ISSN: 1607-8322, e-ISSN: 2220-5799 **Anaesthesia, Pain & Intensive Care**
Vol 28(6); December 2024 DOI:10.35975/apic.v28i6.2622

ORIGINAL RESEARCH **PERIOPERATIVE MEDICINE**

Molecular detection of Toll-like receptors 1, 4, and 5 genes among patients with appendicitis

Zainab Nihad Shaker [1], Dawood S. Mahdi [2], Ihsan Edan AlSaimary [3]

Author affiliations:

1. Zainab Nihad Shaker, College of Health & Medical Techniques, Southern Technical University, Basrah, Iraq; E-mail: zainab.n.shaker@fgs.stu.edu.iq
2. Dawood S. Mahdi, College of Health & Medical Techniques, Southern Technical University, Basrah, Iraq; E-mail: dr.dawds@stu.edu.iq
3. Ihsan Edan AlSaimary, Microbiology Department, College of Medicine, Basrah University, Basrah, Iraq; E-mail: ihsan.alsaimary@uobasrah.edu.iq

Correspondence: Zainab Nihad shaker; **E-mail:** zainab.n.shaker@fgs.stu.edu.iq; **Phone:** +964 773 018 3063

ABSTRACT

Background & Objective: Acute appendicitis is the predominant etiology of emergency cases on a global scale, and it may have serious consequences. If there is no precise diagnosis. Toll-like receptors (TLRs) are a crucial group of receptors that form the initial barrier against infections. The study aimed to investigate the molecular structure of Toll-like receptor (TLRs) genes by conventional Polymerase Chain Reaction (PCR) and deoxyribonucleic acid (DNA) sequencing in acute appendicitis patients and investigate whether any alteration in this gene may act as a risk factor for appendicitis.

Methodology: A case-control investigation was conducted at Al-Sadr Teaching Hospital and Al-Shifaa Teaching Hospital in Basrah, Iraq, from July to November 2023. The study involved 20 blood samples from 15 patients previously diagnosed with appendicitis; and 5 healthy individuals included as a control group. We randomly selected six samples from patients with appendicitis, with two samples for each primer, for DNA sequencing.

Results: The results of conventional PCR and electrophoresis showed different molecular weights at different temperatures for TLR1, TLR4, and TLR5. DNA sequencing results showed several different mutations for each TLRs gene primer for both reverse and forward when compared by the Basic Local Alignment Search Tool (BLAST) to sequences from the National Centre for Biotechnology Information (NCBI). Six new mutations were recorded in the gene bank (NCBI), two mutations per TLR. The study found that TLR1's forward primer had two mutations, while the reverse primer had four. Four alterations were found in TLR4, while one in TLR5 was found in the reverse primer.

Conclusion: Through altering the immune system's reaction to pathogenic stimuli, excessive immunological activation, and inflammation, genetic differences in TLRs may influence the pathophysiology or outcome of disease. These factors can also contribute to the development of appendicitis.

Abbreviations: AA, Acute Appendicitis; AHC, apparently healthy controls, "BLAST, Basic Local Alignment Search Tool"; CD, cluster of differentiation; EDTA, ethylene diamine tetra acetic acid; GBD, Global Burden of Disease Study; GC, gastric cancer; GIT, gastrointestinal tract; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; "MyD88, myeloid differentiation primary response 88"; NCBI, National Center for Biotechnology Information; PRRs, "Pathogen Recognition Receptors; PAMPs Pathogen Associated Molecular patterns"; SNPs, single-nucleotide polymorphisms; TE, Tris-EDTA; TIR, Toll-interleukin (IL)-1 receptor.

Keywords: Appendicitis, TLR1, TLR4, TLR5, PCR, DNA sequencing, Molecular Detection

Citation: Shaker ZN, Mahdi DS, AlSaimary IE. Molecular detection of Toll-like receptors 1, 4, and 5 genes among patients with appendicitis. Anaesth. pain intensive care 2024;28(6):1050-1060; **DOI:** 10.35975/apic.v28i6.2622

Received: August 22, 2024; **Reviewed:** October 06, 2024; **Accepted:** October 18, 2024

1. INTRODUCTION

Acute appendicitis arises from the inflammation of the appendix.[1] It is the primary cause of most cases of emergency,[2] emergency appendectomy makes up one in ten of all emergency surgeries.[3] Impacting a total of 17.7 million individuals on a global scale each year. It is more common among young people.[4] The rise of appendicitis symptoms such as nausea, vomiting and severe pain in the lower right abdomen has been described by Global Burden of Disease Study (GBD) 2019.[5] Although appendectomy is a frequently performed surgical intervention, its etiology and pathogenesis have continued to remain elusive.[6] The etiology of acute appendicitis is not fully understood;[7] the obstruction of the appendix by a hard mass known as an appendicolith is one of the numerous causes of appendicitis. A perforation of the appendix occurs as a result of inflammation, necrosis and pus production, increased pressure against the appendix wall, blood pooling in the abscess may develop as a result, or widespread peritonitis may follow.[8] Septic shock and other severe consequence, such as sepsis and death, can also result from appendicitis.[5] VanPutte *et al*. demonstrated that the formation of appendix secretions causes pain and inflammation.[9] The appendicular epithelium's ability to act as a barrier against commensal microbes is compromised by swelling areas caused by blocked blood and lymphatic flow,[10] as a result, the bacteria infiltrate the submucosal layers, necessitating an immediate immune response. This reaction is a result of microbial infections and their byproducts stimulating the Pathogen Recognition Receptors (PRRs).[11]

