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Plasmid curing pattern of nosocomial pathogens of intensive care units (ICU) in Zagazig University Hospital

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ABSTRACT

Nosocomial infections and antibiotic resistance are serious and growing phenomenon in contemporary medicine and has emerged as one of the public health concerns. A total of seventy isolates of bacteria were collected from patients. The selected isolates include 33 (56.9%) from males and 25 (43.1%) from females.All bacteria were susceptible to imipenem followed by amikacin, ceftazidime, ciprofloxacin and tobramycin, respectively.The multi-drug resistant (MDR) bacterial isolates (33 isolates) divided into four groups named *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *and Staphylococcus aureus* were selected to be identified according to the keys of different identification protocols. *Escherichia coli* found to be the most frequent pathogen within MDR isolates followed b*y Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiellapneumonia*, respectively. The MICs and MBCs of (Imipenem,Amikacin,Ceftazidime and ciprofloxacin) against the most resistant isolates were determined. Generally,*P. aeruginosa* (Strain No. 55) was found to be the highest resistant bacteria. Plasmid profile of *Pseudomonas aeruginosa* was carried out, after incubation at 37°c for 24hr recorded no changes in plasmid and compared with incubation at 43°c (plasmid curing) which showed complete removal of plasmid bands and the tested bacteria became more sensitive to antibiotics. This study therefore determined the prevalence, anti-biotic susceptibility and plasmid patterns of P.aeruginosa strains from clinical specimens obtained from the intensive care units of Zagazig University Hospital in Egypt.

Keywords: Plasmid curing, Nosocomial infection, antibiotic susceptibility, minimum inhibitory concentration, minimum bactericidal concentration.

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INTRODUCTION

Nosocomial infections are a leading cause of morbidity and mortality among hospitalized patients. These infections involve 5% to 15% of hospitalized patients and can lead to complications in 25% to 50% of those admitted to intensive care units (ICUs) [1].

Pathogenic organisms that cause nosocomial infections can be transmitted to the community in a number of different ways and particularly through health staff, visitors and discharged patients. The characteristics of the micro-organisms including their level of antibiotic resistance, innate virulence and patient susceptibility were studied by [2].

Antibiotic resistance is a global problem, but poor and developing nations are more affected due to the fact that antibiotics can be obtained easily over the counter without doctors' prescriptions, whereas in the developed countries access to antibiotics is much more limited [3,4].

The main factors that lead to microbial resistance and the spread of multi-drug resistance organisms are thought to be the misuse of antibiotics by healthcare workers. The term multi-drug resistance (MDR) is used to describe bacteria that are able to resist one or more antibiotics in three or more antibiotic classes, while bacterial strains that are resistant to all antibiotics are termed as extreme drug resistant strains [5].

Multidrug resistant *Enterobacteriaceae* have become an important cause of urinary tract and blood stream infections within the community setting [6]. *Pseudomonas aeruginosa* is a major nosocomial pathogen worldwide, intrinsically resistant to many drugs. There are many challenges in the treatment of (MDR*) P. aeruginosa*, especially considering the absence of new antimicrobials in the drug-development pipeline [7].

Plasmid is extra chromosomal DNA present in the cytoplasm of the cell, double stranded, circular DNA, self reproducing, many plasmids are transferred among bacterial cells such plasmids can lead to dissemination throughout a population, frequently containing genes that convey antibiotic resistance or toxin production [8]. The transfer of resistance genes by plasmids is more effective than chromosomal mutation [9].

The present work was aim to Studying the susceptibility and resistance of isolated bacteria to different antibiotics and determination of MICs and MBCs of some antibiotics against multi-resistant isolates and studying the analysis of plasmid profile for multi-drug resistant isolate.

MATERIALS AND METHODS

Samples Collection:

Seventy isolates of bacteria were collected from Zagazig University hospital's intensive care units (ICUs). The samples were collected from Patients, tools, air, floor, wall and nursery incubators. The patient data (Age, Gender, Type of infection and drugs) were collected. The specimens were collected and transported according to **[10]** under aseptic conditions quickly to the Microbiology Laboratory at Faculty of Science Zagazig University where the study was carried out.

