Analysis of pulsotypes of *salmonella typhi* isolates and their clinical profiles in typhoid fever patients

Retno Kadarsih Soemanto, Lucky Hartati Moehario

Abstrak

*Studi penentuan genotip (pulsotip) terhadap isolat-isolat Salmonella typhi (S. typhi) telah dilakukan menggunakan elektroforesis medan listrik berpulsasi (PFGE = Pulse-Field Gel Electrophoresis). Penelitian ini bertujuan untuk mempelajari diversitas genetik dan hubungan antara karakter genetik dengan manifestasi kliniknya. Sebanyak 66 isolat S. typhi yang berasal dari kasus demam tifoid yang dirawat di rumah sakit telah dianalisis. Empat isolat ditemukan identik dan hasil konstruksi dendogram menunjukkan terdapatnya 33 pulsotip dimana 13 di antaranya dapat dipisahkan dalam 30 subtip. Keragaman genetik di antara mereka relatif tinggi yang ditunjukkan dengan koefisien Dice 0,486-1,000. Pada derajat similaritas 65%, analisis sidik gerombol menunjukkan adanya 2 sidik gerombol utama, sehingga timbul dugaan bahwa S. typhi yang beredar bukan berasal dari klon tunggal. Pada derajat similaritas 90%, dari 9 sidik gerombol yang beranggotakan ≥ 3 isolat, didapatkan manifestasi klinik yang sangat bervariasi dari ringan sampai berat tersebar diantara 9 sidik gerombol tersebut. Walaupun data rekam medis yang didapat kurang lengkap, 2 dari 4 pasien demam tifoid dengan S. typhi yang berasal dari sidik gerombol 1 memperlihatkan kenaikan total bilirubin yang tidak ditemukan pada 19 pasien yang berasal dari 8 sidik gerombol yang lain. Dengan adanya temuan ini, diduga adanya kemungkinan suatu trofisme pada system hepatobilier dari kuman S. typhi pulsotip I_1 dan I_2 yang berasal dari sidik gerombol 1. (**Med J Indones 2003; 12: 13-20**)*

Abstract

*A study of genotyping (pulsotyping) of Salmonella typhi (S. typhi) isolates using pulse-field gel electrophoresis (PFGE) methods was performed to examine their genetic diversity, and relationship between genetic characteristics and clinical outcomes. Sixty-six S. typhi isolates obtained from sporadic hospitalized typhoid fever cases were used in this study. Four isolates were found identical and the dendogram constructed showed 33 pulsotypes in which 13 of them can be divided into 30 subtypes. Diversity among them were high as shown by the Dice coefficients that ranged from 0.486 to 1.000. Cluster analysis showed 2 main clusters with 65% degree of similarity, suggested that they were not originated from one clone. Further, at 90% degree of similarity, 9 clusters containing at least 3 isolates were determined to explore any possible existence of relationship between genetic profile and particular clinical outcomes. Clinical manifestations ranged from mild to severe were in fact distributed diversely among these clusters. Although the clinical data obtained were incomplete, 2 out of 4 patients infected by the S. typhi belonged to cluster 1 showed an elevation of total bilirubin, whereas it was not found in 19 other patients distributed in other 8 clusters. Even though specific clinical manifestations were apparently not found to relate with particular clusters of genotypes, S. typhi isolates grouped in cluster 1 seemed to show trophism to hepatobiliary system. (**Med J Indones 2003; 12: 13-20**)*

Keywords: *S. typhi, typhoid fever, Pulsed-field Gel electrophoresis (PFGE)*

Typhoid fever is still a major problem in public health in many developing countries. Increase of international travel and mobility of people between neighborhood countries are contributing to the emergence of new clones of *S. typhi* in these countries. Annual incidence of the disease is 16-17 million cases and approximately 600,000 deaths, and neighboring countries such as Indonesia, Malaysia and Thailand had one of the highest incidence in the world as more than 1,000 per 100,000 populations.[1] In Indonesia, the mean annual morbidity rate from hospitals and health centers during the period of 1981-1986 was 13.9 cases per 100.000-population and case fatality rate (CFR) was 2.6%.[2] In Jakarta, typhoid fever was the second leading infectious disease after gastroenteritis and caused the highest mortality.[3] *S. typhi* was isolated from 14.9% cases in 1984-1990 while *S. paratyphi* A and other *S. paratyphi* was 3.1% and 1.0% respectively.[4]