The innate immune response is crucial to the prevention of infection, and recognition of pathogens is a major step in this process. Toll-like receptors are known for their ability to recognize microbial components and initiate the early responses that lead to adaptive immunity, as well as being responsible for detecting pathogen associated molecular patterns (PAMPs).[12] The TLR are presents on all types of cells, even those that are not part of the immune system. However, dendritic cells (DC) and macrophages are the greatest source of these receptors, despite the fact their sizes vary within distinct subsets.[13] Considered as the host's first defensive mechanism, TLR's pro-inflammatory reactions help to promote healing and fight infections thereby restoring immunological homeostasis.[14] Numerous synonymous and nonsynonymous single-nucleotide polymorphisms (SNPs) have been identified in the TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 promoter and coding regions. These SNPs have been connected to inflammatory and infectious diseases.[15,16] Upon PAMPs are recognized, TLRs interact with adaptor molecules that contain Toll-interleukin (IL)-1 receptor (TIR) domains, such as myeloid differentiation primary response 88 (MyD88) and TLR domain-containing adaptor inducing interferon β. This interaction initiates signaling pathways that lead to the secretion of inflammatory cytokines, interferon, chemokines, and antimicrobial peptides. The TLRs also detect endogenous damage-associated molecular patters (DAMPs), resulting in the initiation of inflammatory reactions.[17] Confirmed that many indicators were used by researchers in an attempt to get a definite diagnosis of appendicitis.[18]

Objective of study

The study aims to investigate the potential link between genetic difference in Toll-like receptors (TLR1, TLR4, and TLR5) and the development of acute appendicitis.

2. METHODOLOGY

2.1. Study design / subjects

A case-control study was conducted on patients diagnosed with appendicitis at the operating rooms of "Al-Sadr Teaching Hospital and Al-Shifaa Teaching Hospital" in Al-Basrah, Iraq, from July 2023 to November 2023. This study included twenty participants ranging in age from 12 to 63 years, separated into five people as a randomly chosen control group and fifteen patients with appendicitis symptoms. The DNA sequencing samples used for detection TLR-1 gene. The questionnaire was used to capture all relevant information from both the patients and control group participating in this study. Age, sex, smoking, residency, and a family history of appendicitis were among the demographic factors considered for this study. A clinical analysis for patients, singing and clinical symptoms: important symptoms categories (anorexia, nausea, vomiting, shifting pain) and signs (tenderness, rebound tenderness, fever) were distinguished.

2.2. Exclusion and inclusion criteria

Patients with autoimmune diseases or allergic illnesses; patients with chronic inflammation or infectious disease; patients with any type of cancer; patients suffering from chronic diseases; patients receiving medication or chemotherapy; and pregnant or breast-feeding women were excluded from the current study. The present study enrolled patients exhibiting signs of appendicitis in the medical emergency section. Surgeons typically diagnose appendicitis based on a patient's medical history, physical examination, laboratory tests, and imaging studies. The patient typically presents with sever, localized pain in the lower right abdomen, which may have started around the umbilicus and gradually moved to the right lower quadrant. Common symptoms include nausea, vomiting, fever, and loss of appetite. Physical

examination involves locating tenderness in the lower right quadrant and rebound tenderness if the pain worsens when pressure is released. Rovsing's sign test is used to determine if the patient's pain in the right lower abdomen is a positive sign. Laboratory tests include blood tests, C-reactive protein, general urine examination, ultrasound, and CT scans. Ultrasound can visualize the appendix and surrounding organs, but its, diagnostic accuracy depends on the radiologist's experience. A CT scan is often used to confirm the diagnosis, especially if an ultrasound is inconclusive or if other conditions are suspected. The specific diagnostic approach may vary depending on the individual's symptoms, medical history, and other factors. Randomly chosen individuals served as the control group. These individuals included non-smokers, those without a history of appendicitis in their family, those with autoimmune diseases and chronic inflammation, and those not receiving any form of immune treatment.

2.3. Blood samples

Five milliliters (ml) of blood were collected by venipuncture from all patients after diagnosis with appendicitis, as well as healthy controls. The collected blood was put in tubes with ethaline diamine tetraacetic acid (EDTA) as an anti-coagulant agent, gently stirred and shaken, and then stored at a temperature of -70 °C in a deep freeze until detection time.

2.4. DNA Extraction

Deoxyribonucleic acid extraction kit was used for the detection of the titers of TLR1, TLR4, and TLR5 in blood samples of appendicitis patients and control by using the EasyPure®Blood Genomic DNA Kit (Trans Gen Biotech Cat. No. EE121,China). Proteinase K (20µl) and binding buffer 3 are combined with blood in a micro-centrifuge tube, vortexed for 15 seconds, and applied 1000 times gravitational force. The liquid is drained and moved to the spin column, where clean buffer 3 and washing buffer 3 were added. The column is centrifuged 12000 times for two minutes to remove any residue. After sterilization, the spin column was inserted into the centrifuge tube, and one minutes is spent with the sample at room temperature. To elute more genomic DNA, the centrifuge should be set at 12000× g for 1 minute. Either distilled water or Elution buffer can be added for a second elution. For prolonged preservation, pure DNA stored at -20℃. The results confirmed gel electrophoresis; DNA-based methods have been further simplified thanks to the PCR introduction. As shown in Figure 1.

2.5. Polymerase Chain Reaction Technique

The PCR test necessitates the presence of template DNA, primers, nucleotides, and DNA polymerase 19]. Using the primers listed in Table 1, the primer sequences for TLR1 are specifically designed to amplify a region of 403 base pairs. These primers were particularly produced for this research. The size of the PCR product for TLR4 is 143 base pairs. The primer was already published 19]. The primer for TLR5 were specifically designed for this study. They are similar to the primers used for TLR1. The size of the PCR result is 415 base pairs. all samples were tested for the presence of the TLR1, TLR4, and TLR5 genes by PCR. Diluted forward and reverse primers were used for the PCR, and the final working solution (10 pmol\µl) for each primer was prepared by diluting the stock solution with Tris-EDTA (TE) buffer.

