Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed.nahrainuniv.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net\
Iraqi JMS 2025; Vol. 23(2)

Thr399Ile Polymorphism of Toll-Like Receptor 4 Gene and COVID -19 Severity in Samples of Iraqi Patients: Baghdad Province

Marwa F. Jaber *MSC*, **Anfal M. Khudhair** *PhD*

[1]Dept. of Biology, College of Medicine, Al-Iraqiya University, Baghdad, Iraq

Abstract

Background: Toll-like receptor 4 (TLR4) is a pattern recognition receptor involved in the innate immune response, capable of recognizing certain molecular patterns found in bacteria and viruses, including the severe acute respiratory syndrome coronavirus 2 (SARS–CoV2) virus responsible for Corona virus disease 2019 (COVID-19).

Objective: To investigate the potential relationship between the severity of COVID-19 and a specific genetic variation in the TLR4 gene known as Thr399Ile.

Methods: A cross-sectional study conducted on 90 patients with COVID-19 in Baghdad. The researchers genotyping of Thr399Ile polymorphism in DNA extracted from blood samples of patients was done using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses.

Results: Two genotypes were found CC and CT, and no patients exhibiting the TT genotype. The CC genotype was associated with a DNA fragment size of 407 base pairs, while the CT genotype showed a size of 378 + 29 base pairs. In the non-severe infection group, the CC genotype frequency was 95.55%, while the CT genotype frequency was 4.44%. In the severe infection group, the CC genotype frequency was 93.33%, and the CT genotype frequency was 6.66%. This indicates no significant association between Thr399Ile polymorphism and COVID-19 severity. Thr399Ile mutant allele frequency among the 90 COVID-19 patients was found to be 5.5% (5 out of 90).

Conclusion: Functionally effective Thr399Ile polymorphism of TLR4 gene have apparently no influence on the individual susceptibility for COVID-19 severity infection.

Keywords: COVID-19, TLR4, Thr399Ile polymorphism, mutant allele frequency.

Citation: Jaber MF, Khudhair AM. Thr399Ile Polymorphism of toll-like receptor 4 gene and COVID -19 severity in samples of Iraqi patients: Baghdad province. Iraqi JMS. 2025; 23(2): 264-271. doi: 10.22578/IJMS.23.2.9

List of abbreviations: COVID-19 = Corona virus disease 2019, RFLP = Restriction fragment length polymorphism, SNP = Single nucleotide polymorphism, TLR = Toll like receptor

Introduction

Human coronaviruses (HCoVs) belong to the Coronaviridae family in order Nidovirales [(1)] that infect the human respiratory tract. Coronaviruses are a set of large enveloped viruses of RNA, which range in size from 80 to 120 nm. In recent times, there have been three major coronaviruses leading to disease outbreaks, beginning with the severe acute respiratory syndrome coronavirus (SARS–CoV) in 2002, followed by the Middle East respiratory syndrome coronavirus (MERS–CoV) in 2012, and now the severe acute respiratory syndrome coronavirus 2 (SARS–CoV2). The 2019 novel coronavirus (2019-nCoV), began in Wuhan, China [(2)]. The

coronavirus disease 2019 (COVID-19) pandemic in Iraq was a part of the worldwide pandemic of COVID-19. During the pandemic, Iraq reported its first confirmed cases of SARS-CoV-2 infections on 22 February 2020 in Najaf [3].

This pandemic is particularly threatening for certain groups of people, including patients with comorbidities [4].

Pathogen-associated molecular patterns (PAMPs), which are structurally conserved patterns of organisms, are recognized by the innate immune system, which is the first line of defense. The innate defense mechanisms is also responsible for creating an early pro-inflammatory response [5].

Toll-like receptors (TLRs) are crucial for a number of innate immune system processes, including innate immune system stimulation, indirect adaptive immune system triggering, and modulation of cytokine expression, which is primarily accomplished through the identification of PAMPs [6].

