and a non-significant relative risk of 2.68 (95% confidence interval, 0.5–15.0) was calculated for the association between the presence of these “foreign” flagellin genes and severe mental disease.

DISCUSSION

Originally, it was thought that *S. Typhi* unlike other *Salmonellas* produces just one type of flagellum, the Hd antigen. Certain *S. Typhi* strains circulating in Indonesia, however, appeared to be unique and may produce a flagella protein called z66 while in addition some strains from Indonesia contain a mutant Hd antigen named Hj. *Salmonellas* show phase variation depending on the type of flagellum that is expressed and in *S. Typhi* the Hd and Hj flagella are thought to represent phase 1 and the z66 flagella phase 2. We are finding that some *S. Typhi* isolates from Indonesia may express yet another flagellum, which shows extensive homology with z66 and Hd in the conserved amino and carboxyterminal domains but has a significantly shorter heterologous central domain of only 86 amino acids. This flagella protein is named Ind. The partial sequence homology found for the conserved and heterologous domains of Ind with the *fliC* genes of the nosocomial agent *S. marcescens* and of the fish pathogen and a rare cause of human (nosocomial) infections *Edwardsiella tarda* may suggest that this gene has been acquired by *S. Typhi* from an infectious agent related to either of these pathogens and then perhaps further adapted to the specific needs of *S. Typhi*.^{13–17} Whether Ind represents a phase 1 or a phase 2 flagellum remains to be investigated.

We have found that three of the five patients presenting with a severe mental condition harbored either the Ind gene or the z66 gene and it is tempting to speculate that the expression of either of these flagellin genes increases the risk of developing severe disease. Alternatively, a specific *S. Typhi* genotype with a mutation in one of the pathogenic islands leading to increased virulence or pathogenicity has acquired these flagellin genes. As isolates from Papua most often contained a second flagellin gene and three of the five patients with a severe clinical condition included in the study came from this island, and a further study is required to exclude the possibility that the observed association is because of confounding.

Bacteria such as *Salmonella* swim by counterclockwise rotation of helical flagellar filaments propelled by a rotary motor at the base of the filament.¹⁸ The counterclockwise rotation may be alternated by short periods lasting a fraction of a second of clockwise rotation. During periods of counterclockwise rotation several filaments form a bundle that propels the cell; during the clockwise rotation the bundle falls apart. The flagellum is a tubular structure composed of 11 filaments with each filament being an assembly of a single protein, flagellin,

FIGURE 2. Amino acid sequence of the Ind flagellin protein in *Salmonella enterica* serovar Typhi isolates from East Indonesia and comparison with Hd and z66. The conserved amino terminal domains of the (A) Ind gene, (B) Hd gene, and (C) z66 gene are depicted in red, the conserved carboxy terminal domains in blue, and the heterogeneous central domains in black. Amino acid substitutions in the conserved regions of Ind that are unique as compared with z66 are highlighted with yellow, unique substitution compared with Hd are highlighted with green, and substitution at positions that differ from both Hd and z66 are highlighted with blue. A two nucleotides (nt) insertion in the aminoterminal region of the Ind gene is shown in dark blue. A stretch of three amino acids at the junction of the hypervariable central domain and the conserved carboxyterminal regions of Hd and z66 that is not present in Ind is highlighted with red. The Pubmed accession no. of the z66 gene is AB108532 and that of Hd is L21912.

with roughly 11 subunits per two turns of the helix. The subunits may switch between two stages of interaction resulting in different structures of the filament. The flagella protein is composed of four structural domains, D0–D3.¹⁸ The domains D0 and D1 form the central tube of the filament and are highly conserved. Domain D3 is a hyper variable and is exposed to the outside of the filament. D3 is located in the center of the protein and is flanked at both sides by D2, which in turn is flanked at the C- and N-terminal ends by D1 and D0, respectively. Crystallography of the segment of the flagellin protein from *S. Typhimurium* containing D1, D2, and D3 has revealed that this segment has the shape of a boomerang of which one

wing is composed of three rod-shaped hydrophobic cores formed by D1. The hyper variable domain D3 region consisting mostly of β -strands forms the other wing. It has been demonstrated that the flagella protein from *S. Typhimurium* can have its D3 domain deleted without affecting its ability to form filaments.¹⁹ Domain D3 is relatively independently arranged from the other domains and this may explain why its deletion has a minimal effect on the structural integrity. Mutations in the conserved regions may influence the motility of the flagellum and changes in the heterologous region could provide the flagellum with unique antigenic properties. Ind has a unique amino acid composition of the heterologous region and