The PCR amplifications were performed under the specified conditions using a thermocycler (Applied Biosystem, US). The procedure entails an initial denaturation at 94 ℃ for three minutes, followed by 35 cycles of denaturation at the same temperature for thirty seconds of annealing at 57 ℃ for TLR1 primer, 63 ℃ for TLR4 primer, and 55 ℃ for TLR5 primer, and one minute of extension at 72 ℃. At 72 ℃, the ultimate extension period is five minutes. By using agarose gel electrophoresis, the amplified products were visible. The PCR master mix reaction was carried out in accordance with the manufacturer's instructions using (one Taq Quick-load) PCR kit.

2.6. Sequencing Methods

Instructions for sequencing PCR amplicons in both forward and reverse directions were supplied by Seoul, South Korea-based sequencing business Macrogen Inc. To prevent annotations and modifications, additional tests were performed on clear chromatographs from Applied Biosystem sequencing data. The obtained nucleic acid sequences were compared to the returning sequences in terms of virtual locations and attributes. Using DNASTAR's "Bio-Edit Sequence Alignment Editor Software Version 7.1", the PCR sequencing result was adjusted, aligned, and analyzed in conjunction with matching databases. Numerical values were allocated to nucleic acids according to their locations in the reference genome and PCR amplicons.

3. RESULTS

The TLR1, TLR4, and TLR5 were revealed by analyzing the DNA band that appeared on the gel when attachment between DNA template and specialized primer for each toll results through amplified by using conventional PCR when using 1.5% agarose gel electrophoreses for optimization process with different temperature for PCR product. The bands were observed as orange, dense bands under the UV imaging system. This coloration was

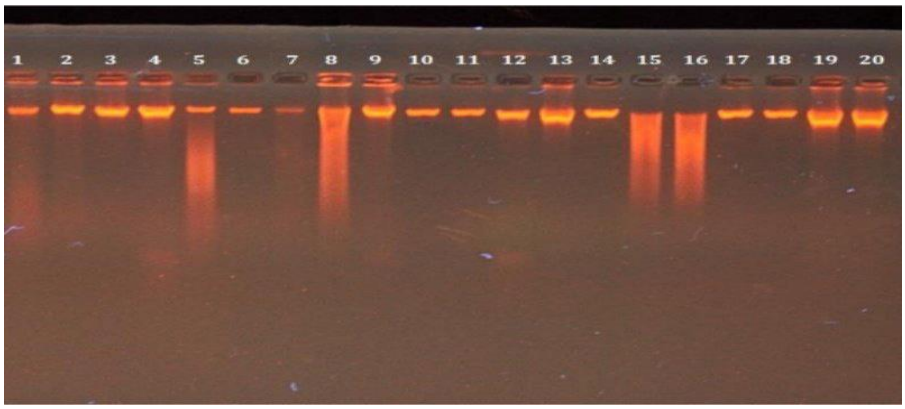

Figure 1: Agarose gel electrophoresis with (1%) concentration at 110 volts and stained with ethidium bromide dye showed a clear band that represented DNA molecule extracted from blood samples.

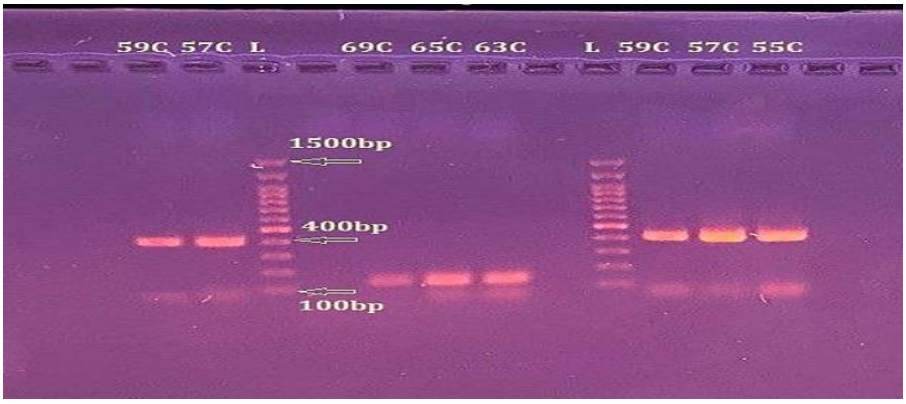

Figure 2: The optimization technique used gel electrophoresis to analyze the PCR result of three primer sets (TLR1, TLR4, and TLR5) at various temperatures. The PCR products showed bands of 403bp, 143bp, and 415bp at temperatures of 57°C, 63°C, and 55°C, respectively. Prepare a solution of agarose with a concentration of 1.5% and incubate it for 15 min at 110 volts first and then reduced to 75 volts for a duration of 60 min. Observed using ultraviolet (UV) light after applying Ethidium bromide stain. Lane L has a DNA ladder ranging from 100 to 1500 base pairs.

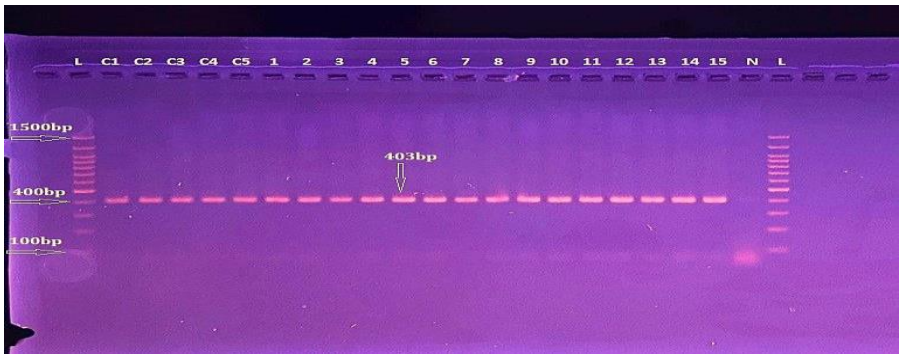

Figure 3: The gel electrophoresis of the PCR product using the TLR1 primer reveals a primer annealing temperature of 57°C and a product size of 403 base pairs. The gel is made of 1.5% agarose and the electrophoresis is run for 15 min. The voltage was first set at 110 volts and then reduced to 75 volts for a duration of 60 minutes. Observed using ultraviolet (UV) light after being treated with ethidium bromide stain. Lane L had a DNA ladder ranging from 100 to 1500 base pairs. Lanes C1 to C15 indicated positive findings, while lane N represented the negative control.

a result of the DNA staining using ethidium bromide as an indicator. The size of the DNA bands retrieved may be determined on gel electrophoresis using a DNA ladder with a range of 100-1500 base pairs. Each band corresponds to a certain size on the ladder. The presence of Toll-like receptors (TLR1, TLR4, and TLR5) can be detected in the amplified DNA findings, as shown in Figure 2.

