

Toll-Like Receptor (TLR2 and TLR4) Polymorphisms: Markers of Innate Immunity in Oral Infection in Children

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Abstract

Introduction: Innate immunity is activated with the participation of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are signal molecules essential for the cellular response to bacterial cell wall components. **Aim:** The aim of the study was to use gene identification of Toll-like receptors (TLR2 and TLR4) to investigate this marker of innate immunity in children with chronic inflammation oral diseases. **Methods:** Three groups of 30 children, one with a history of ear, nose and throat (ENT) infections, one with clinically healthy gingival and no history of ENT infections, and one with gingivitis and no history of ENT infections, were recruited. DNA samples were taken from the children's oral mucosa. The frequency of four different functional polymorphisms for the TLR 2 gene (Arg677Trp, Arg753Gln) and for the TLR 4 gene (Asp299Gly, Thr399Ile) was assessed using polymerase chain reaction. **Results:** Only one polymorphism was found (Arg753Gln) in TLR2 gene in a child suffering from recurrent tonsillitis. **Conclusions:** In the group of Bulgarian children studied, the polymorphism in TLR2 gene occurred in only 1.1% of the sample.

Key Words: Toll-Like Receptors Genes, Polymerase Chain Reaction, Gene Polymorphism, Chronic Infectious Disease, Plaque-Induced Gingivitis, Tonsillitis, Oral Environment

Introduction

The main role of the host immune system is to trigger and innate adaptive immune responses. Innate immunity is activated with the participation of pattern recognition receptors (PRRs) on the dendritic cells, macrophages, polymorphonuclear leukocytes and epithelial cells. They recognise distinguishing pathogen-associated molecular patterns (PAMPs), related to the pathogenic potential of the bacterial cell walls. The most important distinguishing receptors are the Toll-like receptors (TLRs). They represent a primitive defence mechanism against bacteria, fungi and viruses, and play a main role in inflammation initiation and consequent immune response [1-4]. TLRs bind and become activated by different ligands, located on different types of microorganisms or structures. For example, TLR2 binds to multiple lipoproteins, peptidoglycan, and lipoteichoic acid of the cell walls of Gram-positive (G+MO) and Gram-negative (G-MO) microorgan-

isms. TLR4 binds to lipopolysaccharides (LPS) of G-MO and some other ligands of host cells surfaces [5,6]. There is evidence that TLR4 distinguishes and transmits signals of LPS from the bacterial wall of G-MO in the subgingival biofilm, such as *Porphyromonas gingivalis* [7-12].

Gene mutations of these two receptors can lead to a considerable increase in the risk of different oral bacterial diseases [13]. The polymorphisms in the TLRs are encoded on the gene for TLR-2 [14,15], as can be observed in patients with sepsis from G+MO [16,17]. Polymorphisms of TLR-4 are also connected with different anaerobic infectious diseases [18]. The cells of patients with such diseases are incapable of reacting to TLR-stimuli and thus frequent different, current infections with heavy febrile conditions, typical in infancy, develop [19-21].

With oral disease, it is important to evaluate the oral risk environment, which represents a complex system of interactions between local and sys-

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temic factors. The authors hypothesised that the two polymorphisms of the TLR2 and TLR4 receptors are responsible for the high risk of periodontal diseases and frequent throat infections such as tonsillitis, adenoiditis. If this is the case, it could clarify the oral risk environment for oral and systemic disease.

Aim

The aim of the study was therefore to examine gene mutations of Toll-Like receptors (TLR2 and TLR4) as a surrogate for innate immunity in children with chronic inflammatory oral diseases.

Methods

Patients

The study involved 90 children, divided into three clinically distinct groups.

Group 1

Group 1 contained 30 children (11 males and 19 females) with an average age of 5 (± 2.5) years who had experienced frequent current infections and clinically proven chronic throat inflammation (chronic tonsillitis, adenoiditis, etc.).

The children in this group fulfilled four criteria:

- Primary clinical diagnosis of chronic adenoiditis and tonsillitis.
- Minimum duration of this disease of one year.
- Relapse frequency of at least four times per year.
- Indications for tonsillectomy.