TLR4 is a crucial pattern recognition receptor (PRR) that can identify a variety of PAMPs, including those from viruses, bacteria, and other pathogens, primarily lipopolysaccharides (LPS) [7]. TLR4 also detects particular damage-associated molecular patterns (DAMPs) made by dead or lytic cells in response to viral infection or host tissue damage [8].

The TLR4 gene has several polymorphisms that have been identified and rs4986791 is one of the most frequently investigated co-segregating single nucleotide polymorphism (SNP)s of the TLR4 gene in genetic and functional association research [9]. The rs4986791 SNP (replaces a thymine (T) for a cytosine (C) T/C at nucleotide 1196 that alter threonine (Thr) to isoleucine (Ile) at codon 399 [10].

The aim of this study was to investigate the association between TLR4 (Thr399Ile) SNP and COVID-19 severity.

Methods

A cross-sectional study conducted on 90 patients with COVID-19 in Baghdad. The samples collected from Al-Shafaa hospital, Al-Salama hospital, Al-Imamein Al-Kadhimein Medical City and Al-Numan hospital. The world Health organization (WHO) guideline in 2020 on COVID-19 infection divided the cases into mild to moderate, severe and critical according to physician's diagnosis [11]. The cases distributed as 45 non severe, 45 severe.

Sample collection

Taken nasopharyngeal swabs in hospital labs that taken from the patients. RNA confirmed by real-time reverse transcription-polymerase-chain reaction (RT-PCR). Two ml of blood were drawn via venipuncture from each participant when they were admitted, while maintaining stringent aseptic procedures, blood sample was collected in 2 ml ethylene diamine tetraacetic acid (EDTA) tube, then sample were prepared for DNA extraction, amplification and genotyping of TLR4 by PCR restriction fragment length polymorphism (PCR-RFLP) for the detection of genes polymorphism.

DNA extraction

Genomic DNA was isolated from blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System using a DNA extraction kit from Promega, USA.

PCR amplification

Amplifications were performed with 20 µl volumes containing 10 µl GoTaq Green Master Mix (2X); 2 µl of each primer (Forward) (Reverse) (10 pmol); 6 µl nuclease free water and 2 µl of template DNA. PCR cycling was performed with PCR Express (Thermal Cycler, BioRad, USA) with the following temperature program: Initial Denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec; annealing at 65°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation of 7 min at 72°C was included, followed by a 10 min incubation at 10°C to stop the reactions. The primers used in this study were based on those described by Lorenz et al. [11].

Restriction fragments length polymorphism

PCR products were digested with restriction enzyme, 1 µl from Hinf I enzyme was added to 10 µl of PCR product for each sample. RFLP was performed with PCR Express (Thermal Cycler, Veriti, USA) with the following temperature program: 37°C for 1hours; enzyme inactivation at 65°C for 15 minutes followed by 10 min incubation at 4°C to stop the reactions. The restriction enzymes fragments were separated on 2% agarose gel stained with ethidium bromide after prepared it. The ethidium bromide-stained bands in gel were visualized using Gel imaging system.

Table 1. Primer product (Thr399Ile) described by Lorenz et al. [12]

Gene	Primer Sequence 5`-3`
TLR4 Thr399Ile	Forward - GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA
	Reverse - ACCTGAAGACTGGAGAGTGAGTTAAATGCT

Table 2. Restriction enzymes and length of restriction fragments

Gene	Polymorphism	Restriction enzyme fragment	Restriction temp	Length of restriction
TLR4	Thr399Ile	Hinf I	37°C	Wild-type (allele C): 407 bp Thr399Ile (allele T): 378+29 bp

Statistical analysis

The statistical analysis used statistical package for the social sciences (SPSS) software, version 25. The chi-square test was employed to evaluate and compare the genotype frequencies among patient's groups. Statistical significance was determined when the probability value (P value) was less than 0.05

Results

Patients in the severe group were noticeably older. The statistical analysis showed significant differences between the age group and COVID- 19 severity P = 0.014. The highest mean of age in the severely infected group, was 60.28±16.69 yr (Table 3). No significant differences were identified regarding patient's sex and its association with COVID-19 infection severity (P = 0.288) (Table 4).

