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Susceptibility and Resistance for one of the Enterobacteriaceae family to antibiotics according to the data of CLSI 2023

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Abstract :

The study aimed to determine the resistance of *Klebsiella pneumoniae* bacteria isolated from urinary tract infections to different types of antibiotics and to compare it with a previous study to determine the percentages of difference, similarity, or changes occurring in the sensitivity and resistance of bacteria to these antibiotics, by isolating and diagnosing the bacteria and examining the sensitivity and resistance of bacteria to a group. Selected from different antibiotics.The study included collecting (30) pathological bacterial samples from patients with urinary tract infections from various hospitals in Baghdad. *Klebsiella pneumoniae* isolates were diagnosed using microscopic and biochemical methods, and 20 *K. pneumoniae* samples were confirmed. In the accurate diagnostic examination using the polymerase chain reaction (PCR) technique, the sensitivity and resistance of the diagnosed bacterial isolates to 8 antibiotics from different hosts were tested using the disc distribution method. The results showed that there was a variation in the rates of sensitivity and resistance of *K. pneumoniae* isolates, as the highest resistance to Ampicillin reached 90% (18 isolates), a sensitivity of 5% (1 isolate), and an intermediate 5% (1 isolate). On the other hand, the least resistance to Levofloxacin was obtained by 25% (5 isolates), with a sensitivity of 45% (9 isolates), and intermediate of 30% (6 isolates).

And the resistance of bacteria to the antibiotic Cefoxitin reached 60% (12 isolates), with a sensitivity rate of 20% (4 isolates), and an intermediate of 20% (4 isolates). The two antibiotic tablets, Trimethoprim and Tetracycline, recorded a resistance rate of 50% for each of them, and a sensitivity rate to the antibiotic Trimethoprim amounted to 40%, with intermediate of 10%. As for the Tetracycline tablet, the rate of susceptibility (sensitivity) to bacteria was 45%, with intermediate of 5%. Bacterial susceptibility to Azithromycin, Cefepime, and Tobramycin antibiotics was 30%, 25%, and 35%, respectively, with a resistance rate of 70%, 75%, and 65%, respectively, without any medium.

Keys words: *K. pneumoniae* ، PCR ،Antibiotics16 ،SrRNA.

حساسية ومقاومة لأحد أنواع بكتريا العائلة المعوية للمضادات الحيوية وفق بيانات CLSI 2023

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مستخلص:

هدفت الدراسة الى معرفة مقاومة بكتريا الكلبسيلا الرئوية (*Klebsiella pneumoniae*) والمعزولة من التهاب المسالك البولية لأنواع مختلفة من المضادات الحياتية ومقارنتها بدراسة سابقة لمعرفة نسب الاختلاف او التشابه او تغيرات الحاصلة في حساسية ومقاومة البكتريا لهذه المضادات ، عن طريق عزل وتشخيص البكتريا وفحص حساسية ومقاومة البكتريا لمجموعة مختارة من مضادات حياتية مختلفة.

تضمنت الدراسة جمع (30) عينة بكتيرية مرضية من المصابين بالالتهاب المسالك البولية من مختلف مستشفيات بغداد. شُخصت عزلات من بكتريا *Klebsiella pneumoniae* بأستخدام الطرائق المجهرية والكيموحيوية وأكدت 20 عزلة *K pneumoniae*. في الفحص التشخيصي الدقيق بتقنية تفاعل البلمرة المتسلسل PCR اختبرت حساسية ومقاومة العزلات البكتيرية المشخصة تجاه 8 أنواع من المضادات حيوية من عوائل مختلفة بأستعمال طريقة النشر بالاقراص. بينت النتائج ان هنالك تفاوت بنسب الحساسية والمقاومة لعزلات بكتريا K. pneumoniae ، اذ بلغت اعلى مقاومة للمضاد Ampicillin بنسبة بلغت ٪90 (18 عزلة) وبنسبة حساسية ٪5 (1عزلة) و نسبة متوسطة ٪5 (1 عزلة) . ومن ناحية أخرى تم الحصول على اقل مقاومة للمضاد Levofloxacin بنسبة بلغت ٪25 (5 عزلات) وبنسبة حساسية بلغت ٪45 (9 عزلات) و بنسبة وسطية بلغت ٪30 (6 عزلات).