Nineteen (63.3%) of the children suffered from chronic tonsillitis and adenoiditis, of whom six (20%) suffered from an accompanying serous otitis, two (6.7%) with attendant allergic conditions. Seven (23.3%) of the children had chronic rhinitis and rhinopharyngitis, and four (13.3%) suffered recurrence of otitis.

Groups 2 and 3

For groups 2 and 3, 60 children were randomly selected from 180 children aged 15-16 years. These children were subjected to a complete oral examination to assess their periodontal status. They were clinically healthy without systemic diseases. Of the 60 children who were randomly selected, 30 (13 males and 17 females) had healthy gingiva and 30 (11 males and 19 females) had plaque-induced gingivitis. The diagnosis of gingivitis was carried out by using the criteria detailed in *Table 1* for distinguishing the two groups.

Ethical approval

The study was carried out with the approval of the Ethics Commission for Scientific Research at the Medical University, Sofia, and after obtaining informed consent from each participant above 16 years of age or from the parents of children below that age.

Biological material

Epithelial cells were obtained from the oral mucosa of each child by scraping the oral side of both cheeks with a plastic spatula. The material was dispersed in small containers filled with saline solution and after freezing to -20°C the samples were stored.

Genetic analysis

Genetic analysis of the TLR2 and the TLR4 was performed using polymerase chain reaction (PCR), which is used to amplify certain regions of DNA, beginning and ending with certain nucleotide sequences [24]. The process was performed in the following manner:

- Obtaining DNA samples from the oral mucosa
- Preparing the initial DNA samples.
- PCR amplification.

DNA extraction and purification

Two methods were used:

Table 1. Clinical Criteria for Determining Gingival Status of Children

Clinical criteria	For gingival inflammation	For healthy gingiva
Gingival papilla	Oedema, shiny, red, detached, possibly ulcerated	Light pink, tight, fixed
Free gingiva	Swollen, thickened, hyperemic	Light pink, tight, fixed to the tooth
Attached gingiva	Shiny, reddened, smooth contour	Light pink, as orange peel
PBI*	At least 1/4 papilla with bleeding on probing	No bleeding on probing
PSR**	At least one sextant with code 1	All sextants with code 0

* Papilla Bleeding Index (PBI) [22]

** Index for Periodontal Screening and Registration (PSR) [23]

- Purification of DNA by phenol extraction and ethanol precipitation.
- Use of QIAamp® DNA Mini Kit (QIAGEN, Stanford, CA). DNA was diluted in 30-50 µl TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) or ddH₂O with a total concentration of approximately 100 ng/µl [25].

Genetic analysis of polymorphisms Arg677Trp and Arg753Gln in TLR2 and Thr399Ile and Asp299Gly in TLR4 genes

PCR was used for amplification of parts of TLR2 and TLR4 genes, with the following use of primers (Table 2).

Restriction fragment length polymorphism

(RFLP) analysis was used for the detection of the four polymorphisms in all samples. The restriction enzymes, their optimal temperatures, and the size of the fragments are shown in Table 3.

The PCR reactions from 1 to 4 were restricted. The sites of the four enzymes are shown in Table 4. All fragments were tested before and after digestion with 3% agarose gel stained with ethidium bromide.

Results

All samples were amplified and tested for all four polymorphisms in TLR2 (PCR 1 and 2) and TLR4 (PCR 3 and 4) genes. The amplifications were tested with 3% agarose gel stained with ethidium bromide (Figure 1). Group 1 samples are from 1 to 10. The

Table 2. PCR amplification for detection of polymorphisms Arg677Trp and Arg753Gln in TLR2 gene and Thr399Ile and Asp299Gly in TLR2 gene (Folwaczny et al. 2004 [26])