Isolation, Identification and Characterization of bacterial isolates:

Bacterial isolates were isolated and streaked for several consecutive times on nutrient agar medium until pure single colonies were obtained. Preliminary identification of bacteria was based on colonial morphology of the organisms such as hemolysis on blood agar, changes in physical appearance in differential media and enzyme activities of the organisms. Biochemical tests were performed on colonies from primary cultures for identification and characterization of the isolates.

The purified cultures of the selected multi-drug resistant isolates were identified and confirmed after investigating morphological cultural characters and biochemical tests according to standard clinical laboratory methods reported and recommended by Bergey's Manual of determinative bacteriology [11,12] and others [13,14].

Antibiotic susceptibility tests:

Ten of different antibiotics were selected based on the pharmacological action and what antibiotic targets in bacterial cells and each one of them representing different group for carrying out the antimicrobial susceptibility test. The antibiotic discs used in this research were purchased from Oxoid Ltd., England. The name of antibiotic discs, the code, the potency and the standard evaluation of inhibition zones were tested according to [15,16].

Antibiotic susceptibility test for the bacterial isolates was carried out by Kirby Bauer disc diffusion technique according to [17]. Mueller-Hinton agar was used for testing the sensitivity of the experimental isolates to the different antibiotic [18].The appropriate antibiotics were placed in the agar aseptically using sterile forceps the plates were then incubated at 37°C for 24hours.The antibiotics discs used in this study were Amikacin ,30μg; Ceftazidime,30μg; Cefuroxime, 30μg; Ciprofloxacin, 5μg; Gentamycin, 10μg; Imipenem, 10μg;Nitrofurantoin, 300 μg; Oxacillin, 1 μg; Sulphamethoxazole/trimethoprim, 25μg; Tobramycin, 10μg.

Determination of the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of selected antibiotics against selected isolates:

Four antibiotics were used; Imipenem (a member of Carbapenems family), Amikacin (a member of Aminoglycosides family), Ceftazidime (a member of Cephalosporins family) and Oxacillin (a member of Penicillins family) against the most resistantisolates:*E. coli* (59)*, K. pneumoniae*(42), *P. aeruginosa*(55) *and S. aureus*(17). Different antibiotics groups showed that the tested isolates were highly susceptible to imipenem with susceptibility percentage (54.3%) so that it represented the most effective antibiotic followed by amikacin, ceftazidime and ciprofloxacin with 48.6%, 44.3% and 44.3% susceptibility,

The MICs and MBCs were determined by using the standard broth dilution technique according to [19,20]. The stock solution of the antibiotics was diluted with sterile nutrient broth (double strength) to obtain an appropriate dilution needed for each experiment (as micrograms per milliliter).

In Wassermann tubes twofold serial dilutions of one of the antibiotics were made from the diluted stock solution using broth as diluents. To each tube 0.5 ml nutrient broth containing tested organism (24 h. ages) was added. A control tube for each group of tests was un-inoculated medium with and without antibiotic was included to ensure sterility and clarity of the medium. A third control tube containing inoculated medium but without antibiotic was also included to ensure the ability of the organism to grow in the medium. All the tubes were incubated at 37°C for 24 h. and examined for turbidity as an indicator of bacterial growth.

The minimum inhibitory concentration (MIC) is defined as the lowest concentration that inhibits a visible growth in liquid media**.** One hundred micro liters (μl) were taken from each MIC concentration and introduced onto nutrient agar to determine the MBC. The plates were incubated at 37°C for 24 h. The minimum bactericidal concentration (MBC) is the lowest concentration at which no growth occurs on solid media.

Plasmid curing:

Plasmids can be eliminated (cured) from bacteria by several chemical and physical agents.

Plasmid isolation and sample preparation:

Plasmid DNA was extracted using Bacterial Total DNA Isolation by Alkaline Method Kit [21]. In an eppendorf, 1.5 ml from overnight culture were taken, centrifuged at 8,000 x g for 1 min, pellet was kept and 25 μl of solution A was added, mixed by automatic pipette up and dawn. Then 250 μl of solution B was added and mixed by moving up and down three times. Then 250 μl of solution C was added and centrifuged at 13,000 x g for 5 min. Finally, the upper phase was removed into new eppendorf. After extraction of the DNA samples, an appropriate amount was transferred (about 25 μl) of each sample to a fresh eppendorf and 5μl of loading buffer was added.

