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Polymorphisms of *TLR4* Asp299Gly and *TNF-α* -308G/A in Leptospirosis

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ABSTRACT

Background: *TLR4* Asp299Gly and *TNF-α* -308G/A polymorphisms have been shown to be associated with increased susceptibility and severity of infection. *TLR4* Asp299Gly polymorphism could affect the host's ability to respond to *leptospira sp*. *TNF-α* -308G/A polymorphism, is associated with the high producer of *TNF-α*.
Methods: Total of 36 leptospirosis patients (IgM anti leptospira and MAT positive) and healthy individual with equal number were included. The polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using site specific restriction enzyme.
Results: Distribution of homozygous wild-type *TLR4* Asp299Gly polymorphism was higher in both of groups (94.5: 97.2%.) and homozygous mutant allele was absent. There was not significantly difference of TLR4 Asp299Gly in leptospirosis patients and healthy group (ρ=1.00; OR 0.5; 95%CI, 0.04-5.6) and between mild and severe leptospirosis (ρ=0.54; OR 1.54; 95% CI, 1.20-1.98). The presence of homozygous wild-type *TNF-α* -308G/A polymorphism was higher between leptospirosis patients and healthy group (100: 94.5%) and homozygous mutant allele was not found in both of the groups. No significantly different of *TNF-α* -308G/A polymorphism between leptospirosis patient and healthy group (ρ=0.49).
Conclusions: In this study, the polymorphisms of *TLR4* Asp299Gly and *TNF-α* -308G/A are not associated with the susceptibility and severity of leptospirosis.

Keywords: Leptospirosis, *TLR4* Asp299Gly polymorphism, *TNF-α* -308G/A polymorphism.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by *Leptospira interrogans.* Leptospirosis is an endemic health problem in South-East Asia region[1,2]. The clinical manifestations are varying widely, ranging from mild leptospirosis (anicteric) to severe leptospirosis (icteric). Severe leptospirosis known as Weil's disease is characterized by icteric, acute renal failure and bleeding tendency[3,4]. Severity and

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mortalityrates of leptospirosis influence by several factors : types of serovars, and host factors including genetic, age, immunity status, comorbidity, occupation or activity. Genetic factor is one of the main important in susceptibility and severity of infections[1,3].

The pathogenesis of leptospirosis is not fully understood. It seems mimic the pathogenesis of sepsis caused by gram-negative bacteria infection[5]. *Leptospira sp* has immunogenic lipopolysaccaride wall cell (LPS) a potent activator of the natural immune system (innate/nonspecific immunity), which will be responded by *TLR4* (*Toll Like Receptor-4*). *TLR4* is a key receptor for gram negative bacteria, mycobacteria, fungi and parasites of malaria. *TLR4* will stimulate macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6)[6-8].

Polymorphism of *TLR4* with the common frequency >5% is *TLR4* Asp299Gly change from asparagine (Asp) at codon GAT to glysine (Gly) to GGT codon at 299 of amino acid. The presence of these polymorphisms alter the function of *TLR4* receptor, associated with hyporesponsive to LPS and increased the susceptibility of gram negative infection, sepsis, septic shock, tuberculosis and malaria[9-13].

The major cytokines in the acute inflammatory response of gram negative bacteria and microbes is *TNF-α*[14]. There are several type of *TNF-α* polymorphisms that have been studied. *TNF-α* -308G/A is associated with increased of transcriptional activity of *TNF-α*[15]. Several studies showed that the *TNF-α* -308G/A associated with the incidence of infection in old age, respiratory failure, septic shock in community acquired pneumonia, and severe sepsis in burn trauma. There was a significant difference between mutant-type allele A/A of *TNF-α* -308G/A polymorphism with septic shock[15-17]. The study about dengue in Semarang showed that there was correlation of *TNF-α* polymorphism between dengue cases and control[18].

The objective of this study were to know whether polymorphisms of *TLR4* Asp299Gly gene and *TNF-α* -308G/A related to susceptibility and severity of leptospirosis.

MATERIALS AND METHODS

Study Design

The subjects were consecutively derived from an observational cohort study conducted in Dr. Kariadi Hospital and Center for Biomedical Research (CEBIOR) Semarang from 2010 to 2012.