Primer	Polymorphism	Primer sequences (5' - 3')	Amplicon size (bp.)	Primer melting temperature (°C)
TLR2 1F	Arg677Trp	5' -CCCCTTCAAGTTGTGGCTTCATAAG- 3'	152	65
TLR2 1R		5' -AGTCCAGTTCATACTTGCACCAC- 3'		
TLR2 2F	Arg753Gln	5' -CATCCCCAGCGCTTCTGCAAGCTCC- 3'	129	65
TLR2 2R		5' -GGAACCTAGGACTTTATCGCAGCTC- 3'		
TLR4 1F	Thr399Ile	5' -GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA- 3'	124	60
TLR4 2R		5' -GGAAATCCAGATGTTCTAGTTGTTCTAAGCC- 3'		
TLR4 2F	Asp299Gly	5' -AGCATACTTAGACTACTACCTCCATG- 3'	188	62
TLR4 2R		5' -GAGAGATTTGAGTTTCAATGTGGG- 3'		

Table 3. Restriction Enzymes, Optimal Temperatures, and Size of the Fragment (Folwaczny et al. 2004[26])

Gene	Polymorphism	Restriction enzyme	Optimal temperature of the restriction enzymes	Size of the fragments with and without restriction (bp)
TLR2	Arg677Trp	Mwo I	60	Wild type - 130 + 22
				Arg677Trp - 152
	Arg753Gln	Msp I	37	Wild type - 104 + 25 Arg753Gln - 129
TLR4	Asp399Ile	Hinf I	37	Wild type - 124 Asp399Ile - 98 + 26
				Wild type - 188 Asp299Gly - 168 + 20
	Asp299Gly	Nco I	37	

Table 4. The Restriction Site of the Four Enzymes

(1) MwoI 5' - ... G C N N N N N N N G C ... - 3' 3' - ... C G N N N N N N N C G ... - 5'	(2) Hinf I 5' - ... G^A A N T C ... - 3' 3' - ... C T N A^G ... - 5'
(3) Msp I 5' - ... C^A C G G ... - 3' 3' - ... G G C^A C ... - 5'	(4) Nco I 5' - ... C^A C A T G G ... - 3' 3' - ... G G T A C^A C ... - 5'

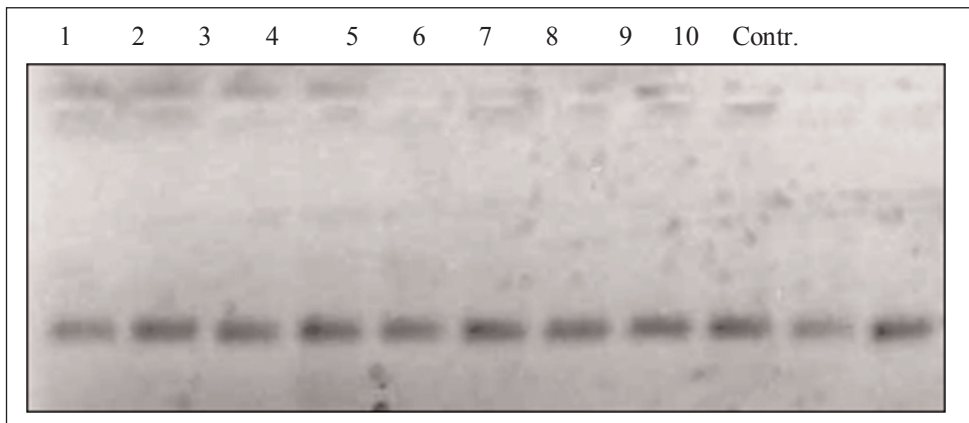


Figure 1. Agarose gel electrophoresis for testing quality of the amplicons.

control sample gave the same PCR amplification when DNA purified from venous blood was used.

Results obtained from group 1 (children with chronic throat inflammation)

Only one individual showed one of the four polymorphisms that were studied. An undigested sample was used as a control sample and compared with all other fragments' mobility. In case of digestion, two fragments were observed. The size of the smaller fragment was similar to the size of the primers (20-26 bp), so it could not be recognised.

Figure 2 shows a heterozygous carrier of Arg753Gln polymorphism in TLR2 gene. After digestion, the carrier had two fragments, one with mobility equal to the undigested control sample, and one equal to the other nine digested samples. The other three polymorphisms were not detected.

Results obtained from group 2 (healthy controls)

This group contained only healthy children. In this group, no carriers of any of the analysed polymorphisms in TLR2 and TLR 4 genes were detected. The results are shown in Figure 3.

