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Association of Typhoid Fever Severity with Polymorphisms NOD2, VDR and NRAMP1 Genes in Endemic Area, Indonesia

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Only small proportions of *Salmonella enterica* serovar Typhi (S. Typhi), in endemic areas, exposing individuals develop typhoid fever symptoms. Identification of polymorphisms NOD2, VDR and NRAMP1 genes is mandatory for a better understanding of typhoid fever molecular pathogenesis and thus to the development of novel strategies for the prevention of infection. The aim of this study was to determine whether genetic polymorphisms in nucleotide oligomerization binding domain 2 (NOD2), vitamin D receptor (VDR) gene and natural resistance-associated macrophage protein 1 (NRAMP1) genes are involved in host susceptibility to severity of typhoid fever. The genotyping of eight regions were applied in the genes; NOD2, VDR and NRAMP1, using PCR-RFLP. A multivariate analysis on 426 mild and 35 severe state of typhoid fever patients. All patients living in the geographically isolated village of South Sulawesi, Middle Sulawesi, Southeast Sulawesi, East Borneo and Papua islands which was an endemic areas in Indonesia. Data were analyzed using Microsoft Excel and Stata 9.2. The G/C and C/C alleles of exon 8 in NOD2 gene were strongly associated and more frequently found in the patients with severe typhoid fever than in mild typhoid fever (p= 0.027and 0.014, odds ratio = 16.7and 27.9, 95% confidence interval = 3.4 - 25.7and 2.6 - 37.1in G/C allele and C/C allele), respectively. In contrast, No evidence for the association of VDR and NRAMP1 genes polymorphisms with severity state of typhoid fever. The polymorphism of exon 8 in NOD2 gene; heterozygotes or homozygotes for a G→C change in codon 2722, was related to susceptibility to typhoid fever in clinical severity of this disease. Polymorphisms of exon 8 in NOD2 gene; heterozygotes or homozygotes for G/C in codon 2722 were related to susceptibility to typhoid fever in clinical severity.

Key words: Nucleotide-binding oligomerization domain 2(NOD2), vitamin D receptor (VDR), natural resistance-associated macrophage protein 1 (NRAMP1), typhoid fever

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INTRODUCTION

Typhoid fever is acute gastrointestinal infection caused by *Salmonella enterica* serovar Typhi (S. Typhi) and related with environmental, behavior, situation and sanitation[1]. The disease could develop to severe state in some of patients. However, the molecular pathomechanisms of severity of typhoid fever are still unclear.

This research was intended to study a well-defined population residing on a small, geographically isolated area in Indonesia, with high typhoid fever prevalence. The inhabitants originate from the South Sulawesi, Middle Sulawesi, Southeast Sulawesi, East Kalimantan and Papua Main Island and the population is rather limited in its genetic variability in three genes of NOD2, VDR and NRAMP1.

Nucleotide oligomerization domain 2 (NOD2) is family of intracellular bacterial sensors and able to recognize highly conserved microbial motifs[2]. The NOD2 gene, located on chromosome 16q12 and functions as an anti-bacterial factor limiting survival of intracellular invasive bacteria and are cytosolic pattern recognition receptors (PRRs) that recognize particular motifs found in bacterial peptidoglycan[3]. The NOD2 are key bacteria-sensing receptors and are important in the early response against enteric pathogens, including *S. typhimurium* and *Citrobacter rodentium*[4]. Mutations in NOD2 in humans have been associated with inflammatory disorders, such as asthma and Cohn's disease, respectively[5]. Previous study, analyzed polymorphism at NOD2 gene of locus exon 4,802 between the leprosy patients of PB, MB types and healthy persons and finding out the relationship between polymorphism of NOD2 gene and antibody titer. The results suggested that no significant differences exist between polymorphism NOD2 gene in the type of leprosy, healthy persons and antibody titer[6].