Samples loading and running:

The first well was loaded with DNA marker. Samples were loaded from right to left, and the gel chamber was closed. The power-source switched on 5V/cm. The run was monitored by reference to the marker dye. Finally, the run was stopped when the bromophenol blue had reached 3/4 the length of the gel.

Staining and examination:

After removing the gel from the chamber, the gel was placed in the ethidium bromide solution inside dark container, leaved for about 30 min then washed by placing the gel in another container having 1xTAE buffer for about 20 min, the gel was examined by UV Trans-illuminator, and photographed

Plasmid curing by elevated temperature:

Bacterial growth in with 10^4 cell/ml nutrient broth was grown to late phase at an elevated temperature 43°C (5-7 °C above the normal growth temperature **,** the culture was diluted 1:20 by taking 0.5 ml of the bacterial growth into 10 ml of nutrient broth and the culture was grown at 43 °C for 24 hour. Two drops of the treated culture were placed on nutrient agar plates to obtain separate colonies, individual colonies were tested for loss plasmid-coded functions (streptomycin and tetracyline resistance) [22].

Antibiotic sensitivity after plasmid curing:

Antibiotic sensitivity tests of the isolated strains after plasmid curing were done by the disc diffusion method with the same antibiotics and the same concentrations as before plasmid curing. The sensitivity of the bacterial organisms to different antibiotics discs were measured according to [23].

RESULTS and DISCUSSION

Nosocomial infections are frequent complications of hospitalizations. In this study seventy isolates of bacteria were collected from patients at Zagazig University hospital's intensive care units (ICUs). The selected isolates include 33 (56.9%) isolates from males and 25 (43.1%) isolates from females whose ages ranged between 25 to 85 years. [24], were reported a significant relationship between age and nosocomial infections.

Antibiotic susceptibility tests:

The susceptibility of the different bacterial isolates which isolated from the tested clinical specimens towards ten of different antibiotics by using a standardized disc diffusion method was carried out. The results in Table (1) and Figure (1) revealed that the tested isolates were highly susceptible to imipenem with susceptibility percentage (54.3%) so that it represented the most effective antibiotic followed by amikacin, ceftazidime, ciprofloxacin and tobramycin with 48.6%, 44.3%, 44.3% and 42.9% susceptibility, respectively. On the other hand the data showed that 61.4% of bacterial isolates were resistant to nitrofurantoin while 55.7% and 52.9% of bacterial isolates were resistant to sulphamethoxazole/trimethoprim and cefuroxime, respectively. These results are in agreement with [25] they reported that maximum sensitivity was seen for imipenem (82%), followed by piperacillin + tazobactam (76%), amikacin (71%), ciprofloxacin (63%), gentamycin (58%), and ofloxacin (63%). High resistance was identified with co-trimoxazole (54%), ceftriaxone (66%), and doxycycline (85%). [26] showed that the susceptibility rate of clinical isolates was the highest for amikacin (87.2%), followed by ciprofloxacin (74.8%), ceftazidime (71.5%) and gentamicin (70.4%). Generally, the tested isolates showed high resistance to β-lactam antibiotics. This is agree with [27] who found that all 94 strains of *P. aeruginosa* and *E. coli* tested isolates were resistant to at least 7 β-lactam antibiotics

Table (1): Comparative susceptibility of bacterial isolates against different antibiotics

