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Toll-Like Receptor Associated Immunomodulatory Changes in Staphylococcal Urinary Tract Infections in Pregnant Women

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ABSTRACT

Objective: *Urinary tract infection is the most common type of bacterial infection during pregnancy, and not treating it leads to serious complications for the mother and fetus. Then, the percentage of pathogenic causes of urinary tract infections was investigated according to the stage of pregnancy, age and miscarriage. The immune protein Toll-like receptors 4 (TLRs-4) level was also measured in the sera of infected and healthy pregnant women using ELISA technology.* ***Method:*** *The current study included collecting 110 urine and blood samples, 88 samples from pregnant women with urinary tract infections and 22 samples from healthy pregnant women as control samples who visited the Maternity, Gynecology and Children's Hospital in Kirkuk, Iraq during the period from 6/1/2024 to 1/1/2025 with ages ranging from 18–50 years.* ***Results:*** *The results showed that the highest incidence of urinary tract infections was in the third trimester of pregnancy at 50% and in the age group 18–29 years at 60%, and the lowest incidence was in the first trimester of pregnancy at 17% and in the age group 40–50 years at 12%. The number of pregnant women infected with the disease who had miscarriages was higher than those who did not have miscarriages at 78% and 22%, respectively. The bacterial culture results showed that 60 urine samples gave positive growth and five types of staphylococcus. Staphylococcus aureus was prevalent at 42%, while Staphylococcus hominis and Staphylococcus haemolyticus were 5% and 3%, respectively. Chloramphenicol was 100% effective, whereas Ampicillin showed 100% resistance. TLRs-4 levels were higher in infected pregnant women, especially in cases caused by Staphylococcus aureus, with no significant differences.* ***Novelty:*** *The study correlates trimester, age, miscarriage rate, bacterial profile, antibiotic sensitivity, and TLRs-4 levels in pregnant women with urinary tract infections within a single clinical investigation.*

INTRODUCTION

Urinary tract infections are widespread among individuals of all ages. However, women are more susceptible to infection than men for several reasons, including the short urethra, the proximity of the urinary opening to the anal and genital areas, and pregnancy, during which physiological changes occur that lead to urinary tract infections [1]. This disease is the most common during pregnancy and comes second after anemia in pregnant women. Infections in pregnant women include urethritis and cystitis, which are the most common during pregnancy and may increase the risk of infection of other parts of the urinary tract, especially in the case of asymptomatic inflammation, which is common in pregnant women [2]. It may develop into acute nephritis if left untreated, which increases the risk of multiple complications for the mother and fetus [3].

Many Gram-positive and Gram-negative bacteria cause urinary tract infections [4], and the predominant Gram-positive bacteria are staphylococci, including Staphylococcus

aureus and Coagulase-Negative Staphylococci (CoNS) [5]. Staphylococcus aureus is a human pathogen that causes urinary tract infections in approximately 1-3% of individuals. This variation in prevalence may be due to the detection methods used in the laboratory and the geographic areas in which the samples were taken [6]. This species is spherical and often arranged in irregular clusters resembling a grape cluster or single or short chains. It is non-motile, non-spore-forming, and non-capsular. It is catalase-positive and oxidase-negative. Its cells are facultative anaerobes that grow readily on many culture media, the most important of which is mannitol saline because of its ability to tolerate high salinity [7].

Antibiotics have contributed and continue to contribute to the treatment of urinary tract infections. However, the effectiveness of these antibiotics has begun to be affected by the increasing ability of bacteria to resist antibiotics in various ways, making treatment useless, as this resistance appears significantly with the random increase in the use of antibiotics [8].

The body's immune defenses play a fundamental role in protecting the human body from diseases, as they provide indicators of inflammation. One of the most important effects is the secretion of cytokines, which are produced in response to the antigen. Cytokines play a pivotal role in regulating the immune response by controlling the activities and functions of cells involved in the immune response [9].

Tol-like receptors (TLRs) are important innate immune receptors that significantly protect the human body from diseases. TLRs recognize different structures of pathogens and stimulate the innate immune response [10].

