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Article

Associations Between Toll-like Receptor (TLR2, TLR3, TLR9) Gene Polymorphisms and Susceptibility to Gastrointestinal Protozoan Infections

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Abstract: Infection with gastrointestinal protozoan like Cryptosporidium spp., Giardia lamblia, and Entamoeba histolytica are responsible for morbidity worldwide. our case-control study aimed to investigate the association between functional polymorphisms in recognition genes of innate immune, specifically Toll-like receptors TLR2 (rs5743708, Arg677Trp), TLR3 (rs3775291, A1234G), and TLR9 (rs5743836, C-1237T) and host susceptibility to Gastrointestinal Protozoan infections. The study enrolled 50 patients with confirmed protozoan infections microscopically (giardiasis: n=24; cryptosporidiosis: n=16; amoebiasis: n=10) and 50 matched healthy individuals. Allele frequency and genotype analyses, along with Hardy-Weinberg equilibrium testing in controls, were investigated. Results demonstrated significant disease-specific associations. Moreover, giardiasis risk (OR = 11.77, 95% CI: 2.13–65.06, p = 0.0012) was highly associated with TLR2 AA genotype. the TT genotype of TLR3, conferred increased susceptibility to cryptosporidiosis (OR = 6.07, 95% CI: 1.29–28.49, p = 0.0144). Additionally, the TLR9 GG genotype showed a notable association with amoebiasis (OR = 34.17, 95% CI: 4.55–256.47, p < 0.001and the AG genotype was associated with giardiasis (OR = 3.68, 95% CI: 1.05–12.92, p = 0.0352). This study underscores significance of variations of hosts genetic in infection outcomes, more investigation is needed into these variants as biomarkers for risk stratification and targets for immunomodulatory strategies. Findings of this study study confirm that these functional TLR polymorphisms are important host genetic factors affecting immune recognition which significantly predispose hosts to the protozoan infections regardless with the environmental exposure.

Keywords: protozoan infections, Giardia, Cryptosporidium, Entamoeba, TLR polymorphisms, Toll-like receptors, gene variants.

Introduction

Among gastrointestinal protozoan infections, Cryptosporidium spp, Giardia lamblia, and Entamoeba histolytica, are still to pose a crucial global health challenge, regions with limited access to clean water and adequate sanitation in particular. These protozoon infections have a significant contribution to the diarrheal diseases burden causing considerable malnutrition, impaired childhood development and morbidity [1]. In their clinical outcomes, individuals exhibit notable heterogeneity despite widespread exposure, ranging from asymptomatic carriage to severe chronic disease suggesting an essential role for host genetic predisposition in determining susceptibility and severity of the disease [2]. In the first line of defense against invasive pathogens, The innate immune system, Toll-like receptors (TLRs) have a pivotal role in the early detection of microbial components, they are a family of pattern recognition receptors recognizing conserved pathogen-associated molecular patterns (PAMPs) and initiate downstream signaling cascades culminating in the production of pro-inflammatory cytokines, interferons and chemokines essential for clearance of the pathogen [3]. TLR2, TLR3, and TLR9 are TLRs that have been specifically implicated in the protozoan element's recognition, including nucleic acids, glycolipids , and CpG DNA motifs [4], [5]. Researches indicated functional polymorphisms within these TLR genes can alter ligand binding affinity, receptor expression or signal transduction efficiency modulating an individual's immune response and risk of protozon infection [6]. For example, the TLR2 rs5743708 (Arg753Gln) polymorphism in the intracellular TIR domain, has been extensively investigated in bacterial infections such as tuberculosis. In their meta-analysis, Guo and Xia (2015) showed that this variant notably increases tuberculosis risk across multiple ethnic groups, highlighting its broader significance in infectious disease susceptibility. Additionally, researches indicated that TLR3 rs3775291 (Leu412Phe) polymorphism impair the receptor's ectodomain and its effect to bind double-stranded RNA, a key viral signature. Silva et al. (2023). Moreover, this SNP significantly increase susceptibility to different viral infections, with effect directions varying by geographic region. additionally, polymorphism of promoter in TLR9 rs5743836 alters NF-κB binding and receptor expression that affects immune responses to bacterial and viral DNA. The genetic variant has been implicated with differential conditions ranging from Hodgkin's lymphoma to viral infections [7], [8]. Despite of the effects of these polymorphisms with bacterial and viral infections are increasingly studied, their specific impacts in susceptibility to gastrointestinal protozoa remain relatively underexplored and inconsistent across studies. Molecular patterns presented by protozoan parasites and the crucial involvement of TLR-mediated pathways in intestinal mucosal immunity still under invastigation, genetic variations in TLR2, TLR3, and TLR9 could notably influence host defense against Cryptosporidium, Giardia, and Entamoeba. Aim this case-control study is to investigate the functional polymorphisms in TLR2 (rs5743708), TLR3 (rs3775291), and TLR9 (rs5743836) and its association with differential susceptibility to major gastrointestinal protozoan infections through genotyping and allele frequencies in well-characterized patients compared with healthy controls.

