

Identification and Characterization of Pathogenic Bacteria in Children Suffering from Urinary Tract Infection in District Mardan

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ABSTRACT

The paper examined prevalence, etiology and antimicrobial resistance of Urinary Tract Infection (UTI) in children at Mardan Medical Complex. There were 20 positive cases of UTI out of the 50 mid stream urine samples with girls (70%), having a much higher prevalence than boys (30%). The prevalence also rose with the age and it was 65 percent at age 10. *Staphylococcus aureus* (15%), *Escherichia coli* (55%), was the most frequent isolate. An API kit characterization and biochemical testing of bacteria demonstrated a high rate of antibiotic resistance; *S. aureus* and *Klebsiella pneumoniae* were those that demonstrated Cefepime and Amoxicillin resistance. The Tissue Culture Plate method of biofilm analysis showed strong biofilm producers to be *K. pneumoniae*, *Serratia marcescens* and *E. coli*. The results show a great load of multi-drug resistant and biofilm forming uropathogens in children of school age, making the development of successful clinical control measures significant.

Keywords: Urinary Tract Infection (UTI), Antibiotic Resistance, Biofilm Formation, Pediatric Health, Uropathogens

Introduction

One of serious physical health problem is urinary tract infection cause by microbes. Microbes, are all those organisms which are too small and we can't see them without microscope. Most of the time the bacteria can caused the uti but in some rare cases the viruse are also responsible for causing UTI. UTIs is one of the most common

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infection in human and its can happen anywhere in the body like as kidneys, bladder and urethra.

Most of the time the UTIs infections occurs in bladder and also in the urethra mean in lower tract. Although it can also occurs in the kidneys means in the upper tract. However the lower tract UTI are more as compared to upper tract UTI, and usually are more severe. It is expected that in the world there are about 150 million cases of UTI reported per year (Stamm & Norrby, 2001)

Urinary Tract Infections (UTI) are painful and uncomfortable, yet preventable. Over 20% have had multiple UTIs and over 50% of female have had at least one UTI. High danger factor for rising UTI's is sexual activity in female (Foxman et al., 1990).

The UTI's are most caused by *E. coli* and when the *E.coli* encounter to the urinary tract from the bowel, the get attachment with the mucosal membrane of urinary using a type of fimbrialadhesin called P fimbriae. These P fimbriae are used by *E. coli* strands and bind to glycoprotein receptors on urothelial cells that colonize the urethra to specifically bind to glycoprotein receptors which have the a mannose residue that is the binding site for the P fimbriae proteins, on urothelial cells and start colonization.

The most common uropathogen is *E.coli* specie, and *Enterobacter* specie but there are other pathogenic bacteria that are involved in uti include *Serratia marcescens* and *Staphylococcus aureus*. The treatment for the UTI caused by the gram-negative bacteria is an antibiotic regiment. And there are several antibiotics for the UTIs treatment but the antibiotic resistant bacteria's increases and the proliferate of this pathogenic bacteria lead the biofilm formation which is an extracellular matrix and this biofilm shows grate resistance to antibiotics.

This is one of the major problem during treating UTIs which can lead to more serious infections, there fore it is important to take the broad spectrum antibiotics for the treatment of UTIs.

Normal flora of Human body

A number of microbes that live in the dermis (skin) and mucus membranes of a normal individual without causing any disease or the group of microbes that inhabit in the epidermis and mucus membranes and protecting the body from the infection of the pathogenic microbes are called normal flora of the body (Brooks et al., 2010). These microbes protect us from all type of disease by fighting with invaded microorganisms for nutrients & space producing bateriocins which acts as a antibiotics, which can't grow other bacteria and kill the harmful bacteria by lowering its PH.

Table 1 Categorization of human body Normal flora (Eckburg et al., 2005).

Human`Body	Normal Flora
Skin	<i>Staphylococci, micrococci, diptheroids.</i>
Respiratory tarct and Oral	<i>Neisseria, Bordetella, and Streptococcus specie</i>
Conjunctiva	<i>Haemophilus and Staphylococcus specie</i>
Gastro Intestinal tract	<i>lactobacillus Enterococci, non-haemolytic streptococcus, E.coli.</i>

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Genital tract	<i>Corynebacterium</i> , <i>Nonpathogenic</i> <i>Neisseria</i> specie, and <i>Lactobacillus</i> specie.
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Classification of UTI

UTI is classified into different categories respective there site of infection such as Cystitis, Pyelonephritis and Bacteriuria.

Bacteriuria: the UTI of the urine.

Cystitis: the UTI of bladder are called Cystitis.

Pyelonephritis: the UTI of the kidney known as Pyelonephritis.

Pyelonephritis is caused as a result of infection that spread from kidney to urinary tract. it create the barrier in pathway of urine that flow back to the kidney and ureters and cause infection (Hay et al., 2011, Hoberman et al., 1993).

Asymptomatic bacteriuria (ABU) is the colonization of urine in the absence of clinical symptoms. In healthy person the asymptomatic bacteriuria occurs in up to 6 % and in elderly persond it occure up to 0 %. As its name indicate that Asymptomatic bacteriuria commonly do not cause symptoms. No treatment is required by most of the patients with ABU, and in more cases are reported that colonizing organism truly helps to avoid infection by further additional of virulent bacteria (Darouiche et al., 2001).

Fig 1 Site of infection for uropathogen that result as urinary tract infection (Image was taken from www.google.com/site+infection+of+UTI+images).

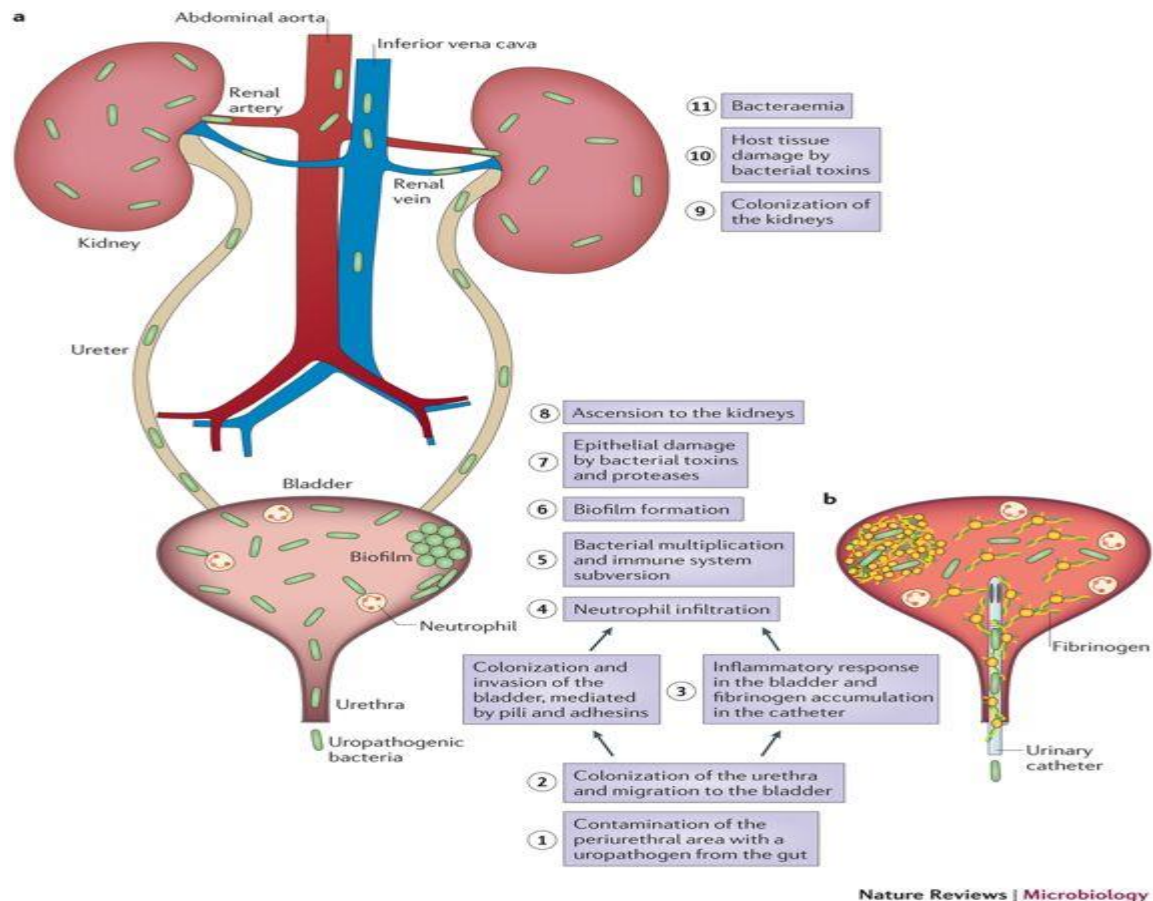
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Causes of UTI

