

TOLL-LIKE (TLRS 1,4,5) IMMUNODETECTION OF SOME TOLL-LIKE RECEPTORS AS AN IMMUNE RESPONSE TO PATIENTS WITH ACUTE APPENDICITIS AND ITS EFFECT ON CLINICAL FEATURES IN BASRA/IRAQ**Zainab Nihad shaker**

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Abstract: Background: Acute Appendicitis is the most common cause of emergency cases worldwide, and it may have serious consequences. If there is no precise diagnosis, it poses a dreadful issue for the community. Researchers tried to use several markers to determine the definitive diagnosis of Appendicitis. Our research thus sought to determine the various Toll-like receptor TLR1, TLR4, and TLR5 concentrations in the blood of patients suspected of Appendicitis as a diagnostic predictor. Method: This study conducted a case-control analysis on patients diagnosed with Appendicitis in the operating room at Al-Sadr Teaching Hospital and Al-Shifaa Teaching Hospital in Basra, Iraq. The control group was randomly chosen between July 2023 and November 2023. a total of 88 samples were enrolled in the study. Seventy of these samples came from patients experiencing signs and symptoms of Appendicitis. In contrast, the remaining eighteen samples were collected from apparently healthy controls (AHC) who were identical to the patient's group in terms of age, gender, habitat, and other characteristics but did not experience any appendicitis symptoms. Ethical approval was obtained from the Basra Health Department for data collection. Results: According to the findings of this investigation, the immunological markers TLR1 and TLR4 were statistically significant at a level of ($P \leq 0.05$) between the serum samples of patients and the control groups. the median concentration of (TLR1 and TLR4) were (14.70 ,10.89 ng/ml) respectively than control group (8.15 ,6.98 ng/ml), statistically show highly significant ($P = 0.000$), while the median concentration of TLR5 in appendicitis patients was lower (3.11 ng/ml) than control (3.77 ng/ml), without any significant differences ($p = 0.185$). Conclusion: The serum concentration of TLR1 and TLR4 has the potential to predict the diagnosis of appendicitis.

Keywords: Appendicitis, TLR1, TLR4, TLR5, ELISA, clinical features



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Introduction

Acute Appendicitis (AA) arises from the Inflammation of the appendix [1]. It is the most common reason of emergency cases [2]; emergency appendicectomy 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 Global Burden of Disease Study (GBD) 2019 has characterized Appendicitis as the Inflammation of the appendix, resulting in symptoms such as nausea, vomiting, and intense pain in the lower right abdomen [5].

The typical symptoms of acute Appendicitis start with poorly localized, cramp-like abdominal pain that is initially felt around the belly button. This is followed by loss of appetite and feelings of nausea. As the inflammatory process advances and affects the outer lining of the appendix, the pain becomes localized to the lower right side of the abdomen [6]. While appendectomy for (AA) is a standard surgical procedure, the pathophysiology and natural history of this condition remain unclear [7]. Little is understood about the etiology of acute Appendicitis [8]; Appendicitis may be caused by several factors that result in the blockage of the appendix by a hard mass called an appendicolith. This blockage occurs due to an increase in the production of mucus and the development of germs. Ultimately, this disease results in the accumulation of blood in the veins, increased pressure on the wall of the appendix, and tissue death, which then advances to the formation of pus, Inflammation, and a hole in the appendix. As a result, generalized peritonitis or the formation of an appendiceal mass/abscess may ensue [9]. Furthermore, Appendicitis has the potential to result in septic shock and other grave consequences, such as sepsis and mortality [5].

The immune response is mediated by cells of the immune system, such as phagocyte and antigen-presenting cells (APCs), which express the type of receptor called pattern recognition receptors (PRRs). PRRs bind to pathogen-associated molecular patterns (PAMPs), which are described in pathogenic organisms [10]. Toll-Like Receptors, a family of PRRs that activate the early response to microbial components generate signals that lead to induce adaptive immune responses and act as a vital role in the innate immune response by recognizing PAMPs [11]. Toll-like receptors (TLRs) are found on several types of cells, including both immune and non-immune cells. However, they are particularly abundant in dendritic cells (DC) and macrophages, exhibiting significant changes in expression levels among distinct subsets of these cells [12]. Although the high incidence of the disease there is a scarcity of evidence about the pathophysiological changes that occur; specifically, the innate immune responses involved are mostly unknown (Arlt et al.,2015).

[13] Confirmed that researchers tried to use several markers to determine the definitive diagnosis of Appendicitis. So this study Aims to:

This study aimed to investigate the TLRs (1, 4, 5) using the ELISA technique to acknowledge the Role of TLR in appendicitis cases for early diagnosis.

