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Screening of Drug Resistant Bacterial Pathogens from UTI Patients and Plasmid Curing Analysis in Ampicillin Resistant *Klebsiella sp.*

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ABSTRACT

In the present study 19 urine samples were collected from Rio Lab, Global hospital located in Salem. The samples were serially diluted and 92 different colonies were isolated and identified. The isolates bacterial colonies were tested for its susceptibility to fourteen different antibiotics. Among the 14 antibiotics screened against the 92 isolates three isolates viz., Staphylococcus aureus (30%), Escherichia coli (70%) and Klebsiella sp. (50%) were resistant to erythromycin, amikacin and tetracycline. The concentration at which organism turns into sensitive was tested by increasing the concentration of the antibiotics. The spectrum of antibiotic activities showed that some antibiotics retain their efficiency on bacterial antibiotics such as amoxicillin, nitrofurantoin and ampicillin. The ampicillin resistant Klebsiella sp., was chosen for plasmid curing analysis. Plasmid curing analysis was performed using ethidium bromide at different concentrations viz., 75, 100 and 125 µg/ml to check whether the drug resistance was plasmid mediated. In the present study it was observed that the ampicillin resistant isolate was converted to sensitive after ethidium bromide treatment proving that resistance was plasmid mediated.

Keywords: Antibiotic, Susceptibility, Plasmid curing, Resistance

INTRODUCTION

Urinary tract infections have been described since ancient times with the first documented description in the Ebers Papyrus dated to 1550 BC [1]. It was described by the Egyptians as "sending forth heat from the bladder" [2]. Effective treatment did not occur until the development and availability of antibiotics in the 1930s before which time herbs, bloodletting and rest were recommended. Urinary Tract Infections (UTIs) can be classified by anatomic site of involvement into lower and upper urinary tract infections. UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi.

The most common bacterial infections, affecting 150 million people each year worldwide. In 2007, in the United States alone, there were an estimated 10.5 million office visits for UTI symptoms (constituting 0.9% of all ambulatory visits) and 2-3 million emergency department visits [2-4]. Currently, the societal costs of these infections, including health care costs and time missed from work, are approximately US $ 3.5 billion per year in the United States alone. UTIs are one of the most common bacterial infections affecting humans throughout their life span [3,4]. UTI has become the most common hospital-acquired infection, accounting for as many as 35% of nosocomial infections, and it is the second most common cause of bacteraemia in hospitalized patients [5,6]. UTI also varies based on severity (i.e., complicated versus uncomplicated). Bacterial UTIs typically result from normal skin and gastro intestinal tract flora ascending the urinary tract and overcoming the normal urinary tract defenses that prevent colonization. Patients suffering from a symptomatic UTI are commonly treated with antibiotics; these treatments can result in long-term alteration of the normal microbiota of the vagina and gastrointestinal tract and in the development of multidrug-resistant microorganisms. Quinolones are commonly used to treat UTI due to *E. coli* [7]. These synthetic antimicrobial agents include nalidixic acid and ciprofloxacin which is a Fluoroquinolone (FQ) with a wide spectrum of antibacterial activity *in vitro*, particularly against Gram-negative bacteria. The extensive use of FQ has led to an increasing resistance in *E. coli*. Resistance to quinolone occurs as a result of chromosome and plasmatic mechanisms. Chromosomes-mediated quinolone resistance concerns an accumulation of mutations in the Quinolone Resistance Determining Region (QRDR) primarily in DNA gyrase (gyrA), then in topoisomerase IV (parC). The FDA recommends against the use of fluoroquinolones when other options are available due to higher risks of serious side effect. These medications substantially shorten the time to recovery with all being equally effective [8].

Habeeb et al., [9] investigated the multidrug resistance of *Klebsiella pneumonia in* children below 5 years. The *K. pneumonia* strains isolated

from patient suffering from intestinal and extra-intestinal infection between the 0-5 years' ages of children showed resistant to the three antibiotics and co-exist with non β-lactam resistance. Out of 110 stains only 9 strains produced Extended Spectrum Beta-Lactamases (ESBL). The plasmid responsible for the antibiotic resistance and ESBL production can be transferred to recipient *Escherichia coli* strains.