Table 3. Comparison of study's sample mean of age according to COVID-19 severity (n=90)

Covid-19 Severity	N	Mean	SD	P value
Non-severe	45	35.22	10.42	0.014
Severe	45	60.28	16.69	

Table 4. Comparison of study's sample sex according to COVID-19 severity

Patients' sex	Non-severe N = 45		Severe N = 45		Total	P value
	N	%	N	%		
Female	28	62.22	23	51.11	51	0.288
Male	17	37.77	22	48.88	39	

Genetic detection

The reaction of PCR was carried for the amplification of SNP (Thr399Ile) site for TLR4 gene in the optimal conditions, using a specific primer and then the PCR product was detected via electrophoresis in agarose gel 2% as shown in figure (1).

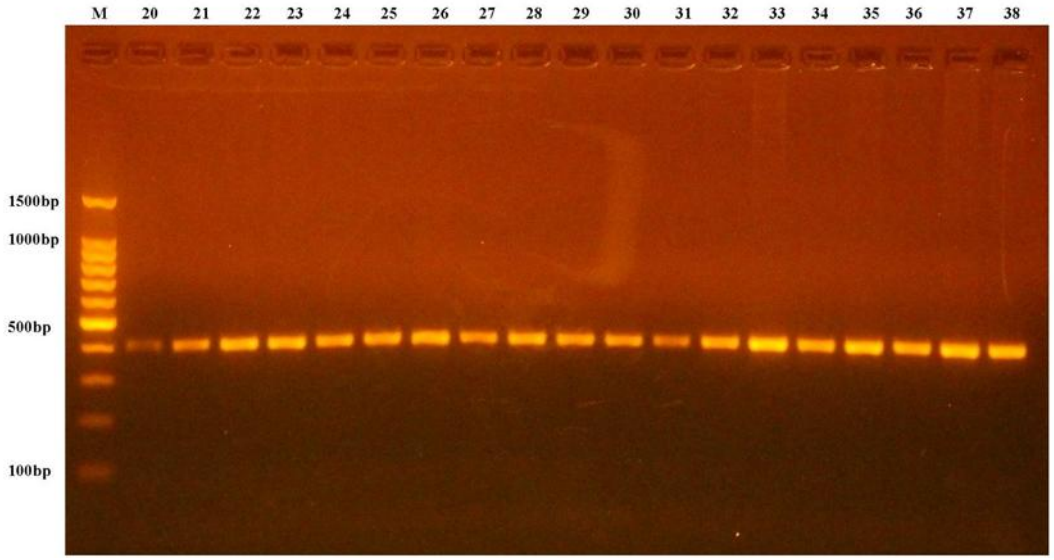

Figure 1. PCR product the band 407 bp of (Thr399Ile) SNP. The product was electrophoresis on 2% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker

The results of SNP (Thr399Ile) are shown in figures (2 and 3) for the patient's samples; where the genotype CC is shown in the size 407 bp, CT is shown in the size 378+29 bp, whereas TT is not shown.