Results obtained from group 3 (children with plaque-induced gingivitis)

No polymorphism in TLR2 and TLR4 genes were detected in children suffering from gingivitis. The results looked like those of group 2 (healthy controls).

Thus of the 90 children, divided in three groups, based on their clinical data, only one heterozygous polymorphism (Arg753Gln) in TLR2 gene in one patient from group 1 was found.

Discussion

The differences in the average age of children in group 1 and those in groups 2 and 3 was because the authors chose a group of unhealthy children with early clinical manifestations (frequent current infections and clinically proven chronic throat inflammation such as chronic tonsillitis, adenoiditis) because they considered that children in this age group were more likely to be carriers of one or more of the polymorphisms that were studied, whereas older children would be more likely to have well developed immune responses.

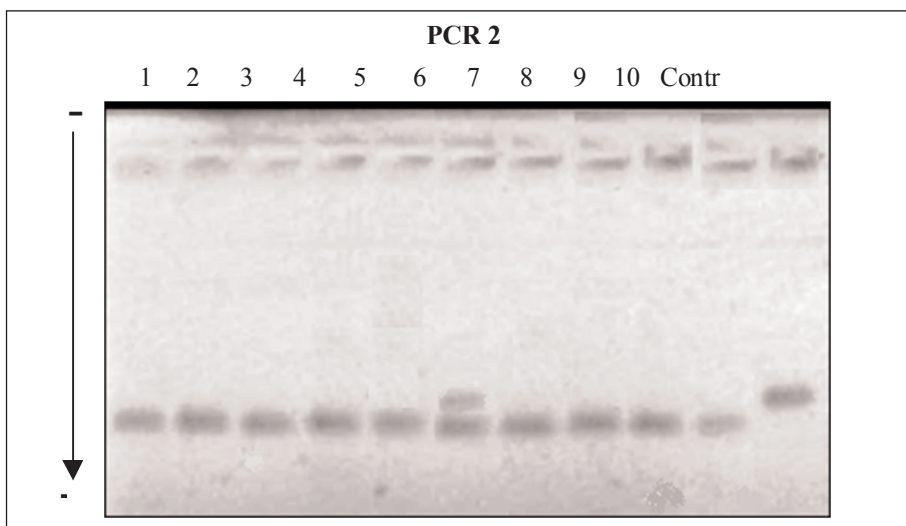


Figure 2. Gel electrophoresis of 10 PCR 2 products after restriction with *Msp I* restrictase enzyme and one undigested control sample. Sample 6 was a heterozygous carrier of Arg753Gln polymorphism in TLR2 gene.

