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Evalution The Levels of some Immunological Markers in Patients with Urinary Tract Infection Infected with Gram Positive and Gram Negative Bacteria and Association with Levels of Zinc and Vitamin D

A Thesis

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بسم الله الرحمن الرحيم

الْحَمْدُ لِلّهِ الَّذِي هَدَإنّا لِهَذَا وَمَا كُنَّا لِنَهْتَدِيَ لَوْلا إنّ هَدَإنّا اللّهُ

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Dedication

This work is reverently dedicated to:

Imam of the time (may God Almighty hasten his appearance),

Imam Hussein (peace be upon him), Who invited me to his side,

Every lover of knowledge,

The soul of my dear father, (may God Almighty show mercy on him)

My loving mother, who prays for me all the time,

My dear sisters, (Wafa, Alaa, Ruqaya, Ghufran),

My virtuous husband, Thaer, and the two children of my life, Maryam and Hassan,

My esteemed supporters: my dear Seham, Enas, Zahraa, Sakina, and the engineers, Sajjad Muyaser and Ali Emad

I hope God

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Abstract

Urinary tract infection (UTI) is a common disease in Iraq and the world, which has serious health effects. Many studies around the world have covered this disease from various aspects related to its causes, immune factors (natural and acquired immunity), and effective treatments .The current study attempts to focus on the immune aspect of infection by studying some factors of innate and adaptive immunity and its relationship to zinc and Vit. D3.

The methods used in this research include drawing venous blood from the patients and controls for the purpose of separating the sera. Then, the immune factors were Immunoglobulin G (IgG), Immunoglobulin M IgM, Toll-like receptor 2 (TLR2) and Toll-like receptor (TLR4) were measured by ELISA. Vitamin D3 and zinc were also measured.

General urine examination of patients and controls along with urine culture was performed on the same day as the venous blood sample was taken. The research design of the current study involved the collection of 80 urine and blood serum samples. Of these, 60 samples were taken from patients who visited Imam Hassan Al-Mujtaba Hospital and Imam Hussein Medical Educational City / Holy Karbala between December 2022 and April 2023 with complaints of urinary tract infections, and 20 samples were taken from healthy individuals during the same period.

A questionnaire specifically designed for the present study and it was used to collect all data related to patients and controls, including age, sex, congenital anomalies, diabetes, autoimmune diseases, pregnancy, elderly, etc. The sex was distributed to 50% females and 50% males. The ages of the participants ranged from 18 to 50 years, where patients and controls groups were divided into age subgroups. 35 (58.3%) of patients were diagnosed with recurrent urinary tract infections while

25 (41.6%) were diagnosed with non-recurrent urinary tract infections. It was found that most of the patients had a significant difference in the mean level of Pus Cells, RBCs, Epithelial Cells compared to the healthy controls.

The Results showed an increased IgG antibodies in UTI patients compared to the control group, with a significant increase in serum IgG (17.68 ng/ml) compared to the average amount of IgG (10.57 ng/ml) in controls.

However, there was a decreased level of IgM antibodies in UTI patients compared to the control group, This decrease was not significant. IgM was (153.7 ng/ml) in patients and (366.8 ng/ml) the controls group. The results of TLRs (2 and 4) showed that the serum level of TLR2 in patients' group increased significantly compared to controls group. The mean level of TLR2 in patients' group was 0.23ng/mL while in the control group was 0.16ng/mL.

Non-significant increase was noticed in the serum level of TLR4 in patients' group compared to healthy controls group. It was 215.6 pg/mL in patients' group and 187.6 pg/mL in the group of controls. Statistical analysis showed no differences between UTI patients and healthy subjects in vitamin D3 levels, but healthy subjects had a higher average preference. UTI patients had lower blood zinc levels than those of controls group.

The infection rates for each type of bacteria were also examined. *E. coli* had the highest percentage, followed by *Staphylococcus epidermidis* (21.66%), *Pseudomonas aeruginosa* (16.66%), *Klebsiella pneumoniae* (5%), and *Staphylococcus aureus* (1.66%). The study also measured the levels of TLR2 and TLR4, based on the types of bacteria, including Gram-negative and Gram-positive. The mean levels of TLR2 in patients with Gram-negative pathogens were lower than

their levels with Gram-positive pathogens, while TLR4 in patients with Gram-positive pathogens was lower than in Gram-negative groups.

In both recurrent and non-recurrent groups, there was a significant difference in the mean levels of IgG compared to the controls group. The mean of IgM was in lowest levels in recurrent infections; however, it was non-significant. The mean level of IgM in recurrent infections, non-recurrent infections and controls were 77.68 ng/ml, 260.1 ng/ml, and 238.4 ng/ml, respectively.

Serum levels of TLR2 were increased in non-recurrent infections, with a significant increase compared to the recurrent infection group and controls group. Serum levels of TLR4 were slightly increased in non-recurrent infections, with no significant differences found among all groups. For vit. D3 and zinc there were no significant differences appear when the three groups of recurrent, non- recurrent, and controls compared among each other. The Spearman rank test analysis was used to show the response relationship among all immune markers (IgG, IgM, TLR2, and TLR4) and among vit. D3 and zinc in UTI patients. One significant correlation was found, and it was between TLR2 and D3.

In conclusion, IgG and TLR2 were significantly high, while IgM was insignificantly decreased, That may indicate to the importance of these factors in UTI pathogenicity and treatment. In general IgG, and TLR2 were dropped significantly in recurrent infections compared to non-recurrent infections and that could be because these factors are consumed during the recurrent infections.

The low levels of vit. D_3 and Zn in patients may have a negative impact on the immune system in general and may have a role in increasing the UTIs. The correlation between D_3 and zinc and UTI still needs much investigation to determine exactly when these two factors can have significant effect on the developing UTI and what other factors that can alter the results.

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List of Abbreviations

Abbreviation	Description
1,25(OH)2D3	circulatory vitamin D3
19-kD	synthetic 19-kD lipopeptide
25(OH)D	25-hydroxyvitamin D
C3	Complement 3
Ca	Calcium
CAMP gene	Human cathelicidin antimicrobial peptide
CAUTI	catheter-associated urinary tract infections
CRP	C-reactive protein
Cu	Copper
CYP27B1 gene	Gene that activate 25-hydroxyvitamin D-1a-hydroxylase
E coli	Escherichia coli
E. faecalis	Enterococcus faecalis
ESBL	extended-spectrum beta-lactamases
HSPs	Heat Shock Proteins
ICAM-1	Intercellular Adhesion Molecule-1
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
IL	interleukins
K. pneumoniae	Klebsiella pneumoniae
LL-37	The cathelicdin anti-microbial peptide
LPS	Lipo-poly saccharide
NF-κB	Nuclear Factor kappa B
NK-cells	Natural killer cells
PAMP	Pathogene-Associated Molecular Patterns
P. aeruginosa	Pseudomonas aeruginosa
PCT	Prolactinin
PRRs	pattern recognition receptors
S. aureus	Staphylococcus. Aureus

S. saprophyticus	Staphylococcus saprophyticus
SAA	Amyloid protein A
TIR	Toll/Interleukin—1 receptor-like
TLR11	toll-like receptor11
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TLR5	toll-like receptor5
TLRs	toll-like receptors
TNF-a	Tumor Necrosis Factor alpha
UPEC	Uropathogenic Escherichia coli
UTIs	Urinary tract infections
VDR	vitamin D receptor

Chapter One

Introduction

1-1 INTRODUCTION

With a frequency ranging from 1.8% to 7.5%, urinary tract infections (UTIs), which include pyelonephritis and cystitis, are a frequent bacterial illness in children of all ages (Zorc *et al.*, 2005). Urinary tract infections are the most common bacterial infections, affecting 150 million people each year worldwide (Stamm & Norrby, 2001).

Escherichia coli is the primary cause (70–90%), with other common pathogenic bacteria including *Klebsiella*, *Proteus*, *Enterococcus*, and *Enterobacter* species all contributing to the illness (Mattoo *et al.*, 2021). Renal scarring and possibly serious renal damage can result from UTIs (Shaikh *et al.*, 2007).

Additionally, a variety of diseases are responsible for it, and it is regarded as a serious public health issue, Uropathogens' rising antibiotic resistance and high recurrence rates pose a serious threat to the economic burden of these illnesses (Flores-Mireles *et al.*, 2015).

In elderly men and women of all ages, UTIs are a major source of morbidity repeated recurrences, pyelonephritis with sepsis, renal impairment in young patients, and consequences from repeated use of antibiotics, such as *Clostridium difficile* colitis and high-level antibiotic resistance, are among the worst sequelae, UTIs are classified as either simple or complex clinically, People who are generally healthy and do not have any anatomical or neurological urinary system abnormalities are usually the ones who have simple UTIs (Hooton, 2012).

Due to the importance of urinary tract infections, as they are among the most common types of bacterial infections, they are economically costly to an individual's health care, Hypotheses were found linking the recurrence of urinary tract infections and host immunity, especially to immunoglobulin IgG and IgM, comparing the level of complement component C3. IgG and IgM are highly important in the control, whether local or systemic, of urinary tract infections (Barwary & Ahmed, 2017).

Immune globulin type IgG is a glycoprotein molecule that has a fundamental effect on human immunity, especially humoral immunity, as its representation of the total immune globulin is seventy-five percent in the plasma of healthy people (Leusen & Nimmerjahn, 2013). In the urinary system, for example, in order to protect itself from the colonization of infectious bacteria, the bladder contains basic passive defenses, such as mucus, as well as IgG, one of the immune globulins (Lacerda Mariano & Ingersoll, 2020).

It has been observed that IgM increases with urinary tract infections when the immune response leads to a humoral increase in immunoglobulins, including IgM, this occurred due to immune resistance to urinary tract infection in preventing the adhesion of pathogenic bacteria to urinary epithelial tissue cells (Rajab *et al.*, 2014).

What IgM does in destroying programmed organisms and maintaining immune balance when controlling inflammation, especially in autoimmunity, is one of the most important roles of secreted or serum IgM as a defender against microbes (Lucuab-Fegurgur & Gupta, 2019).

The discovery of toll-like receptors (TLRs) is crucial for immunology research and was awarded the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffman

and Bruce Beutler, who pioneered the field of innate immunity and played a central role in this research (O'neill *et al.*, 2013).

Toll-like receptor 2 (TLR2), TLR4, TLR5, and TLR11 are just a few of the many pattern recognition receptors (PRRs) expressed by the diverse resident and recruited cells that make up the urinary tract's innate immune system, These cells facilitate early pathogen recognition and transduce this signal to trigger a swift and powerful pro-inflammatory immune response (Abraham & Miao, 2015). TLRs are crucial in innate immune system responses, facilitating pathogen elimination and developing pathogen-specific adaptive immunity, mediated by B and T cells (Kumar *et al.*, 2009).

There are many roles TLRs have, TLR-mediated signaling pathways through genetic and biochemical methods, but mechanisms of signal transduction, regulation, and integration remain unknown, TLR-mediated antimicrobial defense and at last TLR-mediated control of adaptive immunity (Medzhitov, 2007).

TLR2 may identify a wide range of microbiological elements, These include peptidoglycan, lipoteichoic acid representing Gram-positive bacteria, lipoproteins and lipopeptides from different pathogens, the number that acyl chains that compose the lipid A component of these LPS is structurally different from the typical LPS that Gram-negative bacteria identified by TLR4, which likely allows for differential detection (Takeda & Akira, 2005).

The concept of zinc deficiency was known 40 years ago in the Middle East, People there suffered from a severe deficiency when most of them died from it at the age of about twenty-five (Prasad, 2008). One micronutrient that might raise the risk of infectious illnesses is zinc deficiency (Sampaio *et al.*, 2013). The host immune system is regulated by zinc, and immune system malfunction results from

even a little zinc shortage, T lymphocyte growth and function depend on zinc, and low zinc levels result in reduced cellular immunity (Yousefi *et al.*, 2010).

Vitamin D has long been recognized for its antibacterial qualities and plays a significant role in controlling inflammation and the generation of chemokines (Zendehdel & Roham, 2019).

Immune cells including B and T lymphocytes, monocytes, and dendritic cells all have high expression of vitamin D receptors, which allow the nutrient to have immunomodulatory effects, macrophages are directly impacted by vitamin D circulation, which increases their capacity for oxidative processes such as cytokine synthesis and generation, phosphatase, and hydrogen peroxide (Zendehdel & Arefi, 2019). Additionally, vitamin D increases phagocytic activity and neutrophil motility (Maleki-Sadeghi *et al.*, 2022 and Aghsaeifard *et al.*, 2022).

1-2 Aim of The Study

The current research aims to investigate the relationship among the levels of IgG, IgM, TLR2, TLR4, zinc and Vit. D factors in the sera of patients and healthy indivuals and that is achieve by the following:

1. To estimate of the level of serum IgG, IgM, TLR2, TLR4 in UTI patients and control.
2. To measure the level of serum Zn and Vit D in UTI patients and controls.
3. To identify the correlation of above serum biomarkers in this study.

Chapter Two

Literature Review

2-1 Literature Review

Definition: Urinary tract infections (UTIs) are among the most common diseases caused by *Escherichia coli*, through the latest research, it has been found that recurrent urinary tract infections are spread through *Escherichia coli*, which causes urinary tract infections (UPEC), these coli bacteria multiply in the urethra until they are expelled from outside the cells and quiescent sites inside the cells and other aids, lipopolysaccharide and polymicrobial infection (Kim et al., 2021).

The upper part of urinary tract system and lower part of urinary tract system make up the urinary system, the kidneys and ureters make up the top portion of the urinary system, whilst the bladder and urethra make up the bottom portion, consequently, lower tract infections and upper tract infections are the two anatomical subtypes of urinary tract infections (UTI) (Bono & Reygaert, 2021).

Urinary obstruction, neurological disease-related urinary retention, immunosuppression, renal failure, renal transplantation, pregnancy, and the presence of foreign bodies like calculi, indwelling catheters, or other drainage devices are all considered causes of complicated urinary tract infections (UTIs) (Levison & Kaye, 2013).

Cystitis, urethritis, prostatitis, and epididymitis are examples of lower tract infections; pyelonephritis, the most serious urinary tract infection, can be transient or chronic, the inflammation of the renal parenchyma brought on by one of 13 bacteria or fungus is known as acute pyelonephritis (Bono & Reygaert, 2021).

A number of risk factors, such as sexual activity, vaginal infections, diabetes, obesity, history of UTIs, sex, and genetic vulnerability, are linked to cystitis (Foxman, 2014). Both Gram-positive and Gram-negative bacteria can cause UTIs, as listed in Figure (1), in addition to those caused by certain fungus, Uropathogenic

Escherichia coli (UPEC) is the most prevalent causal agent for both simple and complex UTIs (Storme et al., 2019).

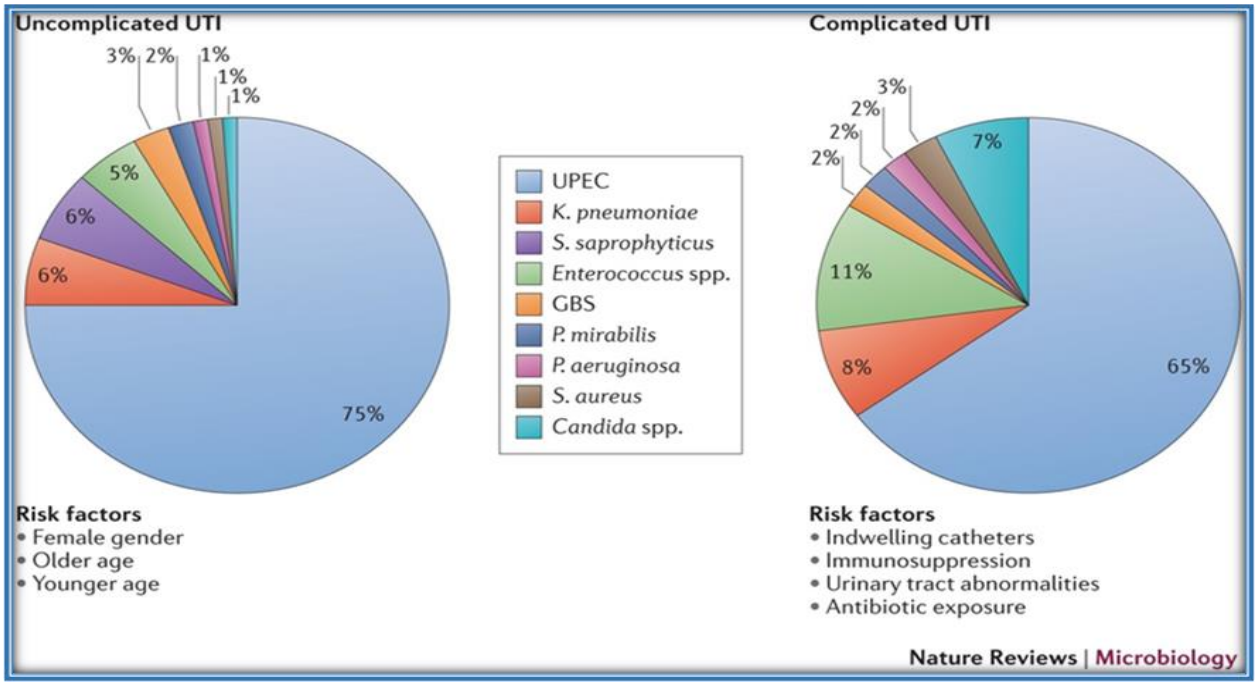

Figure (2-1): The most common infection types as a risk factors of Urinary tract infection. (Flores-Mireles, *et al.*, 2015)

Additionally, UTIs are categorized as basic or complex. A simple or uncomplicated urinary tract infection (UTI) is defined as inflammation of the normal urinary tract when there are no accompanying medical disorders such as diabetes mellitus, sickle cell disease, or immunosuppression, nor any anatomical abnormalities in the urinary system (Hooton, 2012).

When there are anatomical or functional abnormalities in the sick urinary tract, UTIs become more difficult. These anomalies may include renal calculi, bladder calculi, or an indwelling catheter. Acute cystitis is the primary example of a

simple UTI, whereas pyelonephritis is the primary example of a difficult UTI (Sabih & Leslie, 2021).

Although certain fungi can also cause UTIs, bacteria are the primary cause of UTIs. According to a research by Melaku *et al.*, The most frequently found uropathogen (100%) that was isolated from all of the qualifying papers that were examined originated from countries in Asia and Africa was *Escherichia coli* , In 82 and 87% of the eleven Asian and fifteen African countries, the second most prevalent uropathogen was discovered, respectively, was the *Klebsiella* species. *S. aureus* was examined in 82 and 73% of the research conducted in fifteen African and eleven Asian nations, respectively (Belet & Saravanan, 2020).

2-2 Epidemiology

One of the most frequent causes of adult medical consultations worldwide is UTI. An acute simple cystitis is the most prevalent kind of UTI (Behzadi *et al.*, 2016) .

Even though *E. coli* is still the most common pathogen in recurrent urinary tract infections, the relative frequency of infections caused by *Proteus, Pseudomonas, Klebsiella, Enterobacter* species, enterococci, and staphylococci increases significantly in these cases, particularly in the presence of structural abnormalities in the urinary tract (Tang *et al.*, 2019).

2-3 prevalence factors of UTI

1. The physical difference in the length of the urethra between men and women, which causes the female urethra to be shorter and closer to the anus, is the primary reason why UTIs are substantially more common in women than in males. Approximately 50% of women will get a UTI at some point in their lives. After *Escherichia coli*, *Staphylococcus saprophyticus* is the second

most frequent cause among young, sexually active women (Chu & Lowder, 2018).

2. Age prevalence: Certain age groups are more likely to get a UTI. With a lifetime prevalence of 50–60% in adult women, urinary tract infections (UTIs) are the most prevalent outpatient infections. The frequency of UTI rises with age, and in women over 65, it is around twice as high as in the general female population. In this age range, the etiology varies according on health state; for example, catheterization can have an impact on the risk of infection and the bacteria most likely to cause it (Medina & Castillo-Pino, 2019), (Flores-Mireles *et al.*, 2015). There was also a difference depending on age, the issue of circumcision, and gender in the prevalence of urinary tract infections. Male infants who have not yet been circumcised and are less than three months old and female infants who are less than 12 months old have a higher prevalence of urinary tract infections. These prevalence levels help doctors make clear decisions regarding diagnostic testing in a child who has signs and symptoms of urinary tract infections (Shaikh *et al.*, 2008).

2-4 Classifications of UTIs

Understanding UTI categories is crucial when analyzing their epidemiology. In general, UTIs are categorized according to where they are in the urinary system, whether or not they have symptoms, and whether or not there are any relevant complicating factors. Symptomatic or recurrent UTIs increase patient discomfort and stress or cause the relapse of concomitant stones (Wang et al., 2024). Table 1 summarizes definitions of some of the main UTI groups based on the most recent recommendations (Kranz *et al.*, 2018).

Table (2-1): The classifications of UTIs (Kranz *et al*.,2018)

Classification	Definition
Uncomplicated UTI	A UTI in which there are no pertinent kidney function impairments, functional or anatomical abnormalities in the urinary tract, or concurrent conditions that increase the likelihood of developing significant consequences
Acute uncomplicated cystitis	An acute lower urinary tract infection (UTI) is characterized by lower urinary tract symptoms alone, such as urgency, painful voiding (dysuria), pollakiuria, and discomfort above the symphysis
Acute uncomplicated pyelonephritis	A prolonged upper urinary tract infection characterized by flank discomfort, flank soreness, or fever (>38°C)
Asymptomatic bacteriuria	a positive urine culture (>105 colony-forming units/ml) without any symptoms related to the bladder
Recurrent uncomplicated UTIs	A recurrent urinary tract infection (UTI) is defined as two or more symptomatic episodes in a period of six or twelve months.

2-5 Pathogenic Bacteria in UTI

In both simple and complex UTIs, the predominant infecting agent is uropathogenic *Escherichia coli* (*E. coli*) (UPEC). In complex infections, *Enterococcus* species and *Candida* species are significantly more prevalent, but *Staphylococcus saprophyticus* (*S. saprophyticus*) is uncommon *(Flores-Mireles et al.*, 2015).

The diagnosis of a UTI can be made by a combination of symptoms and a positive urine analysis or culture. In most patient groups, the threshold for bacteriuria

is considered to be 1,000 colony-forming units (cfu)/ml, based on studies correlating midstream-urine specimens with catheterized collection to demonstrate bladder bacteriuria{Geerlings, 2016 #247}

fever in those with profound cognitive impairment as well as scrotal or prostate swelling tenderness to be consistent with the updated McGeer criteria by Stone, as well as pregnancy in women{Nace, 2014 #248}`

Bacteria that cause UTI are divided into two main classes: gram-negative and gram-positive bacteria. The gram-negative bacteria that cause urinary tract infections include *Escherichia coli* cause most uncomplicated cystitis and pyelonephritis cases,

Klebsiella pneumonia–notorious for causing bloodstream infections, *Pseudomonas aeruginosa*–causes hospital-acquired UTIs that also could lead to dangerous sepsis. The gram-positive bacteria causing UTI are *Staphylococcus saprophyticus (S. saprophyticus), Group B Streptococcus (GBS), Aerococcus, Enterococcus.*

2-6 *E. coli* UTI

Most often, urinary tract infections (UTIs) in youthful, active-in-sex, and non-pregnant women (75%–80%) are caused by *E. coli* bacteria. These bacteria is gram-negative and prone to becoming resistant to medications. Since *E. Coli* is prevalent in the lower intestines, it is possible to find it in human waste (Flores-Mireles *et al.*, 2015).

2-7 *Proteus mirabilis*

Gram-negative Patients who have used catheters for a long time are preferred by *Proteus mirabilis*. *Proteus mirabilis* can form biofilms and develop drug resistance, making catheter-associated infections of the urinary tract more challenging to treat (CAUTI) (Schaffer *et al.*, 2016).

2-8 *Pseudomonas aeruginosa*

Gram-negative one of the most common bacteria responsible for infections and urinary tract infections associated with catheter use in people with immunosuppressive conditions is *P. aeruginosa* (Newman *et al.*, 2022).

2-9 *Klebsiella pneumoniae*

Adults with hospital-acquired urinary tract infections as well as UTIs and sepsis in neonates are known to be caused by *Klebsiella pneumoniae* (*K. pneumoniae*). It is well known that these gram-negative bacteria may become resistant to several medicines, including carbapenems. Humans normally contain *K. pneumoniae* bacteria, yet how frequently these germs are discovered depends on a number of variables. For instance, in some regions of the world, the community's carrier rates of *K. pneumoniae* are noticeably greater. Patients who identify as Asian in particular are more likely to have these bacteria colonize their intestines. According to some research, healthy adult *K. pneumoniae* stool carrier rates vary from 19% in Japan to 88% in Malaysia (Flores-Mireles *et al.*, 2015).