Study Subjects

Inclusion criteria for patients were individual aged 14 years and older who match clinical criteria of leptospirosis (WHO-SEARO, Chennai 2010) with positive of rapid diagnostic test of IgM anti leptospira and confirmed by MAT (microagglutination test) and agreeing to become a study subject by signing the consent form. Inclusion criteria for control were healthy volunteers not suffering from leptospirosis and signing the consent form. Thirty six controls and 36 leptospirosis patients were selected; 24 patients (72.2%) were classified as severe leptospirosis, while 12 patients (27.8%) were mild.

DNA extraction

DNA extraction used the salting out method as described by previous investigators with slight modification[19].

Analysis of gene polymorphisms

Analysis of *TNF-α* gene polymorphism was performed by amplification of the *TNF-α* -308 gene in the promotor region at position -308, using a PCR cycler (Applied Biosystems). The total volume of each sample mixture (20ul) contained 2ul DNA, 0.2 ul of forward primer (5'AGG CAA TAG GTT TTG AGG GCC AT 3') and reverse primer (5' TCC TCC CTG CTC CGA TTC CG 3') respectively, 0.4 ul dNTP, 0.4 ul MgCl2 and 0.2 ul of the Taq enzyme.

The polymerase chain reaction (PCR) profile was consisted of 5 minutes at 95°C for initial denaturation, followed by final extension for 7 minutes at 72°C. The PCR product of 107 bp was then cut by restriction fragment length polymorphism (RFLP), by incubation at 55°C for 18 hours (overnight) with the *NcoI* restriction enzyme. The PCR product was visualized by 2% agarose gel electrophoresis. In individuals homozygous for the -308 (A/A) allele, the PCR product was not cut, resulting in a band on the gel of 107 bp. In contrast, homozygous G/G yielded fragments of 87bp and 20 bp, while heterozygous G/A yielded fragments of 107 bp, 87 bp and 20 bp.

Analysis of *TLR-4* Asp299Gly gene polymorphism was using a PCR. The total volume of each sample mixture (20ul) contained 2ul DNA, 0.5 ul of forward primer (5' GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG 3') and reverse primer (5' TCC TCC CTG CTC CGA TTC CG 3'), respectively, 1.2 ul dNTP, 2.5 ul MgCl2 and 0.3 ul of the Taq enzyme. The polymerase chain reaction (PCR) cycle was consisted of 5 minutes at 95°C for initial denaturation, followed by final extension for 7 minutes at 72°C. The PCR product of 107 bp was then cut by restriction fragment length polymorphism (RFLP), by incubation at 55°C for 18 hours (overnight) with the *NcoI* restriction enzyme. We used 100 bp for marker ladder. The PCR product was visualized using 2% agarose

gel. In individuals homozygous for the *TLR4* Asp299Gly (A/A) allele, the PCR product was not cut, resulting in a band on the gel 195bp. In contrast, homozygous G/G yielded fragments of 176 bp and 19 bp, while heterozygous A/G yielded fragments of 195 bp, 176 bp and 19 bp.

Data Analysis

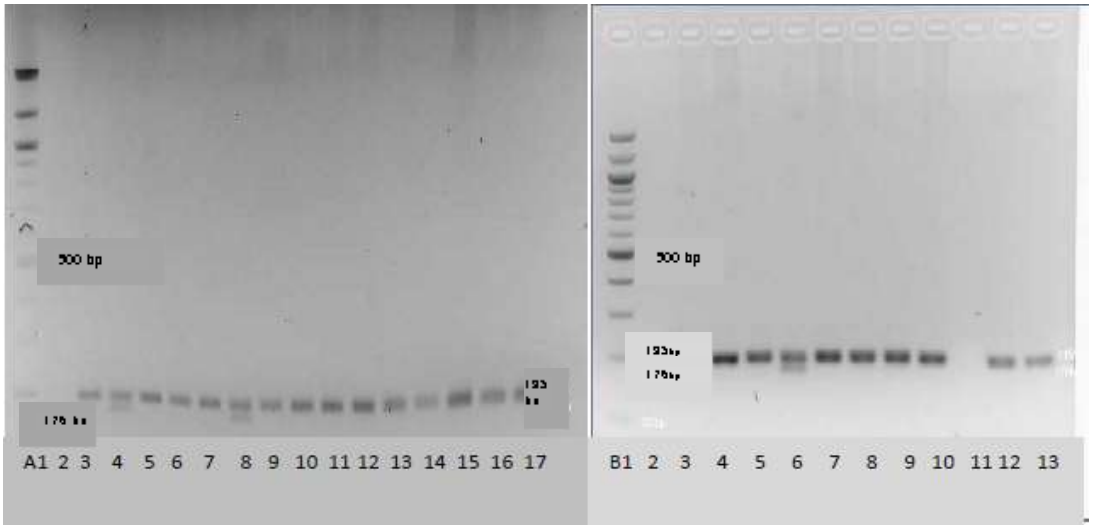
The data were analysed by Mann- Whitney U test, Hardy-Weinberg Equilibrium for allele frequency and Fischer-Exact test. All analyses were performed by means of the SPSS program version 15 for Windows, at a significance level of 5%.

