Determination of the prevalence and antibiotic susceptibility pattern of the members of Enterobacteriaceae family from the urine samples of UTI suspected patients from a diagnostic center in Dhaka city

Inspiring Excellence

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I, Syeda Samiha Haque declare that this thesis and the work entitled “A study on the” submitted to the Department of Mathematics and Natural Sciences (MNS), BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under the supervision of my supervisor, Namista Islam, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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DEDICATED TO MY BELOVED FATHER AND MY MOTHER

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ABSTRACT

Urinary Tract Infection (UTI) defines a condition in which the urinary tract is infected with a pathogen causing inflammation which is a common, distressing and occasionally life threatening condition. UTI affects people of all ages and both gender. In developing countries, UTI is a common experience in clinical practice. UTI can also lead to bladder infection (cystitis) and kidney infection (pyelonephritis). Symptoms from a lower urinary tract include pain with urination, frequent urination, and feeling the need to urinate despite having an empty bladder. Symptoms of a kidney infection include fever and flank pain usually in addition to the symptoms of a lower UTI. Rarely the urine may appear bloody. In the very old and the very young, symptoms may be vague or non-specific. Every year millions of people suffer from UTI worldwide, women are mostly affected from this infection.

Urinary tract infection is increasing day by day and becoming a life-threatening infection due to its resistance against different antibiotics. Antimicrobial resistance is not only increasing the healthcare costs, but also the severity and death rates from certain infections. In most cases the infectious agents are the members of Enterobacteriaceae family members such as *E. coli, Klebsiella spp., Enterobacter spp*. and *Proteus spp.* The aim of this study was to determine the prevalence of the members of Enterobacteriaceae family which are involved in urinary tract infections and the susceptibility of these microorganisms against different antibiotics. 25 urine samples of some UTI suspected male and female patients were collected from a diagnostic center. The samples were plated onto Nutrient agar for total count. Then the isolates were sub-cultured and the selected isolates were grown in MacConkey agar, EMB agar, Hi- Crome agar and Blood agar and Biochemical tests were done for identification of the isolates. About 44 isolates were the members of Enterobacteriaceae family. *Klebsiell*a *spp*. was predominant pathogen while *Enterobacter spp.*, *E. coli* and *Proteus spp*. were also found. Most of the isolates were resistant to Penicillin (90.09%) followed by Erythromycin (84.09%), Ampicillin (75%), Rifampicin (75%) and Cefepime (70.45%), Azithromycin (59.09%), Ciprofloxacin (47.72%) and Tetracycline (31.82%). On the other hand, Streptomycin (2.27%) and Chloramphenicol (9.09%) were found to be effective antibiotics. Considering the above, it is a great matter of concern that the emergence of resistant strains is increasing day by day. However, proper and rational use of antibiotics should be practiced to delay the emergence of the resistant strains.

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UTI	Urinary Tract Infections
MR	Methyl Red
VP	Voges-proskauer
TSI	Triple Sugar Iron
EMB	Eosin Methylene Blue agar
MHA	Muller Hinton Agar
MIU	Motility Indole Urease
µL	Microliter
mm	Millimeter
spp.	Species

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Chapter 1

INTRODUCTION

1.1) Background:

Urinary tract infection is one of the major diseases nowadays. Urinary tract infections are caused by microbes such as bacteria overcoming the body's defenses in the urinary tract. They can affect bladder, kidneys and the tubes of urinary tract. Both men and women can be affected by urinary tract infections but women have more chances of developing a urinary tract infection. In most cases the infectious agents are the members of Enterobacteriaceae family such as *E. coli*, *Klebsiella spp.*, *Proteus spp.* and *Enterobacter spp.* etc. *Chlamydia spp.* and *Mycoplasma spp.* can infect the urethra but not the bladder.

UTIs are given different names depending on where they occur. For example: A bladder infection is called cystitis, a urethra infection is called urethritis and a kidney infection is called pyelonephritis. The ureters are very rarely the site of infection.

There are some symptoms of having Urinary tract infections. The symptoms of a UTI can depend on age, gender, the presence of a catheter, and what part of the urinary tract has been infected. Common symptoms of a UTI include: strong and frequent urge to urinate, cloudy, bloody, or strong-smelling urine, pain or a burning sensation when urinating, muscle aches and abdominal pains, Pain, pressure or tenderness in the area of the bladder and when bacteria spread to the kidneys patient may experience: back pain, chills, fever, nausea and vomiting (https://www.medicalnewstoday.com/articles/189953.php).

Over 50 percent of all women will experience at least one UTI during their lifetime. Pregnant women are not more likely to develop a UTI than other women, but if one does occur, it is more likely to travel up to the kidneys. People of any age and sex can develop a UTI. However, some people are more at risk than others. The following factors can increase the likelihood of developing a UTI: sexual intercourse, diabetes, poor personal hygiene, problems emptying the bladder completely, having a urinary catheter, bowel incontinence, blocked flow of urine, kidney stones, pregnancy, menopause, procedures involving the urinary tract and suppressed immune system.

UTI is the most common bacterial infection accounting for 25% of all infections. It is one of the most important causes of morbidity and also the second most common cause of hospital visit. It occurs in all populations and ages from the neonate to the geriatric age group. However, infection is most common in women, especially sexually active women. Women are more susceptible than men, due to several clinical factors including anatomic difference, hormonal effects and behavioral pattern. In Bangladesh urinary tract infection (UTI) is also one of the most important causes of morbidity and mortality.

Urinary tract infection (UTI) remains as one of the most common bacterial infections and second most common infectious disease in community practice with approximately 150 million diagnosed cases each year. Presence of more than 10^5 organisms per ml in a midstream sample of urine refers to significant bacteriuria and caused mainly by normal bowel flora, *Escherichia coli*, which is responsible for over 75 % of cases. Other members of Enterobacteriaceae and a few Gram-positive bacteria like *Staphylococcus saprophyticus* and *Enterococcus faecalis* are also responsible for UTI (Haque et al. BMC Res Notes, 2015).

Since the initiation of antimicrobial therapy in UTI is empirical, a huge need demand for antimicrobial resistance exists at local, national and international levels (Bassetti 2000). Knowledge on the antimicrobial resistance patterns of common pathogens and the subsequent treatment are thus required to minimize urinary diseases (Prais et al. 2003). Along these lines, the present study was designed to identify the prevalence of Enterobacteriaceae bacteria that causes UTI in males and females of different age groups and to investigate their responses against locally available antibiotics.

1.2) Objectives of the study:

- To isolate and identify organisms that are belong to Enterobacteriaceae family.
- To identify the predominant organisms that are responsible for Urinary Tract Infection.
- To study the antibiotic resistance pattern of these organisms.
- To identify the effective antibiotic for the treatment of Urinary Tract Infection.

1.3) Literature Review:

1.3.1) Overview of Urinary Tract Infection:

A urinary tract infection (UTI) remains a major clinical problem over 50 years after the introduction of anti-microbial therapy. This is partly because of the emergence of increasing rates of drug resistance in UTI. The increasing prevalence of antibiotic resistance has been reported from various countries. Urinary tract infection is the second infection in human. Urinary infections cause fewer complications than nosocomial infections, but they occasionally can cause bacteremia and death. Gram negative bacteria play an important role in UTI. It has been estimated that more than 7 million visits to emergency units and 100,000 in hospitals occurs annually in the USA (Kumar et al. 2013).

Infection of the urinary tract is an extremely common clinical problem (Khanum et al. 2012). Even today urinary tract infection (UTI) is one of the most important causes of morbidity and mortality in the developing countries like Bangladesh. This may be attributed to lack of proper research, faulty diagnostic procedures, abuse of chemotherapeutic agents of the people and little or no preventive measures. The alarming phenomenon is that UTI does not restrict itself to the urinary tract only rather it can spread. UTI infections usually cause inflammation of the affected tissues of the urethra (urethritis) and urinary bladder. The most significant danger from lower urinary tract infections is that they can affect the kidney (causing pyelonephritis) and develop bladder infections subsequently (Nahar et al. 2010). Bacteria carried by blood stream can also infect the kidney and the infections can be very difficult to eradicate, are often chronic, and lead to marked damage of the kidney. Death promptly follows kidney failure unless the patient is lucky enough to be able to use artificial kidneys, or perhaps to receive a kidney transplant.

Urinary tract infection (UTI) involves the infection of kidneys, ureters, bladder, or urethra by pathogenic invasion of the urinary tract, which ultimately leads to an inflammatory response of the urothelium. All individuals may be susceptible to UTIs; however, the prevalence of infection differs with age, sex and certain predisposing factors (Griebling 2001). The incidence of infection is greater in females than in males with two exceptions, infants and the catheter related infections

(Khan et al. 2014). Women tend to get UTIs more often because their urethra is shorter and closer to the anus than men and hence the pathogenic bacteria get quick access to the bladder.

1.3.2) Pathogenesis of Urinary Tract Infection:

Anatomically urinary tract consists of lower urinary parts namely the urethra and bladder while the upper urinary tract is classified as the ureters and kidneys (Heffner and Gorelik, 2008). All part of urinary systems above the urethra in a healthy human is clean and the urinary system erases metabolic wastes from the blood and excretes it as urine to the outside during urination (KİREÇCİ et al. 2015). Symptoms and signs of urinary tract infections may include fever, urinary urgency, dysuria, chills, cloudy and frequency or malodorous urine. Infections are nearly always ascending in origin and caused by bacteria in the distal urethra and the urethral flora. These bacteria colonize the perineal area and inhabit the distal gastrointestinal (GI) tract. UTIs are classified into uncomplicated and complicated infections which have effects on pre-and post-treatment evaluation, duration of antimicrobial therapy, the type and extent of estimation of the urinary tract (Sharma and Bidwai, 2013).

UTIs occur as a result of interactions between the uropathogen and host and their pathogenesis involve several processes. Initially the uropathogen attaches to the epithelial surface; it subsequently colonies and disseminates throughout the mucosa causing tissue damage. After the initial colonization period, pathogens can ascend into the urinary bladder resulting in symptomatic or asymptomatic bacteriuria. Further progression may lead to pyelonephritis and renal impairment. Specific virulence factors residing on the uropathogen's membrane are responsible for bacterial resistance to the normally effective defense mechanisms of the host (Niall F. Davis and Hugh D. Flood Department of Urology, Mid-Western Regional Hospital).

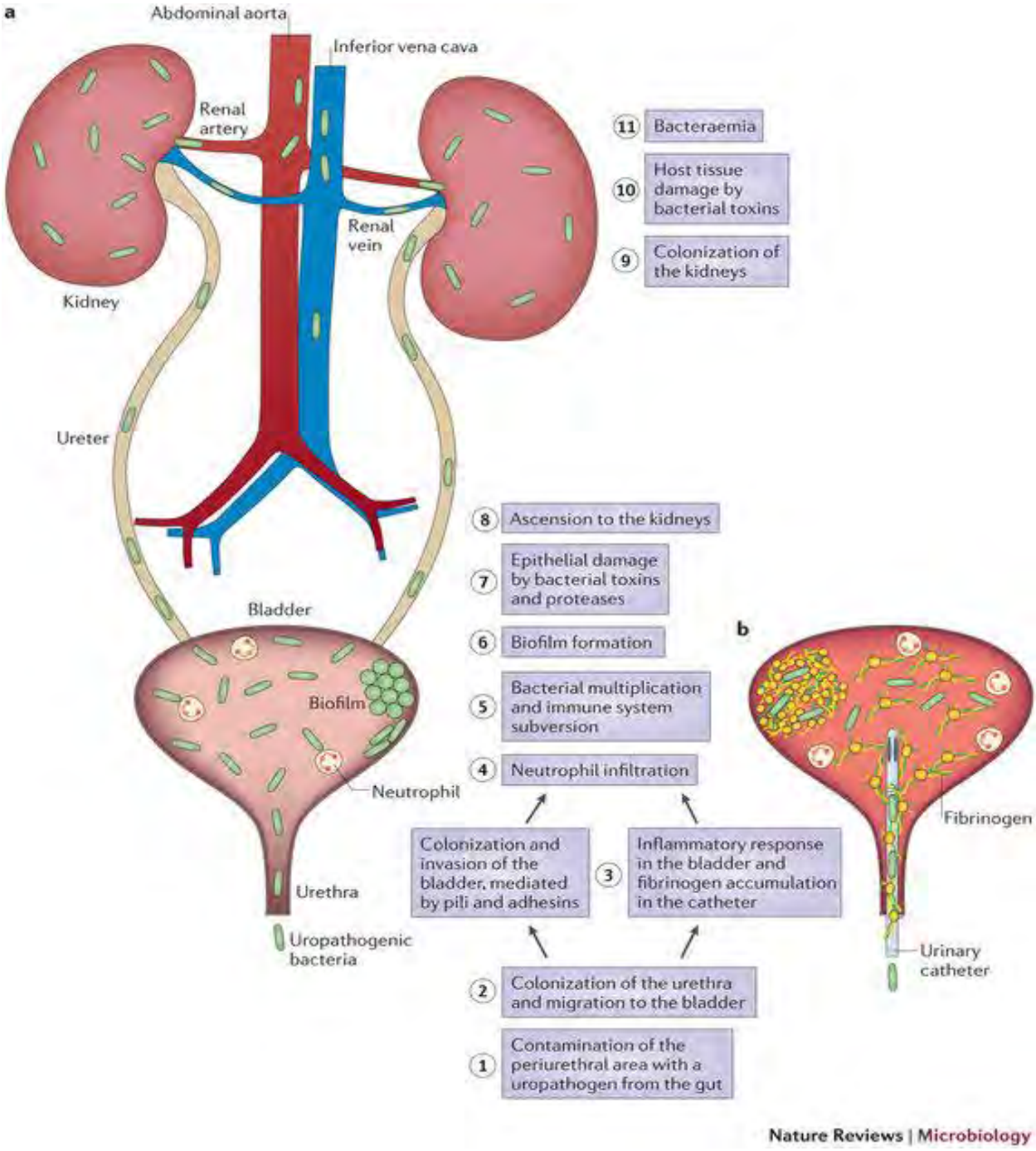


Figure 1.1: Pathogenesis of bacteria causing Urinary Tract Infection

1.3.3) Classification of UTI:

Urinary tract infection is classified into three types: acute pyelonephritis, lower UTI and asymptotic bacteriuria. More than 95% of urinary tract infections are caused by a single bacterial species (Shahab et al. 2017).