RESEARCH METHOD

Sample collection

The current study collected 110 urine and blood samples from pregnant women aged 18-50 from the Maternity, Gynecology and Children's Hospital in Kirkuk Governorate from 6/1/2024 to 1/1/2025. The samples included 88 urine and blood samples from pregnant women who were proven to have urinary tract infections through a general urine examination that included observing pus cells and bacteria in the urine and 22 blood and urine samples from healthy pregnant women as a control group after ensuring that they had undergone a clinical and laboratory examination.

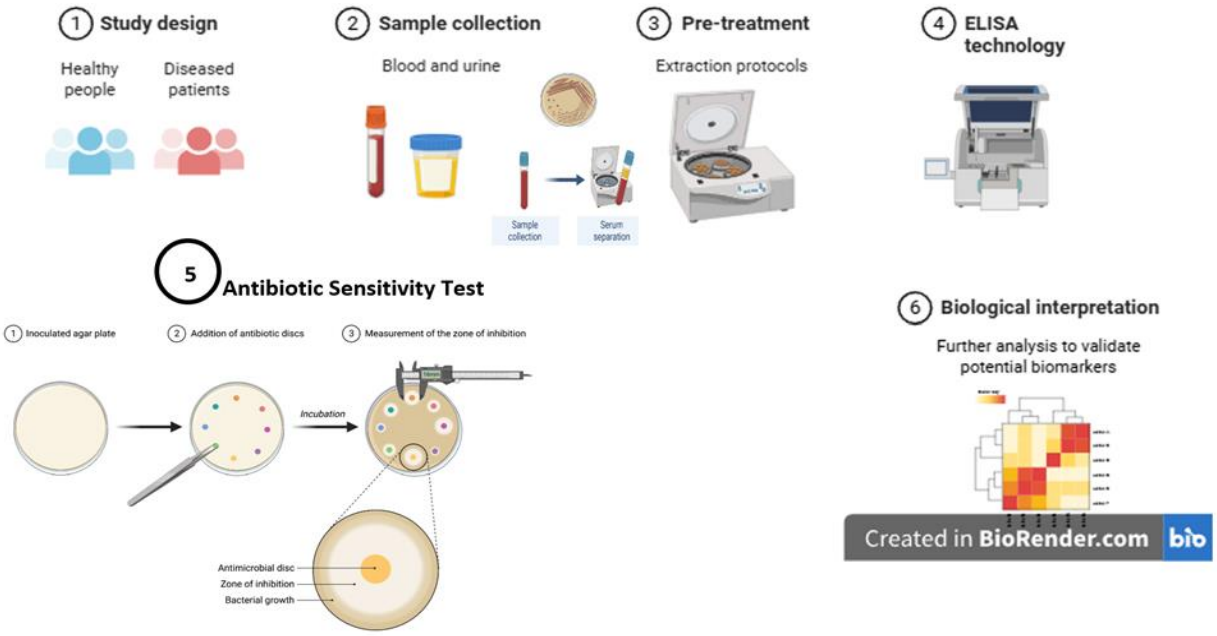

Figure1. Urine and blood samples were collected.

Immunological assays

Enzyme-linked immunosorbent Assay (ELISA) was used to estimate the level of TLR4 immune protein in the serum of the studied samples. The test was performed using a ready-made kit manufactured by Sunlong. The principle of this test is based on adding the studied samples to the holes in the microtiter plate, which was previously covered with monoclonal antibodies of the studied antigen TLR4. After incubation, the plate is washed to remove the unbound proteins in the studied sample. A solution for the detection antibody linked to the enzyme is added. The microtiter plate is washed to get rid of the unbound conjugated antibodies. The base solution is then added, and the linked enzyme reacts with the base molecules, forming a colour complex whose colour intensity is directly proportional to the concentration of the studied antigen**.**

The test was conducted as follows based on the manufacturer's instructions:

1. All samples, materials and reagents were left at room temperature for half an hour before use.
2. Standard concentrations were prepared by performing a series of dilutions in 5 sterile tubes to obtain TLR4 concentrations (300- 600- 1200- 2400- 3600) as shown in Figure (2).