The biological active form of vitamin D31, 25(OH) 2D3, induces differentiation of peripheral mononuclear cell into macrophage, activation of macrophage and granuloma formation. Its effects are exerted by interaction with vitamin D receptor (VDR), which is present on monocyte and activated T and B lymphocyte[7]. The VDR genotype was a genetic factor influencing Th1-Th2 shift in humans. Study on association of VDR genotype with leprosy type suggests that the VDR polymorphism may affect the type and the strength of the host immune response[8]. Study on CARD 15 gene with chronic periodontitis patients were found heterozygote and homozygote mutation variants in codon 802 in exon 4[9].

Natural resistance associated macrophage protein 1 (NRAMP1) encodes a divalent cation transporter protein. Divalent cations include Fe^{2+}, which is a complementary factor to various enzymes. Fe^{2+} is essential for bacterial proliferation, whereas Fe^{2+} is also necessary to produce hydroxyl radical, which will be reacted with nitric oxide (NO) to be biocidal peroxynitrite[10]. In this way, control of divalent cation transport may influence biocidal activity of macrophage. It was reported that NRAMP1 gene polymorphisms affect development of some immune-related diseases such as rheumatoid arthritis, type I diabetes, multiple sclerosis and inflammatory bowel diseases and tuberculosis[11-14]. Previous study, Hatta *et al.*[15], analyzed three locus D543N, 3'UTR and INT4 of NRAMP1 gene in tuberculosis, leprosy and healthy individual from South Sulawesi, Indonesia. It was observed an association of INT4 polymorphism with paucibacillary type of leprosy but not to multibacillary type. No significant association was found in the three locus with tuberculosis in this population[15].

In the present study, investigate the relationship between the genetic polymorphisms of NOD2, VDR and NRAMP1 genes and the host susceptibility to severity of typhoid fever patients living in the geographically isolated area of Indonesia. This study was conducted to explore the candidate gene associated with host susceptibility to severity of this disease.

MATERIALS AND METHODS

Typhoid fever patients enrollment: Total 461 acute febrile patients with clinical suspicion of typhoid fever presented at different hospitals, clinics and primary health care in South Sulawesi, Middle Sulawesi, Southeast Sulawesi, East Kalimantan and Papua were selected with the clinical information recorded between 2014-2016. The population of the area is small and well defined, the inhabitants originate from the selected area with ethnic group and their genetic variability is limited. Thus this area is an ideal area for the study of the polymorphisms of the candidate genes associated with host susceptibility to severity this disease. In this study selected eight regions in three genes, Exon 4 (802 C/T, 2104 C/T), Exon 8 (2722 G/C) and Exon 11 (3020 ins. C) in NOD2 gene, Exon 9 (352 T/C) in VDR gene, D543N (1703 G/A), 3'untranslated region (3'UTR, 1729+55 del.4 TGTG/del.), Intron 4 (469+14 G/C) in NRAMP1 gene, which were involved in modulation of macrophage function and reported to be associated with immune-related diseases infectious diseases. The clinical information was collected prospectively using a structured questionnaire and that was completed by the responsible physicians or nurses and was confirmed by internal medicine specialists.

Blood culture: Blood culture was performed for each of the group of patients with clinically suspected typhoid fever from several hospitals, clinics and primary health care in South Sulawesi, Middle Sulawesi, Southeast Sulawesi, East Kalimantan and Papua, Indonesia. The blood culture was performed by inoculation of 15 mL of bile broth (Merck, Rahway, NJ) with 5 mL of freshly collected blood. Cultures were incubated for 24 h at 37°C. One milliliter culture sample was then plated on *Salmonella shigella* agar. After incubation for 24 h at 37°C, colonies were examined by Gram staining and tested biochemically with the triple sugar iron test, sulfide indole motility, methyl red Voges' Proskauer reactivity, citrate

Table 1: Primers and PCR products in restriction enzyme cleavage in PCR RFLP of NOD2, VDR and NRAMP1 polymorphisms