Both bacteria that is gram positive and gram negative are mainly responsible for creating UTI. The *Staphylococcus* specie, *Streptococcus* specie, *Enterococcus* specie having thick peptidoglycan cell wall and are involved in UTI and among gram negative the *Serratia* specie, *Salmonella* specie, *Enterobacteriaceae* specie, *Klebsiella* specie, *Escherichia* specie, *Pseudomonas* specie, *Proteus* specie, *Citrobacter* specie are commonly cause the un complicated UTI (Mangiarotti et al., 2000, Nuutinen and Uhari 2001).

Although in children *E. coli* infection rate is more with primary urinary tract abnormalities, further pathogens, involved in causing uti are *Enterobacter* specie, *Klebsiell* specie, *Enterococcus* specie, *Pseudomonas* specie, and *Proteus* specie. About 15% of UTI infection in female are because of a coagulase negative *staphylococcus* and *staphylococcus*.

Pyelonephritis and cystitis are hardly caused by methicillinresistant *S. aureus* (MRSA), *Staphylococcus aureus* from the urine *Staphylococcus aureus* recommend an extra site of infection that is, bacterial endocarditis, Osteomyelitis and renal abscess.

Urine Culture

Unsympathetic past and bodily test may recommend the UTI diagnosis. But it should be confirmed by the culture of urine specimen because the cultered urine indicate the

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increasment of pathogenic microbes in the urinary tract (Bauer and Kogan 2008). To avoid the false negative result in childhood, the urine proper collection is very important. It can be performed by cleaning the genital part with soap and water and do not use antibacterial agents (Tullus, 2011). There are four most commonly used methods for obtaining urine from newly born, non toilet trained and kids, with changeable contamination rates (Stein et al., 2015). Most commonly method used in collecting urine in a synthetic bag is attached near to the clean genitalia this method is used most common in daily practice (Bauchner, 2005). The most sensitive method is the suprapubic bladder aspiration (SPA) these are also urine collection method the urine obtained by this method are uncontaminates. When urine is obtained by suprapubic aspiration, a process that bypasses the urethra. Every additional process of the urine collection that is catheterization, a plastic bag collection and mid stream clean catch require pathway of passing urine through the urethra.

Alternative to SPA method is bladder catheterization. Although the contamination rate in the bladder catheters is increase. The sensitivity and specificity of the urine obtained by catheterization method is 95% and 99% respectively when it was compared to the urine that obtained through SPA method (Kramer et al., 1994).

Literature Review

Urinary tract infections are most commonly seen in the girls than boys. UTI are bacterial infections throughout pediatric age (Rushton, 1997). In children Urinary tract infection is a significant source of morbidity. Further investigation and continuing urinary surveillance are required for the children infected with UTI in order to minimize future complications..

In a study that was carried out by Shaikh and his coworker UTI prevalence rate was reported as 7% in children. UTI prevalence rate was reported at high level in female children, especially during first years of their life. UTI prevalence in all age groups from Iran is studied in the current study but in Shaikh investigation UTI has been addressed among children and also throughout the world. According, difference among the results of both studies can be comprehensible (Shaikh et al., 2008).

In another study, Kathy and his coworker evaluated UTI prevalence rate in teenagers, who referred to emergency ward. According to results of the research, UTI prevalence rate in these patients was 3.3% while its prevalence was estimated further among Caucasians and girls (Shawet al., 1998).

Daniels and his coworker stated in their study about prevalence of catheterization-related UTI and its trend on 70.4 million patients, 3.8 million cases of UTI were reported during years 2001-2010, so UTI prevalence total rate was estimated 5.3%. The relevant UTI prevalence rate was reduced by intubation from 9.4 per 100 patients in 2001 to 5.3 per 100 patients in 2010. In this research, gender has been expressed as an independent risky agent for intubation- related UTI (Daniels et al., 2014).

In another study on 945 febrile newborns by Hoberman and his coworker stated that UTI prevalence rate was reported 5.3%. In this study, prevalence rate of UTI was greater in girls and Caucasian newborns than in boys and black newborns (Hoberman et al., 1993).

Urinary tract infections occur in patients with structurally or functionally abnormal tracts have the urinary tract infection (complicated UTI) and also occure in patients

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with anatomically normal urinary tracts (uncomplicated UTI). The pathogens of urinary tract infection involve complex interaction of organisms, the environment and the potential host. Gram negative aerobic organisms are the organisms involved in urinary tract infections. These organisms usually originating from the normal microbial flora and 80-90% of first infection, *E.coli* is usually isolated. The outer genital and periurethral bacterial flora usually reflects the gut flora (Ouno et al., 2013).

on the other hand, various results have reported that the host factors and different environmental condition are responsible for distribution of uropathogens and also report the similarities and differences in the type of uropathogens and also from some practices such as standard hygiene practices, socioeconomic healthcare and education programmers in each country (Shaifali et al., 2012).

In urinary tract infection most familiar isolated pathogenic microbes found were the enterobacteriaceae family. Gram negative bacilli in this study the have been the most common isolates which accounts for 100% of the total isolates present in uti. Among bacilli the *Escherichia coli* (78.45%) was the major bacteria go next by *Klebsiella pneumoniae* (21.56%), which account the second most widespread pathogen of UTI. Beside them, from three cases who infected with *E. coli* or *K. pneumoniae* was found that four type of other bacterial species have been isolated and they were *Morganella morganai* (0.86%), *Enterococcus faecalis* (0.86%) (*Pseudomonas aeruginosa* (2.58%), and a *Proteus mirabilis* (0.86%) (Al-Mijalli, 2017).