TABLE 1

Presence of flagellin genes and gene combinations in *Salmonella enterica* serovar Typhi isolates from different islands in East Indonesia

Island	No. (%) of isolates containing the following flagellin genes		
	Hd	z66	Ind
South Sulawesi	26 (100%)	4 (15.4%)	4 (15.4%)
East Kalimantan	26 (100%)	3 (11.5%)	4 (14.4%)
Papua	26 (100%)	5 (19.2%)	9 (34.6%)
Total	78 (100%)	12 (15.4%)	17 (21.8%)

compared with Hd and z66 has several amino acid changes in the conserved domains. Thus, the expression of Ind may endow *S. Typhi* with various unique properties affecting both motility and antigenicity.

It may be proposed that functional difference provided by the expression of different flagella influence infectivity and or pathogenicity through, for instance, interactions with the mucosal tissue.²⁰ In addition, the expression of different flagella may influence the interaction with immunoreceptors such as toll-like receptors making the bacterium less sensitive to the host immune system.²¹ It also may be proposed that the ability to use different flagella provide the bacterium with unique properties to respond to the presence of antibiotics. Bacteria such as *S. enterica* and *S. marcescens* have the capability to reduce sensitivity to antibiotics by moving at high density in crowded groups, a phenomenon also called swarming.²² Thus, the acquisition of novel flagella by *S. Typhi* in Indonesia may have increased or modified fitness in various manners and resulted in an increased risk of developing severe disease. A larger study will be required to determine whether the presence of either of these flagellin genes poses a significant risk of developing severe clinical illness.

Previously, *S. Typhi* containing the H_j *fliC* or the z66 *fliB* genes has only been isolated from Indonesia. However, their geographic distribution within the Indonesia archipelago has not been studied. We show that all *S. Typhi* isolates from South-Sulawesi, Kalimantan, and Papua collected in 2010 contained the Hd *fliC* gene, that the H_j gene was not detected in any isolate, that 15.4% of the *S. Typhi* isolates from the eastern Indonesia archipelago contain the z66, and that 21.8% contained the novel Ind gene. The Ind gene was most prevalent (34.6%) in *S. Typhi* isolates from Papua and two isolates from Papua contained both the z66 and the Ind gene. Whether the Ind gene is unique for *S. Typhi* isolates circulating in east Indonesia remains to be investigated. Notably, the z66 gene was previously detected in a series of *S. Typhi* isolates from Jakarta all containing the H_j gene.²³

Whereas Hd and H_j are encoded on the circular genome of *S. Typhi*, z66 is encoded by a linear plasmid. Ind was likely obtained by horizontal gene transfer from an unrelated infectious bacterium but whether Ind is encoded on a linear or circular plasmid remains to be investigated. The PCRs designed for the detection of the tirs present on the plasmid containing the z66 gene in *S. Typhi* isolates tested negative for isolates containing the Ind gene. However, this does not exclude the possibility that Ind is encoded on a linear plasmid with different tirs.

Flagella play an important function in the lifecycle of *S. Typhi*. The possibility to express one or more different flagella may provide *S. Typhi* with unique characteristics that help the bacterium to infect and survive. The genome of *S. Typhi* is highly

conserved and has undergone little differentiation because of its emergence as a human pathogen, which is thought to have occurred some 30,000 years ago when humans started to settle and live in larger communities. The unique niche that *S. Typhi* occupies likely endows severe restriction on its genetic flexibility. The collection of genes from other bacteria may help *S. Typhi* to expand or change its niche and further evolve as a human pathogen.

CONCLUSION

Some *S. Typhi* from the eastern Indonesia archipelago harbor a novel flagellin gene, named Ind, which differs from the Hd *fliC* and z66 *fliB* genes by several nucleic acid changes in the conserved domains of the gene and the presence of a short 258 nt long unique hyper variable central domain. The Ind gene showed partial homology in the conserved and heterologous regions of the *fliC* gene of nosocomial agent *S. marcescens*, and we speculate that *S. Typhi* may have acquired this flagellin gene from an unrelated pathogen and may endow the pathogen with unique pathogenic and antigen properties possibly contributing to an increased risk for the patient of developing severe mental illness.

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Disclaimer: The authors declare that they do not have competing interest.

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