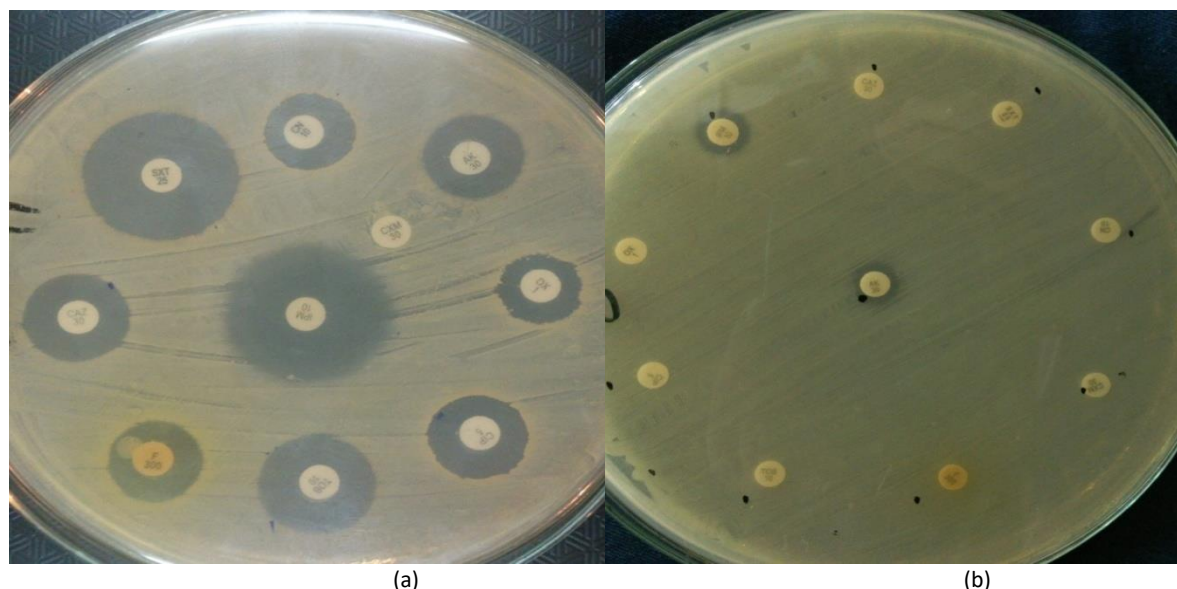| Antibiotic	Symbol	Conc. µg/disc	Resistant (R)		Intermediate (I)		Susceptible (S)	
			No.	%	No.	%	No.	%
Imipenem	IPM	10	27	38.5	5	7.1	38	54.3
Amikacin	AK	30	28	40	8	11.4	34	48.6
Ceftazidime	CAZ	30	35	50	4	5.7	31	44.3
Ciprofloxacin	CIP	5	36	51.4	3	4.3	31	44.3
Tobramycin	TOB	10	36	51.4	4	5.7	30	42.9
Oxacillin	OX	1	30	42.9	11	15.7	29	41.4
Gentamycin	CN	10	37	52.9	4	5.7	29	41.4
Cefuroxime	CXM	30	34	48.6	9	12.9	27	38.5
Sulphamethoxazole /trimethoprim	SXT	25	39	55.7	6	8.6	25	35.7
Nitrofurantoin	F	300	43	61.4	6	8.6	21	30

(a)   (b)

Figure (1): Antibiotic susceptibility test (a), sensitive isolate and (b), resistant isolate.

Frequency of different bacterial groups within multi-drug resistant bacterial isolates:

In our study (table 2) ,the multi-drug resistant (MDR) bacterial isolates (33) isolates were distributed as 23 gram negative bacterial isolates (69.7%) and 10 gram positive bacterial isolates (30.3%), and divided into four groups namely, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Staphylococcus aureus*. *Escherichia coli* was the most frequent pathogen within MDR isolates representing 36.4% of MDR isolates followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa* with frequency percentage 30.3% and 21.2%, respectively. On the other hand*Klebsiellapneumoniae* was the less frequent pathogen within MDR isolates (12.1%). [28] found that the microorganisms most commonly isolated from clinical specimens were *E. coli* (28%), *S. aureus*(11.11%) and *P .aeruginosa* (8.6%). [29] reported that the most causative agents was *E. coli* (87%of cases) followed by *K. pneumonia* (10%). But in other study nosocomial infections were most frequently caused by *Acinetobacter* (34.5%), followed by *Pseudomonas* (32.8%), *Klebsiella* (13.9%), *E. coli* (12.1%) and *Citrobacter* (5%) [30]