Materials and Methods

Study Population and Sample Collection

Our investigation enrolled 50 adult patients with gastrointestinal protozoan infections diagnosed by routine examination at the hospital's laboratory. The control group also included 50 healthy individuals, age and sex matched, they had no clinical history or evidence of gastrointestinal parasitic infection at the time of study or in the preceding six months. Prior to sample collection, written informed consent was obtained from all participants. Samples of Fresh stool were collected from all participants in clean, labeled containers. Samples were processed within one hour of collection or preserved in 10% formalin for later analysis for microscopic examination. At the same time, 5 ml of perepheral venous blood was drawn from each participant into EDTA tubes (BD Biosciences) and stored at -20°C until DNA extraction.

Direct Microscopic Examination

By using 0.9% NaCl (normal saline) and Lugol's iodine solution, direct wet mount slides were prepared. A small amount of stool was emulsified in a drop of the saline and covered with a coverslip then examined under 10x and 40x objectives for diagnosis of trophozoites, cysts, or oocysts. For

suspected Cryptosporidium infection, a modified Ziehl-Neelsen acid-fast staining technique was applied. The diagnosis was confirmed by an experienced parasitologist, and the intensity of infection was semi-quantitatively recorded.

Blood DNA Extraction

Genomic DNA was extracted from 200 µl of whole blood using the DNA Blood Mini Kit (Geneaid, Taiwain), following the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The extracted DNA samples were stored at -20°C until used for genotyping.

Genotyping of TLR2, TLR3, and TLR9 Polymorphisms Using Tetra-Primer Amplification Refractory Mutation System PCR (T-ARMS-PCR)

The polymorphisms TLR2 (rs5743708, Arg753Gln), TLR3 (rs3775291, Leu412Phe), and TLR9 (rs5743836, C-1237T) were genotyped using the T-ARMS-PCR method, a cost-effective and reliable technique for SNP detection. This method employs four primers in a single reaction: two outer primers that amplify a common fragment and two allele-specific inner primers that anneal to the SNP site.

Primer Design and Sequences

Primers were designed using (PRIMER1: primer design for tetra-primer ARMS-PCR) and synthesized by Macrogen (South Korea). The sequences, melting temperatures, and expected product sizes are summarized in Table 1.

Table 1. Sequences and characteristics of primers used for T-ARMS-PCR genotyping of TLR2 (rs5743708), TLR3 (rs3775291), and TLR9 (rs5743836) polymorphisms.

Gene (SNP)	Primer	Sequence (5′→3′)	Tm (°C)	Product Size (bp)
TLR2 (rs5743708)	Forward Outer (FO)	TTGATGAGAACAATGATGCTGCCATTCTCA	72	162 (common)
	Reverse Outer (RO)	TCAGATTTACCCAAAATCCTTCCCGCTGA	72	
	Forward Inner (A allele)	ATTCCCCAGCGCTTCTGCAAGCTTCA	74	104 (A allele)
	Reverse Inner (G allele)	CCAGGTAGGTCTTGGTGTTCATTATCTGCC	70	114 (G allele)
TLR3 (rs3775291)	Forward Outer (FO)	ATTTTTCTTTTCAGTGGCTAAAATGTTT	61	359 (common)
	Reverse Outer (RO)	CTTGTTGTAGGAAAGATAGATTTCGAAA	61	
	Forward Inner (T allele)	ACTTGCTCATTCTCCCTTACACAGAT	62	194 (T allele)
	Reverse Inner (C allele)	TTTTGAGATTTTATTCTTGGTTAGGTTTAG	60	221 (C allele)
TLR9 (rs5743836)	Forward Outer (FO)	AGCCACAGTCCACAGATGGCCAACAAGG	75	300 (common)
	Reverse Outer (RO)	AGACCAGGCAAAGGAGCTCAGGAGTGCC	75	