Genes	Primer sequences	PCR temperature/ Product length	Restriction enzyme
NOD2			
Exon4 802 C/T	Forward: 5'-CAGTCTCGCTTCCTCAGTACC-3'	60°C/187 bp	*Bam*H I
	Reverse: 5'-AGTGTCCGCATCGTCATTG-3'		
Exon4 2104 C/T	Forward: 5'-TTCCTGGCAGGGCTGTTGTCCTGG-3'	60°C/139 bp	*Msp*I
	Reverse: 5'-GGATGGAGTGGAAGTGCTTG-3'		
Exon82722 G/C	Forward: 5'-CCACTCTGGGATTGAGTGGT-3'	60°C/261 bp	*Hha*I
	Reverse: 5'-CTTCACCTGATCTCCCCAAG-3'		
Exon113020 insC	Forward: 5'-GGCAGAAGCCCTCCTGCAGGGCC-3'	60°C/152 bp	*Apa*I
	Reverse: 5'-CCTCAAAATTCTGCCATTCC-3'		
VDR			
Exon9 352 T/C	Forward: 5'-CTGGGGAGCGGGAGTATGAAGGA-3'	60°C/1100 bp	*Taq*I
	Reverse: 5'-GGGTGGCGGCAGCGGATGTA-3'		
NRAMP1			
D543N 1703 G/A	Forward: 5'-GCATCTCCCCAATTCATGGT-3'	57°C/244 bp	*AvaII*
	Reverse: 5'-AACTGTCCCACTCTATCCTG-3'		
3'UTR 1729+TGTG	Forward: 5'-GCATCTCCCCAATTCATGGT-3'	57°C/240 bp	*FoxI*
	Reverse: 5'-AACTGTCCCACTCTATCCTC-5'		
Intron4 469 + 14G/C	Forward: 5'-TCTCTGGCTGAAGGCTCTCC-3'	59°C/624 bp	*ApaI*
	Reverse: 5'-TGTGCTATCAGTTTGAGCCTC-3'		

consumption, urease and decarboxylase activity and carbohydrate fermentation of glucose, lactose, mannitol, sucrose and arabinose to identify S. Typhi positive cultures[16,17].

Widal test: The Widal test procedure using O antigen was performed according to the manufacturer's protocol (Murex Biotech, Ltd., Dartford, UK). Briefly, two-fold serial dilutions (1:20-1:1,280) of the serum sample were prepared. One drop (~25 µL) of the O antigen suspension was added to each tube containing the diluted sample. Antigen and serum was remixed and incubated at 50°C. Tubes were checked for agglutination after 4 h. According to routine diagnostic criteria, a titer ≥1:320 was considered positive for the samples tested[18,19].

Extraction of DNA from blood samples: Genomic DNA extraction from whole blood samples was performed using silica-guanidinium Isothiocyanate[16,19]. Briefly, DNA was extracted from freshly collected whole blood, a 100 µL freshly collected blood sample was mixed with 900 L of lysis buffer (50 mM Tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X-100). To obtain the DNA, samples were lysed by incubation for 15 min at 18°C and 20 µL of diatom suspension was added. The diatom containing the bound DNA was sedimented by centrifugation at 12000 rpm for 15 sec. 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 (analytical grade) and dried by incubation at 56°C for 10 min. The pellet was mixed with 60 µL of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer and the DNA was eluted by incubation at 56°C for 10 min. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until PCR was performed.

