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

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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 TLR1, 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: TLR1 polymorphism was observed in 5% (1/20) of typhoid patients and 6.6% (2/30) of typhoid carriers. TLR2 polymorphism was observed in 10% (2/20) of typhoid patients and 6.6% (2/30) of carriers. TLR4 polymorphism was not observed in typhoid patients, but 6.6% (2/30) of typhoid carriers exhibited a polymorphism. As well, TLR5 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.

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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 [2-5]. 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 TLR4-mediated downstream signaling cascade inducing host immune response [8,9]. Further, membrane remodeling blocks detection by host TLR4 and also increases the resistance of bacteria against host antimicrobial [10]. A study in Malay population has identified 8.9% TLR4 Asp299Gly and 7.2% TLR4 The399Ile polymorphisms in typhoid susceptible populations. In addition, TLR5 mediates the innate immune responses to Salmonella through binding to flagellin [11]. A polymorphism in the TLR5 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 TLR5 gene polymorphism was not associated with susceptibility to typhoid fever, as there was no significant correlation between TLR5 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., TLR1, 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 TLR1, TLR2, TLR4 and TLR5 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 (QiAamp 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 TLR1, TLR2, TLR4 and TLR5 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 (TLR4^{A622T}, TLR5^{75Glu}, 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 TLR1, TLR2, TLR4 and TLR5 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 TLR1, TLR2, TLR4 and TLR5 genes from all subjects were subjected to RFLP using restriction enzymes to determine polymorphisms (Fig. 2). Among the typhoid patients, polymorphism of TLR1, TLR2, TLR4 and TLR5 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 TLR1, TLR2, TLR4 and 13.3% of TLR5 (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 TLR5 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 TLR4 gene might also influence the infection state during typhoid infection [8,9]. The prevalence of 12.5% of TLR4 polymorphism in typhoid-susceptible Malay populations was reported [11]. This TLR4 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 TLR1, 2, 4 and 5 genes, in the present study, revealed the
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 \( TLR4 \) or \( TLR5 \), 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 \( TLR4 \) and \( TLR2 \) 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, \( TLR5 \) 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 \( TLR5 \) have deficient innate immune responses to bacterial flagellins and/or flagellated microbes, clearly demonstrating that \( TLR5 \) recognition of flagellin is involved in innate activation [21,22]. In this study, a lower frequency of \( TLR5 \) polymorphism was observed only in typhoid carrier, but not schoolchildren with typhoid fever, which is

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

<table>
<thead>
<tr>
<th>( TLR ) gene</th>
<th>Primers</th>
<th>Cycling conditions</th>
<th>Restriction enzyme</th>
<th>Allele length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( TLR1 )</td>
<td>F: 5'-GGAAAGTTATA GAGGAACCCCT-3' R: 5'-CCCTACCCCGA AAGAATCGGTC-3'</td>
<td>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.</td>
<td>( AluI )</td>
<td>280, 129, 151</td>
</tr>
<tr>
<td>( TLR2 )</td>
<td>F: 5'-GCTACCTGGTGGAGGAACCT-3' R: 5'-GGCCACCTCC AGGTAGGTCTT-3'</td>
<td>95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 7 min.</td>
<td>( AciI )</td>
<td>340, 265, 75</td>
</tr>
<tr>
<td>( TLR4 )</td>
<td>F: 5'-GATTACCTAGACTACTACCTCCATG-3' R: 5'-GATCAACTCTGA AAAAGCATTCCAC-3'</td>
<td>95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 7 min.</td>
<td>( NcoI )</td>
<td>249, 30, 219</td>
</tr>
<tr>
<td>( TLR5 )</td>
<td>F: 5'-GGTAGCCTA CATTGATTTCG-3' R: 5'-GAGCATCTGGAG ATGAGGTACC-3'</td>
<td>95 °C for 5 min; 35 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 7 min.</td>
<td>( DdeI )</td>
<td>277, 91, 186</td>
</tr>
</tbody>
</table>

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**Figure 1** PCR amplification of (A) \( TLR1 \), (B) \( TLR2 \), (C) \( TLR4 \) and (D) \( TLR5 \) 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.
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 TLR1, 2, 4 and 5 genes among typhoid-susceptible schoolchildren. Asymptomatic typhoid carriers show relatively more TLR gene polymorphism than typhoid patients.

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLR1</th>
<th>TLR2</th>
<th>TLR4</th>
<th>TLR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Restriction site</td>
<td>GC</td>
<td>GC</td>
<td>GC</td>
<td>CT</td>
</tr>
<tr>
<td>(2) Restriction enzyme</td>
<td>AluI</td>
<td>AcI</td>
<td>NcoI</td>
<td>DdeI</td>
</tr>
<tr>
<td>(3) RFLP mutant (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Typhoid patient (n = 20)</td>
<td>5% (1/20)</td>
<td>10% (2/20)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(b) Typhoid carriers (n = 30)</td>
<td>6.6% (2/30)</td>
<td>6.6% (2/30)</td>
<td>6.6% (2/30)</td>
<td>13.3% (4/30)</td>
</tr>
</tbody>
</table>

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.

Acknowledgement

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