



A Study of Urinary Tract Infections Prevalence, Antibiotics Resistance, and Biofilm Formation Capability of the Bacterial Causal Agents

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ABSTRACT

The aim of this study was to evaluate the incidence of Urinary Tract Infections (henceforth, UTI) in Salahadin governorate/ Iraq, identify the causative agents, their antibiotic sensitivity and their ability to form biofilms. Such studies are mandatory to determine the empirical therapy of such cases. A total of 650 urine samples were collected. Then, 250 samples were cultured, as suspected of having UTI, of which 193 samples (77.2 %) showed positive culture, while 57 samples (22.8 %) were negative cultures. Gram-positive bacteria were the predominant cause of UTI as represented by (66.7%), while Gram-negative species were found in (33.3%). *Staphylococcus spp.* bacteria were the predominant Gram-positive genus to isolate from UTI patients, while *E. coli* was the predominant species among Gram-negative bacteria. The isolates were tested for antibiotic susceptibility and biofilm formation. The most effective antibiotics tested on the Gram-positive isolates were Nitrofurantoin followed by Chloramphenicol (with resistance of 19% and 20%, respectively), whereas the least effective one was Azithromycin (with 64% resistance). As for the most effective antibiotic tested on the Gram-negative isolates, it was Amikacin (having only 8% resistance), while the least effective one was Erythromycin (with 98% resistance). Biofilm detection was performed by employing Congo red agar (CRA) method and microtiter plate (MTP) assay. The results showed that by using CRA method, about 81.3% of isolates were biofilm formers. While 96.7% of isolates were detected as biofilm formers by using MTP assay. Generally, biofilm formation was more predominant among Gram-positive isolates than Gram-negative ones.

Introduction

Urinary tract infection (UTI) is the infection that leads to an inflammatory response in the urinary tract epithelium [1]. In communities and hospitals, one of the most commonly encountered infections is UTI. Clinically, UTI can be complicated or uncomplicated. Uncomplicated UTI affects individuals who are otherwise healthy with no abnormalities in the structure of the urinary tract. On the other hand, complicated UTI occurs in individuals with abnormalities in the structure of the urinary tract, immunosuppression, or with indwelling urinary catheters [2]. Most of the UTIs are of bacterial origin,

but some are due to fungi and seldom to viruses' infection [3].

For the last three decades, bacterial resistance has been going on among UTI-causing pathogens. The available reports and data confirm that the elevation in resistance to commonly utilized antibiotics is a sequel of inappropriate use of the antimicrobials. The surfacing of resistance amongst the UTI pathogens is an issue of intense concern and requires urgent attention in order to derive appropriate remedies to overcome the trouble [4].

Bacterial biofilms play a significant role in UTIs, being the cause of both acute and persistent infections. Up to 80% of all infections involve biofilm-forming bacteria, and mainly in the urinary tract, biofilm can become a serious problem. The antimicrobial resistance shown by biofilms is one of the most important concerns of these structures. Biofilm can be resistant to antibiotics up to 1,000-fold more than planktonic cells as a result of several mechanisms [5].

Methodology

Samples Collection and Bacterial Identification

Urine samples were collected during the period from 1/12/2020 to 1/2/2021 from out- and inpatients with different health issues admitted to Al-Alam and Salahadin General Hospitals. The method of urine collection was clarified to patients carefully. Each urine sample cup was labelled with name, age, gender, and time of collection [6]. Samples were

analyzed using the dipstick and sediment microscopic examination, and those suspected of UTI were submitted to the microbial culture method. All samples were inoculated on blood agar as well as MacConkey agar, incubated at 37°C for 24 hours and then inspected for bacterial growth [7]. Bacterial colonies were primarily classified by Gram-stain morphology. Then, they were definitively identified depending on standard culture and biochemical characteristics of isolates by using the Vitek-2 system (bioMerieux) according to the manufacturer's instructions.

Antimicrobial Susceptibility Testing

This test was performed by using the Kirby-Bauer method on Mueller Hinton agar (HiMedia, India) depending on Clinical Laboratory Standards Institute (CLSI, 2021) guide and according to Bahador *et al.* (2019) [8,9]. Antibiotics that were tested in this study are mentioned in table 1.