Urinary tract infection can be find out on the basis of totally negative urinalysis outcome or result among kids who do not obtain instantaneous antibacterial therapy. For this reason, improved urinalysis is preferable. Though, facilities for Gram staining and microscopy of urine by mean of counting chambers, may not be accessible in all the time and everywhere. Result for urine testing agent strip with negative leukocyte esterase and nitrite reaction is enough to find out UTI. Diagnosis of UTI. Two methods were very best to diagnose the uti they are urine culture and urinalysis. As compared with SPA, the culture of urine collected from catheterization patients are practically sensitive and specific but have huge success value and the procedure is not painful. High false-positive result have been expected from cultures of urine collected in bags (Finnell et al., 2011).

The urine dipstick are the biochemical test that received the most attention for diagnosis UTI. The urine dipstick test can identify the occurrence of leucocyte esterase or nitrites (as a surrogate marker of pyuria). Approximately 4 hours are required for the conversion of dietary nitrates to nitrites by the action of gram negative organisms in the urine (Powell et al., 1987). Therefore, the nitrites test which have negative result has less value in finding out UTI.

Many authors in their studies have noted a huge amount of these drug resistances (Banerjee and Padmashri, 2011, Behera et al., Ehinmidu, 2003, Sowmya and Lakshmidhevi, 2013). A major increase has been found worldwide in protection of pathogenic strains to Cotrimoxazole, Amoxicillin & Cephadrin (Wattal et al., 2005).

For treating UTIs one of the mainly broadly established antibiotic families are Fluoroquinolones, even in complex cases, specified their broad-spectrum action, good tolerance, outstanding oral bioaccessibility, post antibiotic effect and bactericidal potency (Hooton, 2003, Tena et al., 2010). still, different studies have describe, these

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antibiotics high resistance rates (Lorente et al., 2005).

As discussed in the previous study that the Urinary tract infection is caused by *Escherichia coli* in kids. The prevalence of antibiotic resistance especially in the countries outer from the Organization for Economic Co-operation and Development (OECD) found that the incidence of resistance is greater in that countries. In countries outside the OECD resistance was 17.0% for nitrofurantoin, 60.3% for ciprofloxacin, and 79.8% for ampicillin (Bryce et al., 2016).

All isolated uropathogen were shown 100% resistant to cephalixin, amoxicillin, and ampicillin. Increased resistance were shown by gram positive bacteria to trimethoprim/sulfamethoxazole (SXT) (75%), amoxicillin clavulanic acid (66.7%) and tetracycline (67%). besides, these gram positive bacteria were shown resistance to gentamicin, oxacillin, and erythromycin, each gram positive isolates account for 92%. and 100%. resistance to penicillin. in the same way, to amoxicillin clavulanic acid (66.7%). SXT (76%). gentamicin (80.1%). and tetracycline (81.0%) the gram negative bacterial isolates had reported higher resistance to these antibiotics (Awoke et al., 2019).

Empirical antibiotic therapy may not always be appropriate because previous study reported that non *E. coli* pathogens are more resistant to most antimicrobial agents like amikacin, cephalosporin, amoxicillin clavulanate and nitrofurantoin. For UTI We have to obtain into consideration the uselessness of oral empirical antibiotic therapy, because of rising *Klebsiella* strains. Growing cases of *Klebsiella* spp, may raise the cost and also hospitalization and parenteral antibiotherapy (Marcus et al., 2005).

In the most likely infecting strain the resistance should not exceed 20% for an antibiotic to be considered a first line empirical treatment for urinary tract infection (Gupta, 2011).

Most of the children with suspected lower UTI are initially treated with co-trimoxazole or oral cephalosporins as first line before culture or where urine culture sensitivity cannot be performed. Due to the reasons outlined above there is moderately high resistance to cotrimoxazole by the gram negative uropathogens in our state *E. coli* 36.95%, *Klebsiella* spp. 15.8%, *Proteus* spp. 52.9% and *Pseudomonas* spp. 40%. (Lalmangaihzuali et al., 2018).

In this study, to penicillin G, Amoxicillin, Ampicillin and cephalixin all gram-positive isolates were 100% resistant and show 92% resistant to oxacillin and erythromycin antibiotic. similar observation from some previous study were reported that was conducted in Ethiopia (Tesfahunegn et al., 2009). In another study conducted in Nigeria and India also show same observation for antibiotic resistance to gram positive bacteria (Bhatta et al., 2019, Taiwo and Aderounmu, 2006). In contrast, the bulk of the Gram-positive bacterial isolates were show protection to amoxicillin-clavulanic acid (67%), gentamicin (92%), and tetracycline (67%). Though, these bacterial strains were fairly weak resistant to ceftriaxone (50%) and ciprofloxacin (33.3%). Dissimilarity with the prior report that was conducted in Ethiopia that showed higher resistance to ciprofloxacin (100%) (Bellifa et al., 2016).

Biofilm production is another significant method in UTI pathogenesis. Compared to their planktonic counterparts, the genetic expression is varies in bacteria-forming biofilm and numerous works have confirmed the significance of this change (Donlan and Costerton 2002). Formation of biofilm is a complex process that comprises different

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stages, and different factors are involved in this process (Klausen et al., 2003).

Although there is required of standardization for the measurement of bacterial biofilm production in vitro globally the characteristic of bacterial strains attachment to form biofilm can be valued by growth of bacterial isolates on microtiter plate made from plastic polystyrene (Christensen et al., 1985, Stepanovic et al., 2007).

In another study, all bacterial strains were about 80% able to form biofilm production, Gram negative isolates were 81% and Gram positive isolates were 75%, capable for biofilm production. This result is similar with studies that was conducted in Iraq and also similar with three studies, in India where biofilm former bacterial isolates were about 69–72%. However, in Egypt the lower biofilm forming isolates were reported about 59.1%. (Ponnusamy et al., 2012, Salih and AL-Ani, 2013, SarojGolia et al., 2012). A study that was carry out in Egypt was contrast of previous study they reported that larger biofilm formation was amongst CONS (57.1%) go after by *Pseudomonas* (50.0%), *Klebsiella pnemumonia* (44.4%), *Staphylococcus aureus* (42.9%), and *E. coli* (31.6%) (Abdallah et al., 2011).

Previous study stated that among urinary catheterized patients, from all the bacterial isolates, Forty three (79.7%) of them were biofilm producer, In between the gram positive isolates and gram negative isolates 9 (75%) and 34 (81%) of them were biofilm producer correspondingly without any importance difference ($p = 0.6516$) (Awoke et al., 2019).

Using the standard plastic micro titer plate-based assays the biofilm formation was estimated in vitro. The result for control was $OD_c = 0.21$ so on the basis of OD values all the bacterial isolates was categorized to OD values which are as follows: the $OD \leq 0.21$ = biofilm formation is absent. $OD \leq 0.40$ = biofilm formation was weak; $0.40 < OD \leq 0.84$ = fair biofilm producer; and $0.84 < OD$ = pathogen was strong biofilm producer. 68 strains (58.6%) was negative for biofilm production. The left over 48 strains were strong biofilm producers (8 strains, 6.8%), fair biofilm producer (21 strains, 18.1%) and weak producer (19 strains, 16.4%).

In previous study, the non biofilm formers bacteria had less antimicrobial resistance to that of biofilm-forming bacterial isolates to ciprofloxacin (46.5% vs. 9.1%) and amoxicillinclavulanic acid (74.4% vs. 36.4%) respectively.