A study was carried out by Saha et al., [10] where he cultivated, isolated and identified bacterial pathogens causing urinary tract infection, antibiotic sensitivity pattern of isolated bacteria was performed, and detected the ESBLs among isolated organisms. This was a hospital based cross-sectional study in which 260 midstream urine sample were collected from March 2012 to February 2013 from clinically suspected UTI patient of various departments. All the samples were processed as per standard microbiological protocol and antimicrobial susceptibility was assessed by Kirby-Bauer method. Further ESBLs was detected by double disk synergy and combined disk diffusion test. A total of 112 bacterial pathogen were isolated. *E. coli* (48.21%) were, the predominant bacteria followed by *K. pneumoniae* (33.92%). Nitrofurantoin, ofloxacin, amikacin showed good sensitivity, whereas, amoxicillin showed very poor sensitive pattern.

Trevors [11] worked with some plasmid-containing bacteria, it is often based on an evaluation of the microbiological data, clinical outcomes, and Pharmacodynamic-Pharmacokinetic (PK-PD) properties for susceptibility breakpoints, the Clinical and Laboratory Standards Institute (CLSI) performance standards in 2010 revised the interpretive criteria for cephalosporin's and aztreonam. The Minimum Inhibitory Concentration (MIC) breakpoints for cefazolin were revised in 2010: the susceptible breakpoint changed from 8-1 mg/ml, the intermediate breakpoint from 16-2 mg/ml, and the resistant breakpoint from 32-4 mg/ml. Desirable to obtain a plasmid-cured derivative, allowing a direct comparison to be made between the plasmid-containing and plasmid-cured cells. Some plasmids undergo spontaneous segregation and deletion, but the majority is extremely stable and requires the use of curing agents or other procedures to increase the frequency of spontaneous segregation [12].

The focus of the current study is to isolate, identify and determine the antibiotic profile in bacterial pathogens isolated from UTI patients and plasmid curing using ethidium bromide. Firstly the urine sample was collected from Rio Lab, Global hospital located in Salem. Then the isolation and identification of the causative agents were identified. Identification of antimicrobial susceptibility (Antibiogram) of isolated pathogens against different standard antibiotics. The MIC of an antibiotic using broth dilution method is determined by using resistant bacteria. Finally the plasmid curing of ampicillin resistant *Klebsiella* sp. was performed using ethidium bromide [13-15].

MATERIALS AND METHODS

Collection of urine samples

Totally 19 urine samples from UTI Patients (Both men & women) were collected using appropriate sterile containers from Rio Lab, Global hospital located in Salem.

Isolation of bacterial species

Isolation of bacterial species was carried out using spread plate technique. The urine samples were diluted 10^6 fold with sterile water and 0.1 ml of the diluted sample was spread on to nutrient agar containing plates. The bacterial colonies with different colony morphology were sub cultured on nutrient agar plate. The plates were incubated at 37°C for 24 h then used for further studies.

Preliminary identification of the isolates

The preliminary identification of the bacterial isolates was performed by biochemical tests (Catalase, Oxidase, Indole, Trytone, MR Test, VP Test, Citrate utilization test and staining procedures such as Gram staining, motility test) were performed.

Antimicrobial susceptibility (Antibiogram) of isolated pathogens against standard antibiotics

The sterilized Mueller Hinton agar medium was poured into a sterile petri plates. After solidification, a lawn culture of the organism was made using cotton swab and it is allowed to dry for 5 min. The standard antibiotic discs were placed on to the surface of the inoculated agar plates (amoxicillin (AML, 10 µg), ampicillin (AMP, 10 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), trimethoprim+sulphamethazole (SXT, 25 µg), gentamicin (CN, 10 µg), ceftriaxone (CRO, 30 µg), nalidixic acid (NA, 30 µg), and nitrofurantoin (F, 300 µ)) and gently pressed using forceps in order to adhere the discs. Then the plates were incubated at 37°C for 18-24 h. After the incubation period the plates were observed for hollow zone of inhibition that will be noted and tabulated in the results. Muller Hinton agar, beef heart infusion-30.0 g, casein acid hydrogen 17.5 g, starch 1.5 g, agar 20.0 g, distilled water 1000 ml, pH 7.3 ± 0.2

MIC of an antibiotic using broth dilution method is determined by using resistant bacteria

Preparation of antibiotic stock solution

Antibiotic stock solution was prepared using commercially available antimicrobial powders (with given potency). The amount needed and the diluents in which it can be dissolved was calculated using either of the following formulas to determine the amount of antimicrobial powder (1) or dilute (2) needed for a standard solution:

$$\text{Weight (mg)} = \text{Volume (ml)} \cdot \text{Concentration (µg)} / \text{Potency (µg)}$$

$$\text{Volume (ml)} = \text{Weight (mg)} \cdot \text{Potency (µg/mg)} / \text{Concentration (µg/ml)}$$

Antimicrobial agent stock solutions at concentrations of at least 1000 µg/ml (Example: 1280 µg/ml) or 10 times the highest concentration to be tested, whichever is greater was prepared.