A urinary tract infection may involve only the lower urinary tract, in which case it is known as a bladder infection. Alternatively, it may involve the upper urinary tract, in which case it is known as pyelonephritis. If the urine contains significant bacteria but there are no symptoms, the condition is known as asymptomatic bacteriuria. If a urinary tract infection involves the upper tract, and the person has diabetes mellitus, is pregnant, is male, or immunocompromised, it is considered complicated. Otherwise if a woman is healthy and premenopausal it is considered uncomplicated. In children when a urinary tract infection is associated with a fever, it is deemed to be an upper urinary tract infection.

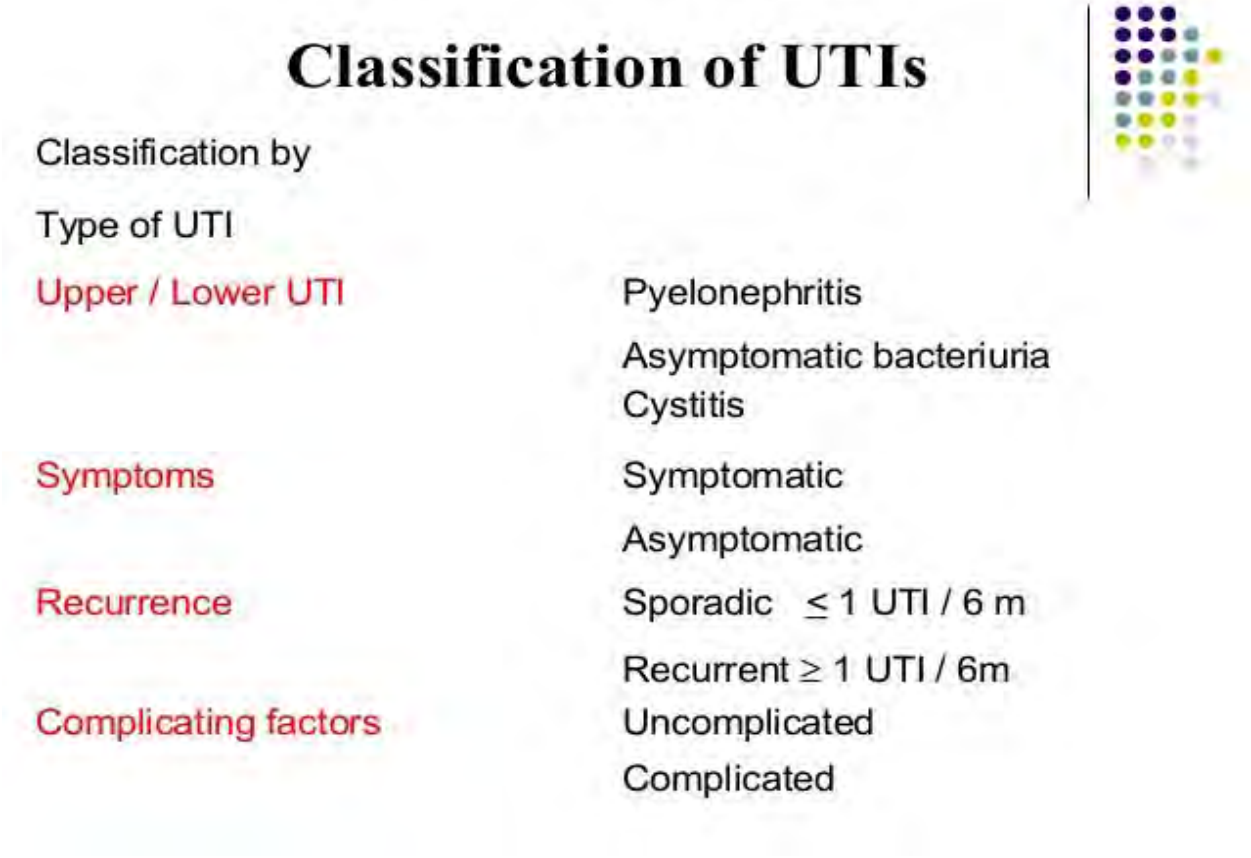


Figure 1.2: Classification of Urinary Tract Infection

1.3.4) Urinary Pathogens:

Urinary tract infection is one of the most common frequently occurring nosocomial infections. Normally UTIs are caused by a variety of Gram-negative and Gram-positive bacteria. The Gram-positive bacteria includes *Staphylococcus spp., Streptococcus spp.* and *Enterococcus spp.* Gram-negative includes a large number of aerobic bacilli such as *Escherichia spp., Klebsiella spp., Enterobacter spp., Citrobacter spp., Proteus spp., Serratia spp., Salmonella spp.* and *Pseudomonas spp.* Among this 80-90% of UTI is caused by *E. coli* (H.G.I Rushton 1997) and in ambulatory patients and of nosocomial infections, *Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, Enterococcus faecalis* are the most frequently isolated (Ouno et al. 2013).

Enterobacteriaceae are the most common cause of urinary tract infections (UTIs) in both community and healthcare settings. Selection of empiric antibiotic therapy for UTIs is therefore often based on the institutional susceptibility profiles of the Enterobacteriaceae. The Enterobacteriaceae are a large family of Gram-negative bacteria that includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella spp.*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella spp.*, and *Shigella spp*. Other disease-causing bacteria in this family include *Proteus spp.*, *Enterobacter spp.*, *Serratia spp.*, and *Citrobacter spp.* (Khawcharoenporn et al. 2013).

Escherichia coli remained the most common causative agent of uncomplicated UTI for many years with 75-90% causes of UTI infection. *Klebsiella pneumonia* accounts for 2nd highest organisms. The other gram-negative pathogens causing UTI are *Proteus mirabilis* and *Pseudomonas aeruginosa*, however, *Enterococci spp.* and coagulase negative *Staphylococci spp.* are the most frequently encountered gram-positive bacteria in UTI (Kumar et al. 2013).

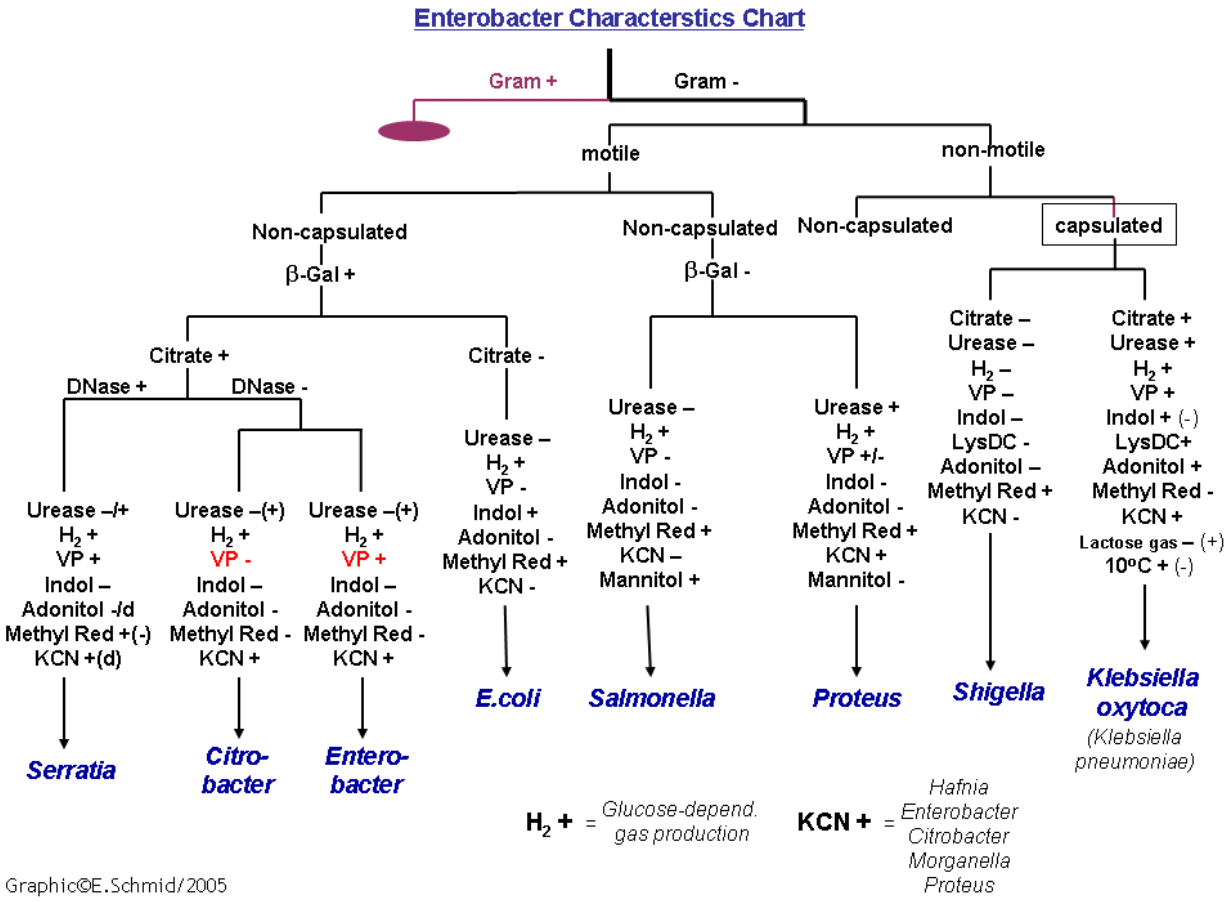


Figure 1.3: Overview of the members of Enterobacteriaceae family

1.4) Antibiotic Resistance:

Different drugs are used to treat the infection. Antibiotic resistance occurs when microorganisms alter when they are exposed to antibiotics. Antibiotic resistant-microbes are prevalent in people, animals, food, and the environment. They can spread between people and animals, and from person to person. Poor infection control, insufficient sanitary conditions and improper food-handling boost the spread of antibiotic resistance (WHO, 2017).

Antibiotic resistance is a worldwide issue. According the World Health Organization (WHO), antibiotic resistance is one of the world's greatest health threats to date (Haddox, 2013). New forms of antibiotic resistance can cross international boundaries and spread between continents easily (CDC, 2013).

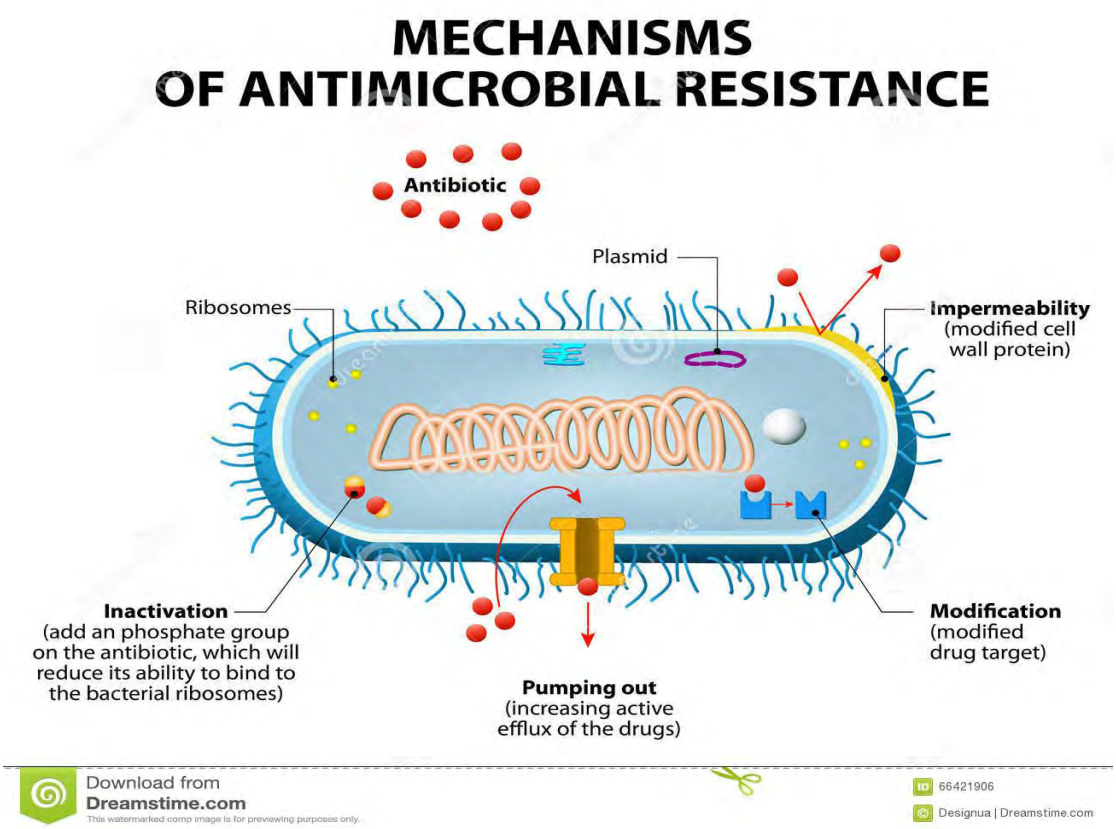


Figure 1.4: Mechanism of Antimicrobial Resistance

Indiscriminate use of antimicrobial agents is a common practice in underdeveloped and many developing countries that often leads to emergence of resistant microorganisms to one or several of these agents with gradual narrowing of scope for effective molecules to combat bacterial infections including UTIs. As a common practice, empirical antimicrobial treatment is initiated before the laboratory results of urine culture are available which may lead to emergence and spread of antimicrobial resistant strains. Factually antimicrobial resistance is one of the principal causes of treatment failure in infectious diseases and a great concern for UTIs. The prevalence and pattern of antimicrobial susceptibility of uropathogens are dependent on many factors and constantly changing with the ever-increasing use of antimicrobials, continuous monitoring of the susceptibility pattern is of paramount importance for not only selecting appropriate drugs but also for rational choice of empirical therapy (Haque et al. BMC Res Notes, 2015).