Methods

Study design

A case-control study was conducted on patients diagnosed with Appendicitis admitted to the operating rooms of Al-Sadr Teaching Hospital and Al-Shifaa Teaching Hospital in Basrah, Iraq. (70 patients) and 18 controls), and the control group (18) randomly between July 2023 to November 2023, inclusive. The ages of the control and patients ranged from (12 to 63) years. The samples of blood

were collected from all patients and controls. Three ml of blood was collected by venipuncture from all patients and controls using gel tubes and placed for 20 minutes at room temperature, then centrifuged to obtain the serum, which was stored in an Eppendorf tube at (-20 C) until used.

Exclusion Criteria:

- All patients who have autoimmune diseases.
- Patients who have an infectious disease.
- All patients who have DM.
- All pregnant women.
- Patients who are suffering from allergic illnesses.

Inclusion criteria:

Patients are showing signs of Appendicitis, which is a medical emergency, and the surgeons at Al-Sadr and Al-Shifa hospitals have given them the all-clear.

Data collection:

The questionnaire documented all patient and control information obtained from participants in the trial. Several Clinical Symptoms and signs (Clinical examination) were considered for inclusion in this study, including: It was separated into important symptomatic categories (Anorexia, Nausea, vomiting, shifting pain) and signs (tenderness, rebound tenderness, fever)

The Kits

The table (1) provides a comprehensive list of all the kits used in the present investigation.

Table (1): The kits used in the study

Item	Description and Company	Country
(Human Toll-like receptor) 1 ELISA kit	Bioassay Tech.Lab. Cat., ELISA catalog No: ELK2734	China
(Human Toll-like receptor) 4 ELISA kit	Bioassay Tech.Lab. Cat., ELISA catalog No: ELK177	China
(Human Toll-like receptor) 5 ELISA kit	Bioassay Tech.Lab. Cat., ELISA catalog No: ELK2736	China

ELISA Procedure:

The titer of TLR1, TLR4, and TLR5 in blood samples of appendicitis patients and control subjects was detected using a Sandwich ELISA kit provided by Bio. Tech. The ELISA kit consists of 96 wells. The operation was performed according to the instructions provided by the kit manufacturer.

Statistical analysis

Statistical analysis in this work was conducted using SPSS (Version 26). An analysis of all variables was conducted using the Mann-Whitney U test procedure. Statistical significance is defined as probability values at or below 0.05 ($p < 0.05$).

Results and Discussion

Clinical study

Clinical features

Table (2) presents the distribution of clinical features among patients. The most frequent symptom was abdominal pain (100%) and the least was Fever (44%)

Table (2): Clinical features of patients

Clinical feature	Frequency	Percent(%)
Abdominal pain	100	100
Nausea	87	87
Anorexia	79	79
Shifting pain	72	72
Tenderness	70	70
Rebound tenderness	70	70
Vomiting	53	53
Fever	44	44

*Patients number =100

Comparison of TLR markers between patient and control groups

The results in Table (3) show that TLR1 and TLR4 were a higher significant in patients with Appendicitis than in control groups. It showed that the median concentration of (TLR1 and TLR4) were (14.70, 10.89 ng/ml) respectively, the control group (8.15 ,6.89 ng/ml). Statistically, these differences were highly significant (P= 0.0001). while in TLR5 the median concentration in appendicitis patients were lower (3.11 ng/ml) than control (3.77 ng/ml), without any significant (p = 0.185).

Table (3): Comparison between the Toll-Like receptor levels in patients and controls

Category		TLR1 (ng/ml)	TLR4 (ng/ml)	TLR5 (ng/ml)
Patient	No.	70	70	70
	Mean	15.49	11.40	3.55
	Median	14.70	10.89	3.11
	SD	6.07	4.55	2.87
	Minimum	6.08	2.68	1.177
	Maximum	29.01	29.90	25.64
Control	No.	18	18	18
	Mean	8.41	7.34	4.16
	Median	8.15	6.98	3.77
	SD	2.98	3.70	2.25
	Minimum	2.87	1.17	1.11
	Maximum	15.56	15.31	9.70

P-value*		0.0001	0.0001	0.185
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*Mann-Whitney U Test

Toll like receptors levels according among patients only to signs and symptoms (Clinical features)

In Table (4) show the TLR's 1,4,5 levels between patients who have nausea (14.69, 10.89, 3.32 ng/ml).and patients who did not have nausea (15.30, 12.63, 2.65ng/ml). There was no a statistical difference of significant in levels of TLRs when compared and categorized according to complaining nausea or not.