Different antibiotics were estimated at different concentrations to evaluate the possible effect on biofilms. To ampicillin there were thirty eight (38) isolates (79.2%) out of forty eight (48) that show strong resistance. (with MICs $\geq 32 \mu\text{g/ml}$). result showed that On biofilm there is no significant effects of ampicillin in all the concentrations evaluated. No significant decrease of the biofilm was obtained even in the case of the bacterial strains that were susceptible to ampicillin.

Twenty one isolates (43.8%) showed resistance to cephalothin antibiotic with MICs ranged from 32 to $\geq 64 \mu\text{g/ml}$. Three dissimilar cephalothin concentrations were assayed over biofilm (1200, 1600 and 2000 $\mu\text{g/ml}$) and a significant decrease of the biofilm biomass $P \leq 0.05$. was produced to all three of them.

Methodology

Collection of Urine Sample

The mid-stream urine samples were collected in sterile bottle from pathology

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laboratory of Mardan medical complex(MMC). The bottle were labeled as patient name, age and sex the time of collection were also be labeled on bottle.

Sample Processing

The physiochemical properties (odor, color, appearance and PH) of urine were observed. The milky, orange or red color, the dirty smell and acidic PH of the urine samples were suspected for UTI infection. The suspected samples were further examined by microscopy. Thereby the presence of the pus cells and RBCs confirmed by microscopic examination called Urine Routine Examination (Urine R/E) test. The urine samples were centrifuged for 10 minutes at 4000rpm. The supernatant were discarded and slide smear were made from the pellet and observed through light microscope at 10X lense. The positive urine samples were cultured for further investigations on general bacterial growth media (Nutrient agar) and specific growth media for gram negative bacteria (MacConkey agar).

Urine Culture

Urine were streaked on the nutrient agar as well as macConkey agar sterile plates (sterile media and plates) as previous demonstrated by (Chowdhury, 1998).Urine were streaked through sterile wire loof into the agar plates inside the LFH (Laminar Flow Hood). These streaked disposable plates were then covered in the aluminum foil and put in the incubator at 37°C for 24 hours

Identification of Isolates

On the next day each of the size, shape, color, and surface quality of colonies were screened through necked eye. The structural dissimilar colonies on both type of growth media ((MacConkey agar & nutrient agar) were observed through nacked eye. The pathogenic bacteria were then further identified by two methods the gram staining and biochemical tests.

Gram Staining

Gram method was determined as previous reported by (Cheesbrough, 2006). A microscopic slide with one drop of normal saline solution was taken and through a tooth pick the pure colony from the nutrient agar or mecConkey agar plate was taken gently mixed it into the normal saline in order to make smear than wait for 5 minutes so that the smear was fixed. when the smear was fixed then used the four gram staining reagents which was Crystal violet, gram Iodine, gram decolorizar and Saffranine respectively. Washing with tap water was performed after each step of gram staining. One drop of imulsion oil was putted on the microscopic slide which had bacterial smear and than observed this slide under microscope at 100x lens. The purple colour of bacterial strain per field indicated the gram positive and the pink colour strain per field showed the bacteria was gram negative. 3.2.7.2. Biochemical test The selected colonies based on the cultural and microscopic were subjected for biochemical examination.

Biochemical test for Isolated bacteria (Gram negative)

Meant for the recognition of gram negative bacteria Api10s kit were used. The normal

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saline were taken in small eppendorf tube. Picked the isolated colony through sterile wireloop and gently mixed it in the eppendorf tube to get the mixture of bacterial isolate then added 1000 μ l of bacterial mixture to each well of Api strip through pipette. When all the wells were filled then added 300 μ l mineral oil into the LDC, ODC, H₂S and URE wells, then placed the Api strip in the incubator for 24 hours at 37°C.

After 24 hours incubation the four reagents that were Nit 1, Nit 2, Indole, and TDA were applied into relevant wells of API 10S strip that is GLU, IND and TDA respectively and after 5 minutes the colour of the wells changed and this colour change showed the positive and negative result for bacteria and thus identified specific bacterial strains.

Biochemical Tests for Isolated Gram positive Bacteria

The Coagulase, Catalase and DNase test were conducted for gram positive bacteria as previously reported by (Cheesbrough, 2006)

Catalase Test

This test was used to distinguish the catalase positive staphylococci from the catalase negative streptococci. Catalase is the enzyme and this enzyme is produced by the aerobic bacteria.

Catalase interferes the collapse of hydrogen peroxide H₂O₂ into oxygen and water.

Put 3 drop of H₂O₂ on a microscopic slide and picked isolated colony through tip and dissolved it in H₂O₂. After few seconds the strong bubbles were produced which shows the bacteria were catalase positive.

Coagulase Test

For the distinguish of staphylococcus aureus (coagulase positive) from staphylococcus (coagulase negative) the coagulase test was used. staphylococcus aureus bacteria can produce the Coagulase enzyme which converts soluble fibrinogen in plasma to insoluble fibrin.

Clean microscopic slide with two circles were taken. One circle was marked as test and other was marked as control. Put 2 drop of normal saline in both the circles and then picked the colony and gently emulsify the colony in the liquid of each circle. placed a drop of human plasma into the circle T and placed a drop of saline water into the circle C wait for 2 minutes and observed for agglutination. After few minutes agglutination was observed which shows the bacteria were coagulase positive.

DNase Test

DNase test was used to distinguish the staphylococcus aureus from other staphylococcus bacteria.

The DNase agar plate was divided and labeled into required number of steps. Through inoculating loop the bacterial colony was inoculated into the DNase agar plate and placed in the incubator at 37°C for 24 hours. After overnight incubation covered the surface of DNase agar plate by pouring HCL, the plate was kept for 10 minutes. After 10 minutes the excess HCL was removed and the clear zone was observed.

Antibiotic Sensitivity.

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The antimicrobial susceptibility test were performed on Mueller Hinton agar (Oxoid Ltd, England) by Kirby–Bauer disc diffusion technique following standard procedures using disc diffusion method.

For antibiotic sensitivity test first prepared the bacterial lawn on the plate containing MHA media (3.8 gram MHA in 100ml distill water).

Antibiotic disk were placed on the plate which had bacterial lawn each disc were put at a distance of 1 inch from other and wrap the plates through parafilm tape tightly. It was than kept at 37°C in the incubator for 24 hours. Organism that showed a clear extension of any of the inhibition zone towards the disc was performed according to the guidelines of CLSI 2015.

Biofilm Assay

The biofilm formation assay for identified strains was determined as previous report by (Kwasny, 2010). The biofilm formation were studied for 48 hours and for one week using 96 wells microtiter plates assay to screen the attachment of microbes to the abiotic surface. 3.2.9.1 Procedure for Biofilm

Day 1

Inoculated each bacteria of intrest in a 4 to 6 ml TSB media and was grown to mobile phase of incubator for one day.

Day 2

Cultured dilution were performed in 1;100 in the TSB media. 100 ul of each diluted culture were pipet into each of the six wells of 96 wells microtiter plate. Coated the plate in the aluminum foil and incubate it for 48 hours at 37°C in a static incubator.