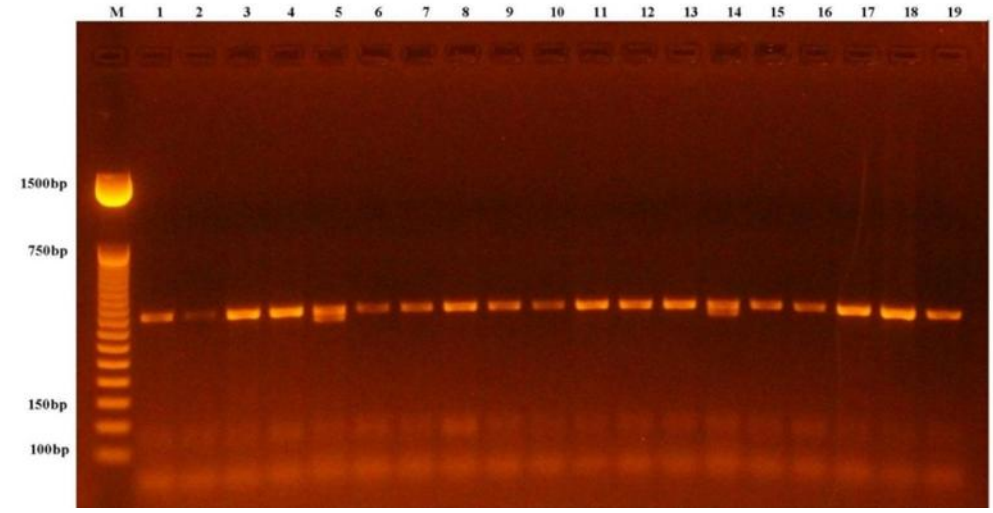

Figure 2. Results of RFLP of TLR4 gene Thr399Ile specific gene region of non-severe patients were fractionated on 2% agarose gel electrophoresis stained with Eth.Br. M: 50 bp ladder marker. lanes 5 & 14, in the case of two people who are heterozygous for the Thr399Ile mutation

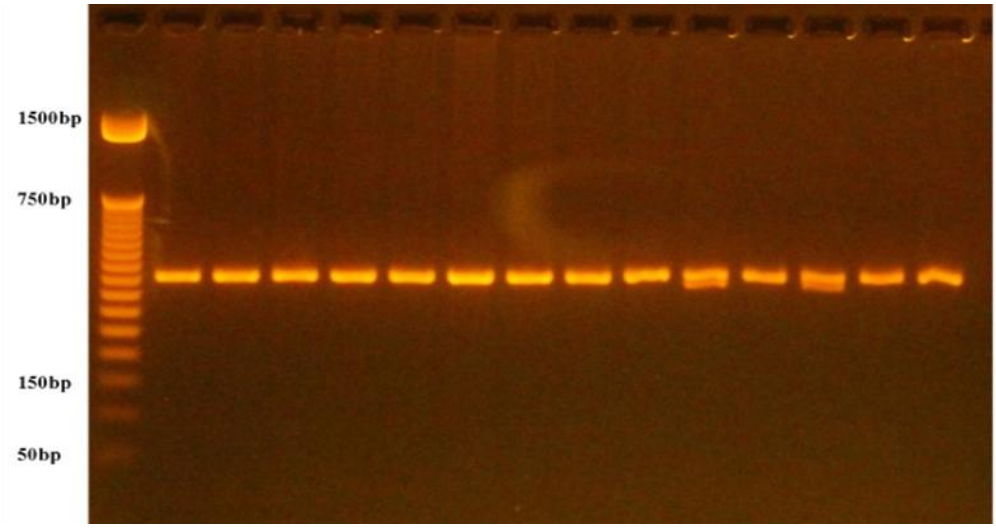

Figure 3: Results of RFLP of TLR4 Thr399Ile specific gene region of severe patient were fractionated on 2% agarose gel electrophoresis stained with Eth.Br. M: 50 bp ladder marker. Lanes 1-9,11,13,14: representative samples positive for the Wild-type (allele C): 407 bp among sever COVID-19 infected patients. Lane 10, 12: Positive for (allele T): 378 + 29 bp

Table (5) shows the genotypes frequency for Thr399Ile of TLR4 gene in severe and non-severe patients of COVID-19. No significant was seen with this SNP Thr399Ile and COVID-19 severity (P = 0.645)

The results of frequency genotypes in non-severe infection of COVID-19 patients were CC (95.55%), CT (4.44%) and TT (0 %), whereas in severe infections of COVID-19 patients, the frequency of genotypes was CC (93.33%), CT (6.66%) and TT (0%). There were no significant differences for the genotypes (CC and CT) when compared between non-severe and severe infection of COVID-19 patients (P >0.05).

