STUDY OF PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITIES OF BACTERIA AND FUNGI ISOLATED FROM PATIENTS WITH URINARY TRACT INFECTIONS IN PATTUKKOTTAI, TAMIL NADU, INDIA

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INTRODUCTION

- Urinary Tract Infections (UTIs) are one of the **most common bacterial diseases** worldwide.

- Urinary tract infection (UTI) is a bacterial infection that affects any part of the urinary tract. Normally, urine is sterile and it is usually free of bacteria and fungi. It contains a variety of fluids, salts, and waste products but the infection occurs when any organisms, usually bacteria from the digestive tract, cling to the opening of the urethra and begin to multiply, the most of these organisms is *E. coli*, which normally lives in the colon.

- In most cases bacteria travel to the urethra and multiply causing urethra infection **(urethritis)** and if the bacteria move to the bladder and multiply, a bladder infection **(cystitis)** can occur. If the infection is not treated promptly, bacteria may then travel further up the ureters to multiply and reach to the kidneys causing kidney infection **(pyelonephritis)** which is much more serious because it leading to kidneys damaged if a UTI is not treated for months or years.

- More than 95% of urinary tract infections are caused by a **single bacterial species.**

Sources of Urinary Tract Infection spread

- Urinary tract infection (UTI) is an infection of one or more structures in the urinary system. The urinary tract includes **urethra, bladder, ureters, prostate** and **kidneys**. So UTI encompasses a broad range of clinical entities namely;

- **Urethritis-** Urethritis is inflammation of the urethra

- **Cystitis-** Symptoms of bladder infection relate to inflammation- frequency, urgency, and dysuria.
- **Acute cystitis** generally occurs in women. Patients usually present with dysuria, frequency, urgency, voiding small amounts of urine, incontinence and suprapubic or pelvic pain. In women, sexual intercourse can introduce larger numbers of bacteria into the bladder, giving us the term **honeymoon cystitis.** Most cases are due to infection by *E.coli* , also secondary bacterial cause is the *Coagulase-negative Staphylococcus saprophyticus*.

- **Pyelonephritis-** Pyelonephritis refers to inflammation of the kidney parenchyma, calices and pelvis

- **Prostatitis** is inflammation of the prostate gland. Prostate gland provides nursing and protective fluid to support sperm survival.

Sex - The female urethra appears to be particularly prone to colonisation with colonic Gram-negative bacilli, because of its proximity to the anus; it has short length and its termination beneath the labia. Sexual intercourse causes the introduction of bacteria into the bladder.

Pregnancy - Symptomatic upper urinary tract infections are common during pregnancy due to decreased urethral tone, decreased urethral peristalsis and temporary incompetence (abnormal backward movement of urine) of the vesicoureteral valves.

Catheterization- A common source of infection is **catheters,** or tubes placed in the urethra and bladder. A person who cannot void or who is unconscious or critically ill often needs a catheter that stays in place for a long time. Some people, especially the elderly or those with nervous system disorders who lose bladder control, may need a catheter for life. Bacteria on the catheter can infect the bladder.

Diabetes - People with **diabetes** have a higher risk of a UTI, because of changes in the immune system. Any other disorder that suppresses the immune system raises the risk of a urinary infection. International studies suggest an increase in *Enterococcus spp*. as uropathogens among elderly and diabetic patient.

Similarly there are a number of factors that increase the risk of developing urinary tract infection. Some of these are: **kidney stones, tumors, urethral strictures (narrowing urethra), neurological diseases, congenital /acquired anomalies of bladder, suppressed immune system, ureteric stresses,** etc.

Objectives of the Present Study

There is no record before 2011 up till date to suggest that the UTI status of the people had been probed into through research. This work is therefore targeted at studying urinary tract infection prevalence among patients of Pattukkottai area in Delta district of Thanjavur, Tamilnadu, with the following objectives:

- To study the **prevalence and the distribution** of different bacterial pathogens isolated from patients with UTIs.
- To **isolate and characterise** of pathogens causing UTIs in patients.
- To study the **antibiotic sensitivity** pattern of the urinary bacterial isolates, so as to provide a basic guideline in treating UTIs.
- To study the **molecular characterisation** of the **ESBL** producing urine isolates of **multi-drug resistance *E.coli*** and describing the epidemiology.
- To isolate and characterise of the uropathogenic ***Candida* species**
- To study the **antifungal susceptibility** pattern of *Candida* isolates from the UTIs

Methodology

Study Population

A total of 2400 midstream urine samples were collected from both male and female patients who had clinically suspected UTIs.

Collection and Transportation of Samples

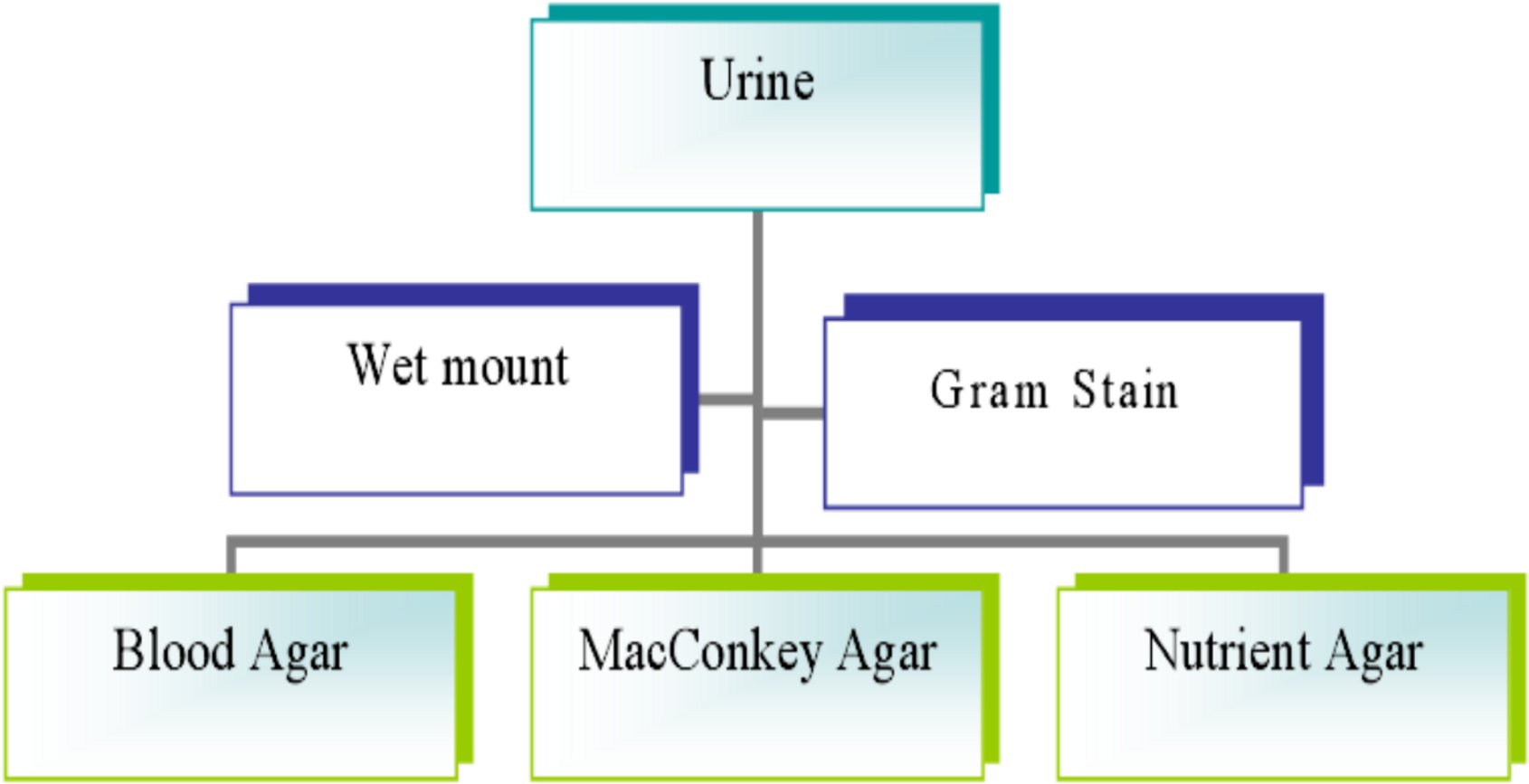
Urine samples were collected into sterile screw-capped containers from patients who had visited in the private hospital, Pattukkottai area to see doctors with various complaints which were diagnosed tentatively as symptoms of urinary tract infection (UTI). The consulting doctors had then referred the patients to the Gangasaras Diagnostic and Research Centre for urine mcs (microscopy, culture and sensitivity) investigation for the purpose of making definite diagnosis.

Processing of samples

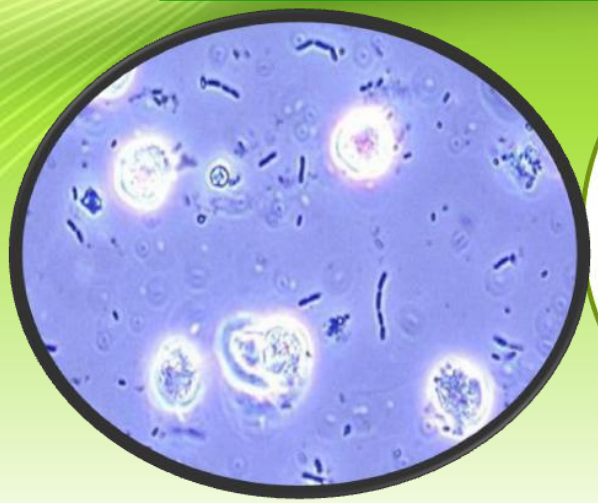
All the uropathogens were identified by standard Biochemical procedures and Morphological features. Antimicrobial and antifungal susceptibility test was done for the bacterial and *candida* isolates. A total of 100 highly multi-drug resistant *E.coli* culture isolates were selected for ESBL activity and molecular characteristics test.