Day 4

Removed the planktonic cells and media by pipetting out into collection beaker containing 10% bleach.

Washed the wells by pipetting in 200µl of water into the wells and pipette out into collection beaker containing 10% bleach. This step was repeated 3 time.

Added 125µl of crystal violet solution (wellson) to each well. Stain for 10 minutes 25°C

Crystal violet solution was removed by pipeting out into the collection beaker containing 10% bleach.

Washed the remaining crystal violet by pipeting in 200 µl water into each well and pipetting out into collecting beaker. This step was repeated 4 times.

To the each stained well add 200µl of DMSO. To solubilize the dye allowed DMSO by covered the plates and incubate for 10 to 15 minutes at 25°C.

For a moment mixed the contents of each well by pipetting and then transfered 125µl of the crystal violet from each well to separate clear well of 96 wells microtiter flat and observed for OD values through spectrophotometer at a wavelength of 500 to 600nm. Again Pour 100µl fresh TSB media into the wells which had bacterial biofilm and again placed it in the incubator to observed biofilm for 72 hours.

Results

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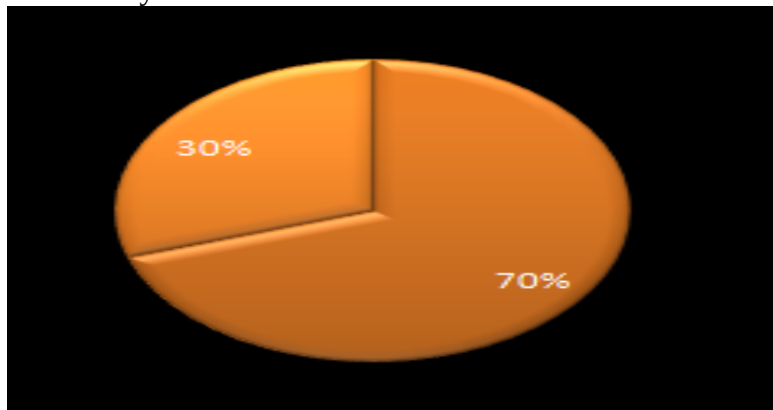
UTI Among the Infected Children.

The total 50 urine samples were collected from children (0-10 years), representing 18 male whereas 32 female individuals. The microscopy results indicated 6 male and 14 female patients having UTI. Our results indicate higher UTI prevalence in female children compared to male children.

Table 4.1 The gender wise positive patients with UTI are shown here.

Total positive patient	Male	Female
20-	6	14

Fig 4.1 UTI Among the Infected Children. Percentage of UTI was 70% in girls and 30% in boys.



Bacterial Growth on Different Media

For characterization of the bacterial strains found in the urine samples of the children, different types of growth media were prepared including Nutrient agar and MacConky agar.

Table 4.2 List of Pathogenic isolates grown on different media were shown here.

Media	Uropathogen	Colony morphology
Nutrient Agar	<i>Escherichia coli</i> <i>Enterobacter aerogenes</i> <i>Klebsiella pneumonia</i> <i>Serratia marcescens</i> <i>Sphingobacterium multivorum</i> <i>Staphylococcus aureus</i>	Circle, convex, small colonies. Small, creamy convex, smooth. Circle, small mucoid colonies. Red Pigmented, round colonies Rod shaped, cream colour. Yellowish round colonies.

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MacConkey agar	<i>Escherichia coli</i> <i>Enterobacter aerogenes</i> <i>Klebsiella pneumoniae</i> <i>Serratia marcescens</i> <i>Sphingobacterium multivorum</i> <i>Staphylococcus aureus</i>	Lactose-fermenting 'shiny, rose pink. Lactose fermented, pink & small. Pink, colony, large & mucous. Circular, pinhead colonies. Yellow colour ,rod shaped. No growth on macConkay agar.
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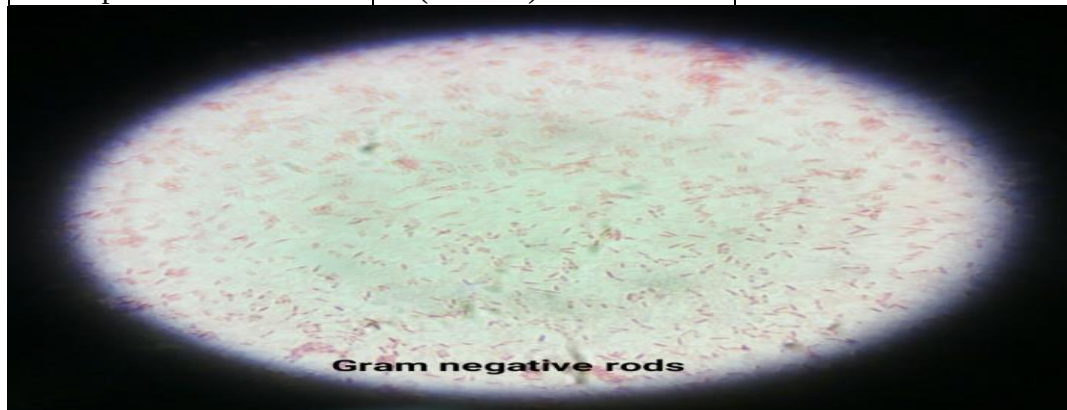
Fig 4.2 Bacterial Growth on Different Media. Streaked the urine of suspected patient with UTI on macConkey agar and nutrient agar plates and incubate for 24 hours at 37°C. Later than overnight incubation the morphology of bacterial colony on different media were observed.

Gram Staining of Bacterial Isolates

Both the Gram negative and Gram positive identified isolates were found. The identified Gram positive and gram negative isolate identified were *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Serratia marcescens*, *Klebsiella pneumonia*, and *Sphingobacterium multivorum* respectively.

Table 4.3 Isolated distribution on basis of gram staining reactions.

Gram staining reactions	No. of isolated bacteria	Percentage(%)
Gram negative	5 (out of 6)	83.33%
Gram positive	1 (out of 6)	16.66%



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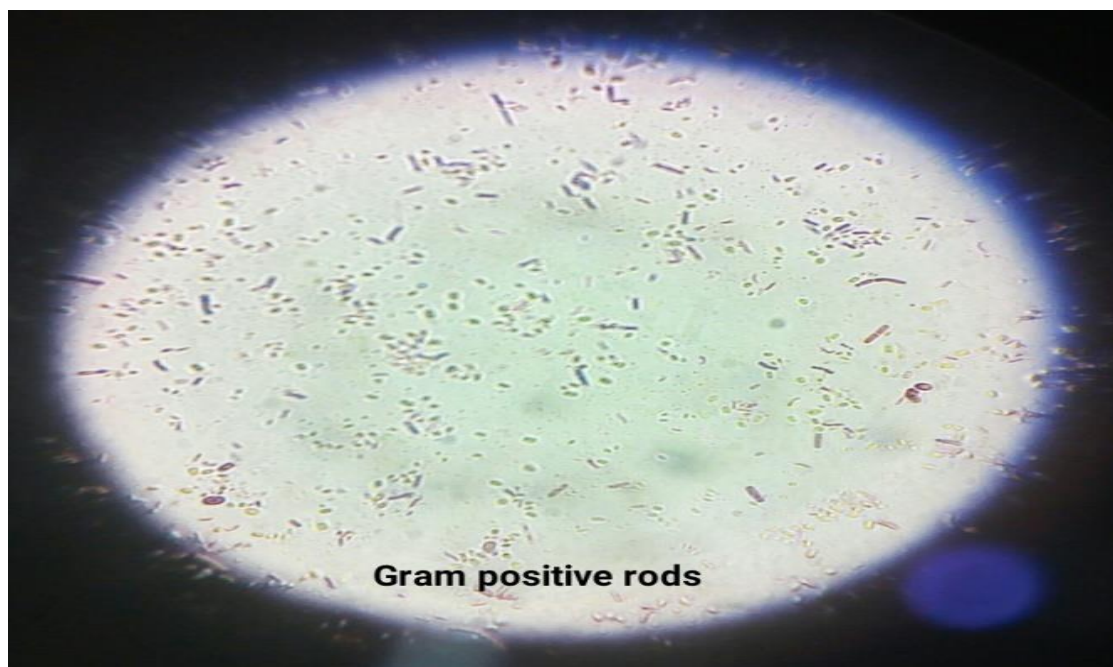