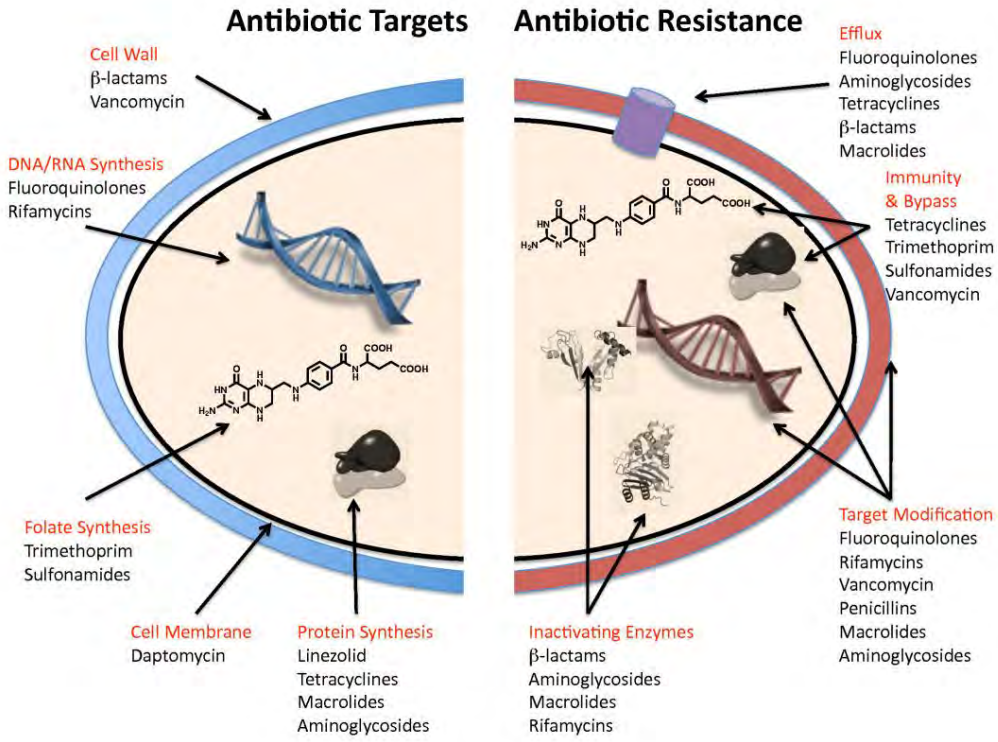


Figure 1.5: Antibiotic Targets and Antibiotic Resistance

In Bangladesh, use of antibiotics by medical practitioners is rampant resulting in increase in resistance to available antibiotics. Random and extensive use of broad spectrum of antibiotics contributed to changes in the microbiological and antibiotic susceptibility patterns of pathogens isolated from UTI. Therefore, for effective management of these infections, selection of antibiotics should be based on antibiotic susceptibility pattern. But it is often hampered by the lack of adequate facilities for proper microbial isolation as well as for their antimicrobial susceptibility testing (Yasmeen et al. 2015).

Chapter 2

Materials & Methods

2.1) Working area:

The entire study was conducted at the Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2) Sample collection:

25 urine samples were collected from a diagnostic center in Dhaka city. The samples were collected mostly at morning. The samples were carried in an ice box to suppress the overload of unwanted organisms. Then it was immediately transported to the laboratory of BRAC University for further processing and analysis. All the samples were collected in the same procedure.

2.3) Sample processing:

Samples were subjected to serial dilution and were spread plated onto Nutrient agar plates for counting the total load of organisms.

2.4) Incubation and selection:

After overnight incubation, the total number of colonies on the Nutrient agar plates were counted. The colony characteristics were observed. Then some colonies were selected by observing their cultural and morphological characteristics for culture, biochemical tests and other tests.

2.5) Isolates naming:

Table 2.1: Sample Collection: Source, Time, Number of the isolates found and their given name in the study

Sample No	Patient ID	Date	Time	Number of the isolates found	Isolates ID
1	R-17	7/5/2017	10:30 am	7	1a, 1b, 1c, 1d, 1e, 1f, 1g
2	R-16	7/5/2017	10:30 am	1	2
3	R-013586	7/5/2017	10:30 am	3	3a, 3b, 3c
4	R-013641	7/5/2017	10:30 am	1	4
5	D69542	11/16/2017	12:30 pm	3	a, b, c
6	D69537	11/16/2017	12:30 pm	3	d, e, f
7	D69522	11/16/2017	12:30 pm	4	g, h, i, j
8	D69551	11/16/2017	12:30 pm	5	k, l, m, n, o, p
9	D69499	11/16/2017	12:30 pm	2	q, r
10	D128346	9/7/2017	11:00 am	2	U_{15}, U_{18}
11	S7659	9/7/2017	11:00 am	6	U_{42}, U_{43}, U_{47}, U_{48}, U_{49}, U_{50}
12	D128496	9/7/2017	11:00 am	2	U_{11}, U_{14}
13	D128497	9/7/2017	11:00 am	7	U_4, U_5, U_6, U_7, U_8, U_9, U_{10}
14	D128358	9/7/2017	11:00 am	2	U_{20}, U_{24}
15	M26367	9/7/2017	11:00 am	2	U_{29}, U_{31}
16	S7710	9/7/2017	11:00 am	8	U_{33}, U_{34}, U_{35}, U_{36}, U_{38}, U_{39}, U_{40}, U_{41}
17	D128537	9/7/2017	11:00 am	1	U_2

18	B-1	10/9/2017	1:00 pm	4	B_3, B_6, B_{10}, B_{13}
19	B-2	10/9/2017	1:00 pm	2	B_{15}, B_{16}
20	E33220	15/10/2017	12:00 pm	7	$I_1, I_3, I_4, I_5, I_6, I_8, I_9$
21	M52249	15/10/2017	12:00 pm	6	$I_{12}, I_{17}, I_{18}, I_{19}, I_{21}, I_{22},$
22	M52923	15/10/2017	12:00 pm	1	I_{24}
23	E33170	15/10/2017	12:00 pm	5	$I_{25}, I_{26}, I_{28}, I_{31}, I_{32}$
24	E33199	15/10/2017	12:00 pm	4	$I_{33}, I_{35}, I_{36}, I_{37}$
25	M54970	15/10/2017	12:00 pm	2	I_{40}, I_{42}

Table 2.2: Sample No., Patient ID, Isolates ID and Number of the isolates that are members of Enterobacteriaceae family

Sample No.	Patient ID	Isolates ID	Number of isolates
1	R-17	1b, 1c,1d	3
5	D69542	a, c	2
6	D69537	d, e	2
7	D69522	g, h, i, j	4
8	D69551	k, n, o, p	4
10	D128346	U_{18}	1
11	S7659	U_{42}, U_{43}, U_{47}, U_{48}, U_{50}	5
12	D128496	U_{14}	1
13	D128947	U_5, U_6, U_7, U_8	4
14	D128358	U_{20}, U_{24}	2
15	M26367	U_{29}, U_{31}	2
16	S7710	U_{33}, U_{34}, U_{35}, U_{36}, U_{38}, U_{41}	6
17	D128537	U_2	1
20	E33220	I_1, I_3, I_4, I_5	4
21	M52249	I_{17}	1
23	E33170	I_{31}, I_{32}	2
			Total= 44

2.6) Inoculation on MacConkey agar:

A loop full colony of selected isolates was then streaked onto MacConkey agar and incubated at 37ºC for 24 hours for the screening of gram- negative enteric bacteria and the differentiation of lactose fermenting gram- negative bacteria and non- lactose fermenting gram negative bacteria.

2.7) Inoculation on Eosin Methylene Blue agar (EMB):

Eosin methylene blue agar (EMB) is a selective and differential medium used to isolate *E. coli*, which produces a characteristic metallic green sheen. Other pathogens such as *Enterobacter aerogenes* and *Klebsiella pneumoniae* can also ferment lactose and grow on EMB media

2.8) Inoculation on Hi Crome agar:

Hi Crome UTI Agar is a differential medium recommended for presumptive identification of microorganisms mainly causing urinary tract infections.

This agar medium is selective for urine infection causing microorganisms such as *Klebsiella pneumonia, Enterococcus fecalis, Staphylococcus aureus*, *Proteus mirabilis, E. coli*, *Pseudomonas aeruginosa* and they produce distinctive different colors on media. *E. coli* gives pink-purple colonies, *Staphylococcus aureus* gives golden yellow colonies, *Proteus spp.* give brown colonies, *Enterococcus faecalis* produce blue colonies, *Klebsiella pneumoniae* produce blue mucoid colonies and *Pseudomonas spp.* give colorless colonies on Hi-Crome agar.

2.9) Inoculation on Blood agar:

Blood Agar (BA) is an enriched medium used to culture pathogens that do not grow easily. It is also a differential medium in allowing the detection of hemolysis (destruction of the RBC). This media is used to see the lysis of red blood cells by the organisms. Usually three types of hemolysis are found including alpha hemolysis, beta hemolysis and gamma hemolysis. Hemolysis is determined by observing the clear zones around the bacterial growth.

2.10) Biochemical Test:

These 44 isolates were thought to be the members of Enterobacteriaceae family but a set of biochemical tests were performed in order to confirm that they were indeed belong to the members of Enterobacteriaceae family. The following biochemical tests were performed:

- Indole test
- Methyl Red (MR) test
- Voges– Proskauer (VP) test
- Citrate Utilization test
- Catalase test
- Oxidase test
- Triple Sugar Iron (TSI) test
- Motility Indole Urease (MIU) test
- Gram staining

2.10.1) Indole test:

Indole production test was done to determine the production of indole by pathogens. Only some pathogens have the ability to produce indole. For the indole test, tryptophan broth was inoculated with bacterial culture to observe the production of indole and incubated at 37°C for 24 hours. Then Kovac's reagent was added to the broth culture to observe the production of indole by observing the colour changes to determine whether the result is positive (cheery red ring) or negative (yellow) (Cappuccino and Sherman, 2005).

2.10.2) Methyl Red (MR) test:

Methyl red test was applied to analyze the bacterial ability to produce stable acid end products. Bacterial cultures were inoculated MR broth in clean test tubes and incubated overnight at a 37°C. Then methyl red reagent was added and the medium was observed for the immediate development of colour. Appearance of a red colour indicates a positive result (Cappuccino and Sherman, 2005).

2.10.3) Voges–Proskauer (VP) test:

The Voges-Proskauer test determines the capability of producing non-acidic or neutral end products. Bacterial cultures were inoculated VP broth in clean test tubes and incubated overnight at 37°C. Then Barrit's reagent A and Barrit's reagent B was added. The tube was then allowed to remain still for 10-15mins and the solution was observed for colour changes to determine whether the result is positive (pink-red) or negative (yellow) (Cappuccino and Sherman, 2005).

2.10.4) Citrate Utilization test:

The Citrate Utilization test was applied to verify the ability of the enteric organism to use citrate as sole source of carbon. The citrate agar was prepared in the vials. A single colony from a 24 hours fresh bacterial culture was streaked into the vials. The vials were incubated at 37°C for 24 hours. The colour of the media was observed after incubation. Colour change to blue is considered to be positive and no colour change was considered to be negative (Cappuccino and Sherman, 2005).

2.10.5) Catalase test:

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide A sterile microscopic slide was placed on a petri dish and a small amount of organism picked using a sterile inoculating loop. Then 1 drop of 3% H_2O_2 was placed on the organism on the microscopic slide by using a dropper. Finally, the positive result was observed for the presence of bubbles of oxygen gas (Cappuccino and Sherman, 2005).

2.10.6) Oxidase test:

The oxidase test detects bacteria that produce cytochrome c oxidase, which is an enzyme of the bacterial transport system. In positive cases, a deep blue or purple stain appears within 5-10 seconds. In this procedure, Kovacs Oxidase Reagent was used (Cappuccino and Sherman, 2005).

2.10.7) Triple Sugar Iron (TSI) test:

Triple sugar iron agar is a differential medium used to determine H_2S production and the type of carbohydrate fermentation. Gas from carbohydrate metabolism can also be detected. To conduct the test, an isolated colony was inoculated in the TSI medium. The results were observed after 24 hours of incubation at 37°C.

Table 2.3: Interpretation of Triple sugar iron (TSI) test result

Result	Interpretation	Symbol
Yellow slant/yellow butt	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt.	A/A
Red slant/yellow butt	Glucose fermentation with acid production. Proteins catabolized aerobically (in the slant) with alkaline products (reversion).	K/A
Red slant/red butt	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products. Not from *Enterobacteriaceae*.	K/K
Red slant/no change in butt	No fermentation. Peptone catabolized aerobically with alkaline products. Not from *Enterobacteriaceae*.	K/NC
No change in slant / no change in butt	Organism is growing slowly or not at all. Not from *Enterobacteriaceae*.	NC/NC
Black precipitate in the agar	Sulfur reduction. (An acid condition, from fermentation of glucose or lactose and/or sucrose, exists in the butt even if the yellow color is obscured by the black precipitate.)	H_2S
Cracks in or lifting of agar	Gas production.	G

2.10.8) Motility Indole Urease test:

In laboratory Motility testing using semi-solid medium is commonly used for the identification of gram negative bacteria of the members of Enterobacteriaceae family. MIU test was done for determining the motility of bacteria, indole production and urea degradation by means of the enzyme urease. Using an inoculating needle, a colony from a 24 hours fresh bacterial culture was picked up and inoculated in the medium. The test tubes were incubated at 37°C for 24 hours. The appearance and colour of the media was observed after incubation (Cappuccino and Sherman, 2005).

2.11) Gram staining:

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram

positive and Gram-negative groups. The morphology of the bacteria can also be checked using this method.

2.12) Antibiotic Susceptibility Test:

Kirby-Bauer antibiotic testing test was done to detect antimicrobial susceptibility in the bacterial isolates. In this study, the effects of 10 different commercially available antibiotics was determined. The list of antibiotics used is as follows:

Serial no	Antibiotic	Disc potency (µg)	Inhibition Zone diameter (in milimeter)		
			Resistant	Intermediate	Susceptible
1	**Penicillin- G**	10	≤23	24-28	≥29
2	**Ciprofloxacin**	5	≤15 / ≤20	16-20/ 21-30	≥21 / ≤31
3	**Chloramphenicol**	30	≤12	13-17	≥18
4	**Ampicillin**	10	≤13 / ≤ 28	14-16	≥17 / ≥ 29
5	**Azithromycin**	15	≤13	14-17	≥18
6	**Rifampicin**	5	≤16	17-19	≥20
7	**Tetracycline**	30	≤11	12-14	≥15
8	**Erythromycin**	15	≤13	14-22	≥23
9	**Streptomycin**	10	≤11	12-14	≥15
10	**Cefepime**	30	≤14	15-17	≥18

2.12.1) Preparation of inoculums of the bacterial isolates:

Saline solutions were prepared in the test tubes and one or two colonies of the bacterial isolates were inoculated to prepare the suspensions. Then all the test tubes were vortexed properly to make the suspension homogenous. The inoculums were then compared with standard McFarland 1.0.