Polymerase chain reaction (PCR) amplification and detection: The PCR mixture for NOD2 and VDR2 gene were each final volume of 25 µL with 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.1 µg of extracted DNA, 9 µM of each primer, 1 U of *Taq* DNA polymerase. For NRAMP1 gene was a final volume of 25 µL with 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.1 µg of extracted DNA, 1 µM of each primer, 2 U of *Taq* DNA polymerase Ampli Taq GOLD (Applied Biosystems, Foster city, California). The reaction for NOD2 and VDR gene were carried out in a program for 40 cycles, each consisting of denaturation for 20 sec at 94°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C. Amplification of NRAMP1 gene was carried out using PCR. The PCR program was 35 cycles. Denaturation at 94°C for 45 sec (D543N and 3'UTR) and denaturation at 94°C for 30 sec (intron 4). Annealing at 57°C for 45 sec (D543N and 3'UTR) and at 59°C for 45 sec (intron 4). Extension for 45 sec at 72°C (D543N and 3'UTR) and for 1 min at 72°C (intron 4) in a thermocycler (Applied Biosystems 2720 Thermal cycler, USA). Each set of primers used is shown in Table 1. Amplified DNA fragment was used for restriction fragment length polymorphism (RFLP) analysis and direct sequencing[6,9,15,20-22]. The PCR was performed without prior knowledge of the classification of the samples.

Restriction fragment length polymorphism (RFLP) analysis: Each region was analyzed for the presence of polymorphisms using PCR-RFLP analysis by each specific restriction enzyme which is shown in Table 1. Each mixture was a final volume of 20 µL with 1 µL of restriction enzyme and 1 µg of DNA amplified by PCR. Activation was performed for 1 h at 37°C. The presence of polymorphism was detected by electrophoresis agarose gel 2%.

Direct sequencing: It was confirmed the presence of polymorphisms by direct sequencing for each band pattern of electrophoresis after PCR-RFLP. Each primer and dNTPs were removed from PCR products using Exo SAP-IT (USB, Cleveland, Ohio). Each mixture for direct sequencing was a final volume of 20 µL with 5 µL of PCR product, 3.2 pmol of forward primer, 1.6 µL of Big Dye Terminator v 3.1 (Applied Biosystems, Foster city, California). Sequencing reaction was carried out in a program for 25 cycles, each consisting of denaturation for 10 sec at 96°C, annealing for 5 sec at 50°C and extension for 4 min at 60°C in Temperature control system PC 701 (Astec, Fukuoka, Japan). And then direct sequencing was performed using ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster city, California, USA).

Statistical analysis: The data were analyzed using Microsoft Excel and Stata 9.2 (Stata Corp LP, College Station, TX, USA). For univariate analysis chi-square tests and Fisher's exact tests were used. For multivariate analysis, a backwards selection multivariate logistic regression model was used. All variables with a $p<0.20$ on univariate analyses were included from multivariate analysis. Significance levels was set at $p<0.05$.

RESULTS

To determine whether genetic polymorphisms in NOD2, VDR and NRAMP1 gene were associated with susceptibility to severity of typhoid fever, it was performed genotyping analyses of 426 patients with mild and 35 severe state of typhoid fever by PCR-RFLP. It was also confirmed the presence of polymorphisms by direct sequencing for each band pattern of electrophoresis agarose gel 2% after PCR-RFLP.

Characteristics of mild and severe state of typhoid fever are shown in Table 2. The mean age of the patients was 21.4 years (range, 7-50) and the male to female ratio was 0.83. The duration of fever at hospital admission was 6.2 days (range, 4-16) and the average body temperature was 38.1°C (range, 37.8-40.5).

The general disease condition was considered severe in 35 patients (7.6%), 34 patients with stupor (7.47%) and one patient with coma (0.21%). It was found that patients with melena and intestinal perforation 15 (3.2%) and 9 (1.9%), respectively. Nine (1.9%) patients with septicaemia. The study also found patients with hepatomegaly, splenomegaly and jaundice 14 (3.0%), 11 (2.4%) and 12 (2.6%), respectively.

None of the patients had respiratory involvement and the main symptoms and signs were malaise (100.0%), coated tongue (98.4%), headache (93.7%), loss of appetite (89.6%) and relative bradycardia (55.9%) (Table 2).

As shown in Table 3, polymorphisms were found in all selected regions. G/C and C/C alleles of exon 8 in NOD2 gene, which located at nucleotide 2722, were more frequent among severe state patients than mild state with typhoid fever. Each p-value was 0.027 and 0.014 and odds ratio was 16.7 and 27.9 (95% confidence interval was 2.422-25.717 and 2.663-37.128). These findings were considered to be significant statistically.