2-10 *Staphylococcus saprophyticus*

Gram-positive *Staphylococcus saprophyticus* bacteria account for 10–15% of UTI cases. In young, sexually active women, *S. saprophyticus* UTIs account for around 40% of cases. While *S. saprophyticus* has several highly uncommon traits, it shares many clinical symptoms with an *Escherichia coli* urinary tract infection. For instance, in the latter part of summer and early fall, they are usually seen within the urine tracts of young girls and women. This particular bacterium seldom causes a UTI in post-menopausal women, and it is less common in the winter and spring. Male *S. saprophyticus* infections are uncommon, however they can strike older or hospitalized men (Djawadi *et al.*, 2023).

2-11 *Staphylococcus aureus*

Contrary to *S. saprophyticus, S. aureus* urinary tract infections typically afflict pregnant women and those using urinary catheters. Furthermore, most *S. aureus* superstrains are methicillin-resistant (Armbruster *et al.*, 2017).

2-12 *Enterococcus*

Gram-positive lactic acid bacteria, or enterococci, can withstand pH ranges of 4.6 to 9 and temperatures between 10 to 45°C. They can even grow and live in the absence of oxygen. Additionally, the third most common cause of UTIs contracted in hospital settings is enterococci (Magill *et al.*, 2014). Enterococcal UTI risk is also increased in diabetics due to impaired immunity and inadequate bladder emptying. Ten percent of men with diabetes may experience prostate irritation as a result of these bacteria, which can also enter the circulation. Regrettably, super strains of Enterococci are becoming increasingly resistant to medicines. In particular, *E. faecalis* forms strong biofilms that are renowned for being challenging for medicines to penetrate and destroy the superbug (Guiton *et al.*, 2013).

2-13 The Causes of Infections in the Urinary Tract

Through the action of certain adhesins, gut-resident uropathogens invade the urethra and then the bladder, causing urinary tract infections (UTIs). If all of the bacteria are not destroyed by the host's inflammatory reaction, they start to grow and produce poisons and enzymes that help them survive. Once the kidneys have been colonized, the infection may progress to bacteremia and subsequently penetrate the kidney epithelial barrier (Flores-Mireles *et al.*, 2015).

The overall pathogenesis of urinary tract infections was illustrated in Figure (2.1). The periurethral region is contaminated by gut-resident uropathogens (step 1), which then have the ability to colonize the urethra and cause simple UTIs.

Colonization and invasion of the superficial umbrella cells occur as a result of subsequent migration to the bladder (step 2) and pili and adhesins being expressed (step 3). Extracellular bacteria are eliminated by the host's inflammatory reactions, which include neutrophil infiltration (step 4).

Some bacteria multiply (step 5) and build biofilms (step 6) in order to elude the immune system. This can happen through host cell invasion or morphological changes that make the bacterium resistant to neutrophils. In order to cause harm to host cells (step 7), these bacteria release toxins and proteases, which release vital nutrients that aid in the survival of the bacteria and their ascent to the kidneys (step 8).

Step 9 kidney colonization leads to step 10 host tissue damage and bacterial toxin production. If the infection penetrates the kidneys' tubular epithelial barrier, untreated UTIs may eventually lead to bacteraemia (step 11). b | Uropathogens that cause complex UTIs proceed through the same early stages-periurethral colonization (step 1), urethral progression, and bladder migration (step 2)-as those that are described for simple infections, but the bladder has to be weakened for the germs to invade it.

Catheterization is the most frequent cause of a compromised bladder. Because catheterization (step 3) triggers a strong immune response, fibrinogen builds up on the catheter, creating a perfect surface for uropathogens that express fibrinogen-binding proteins to adhere to Neutrophils infiltrate the body as a result of infection (step 4). However, following their initial attachment to the fibrinogen-coated catheters, the bacteria multiply (step 5), form biofilms (step 6), encourage damage to the epithelium (step 7), and can also cause kidney infection (steps 8 and 9), which can lead to tissue damage (step 10). Uropathogens that cause complex UTIs have the

ability to breach the tubular epithelial cell barrier and generate bacteraemia if left untreated (step 11) (Flores-Mireles *et al.*, 2015).

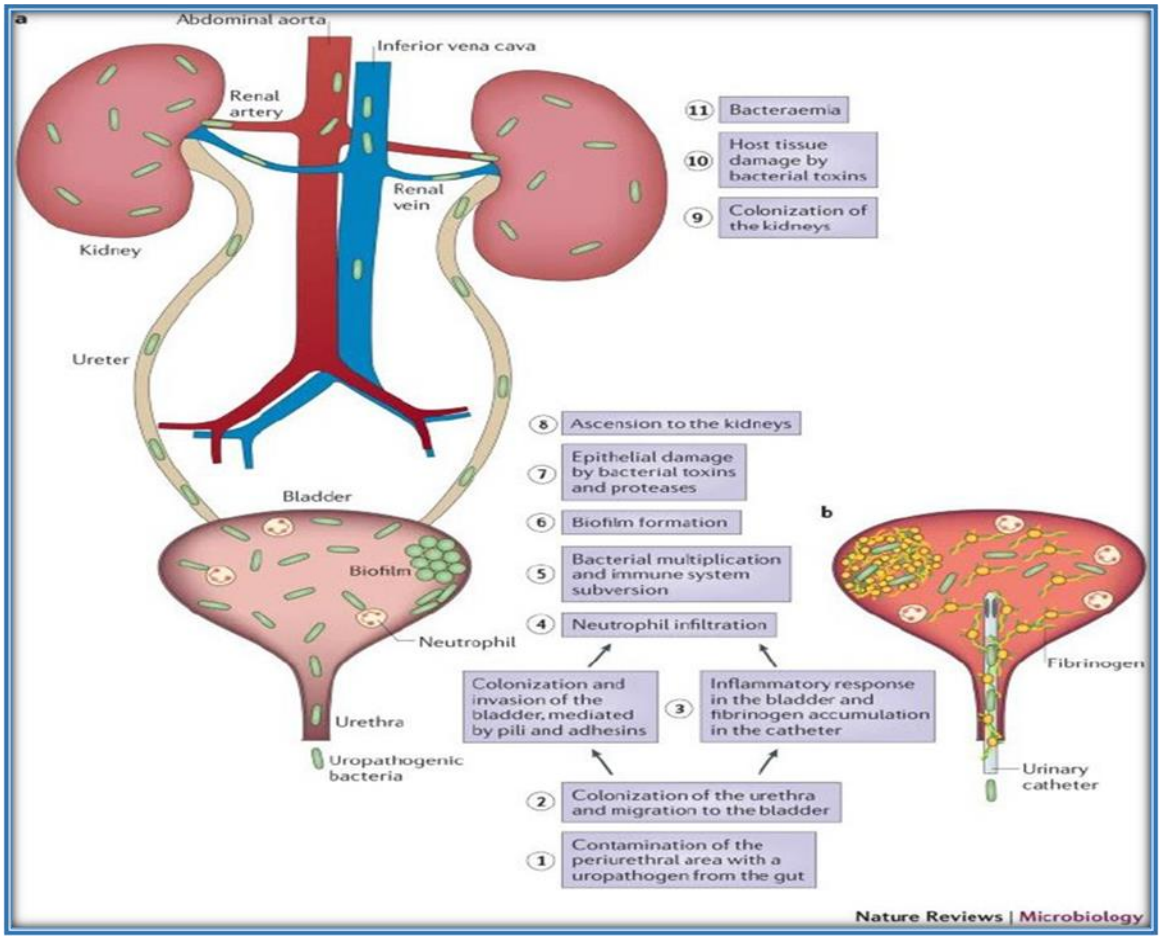

Figure (2-2): Pathogenesis of urinary tract infections. (Flores-Mireles *et al.*, 2015)

2-14 The Role of Immune System in The Urinary Tract Infection

Despite the fact that the urine system is frequently exposed to gastrointestinal tract microbes, infection by these microbes is very uncommon because of the urinary tract's natural immune responses (Abraham & Miao, 2015). Prior research has demonstrated that the immune response is tightly controlled to prevent damage to the epithelial barrier's structural integrity.

In order to attract and trigger neutrophil responses that eliminate germs from the bladder, macrophages and mast cells are essential for immunological control of the urinary tract. Furthermore, these cells play a crucial role in keeping the bladder's tissue from being harmed by an overabundance of neutrophils, which might expose the organ to a prolonged infection (Chhowalla *et al.*, 2013).

Pattern recognition receptors (PRR) are mostly found on immune cells that deliver antigens, such as macrophages and dendritic cells. Nevertheless, various immunological and non-immune cells can also express Pattern recognition receptors. These receptors can be found intracellularly, in the cytoplasm or in endosomes, however they are mostly localized to cell surfaces. Both the pathogen and the location determine the type of PRR that is triggered (Satoh & Akira, 2016).

In general, transcription of genes involved in host defense is triggered by cellular signaling cascades that are started by PRR activation. In particular, PRR trigger downstream cytokine and chemokine production, inflammatory cell recruitment for phagocytosis and bacterial clearance, and Nuclear Factor kappa B (NF-κB) signaling (Chowdhury et *al.*, 2004).

One of the most significant and well-researched families of PRRs is TLRs. TLRs are defined by the presence of a transmembrane segment, a large extracellular domain of leucine-rich repeats, and a cytoplasmic Toll/Interleukin-1 receptor-like (TIR) domain that aids in mediating the relationship between intracellular signaling proteins and ligand binding. Toll like receptors have been closely linked to the initiation of innate defense in the context of UTIs and are crucial for the innate identification of microbial components (Behzadi & Behzadi, 2016).

When uropathogens enter the urinary tract, they cause a conformational shift in the receptor, activating specific adaptor molecules that mediate various cascade

reactions, such as the release of proinflammatory cytokines, chemokines, interferons, and interleukins (IL) figure (3) (Ching *et al.*, 2020).

Recent studies have revealed the existence of a multifaceted innate immune response triggered by toll-like receptor 4 (TLR4) on superficial bladder epithelial cells directed at clearing infection by Gram-negative pathogens, and maybe a less important or a different role of the adaptive immunity. Though previously considered sterile, the bladder microbiome is increasingly thought to have a protective role alongside that of the urethra (Thomas-White *et al.*, 2018).

When TLR4 expression is activated on the urothelium, pro-inflammatory cytokines and numerous chemokines are released, which attracts bloodstream-derived neutrophils to the bladder lumen for phagocytosis. To facilitate cell migration, mast cell-derived substances induce vasodilation, while NK-cells and macrophages also produce cytokines to support this process (Abraham & Miao, 2015).

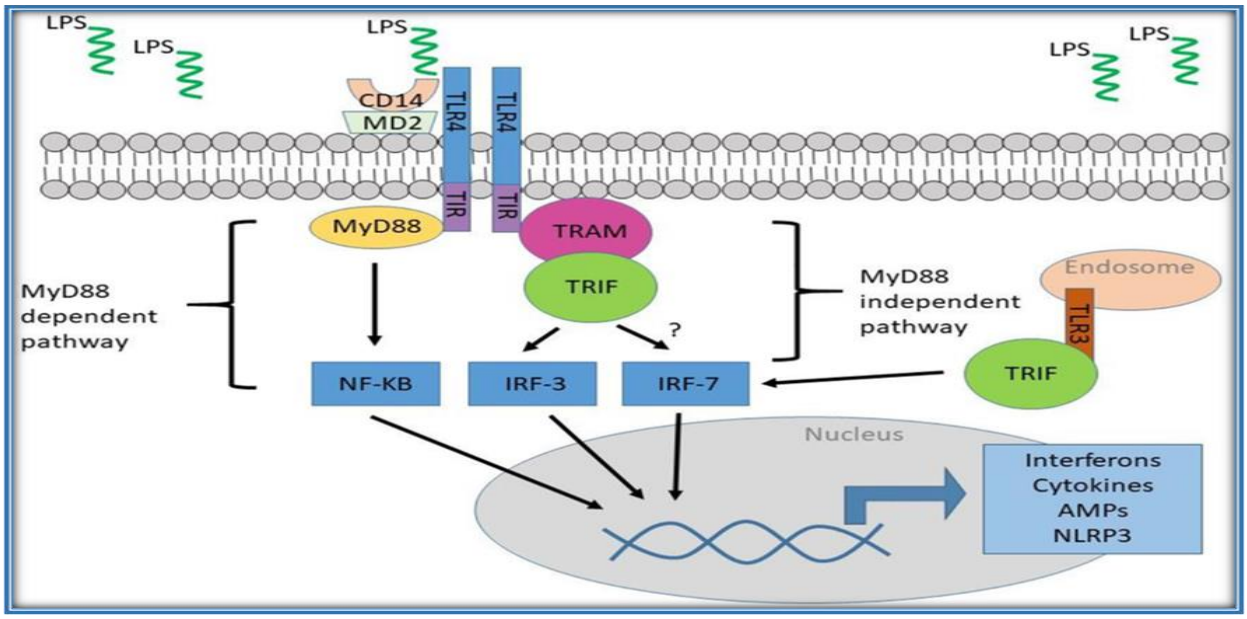

Figure (2-3): The molecular activation of TLR4 signaling cascade in UTI.
(Dobrindt *et al.*, 2016)

TLRs support host defense by triggering an inflammatory response in response to similar microbial patterns shown by both commensal bacteria and invading pathogens. TLR4 appears on the uroepithelium, in which it controls the migration, proliferation, and death of epithelial cells. This plays a factor in the pathophysiology of UTIs, especially when TLR4 activity is unchecked, despite TLR4's distinctive function in host defense due to its activity on immune cells (Dobrindt *et al.*, 2016)

Recent years have seen a significant increase in interest in antibody detection by serological techniques, which is now the cornerstone of diagnosis. There are many different types of immunoglobulins in urine, however, there hasn't been much research on utilizing urine as a diagnosis sample to detect antibodies.

Unlike venipuncture, this non-invasive sample collection technique can help people of all ages. Urine sampling is less risky and more cost-effective than other sample methods. It is discovered that the antibodies are stable in urine at room temperature for an extended amount of time, which facilitates sample transit management (Mohandas *et al.*, 2022).

Pathogen-specific antibodies offer valuable data in many investigations to evaluate infection control strategies, track recurrence, and ascertain the breadth of a pathogen's dissemination and infection intensity (Zhao *et al.*, 2017).

One example of how immune system reactions can become harmful is urinary tract infections (UTIs), which can be symptomatic and partially due to excessive innate immune activation. Infection-associated morbidity is also primarily caused by excessive innate immune activation. Some of these defense systems were initially discovered within the urinary tract, which highlights the critical importance of innate immunity in host susceptibility (Ragnarsdóttir *et al.*, 2011).

Studies have suggested that once the urothelium is exposed to viable bacteria, there can be a rapid antibody response (lgM) detectable in the urine as early as. The

infection may consist of IgM responders to the bacterial inoculum with IgM in the urine during both the acute and chronic phases. As the primary immune line of defense against urinary tract infections, IgM may be crucial. To the best of our knowledge, the existence of early urine IgM responses to UTI in humans has not yet been studied. Urine has been found to contain IgA following an infection. Moreover, it has been suggested that by inhibiting bacterial adhesion, it could contribute to immune defense in a manner akin to that of IgG. Studies demonstrating urinary immunoglobulins among children with UTIs have validated the notion of localized IgG production (Kobayashi *et al.*, 2002).

Hand and his team's research revealed that following urinary tract infections, the patient's bladder experiences a localized immune response. According to Hand, IgA and IgM play a supporting role in the immune response, whereas IgG is the main and most significant immunoglobulin generated, On the tenth day following infection, the infected bladder produced a large amount of protein and immunoglobulin, with the production peaking between days 14 and 129. The rats that Hand investigated had 20 times more IgG production in their bladders than normal between day 14 and roughly day 129 after therapy. Thirty-one percent of the infection was protein complex. In the impacted bladder, there was a little increase in IgM synthesis but no increase in IgA synthesis. (Hand *et al.*, 1970).

2-15 The Role of Zinc in The Urinary Tract Infection

Zinc is an essential trace element that is crucial for both overall human health and a healthy immune system. Zinc is essential for many physiological functions in the human body, including those that impact cell division, proliferation, and apoptosis, all of which have an impact on an organism's ability to develop (Maret, 2017).

Numerous accounts exist regarding the role of zinc for the treatment of infectious diseases (Baker *et al.*, 2018). Research has demonstrated that the element is active against a variety of pathogens, including *E. coli*, and improves response to therapy in many diseases (Consolo *et al.*, 2013).

Many reports have been published on the prevalence of zinc deficiency in infectious illness clinics. Mohsenpour's study (Mohsenpour *et al.*, 2019) found that the serum zinc levels for those suffering recurrent UTIs were lower than those of the control group. As a result, one could think of the zinc level as a risk factor for recurrent UTIs (Mohsenpour *et al.*, 2019).

The results showed that a lower zinc level was linked to a higher risk of UTIs (Javadi Nia *et al.*, 2013) and therefore, Numerous studies have demonstrated the critical role zinc plays in regulating the host immune system and the pathogenic effects of a mild zinc shortage on the immune system. Moreover, its absence results in lower levels of cellular immunity as it is essential for T lymphocyte growth and activation (Yousefi *et al.*, 2010).

However, in order to respond to the bacteria, host cells can alter the amount of zinc in their cytoplasm and lysosomes. Additionally, the buildup of zinc in macrophages and phagolysosomes aids in the regulation of infections. The zinc-induced bacterial poisoning may serve as a defensive mechanism for macrophages to eradicate the infection (Botella *et al.*, 2011). A zinc shortage has been linked to poor outcomes from sepsis and bacterial infections (Liu *et al.*, 2013).

According to Mohsenpour *et al.* (Mohsenpour B, *et al.*, 2019), there was a decrease in serum zinc levels among the recurrent UTI group compared to the control group. The idea that UTI patients had lower blood zinc levels than healthy individuals is supported by this finding. Zinc's antibacterial and anti-biofilm properties against urinary tract infections, *Klebsiella*, and *E. Coli* were investigated, according to Hancock *et al.* It was discovered that the divalent zinc might block the

processes by which the organisms under study produced biofilms in order to use its antibacterial effect (Hancock *et al.*, 2010).

When some antibiotics and zinc are combined, they have a synergistic impact on organisms and may be useful in reducing the frequency of infections, The immune system is less active when serum zinc levels are lower, Zinc is necessary for the production of gamma interferon, interleukin-2, and interferon alpha, all of which are critical for antioxidant defenses and radical detoxification Free radicals are unstable molecules that contain an unpaired electron, making them highly reactive with other molecules in the body. These molecules are formed naturally as a result of metabolic processes, Antioxidant levels are down, and oxidative stress is elevated in UTI patients, In UTIs, serum cations including Cu, Ca, and Zn are reduced while markers of oxidative stress such malondialdehyde are elevated (Elkhatib & Noreddin, 2014).

There are few studies in the literature which investigate the zinc levels with UTIs or the effects of zinc supplementation during UTIs (Mohsenpour *et al*., 2019) or even investigate the effect of zinc on the incidence of UTIs (Amoori *et al.*, 2021).

Generally, researchers indicated that decrease of Zn content is related to the occurrence of urinary tract infection. Zn is directly involved in the formation of the active centers of various catalytic enzymes and can regulate the activity of catalytic enzymes and affect the inflammatory reaction, immune response, oxidative stress and other processes in vivo; when Zn is deficient, it can on the one hand, directly increase the risk of pathogenic bacteria infection.

Further analysis of the relationship between serum Zn content and inflammatory cytokines revealed that the serum Zn content in patients with UTI was negatively correlated with the contents of C-reactive protein (CRP), Amyloid protein A (SAA), Prolactinin (PCT) and Intercellular Adhesion Molecule-1 (ICAM-1). Thus

it indicates that the deficiency of Zn in the course of UTI is closely related to the mass secretion of inflammatory cytokines, and it can be speculated based on the biological links involving Zn in the body that the deficiency of Zn can on the one hand, weaken the body's resistance to bacteria, increase the risk of bacterial infection and stimulate the secretion of inflammatory cytokines, and on the other hand, directly participate in regulating the generation of a variety of cytokines and significantly increase the secretion of inflammatory cytokines (Cao & Jia, 2018).

2-16 The Role of Vitamin D in The Urinary Tract Infection

Vitamin D is known for its immunoregulatory effects, particularly antimicrobial activity. Vitamin D has long been recognized for its antibacterial qualities and plays a significant role in controlling inflammation and the production of chemokines (Zendehdel & Roham, 2019).

Immune cells including B and T lymphocytes, monocytes, and dendritic cells all have high expression of vitamin D receptors, which allow the nutrient to have immunomodulatory effects. Macrophages are directly impacted by vitamin D circulation, which increases their capacity for oxidative processes such as cytokine synthesis and generation, phosphatase, and hydrogen peroxide (Zendehdel & Arefi, 2019). Vitamin D also accelerates neutrophil motility and phagocytic activity (Aghsaeifard *et al.*, 2022).

During bacterial infections, macrophages convert 25-hydroxyvitamin D (25(OH)D) into circulatory vitamin D3 (1,25(OH)2D3), which then alters the expression of antimicrobial peptide genes (Moradniani *et al.*, 2018). These peptides play a key role in the body's defense against microbial pathogens (Anbari *et al.*, 2019). It enhances both immune response and bacteria clearance (Jorde *et al.*, 2016). A study demonstrated a strong correlation between vitamin D deficiency and UTI. Additionally, their investigation revealed a strong correlation between upper urinary

tract infections and blood vitamin D levels in the lower urinary tract (Sherkatolabbasieh *et al.*, 2020). Deng *et al.* conducted a meta-analysis revealed that a child's UTI is linked to a vitamin D deficiency (Deng *et al.*, 2019).

The antimicrobial role of vitamin D modulates the production of cathelicidin (IL-37) and β-defensin against UTIs (Kwon *et al.*, 2015). Yang *et al.* demonstrated that giving children aged one to twelve months a vitamin D supplement can dramatically lower their risk of developing a UTIs (Yang *et al.*, 2016). Because IL-37 is crucial for UTIs as an antibacterial peptides (Nielsen *et al.*, 2014). Ovunc Hacihamdioglu *et al.* demonstrated that kids with low vitamin D levels may not be able to increase the amounts of urine cathelicidin to ward off *Escherichia coli*-caused UTIs (Hacihamdioglu *et al.*, 2016). During the infection, IL-37 mediates the release of cytokines and chemokines to produce immunity. The etiology and severity of illness may be related to the reduction in antimicrobial peptide production by macrophages due to insufficient vitamin D (White, 2010).

Vitamin D also helps to maintain the integrity of the urothelium through its receptor (vitamin D receptor-VDR), which is directly involved in regulating the function of epithelial cell junctions. Uropathogenic *E. coli* directly impacts these epithelial cells by downregulating claudin and occludin, which damages the epithelial barriers (Gurunathan *et al.*, 2015).

There is ongoing debate on the potential impact of vitamin D deficiency or insufficiency on the onset of urinary tract infections. There is currently little research on this topic in children's literature. Low blood vitamin D has been linked in several studies to an increased risk of UTIs (Unkelbach *et al.*, 2018).

In light of current research supporting its critical function in innate immunity, the impact of vitamin D on the urothelium has also been extensively examined (Chun *et al.*, 2008). The majority of research on vitamin D and UTIs that has been done on pediatric populations has demonstrated that low or inadequate vitamin D levels

are associated with an increased risk of developing UTIs. Furthermore, a meta-analysis of nine pediatric research revealed a clear correlation between low vitamin D and a higher incidence of urinary tract infections (Deng *et al.*, 2018).

Vitamin D is a potent stimulator of antimicrobial peptides including cathelicidin IL-37 in innate immunity (Chun *et al.*, 2008). Recently, Hertting *et al.* observed a significant increase in cathelicidin in response to vitamin D in biopsy samples of urinary bladder infected by uropathogenic *Escherichia coli (Hertting et al*., 2010). Vitamin D levels are important for the synthesis of cathelicidin and certain defensins in humans, especially over the course of an illness (Wang *et al.*, 2004). Prior research has demonstrated the significance of vitamin D for innate immunity in the fight against bacterial infections, primarily by enhancing phagocytic and neutrophilic motility (Bikle, 2008).

The immune modulation mechanism that increased antimycobacterial activity has remained a mystery. It was shown that vitamin D increased human monocytes' antibacterial activity (Liu *et al.*, 2006). Others found that the expression of antimicrobial peptide genes was significantly elevated by vitamin D. Later, it was shown in vitro that a synthetic 19-kD lipopeptide generated from *Mycobacterium tuberculosis* stimulates TLR2/1 signaling, which in turn increases the antibacterial potential of monocytes through a route that is reliant on vitamin D and vitamin D receptors (VDR). This required activating the CAMP gene and protein (Martineau *et al.*, 2007).