Table (4): Comparison of TLR's levels between patients who complained nausea and patients did not

Nausea		TLR1 (ng/ml)	TLR4 (ng/ml)	TLR5 (ng/ml)
No	No.	11	11	11
	Mean	15.47	12.97	2.69
	Median	15.30	12.63	2.65
	SD	4.86	5.87	5.62
	Minimum	9.24	5.67	1.17
	Maximum	24.54	25.03	4.34
Yes	No.	59	59	59
	Mean	15.49	11.11	3.72
	Median	14.69	10.89	3.32
	SD	6.31	4.26	3.09
	Minimum	6.09	2.69	1.36
	Maximum	29.01	29.90	25.64
P-value*		0.778	0.513	0.072

* Mann-Whitney U Test

In Table (5) show the TLR's 1,4,5 concentrations between patients who have vomiting (13.43, 10.05, 3.30 ng/ml) and patients who did not have vomiting (15.84 , 12.63, 2.95 ng/ml). Only TLR4 levels showed significant statistical differences (p=0.02), when compared according to complaining vomiting. It was significantly higher in patients who did not have vomiting.

Table (5): Comparison of TLR's levels between patients who complained vomiting and patients did not

Vomiting		TLR1) ng/ml)	TLR4) ng/ml)	TLR5) ng/ml)
No	No.	31	31	31
	Mean	16.39	12.56	3.88
	Median	15.84	12.63	2.95
	SD	5.78	4.36	4.18
	Minimum	6.71	2.89	1.18
	Maximum	28.97	25.04	25.64
Yes	No.	39	39	39
	Mean	14.78	10.48	3.30
	Median	13.45	10.05	3.30
	SD	6.27	4.55	1.00
	Minimum	6.09	2.69	1.94
	Maximum	29.02	29.90	6.11
P-value*		0.204	0.020	0.754

*Mann-Whitney U Test

In Table (6) show that the TLR's 1,4,5 levels between patients who have anorexia (15.84, 10.91, 3.11 ng/ml) and patients who did not have anorexia (11.71, 10.05, 2.91 ng/ml). Complaining anorexia seems not significantly associated with the level of any of the three TLRs.

Table (6): Comparison of TLR's concentration between patients who complained anorexia and patients did not

Anorexia		TLR1 (ng/ml)	TLR4 (ng/ml)	TLR5 (ng/ml)
No	No.	15	15	15
	Mean	13.58	11.42	4.57
	Median	11.72	10.05	2.92
	SD	6.46	5.39	5.90
	Minimum	6.34	2.89	1.36
	Maximum	28.97	25.04	25.64
Yes	No.	55	55	55
	Mean	16.02	11.39	3.29
	Median	15.84	10.91	3.11
	SD	5.91	4.36	1.07
	Minimum	6.08	2.68	1.17
	Maximum	29.01	29.90	6.11
P-value*		0.104	0.737	0.983

* Mann-Whitney U Test

In Table (7) show that the Toll like receptors 1,4,5 levels between patients who have fever (15.95, 10.36, 3.11 ng/ml) and patients who did not have fever (13.43, 11.61, 3.03 ng/ml). fever was not associated with any significant changes in the receptor's levels.

Table (7): Comparison of TLR's levels between patients who complained fever and patients did not

Fever		TLR1 (ng/ml)	TLR4 (ng/ml)	TLR5 (ng/ml)
No	No.	37	37	37
	Mean	15.45	12.07	3.19
	Median	13.43	11.61	3.034
	SD	6.67	5.22	1.08
	Minimum	6.34	2.89	1.35
	Maximum	29.02	29.90	6.11
Yes	No.	33	33	33
	Mean	15.53	10.65	3.96
	Median	15.95	10.36	3.11
	SD	5.43	3.60	4.01
	Minimum	6.09	2.69	1.17
	Maximum	26.59	19.04	25.64
P-value*		0.634	0.309	0.441

* Mann-Whitney U Test

In Table (8) show that the Toll like receptors 1,4,5 levels between patients who have shifting pain (14.15, 10.45, 3.11 ng/ml) and patients who did not have shifting pain (15.88, 12.68, 3.01 ng/ml). Non-significance also noted when the levels of the TLRs were compared according to the presence of shifting pain.