2.12.2) Inoculation on Muller Hinton agar:

Muller Hinton agar plates were prepared to make a lawn culture. A sterile cotton swab was taken and was dipped into the broth culture of the organism. The swab was later onto the MHA plate to

make a lawn culture and to ensure that the cotton swab is touched entirely on the agar surface. After the streaking was complete, the plate was allowed to dry for 5 minutes.

2.12.3) Placement of the Antibiotic disc:

Sterilized forceps were used to place the antibiotic discs. After taking the discs, the discs were gently pressed onto the surface of the MHA agar plates. Once all the discs were properly placed, the MHA plates were inverted and incubated at 37° C for 24 hours.

2.12.4) Measurement of the zone of inhibition:

Following the incubation, the zone of inhibition for each of the antibiotics was observed on the MHA plate. The size of zones for each antibiotic were measured carefully in millimeters (mm). All the measurements were taken viewing the back of the Petri dish. The zone size was recorded on the recording sheet in a chart.

2.13) Stock Culture:

Bacterial glycerol stocks are important for long-term storage. Bacteria on Nutrient agar plate can be stored at 4°C for few weeks. For 1 ml stock, 700µl Nutrient broth was prepared for each isolate and 300µl glycerol was mixed with Nutrient broth for each isolate. The mixtures were autoclaved. After autoclave, sub-cultured isolates were inoculated in that mixture. Then they were kept at -20ºC for long term preservation.

Chapter 3

Results

3.1) Bacterial isolation and identification:

A total of about 25 samples were collected from the urine sample of UTI suspected patients from a diagnostic center. These samples were streaked on various selective, differential and Nutrient media for identifying the organisms present in the urine sample. Both the Gram positive and Gram-negative organisms were found from the samples. But the presence of the members of Enterobacteriaceae family were most in number. All the isolates were identified based on Cultural, Morphological and Biochemical characteristics. Biochemical characteristics of the isolates obtained from the study are shown in Table 3.1 and Table 3.2.

3.1.1) Cultural and morphological characteristics of the bacterial isolates:

In Table 3.1 the color, shape of the colonies on various selective, differential and enriched media and the morphology of the bacterial colonies are explained.

3.1.2) Biochemical test of bacterial isolates:

In Table 3.2 the Biochemical characteristics of the colonies are explained.

Table 3.1: Cultural and Morphological Characteristics and colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched Media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
1	1b	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Undulate	Convex	***Enterobacter spp.***
2	1c	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Undulate	Convex	***Enterobacter spp.***
3	1d	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Small	Off white	Circular	Entire	Raised	***Klebsiella spp.***
4	a	Pink colonies	Purple Colonies	Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Undulate	Convex	***Enterobacter spp.***
5	c	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Irregular	Undulate	Raised	***Klebsiella spp.***
6	d	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Irregular	Undulate	Raised	***Enterobacter spp.***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
7	e	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
8	g	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
9	h	Pink colonies	Purple colonies	Blue colonies	Alpha Hemo-Lysis	Medium	Orange	Circular	Entire	Raised	***Enterobacter spp.***
10	i	Pink colonies		Blue colonies	Beta Hemo-Lysis	Medium	Off white	Irregular	Lobate	Convex	***Enterobacter spp.***
11	j	Pink colonies	Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Small	Off white	Circular	Entire	Raised	***E. coli***
12	k	Pink colonies	Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Irregular	Lobate	Convex	***E. coli***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
13	n	Pink colonies	Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***E. coli***
14	o	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
15	p	Colourless colonies		Brown colonies	Gamma Hemo-Lysis	Small	Off white	Irregular	Undulate	Convex	***Proteus spp.***
16	U_2	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
17	U_5		Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***E. coli.***
18	U_6		Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***E. coli***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
19	U_7		Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Convex	***E. coli***
20	U_8		Metallic green sheen	Purple colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Convex	***E. coli***
21	U_{14}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***
22	U_{18}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
23	U_{20}	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
24	U_{24}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
25	U_{29}	Pink mucoid colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***
26	U_{31}	Pink mucoid colonies	Purple mucoid Colonies	Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
27	U_{33}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Irregular	Lobate	Convex	***Klebsiella spp.***
28	U_{34}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***
29	U_{35}	Pink mucoid colonies	Purple mucoid Colonies	Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***
30	U_{36}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Klebsiella spp.***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

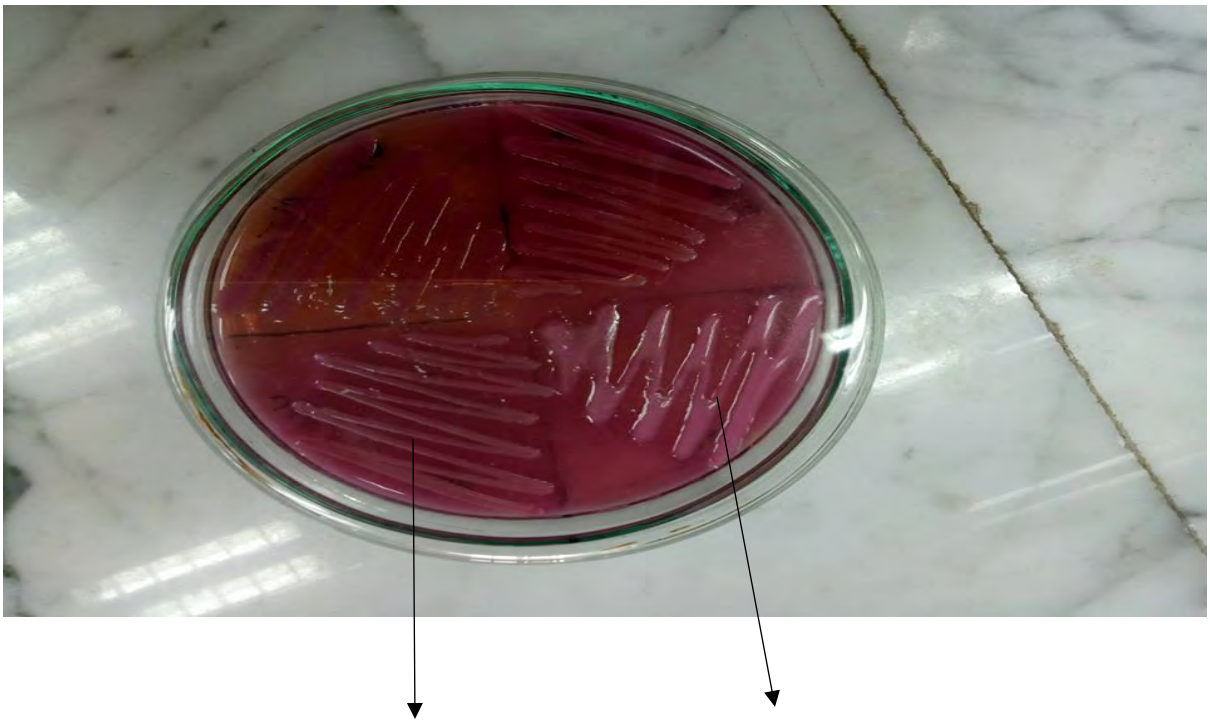
Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
31	U_{38}	Colourless colonies		Brown colonies	Gamma Hemo-Lysis	Small	Off white	Irregular	Undulate	Convex	***Proteus spp.***
32	U_{41}	Colourless colonies		Brown colonies	Gamma Hemo-Lysis	Small	Off white	Irregular	Undulate	Convex	***Proteus spp.***
33	U_{42}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
34	U_{43}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Irregular	Undulate	Convex	***Klebsiella spp.***
35	U_{48}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
36	U_{49}	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Lobate	Raised	***Enterobacter spp.***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates ID	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
37	U_{50}	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
38	I_1	Pink colonies		Blue colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
39	I_3	Pink colonies		Blue colonies	Beta Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
40	I_4	Pink colonies	Purple colonies	Blue colonies	Beta Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
41	I_5	Pink colonies	Purple colonies	Blue colonies	Beta Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Enterobacter spp.***
42	I_{17}	Pink mucoid colonies		Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***

Table 3.1: Cultural and Morphological Characteristics colour of the colonies of the Bacterial Isolates from UTI suspected patients on various Selective, Differential and Enriched media

Isolates No	Isolates Id	Growth on Selective, Differential and Enriched media				Colony Morphology on Nutrient agar					Suspected organism
		MacConkey agar	Eosin Methylene Blue (EMB) agar	Hi Crome UTI agar	Blood agar	Size	Colour	Form	Margin	Elevation	
43	I_{31}	Pink mucoid colonies	Purple mucoid Colonies	Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***
44	I_{32}	Pink mucoid colonies	Purple muoid Colonies	Blue mucoid colonies	Alpha Hemo-Lysis	Medium	Off white	Circular	Entire	Raised	***Klebsiella spp.***



Enterobacter spp. *Klebsiella spp.*

Figure 3.1: Bacterial growth on MacConkey agar

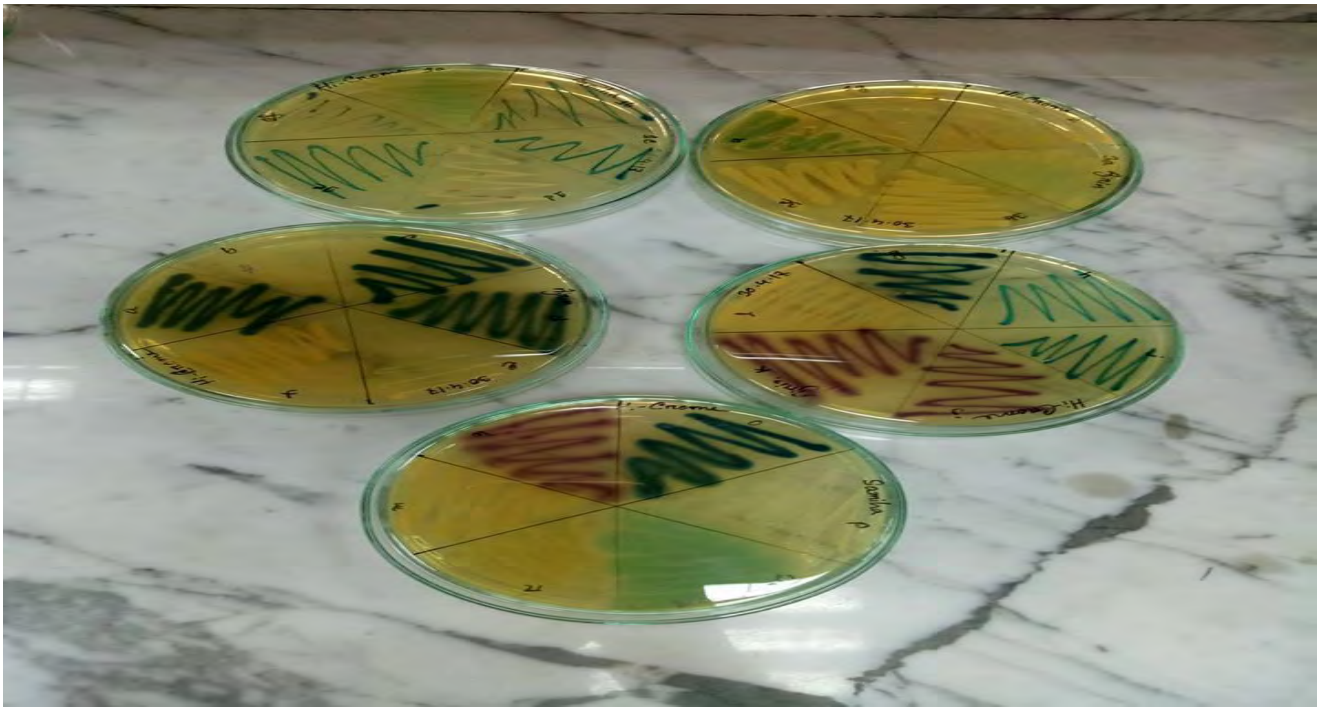
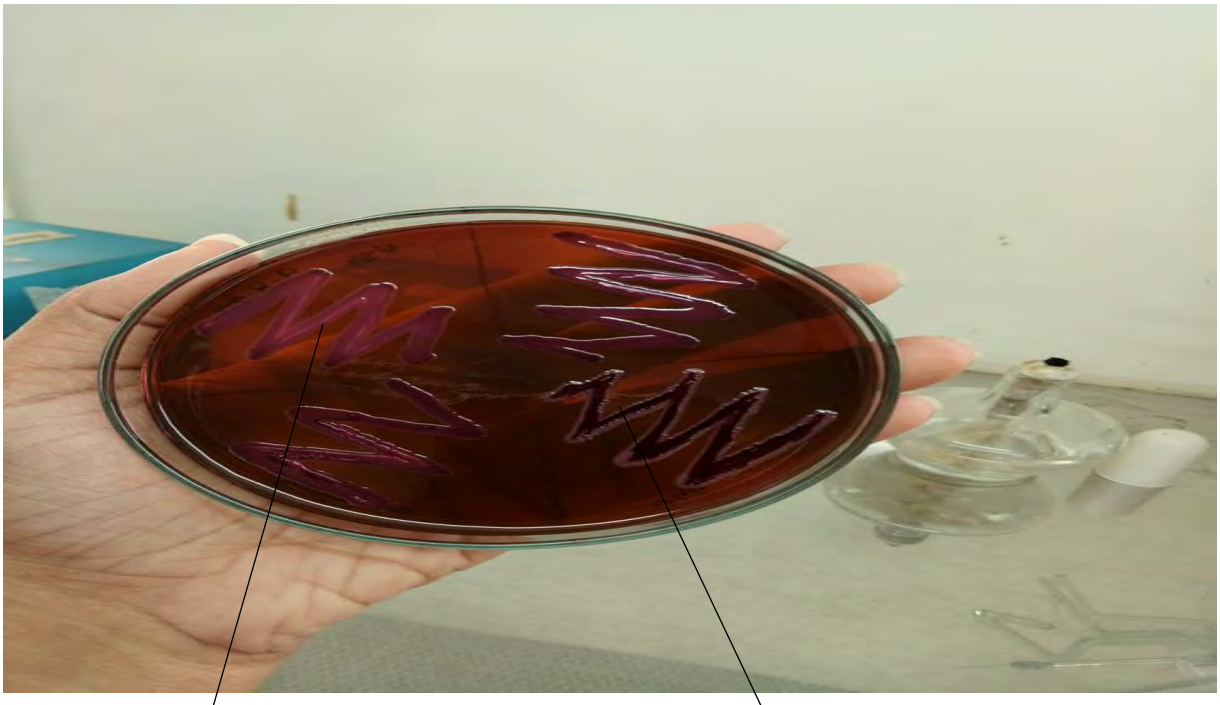