3.1. Toll-like receptors

Gel electrophoresis has validated the PCR-amplification of DNA, which appears as compact separate DNA bands, that result from the precise and unique attachment between the target DNA template and its corresponding primer, the bands seen in UV imaging systems in illuminating orange bands as a result of ethidium bromide staining. Gel electrophoresis demonstrated DNA amplification for the TLR 1 primer with 403 bp, as shown in Figure 3. Figure 4. displays bands specific to TLR4 primer, measuring 143bp. Figure 5 shows TLR5 primer-specific bands with a molecular size of 415 bp.

3.2. DNA sequencing

3.2.1. DNA sequence analysis of TLR 1 gene

The Macrogen Company in Korea has used PCR to sequence two sample from patients. The nucleotide substitutions were found by comparing data from the Gene Bank published, which can be found at

Table 1: TLRs primer sequences and product size.

GENE	PRIMER SEQUENCES	PCR product	Reference
TLR 1	F 5' AAGGGCCTTCTCTGCACAAG '3 R 5' TCTCTTCACCTAATCCCGCC '3	bp 403	This study
TLR 4	F 5'TTGAGCAGGTCTAGGGTGATTGAAC'3 R 5' ATGCGGACACACACACTTTCAAAT '3	bp 143	(19)
TLR 5	F 5' TCACCACTTACCCCTCAAAGG '3 R 5' GCCTCCCTTTGACCACACTA '3	415bp	This study

Table 2: A protocol for One Tag® Quick-Load 2X Master Mix with Standard Buffer

Component	50 µl reaction
10 µM Forward Primer	1 µl
10 µM Reverse Primer	1 µl
Template DNA	Variable
Easy Taq 2X Master Mix with Standard Buffer	1 µl
Nuclease-free water	Variable
Total	1 µl

NCBI https://www.ncbi.nlm.nih.gov. The findings were recorded to NCBI and given the accession number OR924453.

The main mutation types found in the TLR1 forward and reverse gene sequences in this investigation were displayed in Table 3 for sample one, where the transition at position 5767: A missense mutation resulting in a single nucleotide change (GGC to AGC) converts the amino acid lysine to serine. The protein's functioning

may be compromised by this alteration. The table also demonstrated that deletions located at positions 5374 and 5382 cause the gene's reading frame to be disrupted, resulting in the production of the shortened and non-functional proteins. The silent mutation at position 5378: the amino acid sequence remains unchanged due to a synonymous nucleotide change (GGT to GGG), indicating that it is unlikely to directly affect protein function. The second sample, as indicated in Table 4, on the other hand, showed that the transition at position 5589: a single nucleotide shift (AGG to GGG) causes a missense mutation that changes arginine to glycine. The protein's functioning may be compromised by this alteration, which might affect its capacity to identify infections or trigger an immunological response. Furthermore, Tans-version at position 5520: this missense mutation, which changes the amino acid serine to arginine, is caused by another single nucleotide change, from AGT to AGG. The protein's functioning may also be compromised by this alteration, possibly impaired its capacity to identify infections or trigger immunological responses.

3.2.2. DNA sequence analysis of TLR 4 gene

The Macrogen Company in Korea performed PCR sequencing on two patient's samples. Nucleotide substitutions were determined by comparing gene bank published data at NCBI https://www.ncbi.nlm.nih.gov and registering the results under accession number (OR924455). The results for sample 1, which showed a single deletion polymorphism at nucleotide position 17657, are shown in Table 5. The amino acid alanine was lost as a result of this deletion, which could have an impact on the structure and functionality of the protein. Table 6 demonstrates that the TLR4 gene for the second sample had several mutations in both the forward and reverse directions. The addition mutation,

Table 3: Type polymorphism of the Homo sapiens TLR 1 gene sequence for sample one

Sample	Type of substitution	Location	Nucleotide change	Amino acid change	Predicted effect	Source
1 F	Transition	5767	GGC > AGC	lysine > Serine	The gene's protein may exhibit impaired functionality.	Toll Like Receptor (TLR1) Gene
1R	Deletion	5374	AAG > AA-	lysine > No functional code	No functional protein	
	Trans-version	5378	GGT > GGG	Glycine > Glycine	Silent mutation	
	Deletion	5382	ATT > AT-	Isoleucine > No functional code	No functional protein	

Table 4: Type polymorphism of in the Homo sapiens TLR 1 gene sequence for Sample two.

Sample No.	Type of substitution	Location	Nucleotide change	Amino acid change	Predicted effect	Source
2F	Transition	5589	AGG > GGG	Arginine > Glycine	The gene's protein may exhibit impaired functionality.	Toll Like Receptor 1 (TLR1) Gene
2R	Trans-version	5520	AGT >AGG	Serine > Arginine	The gene's protein may exhibit impaired functionality.	

Table 5: Type polymorphism of in the Toll-Like Receptor 4 (TLR4) Gene in sample one.

