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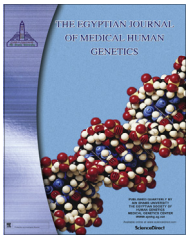

ORIGINAL ARTICLE

Analysis of *TLR* polymorphisms in typhoid patients and asymptomatic typhoid carriers among the schoolchildren

Ilakkia Sivaji [a], **Senbagam Duraisamy** [b], **Senthilkumar Balakrishnan** [a,c,*]

[a] *Department of Biotechnology, Muthayammal College of Arts and Science, Rasipuram – 637 408, Tamil Nadu, India*
[b] *Department of Microbiology, Vivekananda College of Arts and Science for Women, Tiruchengode – 637 205, Tamil Nadu, India*
[c] *Department of Medical Microbiology, College of Health and Medical Sciences, Haramaya University, P.O. Box 235, Harar, Ethiopia*

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Abstract *Background:* *Toll like receptor* (*TLR*) plays a critical role in recognition and activation of both innate and adaptive immune responses against microbial pathogens. Several studies have implicated the genetic variations (polymorphisms) in *TLR* genes to influence the host susceptibility to infectious diseases. However, the available literature on *TLR* polymorphism and susceptibility to typhoid fever is unclear.

Aim: This study aimed to investigate the polymorphism of *TLRs* 1, 2, 4 and 5 in typhoid patients and convalescent phase asymptomatic typhoid carriers among the schoolchildren.

Subjects and methods: *TLR* genes were amplified by PCR from peripheral blood leukocytes of schoolchildren with typhoid ($n = 20$) or asymptomatic typhoid carrier ($n = 30$) state, and normal healthy individuals ($n = 50$). The RFLP analyses for *TLR*1, 2, 4 and 5 genes using restriction enzymes such as *AluI*, *AciI*, *NcoI* and *DdeI*, respectively, were performed to determine the single nucleotide polymorphism.

Results: *TLR*1 polymorphism was observed in 5% (1/20) of typhoid patients and 6.6% (2/30) of typhoid carriers. *TLR*2 polymorphism was observed in 10% (2/20) of typhoid patients and 6.6% (2/30) of carriers. *TLR*4 polymorphism was not observed in typhoid patients, but 6.6% (2/30) of typhoid carriers exhibited a polymorphism. As well, *TLR*5 polymorphism was not observed in typhoid patients, while 13.3% (4/30) of typhoid carriers had polymorphism. None of the control healthy individuals had evidence for *TLR* polymorphisms.

Conclusion: The study reports polymorphisms of *TLR* genes in a lower proportion among the schoolchildren with typhoid or convalescent typhoid carrier state.

* Corresponding author at: Department of Medical Microbiology, College of Health and Medical Sciences, Haramaya University, P.O. Box 235, Harar, Ethiopia. Tel.: +91 9443286292, +251 931907924.
E-mail address: nbsenthilkumar@gmail.com (S. Balakrishnan).

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1. Introduction

Typhoid fever is a major public health problem worldwide and it is considered as a major enteric illness in the developing countries. It is an acute febrile disease caused by *Salmonella enterica* serotype Typhi [1]. Infection with *S*. Typhi occurs by fecal-oral route through ingestion of food and water contaminated with feces of typhoid patients and asymptomatic typhoid carriers [2–6]. The disease is endemic in the Indian subcontinent, Southeast Asia, Latin America and sub-Saharan Africa.

During its intracellular life in macrophages, *Salmonella* induces multiple regulatory components that are responsible for its endurance inside the host [7]. *Salmonella* has evolved remarkable strategies to avoid the host immune response. One of these strategies is a modification in lipopolysaccharide (LPS) structure, which facilitates *TLR*4-mediated downstream signaling cascade inducing host immune response [8,9]. Further, membrane remodeling blocks detection by host *TLR*4 and also increases the resistance of bacteria against host antimicrobial [10]. A study in Malay population has identified 8.9% *TLR*4 Asp299Gly and 7.2% *TLR*4 The399Ile polymorphisms in typhoid susceptible populations. In addition, *TLR*5 mediates the innate immune responses to *Salmonella* through binding to flagellin [11]. A polymorphism in the *TLR*5 gene introduces a premature stop codon ($TLR5^{392STOP}$), which might play a role in variation for binding to flagellin and mediating immune responses [12]. However, in our previous study, we have identified that *TLR*5 gene polymorphism was not associated with susceptibility to typhoid fever, as there was no significant correlation between *TLR*5 gene polymorphism and clinical parameters in typhoid patients and typhoid carrier [13]. As such, we sought to identify whether nucleotide polymorphisms in other *TLR* genes (e.g., *TLR*1, 2, 4 or 5) are involved in susceptibility *Salmonella* infection among the schoolchildren. We have recently reported the clinical correlates and serotyping of *Salmonella* isolates during typhoid fever and asymptomatic carrier state among the schoolchildren [14]. In this study, we report the association between polymorphism of *TLR* genes and occurrence of typhoid fever among the schoolchildren.