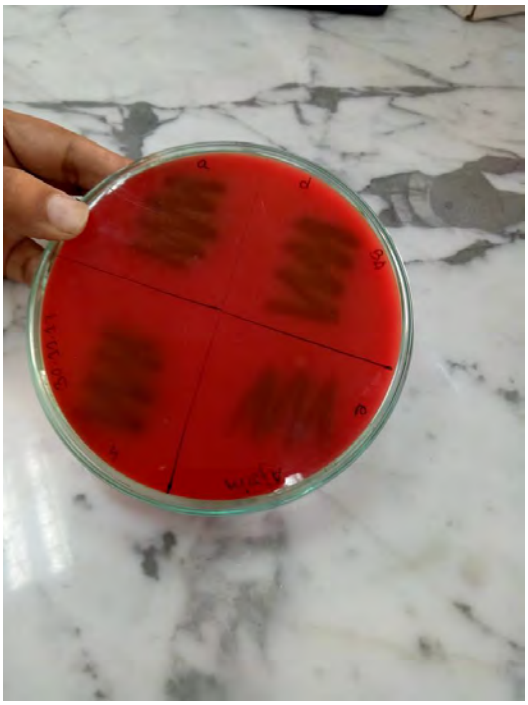
Figure 3.2: Bacterial growth on Hi Crome UTI agar

E. coli



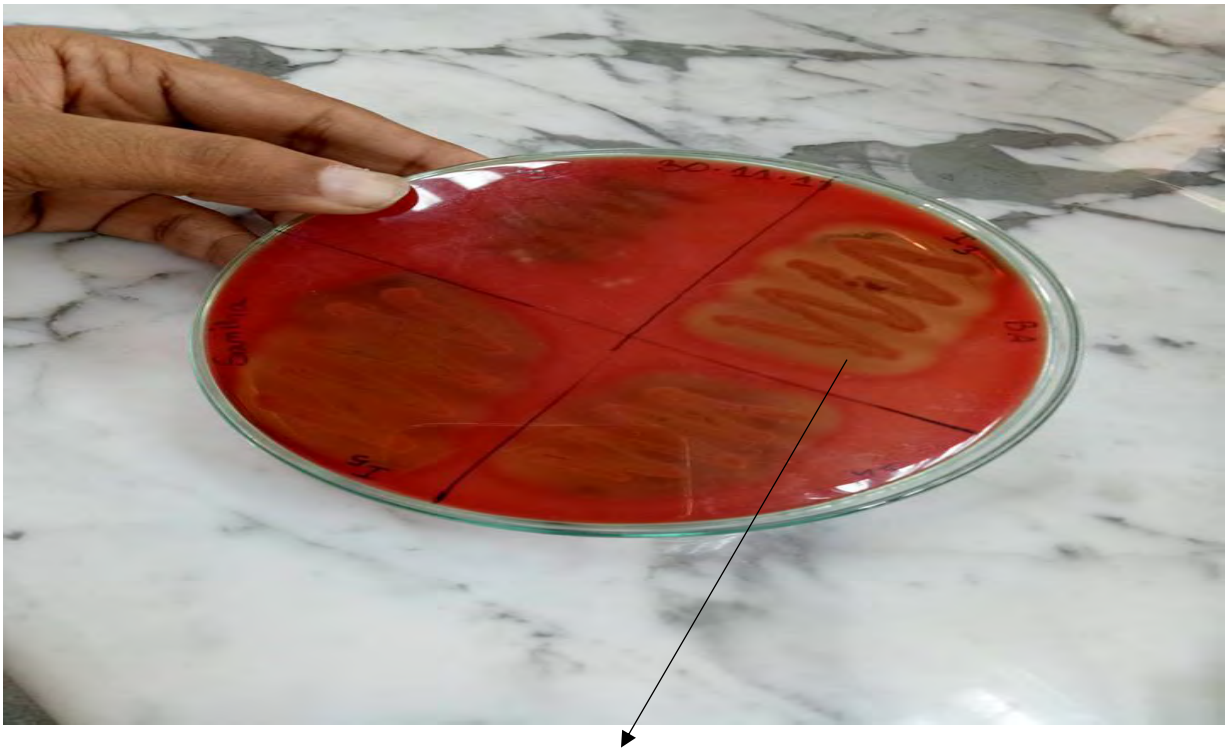
Klebsiella spp. *Enterobacter spp.*

Figure 3.3: Bacterial growth on EMB agar



Alpha Hemolysis on blood agar

Gamma Hemolysis on Blood agar



Clear zone

Beta Hemolysis on Blood agar

Figure 3.4: Bacterial growth on Blood agar

Table 3.2: Biochemical characteristics of the bacteria isolated from urine sample of UTI suspected patients from a diagnostic center

Isolates No	Isolates ID	Indole test	Methyl Red test	Voges-Proskauer test	Citrate test	Triple Sugar Iron test (TSI)						MIU test			Catalase test	Oxidase test	Gram staining		Suspected organisms
						Slant/ Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease			Gram reaction	Shape	
1	1b	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	-	-	Short rods	***Enterobacter spp.***
2	1c	-	-	-	-	Y/Y	+	+	+	-	-	-	-	-	+	-	-	Short rods	***Enterobacter spp.***
3	1d	-	-	-	-	Y/Y	+	+	+	-	-	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
4	a	-	-	+	+	Y/Y	+	+	+	-	+	+	-	+	-	-	-	Short rods	***Enterobacter spp.***
5	c	-	+	+	+	Y/Y	+	+	+	-	+	+	-	+	-	-	-	Short rods	***Klebsiella spp.***
6	d	-	+	+	+	Y/Y	+	+	+	-	-	+	-	-	+	-	-	Short rods	***Enterobacter spp.***
7	e	-	+	+	-	Y/Y	+	+	+	-		+	-	+	+	-	-	Short rods	***Enterobacter spp.***
8	g	+	+	+	+	Y/Y	+	+	+	-	+	+	+	-	+	-	-	Short rods	***Klebsiella spp.***
9	h	-	+	+	+	Y/Y	+	+	+	-	-	-	-	-	-	-	-	Short rods	***Enterobacter spp.***
10	i	-	+	+	-	R/Y	+	-	-	-	-	-	-	+	+	-	-	Short rods	***Enterobacter spp.***
11	j	+	+	-	-	Y/Y	+	+	+	-	-	+	+	+	+	-	-	Short rods	***E. coli***
'+'= Positive, '- '= Negative, Y= Yellow(Acidic), R=Red (Alkaline)																			

Table 3.2: Biochemical characteristics of the bacteria isolated from urine sample of UTI suspected patients from a diagnostic center

Isolates No	Isolates ID	Indole test	Methyl Red test	Voges-Proskauer	Citrate test	Triple Sugar Iron test (TSI)						MIU test			Catalase test	Oxidase test	Gram staining		Suspected organisms
						Slant/ Butt	Glucose	Lactose	Sucrose	Gas	H_2S	Motility	Indole	Urease			Gram reaction	Shape	
12	k	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	-	Short rods	***E. coli***
13	n	+	+	-	-	Y/Y	+	+	+	-	-	+	+	+	+	-	-	Short rods	***E. coli***
14	o	-	+	+	+	Y/Y	+	+	+	-	+	+	-	+	+	-	-	Short rods	***Enterobacter spp.***
15	p	+	+	-	-	Y/Y	+	+	+	-	-	+	+	-	+	-	-		***Proteus spp.***
16	U_2	-	-	+	+	R/Y	+	-	-	-	-	-	-	+	+	-	-	Short rods	***Klebsiella spp.***
17	U_5	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	Short rods	***E. coli***
18	U_6	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	Short rods	***E. coli***
19	U_7	+	+	-	-	Y/Y	+	+	+	-	-	+	+	-	+	-	-	Short rods	***E. coli***
20	U_8	+	+	-	-	Y/Y	+	+	+	-	-	+	+	-	+	-	-	Short rods	***E. coli***
21	U_{14}	+	+	+	+	Y/Y	+	+	+	-	-	+	+	+	+	-	-	Short rods	***Klebsiella spp.***
22	U_{18}	-	+	+	+	Y/Y	+	+	+	-	-	-	-	-	+	-	-	Short rods	***Klebsiella spp.***
'+'= Positive, '- '= Negative, Y= Yellow(Acidic), R=Red (Alkaline)																			

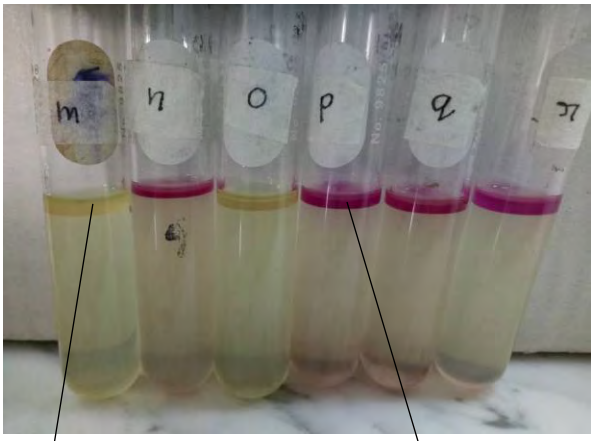
Table 3.2: Biochemical characteristics of the bacteria isolated from urine sample of UTI suspected patients from a diagnostic center

Isolates No	Isolates ID	Indole test	Methyl Red test	Voges-Proskauer test	Citrate test	Triple Sugar Iron test (TSI)						MIU test			Catalase test	Oxidase test	Gram staining		Suspected organisms
						Slant/ Butt	Glucose	Lactose	Sucrose	Gas	H_2S	Motility	Indole	Urease			Gram reaction	Shape	
23	U_{20}	+	+	+	+	Y/Y	+	+	+	-	-	+	+	-	+	-	-	Short rods	***Enterobacter spp.***
24	U_{24}	+	+	+	+	Y/Y	+	+	+	-	+	+	+	+	+	-	-	Short rods	***Klebsiella spp.***
25	U_{29}	-	-	+	+	R/Y	+	-	-	+	-	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
26	U_{31}	-	-	+	+	R/Y	+	-	-	-	-	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
27	U_{33}	-	+	+	+	R/Y	+	-	-	-	-	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
28	U_{34}	+	+	+	-	R/Y	+	-	-	-	-	-	+	+	+	-	-	Short rods	***Klebsiella spp.***
29	U_{35}	+	+	+	+	R/Y	+	-	-	-	+	+	+	+	+	-	-	Short rods	***Klebsiella spp.***
30	U_{36}	-	+	+	+	R/Y	+	-	-	-	-	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
31	U_{38}	+	+	+	-	Y/Y	+	+	+	-	-	+	+	+	+	-	-	Short rods	***Proteus spp.***
32	U_{41}	+	+	-	-	R/Y	+	-	-	-	-	+	+	+	+	-	-	Short rods	***Proteus spp.***
33	U_{42}	+	+	-	+	Y/Y	+	+	+	-	-	+	+	+	+	-	-	Short rods	***Klebsiella spp.***
'+'= Positive, '- '= Negative, Y= Yellow(Acidic), R=Red (Alkaline)																			

Table 3.2: Biochemical characteristics of the bacteria isolated from urine sample of UTI suspected patients from a diagnostic center

Isolates No	Isolates ID	Indole test	Methyl Red test	Voges-Proskauer test	Citrate test	Triple Sugar Iron test (TSI)						MIU test			Catalase test	Oxidase test	Gram staining		Suspected organisms
						Slant/ Butt	Glucose	Lactose	Sucrose	Gas	H_2S	Motility	Indole	Urease			Gram reaction	Shape	
34	U_{43}	+	+	-	+	R/Y	+	-	-	-	-	+	+	+	+	-	-	Short rods	***Klebsiella spp.***
35	U_{48}	+	+	+	+	Y/Y	+	+	+	-	+	+	+	-	+	-	-	Short rods	***Klebsiella spp.***
36	U_{49}	+	+	+	-	R/Y	+	-	-	-	-	+	+	+	+	-	-	Short rods	***Enterobacter spp.***
37	U_{50}	+	+	-	-	R/Y	+	-	-	-	-	+	+	+	+	-	-	Short rods	***Enterobacter spp.***
38	I_1	-	+	+	+	Y/Y	+	+	+	-	-	+	-	+	+	-	-	Short rods	***Enterobacter spp.***
39	I_3	-	-	+	+	Y/Y	+	+	+	-	-	+	-	+	+	-	-	Short rods	***Enterobacter spp.***
40	I_4	-	-	+	+	Y/Y	+	+	+	-	-	+	-	+	+	-	-	Short rods	***Enterobacter spp.***
41	I_5	-	-	+	+	Y/Y	+	+	+	-	-	+	-	+	+	-	-	Short rods	***Enterobacter spp.***
42	I_{17}	-	-	+	+	Y/Y	+	+	+	-	+	+	-	+	+	-	-	Short rods	***Klebsiella spp.***
43	I_{31}	-	+	+	+	Y/Y	+	+	+	-	+	+	-	+	-	-	-	Short rods	***Klebsiella spp.***
44	I_{32}	-	+	+	+	Y/Y	+	+	+	-	+	+	-	+	+	-	-	Short rods	***Klebsiella spp.***

'+'= Positive, '- '= Negative, Y= Yellow(Acidic), R=Red (Alkaline)