Table 1: Antibiotic discs utilized in this study

Antibiotic group	Antimicrobial agent (Code)	Dose /Disc	Antibiotic group	Antimicrobial agent (Code)	Dose /Disc
Penicillins	Oxacillin (OX)	5µg	Aminoglycosides	Amikacin (AK)	10µg
	Ampicillin (AM)	25µg		Gentamicin (CN)	10µg
β_lactamase inhibitor combination	Amoxicillin+ clavulanic acid (AMC)	20/10µg	Fluoroquinolones	Ciprofloxacin (CIP)	10µg
Folate pathway inhibitor	Trimethoprim (TMP)	10µg	Phenicol	Chloramphenicol (C)	30µg
Macrolides	Erythromycin (E)	10µg	Glycopeptides	Vancomycin (VA)	30µg
	Azithromycin (AZM)	15 µg		Carbapenems	Meropenem (MEM)

Detection of Biofilm Formation

a) Congo Red Agar (CRA) Method

CRA plates were inoculated with bacteria and incubated at 37°C /24 hrs. The plates were examined at (24 hrs.) and (48 hrs.) to view the color of grown colonies. Black colonies were recorded as a positive result, whereas non-slime producing strains formed red colonies. Color shades between black and red were interpreted as different slime production intensities [10, 11].

b) Microtiter Plate (MTP) Method

MTP assay is the most broadly used method and is regarded as the standard test for the detection of biofilm formation. The test was done as designated by Christensen *et al.* (1985) with an alteration in incubation time, which was lengthened to 24 hrs., as described by O'Toole and Kolter (1998) [12,13]. Bacterial isolates were incubated in nutrient broth at 37°C/24 hrs. Bacterial growth was diluted in 1:200 and inoculated into micro-titer plates (96 flat-bottom well micro-titer plates). After 24 hrs., PBS buffer was used to wash the wells 2-3 times and left to air-dry. Subsequently, crystal violet solution (0.4 %) was added as a stain for 10 min. After that, the plates were washed-off by sterilized distilled water and left to dry at room temperature. Then, ethanol (70%) was added to quantify bound bacteria. Finally, the absorbance at (490 nm) was determined; an OD of 490 nm > 0.12 was considered as a biofilm-forming sample, described as Heavy biofilm OD > 0.240, Moderate biofilm OD= 0.120-0.240, and No or Weak biofilm OD < 0.120 [14].

Results and Discussion

Incidence of UTI

In the current study, a total of 650 urine samples were collected. After early diagnosis, 250 samples were cultured, as suspected of UTI, of which 193 samples (77.2 %) showed growth of bacteria (positive culture), while 57 samples (22.8%) were negative cultures. According to gender, (220) samples represented by (88 %) were from females and 30 samples represented by (12%) were from males. One hundred and eighty-eight females (75.2%) were married and 37 of them (14.8%) were pregnant. Samples ranged in age from (5) to (62) years (see Table 2). The major rate of UTI (39.2 %) was seen in (21-30 years) age group, followed by (31-40 years) age group by (24%).

Table 2: Age groups in the present study

Age group	Number of samples	Percentage
Less than ten years old	10	4 %
10-20	32	12.8 %
21-30	98	39.2 %
31-40	60	24 %
41-50	25	10 %
51-60	15	6 %
Older than 60	10	4 %
Total	250	100 %

The differences in isolation rate are due to differences in the type and volume of the study population and duration. The percentage of negative cultures despite being positive in urinalysis may be due to infection with anaerobic bacteria, fungi or viruses that are not

obtainable in routine culture methods used in this study [15]. Females are more predisposed to have UTIs than males because the urethra is closer to the anus and much shorter. Sexual contact improves ascending of the organisms. Vaginal microbiota also play a serious role in boosting colonization of coliforms in the vagina and this may cause UTI [16, 17]. In the course of pregnancy, alterations in the urinary tract predispose women to infections. Those alterations include: Ureteral dilation, because of the pressure on ureters from the gravid uterus; Hormonal

effects of progesterone, which also may cause smooth muscle relaxation leading to dilation and vesicoureteral reflux increases; Immunocompromising can be another reason for the increased frequency of UTIs seen in pregnancy [18].