Table 5. The genotype frequency for Thr399Ile TLR4 gene in studied groups

Variation Thr399Ile	COVID-19 infection				Total	P value
	Non-severe N = 45		Severe N = 45			
	No.	%	No.	%		
Wild Homozygous (CC)	43	95.55	42	93.33	85	
Mutated Homozygous (TT)	0	0	0	0	0	0.645
Mutated Heterozygous (CT)	2	4.44	3	6.66	5	

Table (6) shows the frequency of the alleles C and T and its relationship to infection severity. Allele C has a frequency of 0.98 in the non-severe group, and 0.97 in the severe group. While allele T has a frequency of 0.02 in the non-severe group, and 0.03 in severe group.

Table 6. Hardy–Weinberg equilibrium analyses of TLR4 (Thr399Ile) SNP in Severe and non-severe COVID–19 patients(n=90)

Allele frequency	Non-severe	Severe
C	0.98	0.97
T	0.02	0.03

Discussion

Many human genes may be responsible for the observed diversified severity of COVID-19 such as TMPRSS2 gene SNP rs2070788 [13].

This study focused on TLR4 gene as it related to host entry mechanism and immune system, type I and type II interferon systems, as well as pro-inflammatory cytokines, are produced when TLR4 detects pathogen-associated molecular sequences [14].

The TLR4 gene is very variable, the extracellular leucine-rich repeats (LRR) domain, which is responsible for recognizing PAMPs, has the highest frequency of genetic variations; as a result, the evolutionary pressure put on this domain by pathogens may serve as a potential explanation for more frequent polymorphisms in this domain [15].

There are several TLR4 gene polymorphisms found in Caucasian populations [16]. One of them is Thr 399Ile (rs4986791). Nuclear Factor-B (NF-B) activation and the expression of pro-inflammatory cytokines, which are responsible for driving inflammation through interferons, interleukins, and tumor necrosis factor-alpha (TNF-α), which are reduced when Thr399Ile is present [17].

This study showed no association between Thr399Ile SNP of TLR4 gene and severity of COVID-19. It can thus be assumed that Thr399Ile mutation do not influence the individual susceptibility for COVID-19 disease. The outcomes of further researches varied and provided compelling evidence of racial variations. In contrast, Egyptian study proved that Thr 99Ile (T) allele are associated with COVID-19 severity and mortality [18]. It may be because of ethnicity, genetic, and environmental features.

The result of this study shown that The T allele is a relatively rare polymorphism, occurring in only about 2% of the population. This means that even if the T allele does increase the risk of severe COVID-19, it is unlikely to be a major factor in most cases. Also in this study found that the highest mean of age was in the

severely infected group, due to their weak immunity in addition to age-related comorbidities.

In conclusion, functionally effective Thr399Ile polymorphism of TLR4 gene have apparently no influence on the individual susceptibility for COVID-19 severity infection.

Acknowledgement

The authors appreciate the support of Biology Department, College of Medicine, Al-Iraqiya University, Baghdad, Iraq, for their contribution to this research work.

Author contribution

Jaber and Dr. Khudhair: designed the study and contributed to the acquisition of data. Jaber: contributed to sample preparation and was the main person in writing the manuscript. Both authors provided critical feedback and helped shape the research, interpreted the data and read and approved the final manuscript.

Conflict of interest

No potential conflict of interest was reported by the authors.

Funding

Self-funding.