Fig 4.3 Gram Staining of Bacterial Isolates. Colonies were examined under light microscope thus result shows the gram positive (purple rods) and gram negative (pink rods) rods at 100x magnifying lens.

Total Isolates Obtained from the Urine of Infected Children.

Pathogenic isolates	≥1 Years	≥5 year	≥10 years	Percentage
<i>Escherichia coli</i>	1	2	8	55%
<i>Enterobacter aerogenes</i>	0	2	0	10%
<i>Serratia marcescens</i>	0	0	2	10%
<i>Klebsiella pneumonia</i>	0	0	1	5%
<i>Sphingobacterium multivorum</i>	0	0	1	5%
<i>Staphylococcus aureus</i>	0	2	1	15%

The isolates were obtained from the urine of the infected children among different aged groups 'ranged from 0-10 years. Our result showed that UTI reported as minimum aged of 1-

5 years and maximum aged of 6-10 years in girls as compare to boys.

Table 4.4 The isolated pathogens in positive patients with UT I among different age group were shown here.

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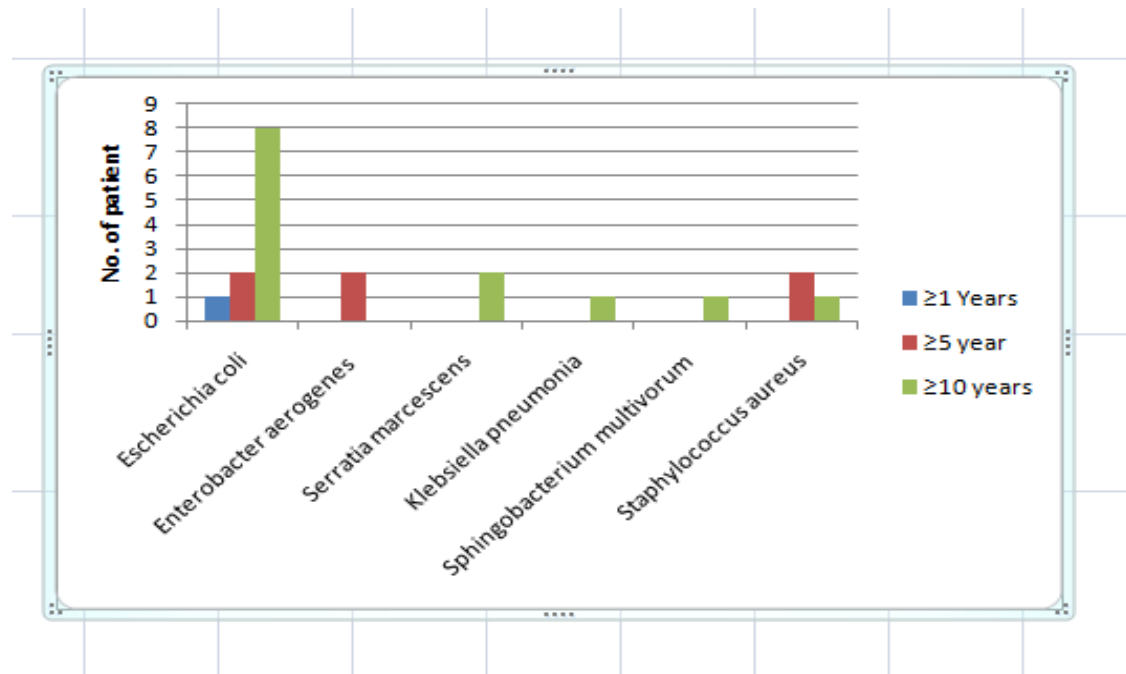


Fig 4.4 Total Isolates Obtained from the Urine of Infected Children. Number of patients having different pathogenic isolates on the basis of various aged groups.

Biochemical Test for Isolated Bacteria.

Several biochemical tests were conducted for both gram positive & negative isolates such as Catalase, Coagulase, DNase and API10S kit(for gram negative) respectively to identified the specific bacteria.

Table 4.5.1 Biochemical test for gram negative isolates are given below in the table.

Biochemical Tests	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>	<i>Serratia marcescens</i>	<i>Klebsiella pneumonia</i>	<i>Sphingobacterium multivorum</i>
Gram staining	-	-	-	-	-
ONPG	+	+	+	+	+
GLU	+	+	+	+	-
ARA	+	+	-	+	-
LDC	+	+	+	-	-
ODC	-	+	+	-	-
CIT	-	+	+	-	-
H ₂ S	-	-	-	-	-
URE	-	-	+	+	+
TDA	-	-	-	-	-
IND	+	-	-	+	-
OX	-	-	-	-	-

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NO2	+	+	+	+	-
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Fig 4.5.1 Identification of Specific Gram Negative Bacterial Strain. The isolated colony were mixed with normal saline solution and pour 1000 μ l into all the wells of Api10S kit. Specific bacterial strain were identified through api10s kit.

Table 4.5.2 Biochemical test for Staphylococcus aureu.(Gram positive).

Catalase Test .

This test were achieved for gram positive. The bubbles were produced which shows that bacteria was catalase positive.



Fig 4.5.2 Catalase Test for Staphylococcus Aureus. The pure isolate was picked through tip and dissolve it on 4 drop of H₂O₂ on clean microscopic slide the bubble formation were shown that bacteria was catalase positive.

Coagulase Test.

This test were achieved for gram positive isolate the result shows agglutination which indicate, Isolate was coagulase positive.



Fig 4.5.2.2 Coagulase Test for Staphylococcus Aureus. Clean microscopic slide with two circle (test and control) were marked. Put 2 drop of normal saline in both the circle and genetly mixed the colony in it. placed a drop of human plasma into circle T and

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drop of saline water into the circle C wait for 2 minutes agglutination was observed.

DNase Test.

DNase test were performed for gram positive isolate the result shows the formation of white zones on DNase agar plate in the presence of HCL which indicate the bacteria was DNase positive.