Etiological Bacterial Agents

After excluding samples that are suspected of being contaminated, Gram staining revealed that 100 isolates (66.7%) were Gram-positive, while 50 isolates (33.3%) were Gram-negative. Types and percentages of isolated bacteria are shown in table 3.

Table 3: Number of bacterial isolates and their percentages

Bacterial species	Number of isolates	Percentage
<i>Staphylococcus aureus</i>	32	21.33 %
<i>Enterococcus faecalis</i>	23	15.33 %
<i>Escherichia coli</i>	20	13.33 %
<i>Staphylococcus haemolyticus</i>	14	9.33 %
<i>Klebsiella oxytoca</i>	11	7.33 %
<i>Staphylococcus epidermidis</i>	11	7.33 %
<i>Staphylococcus saprophyticus</i>	8	5.33 %
<i>Streptococcus agalactiae</i>	6	4 %
<i>Klebsiella pneumoniae subsp. Pneumonia</i>	5	3.33 %
<i>Citrobacter freundii</i>	5	3.33 %
<i>Serratia fonticola</i>	5	3.33 %
<i>Micrococcus luteus</i>	4	2.67 %
<i>Pseudomonas aeruginosa</i>	2	1.33 %
<i>Proteus mirabilis</i>	2	1.33 %
<i>Kocuria rosea</i>	1	0.67 %
<i>Kytococcus sedentarius</i>	1	0.67 %

The results of this study are consistent with those found by Abd-Alwahab and Thalij (2015) whose isolates were 68.5% Gram-positive and 31.5% Gram-negative bacteria from UTI patients in Tikrit city, Iraq [19]. The current study results are close to those of Abdullah (2008) in Mosul city, Iraq, whose isolates were 56.7% Gram-positive and 43.4% Gram-negative bacteria [20]. However, these results contradict those found by Mahdi (2020) who reported that 92% of isolates were Gram-negative and only 8%

were Gram-positive bacteria in Tikrit city, Iraq [21]. Al-Tikrity (2016) had the opposite of our results, he found that 66% of the isolated bacteria were Gram-negative and 34% were Gram-negative in UTI patients in Erbil city, Iraq [22].

Antibiotic resistance of Gram-positive bacteria

Generally, the most effective antibiotics tested on the Gram-positive isolates were Nitrofurantoin followed by Chloramphenicol, and the least effective one was Azithromycin, as illustrated in table 4.

Table 4: Antibiotics Resistance for Gram-positive bacterial isolates

Bacteria	No. of isolates	Antibiotics									
		No. of resistant isolates (%)									
		F	C	OX	MEM	TMP	AK	CIP	AMC	AZM	CN
<i>Staphylococcus aureus</i>	32	5 (15.6)	5 (15.6)	22 (68.8)	6 (18.8)	11 (34.4)	10 (31.3)	4 (12.5)	10 (31.3)	19 (59.4)	15 (46.9)
<i>Staphylococcus haemolyticus</i>	14	4 (28.6)	5 (35.7)	8 (57.1)	4 (28.6)	5 (35.7)	4 (28.6)	4 (28.6)	7 (50)	8 (57.1)	4 (28.6)
<i>Staphylococcus epidermidis</i>	11	2 (18.2)	3 (27.3)	7 (63.3)	2 (18.2)	6 (54.5)	3 (27.3)	4 (36.4)	4 (36.4)	8 (72.7)	4 (36.4)
<i>Staphylococcus saprophyticus</i>	8	3 (37.5)	2 (25)	5 (62.5)	6 (75)	5 (62.5)	5 (62.5)	5 (62.5)	4 (50)	6 (75)	4 (50)
<i>Enterococcus faecalis</i>	23	2 (8.7)	4 (17.4)	9 (39.1)	5 (21.7)	10 (43.5)	11 (47.8)	4 (17.4)	10 (43.5)	14 (60.9)	12 (52.2)
<i>Streptococcus agalactiae</i>	6	0	0	4 (66.7)	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)	1 (16.7)	6 (100)	2 (33.3)
<i>Micrococcus luteus</i>	4	3 (75)	0	1 (25)	0	3 (75)	0	1 (25)	0	2 (50)	0
<i>Kocuria rosea</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Kytococcus sedentarius</i>	1	0	1 (100)	1 (100)	0	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)
Total resistance percentage	100	19	20	57	24	42	36	25	36	64	42