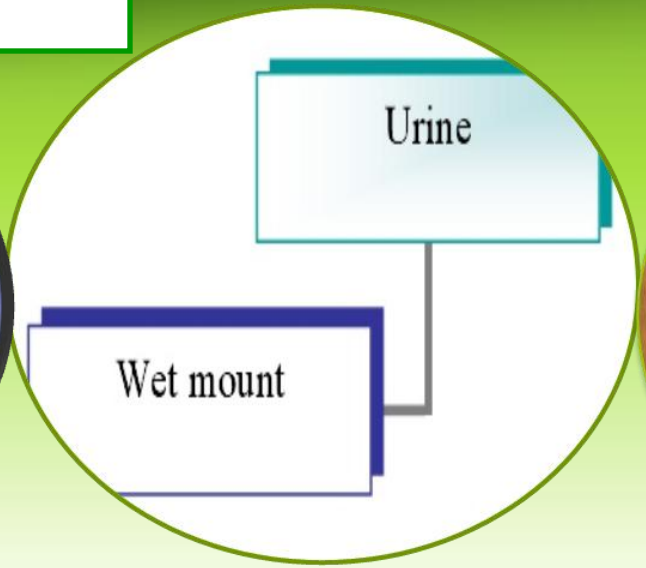
LABORATORY EVALUATION

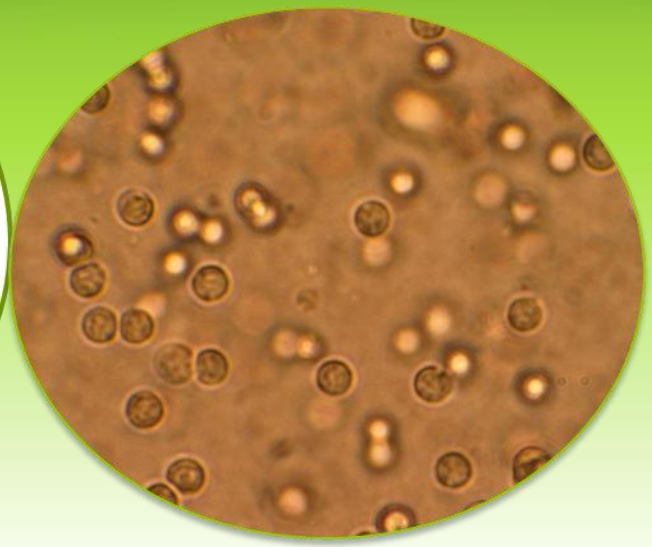
Specimen processing



Initial report







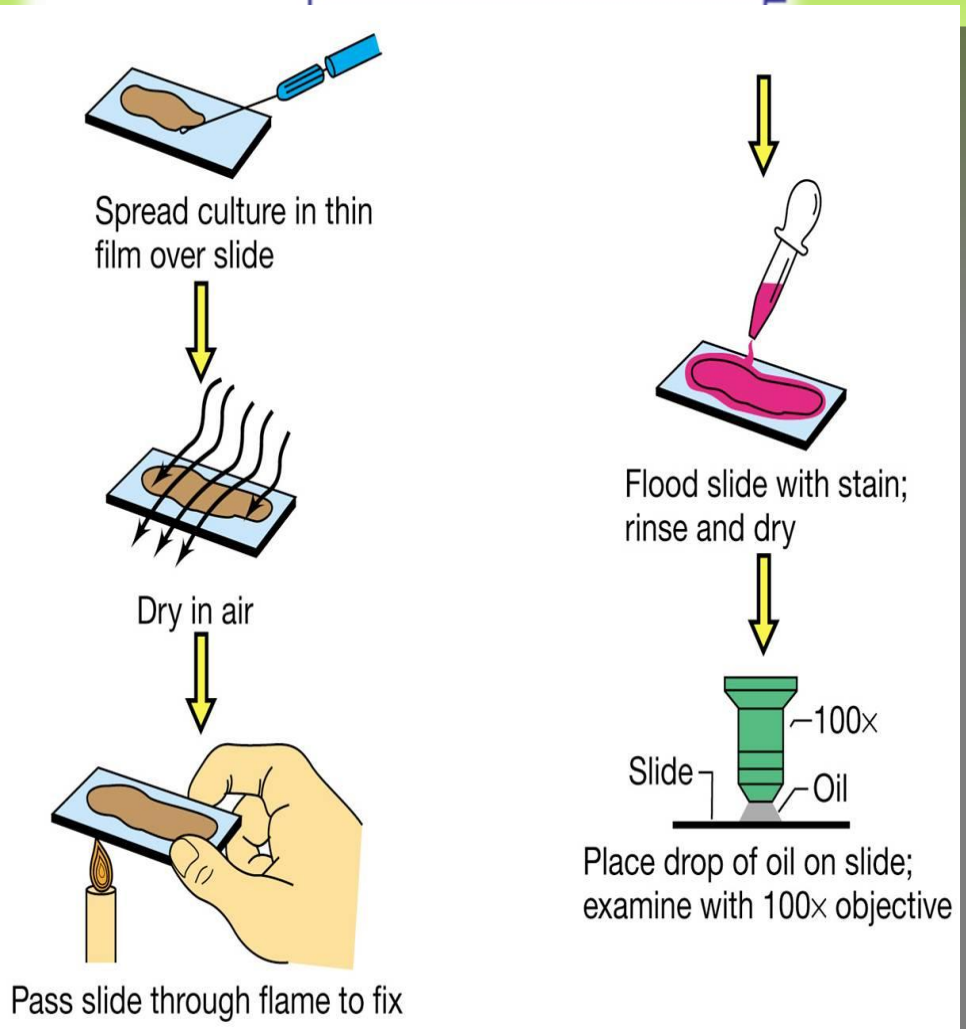
All samples that recorded significant bacterial counts were subjected to urine microscopy test to detect presence of 5-10 pus cells per high power focus (5-10 pus cells/HPF) in urine sediments or deposits

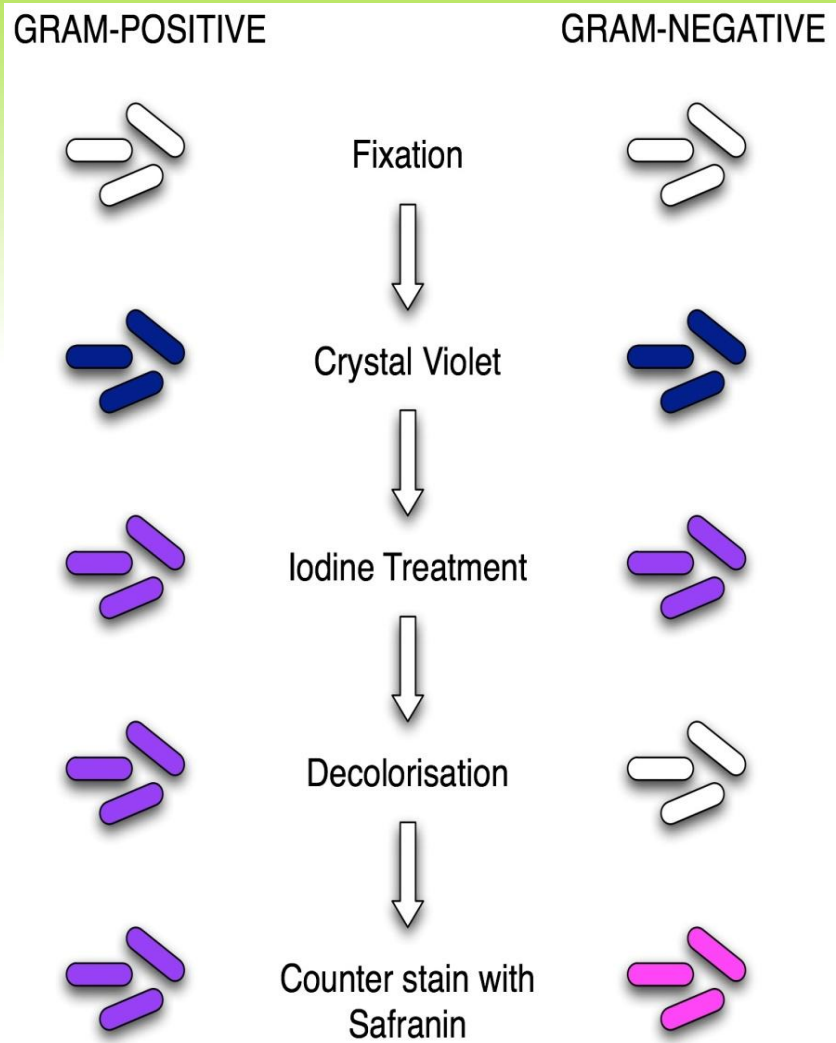
Urine nitrite: The use of dipstick designed to detect the presence of **urine nitrite.** The nitrate test of urine has been used as rapid screening test for significant bacteriuria. A positive nitrite test indicates that cause of the UTI is a **Gram negative organisms**, a most commonly ***E.coli.*** The reason for nitrites existence in the presence of UTI is due to a bacterial conversion of endogenous nitrates to nitrites.

Urine

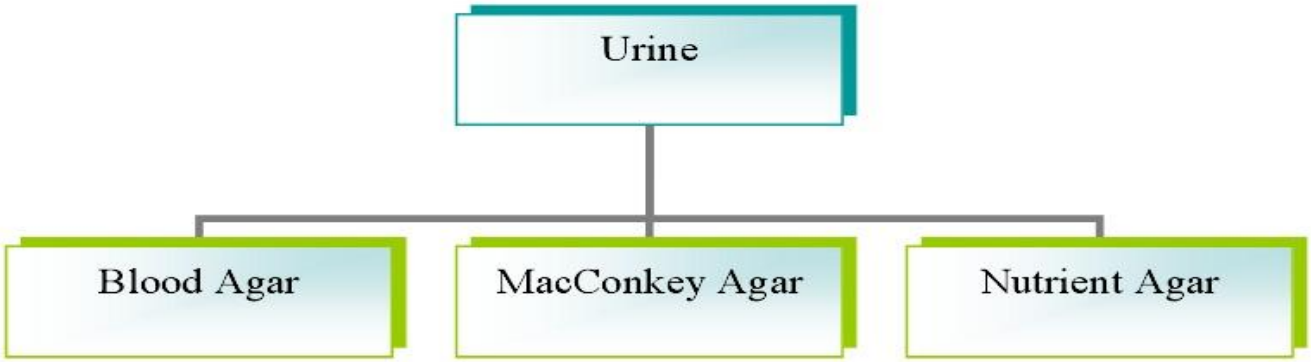
Screening test

Gram Stain





Culturing



UTI Chromagar

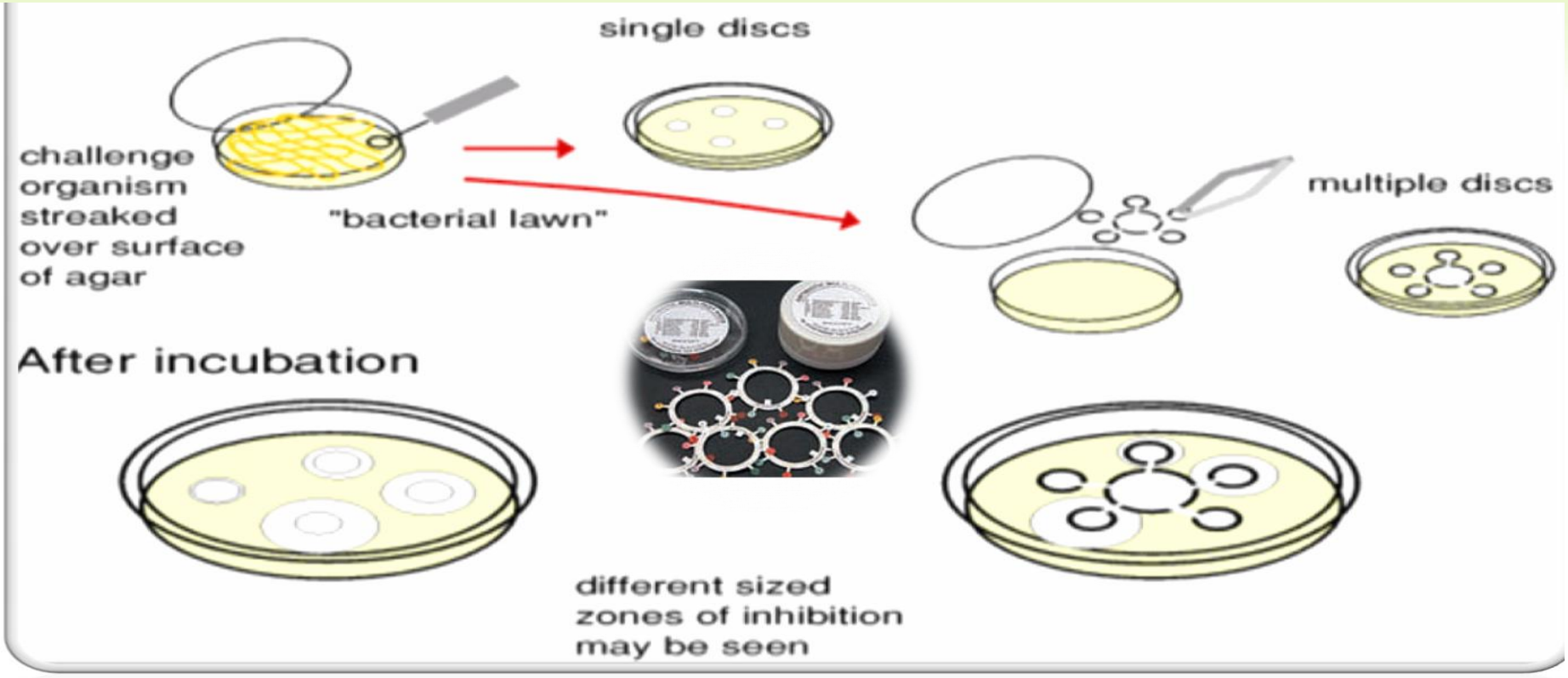
In the laboratory, each sample was inoculated on Blood agar, MacConkey agar, Nutrient agar and UTI Chromagar. All the bacteria were isolated and identified using morphological, microscopy and biochemical tests following standard procedures described by Sharma (2008).

Antibiotic sensitivity test

Antibiotic sensitivity testing of bacteria was carried out by the disc diffusion method by using a commercially available disc (Himedia). The agar diffusion disc technique described by Bauer *et al*. (1996) was applied. Sixteen types of antibiotic discs were used. The antibiotics used were

Amoxicillin,
Ampicillin,
Cefotaxime,
Ceftazidime,
Ceftriaxone,
Cephalexin,
Ciprofloxacin,
Nalidixic acid,
Norfloxacin,
Ofloxacin,
Amikacin,
Gentamicin,
Tobramycin,
Imipenem,
Nitrofurantoin,
Cotrimoxazole.

A colony of each pure isolate was streaked on sterile Mueller Hinton agar plates aseptically using sterile inoculating wire loop. The appropriate multi discs or single disc of antibiotics were aseptically placed firmly onto the surface of the dried plates using sterile forceps. The plates were left at room temperature for one hour to allow diffusion of the different antibiotics from the disc into the medium. The plates were then incubated at 37^0C for 18 hours. Interpretation of results was done using the length of inhibition of zone diameter. Zones of inhibition greater than 10mm were considered sensitive, 5- 10mm moderate sensitive and no zone of inhibition, resistant.



Antibiotics used, their concentration and interpretation of inhibition zones

Antimicrobial agent	Symbol	Concentration	Resistant mm	Intermediate mm	Susceptible mm
Amoxicillin	AX	30 µg	≤ 11	12 - 13	≥14
Ampicillin	AM	10 µg	≤13	14 – 16	≥17
Cefotaxime	CTX	30 µg	≤ 14	15 - 22	≥23
Ceftazidime	CAZ	30 µg	≤14	15 – 17	≥18
Ceftriaxone	CT	30 µg	≤13	14 – 20	≥21
Cephalexin	CL	30 µg	≤ 14	15 - 17	≥18
Ciprofloxacin	CIP	5 µg	≤ 15	16 - 20	≥21
Nalidixic acid	NA	30 µg	≤ 13	14 - 18	≥19
Norfloxacin	NX	10 µg	≤ 12	13 - 16	≥17
Ofloxacin	OF	5 µg	≤ 14	15 - 21	≥22
Amikacin	AK	30 µg	≤ 14	15 - 16	≥17
Gentamicin	GEN	10 µg	≤ 12	13 - 14	≥15
Tobramycin	TB	10 µg	≤ 12	13 - 14	≥15
Imipenem	IPM	10 µg	≤ 13	14 - 15	≥16
Nitrofurantoin	NIT	300 µg	≤ 14	15 - 16	≥17
Cotrimoxazole	COT	30 µg	≤ 10	11 - 15	≥16

ISOLATION AND CHARACTERISATION OF THE PATHOGENIC CANDIDA SPECIES

Twenty two isolates of *Candida* species were used for microscopy analysis through Gram staining and culture on Chromagar media, Sabouraud dextrose agar medium (SDA), supplemented with 50mcg of chloramphenicol. The cultures were incubated at 37^{o}C, for 24-48 hours, under aerobic conditions.