Preparation of antibiotic dilution range

Use sterile 13 × 100 mm test tubes to conduct the test. If the tubes are to be saved for later use, be sure they can be frozen. The tubes with loose screw-caps were closed with plastic or metal closure caps, or cotton plugs. The final two fold (or other) dilutions antimicrobial agent was prepared volumetrically in the broth. A minimum final volume of 1 ml of each dilution is needed for the test.

Preparation of inoculums

The inoculums were prepared by making a direct broth suspension of isolated colonies selected from an 18-24 h agar plate. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard. This results in a suspension containing approximately 1-2 × 10^8 CFU/ml for *E. coli*. The inoculums tubes were compared with 0.5 McFarland standards against a card with a white background and contrasting black lines. Optimally within 15 min of preparation, the adjusted inoculums suspension were diluted in broth so, after inoculation, each tube contains approximately 5 × 10^5 CFU/ml. Within 15 min after the inoculums has been standardized as described above, add 1 ml of the adjusted inoculums to each tube containing 1 ml of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and mix. The inoculated tubes were incubated at 35 ± 2°C for 16-20 h in an ambient air incubator. To maintain the same incubation temperature for all cultures, do not stack micro dilution trays more than four high.

Plasmid curing method

Curing of plasmids was performed by the method of Akhter et al. Ethidium bromide with concentrations 75, 100 and 125 µg/ml were used to cure the plasmids. Amoxicillin resistance was used as the selectable marker. Three isolates were selected randomly for plasmid curing which were *Klebsiella* sp. An overnight culture of each test organism in Luria Broth (LB) containing Ampicillin was diluted to 10^4 cells/ml using freshly prepared sterile LB by serial dilution technique. From this diluted culture, 0.5 ml was added with 4.5 ml LB containing different concentrations of curing agents. Thus, the concentration became 10^3 cells/ml. The cultures were then incubated at 37°C in an orbital shaker at 150 rpm for 48 h. After incubation, the broth culture was again diluted to 1.0×10^3 cells/ml with sterile normal saline. Ten ml of the culture was spread on Luria Agar medium. After 24 h incubation at 37°C, the plates were observed for growth. From this plate culture, some well-isolated colonies were randomly selected and simultaneously patched with sterile tooth pick on one Luria Agar medium without antibiotic and another Luria agar containing ampicillin (21 µg/ml), with a numbered grid line attached on the bottom of each plate. After 24 h incubation at 37°C, plates were observed for the cured cells. The cured plasmid cells were detected comparing the development of bacterial colonies on antibiotic containing plate with that of the normal (without antibiotic) plate. The samples that showed colonies on normal LB agar but failed to grow on LB agar supplemented with ampicillin were the possible cured isolates**.**

Characterization

UV Spectrophotometer

The MIC values of all the samples was performed using the UV spectrophotometer Model No. Cyber Lab 100.

RESULTS

Collection of urine sample

In this study, nineteen urine samples were collected from Rio Lab, Global hospital located in Salem. The enumeration of bacterial population was carried out by serial dilution and plating methods.

Isolation of bacterial strains

The 19 collected samples were serially diluted and three different concentrations were selected for subculture of colonies. Specimens with bacterial concentration of $\geq 10^7$ CFU/ml on nutrient agar plates were treated as positive for urinary tract infection. These bacterial strains showing visible growth were further used for analysis.

Preliminary identification of the isolates

The bacterial isolates were considered for the preliminary tests such as Gram's staining, motility. The results of the bacterial isolates were tabulated in Table 1 and shown in Figures 1-3.