Table 2: Clinical characteristics of typhoid fever patients

Signs and symptoms	Number (%) (n = 461)
Age mean	21.4 year (min 7 year-max 50 year)
Duration illness	6.6 days (min 4 days-max 16 days)
Male/Female ratio	209/252 (82.9)
Fever	38.1 (min 37.8°C-max 40.5°C)
Malaise	461 (100.0)
Coated tongue	454 (98.4)
Headache	432 (93.7)
Loss of appetite	413 (89.6)
Relative bradycardia	258 (55.9)
Nausea	62 (13.4)
Muscle aches	54 (11.7)
Apathy	82 (17.8)
Stupor*	30 (6.5)
Coma*	1 (0.2)
Constipation	26 (5.6)
Abdominal distention	27 (5.8)
Diarrhea	25 (5.4)
Abdominal pain	13 (2.8)
Melena*	15 (3.2)
Septicaemia*	9 (1.9)
Intestinal perforation*	9 (1.9)
Hepatomegaly*	14 (3.0)
Splenomegaly*	21 (4.5)
Jaundice*	12 (2.6)
Widal titer	
<160	13 (2.8)
320-640	413 (89.6)
640-1280*	33 (7.6)
>1280*	2 (0.44)

*Signs and symptoms of severe typhoid fever

On the other hand, at exon 4 (802 C/T and 2104 C/T) and exon 11 (3020 ins.C) in the NOD2 gene, no significant difference was detected. At exon 9 (352 T/C) in VDR gene and D543N (1703 G/A), 3'UTR (1729 +55 del.4), intron4 (469+14 G/C) in NRAMP1 gene, it could not find significant relation either.

DISCUSSION

There are important to determine the severity of typhoid and pathomechanisms of this disease, since clinical signs and symptoms are very fast to change to become severe state of diseases. The variability in host response to S. Typhi infection has been considered to be due to host genetic factors[23]. Though some genes involved in host immune response were examined in past reports[24-26] but the relation between host genetic variability and susceptibility to severity of typhoid fever has not been concluded yet.

The study aimed to elucidate the mechanisms behind severity of typhoid fever in relation with polymorphisms of NOD2, VDR and NRAMP1 genes. The results of this study revealed that polymorphisms G/C and C/C alleles of exon 8 in

Table 3: Distribution of NOD2, VDR and NRAMP1 genotyping among mild and severe state of typhoid fever