Escherichia coli infection is one of the major public health problems in many developing countries and has contributed exceedingly to morbidity, mortality and increased health costs [31]. *K.pneumoniae* pneumonae causes a severe, rapid-onset illness that often causes areas of destruction in the lung and causes

less serious respiratory infections, such as bronchitis, which is usually a hospital-acquired infection [32]. Staphylococcus *aureus* is well documented as a human opportunistic pathogen and one of the most frequently identified pathogens in clinical laboratories [33]. *S. aureus* is notorious for its ability to develop broad antibiotic resistance. The most commonly known resistance of *S. aureus* is (MRSA), the new strain resistant to vancomycin, (VRSA), this organism poses a major threat to human health globally [34].

Pseudomonas aeruginosa is identified as one of the most common pathogen causing hospital acquired infections [35].

Table (2): Frequency of different bacterial groups within multi-drug resistant bacterial isolates

Group no.	Bacterial species	Isolates no.	Total no.	Percentage (%)
I	*Escherichia coli*	2,8,9,11,15,20,23,29,35,38,48 and 59	12	36.4
II	*Staphylococcus aureus*	1,10,16,17,25,37,40,53,65 and 68	10	30.3
III	*Pseudomonas aeruginosa*	18,21,27,28,49,55 and 63	7	21.2
IV	*Klebsiella pneumoniae*	22,24,42 and 70	4	12.1
Total			33	100

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of most effective antibiotics against the most resistant strains:

Four antibiotics (the most effective antibiotics, especially, on gram negative bacteria) were used; imipenem (a member of carbapenems family), amikacin (a member of aminoglycosides family),ceftazidime (a member of cephalosporins family) and ciprofloxacin (a member of fluoroquinolones family) against the most resistant isolates : *E. coli* (59)*, K. pneumonia* (42), *P. aeruginosa* (55) *and S. aureus* (17) .

Values of MICs and MBCs were determined by using the standard broth dilution technique and were shown in table (3), The highest MIC and MBC were observed in ciprofloxacin antibiotic against *P. aeruginosa* (55)(250µg/ml), followed by *E. coli* (59)(131.69 and 197.53µg/ml, respectively). Meanwhile, the lowest MIC and MBC were observed in imipenem and amikacin antibiotics for *S. aureus* (17)**.**

Table (3): The (MICs) and (MBCs) of most effective antibiotics against the most resistantstrains

bacterial isolate	Imipenem (IPM)		Amikacin (AK)		Ceftazidime (CAZ)		Ciprofloxacin (CIP)	
	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
E. coli (59)	62.5	125	87.791	131.687	125	197.53	131.69	197.53
K. pneumoniae (42)	39.018	39.018	62.5	87.791	62.5	125	87.791	125
P. aeruginosa (55)	58.527	87.791	125	125	125	250	250	250
S. aureus (17)	7.813	15.625	15.625	26.012	26.012	31.25	39.018	58.527

Gram negative bacteria were more resistant than gram positive ones, so we used higher concentrations of antibiotics for inhibiting the growth (MIC) or killing (MBC) gram negative bacteria more than that used with gram positive. Most studies show that the high prevalence of gram-negative bacteria isolated from patients in ICUs is associated with a high rate of mortality [36]. Our study documented on the antibiotic resistance develops when microorganisms are exposed to effective doses of an antibiotic within a shorter period or when the microorganisms are exposed to smaller concentrations or residues of the antibiotic over a longer period of time [37].

Plasmid curing

Isolation of plasmid DNA profile of most clinical bacteria multiresistant to antibiotics:

Plasmid is extra chromosomal DNA present in the cytoplasm of the cell, double stranded, circular DNA, self reproducing, many plasmids are transferred among bacterial cells such plasmids can lead to dissemination throughout a population, frequently containing genes that convey antibiotic resistance or toxin production [38].

In our study the plasmid profile of the most multi resistant clinical isolate, *Pseudomonas aeruginosa (55)* were carried out to detect plasmid bands and its molecular weight. Plasmid DNA isolation from our isolate showed that after incubation at 37°c for 24hr harboring a plasmid which recorded no change in plasmid. The DNA profile at lane 3&4 showed three bands at 23.130bp, 1221bp and 72bp compared with tested bacteria incubated at 43°c which showed complete removal of plasmid bands and the tested bacteria become more sensitive to antibiotics.