	Forward Inner (A allele)	CTTGGGATGTGCTGTTCCCTCTGCCGGA	78	170 (A allele)
	Reverse Inner (G allele)	GAGGGGTCATATGAGACTTGGGGGAGTGTC	73	188 (G allele)

PCR Preparation and Thermocycler Conditions

PCR reactions were performed in a total volume of 25 µL containing: 50–100 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each outer primer, 0.4 µM of each allele-specific inner primer, and 1 U of Taq DNA polymerase (Promega. USA). The amplification was carried out in T100 PCR thermal cycler (Bio-Rad/USA) using the following cycling conditions: Initial denaturation: 95°C for 5 minutes, 35 cycles of: Denaturation: 95°C for 30 seconds, Annealing: [Gene-specific temperature] for 30 seconds (TLR2: 60°C, TLR3: 58°C, TLR9: 62°C). Extension: 72°C for 30 seconds, Final extension: 72°C for 10 minutes, and Hold at 4°C.

Electrophoresis and Genotyping

PCR products were separated by electrophoresis on a 2.5% agarose gel (Vivantis) stained with EcoSafe Nucleic Acid Staining Solution (Pacific Science) and visualized under UV light using a Gel Documentation System (Bio-Rad). The genotypes were determined based on the presence and size of the allele-specific bands compared to a 100 bp DNA ladder (Bioneer.Korea).

Data and Statistical Analysis

Genotype and allele frequencies were calculated for both case and control groups. The Hardy-Weinberg equilibrium (HWE) for each SNP was assessed in the control group using the chi-square (χ^2) test. Associations between infection and susceptibility each polymorphism were tested using odds ratios (OR) with 95% confidence intervals (CI) that calculated by unconditional logistic regression under dominant, recessive, and allelic genetic models. The reference genotype for each SNP was defined as the homozygous wild-type (GG for TLR2, CC for TLR3, and AA for TLR9). The statistical analyses were performed by IBM SPSS Statistics version 25 (IBM Corp.), and a two-tailed p-value < 0.05 was considered statistically significant.

Result and Discussions

Parasite Prevalence Distribution

Figure (1) summarizes the prevalence of gastrointestinal protozoan infections of the present study, which visually shows the distribution of 100 total samples across the diagnostic groups. The study revealed that the most prevalent parasitic infection is Giardiasis, accounting for 24 cases (48%), followed by Cryptosporidiosis,16 cases (32%), and 10 cases (20%) of Amoebiasis. Although The figure quantifies the prevalence infection ,it underscores the relative frequency of each parasitic disease in the studied population to provide important context for the subsequent genetic association analysis.

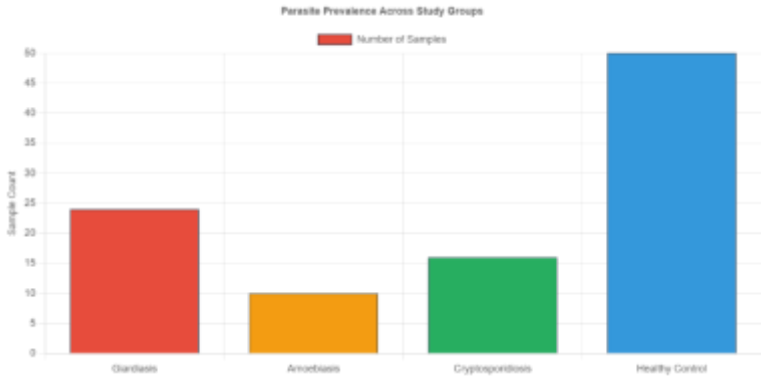

Figure 1. prevalence of gastrointestinal protozoans and control group

Genotype Analysis Results

TLR2 (rs5743708) Analysis

Table 1: TLR2 Genotype Frequencies & Associations

Genotype	Giardiasis	Amoebiasis	Cryptosporidiosis	Healthy Control	OR (Giardiasis)	95% CI	P-value
GG	11 (45.8%)	6 (60.0%)	12 (75.0%)	37 (74.0%)	Reference	N/A	N/A
AA	7 (29.2%)	1 (10.0%)	1 (6.3%)	2 (4.0%)	11.77	2.13–65.06	**0.0012**
GA	6 (25.0%)	3 (30.0%)	3 (18.8%)	11 (22.0%)	1.83	0.55–6.10	0.3183