No of sample	Type of substitution	Location	Nucleotide change	Amino acid change	Predicted effect	Source
1R	Deletion	17657	GCA > GC-	Alanine > No functional code	No protein	Toll Like Receptor 4 (TLR4) Gene

the addition of one nucleotide at position 17618 of the gene, changed it to the amino acid arginine, was the first mutation in the reverse gene sequences. At position 17619 of the gene, a trans-version mutation caused the second mutation to change cysteine to glycine. The third mutation was a transition where the gene's position 17623 was changed from (T > C). The insertion of one nucleotide (> A) at position 17626 constituted the fourth mutation. These mutations may have resulted in improper functioning of the protein produced by these genes. The fifth mutation was a transition that

Table 6: Type polymorphism in the Toll-Like Receptor 4 (TLR4) Gene sequence for sample two.

Sample No.	Type of substitution	Location	Nucleotide change	Amino acid change	Predicated effect	Source
2 F	Addition	17618	-TG > AGG	No functional code > Arginine	The protein made by the gene may not function properly	Toll Like Receptor (TLR) 4 Gene
	Trans-version	17619	TGT > GGT	Cysteine > Glycine		
	Transition	17623	TTT > CTT	Phenylalanine > Lucien		
	Addition	17628	-TG > ATG	No functional code > Methionine		
	Transition	17656	TGC > CGC	Cysteine > Arginine	Silent mutation	
	Addition	17669	-AT > AAT	No functional code > Asparagine	The protein made by the gene may not function properly	
	Trans-version	17723	GCA > CCA	Alanine > Proline		
2 R	Addition	17648	AC- > ACT	No functional code > Threonine		
	Addition	17652	TC- > TCT	No functional code > Serine		
	Addition	17658	AC- > ACC	No functional code > Threonine		

Table 7: Type polymorphism of in the Toll-Like Receptor (TLR) 5 Gene sequence for sample one

Sample No.	Type of substitution	Location	Nucleotide change	Amino acid change	Predicted effect	Source
1F	Deletion	10858	TTG > TT-	Lucien > No functional code	No protein	Toll Like Receptor 5 (TLR 5) Gene

Table 8: Type polymorphism of in the Toll-Like Receptor (TLR) 5 Gene sequence for sample two.

Sample No.	Type of substitution	Location	Nucleotide change	Amino acid change	Predicted effect	Source
2F	Deletion	10857	TGC > TG-	Cysteine > No functional code	NO amino acid	Toll Like Receptor (TLR) 5 Gene
	Transition	10518	AAA > AAG	Lysine > Lysine	Silent mutation	
	Trans-version	10521	GTC > GCA	Valine > Alanine	The gene's protein may exhibit impaired functionality.	
	Transition	10522	GGT > GGC	Glycine > Glycine	Silent mutation	
2R	Transition	10916	TAA > AAA	Stop codon> Lysine	The gene's protein may exhibit impaired functionality.	

Table 9: The recording novel gene mutation in the current study

TLR types	Record genes	Accession number
TLR1	BankIt2774164 Seq1	OR924453
	BankIt2774164 Seq2	OR924454
TLR4	BankIt2774173 Seq1	OR924455
	BankIt2774173 Seq2	OR924456
TLR5	BankIt2774187 Seq1	OR924457
	BankIt2774187 Seq2	OR924458

resulted in a silent mutation (a mutation that is inefficient in protein synthesis) at position 17656 of the gene by swapping (T > C). One nucleotide was added in the sixth mutation, which was a (> A). The gene's location 17669 is the seventh change substitution (G > C) in conversion. The gene's position 17723 caused an alteration in the amino acid; the function of the generated protein was impacted by these alterations. The first mutation in reverse is an addition mutation, which changed the amino acid by being added at position 17648. The inclusion of the second mutation, (> T) at point 17652. The third mutation further adds (> C). Position 17658 caused an alteration in the amino acid that was produced. The protein's functionality is impacted by these other mutations.

3.2.3. DNA sequence analysis of TLR 5 gene

Korea's Macrogen Company sequenced two samples using PCR. By comparing gene bank data from NCBI, nucleotide substitutions were determined and designated with the accession number (OR924457)

https://www.ncbi.nlm.nih. gov/searcg/ all term= OR924457).

Table 7 displays sample 1's findings, which revealed a deletion polymorphism in forward primer at nucleotide position 10858. Due to this deletion, the leucine amino acid was removed, which could have an effect the protein's structure and activity. The results for sample 2, which showed a mix of deletion, transition, and trans-version polymorphism in reverse and forward primers, are displayed in Table 8. Both synonymous (silent) and non-synonymous amino acid changes were the outcome of these modifications. It is noted that significant effects on TLR5 protein function are expected for the trans-version polymorphism at nucleotide position 10521 and the transition polymorphism at nucleotide location 10916.

3.3. Recording new gene

The new gene mutation was recorded in patients with appendicitis in the current study are shown in Table 9. Two sequences, Seq1 and Seq2, were recorded for all

forms of TLR. Each sequence's accession number makes it possible to access and examine these alterations in more detail.

4. DISCUSSION

The presence of inflammation in appendicitis involves the stimulation of several immune cells and their intricate interplay, which is mediated by several pro-inflammatory or anti-inflammatory cytokines predominantly secreted by macrophages and T-lymphocytes.[20] Despite the high incidence of the disease, limited pathophysiology data existed; in particular, the underlying immunological responses that played a role in this condition remain mostly undiscovered.[11] While numerous efforts have been made to discover biomarkers specific to appendicitis, it is unlikely that a single cytokine may function as a conclusive diagnostic indicator for acute appendicitis due to the varied underlying causes of the disease.[21] TLRs, which are part of the PRR family, play a vital role in regulating the body's protective immunological responses. They act as triggers for inflammation. The stimulation of Toll-like receptors in myeloid cells of the innate immune system provides instructions to lymphocytes to develop highly effective responses in order to eliminate infections and maintain the body's internal environment in a state of homeostasis. Acute and chronic inflammation, cancer, and other autoimmune disorders can all arise as a result of inappropriate TLR stimulation.[22] The TLRs' ability to control intestinal homeostasis in the gastric intestinal tract (GIT) is closely correlated with their expression, distribution, and location.[23]