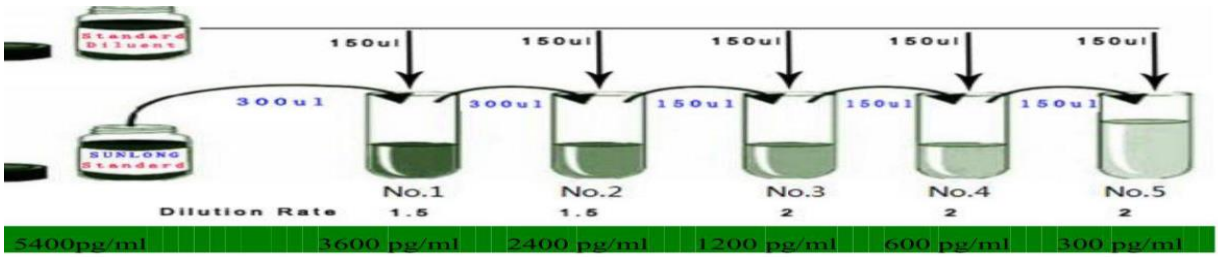

Figure 2. Dilution series of the standard solution of TLR4.

Then, using a pipette, 50 microliters of the diluted standard solution tubes are transferred to the first five holes on the special plate (one hole is used for each tube).

The blank well was left untouched according to the manufacturer's instructions, and 10 µl of serum samples and 40 µl of sample dilution solution were added to the remaining wells and mixed gently. The plate was covered with a cling film and incubated for 30 min at 37 °C. The concentrated wash solution was diluted with distilled water (30 times). The cover was carefully removed from the plate, and the plate's wells were washed with the wash solution. The process was repeated 5 times, and the plate was inverted onto blotting paper to remove the remaining solution. 50 µl of HRP-Conjigate enzyme conjugate solution was added to all plate wells except the blank control well. The cling film was replaced, and the plate was incubated as in step 4. The wells of the plate were washed as in step 6. 50 µl of staining solution A and 50 µl of solution B were added away from direct light to all wells and mixed gently. Then, the plate was incubated for 15 minutes at 37°C. After that, 50 microliters of the stop solution were added to all the wells to terminate the reaction. The absorbance O.D. of the samples in the wells of the calibration plate was read using an ELISA device at a wavelength of 450 nm.

Ethical considerations

The study adhered to the ethical norms for human testing outlined in the Declaration of Helsinki, received prior clearance from the University of Tikrit Board, and was sanctioned by the Research Ethics Committee of the Ministries of Environment, Health, Higher Education, and Scientific Research in Iraq, according to the provisions of the Health Insurance Portability and Accountability Act. The Data Safety Monitoring Board conducted frequent reviews, including sample collection, per the permission of the University of Tikrit and Kirkuk Board and the rules of the National Institutes of Health.

RESULTS AND DISCUSSION

Results

The results of the bacterial culture of 88 urine samples on blood agar and mannitol salt agar collected from pregnant women with urinary tract infections aged 18-50 years show that 60 samples (68%) showed bacterial growth, while 28 samples (32%) did not, as shown in Table (1).

Table 1. Number of isolated samples and their percentages.

Total number of samples	Positive growth	Negative growth
88	60(68%)	28(32)

Distribution of urinary tract infections by stage of pregnancy and age

The results of our study, as shown in Table (2), showed that the highest incidence of urinary tract infection was in the third trimester of pregnancy, at 50%, followed by the second trimester, at 33%, and then the first trimester, at 17%.

Table 2. Shows the number of pregnant women with urinary tract infections by stage of pregnancy and age group.

Age	trimester			Total
	First	twice	Third	
29-18	7	12	17	36(60%)
39-30	2	5	10	17(28%)
50-40	1	3	3	7(12%)
Total	10(17%)	20(33%)	30(50%)	60(100%)

Distribution of UTI According to Abortion

The results showed that the highest incidence of urinary tract infections was among pregnant women who had a miscarriage, reaching 78%. In comparison, the incidence rate was 22% among pregnant women who did not have a miscarriage, as shown in Table (3). This result was consistent with the study conducted by the researcher [11], where the incidence rate of UTI among pregnant women who had previous miscarriages was higher than the incidence rate among pregnant women who did not have a miscarriage, reaching 96.53% and 3.47%, respectively.