TLR3 (rs3775291) Analysis

Table 2: TLR3 Genotype Frequencies & Associations

Genotype	Giardiasis	Amoebiasis	Cryptosporidiosis	Healthy Control	OR (Cryptosporidiosis)	95% CI	P-value
CC	15 (62.5%)	4 (40.0%)	7 (43.8%)	34 (68.0%)	Reference	N/A	N/A
TT	6 (25.0%)	1 (10.0%)	5 (31.3%)	4 (8.0%)	6.07	1.29–28.49	**0.0144**
CT	3 (12.5%)	5 (50.0%)	4 (25.0%)	12 (24.0%)	0.57–1.62	Varies	0.4238–0.4956

TLR9 (rs5743836) Analysis

Table 3: TLR9 Genotype Frequencies & Associations

Genotype	Giardiasis	Amoebiasis	Cryptosporidiosis	Healthy Control	OR (Amoebiasis)	95% CI	P-value
AA	13 (54.2%)	2 (20.0%)	13 (81.3%)	41 (82.0%)	Reference	N/A	N/A
GG	4 (16.7%)	5 (50.0%)	2 (12.5%)	3 (6.0%)	34.17	4.55–256.47	**<0.001**
AG	7 (29.2%)	3 (30.0%)	1 (6.3%)	6 (12.0%)	3.68–10.25	1.05–74.52	0.0352–0.0079

Hardy-Weinberg Equilibrium (HWE) Analysis

Table 4: HWE Test Results in Healthy Controls

Gene	Chi-square	P-value	Equilibrium Status
TLR2 (rs5743708)	0.9419	0.3318	In Equilibrium
TLR3 (rs3775291)	2.1250	0.1449	In Equilibrium
TLR9 (rs5743836)	9.3233	0.0023	**Deviation (p<0.05)**

TLR2 and TLR3 genotypes in controls were in HWE, indicating random mating and no genotyping errors. TLR9 showed significant deviation, suggesting possible population stratification or technical artifacts. The analysis identified significant genotype-specific associations between TLR polymorphisms and susceptibility to gastrointestinal protozoan infections. Key findings include:

- **TLR2 AA genotype**: High risk for Giardiasis (OR=11.77, p=0.0012).
- **TLR3 TT genotype**: Increased risk for Cryptosporidiosis (OR=6.07, p=0.0144).
- **TLR9 GG genotype**: Very high risk for Amoebiasis (OR=34.17, p<0.001).

Discussion

Results obtained by this study demonstrated that there were a significant associations between gene polymorphisms in innate immune receptorsTLR2, TLR3, and TLR9, and susceptibility to gastrointestinal protozoan infections. Our findings came in alignment with other studies that established significance of TLRs in recognizing conserved PAMPs to initiate immune responses (Kawai & Akira, 2010). Findings of the present study highlight how functional genetic variations can modulate individual risk of infection in a pathogen-specific condition. Additionally, our investigation showed a notable strong association between the TLR2 AA genotype (rs5743708, Arg753Gln) and giardiasis (OR=11.77). Wide spectrum of microbial componentssuch as glycolipids, lipoproteins and lipopeptide are recognized TLR2 [9]. Impairment of TLR2-mediated signaling and cytokine production in response to bacterial ligands could mediated by The Arg753Gln substitution, located in the intracellular TIR domain [10]. This functional impairment may similarly hinder effective recognition of Giardia lamblia surface antigens or parasite-induced host cell alterations, compromising the initial inflammatory response and epithelial defense mechanisms that typically limit parasite colonization [11]. Results of the present study are aligned with previous meta-analyses study showing the role of this polymorphism to increase susceptibility to other intracellular infections like tuberculosis [12] and leprosy [10], suggesting a conserved mechanism of immune evasion by diverse pathogens that target TLR2 signaling pathways. Similarly, the association of the TLR3 TT genotype (rs3775291, Leu412Phe) with cryptosporidiosis (OR=6.07) suggests a previously underappreciated role for viral-sensing pathways in defending against this apicomplexan parasite. TLR3 primarily recognizes double-stranded RNA (dsRNA) and signals through the TRIF-dependent pathway to induce type I interferons and inflammatory cytokines [13]. Cryptosporidium parvum may trigger this receptor directly through parasite-derived RNA or indirectly through host-generated dsRNA during the intracellular replication cycle [14]. Studies demonstrated that he Leu412Phe variant locating in the ectodomain near a glycosylation site has been shown to reduce TLR3's affinity for dsRNA and diminish downstream signaling experimentally [15]. Critical interferon-mediated defense mechanisms that have been shown to limit Cryptosporidium infection in murine models could weakened by this attenuated response the