A few research molecular approaches to understanding TLRs and appendicitis exist in literature; hence, the rest of this discussion will revolve around these TLRs as a diagnostic marker for other diseases. Nonetheless, more information is required about the participation of pattern recognition receptors (PRRs) and cytokines in appendicitis. This study assessed TLR1, TLR4 and TLR5 expression as possible indicators for prognosis among stomach cancer specimens they observed that high levels of TLR5 expression were strongly associated with good prognosis but TLR1 was not significantly related to patient outcome.[24] In the present investigation, we confined our self to (TLR1, TLR4 and TLR5) to associate them with Appendicitis. The real-time PCR technique was used by Shirafkan *et al*. [25] to quantify TLR gene expression in gastric biopsy specimens, after they had extracted total RNA from the specimens. The analysis of results to other groups indicated the decreased TLR1 gastric cancer (GC) level. Another probing examined 75% of all the samples for TLR1 (PCR product) and 95% of the samples for TLR2, on an agarose gel of 1% 26].

The role of TLR1 in the innate recognition of the microbiota and its effect on promoting epithelial homeostasis and preventing chronic inflammation was examined in a past research. The results propose that TLR1's potential to identify the microbiota could be important for controlling the colonic epithelium. This limitation assists in inhibiting bacterial adhesion to mucosa, hence curtailing immune system exposure and limiting inflammation.[27] The TLR 1 in the current study, the forward primer when compared with the sequence of NCBI by BLAST showed 96% identities with expected value 1e-152 and there were two mutations: GGC occupies the meaning of AGC and AGG occupies the meaning of GGG in several places which provide new amino acid or protein. On the other hand, TLR1 reverse primer showed 4 mutations: Locations: AGT> AGG, AAG> AA-, GGT> GGG, and AAT > AT- with 98 % identity and expect value 4e-168. The mutation in TLR1 is shown in Tables 3 and 4. Genetic variations in TLRs may impact the pathophysiology or outcome of disease by causing changes in the immune system's response to pathogenic stimulus, excessive immunological activation, and inflammation 28]. Human Toll-like receptors mutations can influence the extent of the innate immune response to microbial stimulus.[29]

Toll Like Receptor 4 is a protein that is attached to the cell membrane that triggers the signaling pathway leading to an inflammatory reaction in response to lipopolysaccharide (LPS) 30]. Activation of TLR4 in the endothelium causes vasoconstriction and intestinal ischemia, which are symptoms of necrotizing small intestine colitis, and TLR4 activation by LPS in the lumen results in the loss of intestinal mucosa.[31] The expression of TLR4 is increased in macrophages in the intestines during inflammation.[32] particularly inside the inflammatory cells located in the lamina propria.[33] Patients diagnosed with inflammatory bowel disease (IBD) had reduced levels of TLR5, while demonstrating elevated expression levels of TLR4 specifically.[34,35] The TLR4 is also more highly expressed in the dendritic cells of IBD patients; there are no distinctions between ulcerative colitis and Crhon's disease.[36] Moreover, intestinal macrophages in inflammatory mucosa express more TLR4 than in a healthy colon.[32] According to the current study's results, the forward primer TLR4, when compared with the sequence of NCBI, showed seven mutations: -TG > AGG, TGT > GGT, TTT > CTT, -TG> ATG, TGC> CGC, -AT > AAT, and GCA > CCA in different locations with 94% identities and 2e-38 expect value. On the other hand, the reverse primer showed four mutations: AC- > ACT, TC- > TCT, GCA > GC-, and AC- > ACC, with 99% identities and a 2e-36 expected

value, the TLR4 mutations represented in Table 5 and 6. The intestinal microbiota is impacted by TLR4 signaling, which has been demonstrated to alter gastrointestinal motility and promote pathogen clearance, commensal population maintenance, The process of goblet cell development and the synthesis of antimicrobial peptides.[37,38] Polymorphic variations of TLR4 are highly valuable in determining the risk and defense of infectious diseases because of their role in inducing an immune response against pathogens. The TLR4 mutation modifies the composition of the receptor and restricts its capacity to bind with LPS, a cluster of differentiation (CD)-14 and MD-2.[39]

The host immunological reply flagellate bacteria and the preservation of intestinal homeostasis is significantly regulated by TLR5 signaling, which is highly expressed in cells.[40] Conversely, a deficiency in TLR5 will result in a disturbance of the gut flora, low-grade inflammation, metabolic syndrome, and an increased risk of colitis.[41] In many ethnic cohorts, TLR5 polymorphisms have been identified and linked to an elevated risk of IBD.[42] The expression of TLR-5 mRNA was measured by quantitative PCR in the study carried out by Falah *et al.* In comparison to individuals without ulcerative colitis, they discovered that the mucosa of those with the disease had less TLR5 mRNA.[43] In comparison to normal controls, Stanislawowski *et al.* reported lower levels of TLR5 protein and TLR5 mRNA in the inflammatory mucosa.[44] The present study's results revealed five mutations in TLR5, the forward primer, when compared to the NCBI sequence: TTG> TT-, TGC> TG-, AAA> AAG, GTC> GCA, and GGT> GGC in different locations with 99% identities and 0.0 expect value. On the other hand, the reverse primer showed one single mutation: TAA> AAA in different locations with 100% identities and 0.0 expected value, the TLR5 mutation in the current investigation is displayed in Tables 7 and 8. Activation of TLR5 signaling promotes the initial defenses against the invasion of host tissues by pathogens by inducing the maturation of inexperienced B-cells differentiate into plasma cells that produce IgA and interleukins IL-17 and IL-22.[45] Theoretically, inflammation-induced damage to the colon's lining may cause TLR5 expression to decrease, maybe as a result of flagellin overexpression in the damaged mucosal barrier epithelium.[43] Dysfunctional TLR5 signaling leads to dysbiotic outgrowth and related diseases, while TLR5 signaling promotes homeostasis at the mucosal surface by delaying the development of microbial dysbiosis.[46]

5. LIMITATIONS

According to their established functions in immune function, this study used a focused strategy, concentrating on particular genes (TLR1, TLR4, and TLR5). Mutations in additional genes or regulatory components, however, might also raise the risk of appendicitis. In order to analyze genetic variants linked to appendicitis in greater detail, future research may use whole-exome or whole-genome sequencing methods. To improve how broadly applicable, the results are, the study's sample size might also be increased. The present investigation employed conventional PCR to identify the DNA gene sequence for TLR1, TLR4, and TLR5, and to evaluate any mutations present in these genes. This method is unable to determine the level of gene expression.