و بلغت مقاومة بكتريا للمضاد الحيوي Cefoxitin ٪60 (12 عزلة) وبنسبة حساسية ٪20 (4عزلات) وبنسبة وسطية ٪20 (4عزلات). وسجل قرصي المضادين الحيوين Trimethoprim و Tetracycline نسبة مقاومة تبلغ 50 ٪ لكل منهما ونسبة حساسية للمضاد Trimethoprim بلغت ٪40 وبنسبة وسطية ٪10 وبالنسبة للقرص الحيوي Tetracycline بلغت نسبة تأثر (حساسية) للبكتريا ٪45 وبنسبة وسطية ٪5. وبلغت نسبة تأثر البكتريا للمضادات الحيوية Azithromycin و Cefepime و Tobramycin ٪30 ، ٪25 و ٪35 على التوالي وبنسبة مقاومة بلغت ٪70 ، ٪75 و ٪65 على التوالي بدون أي نسبة وسطية.

الكلمات المفتاحية : بكتريا الكيبسيلا الرئوية ،تفاعل البلمرة المتسلسل، المضادات الحيوية، فحص الجيني 16SrRNA.

The introduction

Types of bacteria of the intestinal family, including *K. pneumoniae*, are among the most common pathogens that possess large bacterial groups and are Gram-negative bacilli. Causes of hospital-acquired infections, especially in patients with a weakened immune system or those who take drugs that suppress the immune system. This type of bacteria causes many diseases, including pneumonia, urinary tract infections (UTIs), and burn and wound infections. It is described as straight, Gram-negative that contain a thick capsule composed of polysaccharides and contain the somatic antigen (O-antigen), and have the ability to adhere by Pilli mediation, are non-motile and do not form spores, facultative anaerobic, with diameters of 1-0.3 micrometers and a length of 0.6-6 μm, appearing singly or in pairs. The nucleic acid (DNA) contains 58% G+C nitrogenous bases . *Klebsiella* is widespread in nature, and is found in soil, surface water, sewage, and on the surfaces of vegetables, fruits, and grains. It is found naturally in the normal flora of the human body, specifically in the nasopharynx, intestines, and feces. It is the main reason for its spread due to the fact that its rate ranges up to 10^8 bacterial cells per gram of excretion. It can also be found in the respiratory system of healthy people by 10%, and its presence among three types of bacteria in the hands of health workers at a rate of more than 20%. It can also be isolated from all clinical sources, especially from the urinary and respiratory tracts, according to the estimates of the Public Health Organization annually. In America, there are 600 deaths due to *Klebsiella pneumoniae* and *E.coli* resistant to carbapenems, And that the frequent use of antibiotics and the indiscriminate and wrong use of antibiotics leads to bacterial resistance mediated by genetic mutations of these antibiotics, and thus the difficulty of treatment **(1,2).**

Urinary tract infection is a common problem because it is associated with the inability to treat due to the resistance of the bacteria that causes the infection, as it affects different ages and of both sexes and includes infections of the kidneys, bladder and ureters. People often use urinary catheters, and thus they are considered a passage and facilitate the entry of bacteria. The bacteria *K. pneumoniae* is the second