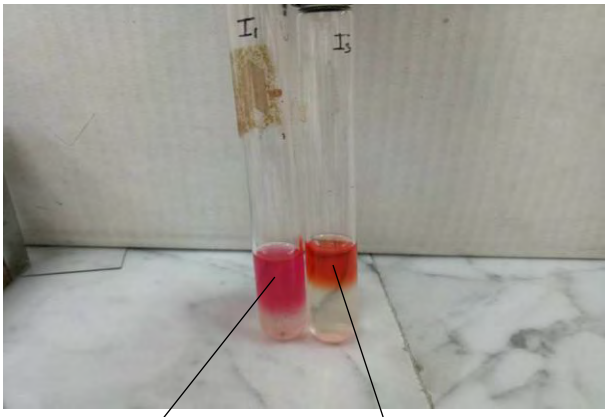


No colour Pink colour

Indole Test

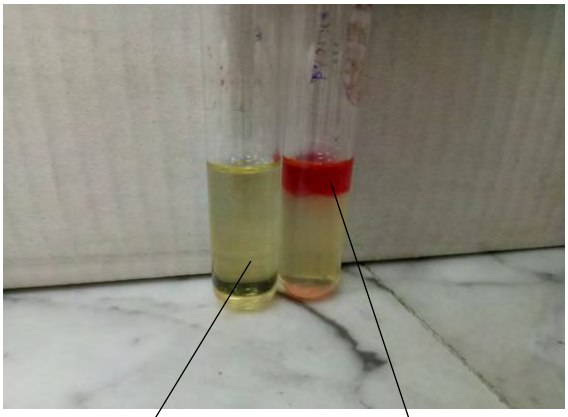
Blue colour Green colour

Citrate Utilization Test



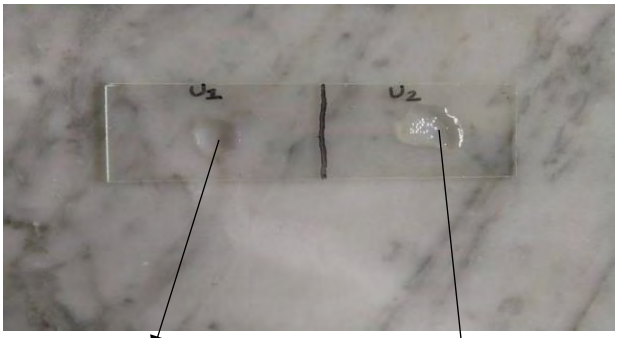
Cherry red colour Orange colour

MR Test



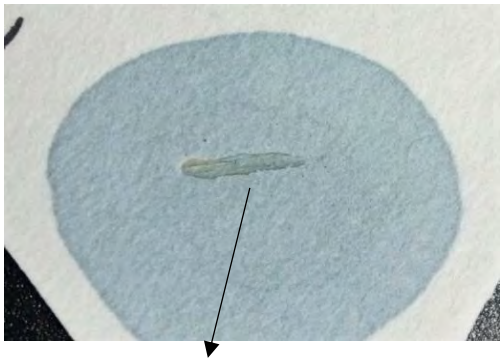
No colour Orange colour

VP Test



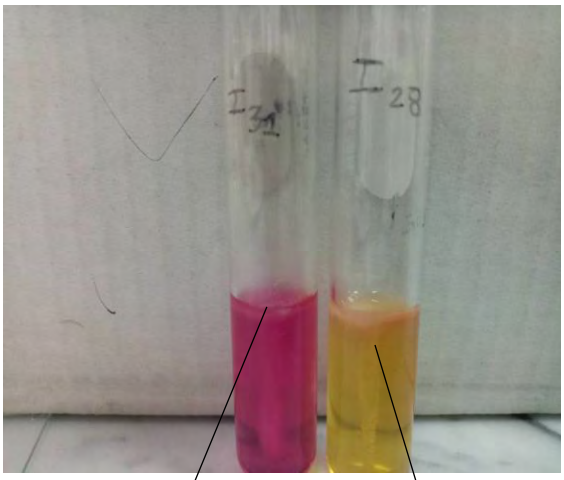
Negative Positive

Catalase Test



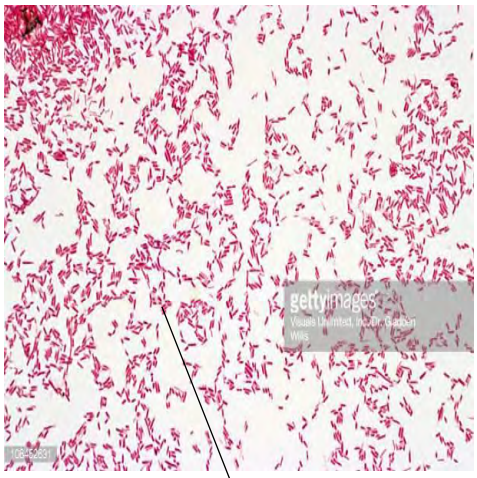
Negative

Oxidase Test



Pink colour (Motile) Yellow colour (Motile)

Figure 18: MIU Test



Gram negative Rod shaped

Figure 19: Gram Staining

Yellow slant, Yellow butt

Yellow slant, Yellow butt (gas produced)

Red slant, Yellow butt

Red slant, Red butt

TSI Test

Figure 3.5: Biochemical results of bacterial isolates

After observing the Cultural and Morphological characteristics of bacterial isolates and performing the Biochemical tests, 44 isolates have been identified from 25 different samples collected from the urine sample of UTI suspected patients from a diagnostic center. The isolates that have been confirmed include *Klebsiella spp.*, *E. coli*, *Enterobacter spp.* and *Proteus spp.* The total number and the percentage of the isolates obtained from the samples are shown in table 3.3 and figure 3.6.

Table 3.3: Prevalence of Enterobacteriaceae species isolated from urine sample

Bacterial isolates	**Number of the isolates**	**Total bacterial isolates**	**% Prevalence**
Klebsiella spp.	19	44	43.18
Enterobacter spp.	15		34.09
E. coli	7		15.91
Proteus spp.	3		6.82

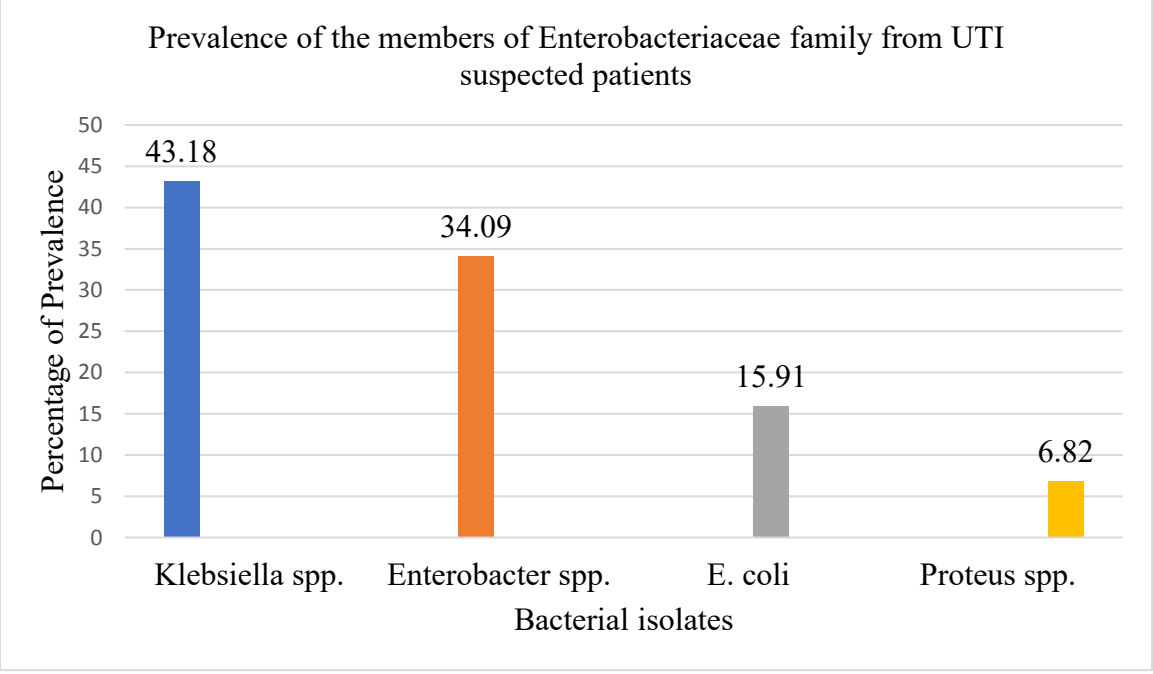


Figure 3.6: Percentage of prevalence of isolated the members of Enterobacteriaceae from UTI suspected patients

3.2) Antibiotic susceptibility test:

After identifying and confirming the organisms, the isolates were selected for antibiotic susceptibility test. About nine to ten antibiotics were used for each of the 44 isolates isolated from urine sample of UTI suspected patients from a diagnostic center. The sensitive and resistance pattern of the isolates to these antibiotics were determined.

In table 8, the zone of inhibition of the isolates according to the zone range for resistance, intermediate and sensitivity to different antibiotics are shown. The zone of inhibition is measured in millimeter. In some cases, no zone of inhibition was observed which means that that particular antibiotic failed to kill the bacterium and the bacterium is resistant to that antibiotic. The interpretation of each bacterium either resistant or susceptible to antibiotic is shown in Table 3.3.

Table 3.4: Antibiotic susceptibility pattern of various organisms isolated from urine sample of UTI suspected patients

Isolates Id	Suspected organisms	Penicillin G		Ciprofloxacin		Chloramphenicol		Ampicillin		Azithromycin		Rifampicin		Tetracycline		Erythromycin		Streptomycin		Cefepime	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
1b	*Enterobacter spp.*	20	R	25	S	33	S	17	S	27	S	20	S	33	S	30	S	25	S	Nil	R
1c	*Enterobacter spp.*	22	R	24	S	30	S	16	I	26	S	20	S	30	S	30	S	24	S	Nil	R
1d	*Klebsiella spp.*	Nil	R	19	I	22	S	Nil	R	17	I	Nil	R	9	R	11	R	12	I	Nil	R
a	*Enterobacter spp.*	Nil	R	23	I	25	S	Nil	R	16	I	Nil	R	24	S	9	R	20	S	34	S
c	*Klebsiella spp.*	Nil	R	33	S	28	S	Nil	R	14	I	Nil	R	22	S	10	R	18	S	31	S
d	*Enterobacter spp.*	Nil	R	Nil	R	21	S	Nil	R	Nil	R	Nil	R	18	S	Nil	R	22	S	Nil	R
e	*Enterobacter spp.*	20	R	35	S	33	S	23	S	Nil	R	37	S	11	R	Nil	R	27	S	28	S
g	*Klebsiella spp.*	Nil	R	Nil	R	22	S	Nil	R	Nil	R	Nil	R	17	S	Nil	R	18	S	Nil	R
h	*Enterobacter spp.*	26	R	Nil	R	11	R	12	R	Nil	R	22	S	Nil	R	12	R	Nil	R	14	R
i	*Enterobacter spp.*	36	S	36	S	24	S	35	S	24	S	22	S	31	S	26	S	24	S	Nil	R
j	*E. coli spp.*	Nil	R	30	I	23	S	8	R	11	R	7	R	24	S	Nil	R	22	S	31	S

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant

Table 3.4: Antibiotic susceptibility pattern of various organisms isolated from urine sample of UTI suspected patients

Isolates Id	Suspected organisms	Penicillin G		Ciprofloxacin		Chloramphenicol		Ampicillin		Azithromycin		Rifampicin		Tetracycline		Erythromycin		Streptomycin		Cefepime	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
k	*E. coli spp.*	Nil	R	32	S	25	S	Nil	R	13	R	9	R	24	S	8	R	20	S	Nil	R
n	*E. coli spp.*	Nil	R	32	S	27	S	19	S	11	R	10	R	22	S	10	R	19	S	32	S
o	*Enterobacter spp.*	Nil	R	24	I	30	S	Nil	R	15	I	8	R	11	R	10	R	20	S	14	R
p	*Proteus spp.*	17	R	37	S	37	S	20	S	Nil	R	38	S	13	I	Nil	R	24	S	23	S
U_2	*Klebsiella spp.*	Nil	R	Nil	R	17	I	Nil	R	Nil	R	Nil	R	16	S	Nil	R	20	S	Nil	R
U_5	*E. coli*	Nil	R	Nil	R	23	S	Nil	R	Nil	R	7	R	13	I	Nil	R	14	I	Nil	R
U_6	*E. coli*	Nil	R	Nil	R	26	S	Nil	R	11	R	9	R	23	S	Nil	R	20	S	Nil	R
U_7	*E. coli*	Nil	R	Nil	R	25	S	Nil	R	9	R	10	R	22	S	Nil	R	20	S	Nil	R
U_8	*E. coli*	Nil	R	Nil	R	26	S	Nil	R	Nil	R	8	R	23	S	Nil	R	18	S	Nil	R
U_{14}	*Klebsiella spp.*	Nil	R	Nil	R	18	S	Nil	R	9	R	9	R	Nil	R	Nil	R	21	S	12	R
U_{18}	*Klebsiella spp.*	Nil	R	Nil	R	16	I	Nil	R	Nil	R	Nil	R	17	S	Nil	R	17	S	Nil	R

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant

Table 3.4: Antibiotic susceptibility pattern of various organisms isolated from urine sample of UTI suspected patients

Isolates Id	Suspected organisms	Penicillin G		Ciprofloxacin		Chloramphenicol		Ampicillin		Azithromycin		Rifampicin		Tetracycline		Erythromycin		Streptomycin		Cefepime	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
U_{20}	*Enterobacter spp.*	Nil	R	Nil	R	16	I	Nil	R	7	R	Nil	R	18	S	Nil	R	17	S	Nil	R
U_{24}	*Klebsiella spp.*	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	18	S	15	I
U_{29}	*Klebsiella spp.*	Nil	R	30	S	26	S	8	R	15	I	8	R	23	S	10	R	18	S	27	S
U_{31}	*Klebsiella spp.*	Nil	R	30	S	26	S	9	R	14	I	9	R	21	R	9	R	18	S	28	S
U_{33}	*Klebsiella spp.*	Nil	R	Nil	R	18	S	Nil	R	Nil	R	Nil	R	16	S	Nil	R	20	S	12	R
U_{34}	*Klebsiella spp.*	Nil	R	Nil	R	24	S	Nil	R	Nil	R	8	R	Nil	R	Nil	R	14	I	20	S
U_{35}	*Klebsiella spp.*	Nil	R	10	R	27	S	Nil	R	Nil	R	7	R	Nil	R	Nil	R	14	I	16	I
U_{36}	*Klebsiella spp.*	Nil	R	Nil	R	15	I	Nil	R	Nil	R	Nil	R	15	S	Nil	R	18	S	Nil	R
U_{38}	*Proteus spp.*	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	10	R	Nil	R	Nil	R	22	S	17	I
U_{41}	*Proteus spp.*	Nil	R	Nil	R	Nil	R	Nil	R	Nil	R	11	R	Nil	R	Nil	R	23	S	17	I
U_{42}	*Klebsiella spp.*	Nil	R	Nil	R	24	S	Nil	R	16	I	9	R	22	S	Nil	R	22	S	Nil	R

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant

Table 3.4: Antibiotic susceptibility pattern of various organisms isolated from urine sample of UTI suspected patients