The obtained results in figure (2) clearly indicated complete curing of plasmid at lane 1 and lane 2, compared with the same tested bacteria incubated at 37°c.
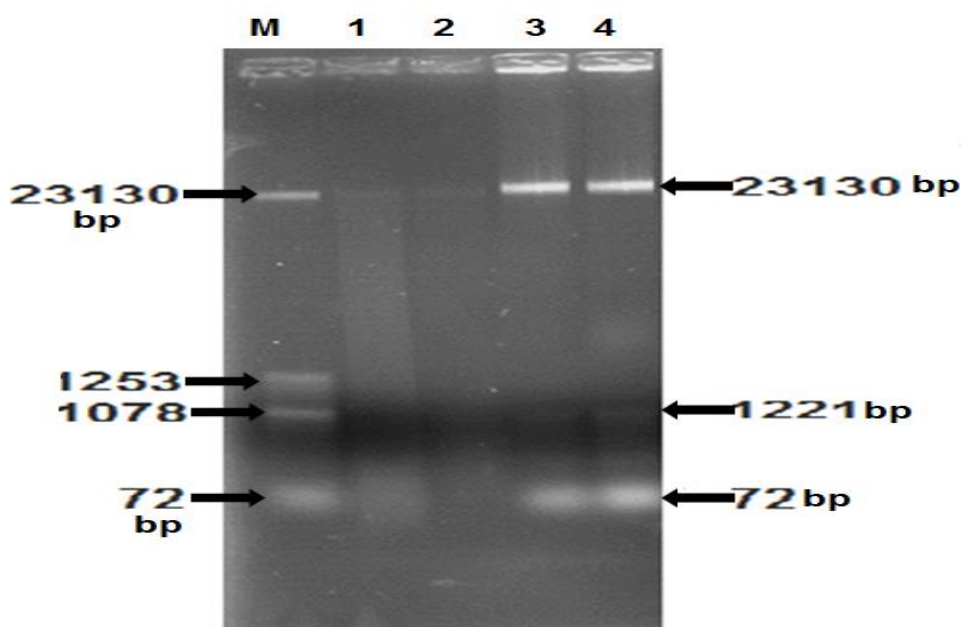

Figure (2): plasmid profile of the most resistant clinical isolate. Lane1&2 : treated bacteria at 43°c Lane3&4 : treated bacteria at 37°c

Some of the resistant determinant are part of the transposable DNA sequences that can even the whole plasmids could also be stably integrated into the undergo translocation to multiple chromosomal and plasmid sites and chromosome [39].

On the other hand, plasmid DNA profile can be used as an epidemiological marker, but in combination with other typing methods and with antibiotic resistance patterns to characterize the circulation of *P. aeruginosa*strains and the spread of resistance in thesebacteria [40]. Plasmids can be spontaneously lost from or readily acquired by a host strain as they are mobile, extra chromosomal elements and not part of the chromosomal genotype that defines the host strain. In addition, there were correlation existed between the antibiotic susceptibility pattern of the most resistant strains and their plasmid profiles [41].

Some antibiotic resistance genes were found on non conjugative plasmids, these genes may also move from one plasmid to another [42]. The unstable nature of plasmids that can spread even to multiple species of bacteria and may be lost or acquired spontaneously [43], make plasmid fingerprinting, which was

the first molecular typing method to be used for epidemiological purposes often poorly reproducible [44]. The emergence and spread of plasmid-mediated ß-lactamases such as extended-spectrum ß-lactamases (ESBLs), and other inhibitor-resistant derivatives have been potentiated by the use and over-use of broad-spectrum ß-lactam antibiotics. The genes encoding these ß-lactamases are3 often located on large plasmids that also encode genes for resistance to other antibiotics, including aminoglycosides, tetracycline, sulfonamides, trimethoprim and chloramphenicol)[45].