ANTIFUNGAL SENSITIVITY TESTING

Antifungal sensitivity testing was carried out using the disc diffusion method following the National Committee for Clinical Laboratory Standards Institute (CLSI, 2004) guidelines, using **fluconazole (25µg), itraconazole (50µg), ketoconazole (10µg),** and **amphotericin B (20µg)** antifungal discs. Supplemented Mueller-Hinton agar [Mueller-Hinton agar + 2% glucose and 0.5 g/mL methylene blue dye] was used for performing the antifungal ssensitivity testing.

PLASMID ANALYSIS OF ESBL PRODUCING *E.coli*

Clinical isolates

A total of 100 highly Multi-drug Resistant (MDR) *E.coli* culture isolates were selected from urine specimens for ESBL (Extended-spectrum beta-lactamases) activity and molecular characteristics test. Only a single positive culture was included in the analysis.

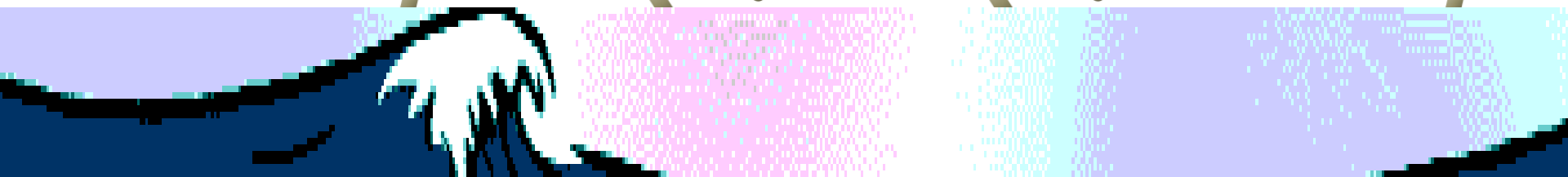
Double disc synergy test

E. coli that exhibited resistance to third generation cephalosporins were screened to detect ESBL production by DDST (Double disc synergy test). Cefotaxime 30µg was placed at a distance of 15mm edge to edge from a centrally placed augmentin disc containing 20µg of amoxicillin+10µg of clavulanic acid. Plates were incubated at 37° C for 18-24 hours and the pattern of zone inhibition was noted. Isolates that exhibited a distinct shape/size with potentiation towards amoxicillin + clavulanic disc were considered potential ESBL producers.

Plasmid analysis

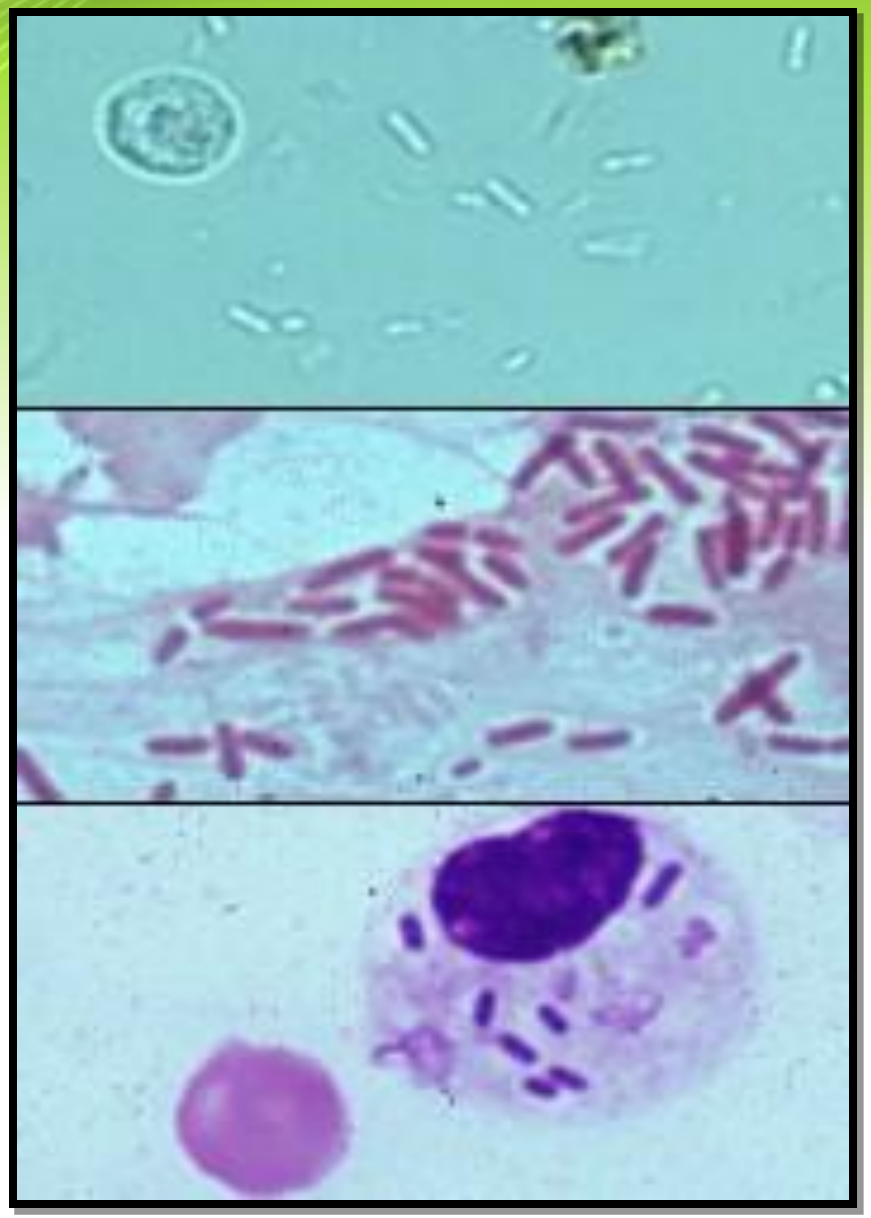
Plasmids DNA were extracted from cultured cells using the alkaline SDS (Sodium Dodecyl Sulfate) method (Johnson, 1998). The DNA was electrophoresed on 0.8% agarose gel stained with ethidium bromide and visualized by UV-transillumination. Plasmid sizes were estimated by comparing with previously characterized plasmids.

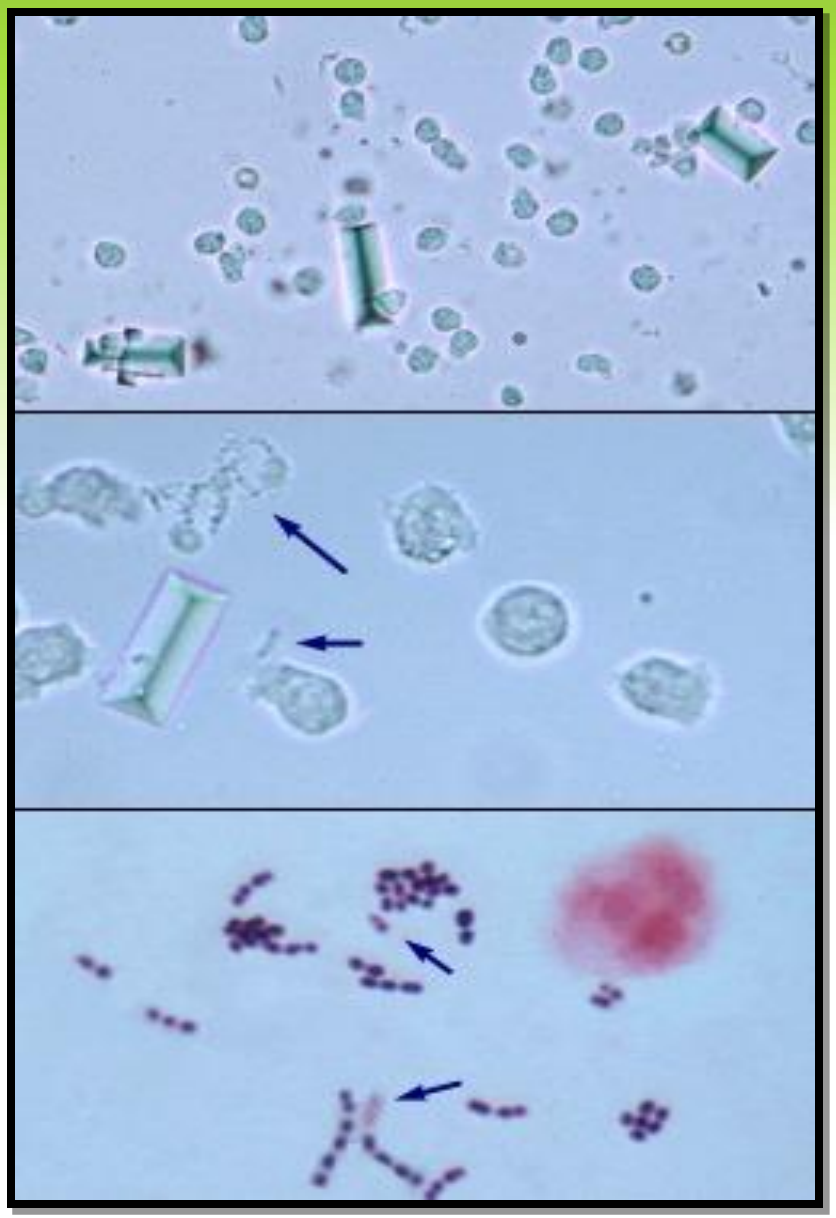
Results



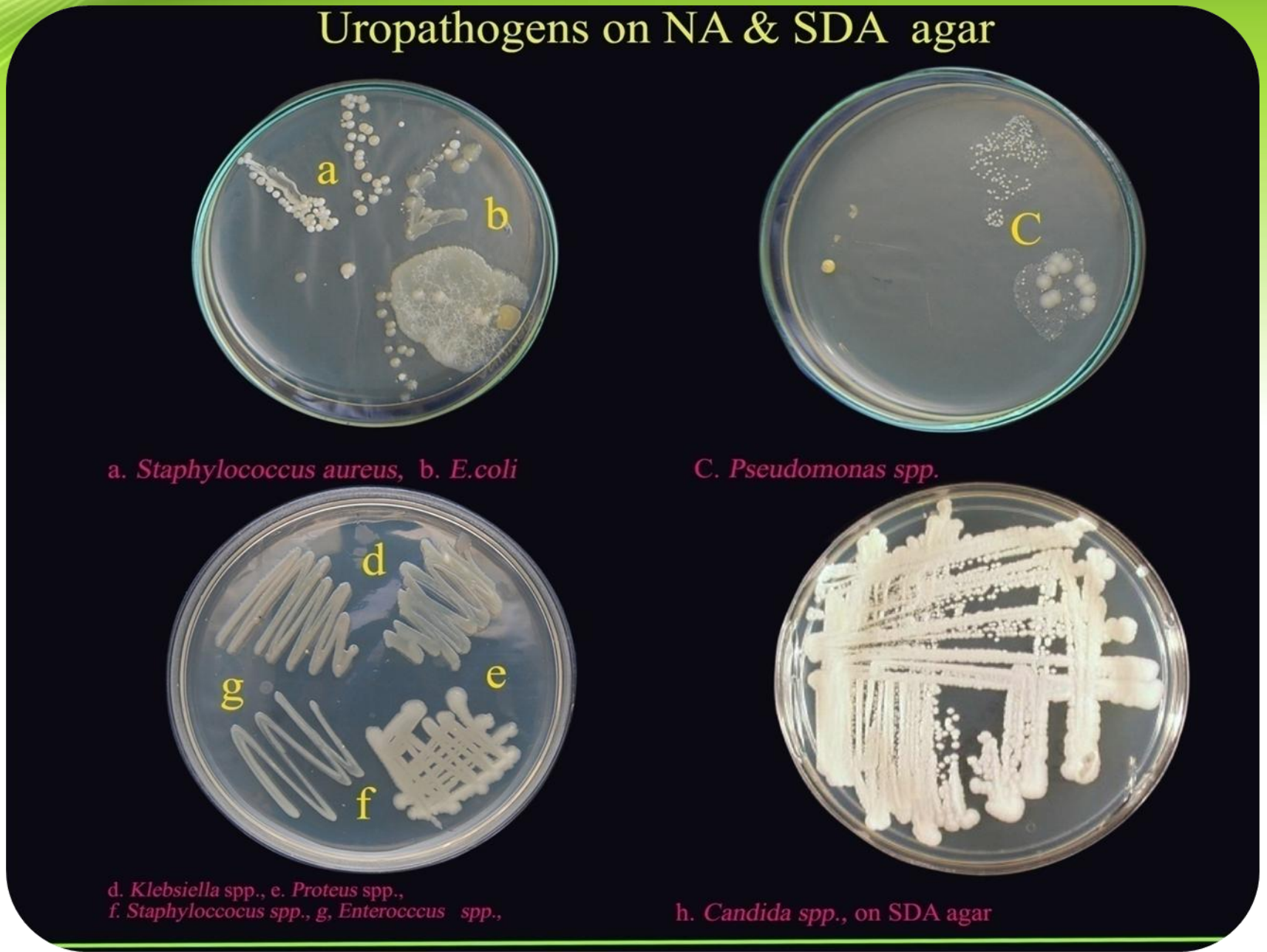
4.1. STUDY OF THE PREVALENCE OF UTIs

Microscopy: Direct wet amount showing Bacteria, Pus cells in urine





Isolation of bacteria on Nutrient agar (a-g) and isolation of *Candida* species on Sabouraud dextrose agar (h).