Isolates Id	Suspected organisms	Penicillin G		Ciprofloxacin		Chloramphenicol		Ampicillin		Azithromycin		Rifampicin		Tetracycline		Erythromycin		Streptomycin		Cefepime	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
U_{43}	*Klebsiella spp.*	Nil	R	Nil	R	28	S	Nil	R	Nil	R	Nil	R	22	S	Nil	R	22	S	12	R
U_{48}	*Klebsiella spp.*	13	R	39	S	20	S	50	S	35	S	33	S	23	S	37	S	28	S	12	R
U_{49}	*Enterobacter spp.*	Nil	R	Nil	R	22	S	Nil	R	Nil	R	8	R	18	S	Nil	R	14	I	Nil	R
U_{50}	*Enterobacter spp.*	Nil	R	Nil	R	22	S	Nil	R	Nil	R	Nil	R	16	S	Nil	R	13	I	Nil	R
I_1	*Enterobacter spp.*	16	R	24	S	35	S	15	S	16	I	36	S	14	I	11	R	27	S	Nil	R
I_3	*Enterobacter spp.*	42	S	37	S	30	S	29	S	33	S	19	I	33	S	32	S	26	S	Nil	R
I_4	*Enterobacter spp.*	32	S	40	S	25	S	30	S	32	S	20	S	33	S	30	S	25	S	Nil	R
I_5	*Enterobacter spp.*	38	S	40	S	22	S	32	S	28	S	23	S	34	S	32	S	26	S	Nil	R
I_{17}	*Klebsiella spp.*	Nil	R	18	I	27	S	Nil	R	18	S	Nil	R	9	R	Nil	R	13	I	Nil	R
I_{31}	*Klebsiella spp.*	Nil	R	19	I	22	S	Nil	R	18	S	Nil	R	Nil	R	12	R	13	I	Nil	R
I_{32}	*Klebsiella spp.*	Nil	R	19	I	23	S	Nil	R	17	I	Nil	R	8	R	10	R	12	I	Nil	R

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant

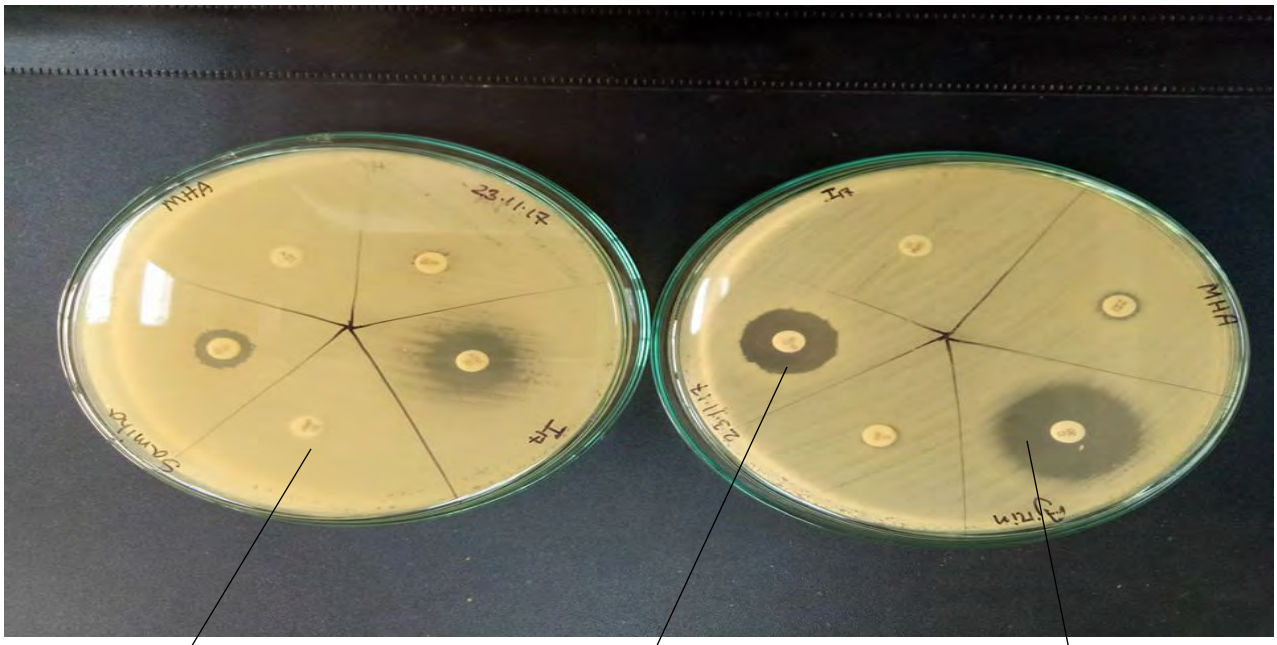


Figure 3.7: Antibiotic susceptibility pattern of *Klebsiella spp.*

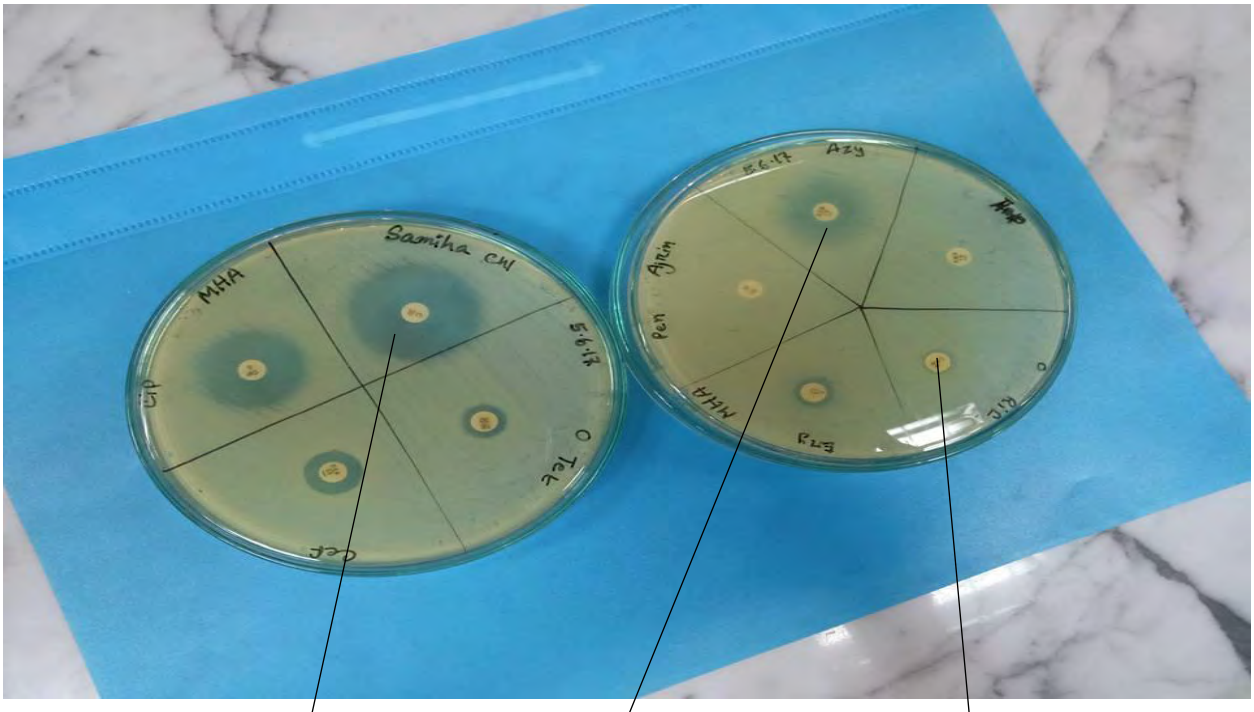


Figure 3.8: Antibiotic susceptibility pattern of *Enterobacter spp.*

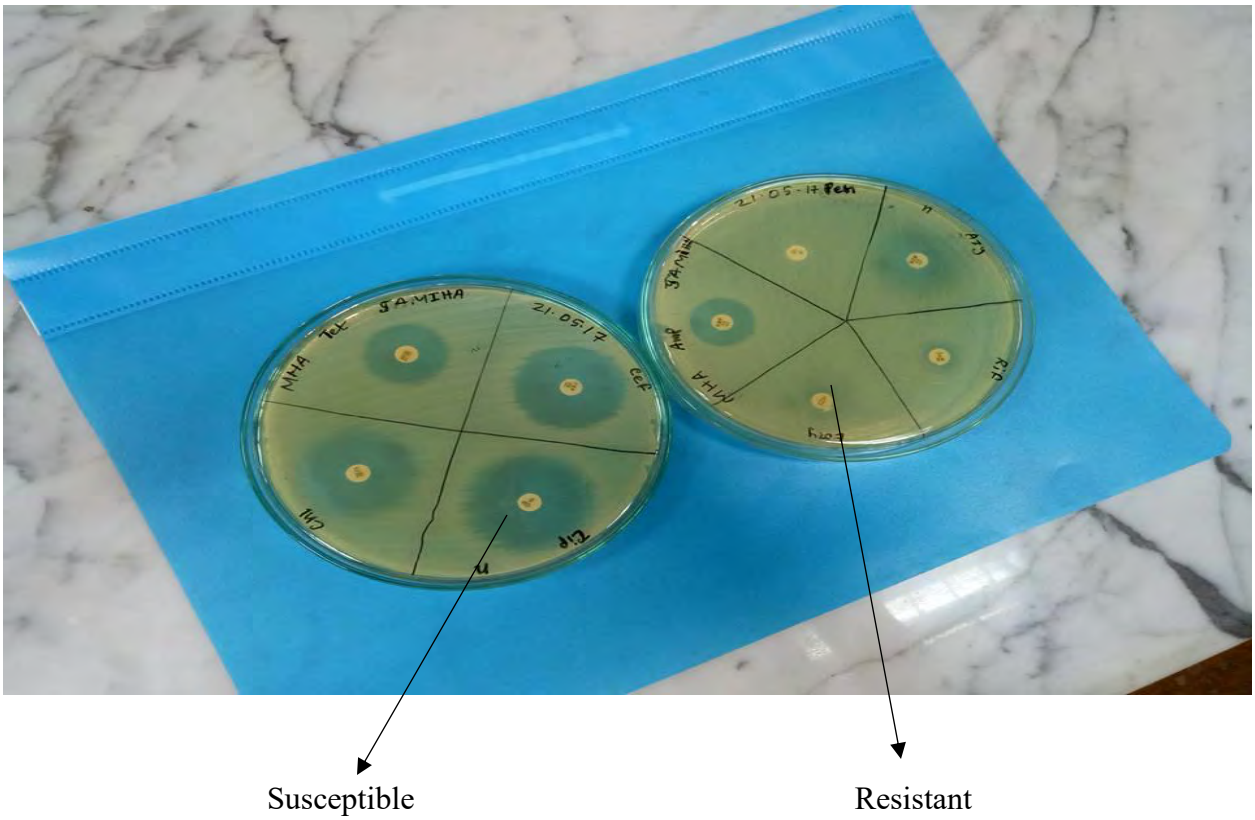


Figure 3.9: Antibiotic susceptibility pattern of *E. coli*

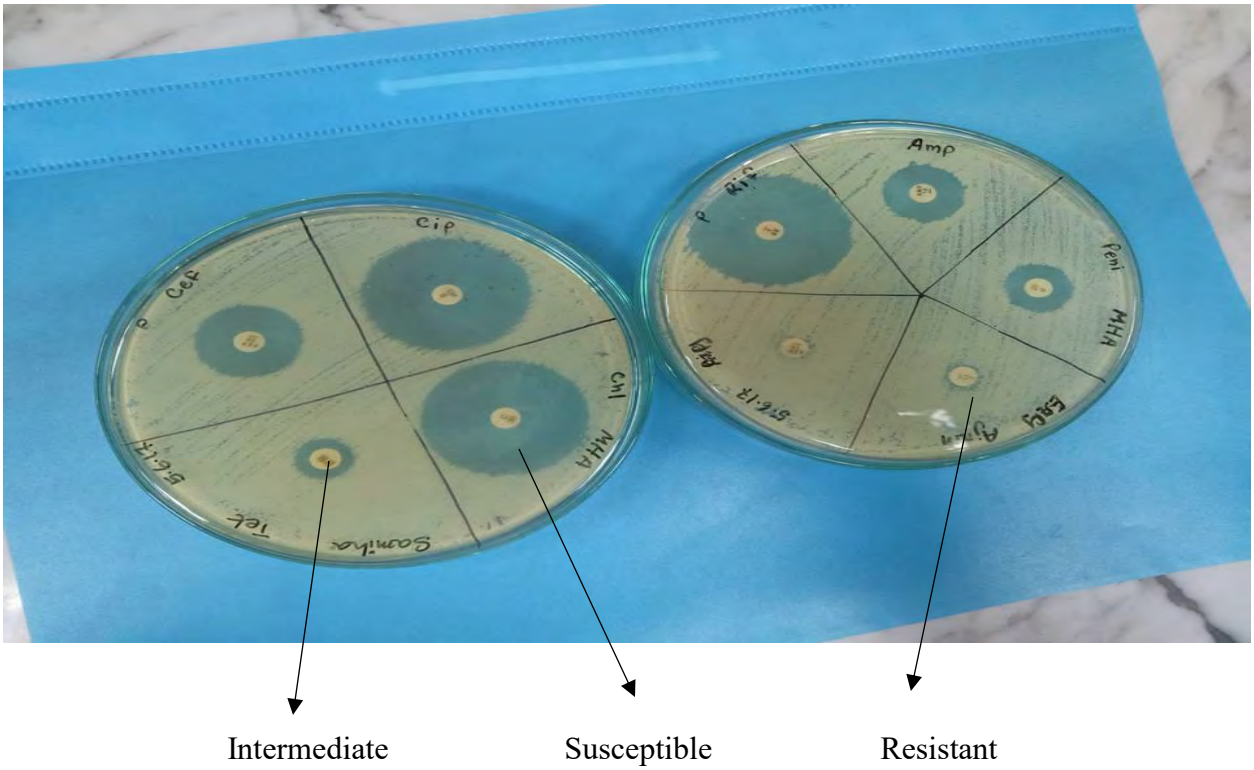


Figure 3.10: Antibiotic susceptibility pattern of *Proteus spp.*

3.2.1) Resistance pattern of the organisms to the tested antibiotics:

After determining the antibiotic resistant organisms, the percentage of the resistant isolates to the tested antibiotics was also determined which are shown in Table 3.5 and in the following figure.