Antibiotic sensitivity after plasmid curing

The sensitivity test for Imipenem, amikacin, ciprofloxacin and ceftazidime, were tested against clinical *Pseudomonas aeur*oginosa before and after plasmid curing and the obtained results illustrated in figure (3.a & 3.B). These results clearly demonstrated that the sensitivity of tested *Pseudomonas aeur*oginosa increasing at degree of inhibition zone formed if compared with non cured plasmid and the highest inhibition zone were obtained with ciprofloxacin followed by ceftazidime.after plasmid curing and the diameter of inhibition zone differs according to antibiotics tested.
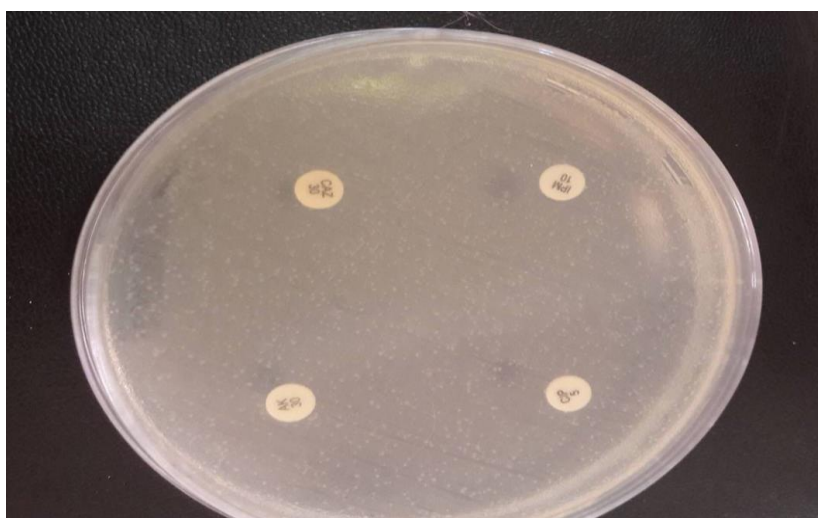

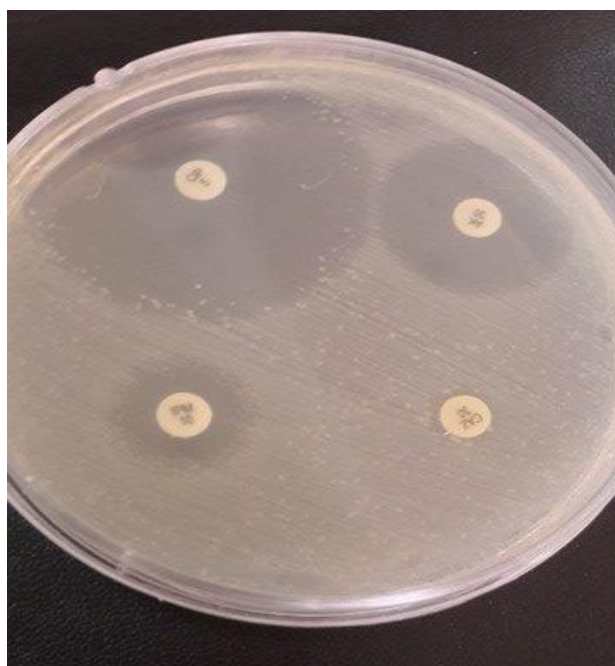**Figure (3.A) Antibiotic sensitivity test for *Pseudomonas aeur*oginosabefore plasmid curing**

Figure (3.B) Antibiotic sensitivity test for *Pseudomonas aeur*oginosa after plasmid curing

CONCLUSION

Since it is evident and established that some of the isolates from hospitals in Egypt, could pose a serious health hazard. Their occurrence and prevalence could be attributed to poor hand hygiene, cross contamination between the patients and hospital personnel, misuse of drug can lead to multidrug resistance mediated by plasmid. Thus indicating the most resistance to antibiotics is plasmid-mediated, this can easily be transferred from one strain to another or from one organism to another within the same environment. Application of strict prevention strategies including changes in antibiotic treatment regimens, hygiene measures, infection prevention and control of horizontal nosocomial transmission of organisms is desirable. On the whole, principle of antimicrobial stewardship is urgently needed to preserve efficacy of available antimicrobial agents.

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