The current results were close to other studies at some points and opposed to others. For example,

Nigussie and Amsalu (2017) found that their Gram-positive isolates were 100% sensitive to

nitrofurantoin, showed a high resistance ratio to ciprofloxacin (50.0%) and trimethoprim-sulphomethoxazole (43.8%); and almost 25% resistance to oxacillin and amoxicillin +clavulanic acid was detected [23]. Woldemariam *et al.* (2019) reported that among Gram-positive organisms, *Staph. aureus* was 100% susceptible to ciprofloxacin and nitrofurantoin; Coagulase negative Staphylococci (CONS) were highly sensitive to amoxicillin +clavulanic acid and nitrofurantoin 6/7 (83.3%). Vancomycin (83.3%) was the drug of choice with superior effectiveness on *Enterococcus spp.*, whereas 2/6 (33.3%) of the *Enterococcus spp.* were nitrofurantoin resistant [24]. Petca *et al.* (2020) agreed with our result that nitrofurantoin showed

major effectiveness against Gram-positive bacteria. They reported 3.3% resistance among *Enterococcus spp.*, and only 0.8% resistance among *Staphylococcus spp.* [25] Al-Asady *et al.* (2022) also reported (80%) sensitivity to nitrofurantoin among Gram-positive uropathogens [26]. Assafi *et al.* (2015) found that nitrofurantoin-positive bacterial isolates showed sensitivity to nitrofurans, while isolates were resistant to the penicillin group [27].

Antibiotic Resistance to Gram-Negative Bacteria

Generally, the most effective antibiotic tested on the Gram-negative isolates was amikacin, and the least effective one was erythromycin, as illustrated in table 5.

Table 5: Antibiotics Resistance for Gram-negative bacterial isolates

Bacteria	No. of isolates	Antibiotics									
		No. of resistant isolates (%)									
		AK	E	VA	CN	F	C	CIP	TMP	AM	AZM
<i>Escherichia coli</i>	20	1 (5)	20 (100)	18 (90)	4 (20)	3 (15)	0	3 (15)	15 (75)	20 (100)	7 (35)
<i>Klebsiella oxytoca</i>	11	0 (100)	11 (100)	11 (100)	6 (54.5)	4 (36.4)	0	2 (18.2)	3 (27.3)	11 (100)	1 (9.1)
<i>Klebsiella pneumonia</i>	5	3 (60)	5 (100)	5 (100)	4 (80)	5 (100)	4 (80)	4 (80)	3 (60)	5 (100)	4 (80)
<i>Citrobacter freundii</i>	5	0 (100)	5 (100)	5 (100)	2 (40)	4 (80)	2 (40)	0	2 (40)	4 (80)	1 (20)
<i>Serratia fonticola</i>	5	0 (80)	4 (80)	5 (100)	4 (80)	2 (40)	1 (20)	0	3 (60)	3 (60)	3 (60)
<i>Pseudomonas aeruginosa</i>	2	0 (100)	2 (100)	2 (100)	1 (50)	2 (100)	1 (50)	0	2 (100)	2 (100)	0
<i>Proteus mirabilis</i>	2	0 (100)	2 (100)	2 (100)	1 (50)	1 (50)	1 (50)	0	2 (100)	2 (100)	2 (100)
Total resistance percentage	50	8	98	96	44	42	18	18	60	94	36

When comparing the current results to other studies, it found that they match or are close to ours at some points and are opposed to others. For example, Al-Zahrani *et al.* (2019) found that the rate of antibiotic resistance was highest for ampicillin (85.6%), nitrofurantoin resistance (48.8%), and resistance to ciprofloxacin (17.9%) [28]. While Demir and Kazanasmaz (2019) reported high resistance to ampicillin (87.1%), while the lowest resistance was to nitrofurantoin (21.4%) and amikacin (4.2%) [29]. Assafi *et al.* (2015) found that Gram-negative bacteria were sensitive to carbapenems and aminoglycosides, and the majority of bacteria were unaffected by penicillins [27]. Gram-negative bacterial isolates were 100% resistant to ampicillin in Nigussie and Amsalu's (2017) study, and highly resistant to gentamicin (58.8%), while all of the Gram-negative isolates were nitrofurantoin sensitive [23]