Within the suggested scenario, monocyte/macrophage TLR2/1 activation triggers the development of CYP27B1 (25-hydroxyvitamin D-1a-hydroxylase), which subsequently results in the generation of bioactive 1,25(OH)2D from circulating inert 25(OH)D. Additionally, there is an increase in VDR expression, which activates the CAMP gene when 1,25(OH)2D levels are high locally Figure (2-3) (Liu *et al.*, 2006).

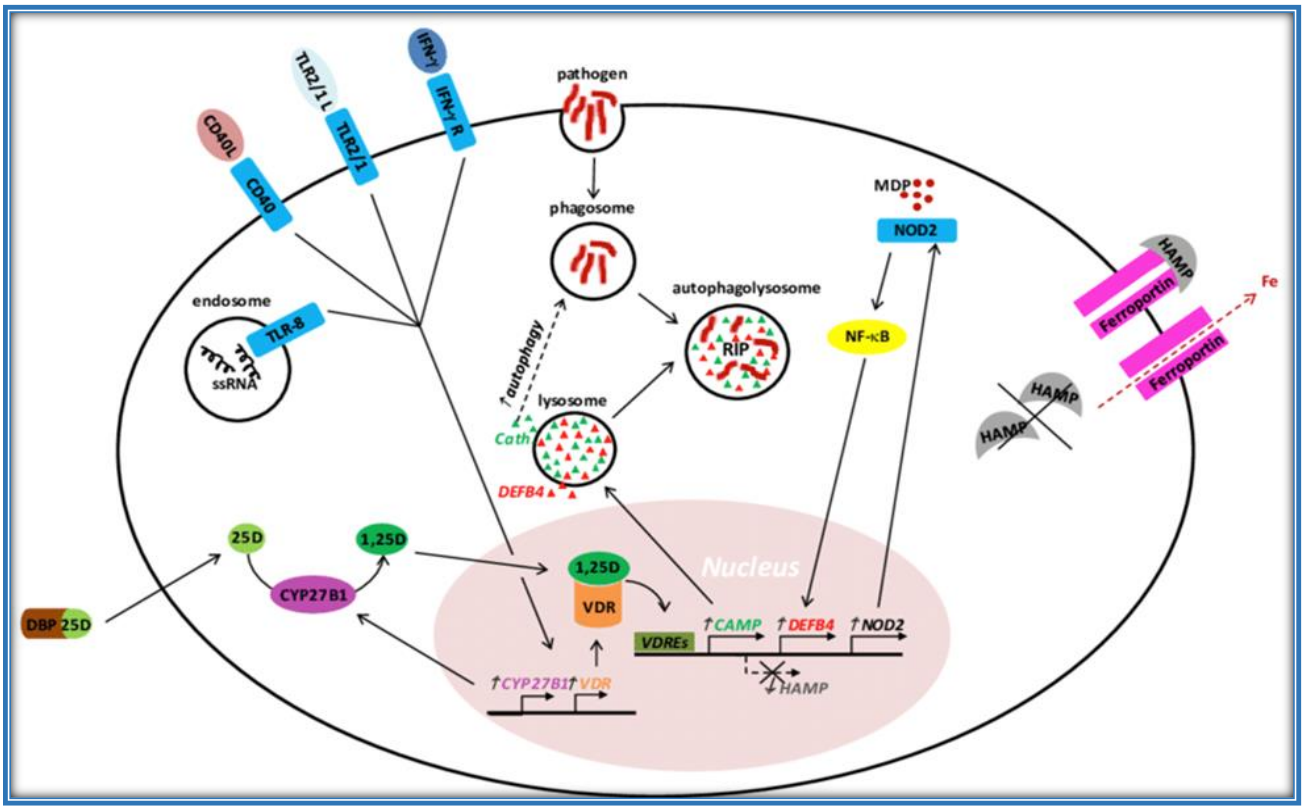

Figure (2-4): Proposed model of the vitamin D-dependent antimicrobial pathway (de Castro Kroner *et al.*, 2015)

Prior research has demonstrated the significance of vitamin D for innate immunity in the fight against bacterial infections, primarily by enhancing phagocytic and neutrophilic motility (Bikle, 2008). Low blood levels of 25-hydroxy vitamin D (25(OH) vitamin D) have been linked to an increased risk of recurring bacterial infections, according to several studies (Mahamid *et al.*, 2013). There is increasing evidence that vitamin D deficiency plays an important role in susceptibility to infections (Juzeniene *et al.*, 2010).

Chapter Three

Materials and Methods

Materials and Methods

3-1. Materials

3-1-1. Apparatuses and Instruments

Table (3-1): Instruments and apparatuses

Instrument	The organization and the origin country
Autoclave device	Japan's Hirayama\ Japan
Benson burner	Germany's Jenway\ Germany
Centrifuge device	Hettich\ Germany
Cooling centrifuge device	Germany's Hettich\ germany
camera of Digital	Sony\ Japan
Distillation device	GFL\ Germany
Eppendorf Centrifuge	Hermle\ Germany
Incubator	Fisher Scientific\ Germany
Inoculating needle	Memmert\ Germany
loop of Inoculation	Loop Shandon\ England
Light Microscope	Genix, (U.S.A)
Laminar Flow Cabinet	Labtech\ Korea
Micropipettes	Slamed\ Germany
Micropipettes	Oxford Germany
Unites of Millipore filter (0.45 and 0.22) µm	Gallenkamp, England
Oven device	Memmert, Germany
PH-meter,Talwan	LKB\ Sweden
Refrigerator	Ozone\ Korea
Sensitive balance	Kern\ Germany
Vortex shaker	Gemmy, USA
Water bath device	GFL, Germany

3-1-2. Materials of single-use

Table (3-2): Table of single-use materials

Single-use material	Company and Country of Origin
Petri dishes	Afco-Dipo/ Jordan
Disposable loop	Afco-Dipo/ Jordan
Eppendorf tubes	Sigma / England
Tubes of 10 ml plane	Afco-Dipo/ Jordan
Tips	Sterellin Ltd/ England
Slides	Sail Brand/ China
Cover slides	Sail Brand/ China
Parafilm	BDH/ England
Cotton	HAD/ China
Latex Gloves	Broche/ Malaysia
Sterilized needles	Afco-Dipo/ Jordan
swabs Sterilized cotton	Afco-Dipo/ Jordan
Gel tubes	Trust lab/ china
EDTA tubes	Trust lab/ china
Reagent strips for urine analysis	Cybow/ Korea
First aid plaster	Life plus/ china

3-1-3. Materials, both chemical and biological

Table (3-3): Table of Materials, both chemical and biological

Material	Company and Country of Origin
Glycerol	Himedia (India)
Gram Stain (Crystal violet, Iodine, Safranin, HCl)	Himedia (India)
Oxidase	Himedia (India)
Catalase	Himedia (India)
Coagulase	Himedia (India)
Human IgG ELISA KIT	Elabscience/ USA
Human IgM ELISA KIT	Elabscience/ USA
Human TLR2 ELISA KIT	Elabscience in USA
Human TLR4 ELISA KIT	Elabscience in USA
Zinc kit	Biosam/ UAE
EDTA (Ethylene Diamine Tetra Acetic acid)	B.D.H\ England
H_2O_2 (Hydrogen Peroxide)	Oxoid/England
Normal saline infusion (sodium chloride)	Oxoid/England
D3	Biosam/ UAE

3-1-4. Culture Media:

Table (3-4): Culture media used in this study

Media	Company and country of origin
Blood agar	Oxoid/England
Brain heart infusion broth	Himidia/ India
Eosin methylene blue agar (EMB)	Oxoid
MacConkey agar	Himidia/ India
Mannitol salt agar	Himidia/ India
Nutrient broth	Himidia/ India
Muller hinton agar	Bilab scince/ USA

3-2. METHODS

The methods are:

3-2-1. STUDY DESIGN

Figure (3-1) shows the steps of the practical part of the current study for patients and healthy people, which begins with the questionnaire step to select the required sample, Next the blood sample is taken and the serum is separated for the purpose of measuring the six immune levels (IgG, IgM, TLR2, TLR4, Zinc, and Vit D) and a urine sample is taken for the purpose of general microscopic examination and then bacterial culture as seen in figure (3-1) below.

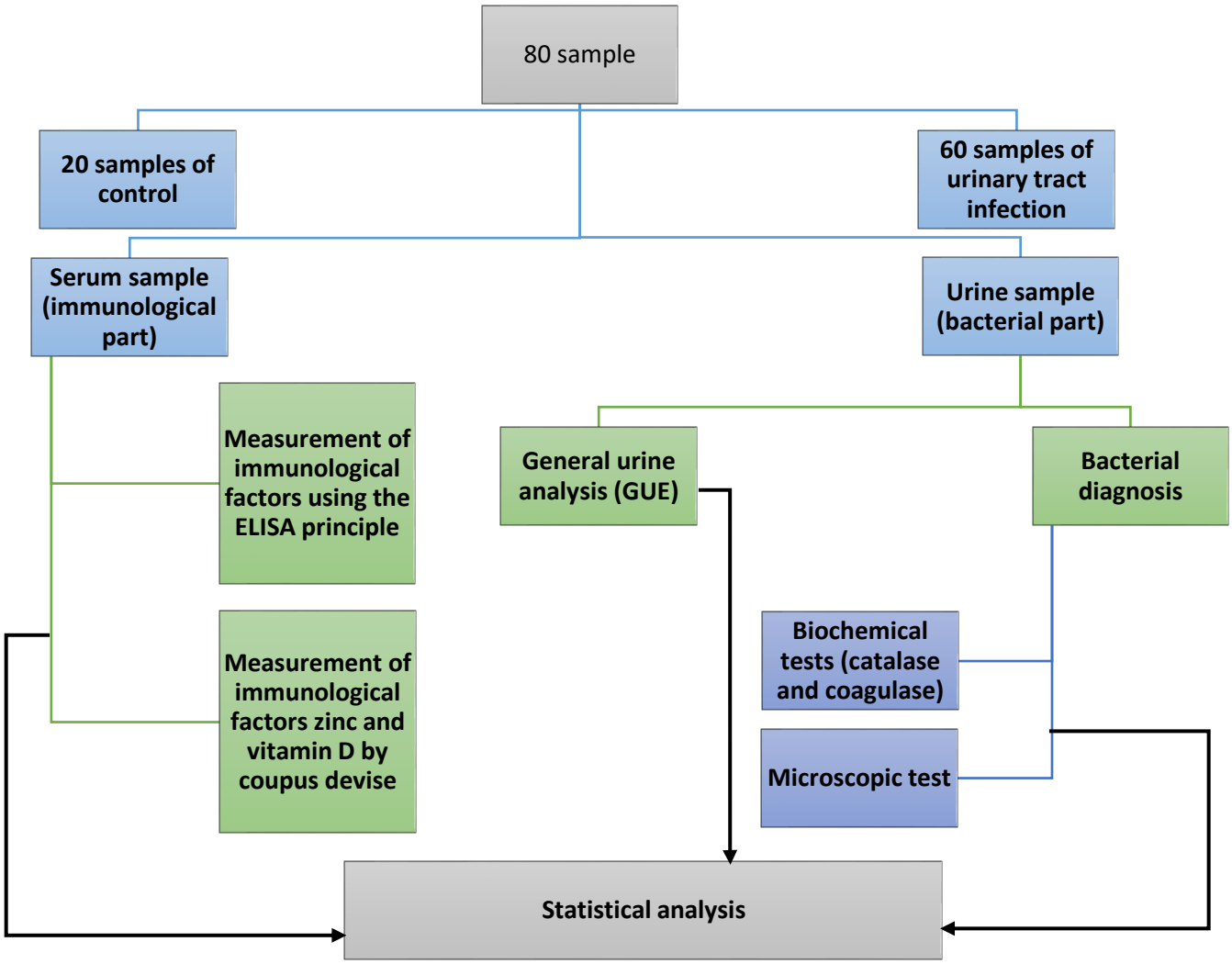

Figure (3-1): STUDY DESIGN

3-2-1-1 Sample population

The study included 60 people with urinary tract infections, about 30 female and 30 male, and the ages ranged from 18 to 55) years, it also included 20 people who did not suffer from urinary tract infections and chronic diseases and were in the same age range.

3-2-1-2 Sample Collection Settings

The Samples were gathered from Imam Hussein Hospital and Imam Hassan Al-Mujtaba Hospital in holy Karbala. As for the process of separating the blood serum, it was simultaneous with the operation plural. The ELISA test was done in one day.

3-2-1-3. Samples Collection of UTI Patients and Control Groups

The current study included 80 urine and blood serum samples, 60 of which were obtained from patients complaining of urinary tract infections, Those came to Imam Hassan Al-Mujtaba Hospital and Imam Hussein Medical Educational City / Holy Karbala in the months of December 2022 through April 2023, and 20 samples from healthy people, who were taken at the same period. All the information about the patients and healthy individuals (age, sex, congenital anomalies, diabetes, autoimmune diseases, pregnancy, aging people, etc.) was collected by a questionnaire prepared for the current study (Appendix 1). Age and sex were included in the questionnaire, while congenital anomalies, diabetes, autoimmune diseases, pregnancy, and elderly people were excluded from the questionnaire.

Clinical diagnosis: all the patients were confirmed with UTI by specialist doctors by doing the urine analysis and microbial cultures. The most typical clinical signs of cystitis include extensive hematuria, suprapubic discomfort, frequent, low-volume urine, and dysuria (pain with urination). There may be some penile discharge in men. Most cystitis patients do not have fever or other systemic infection

symptoms. but when they do, an upper urinary tract infection (pyelonephritis) should be taken into consideration (Levinson, 2013).

3-2-2. Measurement of Immunological Markers IgG, IgM, TLR2, TLR4

3-2-2-1. Measurement of Immunological Marker IgG

3-2-2-1-1. Test principle

Sandwich-ELISA technology is utilized with this ELISA kit. The micro-ELISA plate that comes with this kit has been pre-coated with an antibody that is particular to human IgG. Additionally, samples (or standards) have been added to the wells of micro-ELISA plates. Subsequently, each microplate well receives two additions: firstly, an Avidin-Horseradish Peroxidase (HRP) component, and secondly, a biotinylated detection antibody suitable for human IgG. Free parts are removed during washing. To each well, the substrate solution is applied. Only the wells with Human IgG, biotinylated detection antibody, and Avidin-HRP conjugate will have a blue appearance. The enzyme-substrate process is stopped by the addition a stop solution, and the color turned yellow. Spectrophotometric measurement delivers an optical density (OD) for an approximate wavelength of 450 nm + 2 nm. The OD value and the log level of concentration of human IgG have an inverse relationship. The level of human IgG in the samples can be ascertained by comparing their optical density (OD) with the reference curve.

3-2-2-1-2. Serum seapration process for IgG Immunological Marker

Serum: in advance of 20 minutes of centrifugation at 1000 g at 2–8°C, samples were allowed to clot at room temperature. The supernatant was collected, and the tests were performed using it.

3-2-2-1-3. Dilution Method

In cases when it was necessary to dilute the test samples, the dilution procedure was denoted as follows: One-step dilution is used for a 100-fold dilution. A 100-fold dilution was achieved by adding 5 μL of sample to 495 μL of diluent.

3-2-2-1-4. Reagent Preparation

1. Before use, all required reagents were brought to room temperature (25°C).
2. Washing solution: To make 750 ml of washing solution, 30 milliliters of concentrated washing solution were combined with 720 ml of deionized or distilled water.
3. The sample diluent and 1.0 ml of the standard of reference were added, after ten minutes, give it a few gentle stirs. It was thoroughly combined with a pipette once it had fully dissolved. After reconstitution, 100 ng/mL was the functional solution. As needed, serial dilutions were prepared. The suggested gradient of dilution was as follows: a hundred, 50, 25, 12,500, 6,250, 3,130, 1.56,0 ng/ml. Regarding the dilution procedure, there were seven EP tubes used, and each tube received 500 microliters of the sample dilution as well as the reference standard. To create a 50 ng/mL active solution, pour 500uL of a 100 ng/mL solution that works in the initial tube and stir. In accordance with this procedure, pipette 500 uL about solution from every prior tube into the last tube. This drawing is meant to serve as a guide for you, as an observation the solution was not drawn from the preceding tube and that the final tube is empty. Prior to use, a standard solution for work was produced and progressively diluted.

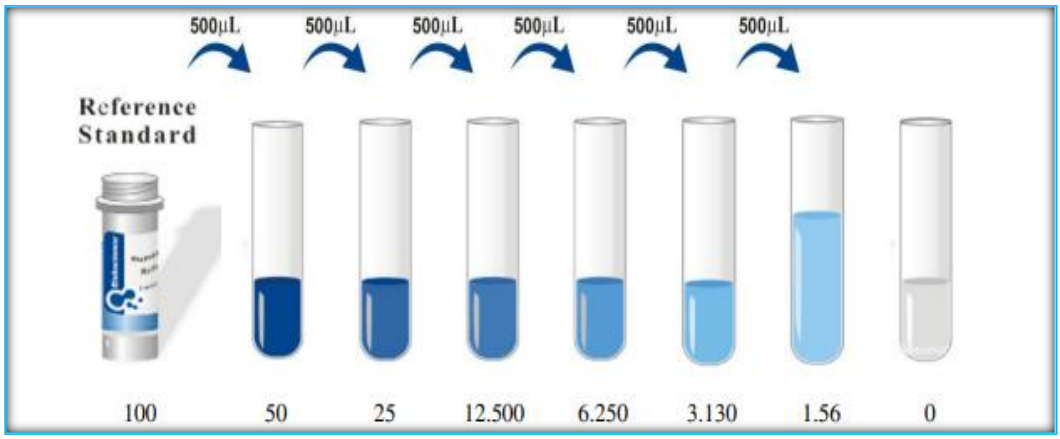

Figure (3-2): Reagent preparation of IgG Concentration according to E-lab science\ USA

4. Biotinylated Ab Operating solution detection: The necessary quantity (100 µl/well) was determined prior to the experiment. A little more was set up than estimated during the preparation phase. For one minute, centrifuge the concentrated biotinylated Antibody around 800 x g, Next, using the highly concentrated biotinylated Ab Dilution (concentrated biotinylated Ab recognition: Diluent = 1:99), dilute 100× from the concentrate biotinylated Ab into 1× working solution.
5. The HRP Conjugate Working Solution's concentration: HRP Conjugate, or avidin conjugated by HRP. Before starting the experiment, calculate the necessary amount (100 µL/well). A little more than anticipated was produced in order to make the concentrated HRP conjugate, which was then diluted 100× into a 1× solution of work using conjugated HRP diluent (Conjugated HRP diluent = 1:99; concentrated HRP conjugate) and centrifuged at 800×g for a minute.

3-2-2-1-5. The Procedure of Assay

1. Wells were designated for diluted, blank, and sample standards. 100 microliters were included for every standard dilution, the blank, as well as the

sample in the appropriate wells, noting that all samples and standards were examined in duplicate, and the sample dilution percentage was determined through preliminary experiments or technical support recommendations). A sealant included in the kit is applied to the plate. The incubation period lasted 90 minutes at 37°C, keeping in mind that the solutions must be added to the microfiber ELISA plate's bottom, in addition to avoiding contact with the inner wall and thus causing the appearance of foam as much as possible.

2. After purifying and washing the liquid from each well, 100 µl of the Biotinylated Detection Ab solution for use was added straight to each well. A fresh sealant was applied to the panel. The incubation period lasted one hour at 37°C.
3. 350 µL from wash material had been added into each well after the solution was drained out of each one. Once a substance had soaked for about sixty seconds, it was taken out of each well, or emptied, and let to dry on new absorbent paper. This step of washing was repeated 3 times ،considering the possibility of using a microplate washer in our step and washing steps, to prevent the wells from drying out, remove the strips of test that are currently in use as soon as the cleaning process is complete.
4. Each well received 100 µL about HRP conjugate solution that worked added to it. A fresh sealant was applied to the plate. The incubation period lasted thirty minutes at 37°C.
5. Each well was filled with the solution, and the washing procedure was then carried out five times, just like the third step.
6. Each well received Ninety µl about substrate-related reagent applied to it. plate was coated using a fresh sealant. The substrate was incubated for fifteen minutes at 37°C and kept out of the light, taking into account as the reader

that was mounted on the microplate had been adjusted to warm up for fifteen minutes before to the OD measurement.

7. Each well received 50 µL of stop solution, which was added in the same sequence as the substrate solution.
8. Each optical density of well, which is termed the OD value, was once measured with a microplate reader set at 450 nm.

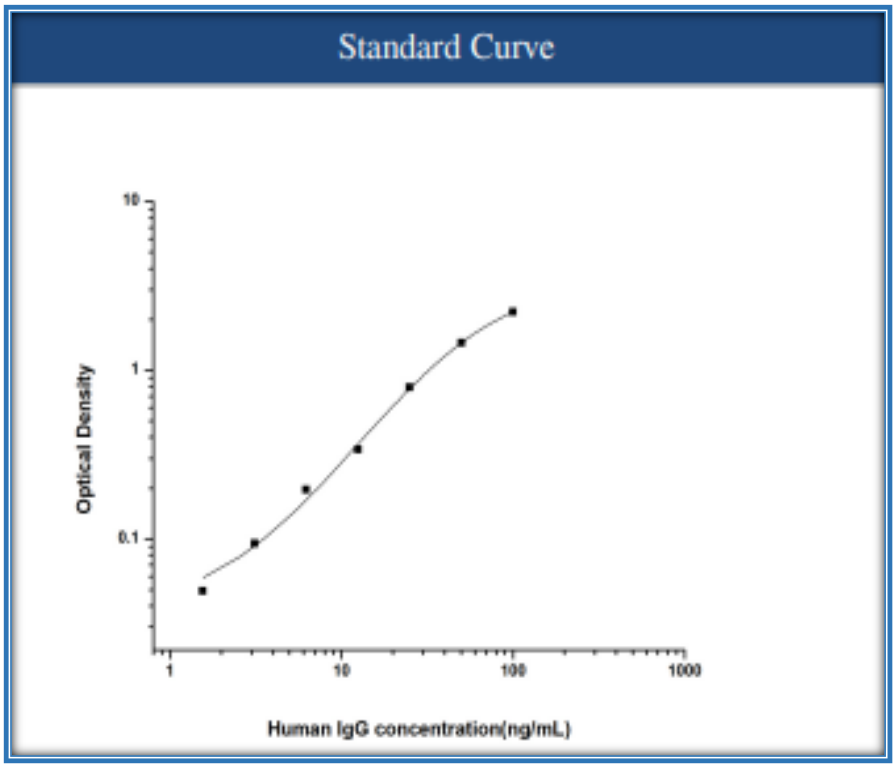

Figure (3-3): standard curve of IgG Concentration according to E-lab science\ USA

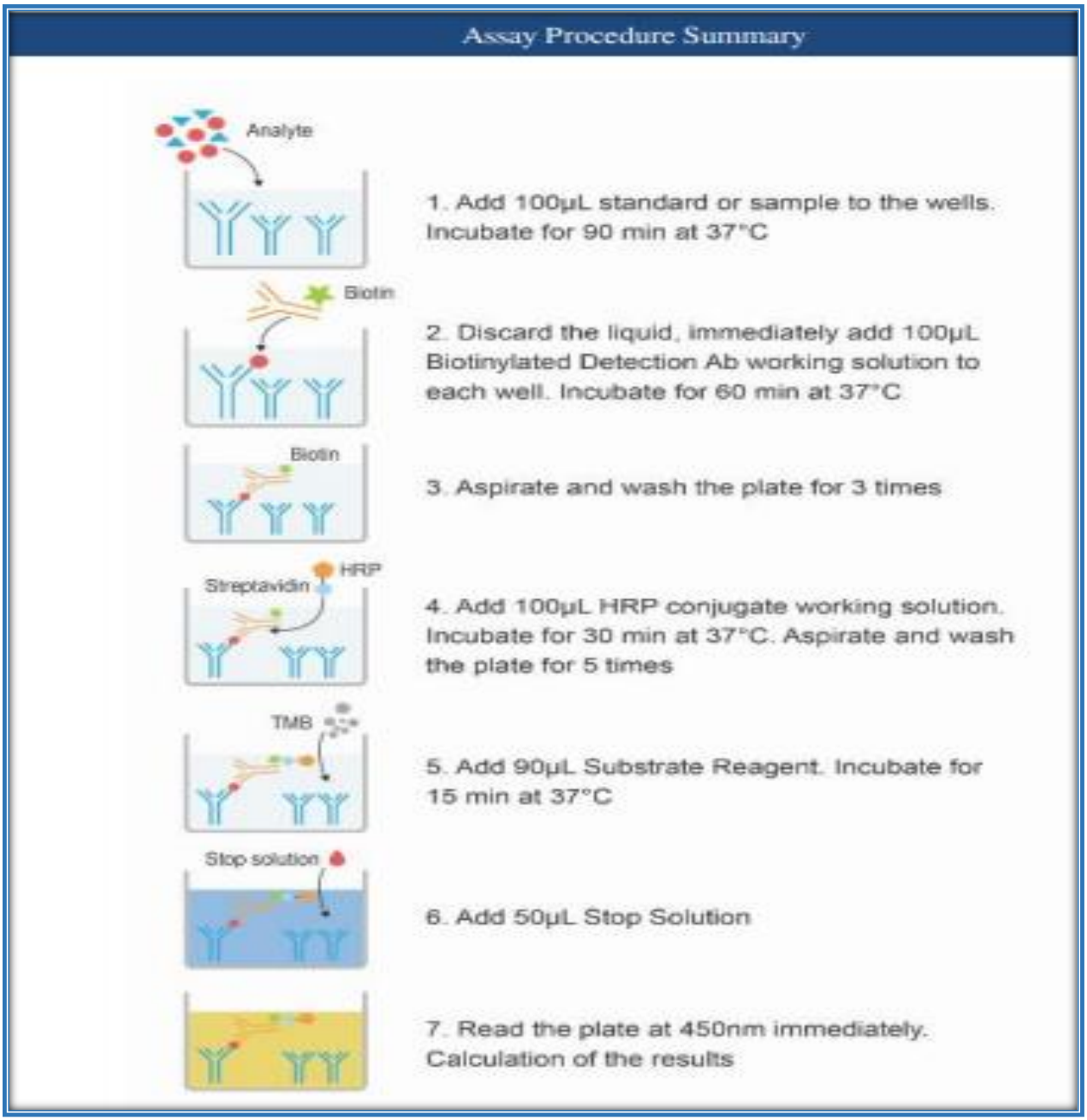

Figure (3-4): ELISA assay procedure summery According to Elabscience/USA specifications of Human IgG (Immunoglobulin G) ELISA Kit

3-2-2-2. Measurement of immunological marker IgM

3-2-2-2-1 Test principle This ELISA kit

The sandwich ELISA concept can be applied to this ELISA kit. This kit includes a micro-ELISA plate that has been pre-coated with an antibody associated with human IgM. Samples or standards were added to the wells of the ELISA microplate, and the proper antibody was then conjugated. After that, every microplate has its own well, which receives successive additions of biotinylated detection antibodies specific for human IgM and the Avidin-Horseradish Peroxidase (HRP) conjugate, which were then incubated. Each well received a substrate solution addition after the free components were removed. The only wells that were blue were those that contained the Avidin-HRP conjugate, biotinylated detection antibodies, and human IgM.