Table 3. Shows the numbers and percentages of pregnant women with urinary tract infections according to Abortion.

Abortion	Number (percentage)	Total
Yes	(%78) 47	60(100%)
No	(%22) 13	

Diagnosis of isolated bacteria

The bacterial isolates isolated from the urine of pregnant women were initially diagnosed by observing the morphological characteristics of the bacterial colonies growing on the culture media used and the microscopic characteristics after staining with Gram stain. The results showed that all isolates could stain with crystal violets, which our study agreed with [12], as Staphylococcus species are the predominant causative agent of urinary tract infection during pregnancy. Then, all isolates were subjected to biochemical diagnosis and the interpretation of the diagnostic results was based on comparing them with [13]. The diagnosis was confirmed using the Vitec device kit.

Diagnosis of gram-positive bacteria

Table 4. Biochemical tests for Gram-positive bacteria.

Bacterial species	Tests						
	Gram Stain	Catalase	Mannitol	Coagulase	Novobiocin	Oxidase	Hemolysin
Staphylococcus aureus	+	+	+	+	-	-	+
Staphylococcus epidermids	+	+	-	-	-	-	V
Staphylococcus saprophyticus	+	+	-	-	+	-	-
Staphylococcus haemolyticus	+	+	-	-	-	-	+

Staphylococcus hominis	+	+	-	-	-	-	V

V: variable, + positive, - negative

A microscopic examination of staphylococci showed they were positive cocci, mono, di, or clusters. This test is considered one of the important tests in diagnosing staphylococci. They were also characterized by being positive for the catalase test. This test served to distinguish them from Streptococcus. It was found that some Staphylococcus aureus colonies were golden yellow, hemolytic, of the beta type on blood agar and mannitol fermenter on mannitol medium, which changed the colour of the medium from pink to yellow. Other isolated staphylococci were not mannitol fermenters and gave a positive result for the coagulase test, while the other isolates gave a negative result. Staphylococcus saprophyticus did not break down red blood cells on blood agar or respond to the antibiotic novobiocin. This test is considered one of the important diagnostic characteristics of this bacterium, differentiating it from the rest of the isolated staphylococci that are sensitive to this antibiotic.