References

1. Woo PC, Lau SK, Lam CS, et al. Discovery of A Novel Bottlenose Dolphin Coronavirus reveals a distinct species of marine mammal Coronavirus in Gammacoronavirus. J Virol. 2014; 88(2): 1318-31. doi: 10.1128/jvi.02351-13.
2. Kim JY, Choe PG, Oh Y, et al. The first case of 2019 novel Coronavirus pneumonia imported into Korea from Wuhan, China: Implication for infection prevention and control measures. J Korean Med Sci. 2020; 35(5): e61. doi: 10.3346/jkms.2020.35. e61.
3. Al-Shaibani AHA-S. Epidemiology of the domestic and repatriation (Covid-19) Infection in Al Najaf province, Iraq. J Fac Med Baghdad. 2020; 62(1.2): 13-9.doi:https://doi.org/10.32007/jfacmedbagdad.621.21738.
4. Russell CD, Lone NI, Baillie JK. Comorbidities, multimorbidity and COVID-19. Nat Med. 2023; 29: 334-43. doi: https://doi.org/10.1038/s41591-022-02156-9.
5. Rasmussen SB, Reinert LS, Paludan SR. Innate recognition of intracellular pathogens: detection and activation of the first line of defense. APMIS. 2009; 117(5-6): 323-37. doi: 10.1111/j.1600-0463.2009.02456.x.
6. Birra D, Benucci M, Landolfi L, et al. COVID 19: a clue from innate immunity. Immunol Res. 2020; 68(3): 161-8. doi: 10.1007/s12026-020-09137-5.
7. Hedayat M, Netea MG, Rezaei N. Targeting of Toll-like receptors: a decade of progress in combating infectious diseases. Lancet Infect Dis. 2011; 11(9): 702-12. doi: 10.1016/S1473-3099(11)70099-8.
8. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010; 11(5): 373-84. doi: 10.1038/ni.1863.
9. Miller RE, Belmadani A, Ishihara S, et al. Damage-associated molecular patterns generated in osteoarthritis directly excite murine nociceptive neurons through Toll-like receptor 4. Arthritis Rheumatol. 2015; 67(11): 2933-43. doi: 10.1002/art.39291.
10. Li Y, Chang M, Abar O, et al. Multiple variants in toll-like receptor 4 gene modulate risk of liver fibrosis in Caucasians with chronic hepatitis C infection. J Hepatol. 2009; 51(4): 750-7. doi: 10.1016/j.jhep.2009.04.027.
11. World Health Organization. Living guidance for clinical management of COVID-19: living guidance, 23 November 2021. Geneva: World Health Organization; 2021. Report No.: WHO/2019-nCoV/clinical/2021.2
12. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000; 25(2): 187-91. doi: 10.1038/76048.
13. Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques. 2001; 31(1): 22-4. doi: 10.2144/01311bm01.
14. Mahdi NZ, Khudhair AM, Khalil NS. Assessing the potential correlation of polymorphisms in the TMPRSS2 gene with severity of COVID 19 patients. Biomedicine. 2022; 42(5): 1034-9. doi: https://doi.org/10.51248/.v42i5.2279.
15. Tsan MF, Gao B. Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors. J Endotoxin Res. 2007; 13(1): 6-14. doi: 10.1177/0968051907078604.
16. Noreen M, Shah MA, Mall SM, et al. TLR4 polymorphisms and disease susceptibility. Inflamm Res. 2012; 61(3): 177-88. doi: 10.1007/s00011-011-0427-1.
17. Laine ML, Crielaard W, Loos BG. Genetic susceptibility to periodontitis. Periodontol 2000. 2012; 58(1): 37-68. doi: 10.1111/j.1600-0757.2011.00415.x.
18. Balistreri CR, Ruvolo G, Lio D, Madonna R. Toll-like receptor-4 signaling pathway in aorta aging and

diseases: "its double nature". J Mol Cell Cardiol. 2017; 110: 38-53. doi: 10.1016/j.yjmcc.2017.06.011.

Correspondence to Marwa F. Jaber
E-mail: marwaf9380@gmail.com
Received Jul. 9th 2023
Accepted Sep. 3rd 2023