The reaction of the enzyme with the substrate was terminated by adding stop solution, which was colored yellow. Optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the human IgM concentration, and it is possible to calculate the human IgM concentration in the samples by comparing the OD of the samples to the standard curve.

3-2-2-2-2. Serum Seapration Process for IgM Immunological Marker

Serum: samples were centrifuged for 20 minutes at 1000 ×g at 2–8°C after letting them clot for one hour at ambient temperature. The supernatant was gathered and used for the experiment.

3-2-2-2-3. Dilution Method

A one-step dilution is used for a dilution of 100 times. This dilution was obtained by including 5 µL of the sample with 495 µL for sample diluent.

3-2-2-2-4. Reagent Preparation

1. Before use, all reagents were kept at ambient temperature (25°C).
2. Washing solution: concentrated washing solution about 30 ml was made into 750 ml of washing solution by diluting it in 720 ml of deionized or distilled water. The concentrate was heated in a water bath at 40°C to prevent crystal formation and mixed gently to ensure complete dissolution.
3. The standard working solution involves centrifuging a standard sample and 1.0 ml of the standard diluent at 10,000 × g for one minute, then leaving the mixture for ten minutes while gently stirring on multiple occasions. Following its total dissolution, a pipette was used to thoroughly mix it. Following a new constitution, the functional solution was 200 ng/ml. Next, make further dilutions as necessary. The following was the suggested dilution gradient: 200, 100, 50, 25, 12,500, 6,250 and 3.13, 0 ng/ml.
4. Dilution protocol: A total of seven EP tubes had been utilized, and 500 µL of the reference standard and sample dilution had been added to each tube. Pipette 500 milliliters of a 200 ng/mL solution into the beginning tube, then

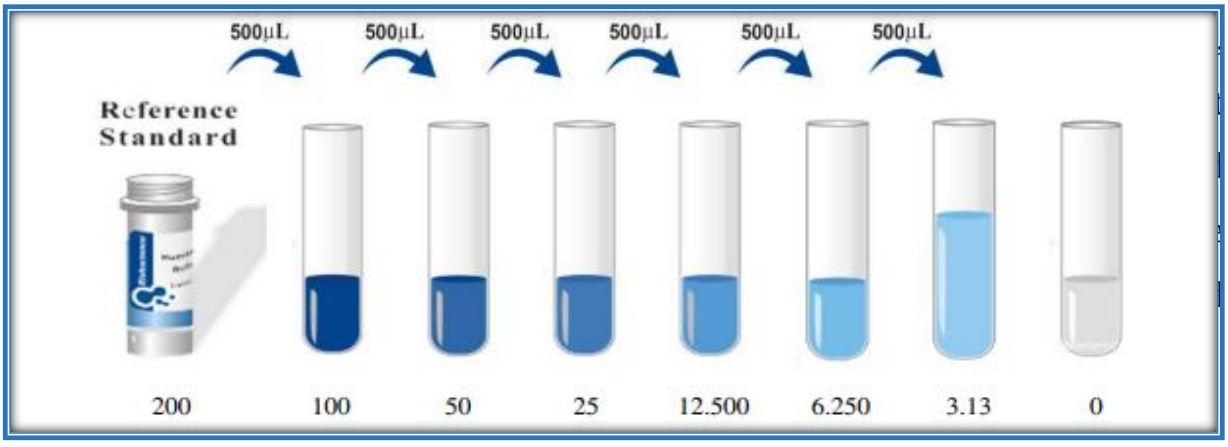

Figure (3-5): Reagent preparation of IgM Concentration according to E-lab science\ USA

5. Working solution for biotinylated Ab detection: Prior to the experiment, determine the necessary quantity (100 μL/well). A bit more was prepared throughout the preparation process than estimated. Using concentrated biotinylated detecting antibodies as a diluent (concentrated biotinylated detecting antibodies: concentrated biotinylated detection antibodies Diluent Equals 1:99), centrifugation is used to identify concentrated biotinylated antibodies for one minute at 800 × g. Condensed biotinylated Ab is then diluted for the purpose of identifying concentrated biotinylated antibodies in a 1× working solution. The solution for the work had been prepared immediately before utilization.
6. Condensed HRP conjugate working solution: That avidin which has been coupled to HRP is known as the HRP conjugate. Prior to the experiment, determine the necessary amount (100 μL/well). A bit more was prepared throughout the preparation process than estimated. Conjugated HRP diluent was used to dilute a concentrated HRP conjugate from 100× into 1× working solution after centrifuging it at 800 × g for one minute (concentrated HRP conjugated: conjugated HRP diluent Equal 1:99). Before usage, a working solution was quickly constructed.

3-2-2-2-5. Assay Procedure

1. The summery procedure of IgM is the in figure 4
2. Sample, blank, and diluted standards all had their own wells. The corresponding wells were loaded with one hundred milliliters of every standard dilution, blank, and sample. Using the sealant plate that comes with the package, covering is completed. The incubation period lasted ninety minutes at 37°C. To maximize foam formation and prevent interaction with the inner wall, the solutions were placed at the bottom of a fine ELISA dish.

3. After being filtered, the liquid was cleaned from each well. Each well received an instant addition of 100 µl for Biotinylated Detecting Ab working solution. After that, a fresh sealant was applied to the panel, and it was incubated for one hour at 37 °C.
4. After the resolution was poured out of every well, 350 µL of washing liquid was included. After one minute of soaking, a solution had been pipetted or emptied from every well, and it was left to finish drying fresh paper that absorbs. Three times was this washing phase carried out. For this and subsequent washing procedures, a microplate washer had been used. After the washing procedure, the tested strips had been prepared to use right away. The holes are not completely dry.
5. Each well received 100 µL about HRP conjugate working solution. Apply a fresh sealant layer on the plate. The incubation period lasted thirty minutes at 37°C.
6. Step 3 involved decanting the solution from each well and repeating the washing procedure five times.
7. Each well received 90 µl about substrate buffer applied to it. A fresh sealant was applied to the plate. At 37°C, incubation lasted for 15 minutes. shielded the artwork from sunlight. Before determining the OD, the microplate reader had been heated for fifteen minutes.
8. To every well, 50 µL of the resolution for stopping was added, being careful to add it in the same sequence of the substrate solution.
9. Each optical density of well (OD measurement) was once measured via a microplate reader that was set to 450 nm.

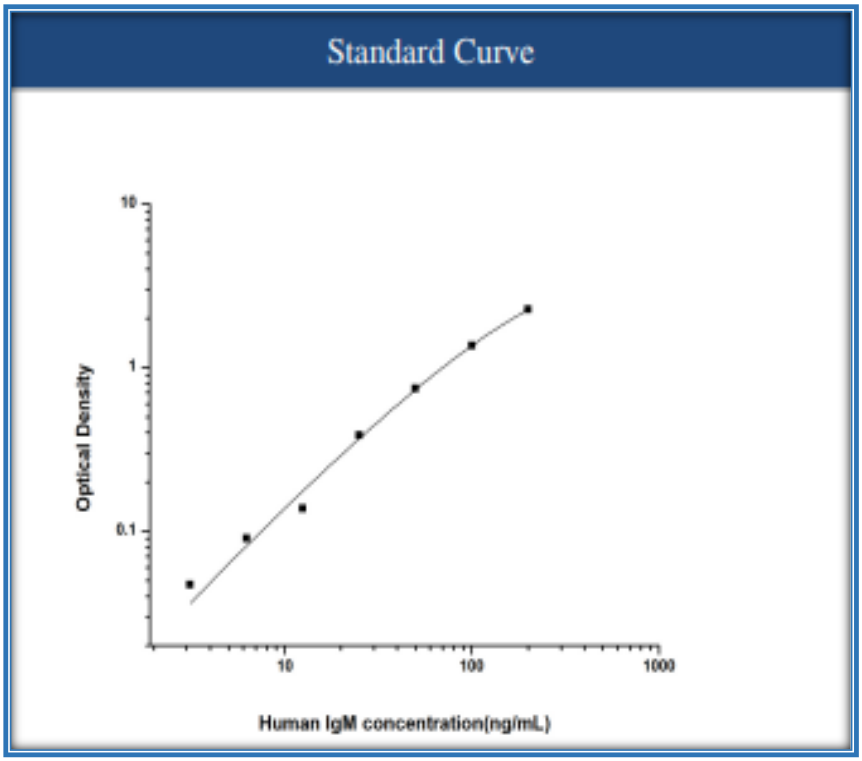

Figure (3-6): standard curve of IgM according to E-lab science\ USA

3-2-2-3. Measurement of Immunological Marker TLR2

3-2-2-3-1. Test Principle This ELISA Kit

The ELISA kit employs the Sandwich-ELISA principle, using a pre-coated micro plate with an antibody specific to Human TLR-2. Samples are combined with the antibody, followed by biotinylated detection antibody and HRP conjugate.

The only wells that are blue in hue are those that contain the Avidin-HRP conjugate, Biotinylated Detection Antibody, and Human TLR-2. When stop solution is added, the reaction between the substrate and the enzyme is stopped and the color changes to yellow. Using spectrophotometry, the optical density, or OD, is measured at 450 nm ± 2 nm wavelength. The Human TLR-2 concentration is directly correlated with the OD value. By comparing the optical density (OD) for the specimens to the standard curve, you may determine the amount of human TLR-2 within the samples.

3-2-2-3-2. Serum Seapration Process for TLR2 Immunological Marker

Serum: samples were centrifuged for 20 minutes at 1000 ×g at 2–8°C after letting them clot for one hour at ambient temperature. The supernatant was gathered and used for the test.

3-2-2-3-3. Dilution Method

A one-step dilution is used for a 100-fold dilution. A 100-fold dilution was obtained by adding 5 μL of sample with 495 μL for sample diluent.

3-2-2-3-4. Reagent Preparation

1. Before use, all reagents were kept at ambient temperature (25°C).
2. Washing solution: 30 ml of concentrated washing solution was diluted with 720 ml of deionized water or distilled water to prepare 750 ml of washing solution. The concentrate was heated in a water bath at 40°C to prevent crystal formation and mixed gently to ensure complete dissolution.
3. Solution for working standard: The standard had been centrifuged for one minute at 10,000 × g. The reference sample and 1.0 ml of the standard diluent were added, allowed to sit for ten minutes, and then repeatedly mixed gently. Use a pipette to thoroughly mix it once it has dissolved. After reconstitution, 20 ng/ml was the functional solution. As needed, serial dilutions were prepared. A dilution gradient of 20, 10, 5, 2.500, 1.250, 0.630, 0.31, and at last 0 ng/mL was advised.
4. Diluting procedure: There were seven EP tubes utilized, and each tube got 500 μL of the sample diluent and reference standard. To create a ten ng/mL work solution, 500uL of a 20 ng/mL solution that worked was injected into the initial tube and combined. In accordance with this procedure, pipette 500 uL for solution from each prior tube into the last tube. For your reference, the illustration is provided below. We did not pipette the solution from the

preceding tube into the last tube since it was assumed to be empty. Just before use, a grade dilution standard solution that worked was made.

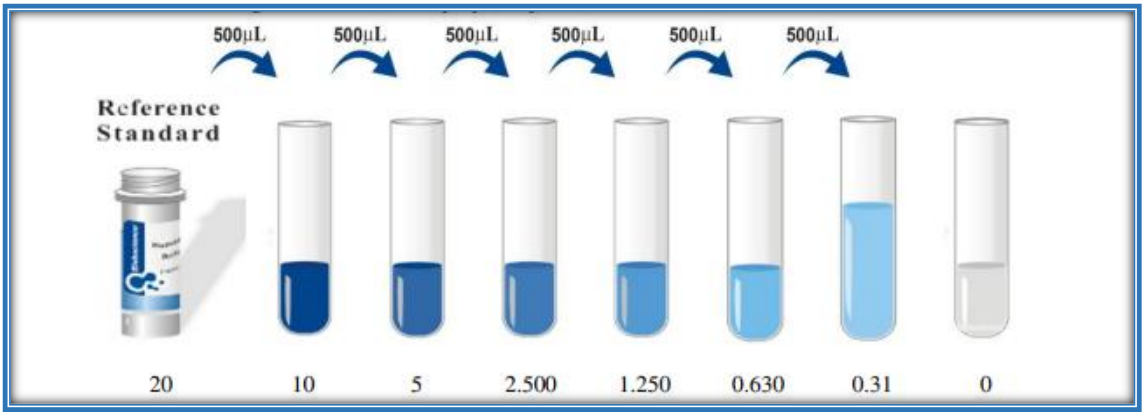

Figure (3-7): Reagent preparation of TLR2 Concentration according to E-lab science\ USA

5. Working solution for biotinylated Ab detection: Determine the necessary quantity (100 µL/well) in advance of the experiment. A bit more was prepared throughout the preparation process than estimated. Using concentrating biotinylated detection Ab Dilution (which is intensely biotinylated observation Ab: concentration detection using biotinylated Ab Diluent Equals 1:99), dilute 100× concentrating biotinylated detecting Ab to 1× work solution. Centrifugation is used for detecting concentrated biotinylated Ab around 800 × g for one minute. Before being used, a working solution was quickly constructed.
6. Working solution for concentrated HRP conjugate: HRP conjugate is an avidin that has been conjugated to HRP. Prior to the experiment, determine the necessary amount (100 µL/well). A bit more was prepared throughout the preparation process than estimated. Conjugated HRP diluent was used to dilute a concentrated HRP conjugate from 100× into 1× working solution after

centrifuging it at 800 × g for one minute (concentrated HRP conjugated: conjugated HRP diluent Equal 1:99). Before usage, a working solution was quickly constructed.

3-2-2-3-5 Assay procedure

1. After marking the wells for diluted standards, blank and sample. 100 µL of each dilution of standard, blank, and sample were added into the appropriate wells. The plate is covered with the sealant provided in the kit. Incubation was for 90 minutes at 37°C, taking care to add the solutions to the bottom of the ELISA micro dish without touching the inner wall and causing foam to appear as much as possible.
2. Following the removal of liquid from each well and a quick wash, 100 µl of the Biotinylated Detecting Ab working mixture was applied to each well. After applying a fresh coat of sealant, the plate was incubated at 37°C for one hour.
3. After the solution had been poured out of each well, 350 µL of additional washing solution was added. Then the solution was soaked about one minute, Next, pipette or dump the mixture from every well, letting it dry on fresh, absorbent paper. Three times was this washing phase carried out. This and subsequent washing stages were carried out in a microplate washer, and testing strips were utilized right away to ensure that the wells did not go dry.
4. 100 µL of HRP conjugated working solution was added to each well and the plate was covered with fresh sealant. Incubate the TM for 30 minutes at 37 $^{\circ}C.$
5. The solution was decanted from each well, and the washing process was repeated for 5 times as performed in step 3.

6. Add 90 µl of substrate reagent to each well. Cover the plate with fresh sealant. Incubate for 15 minutes at 37°C while protecting the plate from light. The microplate reader was heated for 15 min before measuring the OD.
7. Each well received fifty milliliters of stop solution; it should be taken into account that both the stop solution and substrate solution must be supplied in the same sequence.
8. Each optical density of well (OD value) was once measured via a plate reader set to 450 nm.

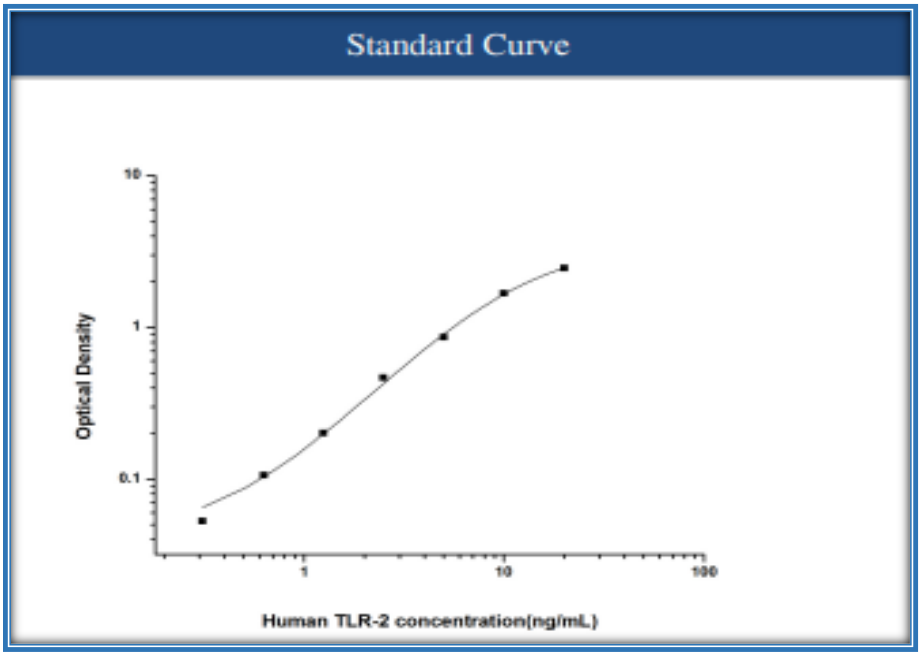

Figure (3-8): standard curve of TLR2 according E-lab science\ USA

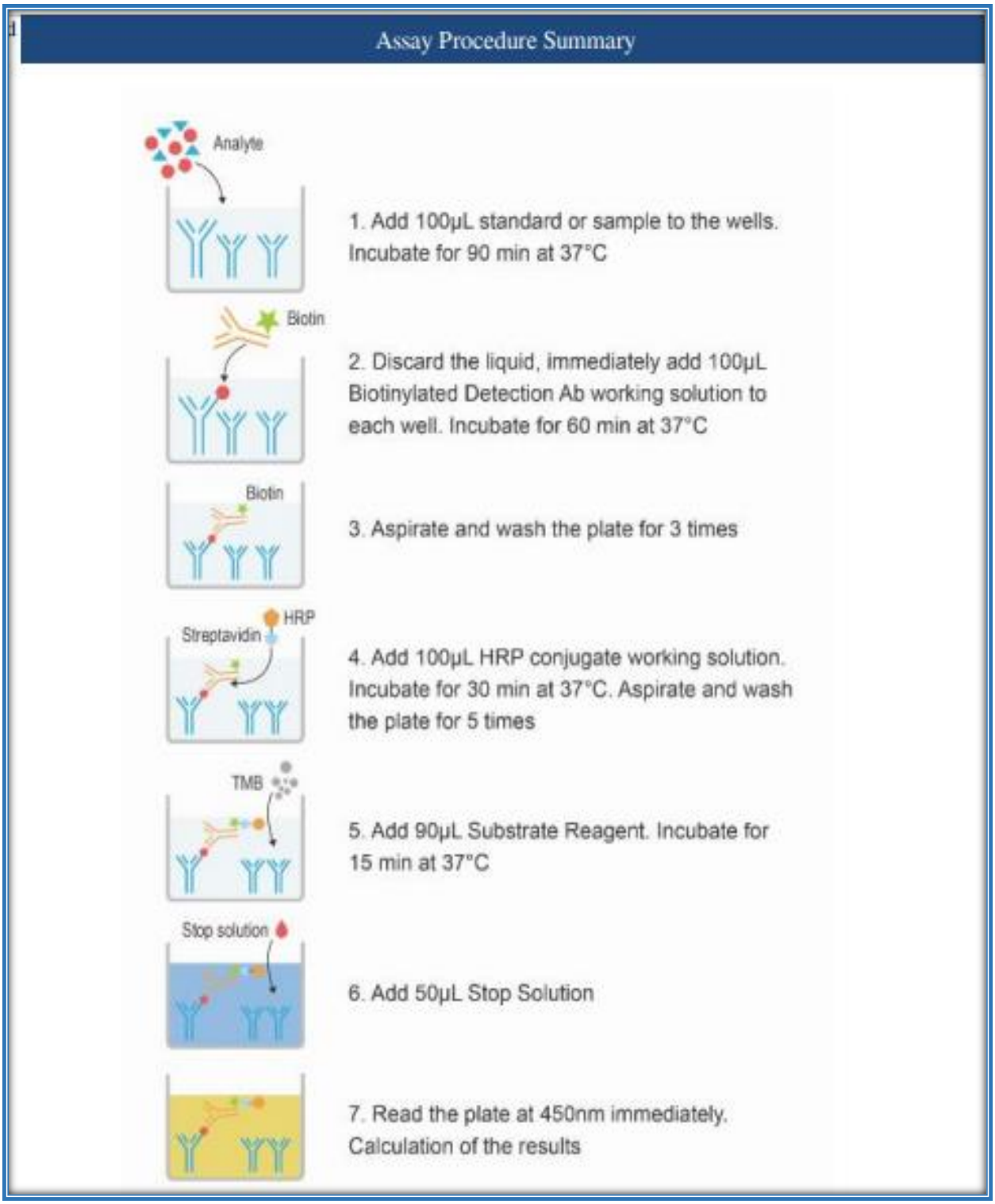

Figure (3-9): ELISA assay procedure summery According to Elabscience/USA specifications of Human TLR-2(Toll-like Receptor 2) ELISA Kit

3-2-2-4. Measurement of immunological marker TLR4

Test principle: This ELISA kit, the sandwich-ELISA concept is applied in this ELISA kit. The antibody designed for human TLR4 has been pre-coated on the micro-ELISA plate included in this kit. A micro-ELISA plate well is filled with samples (or standards) and the appropriate antibody. Subsequently, each microplate well is filled with a biotinylated antibody that is specific for human TLR4 and an Avidin-Horseradish Peroxidase (HRP) conjugate, which is then incubated. Free parts are removed by washing. In each well, a substrate solution is added. The only wells that will be blue in color are those that contain Avidin-HRP conjugate, Biotinylated Detection Antibody, and Human TLR4. When stop solution was added, the reaction between the substrate and the enzyme is stopped, and the color changes to yellow. With a wavelength of about 450 ± 2 nm, the optical density, or OD, is determined spectrophotometrically. The amount of human TLR4 is directly correlated with the OD value. By comparing the sample's optical density (OD) to the standard curve, you may determine the amount of human TLR4 within the samples.

3-2-2-4-1. Serum Seapration Process for TLR4 Immunological Marker

Blood Samples were centrifuged for 20 minutes at 1000×g at 2–8°C after letting them clot for one hour at the temperature of the room. The experiment was performed by collecting the supernatant.

3-2-2-4-2. Reagent Preparation

1. Before use, all reagents were kept at ambient temperature (25°C).
2. Buffer washing solution: 750 milliliters of buffer washing solution were made through thinning out thirty milliliters of focused washing solution as 720 milliliters deionized water. The concentrate had been heated in a water bath at 40°C to prevent crystals from forming, and it was then gently blended till the crystals were totally dissolved.