Detection of Biofilm Formation

A) Congo Red Agar "CRA" Method (Slime Layer Production)

In this study, 122 (81.3 %) of the total bacterial samples showed positive slime formation on CRA. Thirty-four isolates (22.67%) were moderate biofilm formers that appeared as black-dark grey colonies (sometimes with a clear zone around the colonies). While 57 isolates (38%) were weak biofilm formers

that appeared as dark red colonies (maroon), 24 isolates (16%) were considered as non-biofilm producers that appeared as pale red colonies (close to pink).

In addition, 82% of the Gram-positive isolates were biofilm formers, and only 18% were non-biofilm producers. The majority of isolates (53%) were weak biofilm formers, 14% moderate and 15% heavy biofilm formers by using CRA method. While 88% of the Gram-negative isolates were biofilm formers and just 12 % were non-biofilm formers. Moreover, 40% isolates were heavy biofilm formers, 40% isolates were moderate biofilm formers, and only 8% were weak biofilm formers by employing this method.

B) Microtiter Plate (MTP) Method

Totally, 145 isolates (96.7%) were biofilm formers (50% moderate and 46.7% heavy biofilm producers); however, only five isolates (3.3%) were non-biofilm formers by using MTP method. All but 2 isolates (98%) of Gram-positive bacteria were biofilm formers by utilizing this method, and half of them were heavy biofilm formers. All but 3 isolates of the Gram-negative bacteria were biofilm formers (40% heavy and 54% moderate biofilm formers) by using the MTP method. Tables 6 and 7 illustrate the percentages of biofilm formation of each bacterial type by employing both methods.

The difference in thickness of biofilms can be attributed to variations in isolates' capability to synthesize biofilms, the initial number of cells that have the ability for adherence, or differences of the

quality and quantity of auto-inducers quorum sensing signaling molecules (QS) system produced from each isolate [30].

Table 6: Results of biofilm formation of Gram-positive bacteria

Bacterial type	No. of isolates	MTP method Isolates (%)			CRA method Isolates (%)			
		Heavy biofilm OD>0.240	Moderate biofilm OD= 0.120-0.240	No or weak biofilm OD<0.120	Heavy biofilm	Moderate biofilm	Weak biofilm	No biofilm
<i>Staph. aureus</i>	32	17 (53.1%)	15 (46.9%)	-	7 (21.9)	7 (21.9)	15 (46.9)	3 (9.4)
<i>Staph. haemolyticus</i>	14	12 (85.7%)	2 (14.3%)	-	2 (14.3)	-	10 (71.4)	2 (14.3)
<i>Staph. epidermidis</i>	11	7 (63.6%)	4 (36.4%)	-	1 (9.1)	-	5 (45.5)	5 (45.5)
<i>Staph. saprophyticus</i>	8	3 (37.5%)	5 (62.5%)	-	1 (12.5)	1 (12.5)	5 (62.5)	1 (12.5)
<i>E. faecalis</i>	23	10 (43.5%)	13 (56.5%)	-	4 (17.4)	4 (17.4)	14 (60.9)	1 (4.3)
<i>Strep. agalactiae</i>	6	1 (16.7%)	5 (83.3%)	-	-	2 (33.3)	3 (50)	1 (16.7)
<i>M. luteus</i>	4	-	4 (100%)	-	-	-	1 (25)	3 (75)
<i>Kocuria rosea</i>	1	-	-	1 (100%)	-	-	-	1 (100)
<i>Kytococcus sedentarius</i>	1	-	-	1 (100%)	-	-	-	1 (100)
Total percentage	100	50%	48%	2%	15 %	14%	53%	18%