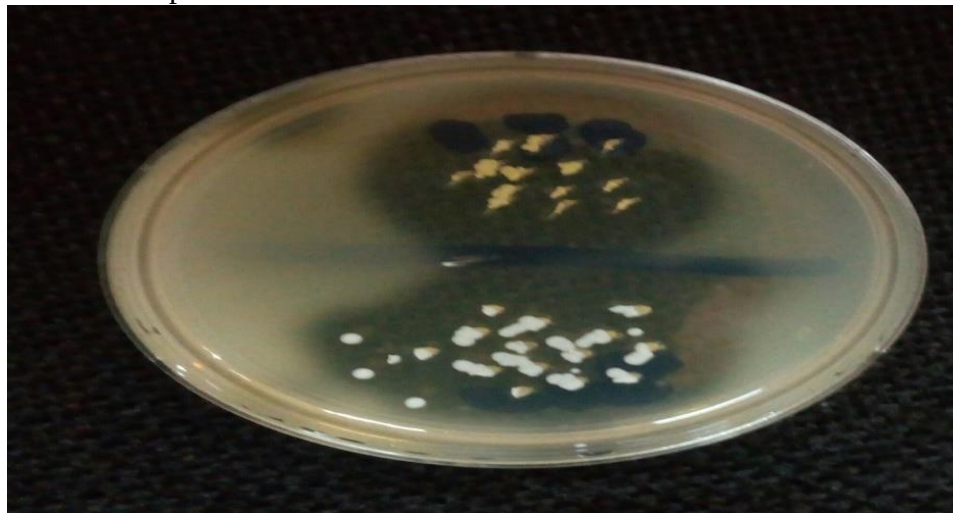


Fig 4.5.2.3 DNase Test for Staphylococcus Aureus. The bacterial colony was inoculated into the DNase agar plate and incubator overnight at 37C. After overnight incubation poured 2ml HCL on it. Wait for 10 minutes then excess HCL were removed and the clear zone were observed.

Resistance of Bacterial Strains toward Antibiotic.

Staphylococcus aureus and klebsella pneumonia were the strongest pathogenic bacteria which shows highest resistance to FEP, CAZ and AMC and very susceptible to AK and SCF antibiotic. Second pathogenic bacteria was Enterobacter aerogene shows 100% resistance to AMC, CN and PIP and very susceptible to AK, FEP, and CIP. Escherichia coli and Serratia marcescens were strongly resistance to AMC and PIP and Sphingobacterium multivorum shows highest resistance to ATM and susceptible to all others antibiotics.

Table 4.6 Resistance and sensitivity of different bacterial isolates to a variety of antibiotics were given in Table 4.5.

Antibiotics Names	<i>Escherichia coli</i>		<i>Enterobacter aerogenes</i>		<i>Serratia marcescens</i>		<i>Klebsiella pneumonia</i>		<i>Staphylococcus aureus</i>		<i>Sphingobacterium multivorum</i>	
	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm
CTX (30µg)	S	26	R	12	S	25	R	10	R	0	S	16
AMC (30 µg)	R	0	R	0	R	0	R	8	R	10	S	18

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FEP (30 µg)	S	25	S	19	S	25	R	0	R	0	S	20
CAZ 30 µg	S	21	S	20	S	20	R	0	R	0	S	15
CIP (5 µg)	S	15	S	21	S	15	S	21	S	18	S	20
AK (30 µg)	S	18	S	19	S	18	S	20	S	17	S	20
CN (10 µg)	S	17	R	0	S	13	S	17	R	0	S	17
PIP (20 µg)	R	0	R	0	R	0	S	16	S	16	S	18
SCF (105 µg)	S	21	S	20	S	20	S	19	S	21	S	16
ATM (30 µg)	S	21	R	10	S	21	R	0	R	0	R	0
MEM (10µg)	S	20	S	15	S	20	S	18	S	16	S	22

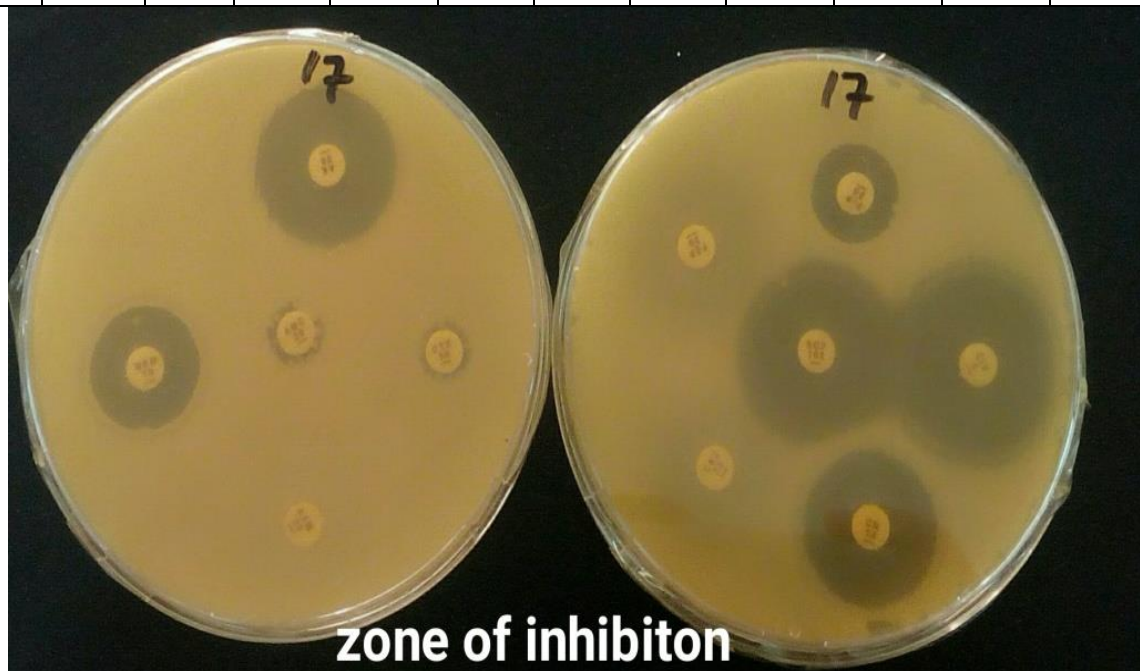


Fig 4.6 Resistance of Bacterial Strains toward Antibiotic. Prepared bacterial lawn on the plate containing MHA media and the antibiotic disk were placed through forceps on bacterial lawn, kept the plate in the incubator for 24 hour at 37°C. The zone inhibition indicate the resistance of antibiotic toward bacteria.

Biofilm Formation of Different Bacteria.

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The biofilm formation of these pathogenic bacteria were studied in the 96 well Elisa plates for 48 hours and for 1 week.