3. Standard working solution: Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 2000pg/mL (or add 1.0mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then the serial dilutions were done as needed. The recommended dilution gradient is as follows: 2000,1000,500,250, 125,62.5,31.25,0pg/mL.
4. Dilution procedure: Seven EP tubes were utilized. All tubes subsequently received 500 µl of the sample diluent plus a reference standard. To create a 1000 pg/mL working solution, pipette 500 µl of a 2000 pg/mL solution into the initial tube and stir. This stage involved taking 500 µl of liquid solution with a pipette that went from the preceding tube through the last tube (figure 3-10).
5. A workable method for identifying biotinylated ab was accomplished by calculating the necessary amount (100 µl/well) prior to the experiment. First, 100× Concentrated Biotinylated Ab Liquid was diluted into 1× working solution using Concentrate Dilution (which is concentrated Biotinylated Ab Dilution: Ab Diluent = 1:99). After that, the diluted biotinylated ab liquid had a centrifugation, beginning about a minute at 800 × g.
6. HRP conjugate work solution was done to determine the needed quantity (100 µl/well) in advance of the experiment. Pure HRP conjugation was diluted from 100× with 1× working solution via conjugated HRP dilution (mainly HRP compound: conjugated HRP diluent Equal 1:99) after being centrifuged at 800 / g for a minute.

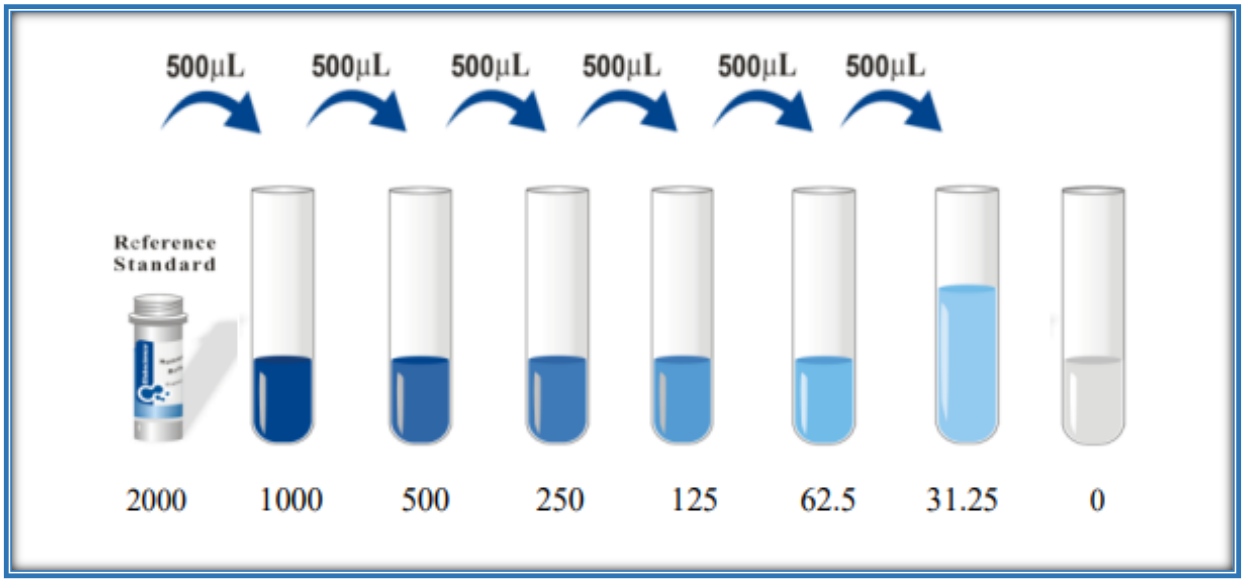

Figure (3-10): Reagent preparation of TLR4 Concentration according to E-lab science\ USA

3-2-2-4-3. Assay Procedure

1. After the wells were identified for the diluted standards, blank, and samples, 100 microliters per each standard dilution, blank and samples were added to the proper wells. The sealant included in the kit is applied to the plate. 90 minutes of incubation were spent at 37°C.
2. The liquid was filtered from each well and washed. A. Immediately add 100 µl of Biotinylated Detection Ab working solution to each well. Cover the plate with new sealant. Incubate for 1 hour at 37 °C.
3. The solution was decanted from each well, each well received 350 µL from washing solution. After soaking for one to two minutes, the solution was pipetted or emptied from each well, and the paper was allowed to air dry on fresh absorbent material. Three times was this washing phase carried out. For these and subsequent cleaning procedures, use a microplate washer. To prevent the wells from drying out, test strips were used right away following the cleaning process.

4. Each well received 100 µL about HRP conjugate solution that worked added to it. Thereafter, fresh sealer was applied to the panel. At 37 °C, incubate for 30 minutes.
5. Each solution of well was decanted, and as instructed in step 3, the washing procedure was carried out five times.
6. 90 µl of substrate reagent to each well was added. The plate was covered with new sealant has been completed. Incubate for 15 minutes at 37 °C. It is necessary to protect the plate from light and make sure to heat the microplate reader for 15 minutes before measuring the OD.
7. To every well, 50 microliters of stop solution had been applied. The substrate solution and the stop solution were added in the same sequence.
8. The optical density of each well was finally determined once. A microplate reader set to 450 nm had been used to evaluate this OD.

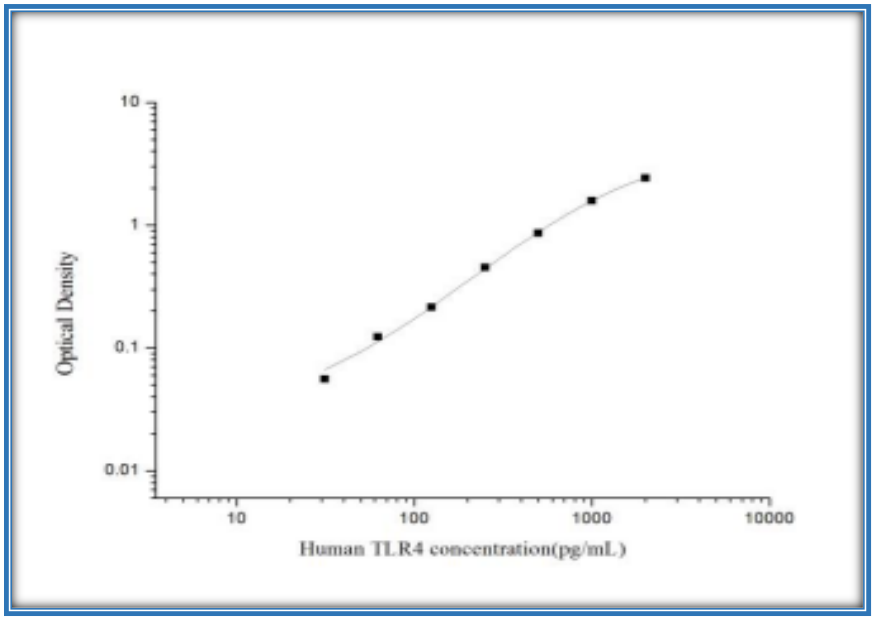

Figure (3-11): standard curve of TLR4 according to E-lab science/USA

3-2-3. Measurement of zinc concentration

3-2-3-1. Principle

Zinc in alkaline medium reacts with Nitro-PAPS to form a purple-colored complex. Intensity of the complex formed is directly proportional to the amount of zinc present in the sample as follow:

Alkaline medium

Zinc + Nitro-PAPS ⟶ Purple Coloured complex

3-2-3-2. Procedure

Using a Mindray apparatus, zinc was evaluated at room temperature with a wavelength and filter of roughly 570 nm/yellow and one centimeter of light path. The materials listed in Table 5 were arranged in blank, standard, and test-designated tubes that were dry and clean, in order. First, 1 milliliter of the working reagent was placed to the blank tube along with 0.05 milliliter of distilled water. Next, 1 milliliter of the working reagent was added to the standard tube along with 0.05 milliliter of zinc standard. Ultimately, the test tube was filled with 1 ml of the working reagent and 0.05 ml of the sample. After mixing was finished, the mixture was incubated for five minutes at room temperature. In order to calculate the zinc content using the following equation, the absorbance was measured in twenty minutes for both the standard absorbance and the test absorbance against the Blanck absorbance.

$200 \times (Abs.T/Abs\ S) = zinc\ mg/dl$

Table (1) refers to Blank, Standard and Test concentrations in ml to evaluate zinc levels in serum.

Table (3-5): components of Zinc kit

Addition Sequence	B(ml)	S(ml)	T(ml)
Working reagent	1.0	1.0	1.0
Distilled Water	0.05	---	---
Zinc Standard	---	0.05	---
Sample	---	---	0.05

3-2-4. Measurement of Vitamin D3

3-2-4-1. Test Principle

The Cobas e 411 system is an electrochemiluminescence (ECL)-based completely autonomous autoanalyzer used for immunological analysis. ECL technology makes use of interference suppression techniques, streptavidin-coated magnetic microparticles, and antigen/antibody interactions as its solid phase.

3-2-4-2. Procedure

Vitamin D evaluation by the Cobas e 411 system was depended on an electrochemiluminescence (ECL)-based completely autonomous autoanalyzer used for immunological analysis. ECL technology makes use of interference suppression techniques, streptavidin-coated magnetic microparticles, and antigen/antibody interactions as its solid phase. The Roche rack cup was adapted within the rack, the samples were loaded and also routine was loaded without barcodes, then it was filled up on supplies and was executed experiments, finally outcomes had gotten.

The process involves programming orders, preparing samples, correct placement on disks and racks, inserting Roche rack cup adapters, loading routines and samples with/without barcodes, loading consumables, running tests, and presenting results.

3-2-4-3. Overview of Orders

A list of all orders the tests that are performed on each sample are listed in orders. The cobas e411 analyzer is connected to a Laboratory Information System (LIS), which is where most laboratories program these assays.

The host connection is used to connect the Cobas e 411 analyzer to the LIS. In this document, the LIS is also referred to as the Host. Make sure you have the test samples, reagents, and calibrators you'll need. Put the data into the Cobas e 411 analyzer. After that, run the tests after loading the samples into the Cobas e 411 analyzer. The Cobas e 411 analyzers save the test findings when they have been completed. After that, you can review the reports, print them, upload the results to the host, and erase any previously printed or uploaded results.

3-2-5. General Urine Examinations

Urine testing remains important to obtain a definitive diagnosis in the management of urinary tract infection (UTI){McNulty, 2015 #249}, Urine samples were obtained from patients with urinary tract infection in an amount of approximately five ml of mid-urine, provided that they had not taken antibiotics within 48 hours, according to the World Health Organization's standard specifications guide.

For urinalysis, the urine sample was evaluated in three ways: visual examination, urine strip test, and microscopic examination.

- **Visual inspection:** The appearance of urine was examined with the assistance of a laboratory technician. The urine was normally clear, but sometimes it was cloudy or had an unusual odor, indicating the presence of a problem, such as an infection. The foamy appearance showed the presence of protein in the urine. The presence of blood in the urine makes it in red or brown colors.

- **Urine Dipstick Test:** A dipstick, a thin plastic strip with chemical strips on it, was placed in the urine. Chemical stripes change color when certain substances are present at high levels. The dip bar test examined the following:

pH: The pH level indicated the amount of acid present in the urine. The pH level indicated the presence of a disorder in the kidneys or urinary tract.

The focus: The concentration measurement indicated the degree of concentration of molecules in the urine. The increase in concentration resulted from the natural degree of concentration due to not drinking enough fluids.

Protein: Normally, protein levels in the urine were low. A slight increase in protein in the urine was not the cause for concern, but a large increase indicated a kidney problem.

Sugar: Normally, the amount of sugar (glucose) in the urine is too low to show up on the test. This test was a screening test to check for diabetes.

Ketones: Similar to sugar, detecting any amount of ketones in the urine was an indicator of diabetes.

Bilirubin: The presence of bilirubin in the urine indicates liver damage or disease.

Evidence of infection: The presence of nitrite or leukocyte esterase, a substance produced by white blood cells, in the urine indicates a urinary tract infection.

The blood: The presence of blood in the urine leads to another test. It is an indication of kidney damage or infection, kidney or bladder stones, kidney or bladder cancer, or blood disorders.

- **Microscopic examination:** This test is sometimes done as part of a urinalysis and involves examining drops of concentrated urine, which is urine that has been vortexed inside a specialized machine, using a microscope. When any of the following levels were higher than normal, further testing was done:

White blood cells (leukocytes): are an indicator of infection.

Red blood cells (erythrocytes): are an indicator of kidney disease, blood disorders, or other underlying medical conditions such as bladder cancer.

Bacteria, yeast, or parasites: are an indicator of infection.

Cylinders (tube-like proteins): due to kidney disorders.

Crystals: that form from chemicals in the urine are an indicator of kidney stones.

3.2.6. Bacterial diagnosis and culture of media

3.2.6.1. Culture of Media Preparation:

The growth medium and the solutions were autoclaved for 15 minutes at 121℃ and 15 lb/inch2, for the purpose of achieving sterilization, additional solutions that had been destroyed through heating were filtered through Millipore 0.22 and 0.45 m filters.

The manufacturer's instructions were followed in the preparation of all the media in the table. Distilled water was used to dissolve the components. All the ingredients were thoroughly melted and blended on the hot plate stirrer. Agar media were placed inside the dishes of Petri with regard to agar media as well as sterile tubes in the case of broth media after being autoclaved at 121 °C to sterilize them. To ensure sterility, the media were incubated for 24 hours at 37°C.

The culture media :MacConkey Agar, Mannitol-Salt Medium, the substances known as Eosin Methylene Blue which referred as (EMB), Peptone Water, Agar of nutrients, Nutrient Broth, Simmon Citrate Agar, the red methyl Voges Proskauer, which is termed MRVP medium, and triple sugar iron agar (TSI). In compliance with the guidelines provided by the manufacturer, the culture medium was prepared as follows:

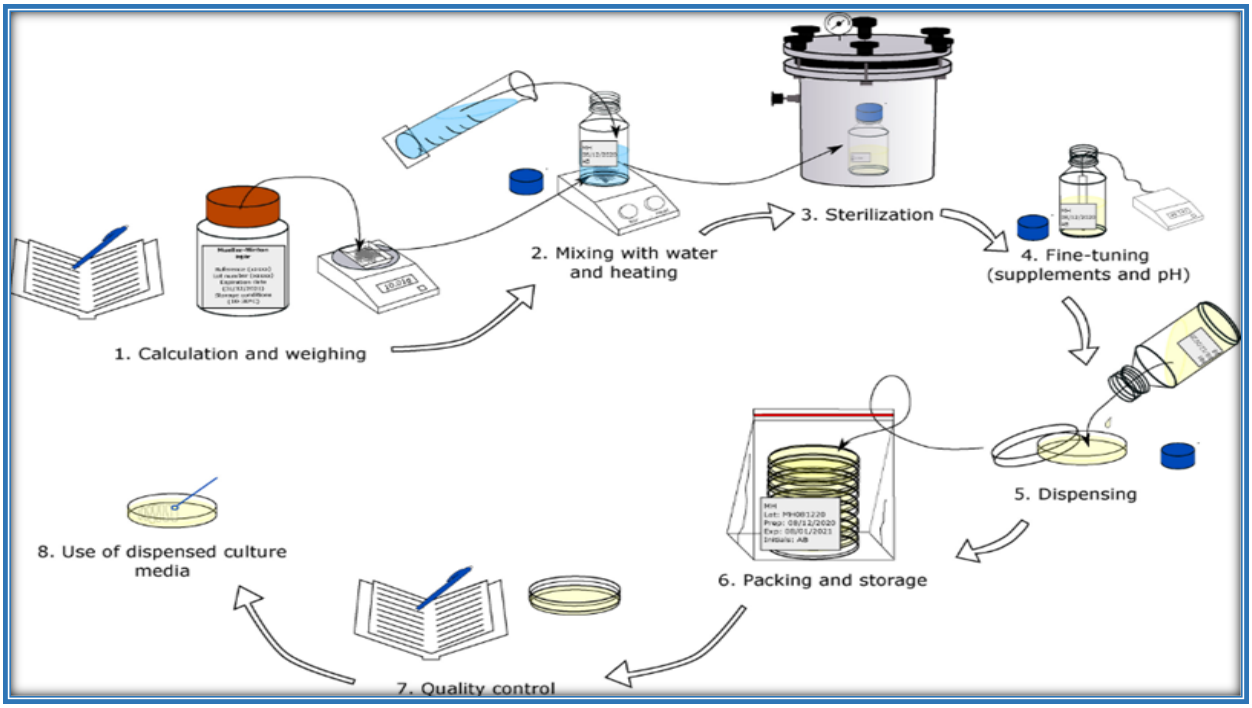

Figure (3-12): Preparation of a non-supplemented dehydrated agar medium (Orekan, *et al.* 2021).

A-Blood Agar Medium

Whole blood cells from humans were added after autoclaving 40g of the medium and a full liter containing distilled water, letting it cool to 45–50 ºC, and then adding the mixture, fully homogenizing it, and then pouring it into Petri dishes. Using this medium, bacteria were first isolated, and their capacity to hemolyze red blood cells (RBCs) was determined.

B-MacConky Agar Medium

The medium was prepared according to the manufacturer's instructions, 50 g was dissolved in 1000 ml of distilled water, A microwave oven was used to dissolve all components completely. The media were sterilized by autoclaving at 121°C, in Petri dishes in case of agar media and in sterile screw-tubes in case of broth media

inside the foil lid, The media were incubated at 37°C for 24 h to ensure sterility {JF, 2000 #250}.

3.2.6.2. Identification of Bacteria

A colony of each growth on the blood plate was taken, the MacConkey, and the mannitol, and we repeated the growth to purify the bacterial colony. The type of bacteria was identified based on the phenotypic characteristics of the shape, size, color, borders, and texture of the colony, as well as other characteristics such as blood hemolysis and fermentation of mannitol sugar. They were examined with an optical microscope after we took a clear colony and placed it on a new slide. We stained with the well-known four-step gram stain, and the period of each dye was adhered while monitoring the interaction of bacteria with each gram stain (Collee, *et al.*,1996; MacFaddin, 2000).

3.2.6.2.1. Vitek Utilization

Automated processes techniques are being used more frequently in microbiology laboratories to identify bacterial infections. A biochemical profile that enables organism identification, for instance, is provided by the Vitek System's compact plastic reagent cards that contain microliter volumes of various biochemical test media in 30 wells (Książczyk, *et al.* 2016).

Automatic introduction of an inoculum made from cultured samples onto the card is followed by sporadic color changes in the card brought on by the organism's metabolic activities. A computerized database is used to analyze, store, and print the data. These automated systems come in a wide variety of commercial variations, and several of them can be utilized for simultaneous identification and antimicrobial susceptibility testing.

The manufacturer's instructions were followed in order to prepare the bacterial suspension. A sufficient quantity of colonies was gathered from pure cultures that were developed for a whole day, transferred to a 12 x 75 mm transparent (polystyrene) test tube, then resuspended with 3 ml of sterile saline solution. A turbidity adjustment of 0.5 McFarland was made. Using the Densi-chek turbidimeter. The GN-ID used the same suspension as a 2-vitek compact system. Lastly, the sample tubes were inserted in the vitek-2 chamber together with the GN-ID cassette.

3.2.6.2.2. Tests of Biochemical Reactions

3.2.3.2.2. Biochemical Tests

Bacteria are rich in biochemical activities because they contain enzymes of a specialized physiological nature for each bacterial species, which facilitates their diagnosis through the chemical reaction in the following tests:

A- Haemolysin Production

It is noticeable that the clearly dissolved blood in the blood medium. We repeated the inoculation of this colony on new blood medium to incubate for a full day at a temperature of 37 ℃ to discover the beta hemolytic type of blood around the colony (De Boy *et al.*, 1980).

B- Catalase Test

Three grams of hydrogen peroxide were dissolved in about 100 ml of distilled water and kept it in a dark bottle (Forbes *et al.*, 2007). The colony was taken to be diagnosed to a drop of 3% H2O2 on a microscope slide. It was found out the formation of gas bubbles, which means that the test is positive for the presence of the catalase enzyme (Collee *et al.*, 1996).

C- Oxidase Test

0.1 g was dissolved of P-paraphenylenediamine tetrahydrochloride in 10 ml distilled water and stored it in a glass jar (Forbes et al., 2007). Filter paper was immersed in the oxidase reagent and distributed the desired colony on the paper, and the color changed from pink to purple, resulting in a positive oxidase test (Collee *et al.*, 1996).

D- Coagulase Test

Using the loop, As many colonies were transferred into a tube containing 0.5 ml of rabbit plasma. The nozzle was covered with a cotton tampon to prevent evaporation, and incubated at 37 ℃overnight. This tube was tilted to observe the clotting as a positive result of the examination. If the plasma remains clear, it is a negative result of the examination (Collee *et al*., 1996).

E- IMVIC TEST

To acquire the results of these four tests-tryptone broths (indole test), methyl red (MR), Voges Proskauer broth (VP broth), and citrate tubes were inoculated. IMViC tests are used to identify and distinguish members of the Enterobacteriaceae family.

Next general guidelines for carrying out IMViC tests and interpreting the results were used. The following tests was run after cultures of any Enterobacteriaceae member have grown for 24 to 48 hours at 37°C:

1- Indole Test:

The required bacteria was activated on a suitable nutrient medium and incubated them for a full day at 37 ℃ in preparation for the indole test by adding about 6-8 drops of Kovach's reagent (p-dimethyl amino benzal dehyde in amyl alcohol). As a

result, a red ring was formed at the top of the nutrient medium as a positive sign, while a yellow ring appeared as a negative sign (MacFaddin, 2000).

2- Methyl Red Test:

About 0.1 g of methyl red was dissolved in a volume of 300 ml of 99% ethanol. Then 200 ml of distilled water was added to bring the total volume to 500 ml (MacFaddin, 2000). 5 ml of MR-VP culture medium were taken and inoculated with the bacteria to be diagnosed and incubated it for a full day at 37 ℃. We added about 6-8 drops of the reagent to the bacterial culture and its color changed to red-orange as a positive result (Collee *et al.*, 1996).

3- Voges Proskauer Reagent:

In compliance with Collee's (Collee *et al*., 1996) methodology, this reagent was employed, which is composed of five grams containing α-naphthol dissolved in 100 milliliters of 99% ethanol (reagent A). According to Collee *et al.* (1996), Reagent B is forty grams of KOH over 100 ml about distilled water. 5 ml of the MR-VP broth that had been cultured with the organism was used for the experiment, and it was left to incubate for 24 hours at 37°C. After adding a total of 15 drops containing 5% α-naphthol (reagent A) and 10 drops for 40% KOH (reagent B), the mixture was thoroughly agitated and let to stand for up to half an hour before the reaction was declared negative. According to Forbes *et al.* (2007), the positive culture would glow red on the liquid's surface and progressively spread throughout the tube.

4- Simon Citrate Test:

After the Simon Citrate material was sterilized in an autoclave at 121 ℃ in a quarter of an hour until we cooled it to about 50 ℃, it was poured in a slanted manner into tubes and inoculated it with the bacterial culture and incubated it for a full day at a

temperature of 37 ℃. A change in the color of the medium to blue appeared as a positive result, but when it remained green it was A negative result (Benson, 1998).

5- Test for Triple Sugar Iron Media (TSI):

The test was used to separate the Enterobacteriaceae based on their ability to produce hydrogen sulfide and ferment carbohydrates. The organism was injected during one day over after being cultured on TSI slant via stab and streak. The medium's color changed from orange-red to yellow as a result of the fermentation of carbohydrates, either with or without gas production at the butt of the slant. Furthermore, a black precipitation was observed near the butt after the generation of hydrogen sulfide (MacFaddin, 2000).

6- Test for Urease:

The urea-based agar was autoclaved for 15 minutes at 121 ℃ to sterilize it. It was cooled to 50°C before the sterilized urea reagent was added and put into sterile tubes. Bacterial cultures were then added, and the mixture was incubated at 37°C for 24 to 48 hours. The outcome was a rich shade of pink. A negative response occurred when the deep pink color failed to develop (MacFaddin, 2000).

7- Statical analysis:

For statistical analysis, Social Science Studies (SPSS, edition 26) was chosen as the statistical software program. The present results are presented as mean ± standard error (mean ± SE). As for charts and graphs, they are drawn using Graph Pad Prism 9 Performing statistical analysis to determine the differences that reach the substantive level using the independent sample T test to examine the two sets of data. The ANOVA test was also used to compare three or more different groups if the data were normally distributed. The Square-Chi square was used to analyze the data statistically and determine the significant differences.