Table7: Results of biofilm formation of Gram-Negative bacteria

Bacterial type	No. of isolates	MTP method Isolates (%)			CRA method Isolates (%)			
		Heavy biofilm OD>0.240	Moderate biofilm OD= 0.120-0.240	No or weak biofilm OD<0.120	Heavy biofilm	Moderate biofilm	Weak biofilm	No biofilm
<i>E. coli</i>	20	7 (35%)	12 (60%)	1 (5%)	9 (45)	10 (50)	1 (2)	-
<i>K. oxytoca</i>	11	6 (54.5%)	5 (45.5%)	-	9 (81.8)	-	2 (18.2)	-
<i>K. pneumonia</i>	5	2 (40%)	3 (60%)	-	2 (40)	-	-	3 (60)
<i>C. freundii</i>	5	3 (60%)	1 (20%)	1 (20%)	-	3 (60)	1 (20)	1 (20)
<i>Serratia fonticola</i>	5	1 (20%)	3 (60%)	1 (20%)	-	5 (100)	-	-
<i>P. aeruginosa</i>	2	1 (50%)	1 (50%)	-	-	-	-	2 (100)
<i>Proteus mirabilis</i>	2	-	2 (100%)	-	-	2 (100)	-	-
Total percentages	50	40%	54%	6%	40%	40%	8%	12%

The results showed that by using the CRA method, 81.33% of isolates were biofilm formers. While 96.7% of isolates were detected as biofilm formers by using MTP assay. In spite of its fame, the CRA method is not recommended as a suitable method for

the detection of biofilm production, as it does not give reliable and dependable results for being based on four colors scale (black, grey, maroon, and red), causing different interpretations from one person to another [31].

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دراسة انتشار التهاب المسالك البولية، مقاومة المضادات الحيوية، و قابلية المسببات البكتيرية على

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الملخص

كان الهدف من الدراسة هو تقييم نسبة الاصابات بالتهاب المسالك البولية في محافظة صلاح الدين/ العراق، تشخيص المسببات البكتيرية ومن ثم معرفة مقاومتها للمضادات الحيوية وقدرتها على تكوين الغشاء الحيوي. هذا النوع من الدراسات مهم جداً لمعرفة العلاجات الاساسية لمثل هذه الامراض. في الدراسة الحالية تم جمع 650 عينة ادرار وتم زرع 250 عينة، اظهرت 193 منها نمواً (77.2%) بينما 57 عينة (22.8%) لم تظهر نمواً. البكتريا الموجبة لصبغة كرام كانت المسبب الرئيس لالتهابات المسالك البولية ضمن عينة الدراسة الحالية، مسؤولة عن 66.7% من الحالات، في حين تسببت البكتريا السالبة لصبغة كرام ب 33.33% من الحالات. كانت بكتريا *Staphylococcus spp.* الاكثر عزلاً من البكتريا الموجبة، و بكتريا *E.coli* كانت الاكثر عزلاً ضمن البكتريا السالبة لصبغة كرام. أظهر المضاد الحيوي Nitrofurantoin أعلى فعالية على البكتريا الموجبة لصبغة كرام يليه المضاد Chloramphenicol (نسبة المقاومة 19 و 20% على التوالي) أما المضاد الاقل فعالية فكان Azithromycin بنسبة مقاومة 64%. أما بالنسبة للبكتريا السالبة لصبغة كرام فكان المضاد الحيوي Amikacin هو الاكثر فعالية تجاهها وبنسبة مقاومة 8% فقط، في حين أظهر المضاد Erythromycin أقل فعالية ضد هذه البكتريا بنسبة مقاومة 98%. تم التحري عن قدرة البكتريا على تكوين الغشاء الحيوي باستخدام طريقتي اكار الكونغو الأحمر وطبق المعايير المتعدد. حيث اظهرت النتائج ان 81.3% من العينات كانت مكونة للغشاء الحيوي بطريقة اكار الكونغو الاحمر بينما اظهرت 96.7% من العينات قابليتها على تكوين الغشاء الحيوي بطريقة اطباق المعايير. بصورة عامة كانت البكتريا الموجبة لصبغة كرام اكثر قدرة على تكوين الغشاء الحيوي من البكتريا السالبة.