6. FUTURE DIRECTIONS

The current study serves as a starting point for researchers in this field. We suggested using real-time PCR to accurately determine gene expression levels and how much effect this mutation on gene expression for TLR. Further information about the discovered mutations' possible role in the pathophysiology of appendicitis would be obtained by functional validation using in vitro tests.

7. CONCLUSION

When comparing the findings of the present study using BLAST with NCBI sequence. TLR1's forward primer showed two mutations, whereas the reverse primer showed four mutations. Four alterations were discovered by the reverse primer in TLR4, compared to seven by the forward primer. Regarding TLR5, there was one mutation in the reverse primer and five in the forward primer. The mutations discovered during this investigation may be considered a risk factor for appendicitis development because critical mutations influence protein production and result in non-functional proteins, which contribute to disease progression. Moreover, the generational transfer of these mutations partly explains the increased occurrence of appendicitis. Future research is necessary to functionally validate these mutations in order to ascertain their effects on ligand recognition, signaling cascades, and possible contribution to appendicitis susceptibility.

8. Conflict of Interest

The contributors affirm that they have no competing interests.

9. Ethical Considerations

The study was approved by the ethical consideration committee of the Training and Human Development Unit, Al-Basra Health Department, "Ministry of Health and Environment, Iraq", in accordance with the Declaration of Helsinki. Written onsent was obtained from every participant.

10. Source of support and findings: privately funded

11. Personal thanks: The author thanks the laboratory staff at the Health Department of Basra's Al-Sadr Teaching Hospital for their great assistance and unalterable dedication during the sample collection.

12. Authors contribution

The manuscript's conception, writing, data analysis, assessment, submission, changes, and final editing were all done in collaboration with the author, Dawood Salman Mahdi. Data collection, composition, and analysis were carried out by Zainab Nihad Shaker. Data collection, the article's concept, and its writing were all done by Ihsan Edan AlSaimary.

13. REFERENCES

1. Shah AA, Al-Zoubi RM, Al-Qudimat AR, Rejeb MA, Ojha LK, Abdulzem S, et al. Daytime versus nighttime laparoscopic appendectomy in term of complications and clinical outcomes: A retrospective study of 1001 appendectomies. Heliyon. 2022;8(12).
2. Di Saverio S, Podda M, De Simone B, Ceresoli M, Augustin G, Gori A, et al. Diagnosis and treatment of acute appendicitis: 2020 update of the WSES Jerusalem guidelines. World J Emerg Surg. 2020;15:1–42.
3. Barman MK, Das K, Mukherjee K. Use of Ripasa Score in the Diagnosis of Acute Appendicitis: A Pilot Study from West Bengal. India J Med Sci Clin Res. 2019;
4. Wickramasinghe DP, Xavier C, Samarasekera DN. The worldwide epidemiology of acute appendicitis: an analysis of the global health data exchange dataset. World J Surg. 2021;45:1999–2008.
5. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020;396(10258):1204–22.
6. Neufeld MY, Bauerle W, Eriksson E, Azar FK, Evans HL, Johnson M, et al. Where did the patients go? Changes in acute appendicitis presentation and severity of illness during the coronavirus disease 2019 pandemic: a retrospective cohort study. Surgery. 2021;169(4):808–15.
7. Lamps LW. Infectious causes of appendicitis. Infect Dis Clin. 2010;24(4):995–1018.
8. Pogorelić Z, Čohadžić T. A bizarre cause of acute appendicitis in a pediatric patient: An ingested tooth. Children. 2023;10(1):108.
9. VanPutte C, Regan J, Russo A. ISE Seeley's Anatomy & Physiology. McGraw-Hill; 2022.
10. Zviedre A, Engelis A, Tretjakovs P, Jurka A, Zile I, Petersons A. Role of serum cytokines in acute appendicitis and acute mesenteric lymphadenitis among children. Medicina (B Aires). 2016;52(5):291–7.
11. Arlt A, Bharti R, Ilves I, Häsler R, Miettinen P, Paajanen H, et al. Characteristic changes in microbial community composition and expression of innate immune genes in acute appendicitis. Innate Immun. 2015;21(1):30–41.
12. Mahmoud FM, Darwish NM, Hassan RA, Abou Shady NM. Evaluation of CD64 detection on neutrophils and TLR-2 on monocytes by flowcytometry as markers for early diagnosis of neonatal sepsis. Int J Adv Res. 2014;2(7):1235–47.
13. Nouri Y, Weinkove R, Perret R. T-cell intrinsic Toll-like receptor signaling: Implications for cancer immunotherapy and CAR T-cells. J Immunother Cancer. 2021;9(11).
14. Mukherjee S, Huda S, Sinha Babu SP. Toll-like receptor polymorphism in host immune response to infectious diseases: A review. Scand J Immunol. 2019;90(1):e12771.
15. Netea MG, Wijmenga C, O'neill LAJ. Genetic variation in Toll-like receptors and disease susceptibility. Nat Immunol. 2012;13(6):535–42.
16. Lee YH, Lee HS, Choi SJ, Ji JD, Song GG. Associations between TLR polymorphisms and systemic lupus erythematosus: a systematic review and meta-analysis. Clin Exp Rheumatol. 2012;30(2):262–5.
17. Kawai T, Ikegawa M, Ori D, Akira S. Decoding Toll-like receptors: Recent insights and perspectives in innate immunity. Immunity. 2024;57(4):649–73.
18. Sevinç B, Turan E, Kurku H, Karahan Ö. Role of CA125 in the Diagnosis of Acute Appendicitis. Eurasian J Emerg Med. 2017;16(2):62.
19. Garibyan L, Avashia N. Research techniques made simple: polymerase chain reaction (PCR). J Invest Dermatol. 2013;133(3):e6.
20. Stankovic N, Surbatovic M, Stanojevic I, Simić R, Djuricic S, Milickovic M, et al. Possible cytokine biomarkers in pediatric acute appendicitis. Ital J Pediatr. 2019;45:1–9.
21. Naqvi SA, Thompson GC, Joffe AR, Blackwood J, Martin DA, Brindle M, et al. Cytokines and chemokines in pediatric appendicitis: a multiplex analysis of inflammatory protein mediators. Mediators Inflamm. 2019;2019(1):2359681.
22. Wang K, Huang H, Zhan Q, Ding H, Li Y. Toll-like receptors in health and disease. MedComm. 2024;5(5):e549.
23. Yu S, Gao N. Compartmentalizing intestinal epithelial cell toll-like receptors for immune surveillance. Cell Mol life Sci. 2015;72:3343–53.
24. Kasurinen A, Hagström J, Laitinen A, Kokkola A, Böckelman C, Haglund C. Evaluation of toll-like receptors as prognostic biomarkers in gastric cancer: high tissue TLR5 predicts a better outcome. Sci Rep. 2019;9(1):12553.
25. Shirafkan F, Shokri-Shirvani J, Morakabati P, Alhooei S, Pirzadeh M, Barari L, et al. Expression of tlr1, tlr3 and tlr7 genes remarkably down-regulated from erosion to peptic ulcer and gastric cancer development. Gene Reports. 2021;24:101229.
26. Shileche EA. Risk based Intervention Capabilities of Health challenges in Kenya a Case of Chikungunya and Dengu fever.