Table 1: Morphological and biochemical characteristics of the isolates

Gram's Staining	Motility	Catalase	Oxidase	Organism
Positive cocci	Motile	Positive	Negative	KY1
Negative bacilli	Non Motile	Positive	Negative	KY2
Negative tiny bacilli	Motile	Positive	Negative	KY3

Biochemical identification of the isolates

Various biochemical characteristics of the isolated strains were shown in Table 2. Based on the biochemical tests, the suspected isolates were identified according to the Bergey's manual of systemic bacteriology.

Table 2: Biochemical characteristics of the isolates

Test	KY1	KY2	KY3
Indole test	Negative	Negative	Positive
Methyl red test	Positive	Negative	Positive
Voges Proskauer test	Positive	Positive	Negative
Citrate utilization test	Negative	Negative	Negative
TSI agar	$A/A,H_2S^-,Gas^-$	$A^+/A^+,G^+,H_2S$	$AG^+/AG^+,H_2S^-$

Cultural characteristics of the isolates

Selective Media

Selective medium were prepared, sterilized and inoculated with the respective organisms such as *S. aureus, Klebsiella* sp. *and E. coli* sp. The inoculated plates were incubated at 37°C for 24 h. After incubation, the plates were examined for morphological characteristics. The results were

tabulated in Table 3.

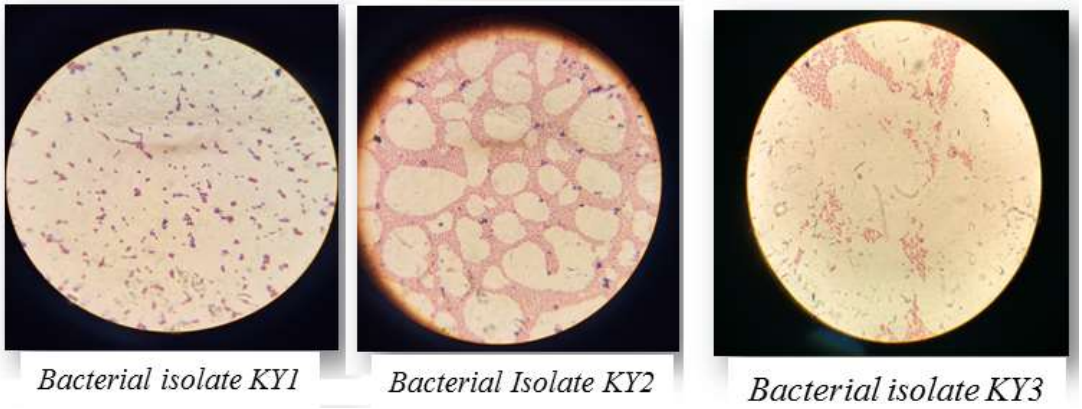

Figure 1: Microscopic appearance of bacterial isolates under light microscope (1000X)