Study population: A total of 2400 midstream urine samples were processed from patients having clinically suspected Urinary Tract Infections (UTIs) attending various hospitals in Pattukkottai area. Out of the 2400 specimens, 650 (27.1%) were culture positive and 1750 (72.9%) specimens were negative

Growth and Sex	No. of patients	Percentage (%)
Growth	**650**	27.1
No growth	1750	72.9
Male	980	40.8
Female	1420	59.2

Distribution of uropathogens according to age groups and gender

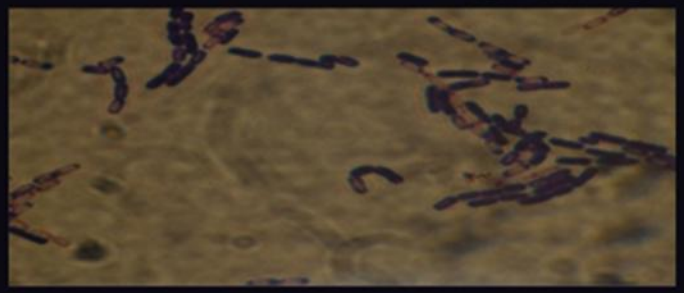
Age groups in years	No of Females infected	%	No of males infected	%
0-10 (Children)	101	15.5	60	9.2
11-20	65	10.0	18	2.8
21-30	105	16.2	40	6.2
31-40	70	10.8	22	3.4
41-50	50	7.7	17	2.6
51-60	28	4.3	13	2.0
61-70	20	3.0	14	2.2
Above 71	15	2.3	12	1.8
Total	454	**69.8**	196	**30.2**

4.2 ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA FROM UTIs

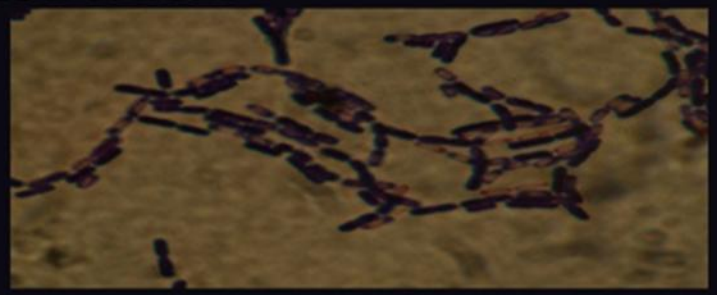
Sources of isolated strains with percentage: Table showed that the sources of the isolated strains from 650 patients with different diseases of urinary tract infections.

Clinical Diagnosis	No of isolated uropathogens	Percentage %
Urinary tract infection	**396**	60.9
Cystitis	63	9.7
Urethritis	51	7.9
Catheterisation	50	7.7
Pyelonephritis	28	4.3
Suspected cancer	25	3.8
Prostatitis	20	3.1
Stone	10	1.5
Ureteric Stone	7	1.1