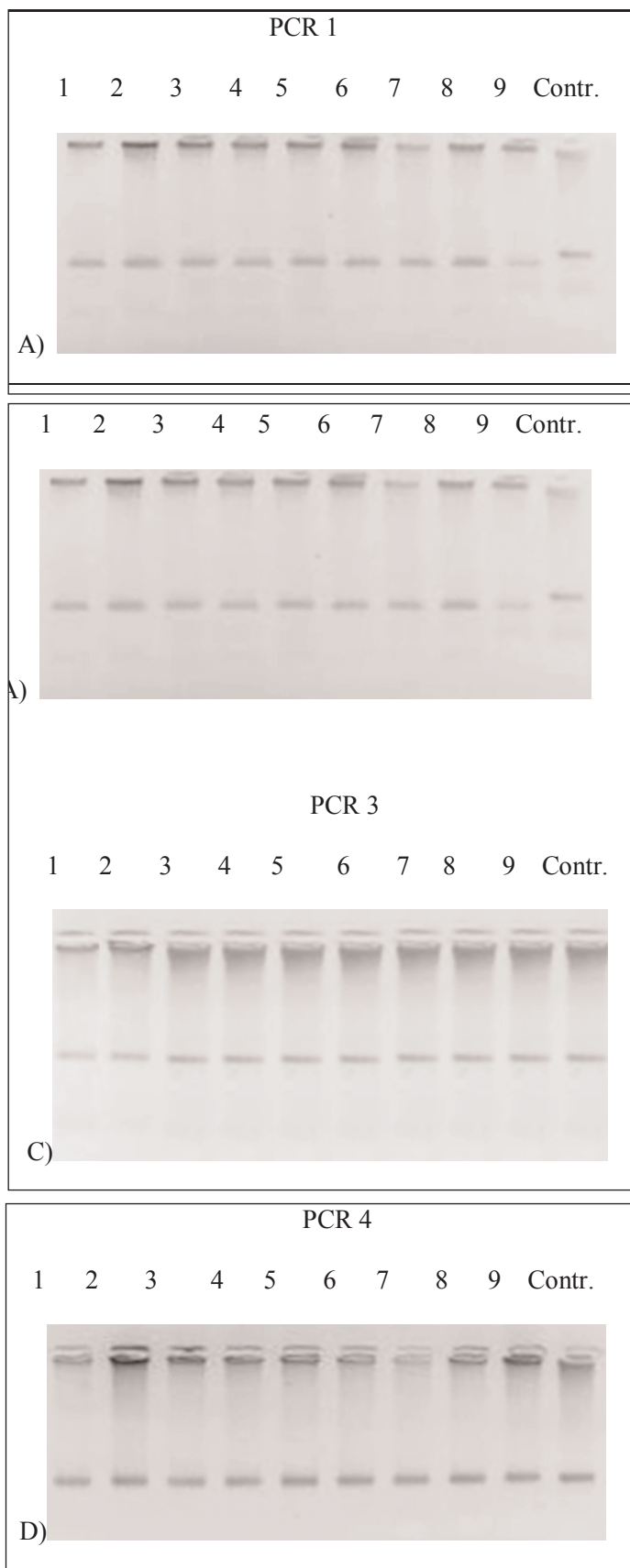


Figure 3. Gel electrophoresis of the PCR 1-4 amplicons after enzyme digestion (A=PCR 1; B=PCR 2; C=PCR 3; D=PCR 4).

Toll-like receptors enable the “barcode” of some oral microorganisms to be decoded while no differentiation of commensal to pathogenic microorganisms occurs. They represent an essential part of innate immunity in the oral environment, targeting oral pathogens such as the G- MO in the subgingival biofilm and the infectious pathogens such as *Staphylococcus aureus* that cause chronic tonsillitis in small children [16,27]. Polymorphisms of the genes on which TLR2 and TLR4 are encoded could bring about an increased risk from these infectious diseases.

The results from this study showed no polymorphisms in TLR4 gene in any of the 90 children and only one polymorphism (Arg753Gln) in the TLR2 gene in a child suffering from chronic tonsillitis, representing a frequency of 1.1%.

The results from different studies in the last few years have shown a different frequency of the polymorphisms in TLR2 and TLR4 genes in patients with different kinds of chronic infection diseases. Folwaczny *et al.* (2004) found the polymorphisms Asp299Gly in the TLR4 gene in 4.1% and Thr399Ile in TLR4 in 4.5% of patients suffering periodontitis. The frequency in their control group was 3.3% and 3.7% [26]. The frequency of the TLR2 gene polymorphisms in the case of patients with priodontal diseases (Arg753Gln) was 2.9 and in their control group it was 4.1%. In this study, Arg677Trp polymorphism in the TLR2 gene was not reported [26].

The polymorphisms Asp299Gly and Th399Ile in the TLR4 gene are found in 10% of Caucasians and are correlated with the gravity of infectious diseases, including septic shock associated with Gram-negative bacteria and respiratory bronchiolitis in children [2,28].

In summary, the results from the current study showed a low rate of this polymorphism in the Bulgarian population that was studied. This variation could be caused by the small group included in the study and because of this, the statistical reliability was possibly low. This was the first

study in Bulgaria and no data was available for the carrier status of the Bulgarian population.

Conclusion

In the four polymorphisms in TLR2 and TLR 4 genes studies in 90 children, only one polymorphism was found (Arg753Gln) in the TLR2 gene in

a child suffering recurrent tonsillitis. This was a 1.1% rate of this polymorphism in this group of Bulgarian children.

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