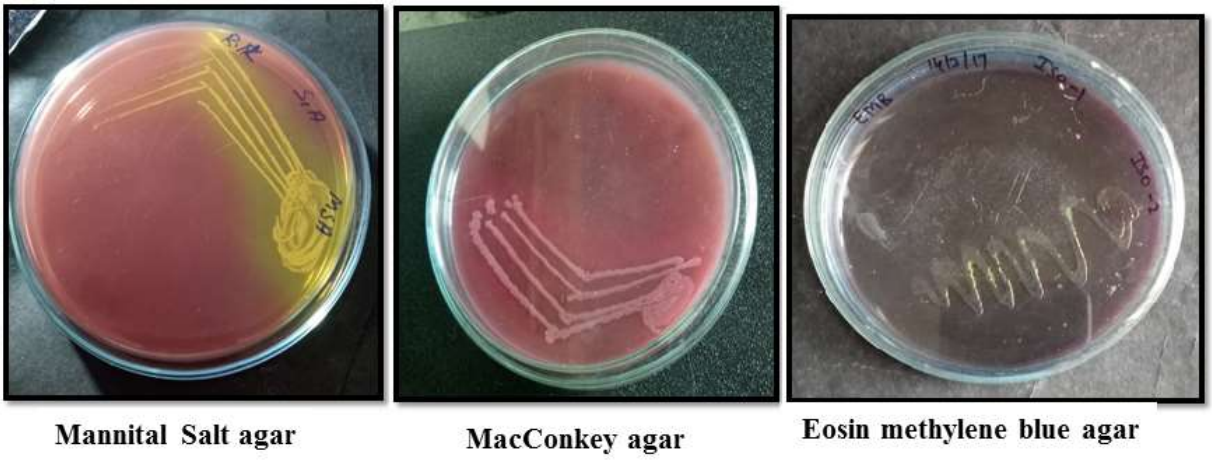

Figure 2: Cultural characteristics of the isolates from bacterial culture

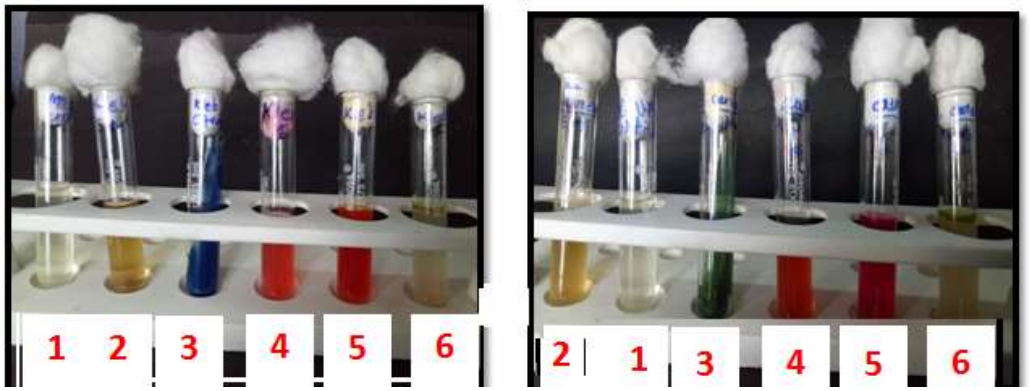

Figure 3: Biochemical tests (1) control, (2) sulfide indole motility, (3) citrate, (4) Voges-Proskauer, (5) methyl red, (6) indole

Table 3: Cultural characteristics of the isolates

S.no	Bacterial isolates	Selective medium	Morphological observation
1	*Staphylococcus aureus*	Mannitol Salt agar	Yellow Zone around colonies
2	*Klebsiella spp*	Mac Conkey agar	Pink colored, lactose fermenting colonies
3	*E. coli*	Eosin methylene blue agar	Blue black metallic sheen colonies

Antibiotic susceptibility test

All the isolated bacterial colonies were tested for antibiotic susceptibility test using fourteen different antibiotics (Table 4). The antibiotics used were amikacin, amoxicillin, ciprofloxacin, co-trimoxazole, erythromycin, gentamycin, levofloxacin, rifampicin and nalidixic acid, nitrofurantoin, piperacillin, rifampicin, streptomycin and tetracycline. Among the 14 antibiotics screened only three bacteria showed resistance, whereas most of the other isolates were sensitive to antibiotic. The resistant organisms were taken for MIC study.

Table 4: Antimicrobial susceptibility of *Staphylococci aureus, Escherichia coli* and *Klebsiella sp*

S. No.	Standard antibiotic	*Staphylococcus aureus* zone of inhibition (mm)		*Escherichia coli* zone of inhibition (mm)		*Klebsiella sp* zone of inhibition (mm)	
		Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
1	Amikacin	-	22-25	09-15	-	08-10	-
2	Amoxyllin	-	25	10	-	-	18-25
3	Ciprofloxacin	-	28-35	-	30	10-12	-
4	Co-Trimoxazole	-	20-40	-	29	-	22-30
5	Erythromycin	15-29	-	08-10	-	-	20-29
6	Gentamycin	-	27	11	-	15	-
7	Levofloxacin	-	27-33	-	29	-	19-25
8	Rifampicin	15-16	-	14	-	-	18-27
9	Nalidic acid	10	-	-	21	-	28
10	Nitrofurantoin	14	-	-	22	-	20-03
11	Piperacillin	-	28	-	23	-	21-27
12	Rifampicin	10	-	11-14	-	-	20-28
13	Streptomycin	-	18-25	-	21	-	23
14	Tetracycline	-	29-31	11-12	-	10-13	-