Gram's stained smear

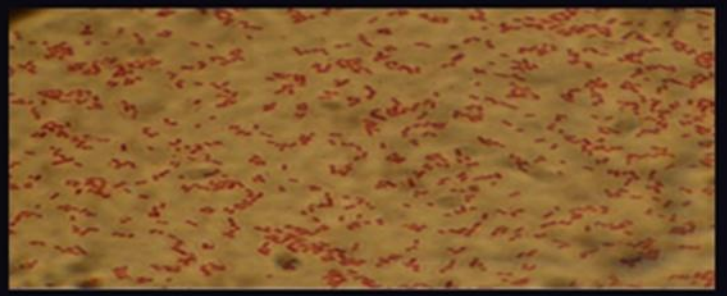


a. *Staphylococcus aureus*

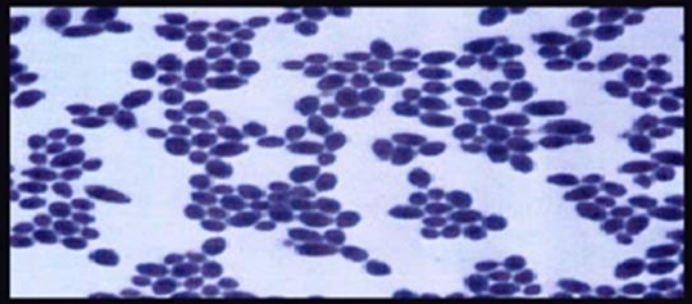


b. *Staphylococci saprophyticus*

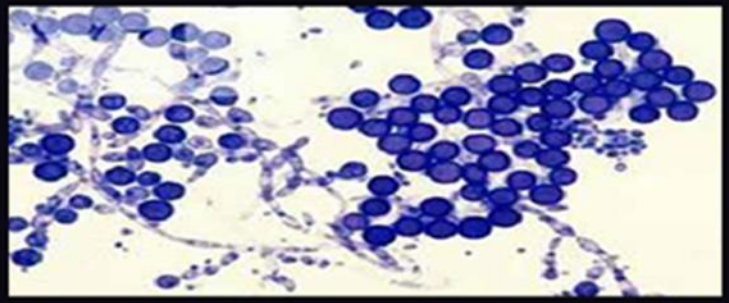
c. *E.coli*



d. *Klebsiella spp.*



e. *Candida albicans*



f. *Candida albicans* with pseudo hyphae

g. Wet mount preparation-Pus cells & *Candida spp.*

h. *Candida spp.* - Germ tube test

Identification of bacteria on MacConkey agar and Blood agar

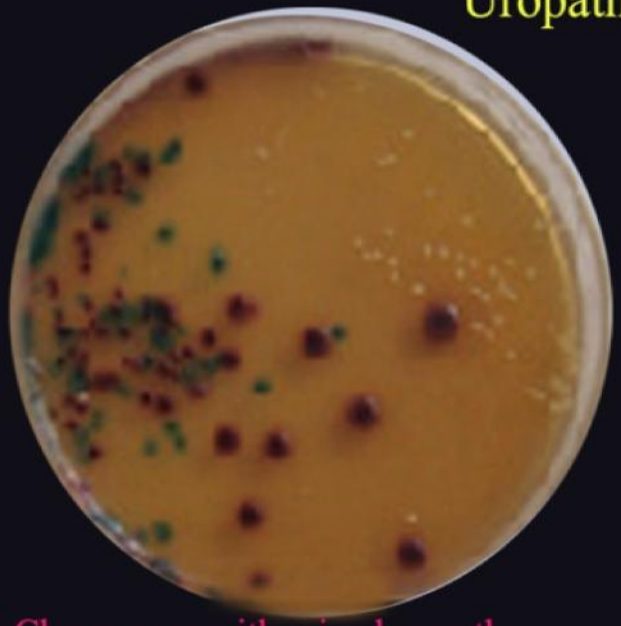
e. LF *E.coli* on MacConkey agar

f. LF *Klebsiella spp.* on MacConkey agar

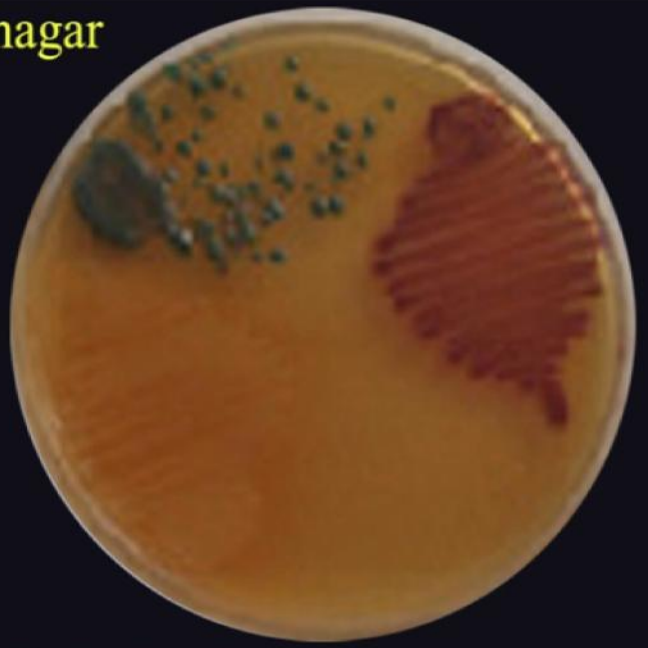
g. NLF *Proteus spp.* on MacConkey agar

h. NLF *Pseudomonas & Stathylococcus spp.* on MacConkey agar

Uropathogens on Chromagar

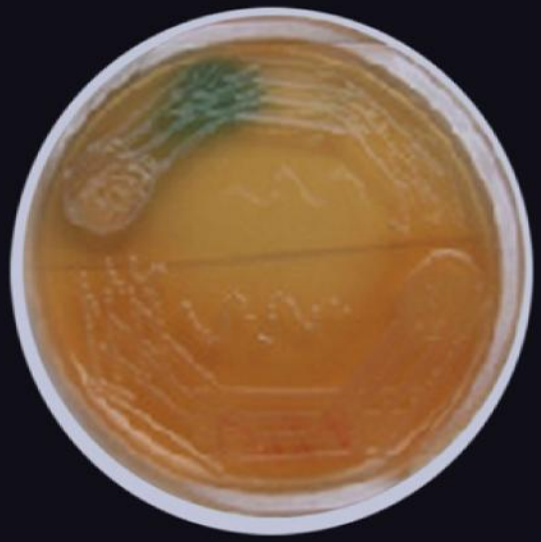


a. Chrom agar with mixed growth

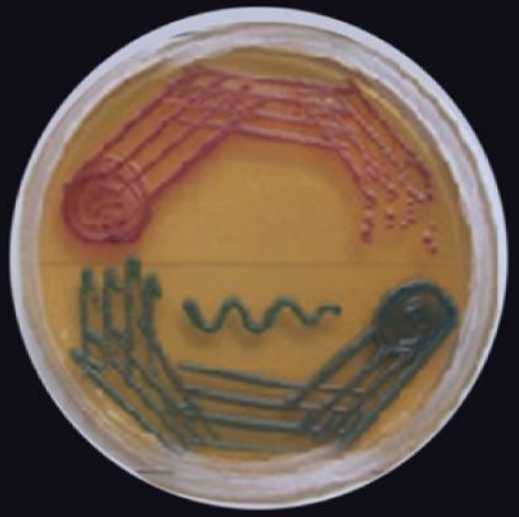


b. *Klebsiella, E.coli* and *Proteus* on Chrom agar

c. *Enterocccus, S.aureus, CoNS*

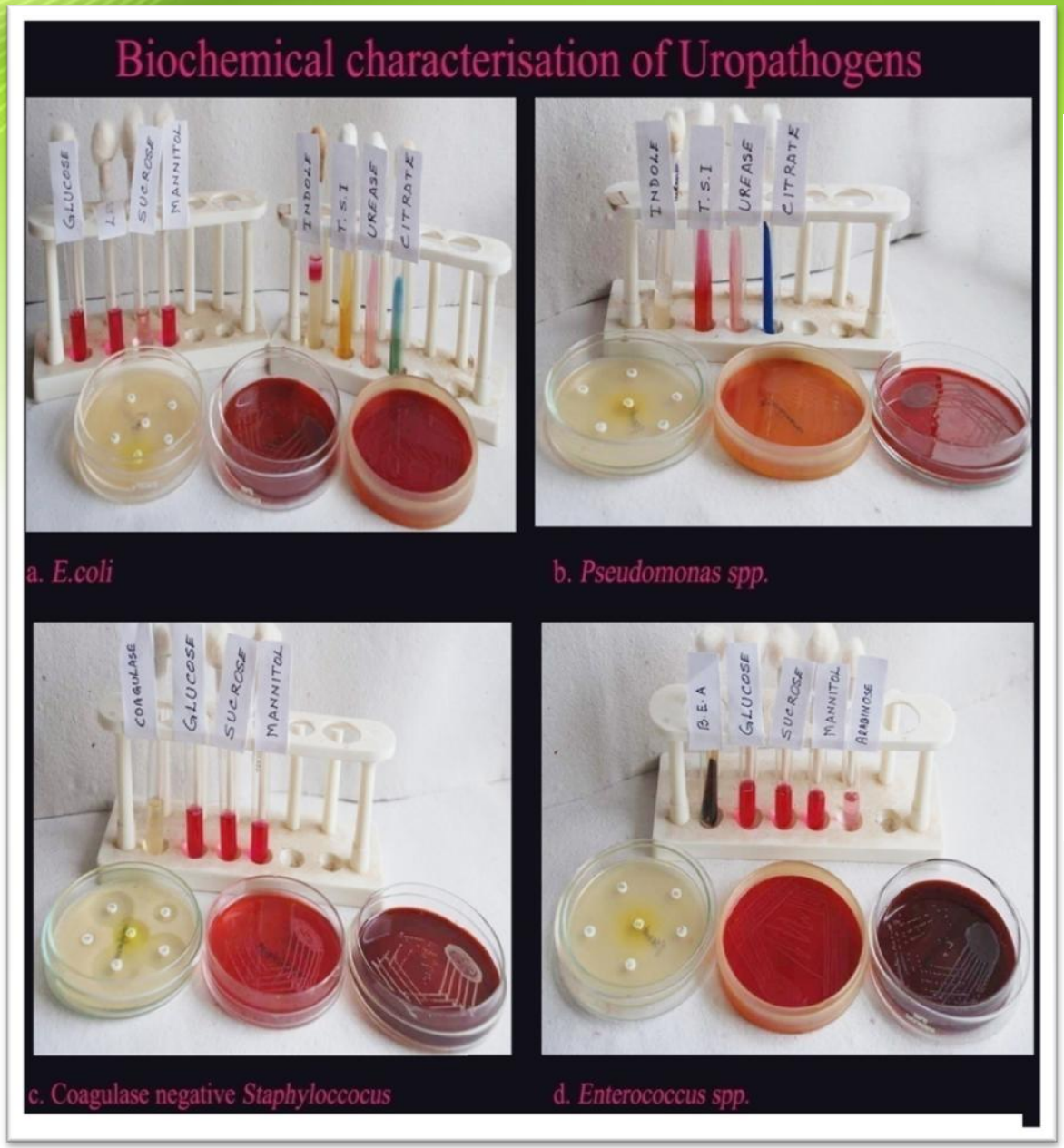


d. *Pseudomonas & Proteus* spp.,



e. *E.coli, Klebsiella*

Identification of bacteria through biochemical testing and selective media



Distribution of isolates among positive urine specimens

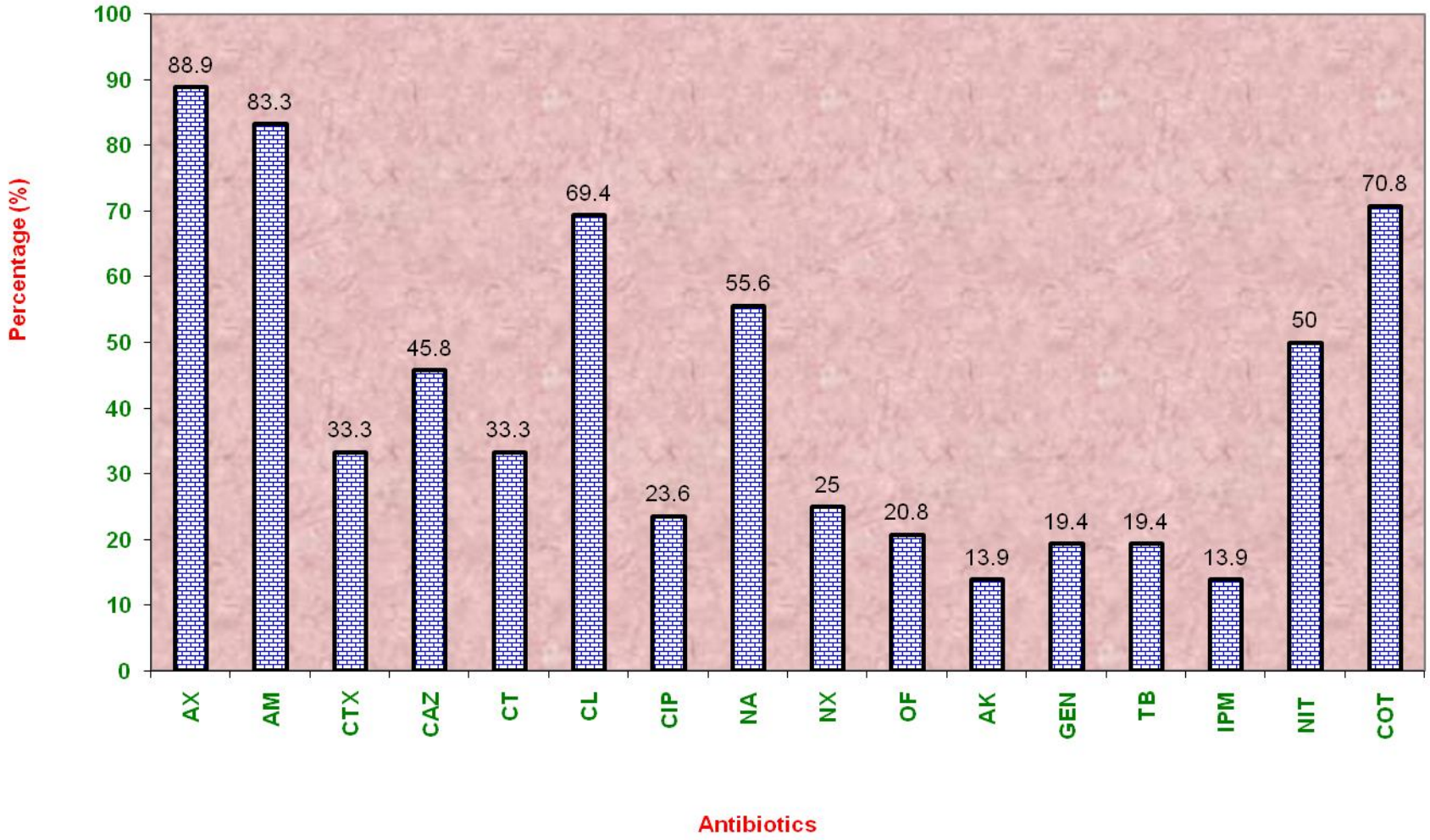
Microorganisms	Frequency	Percentage
Escherichia coli	**355**	54.6%
K. pneumoniae	**72**	11.2%
Pseudomonas spp.	**68**	10.5%
Proteus spp.	44	6.8%
Enterobacter spp.	10	1.5%
Citrobacter spp.	4	0.6%
Acinetobacter spp.	2	0.3%
GNB Total	**555**	**85.4%**
CoNS	**27**	4.1%
Enterococcus spp.	24	3.7%
S. saprophyticus	15	2.3%
S.aureus	7	1.1%
GPC Total	**73**	**11.2%**
Candida spp.	**22**	3.4%
Total	650	100%

4.3. ANTIBIOTIC SENSITIVITY PATTERN OF BACTERIAL ISOLATES FROM THE UTIs

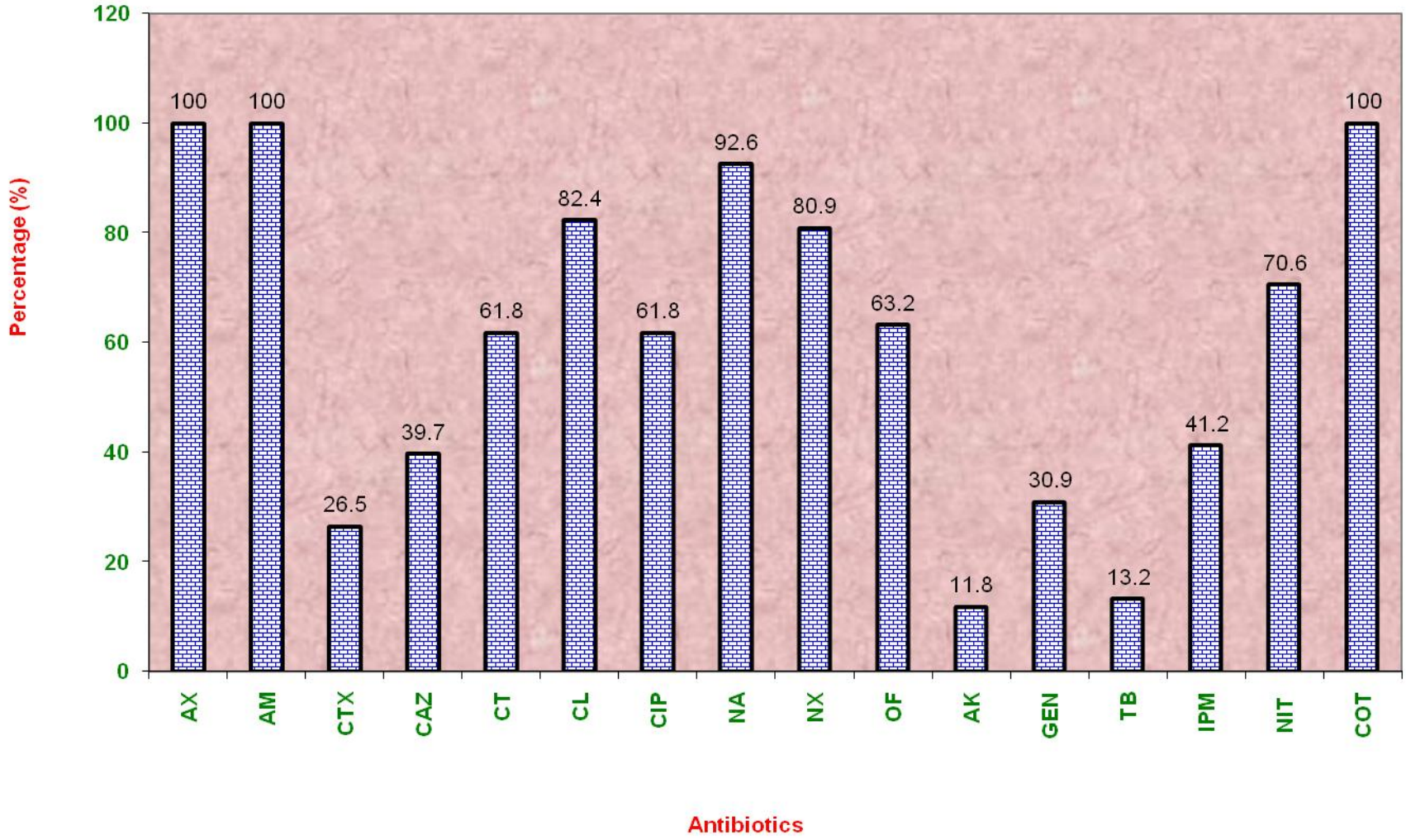
Characterisation and susceptibility pattern of *E.coli*

Antibiotic	Sensitive No (%)	Moderately Sensitive No (%)	Total Sensitive (%)	Total Resistant No (%)
Amoxicillin	20 (5.6)	9 (2.6)	29 (8.2)	**326 (91.8)**
Ampicillin	30 (8.5)	10 (2.8)	40 (11.3)	**315 (88.7)**
Cefotaxime	190 (53.5)	16 (4.5)	206 (58.0)	149 (42.0)
Ceftazidime	188 (53.0)	6 (1.7)	194 (54.7)	161 (45.3)
Ceftriaxone	163 (45.9)	4 (1.1)	164 (47.0)	188 (53)
Cephalexin	39 (10.9)	2 (0.6)	41 (11.5)	**314 (88.5)**
Ciprofloxacin	239 (67.3)	6 (1.7)	245 (69.0)	**110 (31.0)**
Nalidixic acid	78 (22.0)	3 (0.8)	81 (22.8)	274 (77.2)
Norfloxacin	237 (66.8)	3 (0.8)	240 (67.6)	115 (32.4)
Ofloxacin	227 (63.9)	2 (0.6)	229 (64.5)	126 (35.5)
Amikacin	297 (83.7)	16 (4.5)	313 (88.2)	**42 (11.8)**
Gentamicin	212 (59.7)	10 (2.8)	222 (62.5)	133 (37.5)
Tobramycin	269 (75.8)	4 (1.1)	271 (76.9)	**82 (23.1)**
Imipenem	327 (92.1)	5 (1.4)	332 (93.5)	**23 (6.5)**
Nitrofurantoin	303 (85.3)	6 (1.7)	309 (87.0)	**46 (13.0)**
Cotrimoxazole	81 (22.8)	3 (0.9)	84 (23.7)	**271 (76.3)**

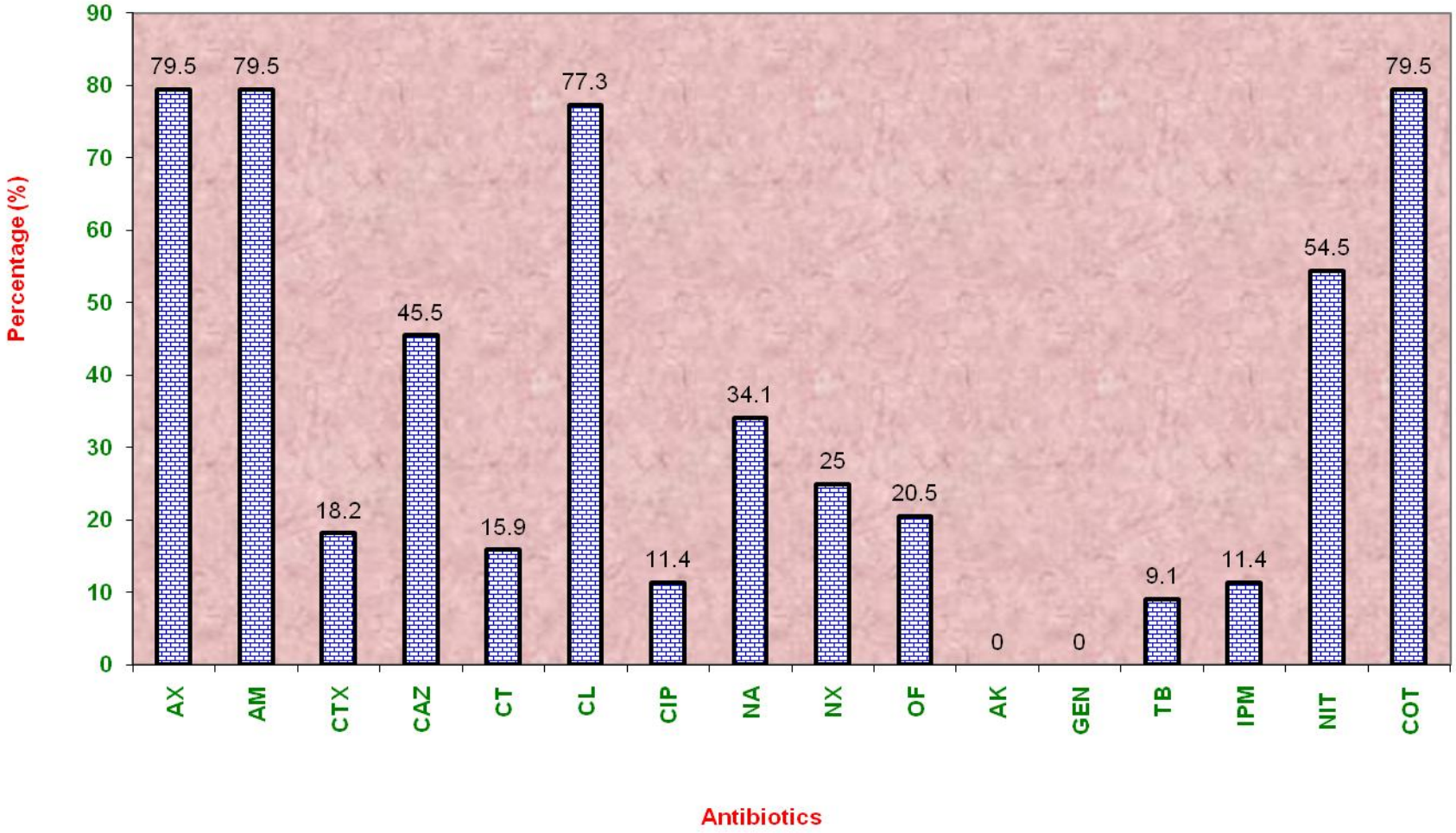
Antibiotic resistance of *Klebsiella* strains from patients with UTIs.