2. Subjects and methods

2.1. Demography

Venous blood samples collected from a cohort of schoolchildren previously reported to be positive for typhoid fever ($n = 20$), convalescent phase asymptomatic carriers ($n = 30$) and healthy control individuals ($n = 50$) among the schoolchildren in northern part of Tamil Nadu, India were used for identifying polymorphism in *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes [14].

2.2. PCR-RFLP analysis of TLR genes polymorphism

Genomic DNA was isolated from 2 ml of EDTA-treated blood using a DNA isolation kit (QlAamp DNA Blood Minikit Cat. No. 51106, Qiagen, Germany) and stored frozen at −20 °C for molecular analysis. The template DNA (200 ng) was amplified in a 50 μl PCR master mix (New England Biolabs) in Thermocycler (Techne, UK) using primers specific for *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes (Table 1) with slight modification of a procedure described previously. The PCR products were analyzed by electrophoresis on 1.5% agarose gel (Biotech, India). *TLR* genes nucleotide polymorphisms ($TLR1^{602I}$, $TLR2^{753Glu}$, $TLR4^{299D}$ and $TLR5^{392STOP}$) were identified by restriction enzyme (RE) digestion of PCR products with gene-specific RE (Table 1). For RFLP analysis of *TLR* genes, RE digestion of PCR products and analyses for size-specific products by electrophoresis were performed as described previously [13].

3. Results

The normal expression patterns of *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes were detected in genomic DNA from all the individuals of three groups. The amplified PCR products of *TLR* genes were observed on 1.5% agarose gel electrophoresis (Fig. 1). The PCR products of *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes from all subjects were subjected to RFLP using restriction enzymes to determine polymorphisms (Fig. 2). Among the typhoid patients, polymorphism of *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes were found to be 5%, 10%, 0% and 0% respectively. In contrast, the typhoid carriers exhibited a less variations of *TLR* genes polymorphism Viz. 6.6% of *TLR*1, *TLR*2, *TLR*4 and 13.3% of *TLR*5 (Table 2). Consequently, the control healthy individuals had no evidence for *TLR* polymorphisms.

4. Discussion

TLR plays a critical role in recognition and activation of both innate and adaptive immune responses against microbial pathogens [16]. Several studies have implicated the genetic variations (polymorphisms) in *TLR* genes to influence the host susceptibility to infectious diseases [17]. However, the available literature on *TLR* polymorphism and susceptibility to typhoid fever is unclear. *Salmonella* flagellin is one of the major virulence factors and thus genetic polymorphism on it cognate receptor, *TLR5*, might be critical for susceptibility to infection [15]. The study in *TLR*5 gene polymorphism at mature stop codon ($TLR5^{392STOP}$) has implicated for variation in binding ability to flagellin and differing host immune responses during typhoid fever [12]. However, in our recent study with a small cohort of typhoid patient and asymptomatic typhoid carriers, we have identified that *TLR5* gene polymorphism was not associated with susceptibility to typhoid fever, as there was no significant correlation between *TLR5* gene polymorphism and clinical parameters associated with typhoid fever [13]. Interestingly, *Salmonella* LPS is potent stimulator of innate immune cells and thus genetic variation in *TLR*4 gene might also influence the infection state during typhoid infection [8,9]. The prevalence of 12.5% of *TLR*4 polymorphism in typhoid-susceptible Malay populations was reported [11]. This *TLR*4 gene polymorphism in Malay population has been suggested to be a higher risk for typhoid infection. Taken together, it is possible that genetic variations in more than one *TLR* might render the higher susceptibility to typhoid infection. However, a RFLP analysis for polymorphisms in *TLR*1, 2, 4 and 5 genes, in the present study, revealed the

Table 1 Summary of primers, PCR conditions, RE digestion and RFLP allele location of *TLR* genes.