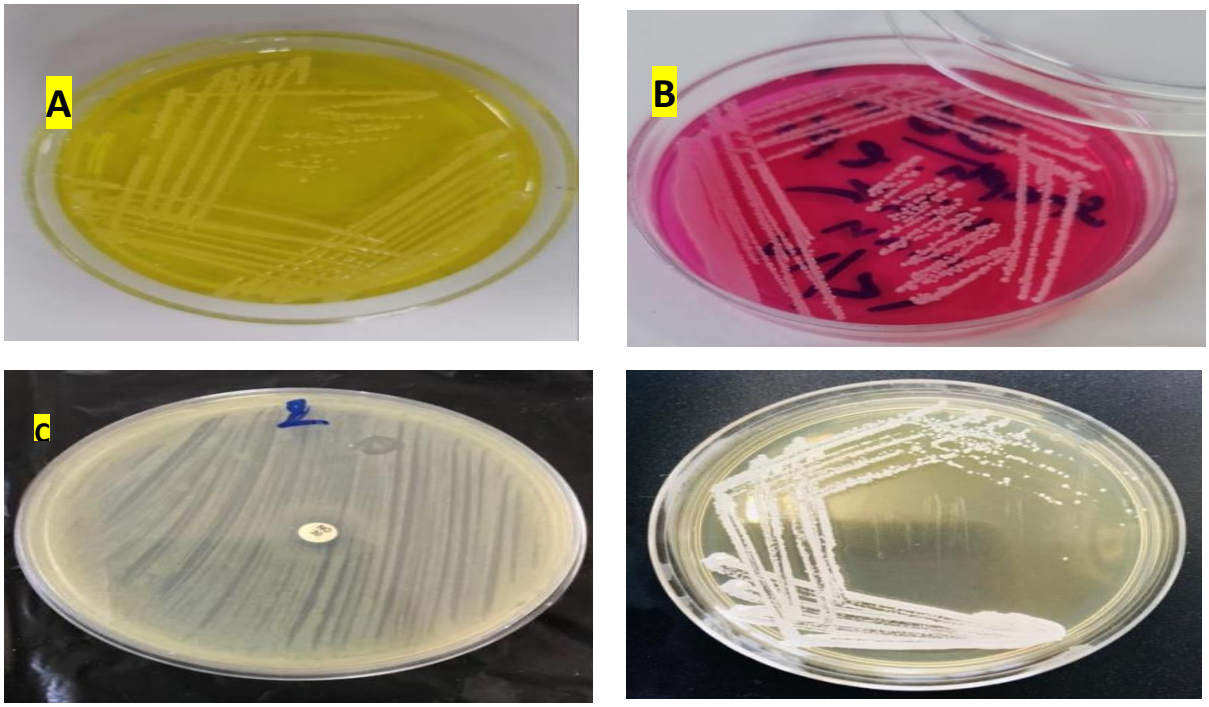

Figure 3. Shows the characteristics of the types of the genus Staphylococcus.
A) *Staphylococcus aureus* on mannitol medium ferments to sugar
B) *Staphylococcus epidermidis* on mannitol medium
C) *Staphylococcus saprophyticus* resistance to novobiocin
D) *Staphylococcus aureus* on chromogenic agar

Number of isolated bacterial species and their percentages

The current study included isolating 60 samples of urinary tract infections in pregnant women. The results showed five types of the genus Staphylococcus in varying proportions, as shown in Table 5.

Table 5. Shows the number of isolated species and their percentages.

Bacteria	Number of isolates	Percentage %
Staphylococcus aureus	25	42 %
Staphylococcus epidermidis	20	33 %
Staphylococcus saprophyticus	10	17 %
Staphylococcus homolyticus	3	5 %
Staphylococcus haemolyticus	2	3 %
Total	60	100 %

Antibiotic sensitivity testing

Three antibiotics that are commonly used to treat urinary tract infections were used to test the sensitivity of 88 bacterial isolates. This was done by spreading disks on Mueller-Hinton agar to see how the antibiotics affected the isolated bacteria. As stated in [14], Table (6) shows the classification of the isolates as sensitive, moderately sensitive, or resistant.

Table 6. Shows the resistance and sensitivity of isolated bacteria to antibiotics.

Bacterial species (number)	Type of antidote								
	Chloramphenicol(C)			Ciprofloxacin (CIP)			Ampicillin (AMP)		
	S%	I%	R%	S%	I%	R%	S%	I%	R%
Staphylococcus aureus (25)	90	0	10	80	0	20	0	0	100
Staphylococcus epidermidis (20)	100	0	0	88	0	12	0	0	100
Staphylococcus saprophyticus (10)	100	0	0	100	0	0	0	0	100
Staphylococcus homolyticus (3)	100	0	0	64.8	0	35.2	0	0	100
Staphylococcus haemolyticus (2)	100	0	0	100	0	0	0	0	100

S: Sensitive, I: Intermediate, R: Resistant

It is clear from the above table that all Staphylococcus isolates showed 100% absolute resistance to ampicillin and sensitivity to chloramphenicol, while the isolates were moderately resistant to ciprofloxacin.

The results indicated that Staphylococcus aureus isolates were completely resistant to ampicillin, but they were 90% sensitive to chloramphenicol and 80% moderately sensitive to ciprofloxacin. This conclusion differed from what we found in [15], where the cocci were only 14.29% sensitive to ampicillin. Staphylococcus epidermidis isolates had complete resistance to ampicillin, absolute sensitivity to chloramphenicol, and high sensitivity to ciprofloxacin (88%). The strains of Staphylococcus saprophyticus were also completely resistant to ampicillin but sensitive to chloramphenicol (C) and ciprofloxacin (CIP).