Department of Microbiology, Medical Faculty, University of Indonesia, Jakarta, Indonesia

As a systemic disease, typhoid fever affects gastro-intestinal tract and also other human organs.[5] Many factors such as individual immune responses, personal hygiene and environmental sanitations, increasing incidence of antibiotic-resistant strains have been considered to play a role in the outcomes of the disease in which the clinical syndromes vary from mild to severe, and sometimes with complications.[6,7,8,9] Nonetheless, plasticity of Salmonella chromosomes have been suggested to be associated with bacterial virulence, and the presence of differences in clinical manifestations.[7,10] Franco et al (1992)[11] found a unique flagellar type in Indonesian strains designated as j genotype and a phase II variant, z66. Those strains had been isolated from typhoid fever patients with severe clinical signs. Moehario et al, (1998)[12] also reported different serum reactivity to outer membrane proteins isolated from local *S. typhi* strains in comparison to those reported by other investigators,[13,14] suggested a possible presence of certain strains in the area which might be related to more severe disease manifestations. Molecular studies of this microorganism using pulsed-field gel electrophoresis (PFGE) suggested that a certain PFGE type (pulsotype) of *S. typhi* was associated with the ability to cause fatal disease.[15] Those studies suggested the existence of characteristic differences of *S. typhi* that might be responsible to the presence of various clinical outcomes of typhoid fever. However, relationship, if any, of the symptoms of the disease and genome characteristics of *S. typhi* is still unclear, and thus needed to be elucidated. In this study, we examined 66 *S. typhi* isolates from sporadic hospitalized typhoid fever cases using PFGE methods, and analyzed clinical manifestations from each patients to observe possible relationship between the pulsotype profiles and clinical outcomes.

METHODS

Samples

Sixty-six *S. typhi* isolates were obtained from Dr. RHH Nelwan, Department of Internal Medicine, Medical Faculty, University of Indonesia, Jakarta. The isolates were originated from sporadic cases of typhoid fever admitted in Persahabatan Hospital Jakarta in 1998. From each of these cases, clinical data that included clinical symptoms, laboratory findings and complications of the diseases were obtained from Medical Record Department of the Hospital.[16] Values of laboratory findings were as shown in table 4. Confirmation tests were carried out for all *S. typhi* isolates using biochemical and serological techniques. Antibiotic susceptibility tests were performed by disk diffusion method according to NCCLS standard.[17] Antibiotics used were as follows: amoxycillin, amoxycillin-clavulanic acid, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftriaxone.

PFGE and Genome Analysis

Chromosomal DNA was prepared as described by other investigators,[18,19] and electrophoresis of *Xba*I digested chromosomal DNA was conducted as described by Moehario and Soemanto.[20] Gels were stained with ethidium bromide, destained and photo-graphed under UV light. PFGE patterns were assessed as previously described,[21,22,23,24,25,26,27] interpretation of gel patterns was based on criteria described earlier by Tenover et al.[27,28] and Zadoks et al.[29] Isolates showing the same PFGE pattern or differing by one to three bands were grouped in a same type (clone), isolates that differed in up three bands were classified in subtype. Different but closely related types were grouped if isolates differed by four to six bands and different or unrelated types were assigned if differed by seven bands and more. All of those types were then compared by calculating similarity coefficient Dice.[30,31] Electrophoretic patterns were analysed for relatedness using a computer program Numerical Taxonomy and Multivariate Analysis (NTSYS-pc) version 1.80.[31] Dendograms were constructed by using Dice coefficients and clustering by using unweighted pair group arithmetic means method (UPGMA).[31]