TLR gene	Primers	Cycling conditions	Restriction enzyme	Allele length (bp)
*TLR*1	F:5′GGAAAGTTATA GAGGAACCCT-3′ R: 5′CTTCACCCAGA AAGAATCGTGCC-3′	95 °C for 5 min; 35 cycles at 95 °C for 30 s; 55 °C for 30 s; and 72 °C for 30 s; followed by 72 °C 7 min.	*AluI*	280 129, 151
*TLR*2	F: 5′-GCCTACTGGG TGGAGAACCT-3′ R: 5′-GGCCACTCC AGGTAGGTCTT-3′	95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; followed by 72 °C for 7 min.	*AciI*	340 265, 75
*TLR*4	F:5′GATTAGCATACTTAG ACTACTACCTCCATG-3′ R: 5′-GATCAACTTCTGA AAAAGCATTCCCAC-3′	95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; followed by 72 °C for 7 min.	*NcoI*	249 30, 219
*TLR*5	F: 5′-GGTAGCCTA CATTGATTTGC-3′ R: 5′GAGAATCTGGAG ATGAGGTACCCG-3′	95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; followed by 72 °C for 7 min.	*DdeI*	277 91, 186

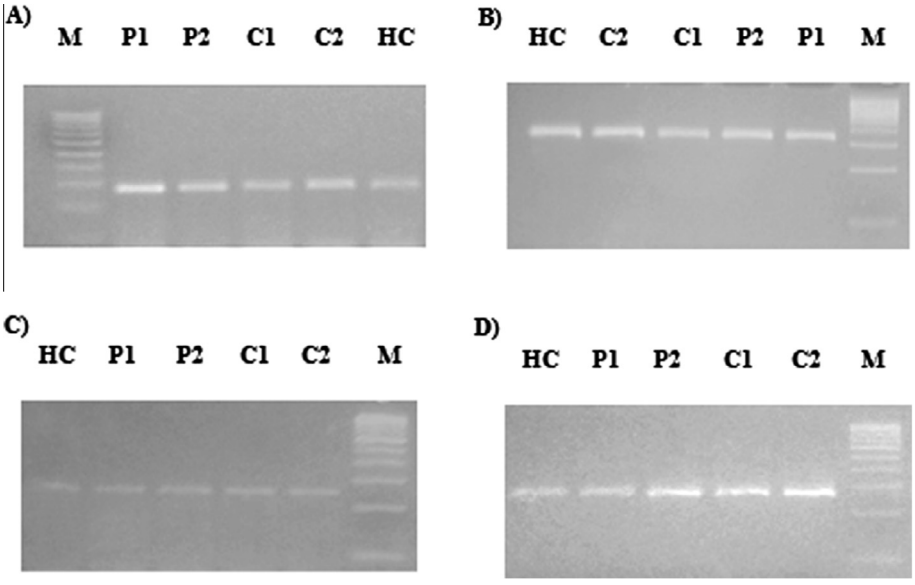

Figure 1 PCR amplification of (A) *TLR*1, (B) *TLR*2, (C) *TLR*4 and (D) *TLR*5 genes. Lane M represents DNA ladder (100 bp); lane P1 and P2 represents patients (typhoid) fever; lane C1 and C2 represents carriers (typhoid); lane HC represents healthy control individuals.

genetic variations in these genes in a marginally lower proportion (5–13%) of schoolchildren that are susceptible to typhoid infection when compared to healthy individuals. Curiously, none of the schoolchildren positive for typhoid fever had polymorphism in either *TLR*4 or *TLR*5, when compared to some of the typhoid carriers. This suggests that genetic variation in *TLR* genes is less critical for susceptibility in this selected cohort of typhoid-susceptible population. This, however, does not exclusively rule out the major *TLR* locus for susceptibility to typhoid infection.

The roles of *TLR*4 and *TLR*2 in host defense have been well established in mouse model of lethal infection with *S.* Typhimurium [18–22] but less is known about how these receptors play a role in immune responses that control and clear *Salmonella* infections. Interestingly, *TLR*5 polymorphism does not make human carriers commonly susceptible to the disease with flagellated bacteria as it has no quantifiable impact on susceptibility to typhoid fever caused by *S.* Typhi [23]. However, mice lacking *TLR*5 have deficient innate immune responses to bacterial flagellins and/or flagellated microbes, clearly demonstrating that *TLR*5 recognition of flagellin is involved in innate activation [21,22]. In this study, a lower frequency of *TLR*5 polymorphism was observed only in typhoid carrier, but not schoolchildren with typhoid fever, which is