Table (8): Comparison of TLR's levels between patients who complained shifting pain and patients did not

Shifting pain		TLR1) ng/ml)	TLR4) ng/ml)	TLR5) ng/ml)
No	No.	18	18	18
	Mean	17.59	13.57	4.41
	Median	15.88	12.68	3.01
	SD	6.38	6.55	5.38
	Minimum	9.07	4.69	1.94
	Maximum	28.81	29.90	25.64
Yes	No.	52	52	52
	Mean	14.76	10.65	3.26
	Median	14.15	10.45	3.11
	SD	5.85	3.39	1.086
	Minimum	6.09	2.69	1.18
	Maximum	29.02	18.94	6.11
P-value*		0.122	0.136	1.000

* Mann-Whitney U Test

In Table (9) show that the Toll like receptors 1,4,5 levels between patients who have tenderness (13.43, 10.34, 3.11 ng/ml) and patients who did not have tenderness (11.18, 14.46, 2.40 ng/ml). The presence/absence of tenderness did not significantly affect the levels of TLRs.

Table (9): Comparison of TLR's levels between patients who complained tenderness and patients did not

Tenderness		TLR1) ng/ml)	TLR4) ng/ml)	TLR5) ng/ml)
No	No.	2	2	2
	Mean	11.18	14.46	2.40
	Median	11.18	14.46	2.40
	Minimum	11.184	14.460	2.402
	Maximum	11.184	14.460	2.402
	Yes	No.	68	68
Mean		14.52	10.51	3.74
Median		13.43	10.34700	3.11
SD		6.01	3.88	3.49
Minimum		6.09	2.69	1.18
Maximum		28.97	19.04	25.64
P-value*		0.792	0.243	0.386

*Mann-Whitney U Test

Finally, in Table (10) show that the Toll like receptors 1,4,5 levels between patients who have rebound tenderness (12.77, 10.89, 3.31ng/ml) and patients who did not have rebound tenderness (13.43, 9.99, 2.44 ng/ml), the presence of rebound tenderness, also, was not associated to significant changes in the TLRs levels.

Table (10): Comparison of TLR's levels between patients who complained rebound tenderness and patients did not

Rebound tenderness		TLR1) ng/ml)	TLR4) ng/ml)	TLR5) ng/ml)
No	No.	17	17	17
	Mean	15.11	10.24	2.74
	Median	13.43	9.99	2.44
	SD	5.79	2.82	9.76
	Minimum	9.31	5.67	1.96
	Maximum	28.97	14.46	4.48
Yes	No.	53	53	53
	Mean	14.24	10.70	4.02
	Median	12.77	10.89	3.31
	SD	6.08	4.19	3.91
	Minimum	6.09	2.69	1.18
	Maximum	26.59	19.04	25.64
P-value*		0.545	0.709	0.041

*Mann-Whitney U Test

Discussion

Inflammation in Appendicitis included the activation of multiple immune cells and their complex interaction, which was mediated by a number of pro-inflammatory or anti-inflammatory cytokines predominantly secreted by macrophages and T-lymphocytes [14].

More knowledge is needed on the involvement of the innate immune system, pattern recognition receptors, and cytokines in appendicitis. In the current study, we selected (TLR1, TLR4, and TLR5) to study their association with Appendicitis.

Clinical factors

In the current study, the most clinical symptoms were abdominal pain (100%) and the least were Fever (44. %), Nausea (87%), Anorexia (79%), Shifting pain (72%), Tenderness (70%), Rebound tenderness (70%) and vomiting (53%) from total cases 100(100%). As in table (2)

These results are similar to [15] that The most common clinical presentation was pain in the abdomen and the least common symptom was fever. [16] show that the Pain in the right iliac fossa was (100%), fever (42%). Similarly, generalized pain was also reported in 100% of patients [17].

Immunological studies:

The present study shows the median concentration of TLR1 (14.70 ng/ml) among patients and the mean concentration of control (8.15 ng/ml), which indicates there is statistically significant between patients and control groups ($P= 0.0001$), As in table (3). No similar studies have been done using the ELISA technique on blood serum in appendicitis patients. However, there was another study done by expressed TLRs in the analyzed tissues of inflamed appendices compared with control appendices Arlt et al., (2015), which used real-time PCR analysis of TLR1, mRNA from specimens of acute Appendicitis ($n=24$), normal appendices ($n=12$), and observed that TLR1 were significantly higher in acute appendicitis patients than other who had with non-inflame appendices ($P= 0.035$), The abundance of TLR1 rose from 2484 ± 391 to 5300 ± 545 copies per 20 ng of mRNA. In contrast, the observed abundance of TLR8 increased from 472 ± 133 to 1354 ± 318 copies per 20 ng of mRNA ($P= 0.02$) [18].