Isolates of Staphylococcus homolyticus were completely resistant to ampicillin and only slightly sensitive to ciprofloxacin (CIP) at a rate of 64.8%. Isolates of Staphylococcus haemolyticus werecompletely sensitive to ciprofloxacin (CIP) and chloramphenicol (C), but not at all to ampicillin (AMP) .Figure (4) shows the susceptibility of Staphylococcus

aureus to antibiotic resistance, as resistance occurs as a result of mutation or modification of antibiotic targets, inhibition of beta-lactamase inhibitors, decreased membrane permeability, or increased activity of efflux pumps.

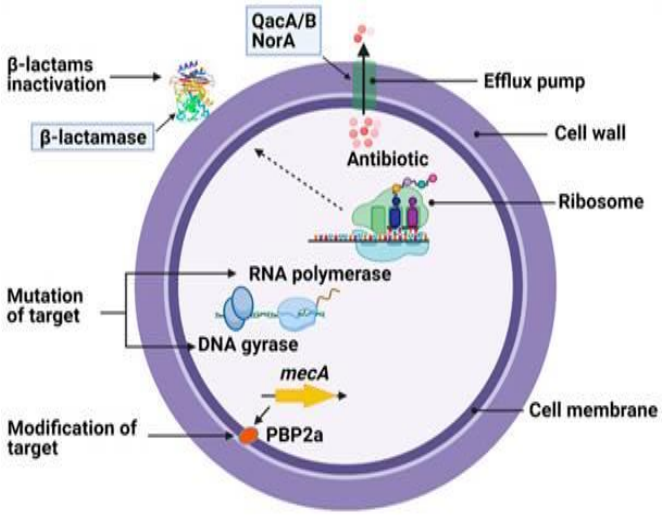

Figure 4. Molecular mechanisms of antibiotic resistance in *Staphylococcus aureus*. Antimicrobial resistance occurs due to the mutation or modification of antibiotic targets, inactivation of β-lactam antibiotics by β-lactamase, a reduction in membrane permeability, or increased activity of efflux pumps (16).

Measurement of TLR-4 concentration

According to the current results, there was more TLR-4 in the sera of pregnant women with urinary tract infections than in the sera of healthy pregnant women. The levels were 465.25 ng/ml for the healthy women and 212.16 ng/ml for the sick women. These differences were significant ($P < 0.001$), as shown in Figure 5. A study by [17] agreed with ours in that it found a significant rise in the level of TLR-4 in pregnant women with urinary tract infections. There were also giant differences between the healthy control group and the patients. For example, TLR-4 levels in the patient's serum reached 486.38 ng/ml, while levels in the healthy control group's serum were only 219.35 ng/ml .The figure (6) shows the standard trend of TLR4 protein and the figure(7) shows the difference between the control groups and the groups of pregnant women with urinary tract infections.

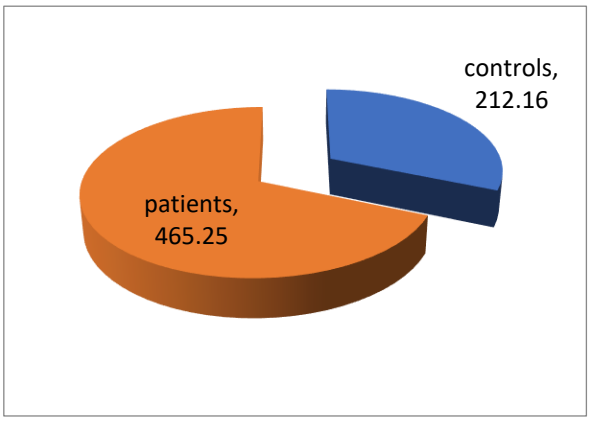

Figure 5. The average value of TLR-4 in patients and controls.