Table 3.5: Antibiotic resistance pattern of total 44 bacterial isolates

Antibiotics	Penicillin	Ciprofloxacin	Chloramphenicol	Ampicillin	Azithromycin	Rifampicin	Tetracycline	Erythromycin	Streptomycin	Cefepime
No of isolates resistant to tested antibiotics	40	21	4	33	26	33	14	37	1	31
Percentage of isolates resistant to antibiotics	90.90	47.72	9.09	75.00	59.09	75.00	31.82	84.09	2.29	70.45

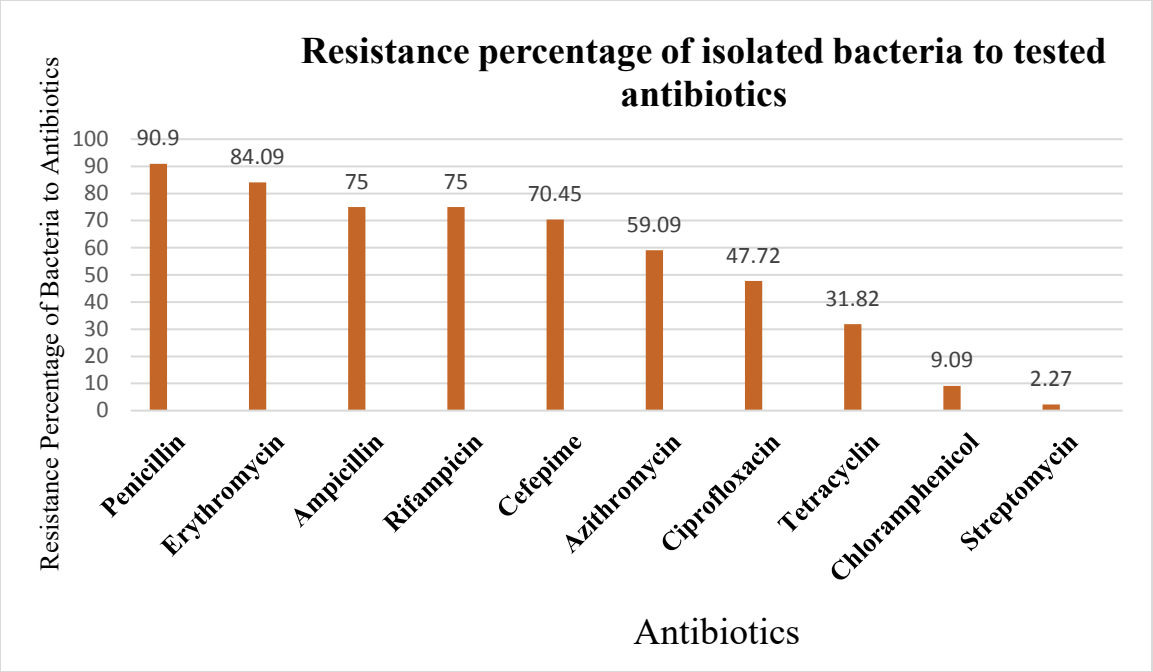


Figure 3.11: Resistance percentage of the isolated bacteria to tested antibiotics

3.2.2) Prevalence of antibiotic resistant organisms:

In this study, most of the bacterial isolates were found resistant to one, two or more than two antibiotics after observing the antibiotic resistance pattern of the organisms. So, the bacterial isolates obtained from this study are divided into three categories in this study: one that are resistant to one antibiotic, that are resistant to two antibiotics and one that are resistant to more than two antibiotics Their total number and percentage are given below in Table 3.7 and Figure 3.12.

Table 3.6: Total number and percentage of the isolates resistant to one antibiotic, the isolates resistant to two antibiotics and the isolates resistant to more than two antibiotics

Total bacterial isolates	Number of isolates Resistant to more than two antibiotics	Percentage of isolates Resistant to more than two antibiotics	Number of isolates Resistant to two antibiotics	Percentage of isolates Resistant to two antibiotics	Number of isolates Resistant to one antibiotics	Percentage of isolates Resistant to one antibiotics
44	37	84.09	3	6.81	4	9.10

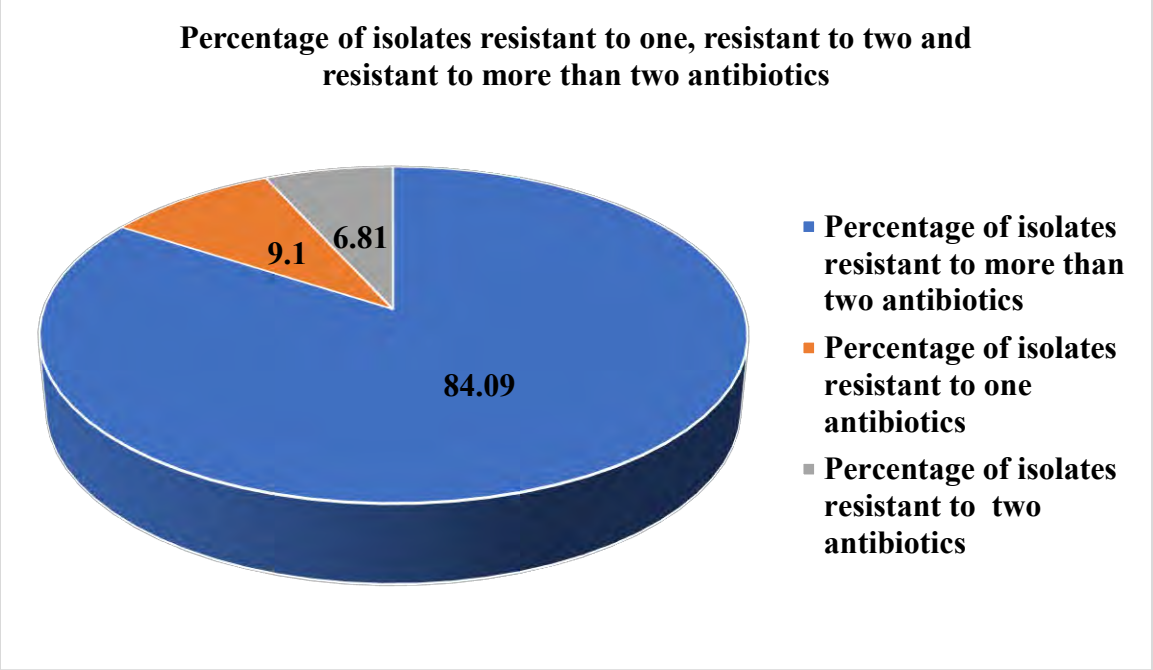


Figure 3.12: Total percentage of the isolates resistant to one antibiotic, the isolates resistant to two antibiotics and the isolates resistant to more than two antibiotics

Chapter 4
Discussion & Conclusion

4.1) Discussion:

Urinary tract infection is one of the most common infection in different countries of the world and that infects human of various age groups, and that 95% of these infections caused by many types of bacteria (Shahab et al. 2017).

Urinary tract infection is emerging as an important community acquired and nosocomial bacterial infection. Moreover, antimicrobial resistance to various classes of antimicrobials to uropathogens continues to be a major health problem in different parts of the world (Haque et al. BMC Res Notes, 2015). According to, Emergency Medicine International, the Enterobacteriaceae are the major causes of UTIs. This study aimed at isolating, identifying the bacterial contaminants, determining the antibiotic resistance pattern from urine sample, collected from a diagnostic center. High level of bacterial contaminants were found from urine sample of UTI suspected patients. These samples were contaminated with different bacteria. Among all the contaminants, the members of Enterobacteriaceae family were found most. This study showed a statistically significant difference in this regard.

Out of 25 samples, the members of Enterobacteriaceae were found in 16 (48.35%) samples. In this study, *Klebsiella spp.* (43.18%) were found most predominant followed by *Enterobacter spp.* (34.09%) and *E. coli* (15.91%) and *Proteus spp.* (6.82%) were found less among all Enterobacteriaceae isolates.

Enterobacteriaceae: (enteric) are Gram-negative bacteria that grow in the intestinal tract of humans and other animals. The IMViC tests are frequently employed for identification of this group of microbes which includes such microorganisms as *Klebsiella spp.*, *Enterobacter spp.* and *Escherichia coli*. The presence of *E. coli* is used by public health officials as an indicator of fecal contamination of food and water supplies. While *Enterobacter spp.* and *Klebsiella spp.* resemble *E. coli* in being lactose fermenters. The IMViC tests can be used to differentiate these three organisms (Euro. J. Exp. Bio., 2011). After overnight incubation, bacterial colonies were selected for biochemical test from Nutrient agar. The isolates were then subjected to a set of Biochemical tests according to Cappuccino, & Sherman. (2005) for confirmation.

Most of the studies state that there is a resistance of gram negative bacteria especially members of Enterobacteriaceae to antibiotics in their different kinds especially β-lactams antibiotics (Belongia

et al. 2005). This increases the importance of these bacteria and the infections they cause are often available at the hospitals with the patients who are inhibited immunologically. β-lactamases are regarded as one of the important and most common among members of this family for being able to move between the different species through plasmids that carry encoded genes of the enzymes. Moreover, the increased amount of these enzymes in quantity and quality had expanded and complicated the problem (Salih et al. 2016).

Resistance to antimicrobial agents has been noted and is an increasing world-wide problem. This study revealed that a higher prevalence rate of resistance to some antibiotic agents. Among the 44 bacterial isolates, 37 (84.09%) of them were found to be resistant to more than two antibiotics, 3 (6.81%) of them were found to be resistant to at least two antibiotics and 4 (9.10%) of them were found to be resistant to less than two antibiotics. Penicillin was found to be less effective out of tested ten antibiotics because out of 44 isolates, 40 (90.90%) isolates showed resistance to penicillin. Erythromycin can be considered less effective as 37 (84.09%) isolates were resistant to this antibiotic. Followed by these two antibiotics, 33 (75%) isolates were found to be resistance to ampicillin and rifampicin. Then 31 (70.45%) isolates were indicated resistance to cefepime, 26 (59.09%) isolates were also resistant to azithromycin, 21 (47.72%) isolates were resistant to ciprofloxacin and 14 (31.82%) isolates were exhibited resistance to tetracycline. Streptomycin was found to be more effective as only 1 (2.27%) isolate was resistant to this antibiotic. Followed by streptomycin, chloramphenicol was also found to be effective as only 4 (9.09%) isolates were resistant to this antibiotic.

This observation is consistent with the findings of other researchers. Manaal Zahera, Chetan Rastogi, Pushpendra Singh, Sana Iram, Shumaila Khalid and Akhilesh Kushwaha found that penicillin and ampicillin were less effective in their research (Euro. J. Exp. Bio., 2011, 1(2):118-124). According to the research of Karzan Mohammed K, Faeza Burhan O and Shahida Nooruldeen Y, Chloramphenicol was found more effective (Karzan Mohammed et al., J Microb Biochem Technol 2017).

4.2) Concluding remarks:

The findings of this research work indicate that the members of Enterobacteriaceae family are mostly responsible for causing Urinary Tract Infection. Although, urine is considered to be sterile and believed to be germ-free but any source of possible infection occurs through urethra which can initiate the incidence of infection. Urinary tract infection (UTI) remains a worldwide therapeutic problem, not only as a nosocomial disease but also as a community-acquired infection. Though pattern of uropathogens doesn't vary too much in different settings but increasing antimicrobial resistance to bacteria causing UTI is a great concern all over and under developed and developing countries in particular. Development of resistant strain is a common problem in antimicrobial chemotherapy. The rate of resistance is high among uropathogens. These resistance properties are easily transferred between bacteria of different genera through plasmids and other means. To ensure appropriate treatment, knowledge of the organisms that cause UTI and their antibiotic susceptibility is mandatory. Frequency of resistance to antibiotics and drug is directly linked to consumption of antibiotics. Antibiotic resistance of urinary tract pathogens has increased worldwide. In fact, the irrational and inappropriate use of antibiotics is responsible for the development of resistance of the members of Enterobacteriaceae family. In order to prevent or decrease resistance to antibiotics, the use of antibiotics should be kept under supervision, should be given in appropriate doses for an appropriate period of time. An effective national and state level antibiotic policy and draft guidelines should be introduced to preserve the effectiveness of antibiotics and for better patient management. On the other hand, drinking plenty of water daily, wipe from front to back to prevent bacterial around the anus from entering the vagina or urethra, avoid smoking, clean genital area before sexual intercourse, avoid using feminine hygiene sprays and scented douches which may irritate urethra should be applied to avoid Urinary Tract Infection.

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APPENDIX – 1

Media composition:

The composition of the media used in the study has been given below. Unless, all the media were autoclaved at 121℃ for 15 minutes.

a. Nutrient Agar:

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

b. Nutrient broth:

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH	7.4±0.2 at 25ºC

c. Saline:

Component	Amount (g/L)
Sodium Chloride	9.0

d. Eosin Methylene Blue Agar (EMB):

Component	Amount (g/L)
Peptone	10.0
Dipotassium Phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin yellow	0.14
Methylene Blue	0.065
Agar	13.50
Final pH	7.1 ± 0.2 at 25°C

e. MacConkey Agar:

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymatic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salt	1.50
Crystal violet	0.001
Neutral red	0.03
Agar	15.0
Final pH	7.1 ± 0.2 at 25°C

f. Blood Agar Base:

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

g. Hi Crome UTI Agar:

Component	Amount (g/L)
Peptic digest of animal tissue	15.0
Chromogenic mixture	26.80
Agar	15.0
Final pH	7.1 ± 0.2 at 25°C

h. Muller Hinton Agar:

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.3± 0.1 at 25°C

i. Methyl Red -Voges Proskauer (MR-VP) Media:

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

j. Simmon's Citrate Agar:

Component	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

k. Triple Sugar Iron Agar (TSI):

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

l. Motility Indole Urease (MIU) Agar:

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

m. Indole broth:

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

APPENDIX – 2

Reagents:

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

APPENDIX – 3

Instruments:

The important equipment used through this study are listed below

Autoclave	Model: WIS 20R Daihan Scientific Co. ltd, Korea
Sterilizer	Model no: NDS-600D, Japan
Balance machine: Adam	UK
Freezer (-20^{o} C)	Siemens Germany
Incubator	Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan
Laminar Airflow Cabinet	Model-SLF-V, vertical, SAARC group Bangladesh
Micropipettes	Eppendorf, Germany
Oven (Universal drying oven)	Model: LDO-060E , Labtech, Singapore
Refrigerator	Samsung
Vortex mixture	Digi system Taiwan, VM-2000