MIC of antibiotic using broth dilution method

The MIC of all the three antibiotic resistant isolates was performed using different antibiotics such as amoxicillin, erythromycin, nitrofurantoin and ampicillin. The different concentration of antibiotics used for all the isolates were (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 40 128 and 256 µg). On UV spectrophotometer analysis the MIC values of the broth are as follows: *S. aureus*: Amoxicillin 64 µg, erythromycin-resistant up to 256 µg and nitrofurantoin 32 µg, *E. coli*: Amoxicillin-resistant up to 256 µg, erythromycin-resistant up to 256 µg, nitrofurantoin 128 µg, *Klebsiella* sp.: Ampicillin 32 µg. The MIC results showed that three bacteria showed minimum inhibitory concentration at concentration ranging from 30-130 µg. The visible bacterial growth was seen up to 64 µg/ml antibiotic concentration staining from the lowest level of 0.125 µg/ml, since no visible growth of *Staphylococcus* sp., *E. coli* and *Klebsiella* sp culture was seen from 32-128 µg/ml antibiotic containing tubes the MIC of amoxicillin, erythromycin, nitrofurantoin, ampicillin antibiotic was considered to be the lowest inhibitory concentration namely 32 µg/ml for the *S. aureus* and *Klebsiella* sp and 128 µg/ml for *E. coli*.

Plasmid curing

The Plasmid curing was carried out using Ethidium bromide with *Klebsiella* sp. which showed a MIC value of 32 µg/ml for ampicillin. The broth containing both Antibiotic and Ethidium bromide with concentration 75, 100 and 125 µg/ml, were kept in orbital shaker on 150 rpm for 48 h. The grown colonies were serially diluted to 10^4 cells and plated on LB Agar plates with and without antibiotic. No colonies were grown in plates with antibiotics whereas plates without antibiotics showed growth. It was observed that the ampicillin resistance in *Klebsiella* sp. was plasmid mediated and not chromosomal mediated.

DISCUSSION

The current study is focused on the screening of urinary tract infections for antibiotic susceptibility to screen the resistant strains and their MIC. We have also analyzed the highly resistant strains for their plasmid curing ability using ethidium bromide at varying concentrations. The antimicrobial resistance profile of isolates in our study revealed a higher resistance rate than reported in European studies. Penicillin's study exhibited a resistance rate higher than cephalosporin, especially those of the third generation. We observed that resistance rates of *E. coli* from UTI to amoxicillin were the highest (85%). Such a strong resistance was observed worldwide. This rate rose from 67% in 2000-91% in 2010. With *E. coli* strains, the rate of resistance increased from 62.5% in 2000-88.6% in 2010. Similar trend had also been observed in Dakar, Senegal. All the isolated bacterial colonies were tested for antibiotic susceptibility test using fourteen different antibiotics. The antibiotics used were amikacin, amoxicillin, ciprofloxacin, Cotrimoxazole, erythromycin, gentamycin, levofloxacin, rifampicin, and nalidic acid, nitrofurantoin, piperacillin, rifampicin, streptomycin and tetracycline. Among the 14 antibiotics screened only three bacteria showed resistance, whereas most of the other isolates were sensitive to antibiotic. The resistant organisms were taken for MIC study. Plasmid cured cells were achieved with 100 and 125 µg/ml ethidium bromide 10% w/v SDS and 50 and 75 µg/ml acridine orange although the frequencies of cured colonies were quite low. The results from Tables 1-3 show that amongst the three curing agents, ethidium bromide was able to cure plasmids successfully at a higher rate than the other two agents. The frequencies of cured cells were 5.55% (with 50 µg/ml) and 11.76% (with 75 µg/ml) for acridine orange, 21.05% (with 100 µg/ ml), 17.65% (with 125 µg/ml) for ethidium bromide and 7.4% (with 10% w/v) and 6.67% (with 10% w/v) for sodium dodecyl sulfate. On the other hand, no cured cells were obtained from 100 µg/ml acridine orange, 75 µg/ml ethidium bromide and 8 and 12% SDS.

CONCLUSION

Our current study had given an overview of the common urinary tract infection causing bacteria found in the urine samples of affected patients. The spectrum of antibiotics activities showed that some antibiotics retain their efficiency on urinary tract infections such as amoxicillin, nitrofurantoin and ampicillin. The isolated Bacterial colonies showed resistance towards some antibiotics but it was sensitive to many antibiotics. They cause this disease either by chromosome mediated or by plasmid mediated mechanism. Plasmid mediated resistance that can be transferred between cells enable rapid spread of the disease. The present study investigated the efficiencies of different curing agents on the *E. coli* isolates from UTI specimen. For this purpose, ethidium bromide was used as curing agents and the results obtained was a preliminary indication of association of drug resistance of the clinical isolates of *Klebsiella* sp. with plasmids.

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