RESULTS

All 66 *S. typhi* isolates were sensitive to antibiotics tested. Not all patients have complete data for clinical symptoms. Out of 66 cases recorded only 44 data were available. The clinical symptoms found were as shown in Table 1: age ranged from 14–74 years old, most of the patients were female (60%), days of fever before admissions less than a week (53.19%), with the following clinical symptoms: fever (93.18%), coated tongue (54.54%), diarrhea (43.18%), constipation (29.54%), hepatomegaly (22.73%). Laboratory findings were as follows: anemia (25%), leucopenia (54,54%), thrombocytopenia (63.64%), elevated erythrocyte sedimentation rate (82.86%), elevated Serum Glutamic Oxaloacetic Transaminase (SGOT) (89.47%), elevated Serum Glutamic Pyruvic Transaminase (SGPT) (47.37%),

hyperbilirubinemia (13.89%), hypoalbuminemia (56.52%), and elevated serum alkaline phosphatase (37.50%) as shown in Table 2. Complications of the disease were shown in Table 3, in which pancytopenia, encephalopathy and typhoid hepatitis apparently were the most common.

Table 1. Clinical symptoms of Typhoid Fever patients

No.	Clinical symptoms	Cases	%	n
1.	Cephalgia	28	63.64	44
2.	Febrile (> 37.2 °C)	41	93.18	44
3.	Nausea	36	81.82	44
4.	Epigastric pain	25	56.82	44
5.	Anthralgia	12	27.27	44
6.	Anorexia	20	45.45	44
7.	Coated tongue	24	54.54	44
8.	Vomitus	23	52.27	44
9.	Cough	20	44.44	44
10.	Relative Bradycardia	20	44.44	44
11.	Diarrhea	19	43.18	44
12.	Hepatomegaly	10	22.73	44
13.	Constipation	13	29.54	44
14.	Ronchi	5	11.36	44
15	Splenomegaly	1	2.27	44

n: Total patient's data recorded

Table 2. Laboratory findings of typhoid fever patients

No.	Laboratory findings	Cases	%	n
1.	Elevated SGOT	34	89.57	38
2.	Elevated erythrocyte sedimentation rate	29	82.86	35
3.	Thrombocytopenia	28	63.64	44
4.	Proteinuria	17	62.96	27
5.	Hypoalbuminemia	13	56.52	23
6.	Leucopenia	24	54.54	44
7.	Elevated SGPT	18	47.37	38
8.	Normoleucocyte	8	40.90	44
9.	Serum Alkali Phosphatase	9	37.50	24
10.	Anemia	11	25.00	44
11.	Elevated Amilase	1	25.00	4
12.	Elevated Lipase	1	25.00	4
13.	Elevated Total Bilirubin	5	13.89	36
14.	Leucocyturia	2	5.71	35
15.	Leucocytosis	2	4.54	44

n: Total patient's data recorded

The *Xba*I (5'-TCTAGA-3') digested chromosomal DNAs of 66 *S. typhi* isolates showed 10 to 20 fragments with molecular weight ranging from 30 kb to 650 kb, which are in agreement with our previous report,[13] and had various PFGE patterns. Further, those isolates were classified in 33 different pulsotypes, and 13 of them were showing 30 subtypes (Figure 1). Genetic diversity of these isolates was found to be relatively high, as shown by their F values: 0.486-1.000. Cluster analysis showed 2 main clusters at 65% degree of similarity designated as main cluster A, which consisted of 44 isolates, and main cluster B, consisted of 22 isolates. Further, at 90% homology, 9 clusters designated cluster 1 to 9, were found and each clusters consisted of 4, 10, 5, 3, 3, 3, 4, 3 and 3 isolates respectively. Dice coefficients among isolates in each of these clusters were as follows: 0.941-0.971, 0.882-1.000, 0.865-0.971, 0.882-0.944, 0.882-1.000, 0.914-0.944, 0.903-0.970, 0.903-0.968 and 0.759-0.966. Pulsotype II was the most frequent type found, consisted of 10 isolates, in which 4 of them were identical (F value 1.000). Pulsotype VII consisted of 5 isolates, type I, type XXIV and XXVI consisted of 4 isolates each, type VIII, XVIII, XIX and type XXV consisted of 3 isolates each, whereas 2 isolates of type XVIII were actually identical. Type IV, XXIII, XXVIII were consisted of 2 isolates each. Meanwhile, single isolates were observed in type III, V, VI, IX, X, XI, XII, XIII, XIV, XV, XVI, XX, XXI, XXII, XXVII, XXIX, XXX, XXXI, XXXII and XXXIII subsequently.