Ethical Clearance

The study was approved by Ethics Committee of Medical Research and Health Faculty of Medicine, Diponegoro University/Dr Kariadi Hospital, Semarang, with ethical clearance No 102/EC/FK/RSDK/2010.

RESULTS

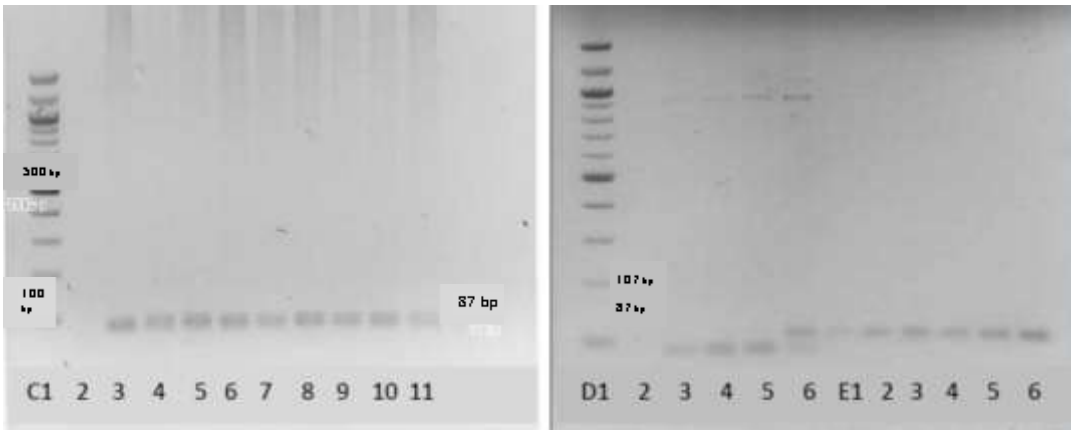
Thirty six leptospirosis patients were included in this study, consisted of male 67.2% and the mean of age was 47.25 (SD 12.52) years. The distribution of homozygous wild-type *TLR4* Asp299Gly polymorphism was high in leptospirosis patients and control groups (94.5 vs 97.2%) and homozygous mutant allele was absent in both studied groups. The heterozygote allele of *TLR4* Asp299Gly was 5.5% while in control was 2.8%. The comparison between leptospirosis patients and control showed that there was not significantly difference in patients than control group (ρ=1.00; OR 0.5; 95%CI, 0.04-5.6). Frequency for G allele in each group was 2.8 : 1.4 % for leptospirosis group and control. There was no significantly difference of *TLR4* Asp299Gly polymorphism between mild and severe leptospirosis (ρ=0.5;OR 1.5; 95%CI 1.20-1.98).



A **B**

Figure 1.A. PCR-RFLP results for *TLR4* Asp299Gly in Leptospirosis
Lane A1 marker 100 bp ladder, lane A2 blanks. Lanes A3, 5-7, 9-17 homozygous A/A Lanes A4,8 heterozygous A/G. PCR product digested using *Ncoi* restriction enzyme.