		Mild (N = 426)			Severe (N = 35)		
Genes	Polymorphisms	n (%)	Odd ratio* (95% CI)	p-value	n (%)	Odd ratio*(95% CI)	p-value
NOD2							
Exon4 802	C/C	218 (52.9)			20 (57.1)		
	C/T	125 (29.3)	1.225 (0.473-1.342)	0.837	9 (25.7)	0.934 (1.238-2.293)	0.872
	T/T	83 (17.8)	0.486 (0.529-1.476)	0.452	6 (17.2)	0.944 (0.452-0.993)	0.917
Exon4 2104	C/C	278 (65.2)			22 (62.8)		
	C/T	116 (27.2)	1.045 (0.347-3.417)	0.734	9 (25.7)	1.325 (1.293-4.521)	0.475
	T/T	32 (7.6)	1.271 (1.024-2.996)	0.925	4 (11.5)	1.698 (0.489-2.754)	0.284
Exon8 2722	G/G	243 (57.0)			2 (5.7)		
	G/C	103 (24.2)	0.876 (1.45-3.281)	0.768	14 (40.0)	16.7 (3.422 - 25.717)	0.027
	C/C	80 (18.8)	0.462 (0.364-1.927)	0.572	19 (52.3)	27.9 (2.663 - 37.128)	0.014
Exon11 3020	C/C	407 (95.5)			33 (94.3)		
	C/del	19 (4.5)	0.126 (0.365-236.323)	0.897	2 (5.7)	1.854 (0.643-21.634)	0.548
	del/del	0 (0.0)			0 (0.0)		
VDR							
Exon9 352	T/T	364 (85.2)			29 (82.8)		
	T/C	49 (11.5)	0.489 (1.958-37.539)	0.367	4 (11.5)	1.528 (0.451-3.237)	0.435
	C/C	13 (3.3)	1.578 (1.209-46.013)	0.465	2 (5.7)	1.946 (0.328-4.291)	0.869
NRAMP1							
D543N 1703	G/G	268 (62.9)			23 (65.7)		
	G/A	136 (31.9)	0.835 (0.254-1.872)	0.683	11 (31.4)	0.429 (0.969-2.478)	0.584
	A/A	22 (5.2)	1.239 (0.493-2.394)	0.392	1 (2.9)	1.217 (0.363-2.471)	0.549
3'UTR 1729	TGTG+/+	269 (63.1)			21 (60.0)		
	TGTG +/del	129 (30.3)	2.023 (0.236-13.749)	0.756	10 (28.5)		
	TGTG del/del	28 (6.6)	1.288 (1.273-19.284)	0.430	4 (11.5)	1.575 (1.281-4.363)	0.768
Int4 469+14	G/G	396 (92.9)			32 (91.4)		
	G/C	30 (7.1)	2.384 (1.204-5.384)	0.839	3 (8.6)	2.426 (1.284-3.529)	0.575
	C/C	0 (0.0)			0 (0.0)		

*Odds ratio value using multivariate analysis

NOD2 gene, which located at nucleotide 2722, were more frequent among severe state patients than mild state with typhoid fever. S. Typhi is bacterial resistant to intracellular germicide mechanisms of usual macrophage. To fight against such pathogen, activation of macrophage is required[27]. It was thought that macrophage function affects development of severity typhoid fever and performed the genotyping of eight regions in three genes (NOD2, VDR and NRAMP1) which were involved in modulation of macrophage function and might be associated with immune-related diseases including severity of symptoms this disease.

On other hand, previous study revealed that predominant in severity state of typhoid fever belong Hd+ and Hd+z66 Ind+ of S. Typhi bacterial in Indonesia but in the endemic areas of typhoid fever, only a small proportion of individuals exposed to S. Typhi develop the severe symptoms[28].

In this study, it was found significant findings in polymorphism of exon 8 in NOD2 gene. The G/C alleles and C/C alleles were more frequent among severe state than mild state of typhoid fever. These alleles may facilitate development of the disease possibly. Recognition of pathogen by macrophage is important for innate immunity. There are receptors specific to pathogen associated molecular pattern (PAMP), of those nucleotide-binding oligomerization domain (NOD) are in the cytoplasm. The NOD2, which mainly exists in macrophage, is a receptor to muramyl dipeptide (MDP) on peptidoglycan of gram positive and negative bacteria[10]. There has been no report about relation between NOD2 gene and susceptibility to severity state of typhoid fever. Meanwhile, other study indicated that NOD2 mutation and increased activities of NF-κB were related to early onset sarcoidosis[29].

Since too many hypothesis in molecular pathomechanisms of infectious diseases related with genetic host susceptibility, therefore clarification of more detailed relationship between the genetic polymorphisms and the host susceptibility to severity typhoid fever was needed, further investigation involving much larger patients population and more candidate genes is warranted.

CONCLUSION

The present study revealed that the G/C genotype in codon 2722 of exon 8 NOD2 gene could be associated with increased risk of clinical severity of typhoid fever.

SIGNIFICANCE STATEMENTS

This study discovers the polymorphism of NOD2, VDR, NRAMP1 genes to susceptibility for typhoid fever severity. This study will help researchers to understand in more detail the molecular pathomechanisms typhoid fever. Thus novelty of this study suggest that the polymorphisms G/C and C/C alleles of exon 8 in NOD2 was related to susceptibility to typhoid fever in clinical severity.

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