Clinical features of 9 clusters that shared 90% degree of similarity were elaborated to find whether there is a relationship between characteristic symptoms of the disease and any particular clusters. The symptoms observed were as followed: fever, enlargement of the liver, leucocyte count, thrombocyte count, SGOT, SGPT and total bilirubin. Cluster 1, type I PFGE, consisted of 4 isolates showed: 100% fever, 25% hepatomegaly, 50% leucopenia, 75% thrombocytopenia, 100% elevated SGOT, 50% elevated SGPT, 50% elevated total bilirubin (see Table 4). Cluster 2, type II PFGE, consisted of 10 isolates showed: 75% fever, 25% with hepatomegaly, 75% with leucopenia, 62.50% thrombocytopenia, 87.50% elevated SGOT, 62.50% normal SGPT, 100% normal total bilirubin (Table 4). The clinical features of patients infected with *S. typhi* originated from cluster 1 to cluster 9 were as shown in Table 4.
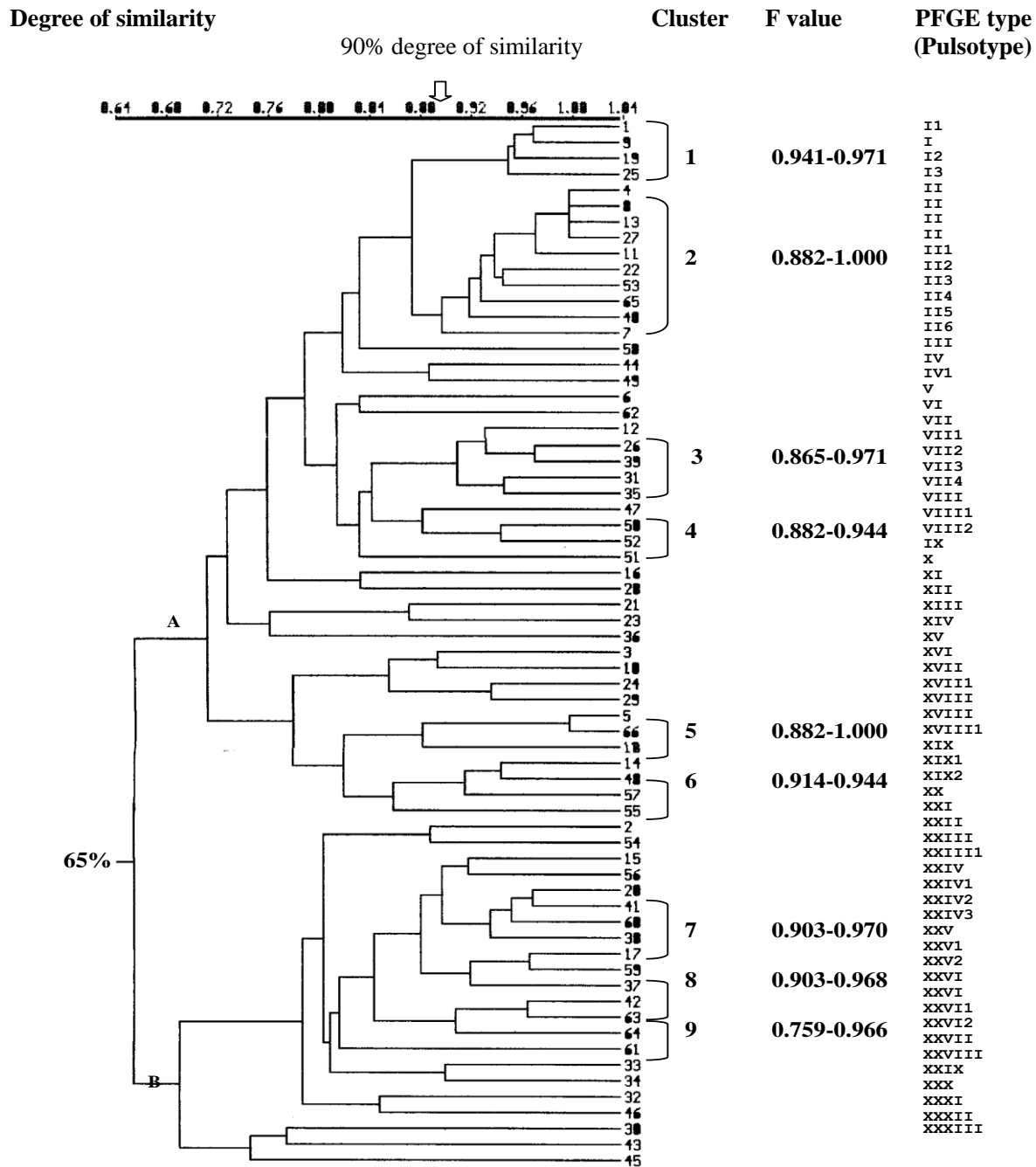
Degree of similarity   **Cluster**   **F value**   **PFGE type**
                                  90% degree of similarity   **(Pulsotype)**