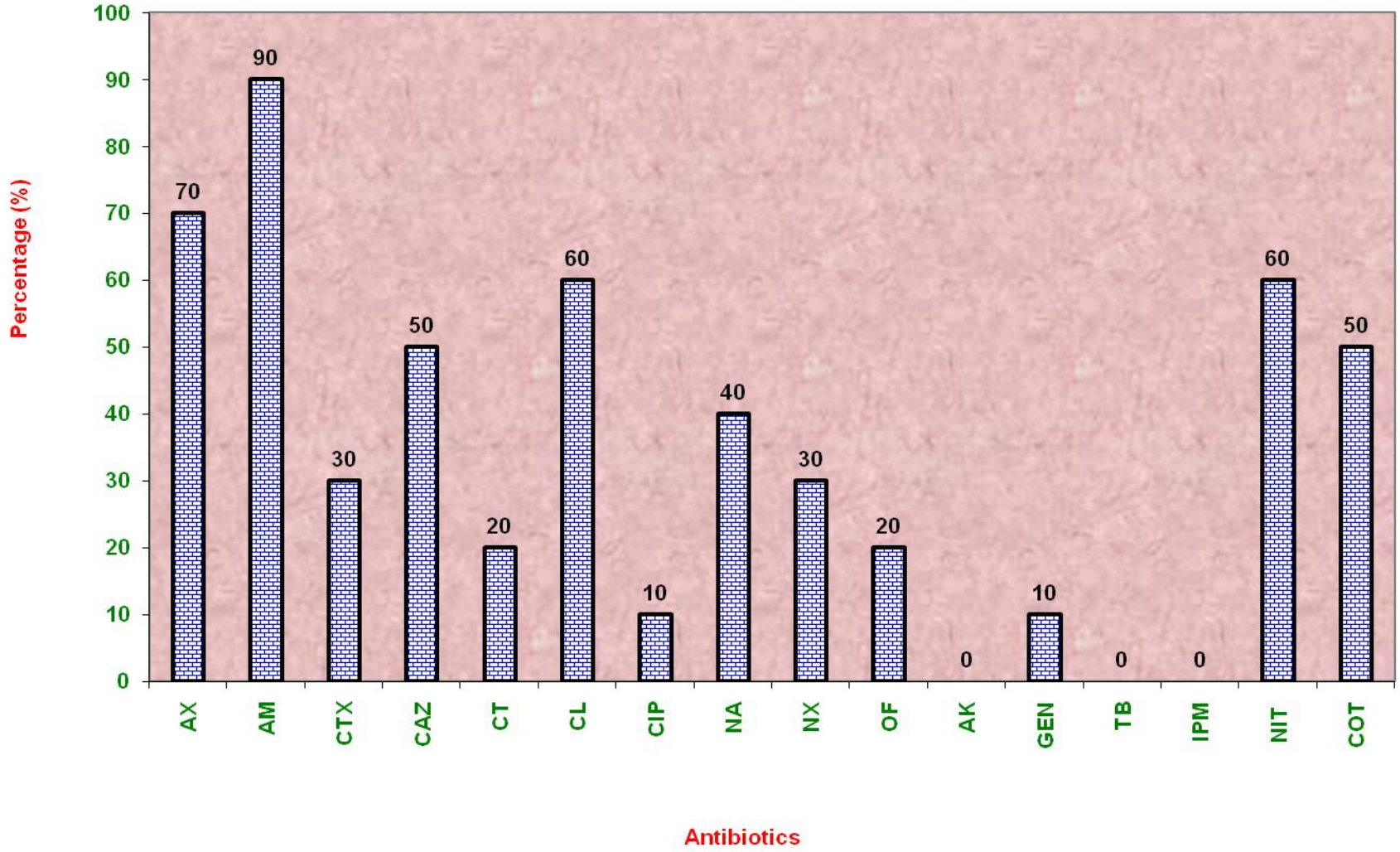
Antibiotic resistance of *Pseudomonas* strains from patients with UTIs.



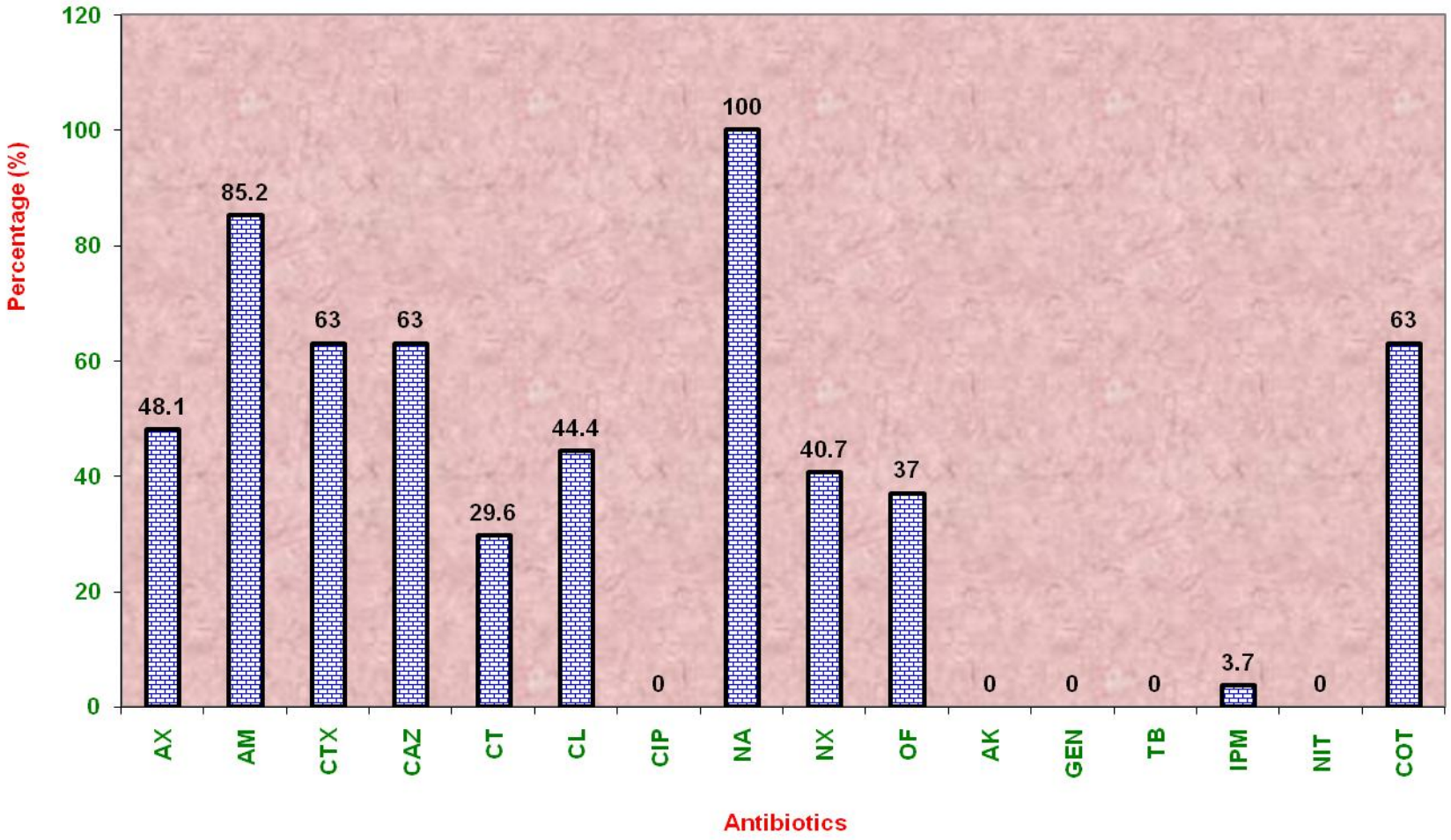
Antibiotic resistance of *Proteus* strains from patients with UTIs.



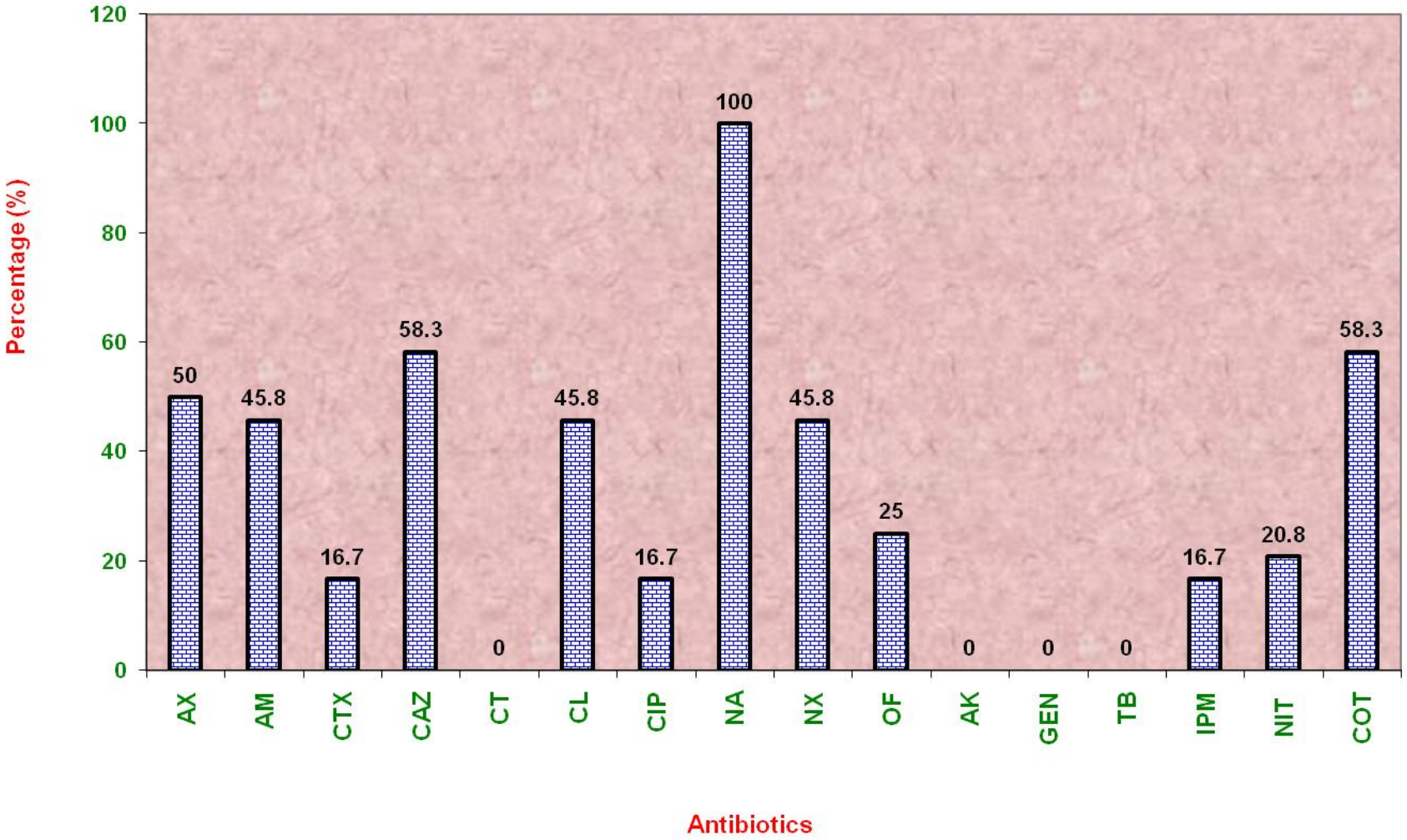
Antibiotic resistance of *Enterobacter* strains from patients with UTIs.