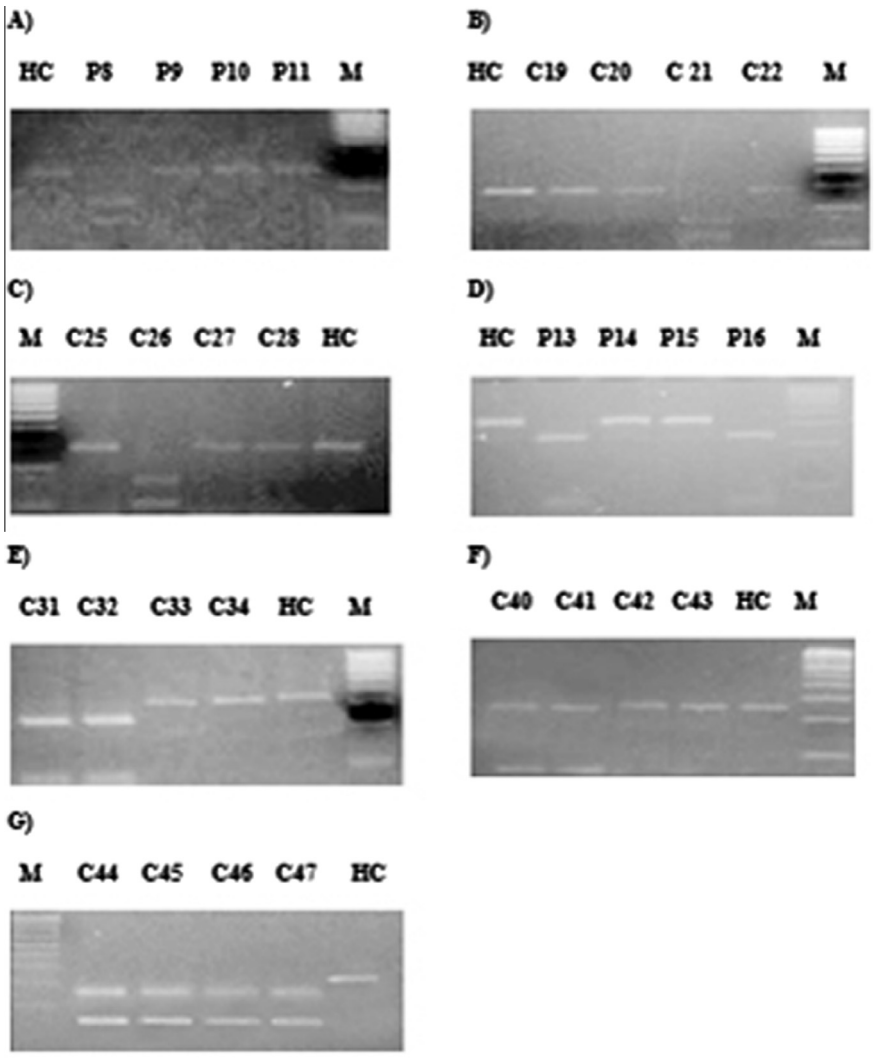

Figure 2 Restriction fragment length polymorphism of *TLR*1, *TLR*2, *TLR*4 and *TLR*5 genes. Gel (A), (B), and (C) shows *TLR*1 polymorphism; gel (D) and (E) shows *TLR*2 polymorphism; gel (F) shows *TLR*4 polymorphism; gel (G) shows *TLR*5 polymorphism. Lane M represents DNA ladder (100 bp); sample number starts with P shows as patients (typhoid); C with number as carriers (typhoid); HC represents healthy control individuals.

Table 2 Summary of RFLP for *TLR* genes in typhoid patients and asymptomatic typhoid carriers among the schoolchildren.

Parameters	*TLR*1	*TLR*2	*TLR*4	*TLR*5
(1) Restriction site	GC	GC	GC	CT
(2) Restriction enzyme	*AluI*	*AciI*	*NcoI*	*DdeI*
(3) RFLP mutant (%)				
(a) Typhoid patient (n = 20)	5% (1/20)	10% (2/20)	0%	0%
(b) Typhoid carriers (n = 30)	6.6% (2/30)	6.6% (2/30)	6.6% (2/30)	13.3% (4/30)

consistent with our previous report [13]. Thus, it is suggested that *TLR5* may not play a role in susceptibility to typhoid fever [23].

In conclusion, this study shows a variable degree of genetic polymorphism in *TLR*1, 2, 4 and 5 genes among typhoid-susceptible schoolchildren. Asymptomatic typhoid carriers show relatively more *TLR* gene polymorphism than typhoid patients.

Ethical consideration

The work has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments in humans. The informed consent was obtained for experimentation with human subjects.

Conflict of interest

None.

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