Figure 1. Dendogram and pulsotype of 66 S. typhi isolates
Dendogram showing the cluster among the 66 S. typhi isolates. Scale indicates level of genetic relatedness within this collections of isolates.
Nine clusters (cluster 1 to 9) were determined at 90% degree of similarity. Pulsotype was determined as described by Tenover (1997).[13]

Four identical isolates of pulsotype II were showing diverse clinical appearance as shown in Table 3 and Table 4. Similar conditions were also observed to the other 6 subtypes in the same pulsotype. On the other hand, distinct clusters happened to share several specific clinical manifestations. All typhoid complications apparently were derived from genetically diverse isolates. Below were 4 most common complications found from the patients and their etiologic pathogen in their pulsotypes and clusters:

Table 3. Disease complications and the distribution of *S. typhi* in pulsotypes and clusters

No.	Complications	Cases	Pulsotype	Cluster at degree of similarity 90%	65%
1	Pancytopenia	6	I_1, II, II_6, III, $XXIV_3$,$XXVI_1$	1, 2, 7, 9	A, B
2	Encephalopathy	5	I_1,I_2,II, XXI,	1, 2	A, B
3	Typhoid hepatitis	4	I_1, I_2, XIX, XXIII	1, 6	A
4	Bronchopneumonia	3	II, XI, XIX_1	2, 6	A
5	Bicytopenia	2	II, XXV_2	2, 8	A, B
6	Paralysis of N.VII	2	II_2	2	A
7	Hematemesis & melena	2	XXIII,$XXIV_3$	7	B
8	Acute Pancreatitis	1	XVII	-	A
9	Peritonitis e.c perforation of the bowel	1	XXIII	-	B
10	Fatal	1	XXIII	-	B
11	Relaps	1	VII_4	3	A

Table 4. Clusters of closely related with 90% degree of similarity of *S. typhi* and their clinical presentations in Typhoid Fever patients