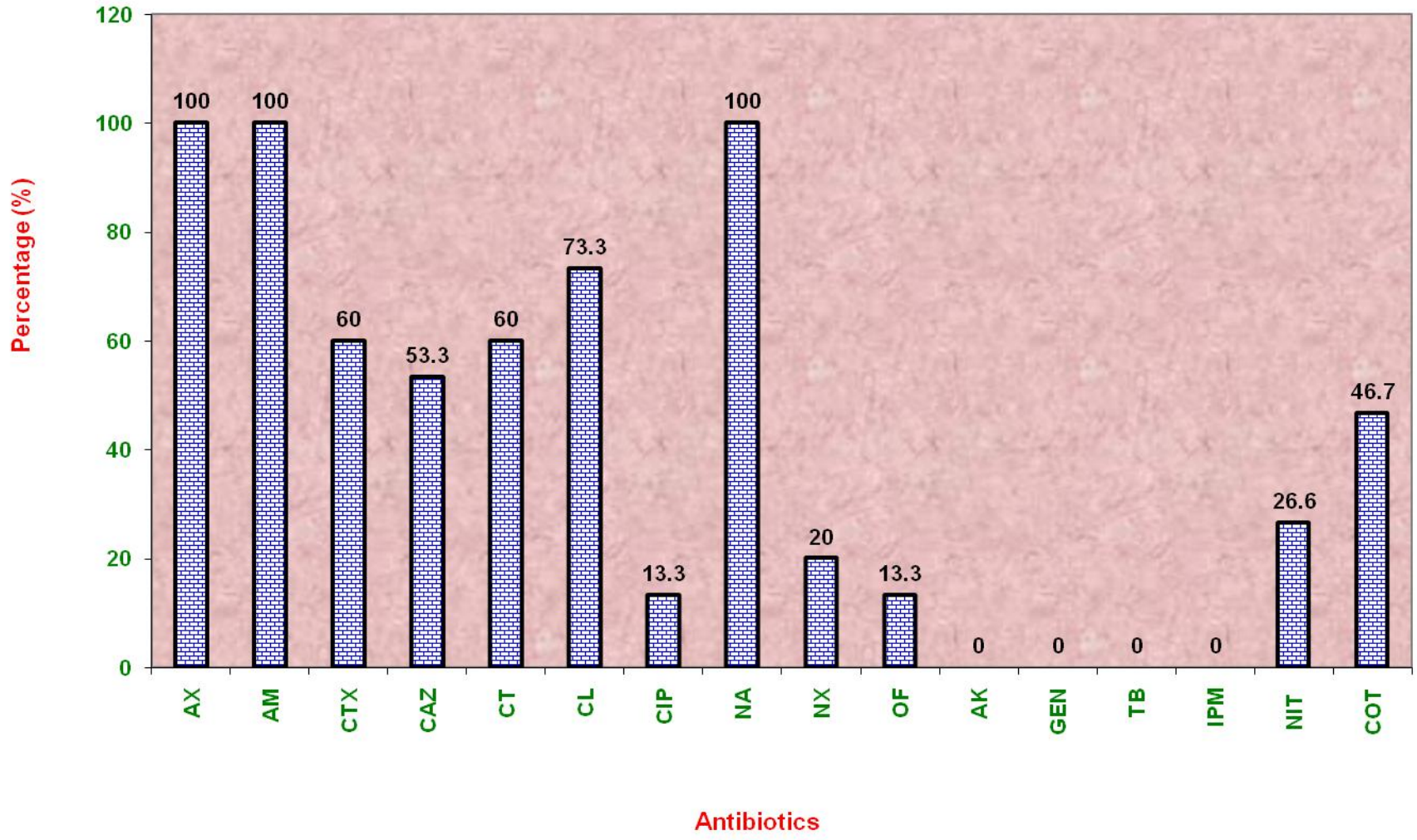
Antibiotic resistance of CoNS strains from patients with UTIs.



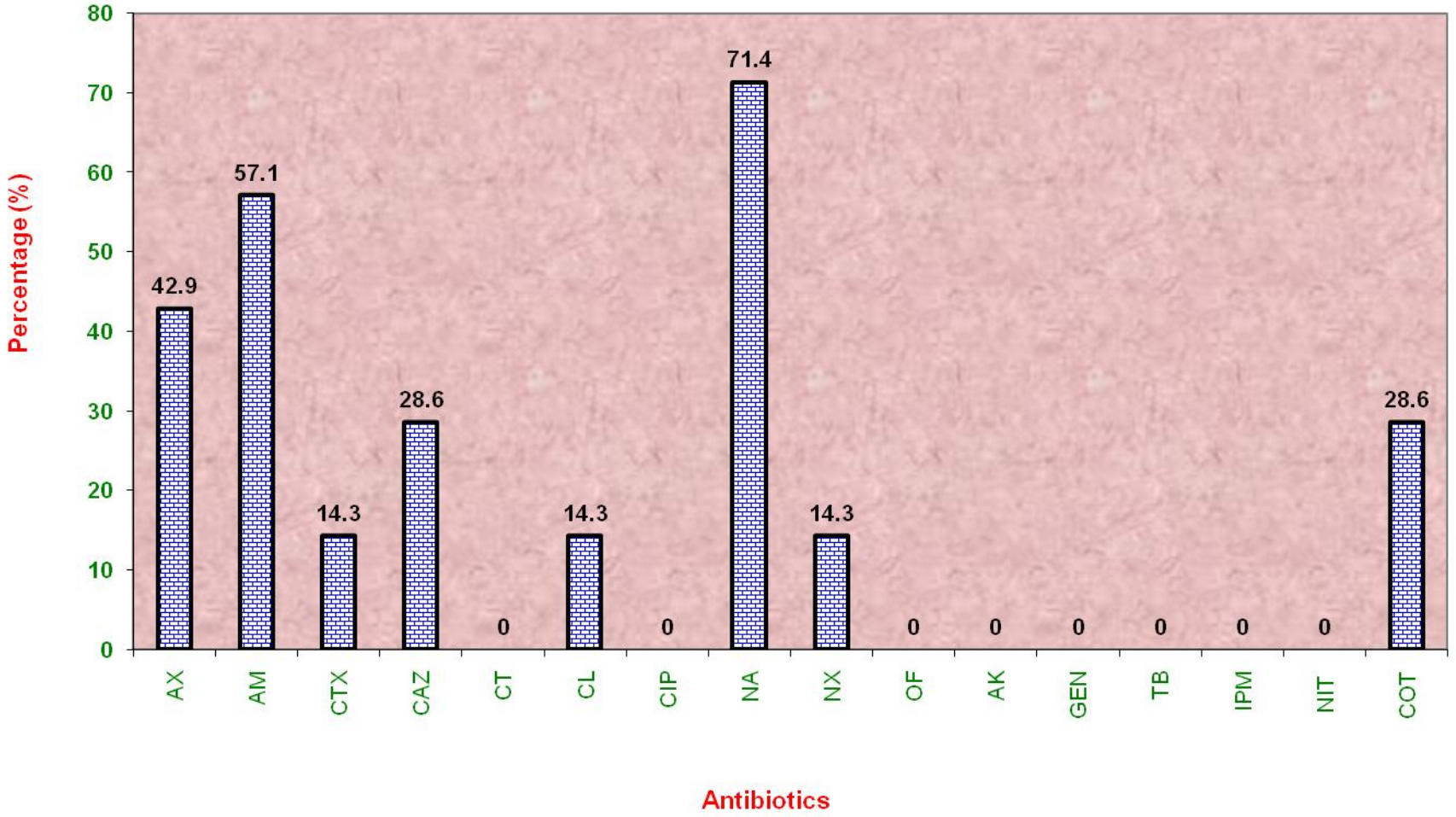
Antibiotic resistance of *Enterococcus* strains from patients with UTIs.



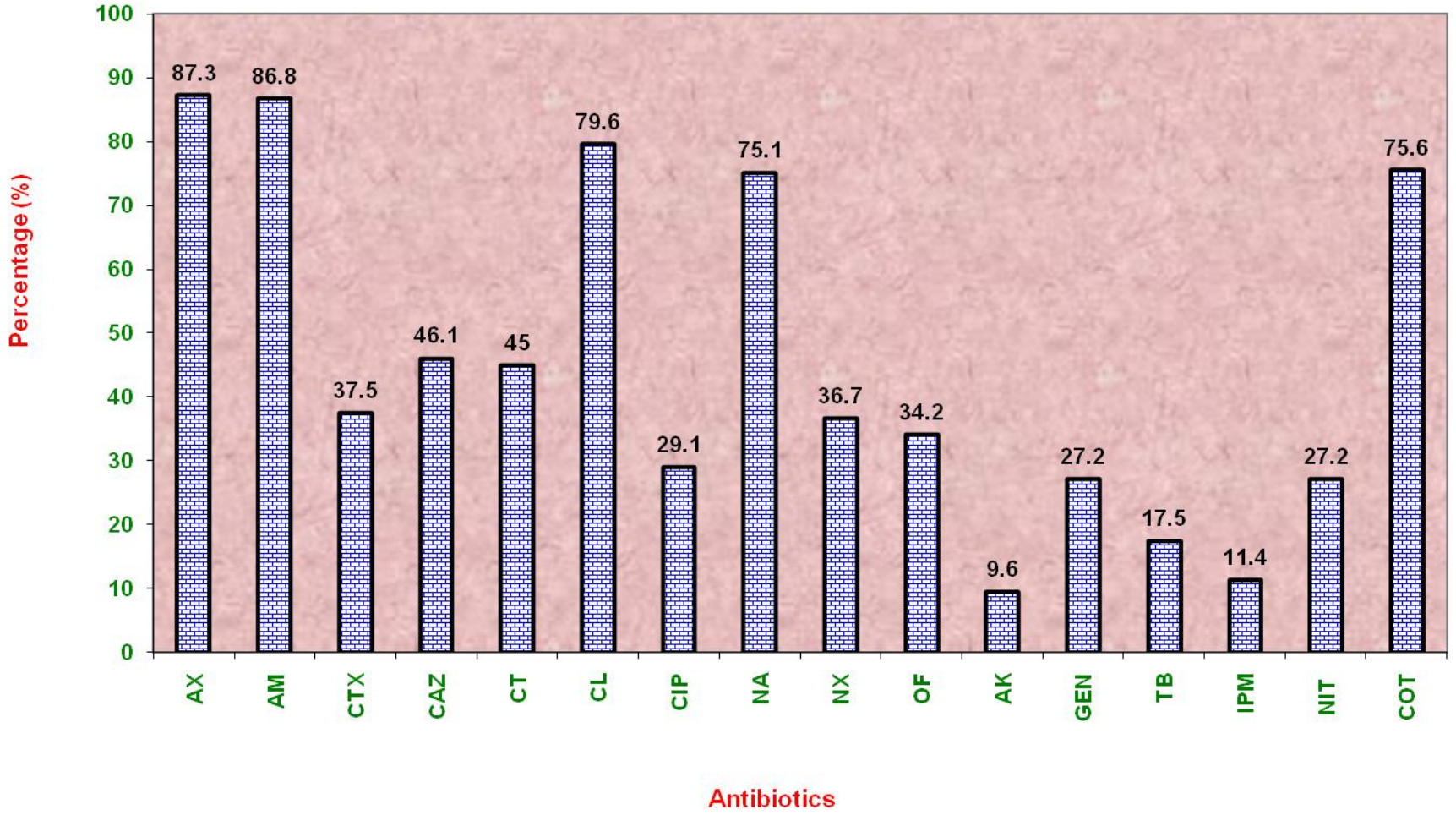
Antibiotic resistance of *S.saprophyticus* from patients with UTIs.



Antibiotic resistance of *S. aureus* from patients with UTIs.

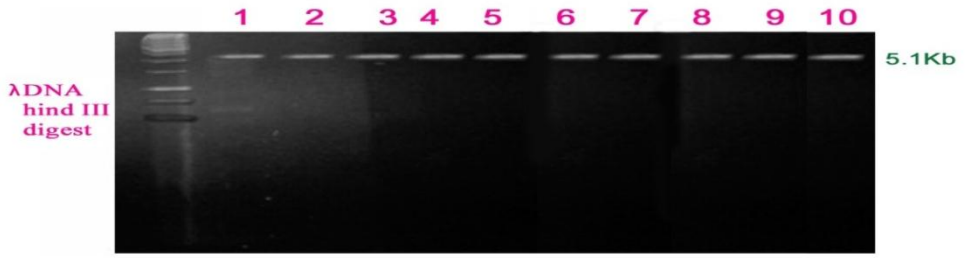


Antibiotic resistant of all bacterial strains isolated from UTIs.

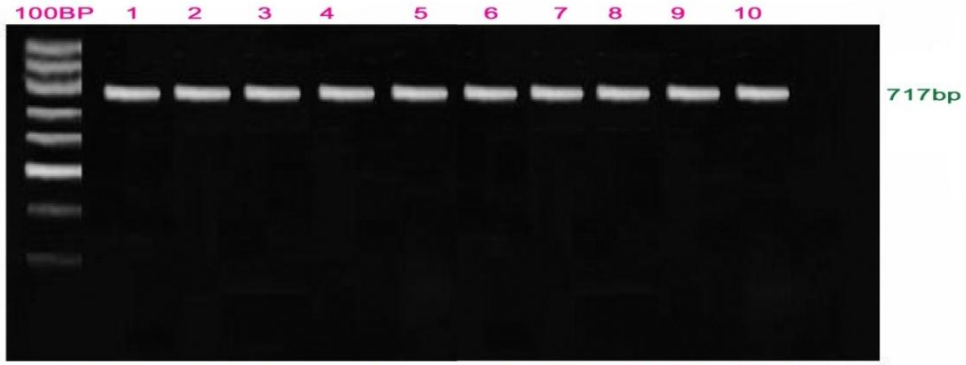


4.4 Plasmid Analysis-Molecular Characterization Of ESBL Producing *E.Coli*

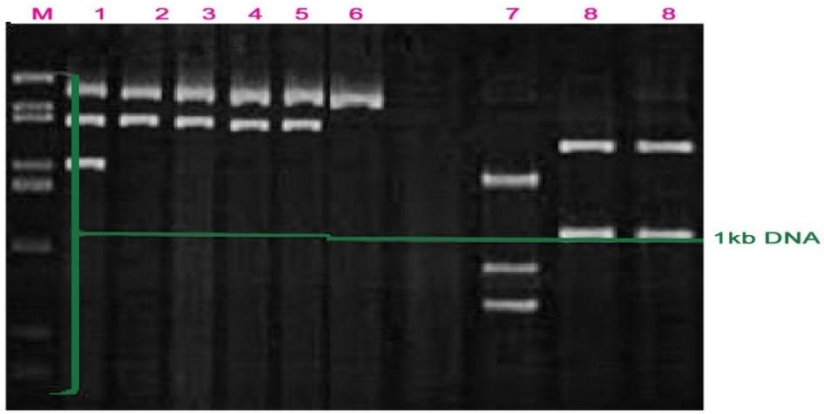
PCR and RFLP analysis



Genomic DNA of ESBL producing E.coli samples



PCR products showing Tem gene of 717bp molecular weight



Digested DNA of E. coli samples

ESBL Positive *E.coli*

Totally 100 MDR *E. coli* isolate were isolated from different source of urine specimens. Ten isolates were confirmed positive for ESBL production. Using the DDST method, ESBL was confirmed in 10 isolates (10%) of which two from cystitis samples (20%), four from pyelonephritis samples (40%) and the rest were normal urine samples (40%).