Lipoproteins are integral components of the cellular membrane found in almost all living

organisms, and they possess potent stimulating effects on TLR1 [19]. Therefore, the TLR1 could be a sensor of bacterial invasion in the course of appendicitis. Thus, the increased expression of TLR1 may serve as a detector of bacterial infiltration during the progression of appendicitis. Recently, the function of TLR1 as both an activator and a target of Antimicrobial peptides (AMPs) has been revealed [20], Both the small and large intestines include epithelial cells that have toll-like receptors1, These receptors play a key role in controlling the gut microbiota and identifying infections [21].

TLR4 is an exceptional pattern recognition receptor that is part of the innate immune system. This process detects and distinguishes different pathogen-associated molecular patterns originating from bacteria, viruses, and other illnesses [22]. The current investigations observed that the median concentrations of TLR4 (10.89 ng/ml) among patients of Appendicitis and (6.98 ng/ml) among control significantly between patient and control groups P (0.0001). There were no similar studies done by the ELISA technique on blood serum in appendicitis patients, but there was another study done by expressing TLRs in the analyzed tissues of inflamed appendices compared with control appendices Arlt et al., (2015) which used real-time PCR analysis of TLR4, which observed that TLR4 were significantly higher in acute appendicitis patients than other who had with non-inflame appendices ($P=0.01$), TLR4 abundance from 2486 ± 381 to 4694 ± 963 copies/20 ng mRNA. In another study done on rabbits [23], Relative mRNA expression of TLR4 was detected by qPCR (Liu et al. 2017). In the acute phase of Inflammation, there was an increase in the relative expression of TLR4, MYD88, and nuclear NF- κ B. The findings indicate that the TLR4/MYD88/NF- κ B signaling pathway was activated during acute Inflammation. However, it did not play a role in regulating the intestinal immunological function of rabbits.

TLR4 specifically detects lipopolysaccharides derived from gram-negative bacteria [24]; there is a recent suggestion that bacterial lipopolysaccharide (LPS) could have a significant effect in causing Appendicitis by triggering a widespread anti-inflammatory response in the body [25] TLR4 is a transmembrane protein that triggers the signaling cascade responsible for generating an inflammatory response to LPS [26]. Additionally, the toll-like receptor family, mainly TLR4, has been implicated in recognizing (PAMPs) and the subsequent activation of pro-inflammatory signaling cascades [27].

Toll-like receptors are a group of 13 preserved receptors located on the cell membrane. They have a significant impact on controlling the body's natural and learned immune responses to protect against harmful bacteria, fungi, viruses, and parasites [28]. In the current study, the mean concentrations for TLR5 were (3.11 ng/ml) among patients with Appendicitis and (3.77 ng/ml) among control without statistical significance between patient and control groups. No similar studies have been done using the ELISA technique on blood serum in appendicitis patients. However, there was another study done by expressing TLRs in the analyzed tissues of inflamed appendices compared with control appendices Arlt et al., (2015), which used real-time PCR analysis of TLR5, which observed that TLR5 was significantly higher in acute inflamed appendices than other who had with non-inflame appendices ($P<0.05$). And observed the abundance of TLR2 rose from 302 ± 84 copies/20 ng mRNA in normal appendices to 1447 ± 367 copies/20 ng mRNA. Similarly, the abundance of TLR10 doubled from 2483 ± 875 to 7297 ± 2342 copies/20 ng mRNA. However, these changes were not statistically significant ($P>0.05$).

In the current study documented that most appendicitis patients according to Clinical features in TLR concentrations were Statistically non-significant with different P -values. except vomiting in only TLR4, there was significantly higher in patients who did not have vomiting (12.63) ng/ml, than in patients who had vomiting (10.05) ng/ml with ($p=0.02$). As in table (5).

No previous studies on the association between TLR (1,4 and 4) concentration and appendicitis clinical symptoms.

Conclusion

The concentration of toll-like receptors TLR1 and TLR4 increased significantly in the plasma of patients with Appendicitis compared to control groups, while TLR5 was not statistically significant. It's not considered a biomarker predictor for acute appendicitis. Furthermore,. The serum concentration of TLR1 and TLR4 can be a diagnostic tool for identifying Appendicitis. The current study found that Toll like receptor 1,4,5 It does not affect the clinical features (symptoms and signs)of appendicitis. There was no statistical difference or statistical significance between the concentration of these receptors and the clinical symptoms of the disease, but it was noted that TLR4 It was significantly higher in patients who did not have vomiting

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