pathogen of urinary tract infection after *E.coli* bacteria, and in Iraq it constitutes 32% of the total number caused by the enteric family **(3)**. *Klebsiella pneumoniae* have factors called virulence factors. These factors use the mechanisms of causing disease in the patient's body and represent a measure of the bacteria's ability to cause disease in the host and contribute to making the bacteria more more pathogenic, able to bypass the basic defense lines in the body, as well as increasing resistance to manufactured antibiotics **(4)** . The acquisition of genetic material or the addition of a genetic mutation is an essential part of the habituation and development of bacteria. If bacteria acquire a gene that is resistant to an antibiotic, this means survival from that antibiotic after it was dying from its effectiveness. The Centers of Disease Control and Prevention (CDC) estimates in America, there are 2 million infections with bacteria that are resistant to several MDR antibiotics, and this leads to 20,000 deaths annually, and there are foundations and rules for the use of antibiotics, including not using antibiotics in unnecessary cases, using the antibiotic that is most appropriate for the disease and is one of the narrow-spectrum antibiotics, and reducing the administration of antibiotics with Reverse effect: K. pneumoniae is characterized by widespread resistance to antibiotics such as Carbapenems, Cephalosporins, Fluoroquinolones, and Aminoglycosides **(5,6,7)**.

MATERIALS AND METHODS

1- Bacterial isolates

1-1 Preparation of reagents used in the diagnosis of bacteria

The reagents used in the diagnosis of bacteria were prepared, then these solutions were autoclaved at a temperature of 121 °C and a pressure of 15 pounds / inch² for 15 minutes, as follows: **(8)**

1-1-1 oxidase reagent solution:

This reagent was prepared by dissolving 1 gram of Tetramethyl-P-phenylene diamine dihydro chloride in 90 milliliters of distilled water, then completing the volume to 100 milliliters and used to detect the production of oxidase enzyme. **(8)**

1-1-2 Catalase reagent solution:

The solution was prepared by diluting hydrogen peroxide H_2O_2 (6%) with distilled water to obtain a concentration of 3%, and was used to detect the ability of bacterial isolates to produce catalase enzyme by slide method and

kept in a dark vial. **(8)**

1-2 Collection of bacterial isolates:

30 clinical samples were collected from the urine of patients with urinary tract infections of different ages from Al-Yarmouk Teaching Hospital and the Medical City Hospitals in Baghdad during the month of June 2022, and examinations were conducted on them.

1-3 Isolation and diagnosis of bacterial samples:

Bacterial isolates were initially diagnosed based on the microscopic characteristics that were performed on them, as follows:

1-3-1 Microscopic characteristics:

The bacterial isolates were subjected to microscopic examination by taking a small smear from the colony, transferring it to a microscopic slide, staining it with a gram stain, and examining it under an oil lens to distinguish the shape of the cells, the way they are grouped and arranged, and whether they are positive or negative for the dye **(9).**

1-4 Biochemical tests:

The following biochemical tests have been chmical tests were carried out for the purpose of primary diagnosis:

1-4-1 Oxidase test:

This test was conducted on a circular and sterile filter paper placed on a clean Petri dish by transferring part of the growing colonies on the nutrient agar to the filter paper and then placing 2-3 drops of the oxidase reagent over the placed colonies and mixing them with the colonies with sticks. Sterilized wood, if the bluish-violet color appears within 10-15 seconds, indicating a positive test result. **(10)**

1-4-2 The catalase test: a smear was taken from the single bacterial colony growing on the nutrient medium onto a clean and dry glass slide using sterilized wooden sticks, then a drop of 3% hydrogen peroxide detector was placed on top of it. The appearance of bubbles on the surface of the slide indicated the production of catalase enzyme. **(10)**

1-4 Conservation on agar agar (short and medium term): Bacterial isolates were inoculated, after diagnosis, into tubes containing 5 ml of brain and heart infusion medium, and on nutrient agar media prepared in an oblique manner and by the method of planning inside the agar tubes, and incubated at a temperature of 37 °C. For a period of 24 hours for daily or weekly use, and it was taken into account to renew the

isolates monthly by activating them on the medium of nutritious broth and the broth of the heart and brain, and then replanting them on a new oblique medium, to ensure that they remain active throughout the study period. **(10)**

1-5 Sterilization Methods (10)

1-5-1 Sterilization by dry heat:

The vaccination needles were sterilized until they reached redness using a direct Benzene flame.