[16], potentially explained the increasing of susceptibility observed in carriers of the variant allele. this polymorphism has shown notable geographical variability in associations with viral infections [17], that suggest its effects which may be context-dependent, influenced by both host genetic background and pathogen factors. The most remarkable association obtained in this study was association between the TLR9 GG genotype (rs5743836) and amoebiasis that had a high odds ratio (34.17). This TLR detects unmethylated CpG DNA motifs prevalent in parasitic and bacterial genomes [18]. The rs5743836 variant of promoter creates a crucial NF-κB binding site which lead to expression of enhanced receptor and hyper-responsiveness to ligand stimulation [19]. In contrast, rather than providing protection, this exaggerated inflammatory immune response may initiate a tissue environment favorable to pathogenesis of Entamoeba histolytica which is known to flourish in inflammatory conditions and modulates host immune responses actively [20]. Studies showed that increased TLR9 signaling could worsen tissue damage through increase production of pro-inflammatory cytokines like TNF-α and IL-1β which led to compromise integrity of mucosal barrier to facilitate dissemination and invasion trophozoite. However, this result agree with studies showed that the polymorphism in increasing risk of autoimmunity and inflammatory conditions [8] that suggest dysregulation of immunity rather than immunodeficiency that may responsible for susceptibility in certain genetic contexts.Results of the present study exhibited that Hardy-Weinberg equilibrium deviation of TLR9 in controls had warrants consideration which may indicate population recent admixture, stratification, or technical artifacts in genotyping, furthermore, it may reflect selective pressure in the population that certain TLR9 variants enhance survival or reproduction in the endemic pathogens. This finding was also reported in other populations [21]. Results of our study may contribute to the increased evidence that host genetic diversity is an important to determine outcomes of infectious disease that extend the paradigm beyond viral and bacterial pathogens to include important eukaryotic parasites. These associations highlight the crucial role of TLR polymorphisms as biomarkers for infection risk evaluation especially in endemic areas, where these parasites responsible for notable public health burdens. Further studies should focus on larger and multi-ethnic populations to verify these associations which explain the molecular mechanisms by which these variants dysregulate immunity in the gut. Additionally, correlation of genetic data with environmental factors like microbiome composition, and parasite genotypes will provide a more comprehensive explanations of protozoan disease pathogenesis.

Conclusion

This study demonstrates a significant association between genetic polymorphisms in toll-like receptors (TLR2, TLR3, and TLR9) and susceptibility to gastrointestinal protozoan infections, such as Giardia lamblia, Cryptosporidium spp., and Entamoeba histolytica. The findings confirm that individual genetic variations play an essential role in determining susceptibility to these infections. The TLR2 (rs5743708) polymorphism is strongly associated with an increased risk of giardiasis, while TLR3 (rs3775291) is associated with cryptosporidiosis, and TLR9 (rs5743836) shows a strong link with amoebiasis. This study also highlights that genetic polymorphisms in TLR receptors not only influence the body's immune response but could also serve as biomarkers for identifying individuals at higher risk of protozoan infections, especially in endemic areas. Further research is needed to explore the molecular mechanisms underlying the impact of these polymorphisms and their role in the pathogenesis of protozoan diseases.

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