Figure 1.B. PCR-RFLP for *TLR4* Asp299Gly in healthy control
Lane B1 marker 100 bp ladder, lane B2 blanks. Lanes B3-5, 7-13 homozygous A/A. Lane B6 heterozygous A/G. Lane B11: blanks. The presence of *TNF-α* -308G/A polymorphism between leptospirosis patients and control group were as follow: homozygous wild-type (100 vs 94.5%) , heterozygous allele (0.0 vs 5.5%) and homozygous mutant allele were not found in both of the group. There was not significantly different of *TNF-α* -308G/A polymorphism between leptospirosis patient and control (ρ=0.49).



A **B**

Figure 2.A. PCR-RFLP *TNF-α* -308G/A polymorphism in leptospirosis.

Lane C1: marker 100 bp ladder, lane C2: blanks Lanes C3-11: homozygote allele G/G. PCR product digested using *Ncoi* restricyion enzyme.

Figure 2.B. PCR-RFLP *TNF-α* -308G/A polymorphism in healthy control.

Lane D1: marker 100 bp ladder, lane D2: blanks Lanes D3-5: homozygote alleleG/G. Lane D6: heterozygote A/G. Lanes E1-E6: PCR product undigested by enzim NcoI.

DISCUSSION

In this study, we found that there were no significant differences between *TLR4* Asp299Gly polymorphism of leptospirosis patients and healthy control, or between mild and severe leptospirosis. It was contrast with previous studies that the polymorphism *TLR4* Asp299Gly gene was associated with susceptibility and severity of gram negative infections[5,10]. Other studies showed similar result that there were no significant differences of *TLR4* Asp299Gly between DHF/DSS and control group, or severity of meningococcal infection and outcome of gram negative sepsis[20-23]. Metaanalysis studies in Caucasian and in China found that there were not strong correlation between sepsis and *TLR4* Asp299Gly polymorphism[24-27].

Race, ethnic and geography seem to affect the frequency of *TLR4* Asp299Gly polymorphism. In African, frequency of *TLR4* Asp299Gly haplotype was higher than Caucasian that had co-segregation of *TLR4* Asp299Gly/Thr399Ile, while in Asia was less. Environmental pressure when human migration to Europe, Africa, Asia and New World affected the frequency of *TLR4* Asp299Gly[28].

Distribution of *TNF-α* -308 G/A in leptospirosis patients was dominant allele homozygot G/G. This study found that there were no significant difference in *TNF-α* -308G/A between leptospirosis and control groups. *TNF-α* -308G/A causes increased production of *TNF-α* that could affect susceptibility and outcome of infection[29,30]. The A allele causes increased production of *TNF -α* in a higher level than allele G[31]. Study of sepsis and severe sepsis in the Chinese population obtained that allele A was higher but was not influence on mortality. *TNF-α* serum level was high in leptospirosis but did not correlated with *TNF-α* -308G/A[32].

A meta-analysis study showed that *TNF-α* -308G/A was associated with the incidence of sepsis not in death, and Asian had a strong contribution compared with other races[33]. In Caucasian study showed that allele homozygotes A/A *TNF-α* -308 G/A effected on susceptibility and poor outcome and duration of ventilator used in critical illness. [34] Other studies showed that polymorphism of *TNF-α* -308G/A was not correlated with the outcome of sepsis gram negative and malaria[35-36].

Study in Aceh revealed that no significant association between genotype distributions and allele frequency of SNP *TNF-α* -308G/A and spontaneous preterm birth in the Acehnese ethnic group[37]. Study in endemic area in Indonesia showed that *TNF-α* -308G/A was not associated with typhoid fever[38]. Ethnic affected the variation of allele and haplotypes of *TNF-α* -308G/A in three ethnic populations of Sulawesi. Some haplotypes might be restricted to Asian or Asian-derived populations[39].

This study was not consistent with the law of Hardy - Weinberg Equilibrium might be due to the small sample size and homogeneous population of Javanese.

CONCLUSION

TLR4 Asp299Gly and *TNF-α* - 308G/A genes polymorphism were not associated with susceptibility and severity of leptospirosis.

SUGGESTION

Further research is needed with a heterogeneous population including such ethnics and a large number of cases and the controls. Sequencing of *TLR4* and *TNF-α* genes are needed to determine the possibility of other polymorphisms.

Competing interest: The authors have declared that no competing interests exist.

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