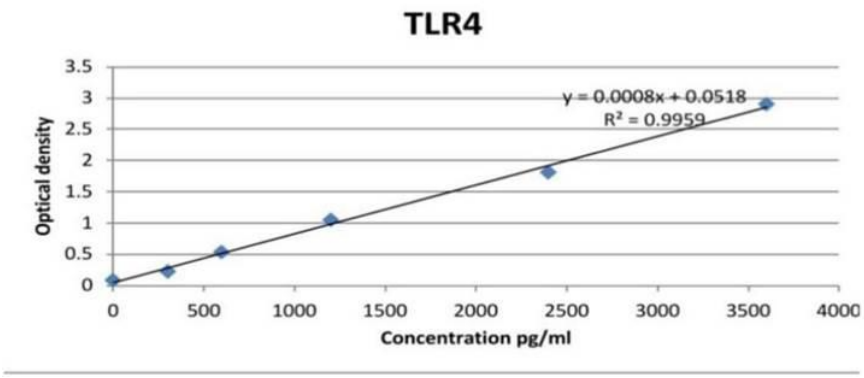

Figure 6. Standard Curve of Immune Protein TLR4.

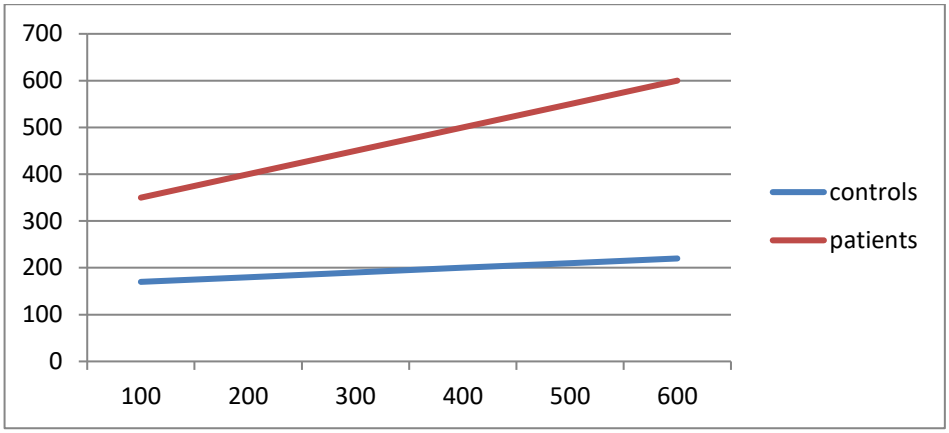

Figure 7. TLR-4 expression in the context of differentiation between patients with urinary tract infections and the control group.

Discussion

Even though pus cells are present, there is no bacterial growth. This is because the cause could be a fungal infection, a parasite, a virus, or anaerobic bacteria. These bacteria are difficult to separate from aerobic bacteria because they need different media and conditions to grow [18]. Our results differed from the results of [19], as the percentage of pregnant women with urinary tract infections reached 8.9%. The difference in the rates of infection with this disease may be due to the study's differences in terms of geographical location, number of samples, isolation methods, or age of patients.

Urinary tract infections are more common during pregnancy because more proteins and sugars are in the urine, making it easier for bacteria to grow and cause infections. The urinary tract also changes physiologically, anatomically, and functionally during pregnancy. The bacterial culture of 22 urine samples from healthy pregnant women aged 18 to 50 on blood agar and mannitol did not show any bacterial growth, meaning there was no urinary tract infection. The highest incidence of urinary tract infections in the third trimester of pregnancy may be caused by increased pressure on the urinary tract from the growing fetus. During the third trimester of pregnancy, the uterine pressure on the ureter and bladder, along with the relaxation of smooth muscles due to pregnancy hormones, may make it hard to urinate and leave the bladder partially empty. These conditions can cause urine to pool, which raises the risk of infection and the growth of bacteria [20].

The results of our study are consistent with the study conducted by the researcher [21], where the incidence of UTI among pregnant women who had a miscarriage was higher than the incidence among pregnant women who did not have a miscarriage, as the rates reached 96.53% and 3.47%, respectively.