2020;

27. Kamdar K, Johnson AMF, Chac D, Myers K, Kulur V, Truevillian K, et al. Innate recognition of the microbiota by TLR1 promotes epithelial homeostasis and prevents chronic inflammation. J Immunol. 2018;201(1):230–42.
28. Corr SC, O'Neill LA. Genetic variation in Toll-like receptor signalling and the risk of inflammatory and immune diseases. J Innate Immun. 2009;1(4):350–7.
29. Feterowski C, Emmanuilidis K, Miethke T, Gerauer K, Rump M, Ulm K, et al. Effects of functional Toll-like receptor-4 mutations on the immune response to human and experimental sepsis. Immunology. 2003;109(3):426–31.
30. Smirnova I, Poltorak A, Chan EKL, McBride C, Beutler B. Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4). Genome Biol. 2000;1(1):1–10.
31. Sodhi CP, Wipf P, Yamaguchi Y, Fulton WB, Kovler M, Niño DF, et al. The human milk oligosaccharides 2'-fucosyllactose and 6'-sialyllactose protect against the development of necrotizing enterocolitis by inhibiting toll-like receptor 4 signaling. Pediatr Res. 2021;89(1):91–101.
32. Hausmann M, Kiessling S, Mestermann S, Webb G, Spöttl T, Andus T, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. 2002;122(7):1987–2000.
33. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun. 2000;68(12):7010–7.
34. Vamadevan AS, Fukata M, Arnold ET, Thomas LS, Hsu D, Abreu MT. Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. Innate Immun. 2010;16(2):93–103.
35. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 2010;10(2):131–44.
36. Hart AL, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel A V, Knight SC, et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. Gastroenterology. 2005;129(1):50–65.
37. Frosali S, Pagliari D, Gambassi G, Landolfi R, Pandolfi F, Cianci R. How the intricate interaction among toll-like receptors, microbiota, and intestinal immunity can influence gastrointestinal pathology. J Immunol Res. 2015;2015(1):489821.
38. Anitha M, Vijay–Kumar M, Sitaraman S V, Gewirtz AT, Srinivasan S. Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology. 2012;143(4):1006–16.
39. Silva MJA, Santana DS, de Oliveira LG, Monteiro EOL, Lima LNGC. The relationship between 896A/G (rs4986790) polymorphism of TLR4 and infectious diseases: A meta-analysis. Front Genet. 2022;13:1045725.
40. Feng T, Cong Y, Alexander K, Elson CO. Regulation of Toll-like receptor 5 gene expression and function on mucosal dendritic cells. PLoS One. 2012;7(4):e35918.
41. Chassaing B, Ley RE, Gewirtz AT. Intestinal epithelial cell toll-like receptor 5 regulates the intestinal microbiota to prevent low-grade inflammation and metabolic syndrome in mice. Gastroenterology. 2014;147(6):1363–77.
42. Gupta SK, Bajwa P, Deb R, Chellappa MM, Dey S. Flagellin a toll-like receptor 5 agonist as an adjuvant in chicken vaccines. Clin Vaccine Immunol. 2014;21(3):261–70.
43. Falah Alshimerry A, Ghalib Farhood R, Hasan Shakir H. Role of Toll-Like Receptor 5 in the Development of Ulcerative Colitis. Arch Razi Inst. 2021;76(6):1639–43.
44. Stanislawowski M, Wierzbicki PM, Golab A, Adrych K, Kartanowicz D, Wypych J, et al. Decreased Toll-like receptor-5 (TLR-5) expression in the mucosa of ulcerative colitis patients. J Physiol Pharmacol. 2009;60(Suppl 4):71–5.
45. Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol. 2008;9(7):769–76.
46. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science (80-). 2010;328(5975):228–31.