Chapter Four

Results

4. Results

The study given some results:

4-1 Study population and demographics of subjects

This study included 80 participants (60 patients and 20 controls), about 30 female and 30 male for patient, and the ages ranged from 18 to 55 years the sex distribution was 50% females and 50% males, the ratio of females to males was 1:1, while in control there was 6 female and 14 male, The number of both sexes in UTI cases and healthy control groups were demonstrated in Figure (4-1). The ages of the participants (patients and controls) ranged from 18 to 50 years. Patients group and controls were divided into three age groups, which were 18-28, 29-39, and 40-50. There were no significant differences between females and males regarding sex groups in figure (4-1) and there were also no significant differences among age subgroups of patients in figure (4-2) & Table (4-1).

Table (4-1): Number of the participants (patients and controls) based on the age groups and sex type

Age Groups	Patients	Controls	Sex	Patients	Controls
18-28	20	7	Females	30	6
29-39	22	7	Males	30	14
40-50	18	6	-----	-----	-----
The total	60	20	-----	60	20
Chi-square= 0.02 P value= 0.99			Chi-square= 2.42 P value= 0.12		

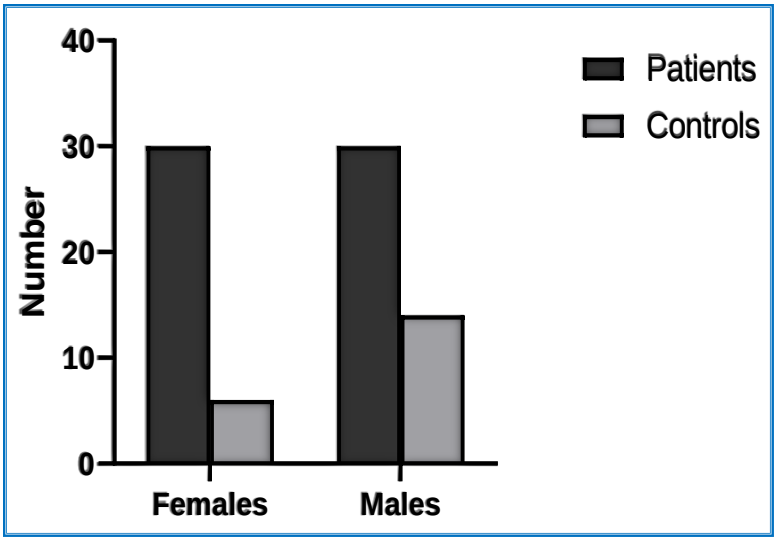

Figure (4-1): Number of the participants in each UTI cases and healthy controls based on sex groups.

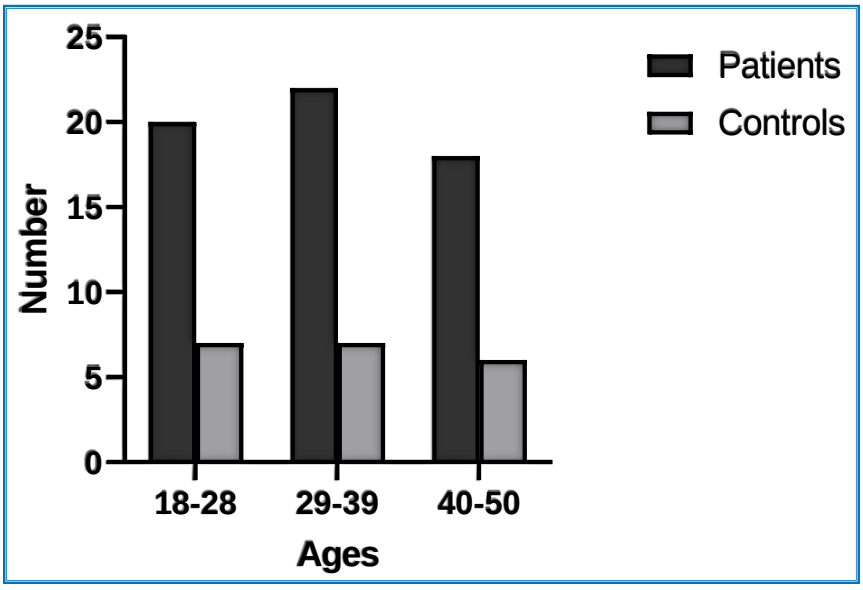

Figure (4-2): Number of the participants in each UTI cases and healthy controls based on age groups.

Table (4- 2) demonstrated the demographics characteristic of the study groups; the age range of both groups was (18- 50) years. It was found that most of the patients had a significant differences in the mean level of pus cells , RBCs, epithelial cells , P value were <0.001, as presented in Table (4-2).

Table (4-2): Demographics of the subjects (patients and controls) included in the study.

Characteristics	Patients		Controls		P- value
	Number, (%)	Mean±SE	Number	Mean±SE	
Number	60	-----	20	-----	-----
Age range	18-50	-----	18-50	-----	X^2= 0.02 P = 0.99
Sex: Females **Males**	30 (50%) 30 (50%)	-----	6 (30%) 14 (70%)	-----	X^2= 2.42 P = 0.12
Recurrent **Non-recurrent**	35 (58.3%) 25 (41.6%)	-----	-----	-----	-----
Pus Cells	-----	17.78± 2.163	-----	1.750± 0.099	<0.0001***
RBCs	-----	5.850±1.223	-----	2.000±0.2052	0.0041**
Epithelial Cells	-----	13.08±1.366	-----	4.500±0.5000	<0.0001***
Specific Gravity	-----	1.040±0.0163	-----	1.025±0.0019	0.6463 (NS)

4-2 Levels of immune markers (IgG, IgM, TLR2, TLR4) in patients and controls groups

4-2-1 Concentrations of IgG and IgM in patients and controls

The prevalence of antibodies (IgG and IgM) in UTI patients and controls was the subject of this investigation. IgG levels were found to be high in all UTI patients during the comparison with the group of controls. The IgG mean concentration in sera of the patients was (17.68) ng/ml, while in control group was (10.57) ng/ml with a significant differences, p value was (0.02), as presented in figure (4-3) and table (4-3).

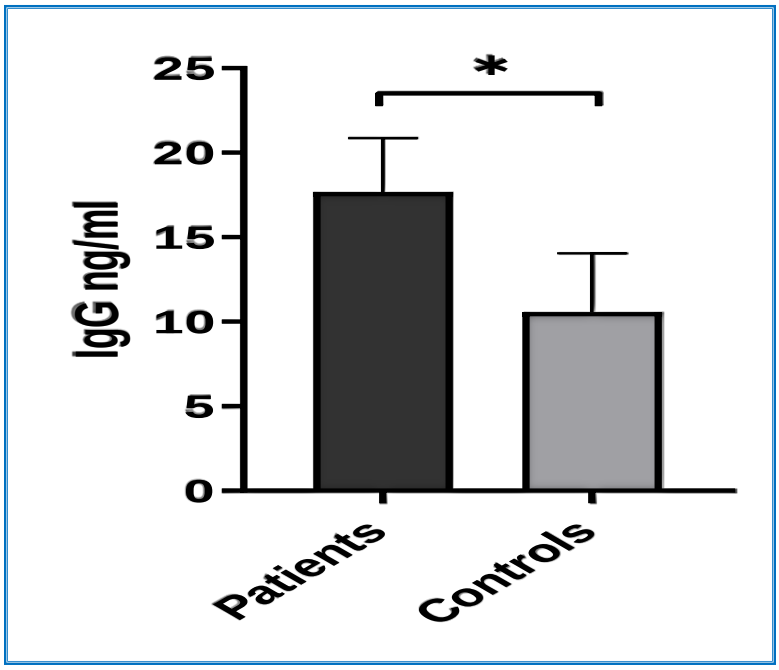

Figure (4-3): The Mean ± SE of IgG level between patient's urinary tract infection and control groups.

On the other hand, IgM levels were found to be lower in UTI patients as a comparison with the group of controls. The IgM mean concentration in sera of the UTIs patients was (153.7) ng/ml, while in controls group was (366.8) ng/ml, p value was (0.34) without significant differences, as presented in figure (4) and table 3.

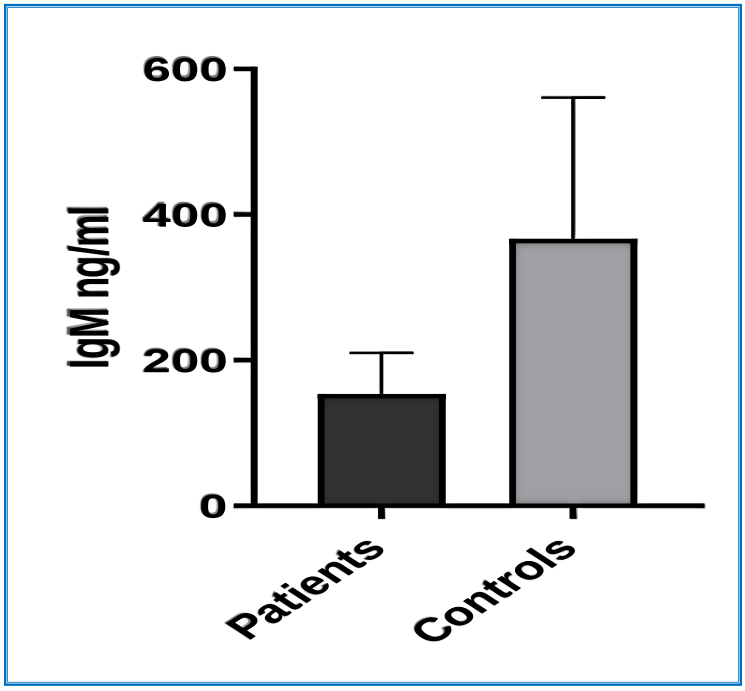

Figure (4-4): The Mean ± SE of IgM level between patients of urinary tract infection and controls.

Table (4-3): The mean differences of IgG and IgM level between patients of urinary tract infection and controls.

The Groups	Mean ± SE	
	IgG (ng/ml)	IgM (ng/ml)
Patients	17.68± 3.18	153.7±56.32
Controls	10.57± 3.49	366.8±193.8
P-value	0.02 *	0.34 (NS)
*** (P<0.05), NS: Non-Significant**		

4-2-2 Concentrations of TLR2 and TLR4 in patients and controls

This study was evaluated the level of TLR2 and TLR4 in patients of urinary tract infections. The results indicated that the serum level of TLR2 in patient group was increased about two times significantly compared to controls group. The mean level of TLR2 in patient group was 0.23ng/mL while in the control group was 0.16ng/mL, the p value was (0.04), as presented in figure (5) and table 4.

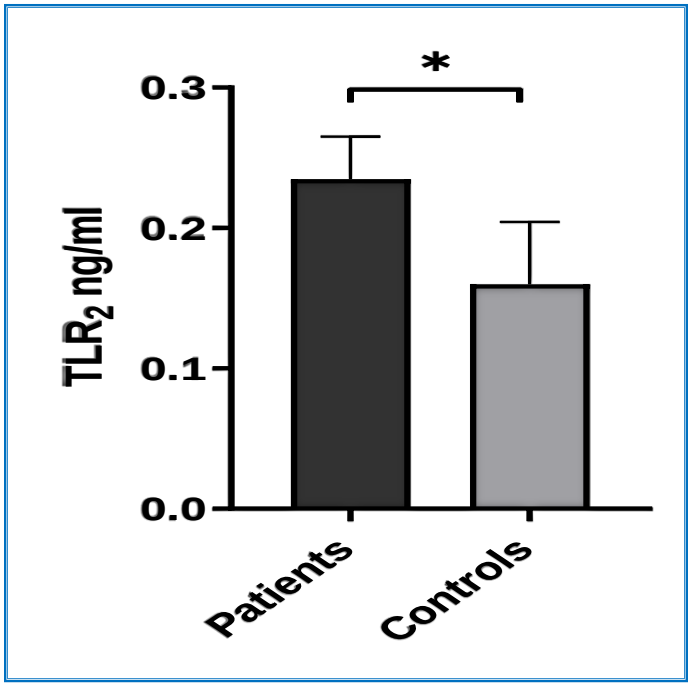

Figure (4-5):The ean ± SE of TLR2 level between patients of urinary tract infection and controls group.

While a non-significant increase in the serum level of TLR4 was seen when compared the mean level of TLR4 in UTI patients group with a group of healthy indivuals. levels of mean as TLR4 in patient group was 215.6 pg/mL while it was 187.6 pg/mL in the group of healthy individuals, the p value was (0.39), as presented in figure (6) and table (4).

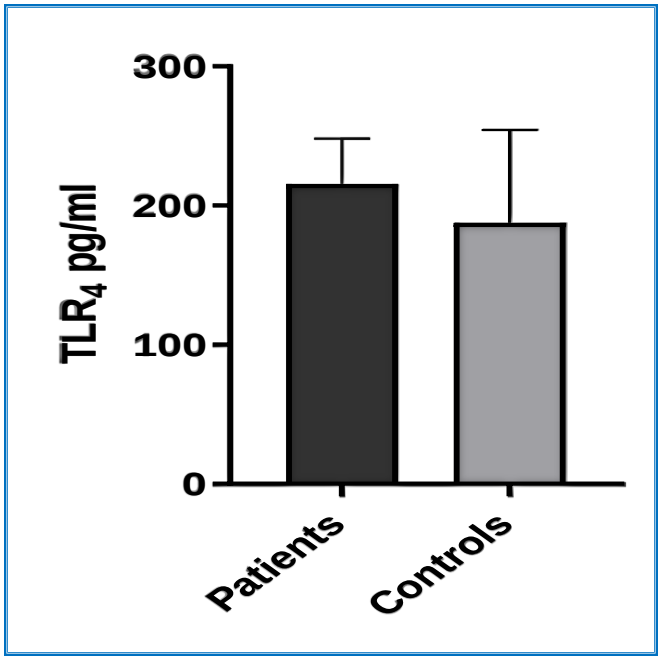

Figure (4-6): The Mean ± SE of TLR4 level betwen patients of urinary tract infection and controls group.

Table (4-4): The Mean differences of TLR_2 and TLR_4 levels between patients of urinary tract infection and controls group.

The Groups	Mean ± SE	
	TLR_2 (ng/ml)	TLR_4 (pg/ml)
Patients	0.23±0.03	215.6±32.64
Controls	0.16±0.045	187.6±66.76
P-value	0.04*	0.39 (NS)
*** (P<0.05), NS: Non-Significant**		

4-3 Concentrations of Vit. D_3 and Zn in patients and controls

The statistical analysis did not show any differences between UTI patients and healthy controls in vitamin D3 levels. Figure 7 and table 5 below show the mean of D3 in the groups of patients and healthy individuals. The differences between the two groups were insignificant, p value > 0.05.

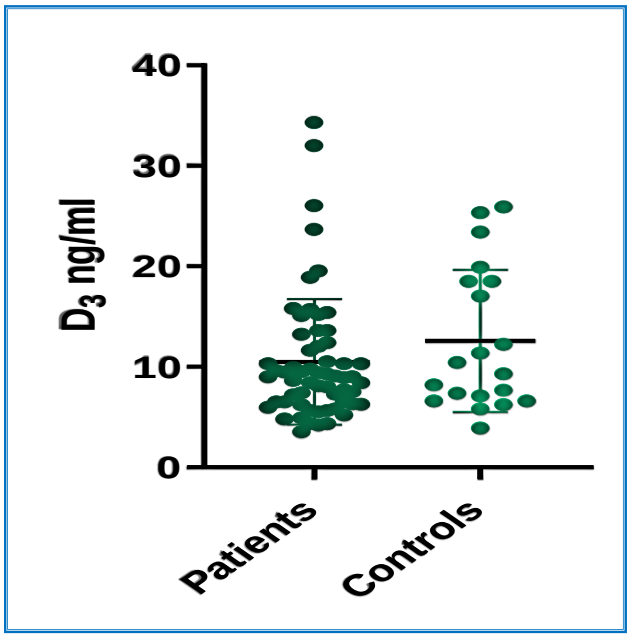

Figure (4-7): The concentration of Vit D3 level between patients of urinary tract infection and controls group.

UTI patients had mean serum zinc levels that were lower than those of controls. The levels mean of Zn in patients' group was 83.0 mg/dL while in the healthy individuals group was 86.27 mg/dL, the p value > 0.05, as presented in figure (8) and table (5).

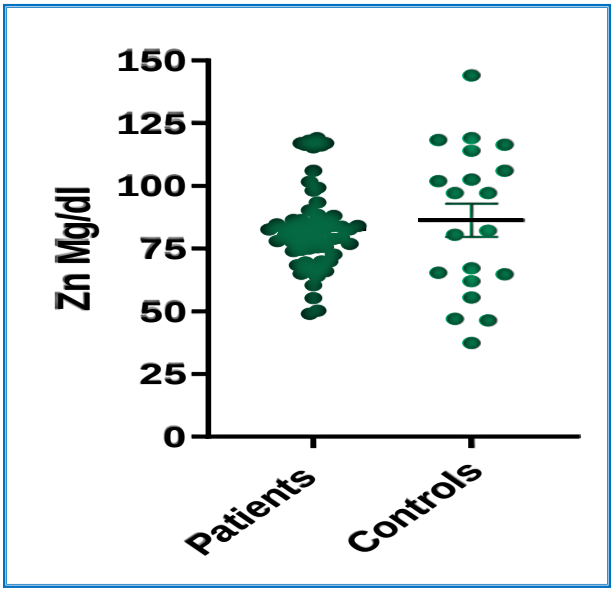

Figure (4-8): The concentration of Zn level between patients of urinary tract infection and controls group.

Table (4-5): The Mean differences of Vit D3 and Zn level between patients of urinary tract infection and controls group.

The Groups	Mean ± SE	
	Vit. D_3 (ng/ml)	Zn (mg/dl)
Patients	10.50±0.81	83.00±2.136
Controls	12.58±1.58	86.27±6.612
P-value	0.28 (NS)	0.77 (NS)
NS: Non-Significant		

4-4 The types of bacteria that were registered in UTI patients

Uropathogens have a well-established global prevalence. The common uropathogen's distribution is largely influenced by geographical location, with each region displaying a unique pattern of uropathogens. In the current study, results showed that the *Escherichia coli* was the most common type of bacterial that might be involved in the UTI with about 38.33% of the total, followed by *Staphylococcus epidermidis* (21.66%). *Klebsiella pneumoniae* also was registered in the current study by 5%. The less common type was *Staphylococcus aureus* by (1.66%). The percentage of the patients who did not show any bacterial growth was 16.66, as presented in table (6).

Table (4-6): The percentage of infection for the type of bacteria in UTI patients.

Type of Bacteria	Number (%)
Escherichia coli	23 (38.33%)
Staphylococcus epidermidis	13 (21.66%)
Pseudomonas aeruginosa	10 (16.66%)
Klebsiella pneumoniae	3 (5%)
Staphylococcus aureus	1 (1.66%)
No growth	10 (16.66%)
The total	60 (100%)

4-5. Concentration ofTLR2 and TLR4 according to the distribution of gram (-) and gram (+) bacteria

This study was also involved measurement of the TLR 2, and TLR4 levels based on the types of bacteria included Gram-negative pathogens and gram positive The types of the bacteria was presented in the table (4-6). Figure (4-9) demonstrated that the mean level of TLR 2 in Gram-negative pathogens which was lower than their level in gram positive.

In gram positive pathogens, the serum level of TLR2 was 0.35ng/mL, while in gram negative pathogens, the serum level of TLR2 was 0.2ng/mL.

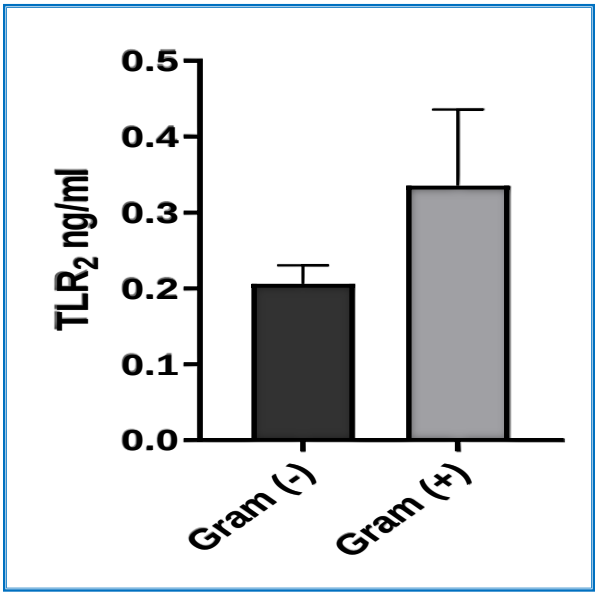

Figure (4-9): The Mean ± SE of TLR2 level according to the distribution of gram (-) and gram (+) bacteria in patients with Urinary tract infection.

On the other hand, in figure (4-10) the mean level of TLR 4 in Gram- positive pathogens was lower than their level in Gram-negative group.

The mean level of TLR4 was 190pg/mL in Gram- positive infection, while TLR4 was 210 pg/mL in Gram-negative group, there were not significant differences between both groups since the p value > 0.05.

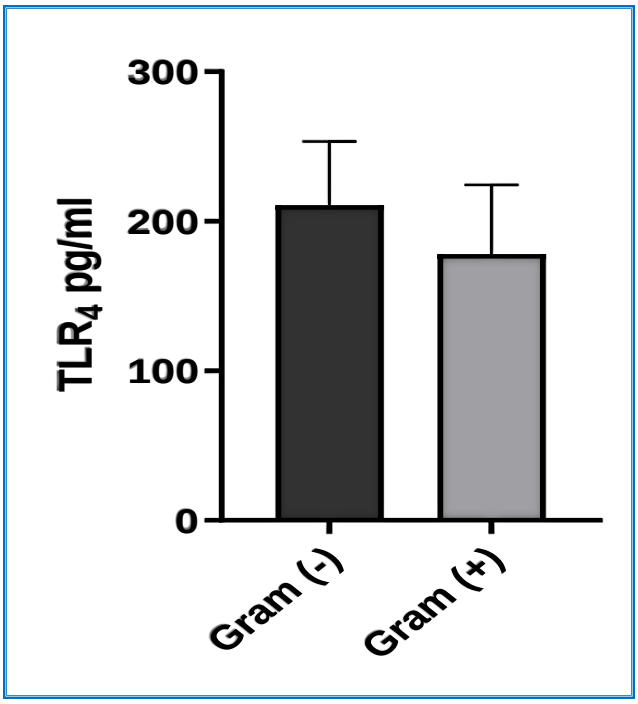

Figure (4-10): The Mean ± SE of TLR4 level according to the distribution of gram (-) and gram (+) bacteria in patients with urinary tract infection.

4-6. Levels of immune markers (IgG, IgM, TLR2, TLR4) recurrent and non-recurrent infections

4.6.1 Concentration of IgG and IgM in recurrent and non-recurrent infections.

The following immunological investigations were also carried out as part of the assessment included identifying the association between IgG and IgM in serum of recurrent and non-recurrent urinary tract infections patient groups. In both recurrent and non-recurrent groups, there was a massive significant difference in the mean level of IgG compared to the controls group, as shown in table (7) & figure (11) , The mean level of IgG in recurrent infections was (14.13) ng/ml, in non-recurrent infections was (22.65) ng/ml and in healthy individuals were (10.57) ng/ml, the p value was (0.038).

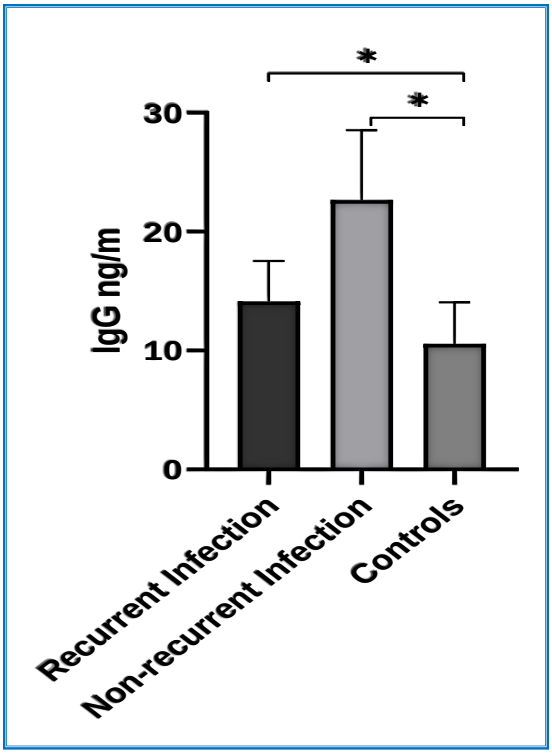

Figure (4-11): The Mean ± SE of IgG level in recurrent and non-recurrent urinary tract infection groups compared to controls group.

while in figure (12) IgM was shown non-significant increase in non-recurrent group compered to recurrent infection and healthy individuals. The mean level of IgM in recurrent infections was 77.68 ng/ml, in non-recurrent infections was 260.1 ng/ml and in control was 238.4 ng/ml, the p value > 0.05.