1-5-2 Moist heat sterilization:

All used culture media are autoclaved at 121 °C for 15 minutes, and all glass tools are sterilized by dry sterilization in the oven at 180 °C for two hours.

1-6 assy genetic (16SrRNA) using the polymerase chain reaction technique (Molecular identification)

1-6-1 Extraction of bacterial DNA

The steps of DNA extraction were according to the manufacturer of the laboratory kit

30 isolates (initially diagnosed as Klebsiella pneumoniae) that are to be examined by PCR technique have been activated in infusion broth brain heart and we follow the laboratory kit method according to the instructions of the supplied company as follows:

A. The first step: Sample preparation

• Add 1 ml of these activated samples to Eppendorf tubes and put them in the centrifuge (14,000 rpm for 1min), the purpose of getting rid of the filtrate.

• Add 180 microliters of GT buffer to these tubes

• Add 20 microliters of proteinase K enzyme, then add distilled deionized water and mix it with a Vortex device.

We put it in a water bath (60°C for 10 min) and shake it every three minutes during the bath.

B. The second step: Lysis and purification

Adding 200 microliters of GB buffer solution and then placing it in the Vortex shaker for 10 seconds.

Put it back in a water bath (70°C for 10min).

• Add 200 microliters of 99% concentrated ethanol to it and shake vigorously.

• Put GD Columm filters in 2ml of each sample of the mixture above, after transferring the mixture into new special sterile tubes with a cover called Collection tubes), and then put them in a centrifuge at (15,000 Rpm for 2Min)

• Add 400 microliters of W1 buffer to the GD columm filter and put it in the centrifuge (15,000 rpm 30s) to get

rid of the filtrate

• Add 600 microliters of Wash Buffer solution to the GD tube and put it in the centrifuge (15,000 rpm 30s) to get rid of the filtrate.

• Add 100 microliters of previously prepared Elution buffer at room temperature (25°C) for 3min, then put it in the centrifuge (15,000 rpm 30s).

• Disposing of the GD filter and keeping the tube with the cap containing the DNA mixture of Klebsiella pneumoniae.

1-6-2 Preparation of polymerase chain reaction medium

A. Primers permeation

The primers were prepared according to the supplying company, as follows:

• Adding 150 microliters of distilled deionized water to initially dissolve the initiators according to the method of the supplying company, and this is the Stock

• Take 20 microliters of each starter (according to the company's stock) and add 180 microliters of deionized water in Eppendorf tubes, for the first starter (Forward) and for the second starter (Revisble).

B. Master Mix content

• F (Forward) represents the first initiator

• R (Revisble) represents the second prime

• Deionized water

- Putting the master mix in the tubes of the Eppendorf and then shaking it with the electric shaker, transferring the content to the tubes of the Eppendorf premix and completing the mixture of the polymerase chain reaction (PCR mixture) according to the manufacturer's instructions (Table 1), and then entering it into the thermal cycler cycler for amplification.

Table (1) shows the content of the PCR mixture for the diagnosis of S16 of K. pneumoniae by the polymerase chain reaction (PCR) technique.

stage	The content of the PCR Mixture	measured in microliters
1	Forward primer	2
2	Reverse primer	2
3	Deionized Water	12
4	DNA Extracted	4
5	Premix	5
	Total Volum	25

1-6-3 DNA amplification by thermal cycler

Place the samples of the PCR mixture prepared according to paragraph inside the Thermal Cycler, and according to the programming for the *Klebsiella pneumoniae* table . Upon completion of the DNA amplification (Table 2), we transfer these samples by means of a micropipette to the tubes of the PCR system. Electrophoresis on an agarose gel.

Table (2) shows the programming of the thermocycler for the amplification of DNA extracted from bacteria *K. pneumoniae*

Stage	Stage name	Temperature	Time	Cycles
1	Initial Denaturation	95°C	3 min	35x
2	Denaturation	95°C	30 sec.	35x
3	annealing	58°C	30 sec.	35x
4	extension	72°C	40 sec.	35x
5	Final extension	72°C	7 min.	35x

1-6-4 Detection of amplified DNA by electrophoresis

1- Method for preparing Tris-Borate-EDTA Buffer (TBE) 10X solution

TBE was prepared according to the manufacturer's instructions. Take 100ml of TBE Buffer and add 900ml of distilled water to it (amount according to the number of samples to be tested).