Cluster (Pulsotype)	1 (I)		2 (II)		3 (VII)		4 (VIII)		5 (XVIII)		6 (XIX)		7 (XXIV)		8 (XXV)		9 (XXVI)	
No. Isolates	4		10		5		3		3		3		4		3		3	
Reported cases	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Fever	4/4	100	6/8	75	3/3	100	Nd	Nd	1/2	50	2/2	100	2/2	100	2/2	100	2/2	100
Hepatomegaly	¼	25	2/8	25	2/2	100	Nd	Nd	0/2	0	1/2	50	1/2	50	0/2	0	0/2	0
Leucopenia < 5,000 /mm3	2/4	50	6/8	75	0/3	0	1/1	100	½	50	2/2	100	½	50	½	50	2/2	100
Leucocytosis > 10,000/mm3	¼	25	0/8	0	0/3	0	0/1	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0
Normal leucocyte	¼	25	2/8	25	3/3	100	0/1	0	½	50	0/2	0	½	50	½	50	0/2	0
Thrombocytopenia <150,000/mm3	¾	75	5/8	62.50	2/3	66.67	0/1	0	½	50	2/2	100	2/2	100	½	50	½	50
Normal Thrombocyte	¼	25	3/8	37.50	1/3	33.33	1/1	100	½	50	0/2	0	0/2	0	½	50	½	50
Thrombocytosis > 500,000/mm3	0/4	0	0/8	0	0/3	0	0/1	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0
Increase SGOT > 37μmol/l	4/4	100	7/8	87.50	2/2	100	0/1	0	2/2	100	2/2	100	1/1	100	1/1	100	2/2	100
Normal SGOT	0/4	0	1/8	12.50	0/2	0	1/1	100	0/2	0	0/2	0	0/1	0	0/1	0	0/2	0
Increase SGPT > 41 μmol/l	2/4	50	3/8	37.50	½	50	0/1	0	2/2	100	2/2	100	0/1	0	1/1	100	½	50
Normal SGPT	2/4	50	5/8	62.50	½	50	1/1	100	0/2	0	0/2	0	1/1	100	0/1	0	½	50
Increase Bilirubin Total > 1.1 mg%	2/4	50	0/8	0	0/2	0	0/1	0	0/2	0	0/2	0	0/1	0	0/1	0	0/2	0
Normal Bilirubin Total 0.1 – 1.1 mg%	2/4	50	8/8	100	2/2	100	1/1	100	2/2	100	2/2	100	1/1	100	1/1	100	2/2	100

1. Pancytopenia was detected from patients harboring *S. typhi* originated from pulsotypes I_1, II, II_6, $XXIV_3$ and $XXVI_1$ in which at 90% degree of similarity gain in clusters 1, 2, 7 and 9

2. Encephalopathy was found in patients infected by *S. typhi* isolates designated by pulsotypes I_1, I_2 and II from clusters 1 and 2 at 90% degree of similarity

3. Typhoid hepatitis was found in pulsotypes I_1, I_2 and XIX or clusters 1 and 6 at 90% degree of similarity

4. Bronchopneumonia may also was found in typhoid fever patients harboring pulsotype II and pulsotype XIX_1 *S. typhi* from cluster 2 and 6 at 90% degree of similarity.

These observations thus far suggested no predominant symptoms were characteristic to any particular clusters. The same clinical symptoms could be found in different clusters. Even in other main cluster A, fatal case due to peritonitis caused by perforation of the bowel was found in patient infected by isolate designated by pulsotype XXIII originated from main cluster B. Four identical isolates, with pulsotype II belonged to cluster 2, showed diverse clinical symptoms pancytopenia, encephalopathy, bronchopneumonia and bicytopenia.

Beyond all these indistinctive clinical symptoms in relation to their genome characteristics of the pathogens, interestingly, 2 out of 4 cases infected by *S. typhi* belonged to cluster 1 were found to have an elevation of total bilirubin, while that same symptom was absent in other 19 patients harboring *S. typhi* originated from other 8 clusters.