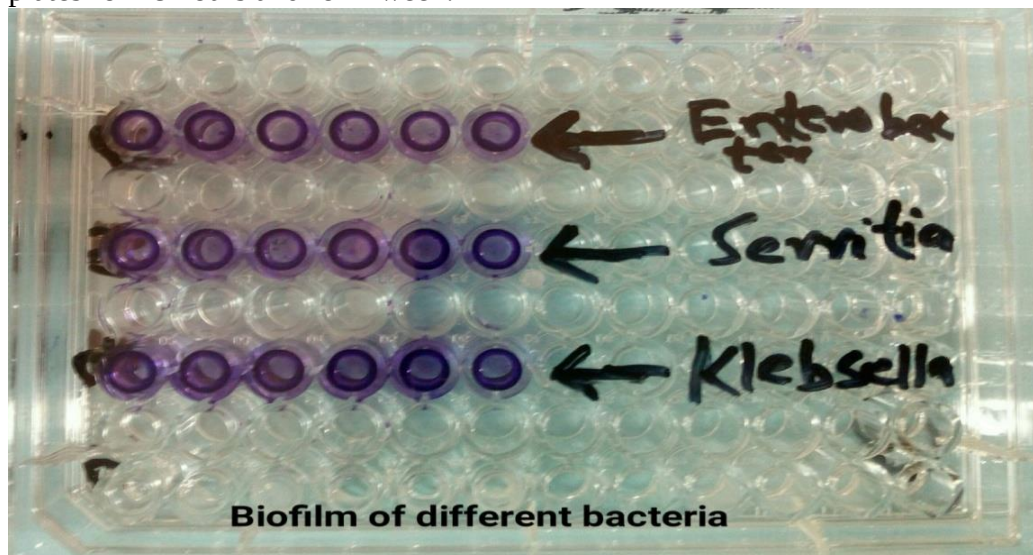


Fig 4.7.1 Biofilm Formation of Different Bacteria. Circular crystal violet stained bacterial biofilm rings are able to be seen in the wells of a 96 wells micro titer plate (indicated by arrow)

Fig 4.7.2 Biofilm Formation of Isolated Bacteria for Different Time. The result of OD value for control was $OD_c = 0.13$ So on the basis of OD values the bacteria isolates were classified as followed: $OD \leq 0.13$ = no biofilm forming bacteria; $0.13 < OD \leq 0.50$ = weak biofilm forming bacterial $0.50 < OD \leq 0.70$ = intermediate biofilm forming bacteria; and $OD > 0.70$ = strong biofilm forming bacteria.

Discussion

Most frequently Urinary tract infections are seen in the girls than in the boys. UTI are bacterial infections throughout pediatric age (Rushton, 1997). nearly all well-known microorganism isolated of Urinary tract was the Enterobacteriaceae family. The thick wall peptidoglycon bacilli (gram negative) which account for 100% of the total isolates are most frequently uropathogens (Al-Mijalli, 2017).

In our study Urine sample of 50 patients were collected from Mardan medical complex hospital. Samples were physically and microscopically examined. The urine color, smell and PH were observed. Microscopically the RBCs, crystal, epithelial cells were also observed in the urine . Out of 50 patient 20 patients were positive for UTI in which 14 were girls (70%) and 6 were boys (30%) aged range from 0 -10 years. Our result indicate that the occurrence rate of UTI was more in girls than in boys, which is similar to the study showed that in children UTI was more ordinary in females(64.8%) than male (35.2%) patients. (Lalhmangaihzuali et al., 2018) further study that was conducted in Bangladesh stated that prevalence of UTI in boys and girls was (38.32%)and (61.68%) respectively (Setu, 2016). UTI . In our study the frequency of UTI in children to the age of ≥ 1 year is 5% at the age of ≥ 5 years is 30% and at the age of ≥ 10 years is 65%.

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Positive UTI Samples were cultured on different media such as nutrient agar and macConkey agar. Bacteria were characterized on the basis of colony morphology and biochemical tests to recognize the isolate more accurately the colony characteristics were recorded. Numerous biochemical tests were achieved for the characterization of gram positive and gram negative isolates such as Catalase, Coagulase, DNase and API 10S kit respectively.

In our result Six different bacteria were obtained from the urine culture of positive patients. Among these bacteria *Escherichia Coli* was the most common uropathogen accounting 55% in UTI, which is similar to the previous report that stated, In UTI the most prevalent microorganism is *Escherichia Coli* (62.1%). (Bimanand et al., 2017) In our study the *klebsella pneumonia* frequency in UTI causing is 5% which resembles to previous work that stated *K. pneumoniae* frequency in UTI is (6.9%) (Asoufi et al., 2017). In our result the *staphylococcus* account 15% in UTI which is similar to previous work that stated *Staphylococcus aureus* report (15%) for the infection of urinary tract (AL-Geborri, 2010)

In our result *Staphylococcus aureus* and *klebsella pneumonia* is the strongest pathogenic bacteria shows highest resistance to cefepime, ceftazidime and Amoxicillin which is similar to previous report that stated highest percentage of resistance is showed by *K. pneumoniae* to ceftazidime, Amoxicillin, and cefepime, (Al-Mijalli, 2017). In our study *Escherichia coli* and *Serratia marcescens* shows resistance to Amoxicillin and Pipemidicid. which is similar to the report that stated *E. coli* confirmed the highest proportion of resistance to Amoxicillin, and ceftazidime (Al-Mijalli, 2017). In our study *Enterobacter aerogenes* show resistance to cefepime, Amoxicillin, Gentamicin and Pipemidicid and *Sphingobacterium multivorum* show strongly resistance to Aztreonam.

In our result the *Escherichia coli*, *klebsella pneumonia*, *Enterobacter aerogenes* and *Serratia marcescens* were very susceptible to meropenem, and Amikacin, which correlate that *Escherichia coli* showed highly susceptibility (98.90%) to each of amikacin, meropenem and *K. pneumoniae* was highly susceptibility to Meropenem (96%) and Amikacin (Al-Mijalli, 2017).

In our result OD value for control was $OD_c = 0.13$. So on the basis of OD values the bacteria isolates were classified as follows: $OD \leq 0.13$ = no biofilm created bacteria; $0.13 < OD \leq 0.50$ = weak biofilm created bacteria; $0.50 < OD \leq 0.70$ = intermediate biofilm created bacteria and $OD \leq 0.99$ = strong biofilm created bacteria

In my study both the gram negative and positive isolated bacteria were able to make biofilm. which associated that about 80% of all bacterial isolates include both Gram negative and Gram positive isolates were capable to form 81% and 75%, biofilm respectively (Bhatta, 2019) However the strong biofilm producer bacteria than that in our result were reported *Escherichia Coli* and *Serratia marcescens* ($OD \leq 0.99$) Intermediate biofilm producer were *Enterobacter* ($OD \leq 0.70$) and the lower biofilm producer bacteria were *Staphylococcus aureus* ($OD \leq 0.50$) and *sphingoporum* was non biofilm producer bacteria ($OD \leq 0.13$).

Conclusion

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Our result show that in district Mardan the urinary tract infection is very common in the girls children than in boys and most of the school aged children are infected from UTI. These infection are caused by pathogenic bacteria and the most common pathogen involved in this infection are Escherichia coli, Staphylococcus aureus, Enterobacter aerogene, Serratia marcescens, klebsella pneumonia and. The Staphylococcus aureus, klebsella pneumonia are the strongest pathogenic bacteria which show resistance to many antibiotic like Amoxicillin, cefepime, and ceftazidime. These pathogenic bacteria are also biofilm producer except Sphingobacterium multivorum. Among these pathogenic bacteria Enterobacter aerogene, Serratia marcescens, klebsella pneumonia are strong biofilm forming bacteria. Biofilm is a virulence factor and this biofilm forming bacteria show resistance to many antibiotic which is a major problem for treating uti. therefore further studies analysis of formation and the associated genes detection in these isolates are required. Further study are needed to see the virulence gene which is involved in biofilm formation.

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