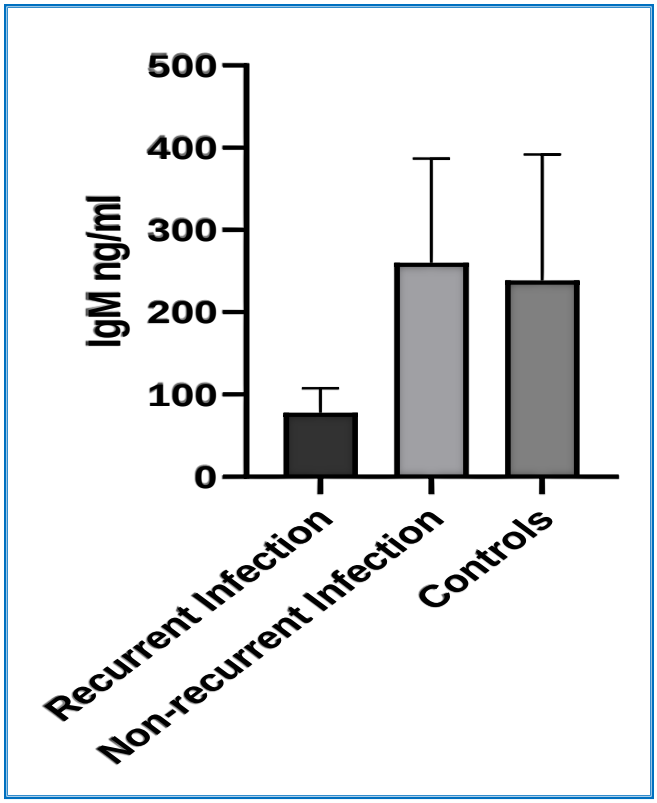

Figure (4-12): The Mean ± SE of IgM level in recurrent and non-recurrent groups of urinary tract infection compared to group of controls.

Table (4-7): Mean differences of IgG and IgM level in recurrent and non-recurrent groups of urinary tract infection compared to group of controls.

The Groups	Mean ± SE	
	IgG (ng/ml)	IgM (ng/ml)
Recurrent infections	14.13± 3.45	77.68± 30.11
Non-recurrent infections	22.65± 5.89a	260.1± 126.9
Controls	10.57± 3.49a	238.4± 153.5
P-value	0.038 *	0.32 (NS)
*** (P < 0.05)** **NS: Non-Significant**		

4-6-2. Concentration of TLR2 and TLR4 in recurrent and non-recurrent infections.

In this study, as shown in table (8) both serum level of TLR2 and TLR4 were increased in the non-recurrent infections , and only TLR2 was indicated a significant increase compared to the recurrent infection group and healthy individuals group, the p value was (0.006) and individual susceptibility to infection can vary based on the frequency and severity of the disease. The serum level of TLR2 in recurrent infections was 0.22ng/mL, in non-recurrent infections was 0.27n g/mL and in healthy individuals group was 0.17ng/ml, as presented in figure (13).

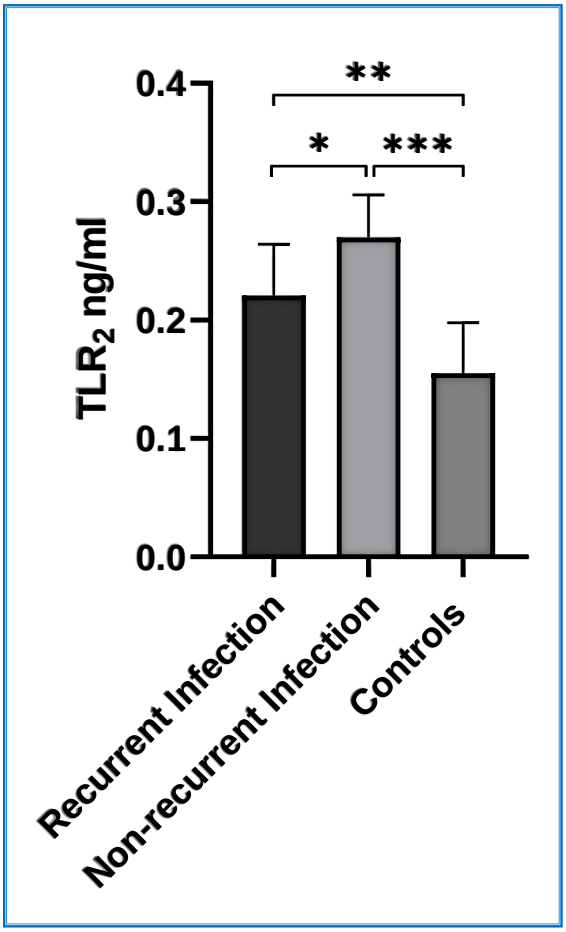

Figure (4-13):The Mean ± SE of TLR2 level in recurrent and non-recurrent groups of urinary tract infections compared to group of controls.

Mean level of TLR4 was 210.1pg/mL in recurrent infections, and 214.3pg/mL in non-recurrent infections and in healthy individuals group was 187.6 pg/ml, there were not significant differences among all groups since the p value > 0.05, as presented in figure (14).

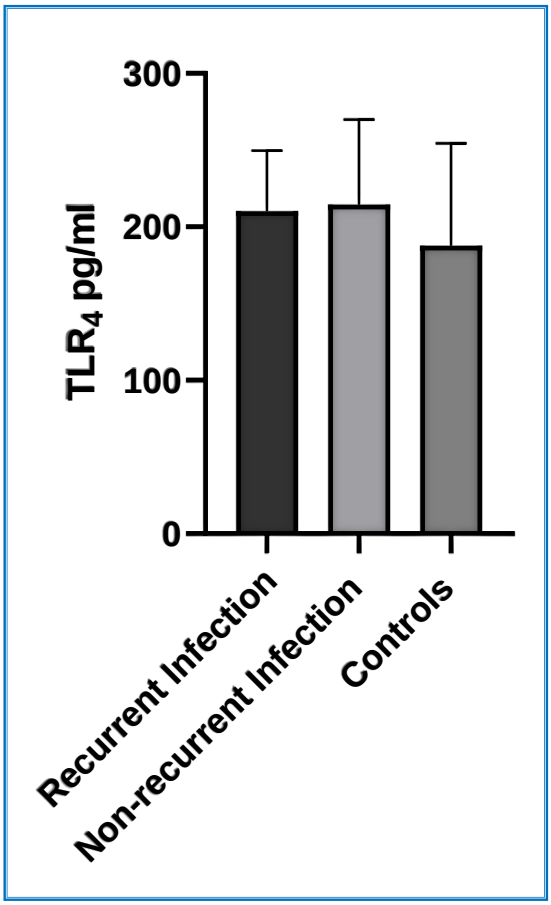

Figure (4-14): The Mean ± SE of TLR4 level in recurrent and non-recurrent groups of urinary tract infections compared to a group of controls.

Table (4-8): Mean differences of TLR2 and TLR_4 level in recurrent and non-recurrent groups of urinary tract infections compared to a group of controls.

Groups	Mean ± SE	
	TLR_2 (ng/ml)	TLR_4 (pg/ml)
Recurrent infections	0.22±0.044	210.1±39.65
Non-recurrent infections	0.27±0.036	214.3±55.57
Controls	0.17±0.042	187.6±66.76
P-value	0.006**	0.76 (NS)
****(P<0.01) * (P < 0.05) NS: Non-Significant**		

4.7 Concentration of Vit. D3 and Zn in recurrent and non-recurrent infections.

In the table (4-2) about demographic of subjects included in the study, patients were divided into subgroups: 35 (58.3%) of them were diagnosed as a Recurrent Urinary tract infection while 25 (41.6%) were diagnosis with Non-recurrent Urinary tract infections an important role for vitamin D in innate immunity has been identified. It has received a lot of attention lately because of its role in the pathophysiology of UTIs. In this study, the mean level of both Vit. D3 and Zinc were measured in recurrent and non-recurrent urinary infections groups. In study group subjects' vitamin D levels were non-significantly lower than those of the healthy individuals. Patients with non-recurrent UTIs presented non significantly lower levels of vitamin D than those with recurrent infection and controls as presented in table (9). The mean level of vitamin D in the recurrent infections was 11.50 ng/ml, in non-recurrent infections was 9.085 while in healthy individuals group was 12.58 ng/ml, as shown in figure (15).

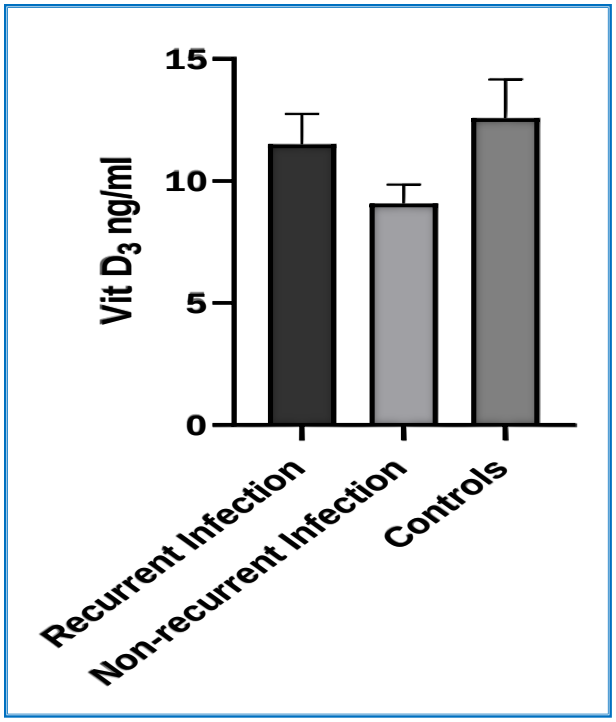

Figure (4-15): The Mean ± SE of Vit D level in recurrent and non-recurrent groups of urinary tract infections compared to group of controls.

On other hand, the mean level of zinc in the recurrent infections was (83.27) mg/dl, while in the non-recurrent infections group was (82.63) mg/dl and it was (88.84) mg/dl in healthy individuals group, for both biomarkers the p value were > 0.05 , as shown in figure (16) and table 9.

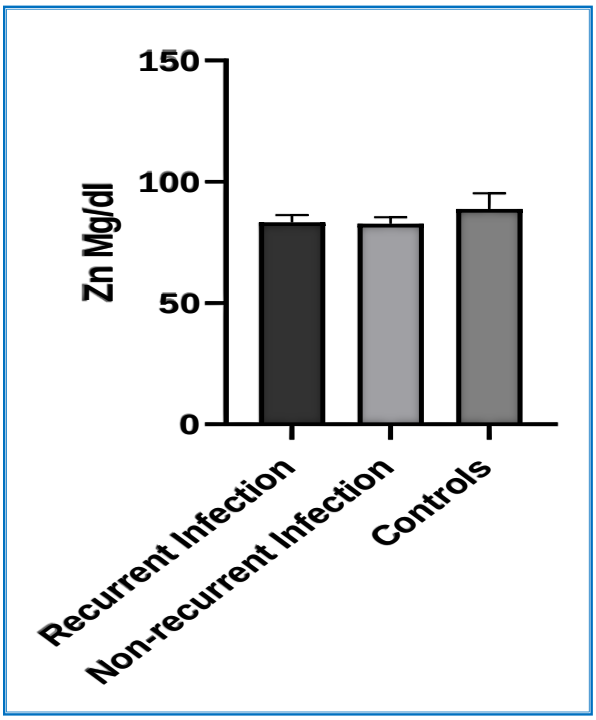

Figure (4-16): The Mean ± SE of Zn level in recurrent and non-recurrent groups of urinary tract infections compared to group of controls.

Table (4-9): Mean differences of Vit D and Zn level in recurrent and non-recurrent groups of urinary tract infection compared to group of controls.

Groups	Mean ± SE	
	Vit. D3 (ng/ml)	Zn (Mg/dl)
Recurrent Infections	11.50±1.26	83.27± 3.076
Non-recurrent Infections	9.085±0.77	82.63± 2.851
Controls	12.58±1.58	88.84± 6.421
P-value	0.32 (NS)	0.5377 (NS)
NS: Non-Significant		

4-8. Correlations among the different parameters in patients with UTI

Considering the important role of the measured parameters in UTI patients, the Spearman rank test analysis of such patients was used to show the response relationship between parameters.

Identifying patients using biomarkers is a challenging task, and numerous studies and research efforts have been conducted to address this issue.

TLRs, found in numerous immune and nonimmune cells, play a crucial role in host defense and are considered the center of the immune response . Regarding the TLRs 2 & 4, it was shown a weak non-significant relationship with the most measured parameters included: (IgG, IgM and Zn). The correlation study demonstrated a significant relationship only between TLR2 and Vit D3, *P* values were < 0.05, as shown in table (10).

Table (4-10): The correlation coefficients and p-value among the measured parameters.

Parameters	Correlation coefficient (R)	P -value
IgG vs. TLR_2	0.035	0.796
IgG vs. TLR_4	0.059	0.657
IgM vs. TLR_2	0.056	0.683
IgM vs. TLR_4	-0.019	0.890
IgG vs. D_3	0.002	0.988
IgG vs. Zn	0.003	0.983
IgM vs. D_3	-0.043	0.746
IgM vs. Zn	-0.045	0.733
TLR_2 vs. D_3	-0.277	0.040 *
TLR_2 vs. Zn	-0.048	0.723
TLR_4 vs. D_3	-0.098	0.469
TLR_4 vs. Zn	0.041	0.763
***(P value <0.05)**		

Chapter Five

Discussion

5. Discussion

5-1. Study population and demographics of subjects

Biological sex has an important effect for females and males regarding phenotypically diverse immune patterns, such as the immune response to diseases on mucosal surfaces. The difference between the sexes plays an important role in the mechanism of response to urinary tract infections (Deltourbe *et al.*, 2022) . The importance of urinary tract infections is a big difference between females and males in terms of infectious diseases, and the evidence for this is that women before menopause are 20-40 times more susceptible to urinary tract infections when compared to men of similar age (Ingersoll, 2017).

One of the most important theories explaining the difference between the sexes is the anatomical difference, The short distance between the urethral and anal openings in females compared to the length of the urinary tract in males is the best example of the difference between the sexes, and therefore it is one of the most important influences causing the spread of the urinary tract in humans (Lacerda Mariano & Ingersoll, 2020).

The susceptibility to infection, taking into account the sex difference in urinary tract infection, is highest among adults under fifty years of age, according to the association with the level of the hormones estrogen and testosterone, which are at the highest level, respectively, among females and males (Foxman & Brown, 2003). The incidence of urinary tract infections increases after puberty for women, when the level of estrogen rises, compared to high testosterone and low estrogen in adult men, which protects them from the risk of exposure to urinary tract infections (Ober *et al.*, 2008).

In general, men exposed to urinary tract infections are at risk of chronic urinary tract infections, in addition to the fact that morbidity and mortality rates increase due to

complicated urinary tract infections (Ki *et al.*, 2004). Then, as men and women experience declines in estrogen and testosterone levels following menopause and andropause, respectively, the prevalence of UTI increases in both sexes, making males at roughly equal risk with women at the same age (Foxman, 2010).

5-2. Levels of immune markers (IgG, IgM, TLR2, TLR4) in patients and controls groups

5-2-1 The level of immune markers IgG and IgM in UTI patients and controls

Even in the absence of established risk factors, recurrent urinary tract infections (rUTI) pose a significant healthcare and financial burden as well as an impact on patient morbidity and quality of life, Immunoglobulins are a class of serum proteins that are important for their antimicrobial activity in UTIs, with the IgM type serving as an indicator of a new infection with pathogens (Abraham & Miao, 2015).

Reduced IgM levels in the current study were consistent with prior studies, which reported that IgM titers dropped precipitously, it is also feasible that inadequate IgM detection in human research is related to the timing of collection and the challenge of anticipating the early beginning and reaction to bacteriuria and infection. This might indicate that IgM may not react immunologically in a UTI. Serum immunoglobulins IgG was 700-1600 mg/dl and IgM level were 40- 230 mg/dl(Gonzalez-Quintela et al., 2008)

While the elevated levels of IgG in UTI patients agreed with other research, it has been shown that women with recurrent UTI had noticeably higher blood levels of IgG, the greatly higher levels of IgG seen in UTIs can be explained by the crucial and protective role that IgG plays against infections, IgG is synthesised during the secondary immune response, the activation of inflammatory mediators was linked by UTI infection to an increase in complement component synthesis (Abraham & Miao, 2015).

Recently difermion of IgG was used as a useful predictor of the prognosis of complicated patients with high proportion global glomerulosclerosis (Coppo, 2017).

IgG may worsen tubule interstitial damage and may indicate the extent of tubule atrophy and interstitial fibrosis. It was also confirmed that increased IgG is related to abnormal renal function, which is consistent with the previous studies who indicated that increased IgG can reflect the degree of chronic renal pathological damage (Xu *et al.*, 2022).

5-2-2 The Concentrations of Toll-Like Receptors 2 & 4 in UTI patients and controls according to distribution of gram negative and gram positive bacteria

Since Toll-Like receptors (TLRS) contribute to the innate immune system, it has been established that it is a member of the class of pattern recognition receptors (PRR) that identify pathogen-associated molecular patterns (PAMP), which are constant components of the microbial structure, a class of membrane receptors known as toll-like receptors (TLRs) is responsible for triggering the innate immune response by identifying conserved pathogen-associated chemical patterns. TLRs are expressed in non-immune cells as well as immune cells, such as the urogenital tract's epithelial cells, which are responsible for the first detection of bacteria (Medzhitov, 2007).

It seems that UTI is more pertinent to TLR2 and TLR4, with their quick reaction to UTIs, they may help activate innate immunity and defend the mucosal barrier against bacterial invasions (Karananou *et al.*, 2016).

Increasing both Toll-like receptor 2 (TLR2), TLR4, might be due to their vital role as an early recognition of the pathogen and transduce this signal to induce a rapid and robust pro-inflammatory immune response (Gupta *et al.*, 2022).

The mean serum TLR2 values in UTI cases of the present study was significantly higher compared to the control healthy subjects, these findings corroborated earlier research (Fischer *et al.*, 2006) and Zanoni and Granucci (Zanoni & Granucci, 2013).

It is now known that TLR2 collaborates with other TLRs, such as TLR1 and TLR6, to distinguish between certain patterns, which explains how TLR2 recognizes a broad variety of microbial components (Akira *et al.*, 2006).

The urinary tract is actively protected against UPEC by TLR2, which is expressed on tubular cells. When TLR2 ligands stimulate tubular cells in vivo, polarized secretion of inflammatory mediators like TNF-κ occurs, suggesting that TLRs can also fight upper urinary tract infections (Tsuboi *et al.*, 2002), (Choi *et al.*, 2016).

The TLR 2 recognize and engage with different gram-positive bacterial structures, TLR2 heterodimers are able to detect different bacterial PAMPs and peptidoglycans in Gram +ve bacteria and released bacterial HSPs. The production of proinflammatory cytokines may occur upon detection of the aforementioned target ligands. It appears to be essential for identifying significant microbiological UTI causing culprits (Höfs *et al.*, 2016).

When intracellular UPEC is detected, toll-like receptor 4 (TLR4) raises intracellular cyclic AMP (cAMP) levels. Autophagy targets the bacteria and delivers them into lysosomes, which are then modified by UPEC to lose their ability to degrade. When TLR4 detects the presence of pathogens and signals, a variety of soluble substances are also secreted including antimicrobial peptides (Abraham & Miao, 2015). Many different mechanisms were proposed the reason behind increasing TLR4 in case of UTI (Abraham & Miao, 2015), (Spencer *et al.*, 2014).

TLR4 molecules are expressed in the presence of released microbial HSPs pertaining to UPEC, *C.albicans*. The majority of UPEC strains encompass the virulence factors of type I fimbriae and FimH, (the FimH adhesion is normally located on the top of type I fimbriae). FimH enables UPEC to adhere to uroplakin 1a molecules. The uroplakin 1a molecules are located on the surface of urothelial cells, which line the inner side of the bladder in human urinary tract. So, the attachment of UPEC cells onto the uroplakin 1a molecules induces the TLR4 molecules to eliminate the foreign pathogens from the bladder (Jahandeh *et al.*, 2015).

In a study done by Aksu *et al*., it was confirmed that TLR4 may be used as a useful biomarker in predicting UTI, it was higher in the UTI group than in the control group (Aksu *et al*., 2024).

Just TLR4, TLR5, and TLR11 have been demonstrated to support the immune system against bacterial infection in vivo to yet, despite the fact that other TLRs are expressed on the cells lining the urinary tract. (Behzadi & Behzadi, 2016).
Increasing level of TLR4 might be due to an instructive role in UTI, TLR4, expressed both by epithelial and non-epithelial cells, initiate appropriate immune and inflammatory responses to overcome microbial invasion and infection (Scherberich & Hartinger, 2008).

The current results also indicated that the mean level of TLR 4 in the patients with Gram- positive pathogens was lower than their level in Gram-negative group. This result was completely agreed with the study of Gluba *et al*., 2010. It has been documented that the expression of TLR4 molecules is triggered by the LPS molecules found in the outer membrane of gram-positive bacteria. The co-receptor molecule MD-2, which is soluble in serum and/or attached to the cytoplasmic membrane and/or bound to TLR, LPS binding proteins (LPSBP soluble in serum), and CD14, which is soluble in serum, attached to the cytoplasmic membrane, and/or bound to TLR, collaborate with the active and effective TLR4 molecules. In fact, TLR4 is able to successfully exclude UPEC cells from the urinary tract region because to the presence of this complex. The hydrophobic region of LPS attaches itself to the extracellular portion of TLR4 through the action of the MD-2 component. It's interesting to note that the amount of fatty chains in lipid A that are linked to the LPS molecule controls the production of TLR4 molecules and, in turn, the degree of inflammatory reactions. TLR4 molecules are expressed by the invasion of bladder and kidney urothelial cells by UPEC cells. Normally, TLR4 molecules enable the invading urothelial cells to selectively exclude UPEC cells. Type I fimbriae provide UPEC with an evolutionary defense against immune responses such as TLR4. This virulence factor promotes the intracellular growth of UPEC cells in the urinary tract and may control the TLR4 defensive mechanism. Consequently, the urinary tract of the host recognizes the bacterial components of UPEC, such as LPS, FimH adhesin, type I, and P fimbriae, as TLR4 inducers (Botos *et al.*, 2011), (Spencer *et al.*, 2014).

Interestingly, very few studies were examining the link between protective immunity and pathological inflammation during Infection. In the current study, results demonstrated a significant relationship between TLR2 and Vit D3. Innate defense

against intracellular bacteria is greatly aided by vitamin D signaling, which also directly binds to and eliminates a variety of infections. Recently, the newest mechanism for the relationship was proposed indirectly, in term of cathelicidin. During an infection, cathelicidin functions as a secondary messenger that propels vitamin D-mediated inflammation. Cathelicidin's biological and therapeutic roles have been clarified in relation to vitamin D signaling. The activation of autophagy boosts antimicrobial activities against a variety of pathogens and is mediated through the vitamin D-cathelicidin axis. Studies on vitamin D have also shown that cathelicidin regulates inflammatory responses to pathogenic stimuli in both positive and negative ways. The involvement of cathelicidin activity in cell-autonomous effector systems is enhanced by the interaction of functioning vitamin D receptor signals with a variety of innate and adaptive immunological signals (Chung *et al.*, 2020).

By binding to vitamin D response sites in target genes, VDR signaling activation during infection coordinates innate immunological signals for the generation of AMPs, such as human cathelicidin AMP (CAMP) and β-defensin 2 (HBD2) (Zittermann *et al.*, 2016). While the primary role of AMPs in VDR signaling has been thought to be their antimicrobial effects, more recent research has shown that AMPs are also important signaling nodes that control immune pathways by regulating autophagy/xenophagy, the production of cytokines, chemokines, and reactive oxygen species (ROS), as well as IFN signaling (Clark & Mach, 2016).

According to Liu *et al.* TLR2/1-mediated cathelicidin induction is essential for human monocytes and macrophages to exhibit anti-mycobacterial action (Liu *et al.*, 2006). Numerous studies have demonstrated the beneficial effects of vitamin D-

mediated cathelicidin in antimicrobial actions against infections (Dimitrov & White, 2016).