DISCUSSION

Typhoid fever as an important public health problem is still an interesting topic to be explored extensively. Many epidemiological marker techniques to investigate the disease have been involved, and in the last few years it seemed that the applications of molecular techniques, especially PFGE, is currently favored for this etiological pathogens.[15,19,20,21,30,32,33]

Although this technique is time and cost consuming, PFGE showed high discriminatory ability and reproducibility and therefore is chosen to be an effective means of differentiating *S. typhi* strains.[27,28,33]

We analyzed 66 isolates of *S. typhi* obtained from sporadic hospitalized typhoid fever cases. As all of the isolates used in this investigation were sensitive to antibiotics tested, further study of this particular phenotype characteristic in relation to severity of typhoid fever was not further continued.

The results of the electrophoresis of all *S. typhi* DNA genome digested with *Xba*I were not different from our earlier investigation.[20] Although, these isolates were collected from one hospital, and most isolates were designated as belonged to pulsotype II, a wide range of different pulsotypes have been found. Genetic relatedness among them varied which were shown by their Dice coefficients from 0.667 to 1.000. Further, at 65% or above degree of similarity they were divided into 2 main clusters suggested they were genetically highly diverse and were not originated from a single clone. This finding supported the previous reports. Thong et al,[30] provided some indications that there were considerable diversity among *S. typhi* strains circulating in Southeast Asia regions, particularly Malaysia, Indonesia and Thailand due to increasing mobility of travelers and migrant workers among these countries. The extent of genetic diversity among strains is actually greater than previously thought. The report also suggested that multiple clones of *S. typhi* were endemic in these regions, thus contrasting the concept that *S. typhi* is derived from a single clone.[34,35]

Clinical manifestations of those patients varied as described above. Variations of clinical manifestations in typhoid fever were assumed to be related to many intrinsic and extrinsic factors of the strains. Regarding to the intrinsic factors, we explored the clinical syndromes from patients within the same clusters. As there is no set criteria that are appropriate for every organisms and circumstances worldwide, 90% or above degree of similarity was chosen in this research to be a cut-off point to evaluate any possible existence of relationship between genetic profile and the varied clinical outcomes.

The symptoms observed in each of the 9 clusters with 90% or above degree of similarity were actually varied. Further, some of the disease complications such as pancytopenia, encephalopathy, typhoid hepatitis, bronchopneumonia were distributed diversely in all those clusters. Thong et al.[14] suggested a correlation between certain pulsotypes of *S. typhi* from Papua New Guinea and the ability to cause fatal typhoid fever, while Franco et al.[11] found specific flagellar types of *S. typhi* in Indonesia that were associated with severe outcomes of the disease. Nonetheless,

the association between pulsotypes and clinical manifestations was statistically not significant.

Although there was seemed no clinical symptoms specifically related to any particular cluster in cluster 1, we found 50% cases with elevated total bilirubin in which were absent in other 19 isolates from other 8 cluster. This finding assumed us that tendency of hepatobiliary symptoms was found in cluster 1.

Acute pancreatitis has been observed in this study as shown by elevated serum amylase and lipase, found in patient infected by *S. typhi* isolate designated by pulsotyope XVII originated from main cluster A. Although typhoid pancreatitis were reported as a very rare complication of typhoid fever, but this complication sporadically appeared in the world reports in the last several decades. It seems that with the advance of modern technology such as improvement of blood amylase and lipase analysis and the availability of ultrasonographic and computer tomographic examinations, this rare complication was diagnosed more easily. Since the first report of typhoid pancreatitis in Indonesia in 1992,[36] the presence of this complications were reported more frequently.[37] One fatal case was found in this study from patients harboring *S. typhi* pulsotype XXIII from main cluster B.

This study, thus far, showed no apparent relationship between pulsotype and the clinical symptoms, and no plausible explanations as yet to suggest the presence of diverse range of symptoms produced by the closely related microorganisms belonged to particular clusters to each host. Nonetheless, it is interesting to further elaborate the possibility of the presence of characteristic pulsotypes of *S. typhi*, which showed hepatobiliary trophism as suggested by pulsotypes I_1 and I_2 of *S. typhi* in cluster 1.

However, the mystery of pathogenesis of this microorganism is still interesting to be explored extensively and some promising and innovative approaches are still needed for further study.

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