Having many miscarriages during pregnancy can make a woman less resistant to getting urinary tract infections. This is because the stress of labor causes blood loss, which causes the lining of the uterus to expand. This process increases the uterus's surface area, making it easier for bacteria to stick and cause an infection. These microorganisms will move upward to the urethra, bladder, and kidneys and may cause cystitis or pyelonephritis [22]. Our results showed that *Staphylococcus aureus* was dominant over other staphylococci causing urinary tract infections in pregnant women, reaching 42%. This result is close to the study of [23]where the isolation rate was 55.9%, and our study contradicts the study of(17), where the percentage reached 14.2%. As for the coagulase-negative staphylococci such as *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus homolyticus*, and *Staphylococcus haemolyticus*, they were isolated in our study at rates of 33%, 17%, 5%, and 3%, respectively. As for the isolation rate of *Staphylococcus epidermidis*, it contradicts the study of [24], where the isolation rate was 11.7%, Staphylococcus aureus is the most common type of staphylococci that causes urinary tract infections. This is because it has many virulence factors, such as a capsule and a sticky layer, that make it resistant to the body's defenses, antibiotics, and disinfectants and make it easier for the bacteria to stick to foreign bodies. Because isolates have multiple resistances, they are not susceptible to antibiotics. This is one of the most important and serious issues in treating infections caused by this bacterium, and it is very difficult to find the proper treatment [25].

The results of our study were consistent with the study of [17], which showed a significant increase in the level of TLR-4 in pregnant women with urinary tract infections, and recorded highly significant differences in the concentration of TLR-4 in the serum of patients compared to the healthy control group, reaching 486.38 ng/ml and 219.35 ng/ml, respectively.

Many studies around the world have shown that pregnant women with urinary tract infections have a high level of TLR-4 in their blood. This is because TLR-4 is important in infectious diseases that affect the urinary system, and TLR-4 receptors are the best molecules for protecting the urinary system against pathogens [26]. In the same way, other studies have shown that measuring TLR-4 levels in the blood can help find urinary tract infections in people who are getting a kidney transplant. More specifically, measuring TLR-4 levels in the urine can help doctors determine which patients are most likely to get these infections so they can give them the right antibiotics to stop them [27].

The current study found that pregnant women with urinary tract infections caused by Staphylococcus aureus had the highest level of TLR-4. There was no significant difference between the groups of bacteria. This conclusion is consistent with the study [28], which found that TLR-4 can recognize and interact with many bacterial components, such as lipopeptides, peptidoglycans, and lipoteichoic acid in gram-positive bacteria and lipoproteins in Mycoplasma and Mycobacteria [29].

Despite substantial barriers made by urothelial cells in the human urinary tract, sometimes the uropathogenic microorganisms successfully enter the urinary tract. When

uropathogenic microorganisms enter the urinary tract, the body's first line of defense is triggered, causing the urothelial cells in the bladder (cystitis) and kidneys (nephritis) to activate specific TLRs. This activation of related TLRs triggers a chain of events that includes the release of chemokines, interferons, interleukins, antimicrobial substances, and proinflammatory cytokines[30].

CONCLUSION

Fundamental Finding : The study identified *Staphylococcus aureus* as the most prevalent bacterium among pregnant women with urinary tract infections, determined chloramphenicol as the most effective antibiotic among those tested, found that infection rates were highest in the third trimester particularly among women aged 18–29 and those with a history of miscarriage, and revealed elevated serum TLR-4 levels in infected pregnant women compared to the control group. **Implication :** These findings suggest the importance of targeted screening during the third trimester and among high-risk groups, support the clinical consideration of chloramphenicol sensitivity patterns in treatment planning, and highlight TLR-4 as a potential immunological marker associated with infection in pregnancy. **Limitation :** The study was limited to the antibiotics tested, the specific population examined, and did not explore broader microbial diversity or longitudinal immune responses across pregnancy stages. **Future Research :** Further studies should investigate larger and more diverse populations, evaluate additional antibiotic resistance patterns, and explore the mechanistic role of TLR-4 in the pathophysiology of urinary tract infections during pregnancy.

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