5-3. The Concentrations of Zinc and Vit D3 in UTI patients and controls

Since it promoted the local synthesis of antimicrobial peptide (AMP), Vitamin D has long been thought of as an antibacterial agent that protects the urothelium, according to the study hypothesized that low level of vitamin D3 and Zn might act as a risk factor for UTI (Gao *et al.*, 2023). This study thus examined the relationship between serum vitamin D3 level, Zn and UTI patients. The results indicated decreased levels of both markers which might reflect their role as a risk factor of UTI. These results were agreed with other who confirmed by evidence the relationship between the risk of UTI and blood vitamin D3 level. They demonstrated a strong correlation between low blood vitamin D levels and children's and adults' risk of UTIs, as shown by Li & Lu (Li & Lu, 2021) who have been reported their main finding showed the risk of urinary tract infection (UTI) is negatively correlated with low blood vitamin D levels. The reason behind that might be their regulator role of the innate and adaptive immune function through various mechanisms and regulate the inflammatory response to the pathogenic agents (Kearns et al., 2015; Mamani et al., 2017)

By altering cytokine responses and activating toll-like receptors in immune cells, low vitamin D3 levels reduce both local and systemic inflammatory responses (Ao *et al.*, 2021), (Calton *et al.*, 2015). In addition to lowering immune cell activities by inducing hypocalcemia, vitamin D shortage also lowers the synthesis of b-defensin-2 and cathelicidin (Wang *et al.*, 2023), (Youssef *et al.*, 2011). Vitamin D3 receptors on immune cells, including T cells, dendritic cells, macrophages, and monocytes, control their antimicrobial activity (Athanassiou *et al.*, 2022), (Mahyar *et al.*, 2018).

Aslan *et al.*'s research (Aslan *et al.*, 2012) demonstrated the role of vitamin D3 receptor in the pathophysiology of UTI. Since the urinary tract epithelium is one of the barrier regions where endogenous antimicrobial peptides (AMPs) are widely produced, they serve as the innate immune system's first line of defense against pathogenic pathogens, these are multifunctional peptides found on the skin, gastrointestinal, respiratory, and urine tract epithelial surfaces. By damaging the bacterial membrane, AMPs exhibit antibacterial action. They also prevent the formation of biofilms and alter a number of immunological processes. Conversely, the intensity of UTI is linked to AMPs activity in the epithelial surfaces (Terlizzi et al., 2017; Yasir et al., 2018) . According to available data, malnutrition may increase a person's risk of UTI (Storme *et al.*, 2019), (Noorbakhsh *et al.*, 2019).

One of the main micronutrients having immune action is zinc, Zinc regulated the host immune system and moderate shortage causes immune system malfunction, deficientness in zinc can raise the risk of infectious illnesses, It is essential for cellular metabolism and development (Brunton et al., 2018; Pecora et al., 2020).

Numerous investigations have examined the significance of this trace element in various infectious illnesses and discovered that it also possesses antibacterial characteristics (Bohan, 2018). Changes in zinc levels seem to have a special effect on the immune system, zinc levels seem to be directly or indirectly tied to every reaction. Cytokines, free radicals, and other chemicals that damage cell membranes can leak into the environment and have a cytotoxic impact (Skrajnowska & Bobrowska-Korczak, 2019).

Nutritional immunity has been the primary factor taken into account when it comes to zinc at the host-pathogen interaction. In this case, a bacterial pathogen cannot

acquire zinc due to the action of the innate immune system. Zinc is reallocated to other tissues during this process, which lowers the amount of zinc in the serum. The zinc transporter ZIP14, which is in charge of collecting zinc in the infected site, can be up-regulated in an IL-6-dependent manner, which can trigger this process (Liuzzi *et al.*, 2005).

The mechanism behind the depletion of plasma zinc also seems to include metallothionein, which is stimulated by inflammatory cytokines like IL-1 and accumulates in the liver in its Zn-bound form, the function of the human protein calprotectin serves as another illustration of zinc nutritional immunity, this stows away zinc and deprives the bacterial infection of these trace metal resources, neutrophil extracellular traps, a crucial mechanism of neutrophil extracellular bactericidal killing, are rich in calprotectin, it is important to highlight that calprotectin likely only starts to work when it is released from the neutrophil (Brophy *et al.*, 2012).

In addition to receiving less sunshine due to hot weather, the Middle East is recognized for having a higher prevalence of vitamin D insufficiency. All of these circumstances add interest to the study, as seen by the findings that clearly relate urinary tract infections to vitamin D deficiency, such results were agreed with other research who reporting same finding (Hewison, 2012).

Even mild zinc deficiency impairs the function of the innate and adaptive immune systems (Roohani *et al.*, 2013). Reduced immunoglobulin synthesis, thymic atrophy, lymphocyte depletion, poor phagocytic function, and decreased interleukin (IL)-2 production are all linked to zinc deficiency (Wessels *et al.*, 2021).

Furthermore, because of damage to the epithelial line of defense and a decrease in antioxidant activity, those who are zinc deficient may be more susceptible to certain diseases (Mahyar *et al.*, 2015). Furthermore, it was confirmed that Zinc deficiency

is associated with bad outcomes in response to bacterial infections and sepsis (Liu *et al.*, 2013).

Regarding the influence of zinc deficiency on raising the risk of UTI, a firm opinion cannot be expressed. generally, numerous factors, including dietary modifications and lifestyle adjustments that impact an individual's blood levels of minerals and vitamins, may impact an individual's zinc levels.

5-4. The types of bacteria that were registered in UTI patients and Concentration ofTLR2 and TLR4 according to the distribution of gram (-) and gram (+) bacteria

Geographical location has a significant impact on the distribution of prevalent uropathogens, with distinct uropathogen patterns found in each region. The findings of the current study indicated that, with around 38.33% of the total, Escherichia coli was the most prevalent form of bacteria that could cause a UTI, followed by Staphylococcus epidermidis (21.66%). Five percent of the participants in the current study also had Klebsiella pneumoniae. Staphylococcus aureus was the less prevalent kind by 1.66%. Table (6) shows the 16.66 percent of patients who did not exhibit any bacterial growth, this agreed with the study confirmed that *E. coli* isolates were the most frequent pathogens causing UTI in Iraq and in recent reports carried out in Iraq, it was found that E. coli, Staphylococcus spp., and K. pneumonia were the most common infectious agents causing UTI, which were also resistant to the most commonly used antibiotics (Naqid et al., 2020). While in babylon E.coli was the most predominant bacteria with 309 isolates followed by K.pneumoniae 112, S.saprophyticus 62, E.faecalis 48, P. aeruginosa 31, S.aureus 20, S.haemolyticus 19, St.agalatiae 16 and C. freundii 8 isolates only, this may be due to overusing antibiotics or ignoring the treatment of UTIs. Imipenem 10g is still the best choice for inhibiting all bacterial growth. As a result, recurrent UTI infections should be treated with more caution since they may lead to serious complications such as renal

failure or chronic kidney disease (Ali & Aljanaby, 2023), the current study was also involved measurement of the TLR 2, and TLR4 levels based on the types of bacteria included Gram-negative pathogens and gram positive The types of the bacteria was presented in the table (4-6). Figure (4-9) demonstrated that the mean level of TLR 2 in Gram-negative pathogens which was lower than their level in gram positive, while in TLR4 the mean level of TLR 4 in Gram-negative group was higher than Gram- positive pathogens in their level, this was agreed with the study mentioned that the mean level of TLR4 was higher in gram negative bacteria (Abdalhussin et al., 2022).

5-5. Level of immune markers in recurrent and non-recurrent UTI

It is commonly recognized that individuals with immunodeficiency frequently get severe, recurring UTIs (Chapel & Cunningham-Rundles, 2009). Nonetheless, the host defense system may also be the cause of recurrent UTIs in certain immunocompetent people. Innate immune responses and urothelium barrier function are the two primary mechanisms that make up the host defense in lower UTI (Abraham & Miao, 2015).

IgM deficiency may be asymptomatic, symptomatic patients present with recurrent bacterial infections. Although generally not considered increased frequency of UTI in antibody deficiency diseases, but in a study done by Fegurgur and Gupta 2019 Was confirmed that about 16% of patients who reporting IgM deficiency had recurrent UTI.

TLRs have been investigated as possible predictors of UTI recurrence from gene to protein level as they have been recognized as a crucial component for bacterial activation of immune response in the urinary tract, certain TLR gene polymorphisms

have been proposed to cause a pathogen detection deficit in the urinary tract, which may therefore be linked to recurrent UTIs (Yin *et al.*, 2010).

Different inflammatory cells and cells with recognition receptors, such as toll-like receptors (TLRs), which may identify infections and trigger a strong inflammatory immune response, make up the bladder's innate immune response. TLRs may be linked to recurrent UTIs and are necessary for the immune system to activate. In a cross-sectional investigation, women suffering a history of recurrent UTIs had their genotyped polymorphisms examined (Hawn et al., 2009).

A variation of TLR2 linked to reduced lipopeptide-induced signaling was found to be polymorphic and linked to a higher risk of bacteriuria. Adult women who have TLR5, which encodes a variation that blocks flagellin-induced signaling, have been linked to a higher risk of recurring UTI, TLR polymorphism, which includes TLR4 and TLR1, on the other hand, may have a part in preventing recurrent UTI (Hawn *et al.*, 2009). Individuals with particular TLR polymorphisms may not be able to recognize pathogens in the bladder, which increases the likelihood of recurring urinary tract infections (Hawn *et al*., 2009).

However, the first potential biomarker for recurrent UTIs was discovered to be serum antibodies. In a 2001 prospective trial, women with recurrent UTIs who had received full antibiotic therapy were enrolled, the research patients had considerably greater serum levels of IgG, IgM, and IgA than did the healthy controls (Suman *et al*., 2001).

5-6 Concentration of Vit D and Zinc in recurrent and non-recurrent UTI

Evidence on the correlation between blood levels of vitamin D and UTI risk has been gathered by prior research (Deng *et al.*, 2019). Through a variety of methods, vitamin D either directly or indirectly controls the functioning of both adaptive and innate immunity. It has the ability to control the inflammatory reactions to pathogenic substance (Kearns *et al.*, 2015). A sufficient amount of vitamin D diminishes both local and systemic inflammatory reactions by regulating cytokine responses and decreasing the activation of toll-like receptors in immune cells (Calton *et al.*, 2015). In addition to reducing immune cell activities through hypocalcemia, a vitamin D shortage also lowers the synthesis of b-defensin-2 and cathelicidin (Youssef *et al.*, 2011). Vitamin D receptors on immune cells such as T cells, dendritic cells, macrophages, and monocytes control their antimicrobial activities (Mahyar *et al.*, 2018).

On the other hand, the deficiency of Vit D either in recurrent or non-recurrent infections might be due to kidney impairment. Given that the kidneys are crucial in converting vitamin D into a form the body can use, it has been shown that vitamin D levels in chronic renal disease are below normal, and occasionally they are extremely low, this can happen because damaged kidneys have a decreased capacity to convert vitamin D into its active form (van Etten & Mathieu, 2005).

Vitamin D is necessary for the innate and adaptive immune systems to operate properly and respond to inflammation and infection (Winzenberg & Jones, 2013). In order to stimulate white blood cells and boost the removal of infected cells by interleukins and cytokines, the immune system requires vitamin D (Keflie e*t al.*, 2019).

Serum zinc levels were not statistically significantly related to study groups. According to the results of this study, group with non-recurrent UTIs had lower serum zinc levels than the recurrent UTIs and control group; that might be due to having Zinc supplementation for treated previous infection since Zn has been shown to reduce the duration and limit the complications of UTI (Roohani *et al.*, 2013) .

Even so, these results were disagreed with Mohsenpour *et al.*, 2019 who shown opposite results when reported that recurrent UTIs had lower serum zinc levels.

A zinc shortage may make infectious illnesses more likely, Considering that recurrent UTIs are a frequent side effect, antioxidant defenses and radical detoxification also require it. Antioxidant levels are down and oxidative stress is elevated in UTI patients (Elkhatib & Noreddin, 2014).

5-7 Conclusions

1. The results of the current study shed light on the role of IgG, IgM, TLR2, and TLR4 in UTI infections. IgG and TLR2 were significantly higher, while IgM was insignificantly decreased to indicate to the importance of these factors in UTI.
2. In general IgG, and TLR2 were dropped significantly in recurrent infections compared to non-recurrent infection and that could be because these factors are consumed during the recurrent infections.
3. However, the result was not significant, the low levels of vit. D3 and Zn may have a negative impact on the immune system in general and may have a role in increasing the UTIs and affect the recurrent infections.
4. E.coli is the predominant isolate in patients with UTIs In Sacred Karbala.
5. TLR4 can be considered as good immunological marker for UTT infections.

5-8 Recommendations

It is important to recommended that :

1. Study of other immune factors related to urinary tract infections, such as the other types of TLRs.
2. It is recommended to study of the same immunological factors, but for the upper urinary tract infections, such as kidney infection.
3. An in-depth study on the importance of immunity against urinary tract infection, could be done especially against types of highly virulent bacteria such as *E. coli* bacteria.
4. More detailed study on the factor zinc and vitamin D to resist other diseases immunologically would be carried out.
5. The correlation between D3 and zinc and UTI still needs much investigation to determine exactly when these two factors can have significant effect on the developing UTI and what other factors can alter the results.
6. The study focuses on cell death, specifically apoptosis, and its relationship with TLR2 and TLR4.

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Appendixes

Appendixes

Urinary tract infection questionnaire

A sample document containing the questionnaire that each participant in the study completed is provided below:

1. Name: No. The date:
2. Gender: Male Female
3. Age (18-50):
4. Urine profile:

Microscopical examination	Culture (register types of bacteria and other pathogens)	Discharges associated with the infection (describe colour and texture)

5. Does the participant have symptomatic UTI? Yes No
6. Status of cigarette-smoking Yes No
7. Recurrent UTI Yes No
   - ❖ Time duration between the recurrent UTIs
8. Other infections around the body associated with UTIs:
9. Other notes (other diseases):
10. Excluded cases (patients with): Congenital anomalies, Diabetes, Autoimmune diseases, Urinary tract stones, Pregnant women, Aged people, Pervious urological surgeries.

(Appendix 1). A questionnaire contains information for the current study about the UTI patients and controls (Age, sex, Congenital anomalies, Diabetes, Autoimmune diseases, Pregnancy, Aged people….)

Table (3-5): components of IgG ELISA kit.

Item	Specifications
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips
Reference Standard	96T: 2 vials
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 µL
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL 96T*5: 5
Biotinylated Detection Ab Diluent	96T/48T/24T: 1 vial, 14 mL
HRP Conjugate Diluent	96T/48T/24T: 1 vial, 14 mL
Wash Buffer Concentrated (25×)	30 mL vial for 96T, 48T, and 24T
Substrate Reagent	10 mL vial for 96T, 48T, and 24T
Stop-Solution	96T/48T/24T: 1 vial, 10 mL
Plate Sealer	96T/48T/24T: 5 pieces
Product Description	1 copy
Certificate of Analysis	1 copy
Additional materials needed Microplate reader with wavelength filter set to 450 nm Disposable pipette tips, EP tubes, and high-precision transfer pipettes an incubator that can sustain 37°C distilled or deionized water Receptive paper loading aperture	

Table (3-6): components of IgM ELISA kit

Item	Specifications
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips
Reference Standard	96T: 2 vials
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 µL
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL
Diluent with Biotinylated Detection Ab	96T, 48T, and 24T: 1 14 mL vial
HRP Conjugate Diluent	96T, 48T, and 24T: 1 14 mL vial
Wash Buffer Concentrated (25×)	30 mL vial for 96T, 48T, and 24T
Substrate Reagent	10 mL vial for 96T, 48T, and 24T
Stop-Solution	10 mL vial for 96T, 48T, and 24T
Plate Sealer	96T, 48T, and 24T: 5 units
Product Description	1 copy
Certificate of Analysis	1 copy
Additional materials needed Microplate reader with wavelength filter set to 450 nm Disposable pipette tips, EP tubes, and high-precision transfer pipettes An incubator that can sustain 37°C distilled or deionized water Receptive paper loading aperture	

Table (3-7): components of TLR2 ELISA kit

Item	Specifications
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips
Reference Standard	96T: 2 vials
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 µL
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL
Diluent for Biotinylated Detection Ab	96T, 48T, and 24T: 1 14 mL vial
HRP Conjugate Diluent	96T/48T/24T: 1 vial, 14 mL
Wash Buffer Concentrated (25×)	30 mL vial for 96T, 48T, and 24T
Substrate Reagent	10 mL vial for 96T, 48T, and 24T
Stop-Solution	10 mL vial for 96T, 48T, and 24T
Plate Sealer	96T, 48T, and 24T: 5 units
Product Description	1 copy
Certificate of Analysis	1 copy
Additional materials needed Microplate reader with wavelength filter set to 450 nm Disposable pipette tips, EP tubes, and high-precision transfer pipettes An incubator that can sustain 37°C distilled or deionized water Receptive paper loading aperture	

Table (3-8). components of TLR4 ELISA kit

Item	Specifications
Micro ELISA Plate (Dismountable)	
Reference Standard	96T: 2 vials
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 μL
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 μL
Reference Standard & Sample Diluent	96T,48T,24T: 1 vial and 20 mL
Diluent for Biotinylated Detection Ab	96T, 48T, and 24T: 1 14 mL vial
HRP Conjugate Diluent	96T,48T,24T: 1 vial and 14 mL

الخلاصة

تعد التهابات المسالك البولية من الأمراض الشائعة في العراق والعالم، ولها آثار صحية خطيرة. وقد تناولت العديد من الدراسات حول العالم هذا المرض من جوانب مختلفة تتعلق بالاسباب والعوامل المناعية (المناعة الطبيعية والمكتسبة) والعلاجات الفعالة. حاولت الدراسة الحالية التركيز على الجانب المناعي للإصابة من خلال دراسة بعض عوامل المناعة الفطرية والتكيفية (IgG, IgM, TLR2 and TLR4) وعلاقتها بالزنك وفيتامين د.

تضمنت طرائق العمل في هذا البحث سحب الدم الوريدي من المرضى والاصحاء لغرض فصل الأمصال. ثم قياس العوامل المناعية المذكورة أعلاه بواسطة ELISA، كما تم قياس مستويات فيتامين د والزنك وتم إجراء تحليل البول للمرضى والضوابط مع زرع البول في نفس يوم أخذ عينة الدم الوريدي. شمل تصميم الدراسة الحالية جمع 80 عينة من البول ومصل الدم. تم اخذ 60 عينة من المرضى الذين راجعوا مستشفى الامام الحسن المجتبى ومدينة الامام الحسين التعليمية الطبية / كربلاء المقدسة بين كانون الاول 2022 ونيسان 2023 حيث كانو يعانون من اعراض لالتهابات المسالك البولية، وتم اخذ 20 عينة من افراد اصحاء خلال نفس الفترة الزمنية. تم استخدام استبيان مصمم خصيصًا لهذه الدراسة لجمع البيانات المتعلقة بالمرضى والضوابط، بما في ذلك معلومات العمر والجنس والتشوهات الخلقية والسكري وأمراض المناعة الذاتية والحمل وكبار السن وما إلى ذلك.

شملت هذه الدراسة 80 مشاركا؛ وتوزع الجنس بنسبة 50% إناث و50% ذكور. تراوحت أعمار المشاركين من 18 إلى 50 سنة، حيث تم تقسيم المرضى والمجموعات الضابطة إلى مجموعات فرعية عمرية. تم تشخيص إصابة 35 (58.3%) من المرضى بالتهابات المسالك البولية المتكررة بينما تم تشخيص 25 (41.6%) بالتهابات المسالك البولية غير المتكررة. وقد وجد أن معظم المرضى لديهم اختلاف معنوي كبير في المستوى المتوسط لخلايا القيح، كريات الدم الحمراء، والخلايا الظهارية مقارنة مع الأصحاء.

أظهرت النتائج زيادة الاجسام المضادة IgG في مرضى التهاب المسالك البولية مقارنة بمجموعة السيطرة، مع زيادة ملحوظة في IgG في المصل (17.68 نانوجرام/مل) مقارنة بمتوسط IgG (10.57 نانوجرام/مل) في مجموعة التحكم. كان هناك انخفاض في مستوى الاجسام المضادة IgM لدى مرضى التهاب المسالك البولية مقارنة بمجموعة السيطرة، ولم يكن هذا الانخفاض معنوي حيث كان مستوى IgM (153.7 نانوجرام / مل) في المرضى مقارنة بمجموعة السيطرة (366.8 نانوجرام / مل). أظهرت نتائج TLRs (2 و 4) أن مستوى

TLR2 في مجموعة المرضى ارتفع بشكل ملحوظ مقارنة بمجموعة السيطرة. كان متوسط مستوى TLR2 في مجموعة المرضى 0.23 نانوجرام/مل بينما كان في المجموعة الضابطة 0.16 نانوجرام/مل. كما كانت هناك زيادة غير معنوية في مستوى TLR4 في مجموعة المرضى مقارنة بمجموعة السيطرة. حيث كانت 215.6 بيكوغرام/مل في مجموعة المرضى و187.6 بيكوغرام/مل في مجموعة السيطرة. أظهر التحليل الإحصائي عدم وجود فروق بين مرضى التهاب المسالك البولية والأشخاص الأصحاء في مستويات فيتامين د، ولكن كان لدى الأشخاص الأصحاء متوسط أعلى من هذا الفيتامين. كان لدى مرضى التهاب المسالك البولية مستويات منخفضة من الزنك في الدم مقارنة بالمجموعة السيطرة.

كما تم فحص معدلات الإصابة لكل نوع من البكتيريا. وكانت الإشريكية القولونية هي الأعلى نسبة، تليها المكورات العنقودية الجلدية (21.66%)، والزائفة الزنجارية (16.66%)، والكلبسيلا الرئوية (5%)، والمكورات العنقودية الذهبية (1.66%). تم في الدراسة قياس مستويات TLR2 وTLR4 على أساس أنواع البكتيريا، بما في ذلك سالبة الجرام وإيجابية الجرام. واظهرت النتائج بان المستويات المتوسطة لـ TLR2 في المرضى الذين يعانون من مسببات الأمراض سلبية الجرام أقل من مستوياتهم مع مسببات الأمراض إيجابية الجرام، في حين كانت مستويات TLR4 في المرضى الذين يعانون من مسببات الأمراض إيجابية الجرام أقل منها في المجموعات سلبية الجرام.

في كل من المجموعتين المتكررة وغير المتكررة، كان هناك اختلاف كبير في متوسط مستويات IgG مقارنة بالمجموعة الضابطة. كان متوسط IgM في أدنى مستوياته في الإصابات المتكررة، إلا أنه لم يكن معنويا. كان متوسط مستوى IgM في الالتهابات المتكررة والالتهابات غير المتكررة والضوابط 77.68 نانوغرام / مل، 260.1 نانوغرام / مل، و238.4 نانوغرام / مل، على التوالي. كانت هناك زيادة في مستويات TLR2 في المصل في حالات العدوى غير المتكررة، مع زيادة كبيرة مقارنة بمجموعة العدوى المتكررة ومجموعة السيطرة. ارتفعت مستويات TLR4 في المصل بشكل طفيف في حالات العدوى غير المتكررة، مع عدم وجود فروق ذات دلالة إحصائية بين جميع الفئات. بالنسبة لفيتامين D3 والزنك لم تكن هناك فروق ذات دلالة إحصائية تظهر عند مقارنة المجموعات الثلاث المتكررة وغير المتكررة والضابطة فيما بينها.

تم استخدام تحليل رتبة سبيرمان لإظهار علاقة الاستجابة بين جميع العلامات المناعية (IgG، IgM، TLR2، وTLR4) وبين فيتامين D3 والزنك في مرضى التهاب المسالك البولية. أظهرت نتائج الارتباط وجود علاقة بين TLR2 وD3.

الاستنتاجات. كانت مستويات IgG وTLR2 أعلى بشكل ملحوظ في عينات المرضى مقايسة مع مجموعة الاصحاء، في حين انخفض IgM بشكل غير معنوي مما قد يشير إلى أهمية هذه العوامل في إمراضية التهاب المسالك البولية وعلاجها. وبشكل عام، انخفض مستوى IgG وTLR2 بشكل ملحوظ في حالات العدوى المتكررة مقارنة بالعدوى غير المتكررة وقد يكون ذلك بسبب استهلاك هذه العوامل أثناء حالات العدوى المتكررة. كانت مستويات فيتامين D3 و Zn منخفضة عند المرضى وقد يكون لهما تأثير سلبي على الجهاز المناعي بشكل عام ويكون لهما دور في زيادة التهابات المسالك البولية والتأثير على الالتهابات المتكررة. لا تزال العلاقة بين D3 والزنك والتهاب المسالك البولية بحاجة إلى الكثير من البحث لتحديد متى يمكن أن يكون لهذين العاملين تأثير كبير على تطور التهاب المسالك البولية وما هي العوامل الأخرى التي يمكن أن يكون لها تاثير على النتائج.

الخلاصة

جـــــــامعـــــة كـــــربـــــلاء

كليــة العلـوم

قسـم علـوم الحيـاة

تقييم مستويات بعض العوامل المناعية لدى مرضى المجاري البولية المصابين بالبكتيريا الموجبة والسالبة وعلاقتها بمستويات الزنك وفيتامين.D

رسالة مقدمة

إلى مجلس كلية العلوم / جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

قدمتها

أسماء فيصل روضان

بكالوريوس علوم حياة – جامعة بغداد 2002

بإشـــــــــــراف

ا.م.د ساجدة فليح حسن

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