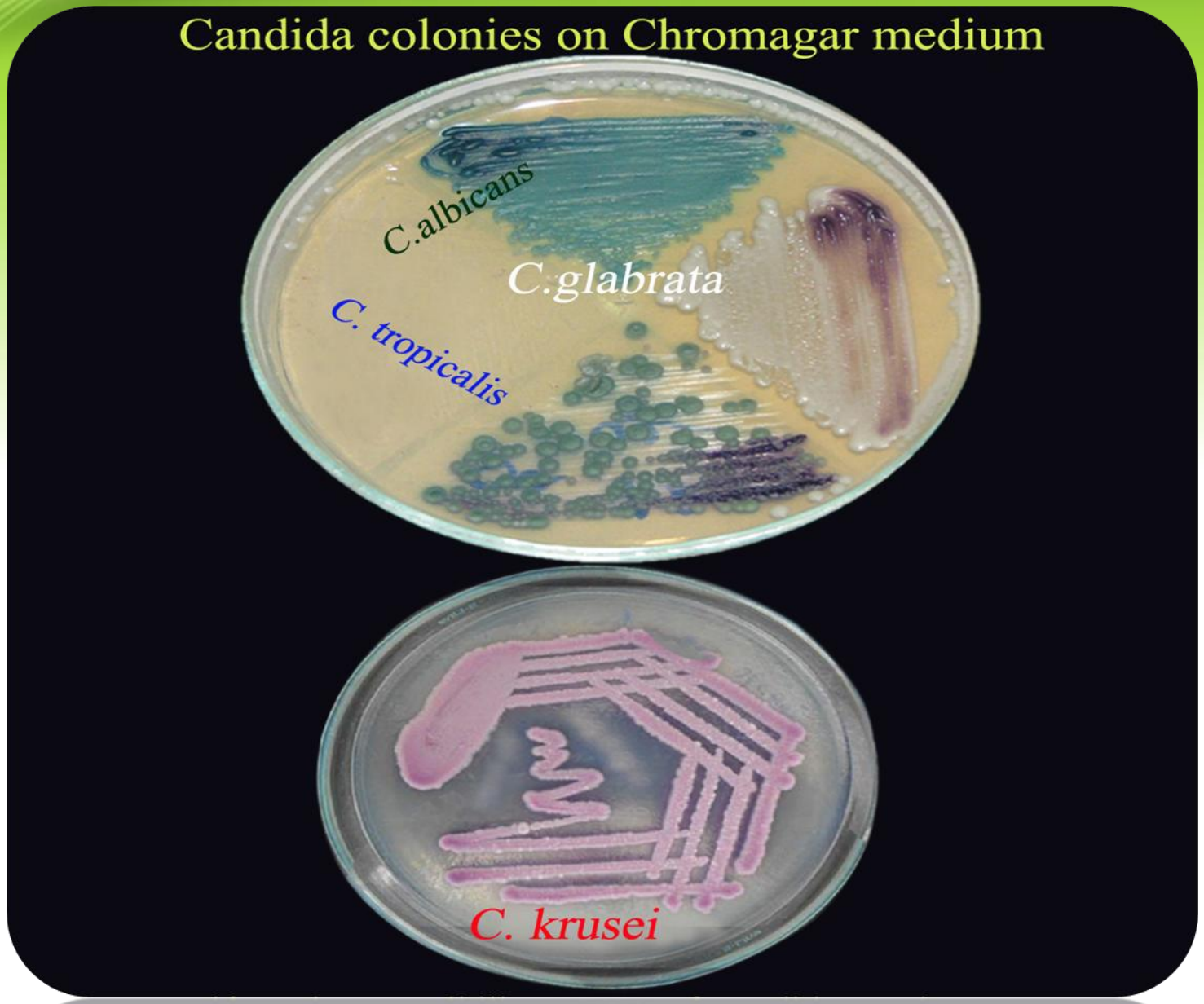
PCR analysis

Confirmed ESBL samples were checked for the presence of TEM (Temorina) gene by PCR amplification and it was found to be positive. PCR products of molecular size 717 bps showed that it was TEM gene and it resulted in 10% of isolates contained bla_{TEM} genes. The results showed that 10% of isolates were positive for ESBL both by **phenotypic** and **genotypic** methods.

RFLP analysis

RFLP analysis showed **eight different** banding patterns for the ten samples among which four samples showed **identical banding** pattern and **other two samples** also had the **similar banding pattern**, indicating same source of origin. Other samples had different patterns indicating that they are not from same samples. Thus ten samples showed eight different banding patterns concludes that the samples are not from same origin.

4.5. ISOLATION AND CHARACTERISATION OF THE UROPATHOGENIC *CANDIDA* SPECIES



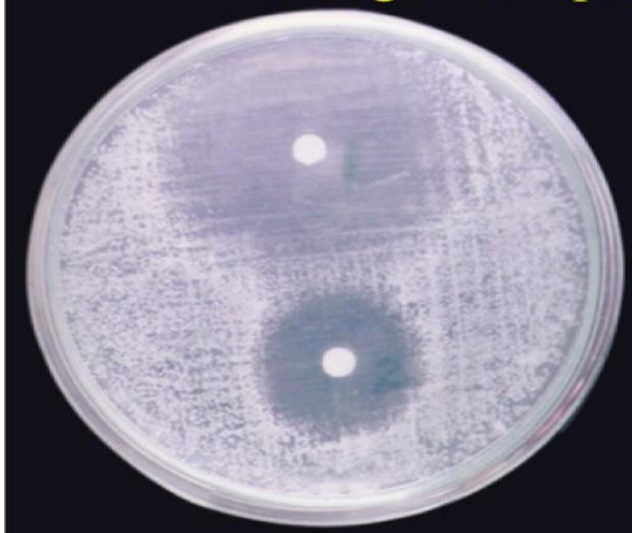
Characteristics of *Candida* species on Chromagar *Candida* media.

Species	Colony characteristics on CHROMagar *Candida*	Total number. of isolates
C. albicans	Apple green colonies; consistent	10 (45.5%)
C. glabrata	White large glossy pale pink to violet colonies	5 (22.7%)
C. tropicalis	Dull blue, to purple color that diffused into surrounding agar with pale pink edges	4 (18.2%)
C. krusei	Large, flat, spreading, pale pink colonies with matt surfaces	3 (13.6%)

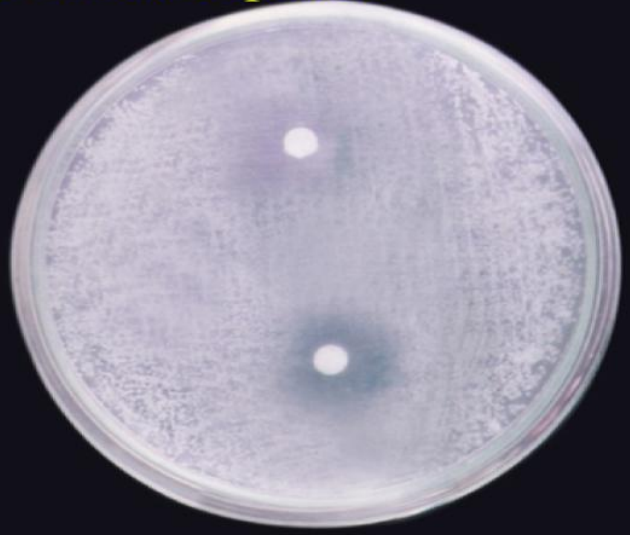
Distribution of isolated *Candida* species (n=22).

Species	No. of organisms	Male	Female
C. albicans	10 (45.5%)	2	8
C. glabrata	5 (22.7%)	1	4
C. tropicalis	4 (18.2%)	0	4
C. krusei	3 (13.6%)	1	2
Total	22 (100%)	4 (18.2%)	**18 (81.8%)**

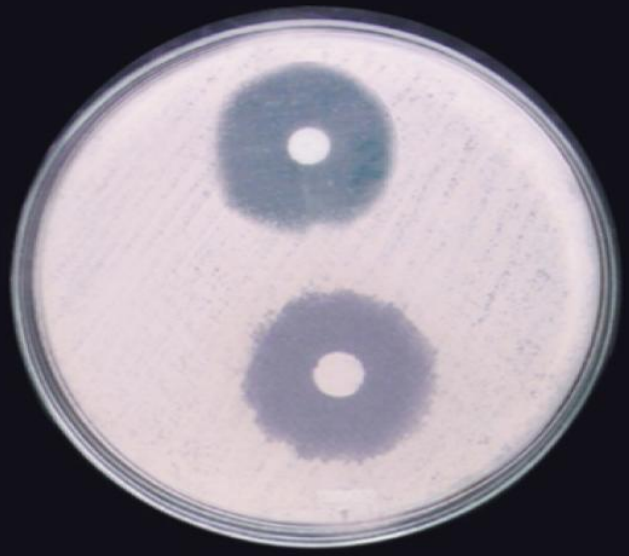
Antifungal susceptibility pattern of Candida species



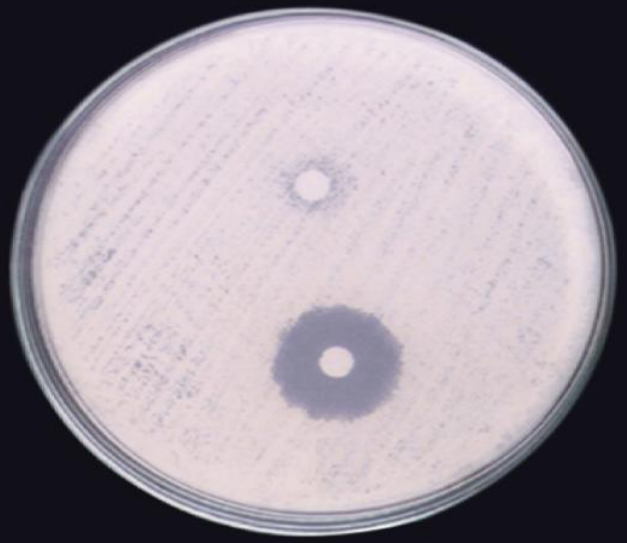
hotericin B -S (≥24 mm), Fluconazole -S (≥18 mm)



Ketoconazole -R (≤8mm), Itraconazole -S (≥14mm)



photericin B -S (≥25 mm), Fluconazole -S (≥24 mm)



Ketoconazole -R (≤8mm), Itraconazole -S (≥20mm)

4.6. ANTIFUNGAL SUSCEPTIBILITY PATTERN OF *CANDIDA* SPECIES CAUSING UTIs

Candida Species	Amphotericin B (20µg)		Fluconazole (25µg)		Itraconazole (50µg)		Ketoconazole (10µg)	
	S	R	S	R	S	R	S	R
C. albicans	10 (100%)	0 (0.0%)	3 (30%)	7 (70%)	10 (100%)	0 (0.0%)	2 (20%)	8 (80%)
C. glabrata	4 (80%)	1 (20%)	1 (20%)	4 (80%)	5 (100%)	0 (0.0%)	2 (40%)	3 (60%)
C. tropicalis	4 (100%	0 (0.0%)	0 (0.0%)	4 (100%)	4 (100%)	0 (0.0%)	0 (0.0%)	4 (100%)
C. krusei	3 (100%)	0 (0.0%)	0 (0.0%)	3 (100%)	2 (66.7%)	1 (33.3%)	0 (0.0%)	3 (100%)
Total	21 (95.5%)	1 (4.5%)	4 (18.2%)	18 (81.8%)	21 (95.5%)	1 (4.5%)	4 (18.2%)	18 (81.8%)

SUMMARY AND CONCLUSION

•Out of the **2400 specimens**, 650 (27.1%) were culture positive and 1750 (72.9%) specimens were negative.

•Major Gram negative isolates were *E.coli* (54.6%); *K. pneumoniae* (11.2%) was the second major isolates and *Pseudomonas* spp., (10.5%) was the third major isolates.

•Overall incidence of UTI in the present study was higher in females (69.8%) than in males (30.2%).

•This study showed that antibiotic like imipenem, amikacin, nitrofurantoin and gentamicin which is still very effective in our environment while conversely an increased resistance to amoxycillin, ampicillin, cotrimoxazole and norfloxacin were observed.

•A total of twenty two numbers of *Candida* species were isolated, of which *C. albicans* showed the highest number of isolates.

•The multidrug resistant ESBL producing *E.coli* isolates were present in these area hospitals with a prevalence of 10%. There were two predominant genes TEM (Temorina) and SHV (Sulfhydryl variant) detected among MDR *E.coli* uropathogenic strains circulating in these area hospitals.

•Hence, our data will help clinician in this region provide safe and effective empiric therapies and could contribute to decrease in emergence of resistance.