2- Method of preparation of agarose solution

Dissolve 1.5g of agarose in 200ml of TBE buffer

• Melt it in the microwave at 60-70 degrees for two minutes (intermittently until it becomes transparent) and then leave it until it cools.

• Add 10-15 microliters of Ethedium bromide solution to a solution, then stir it slightly.

We put the combs and pour the solution into the tank of the relay device. The teeth of the comb are covered with the tank cover, then left until it hardens a little, then we transfer it to the refrigerator for 15 minutes.

• Remove the combs, then add 1 ml of TBE until the holes are covered

• Mix the existing content by means of a micropipette, then put it in the holes of the agarose gel

We put the cover of the box and form the electrodes, the negative electrode (black color) near the side that contains the samples and the positive electrode (red color) on the side far from the samples

• Use the device at 45 volts for 10 minutes, then at 75 volts for 30 minutes

• Placing the samples in an ultraviolet light device to detect the presence of the 16S RNA gene of the bacteria to be examined.

• The standard negative control contains all contents except the extracted DNA.

1-6-5 UV-rays source

• Samples are placed in it after electrophoresis, and the beams of bacteria will radiate, giving the final output of the detection.

2- Examination of the sensitivity and resistance of isolated bacteria to standard antibiotics

The antibiotic disk diffusion method was used to test the sensitivity and resistance of bacterial samples diagnosed to 8 different antibiotics using the Kirby-Bauer disk diffusion method, as follows: **(11)**

Taking 100 microliters of bacterial growth for each tube and activating them with heart-brain infusion medium (BHIB) and then preparing acar Mueller-Hinton medium by dissolving 41 g of the medium powder in 1050 ml of distilled water and using normal saline to dilute the bacterial isolates identical to the standard turbid solution McFarland 0.5 standard, and then add 10 microliters of Activated Bacterial Growth to 5 ml of saline solution for each tube and then immerse the cotton swab under sterile conditions inside the sterilization chamber ((HOOD) in the diluted bacterial growth and spread on all the plate by brushing in all directions To grow the bacteria on acar Mueller-Hinton medium plates, and then leave the culture for some minutes, then put the selected standard antibiotic tablets and distribute them in a diffusion manner, using sterile tweezers at the rate of 8 tablets per plate and gently press on them for fixation, and keep them inside the incubator for 24 hours at 37 degrees Celsius, the results were interpreted And compare them according to the specifications mentioned in the Clinical Laboratory Standards Institute (CLSI) for the year 2023.

Results and discussion

Microscopic examination showed that K. pneumoniae bacteria were in the form of pink sticks, being negative for Gram stain, and were short, arranged singly or in the form of pairs, chains, or spores, according to what was stated in **(8, 9).**

The biochemical examination by oxidase and catalase reagents(Table3) showed that there were 22 isolates that were identical to what was mentioned in **(12),** where the isolates were negative for the oxidase test and positive for the catalase test.

Table (3) shows the biochemical tests for bacterial isolates

	Catalase	Oxidase
K. pneumoniae	+	-

It is clear from what was previously mentioned that *K. pneumoniae* bacteria are positive for the catalase test, by the appearance of air bubbles of oxygen produced by the action of the catalase enzyme, which converts hydrogen peroxide into oxygen and water, and it gave a negative result in the oxidase test, due to its lack of a terminal oxygen receptor such as cytochrome C.

The diagnosis was confirmed by polymerase chain reaction (PCR) using the diagnostic gene 16SRNA, as 20 isolates belonged to the type of *K. pneumoniae* except for two isolates, as the PCR examination gave the result of the diagnosis of 20 confirmed bacterial isolates.

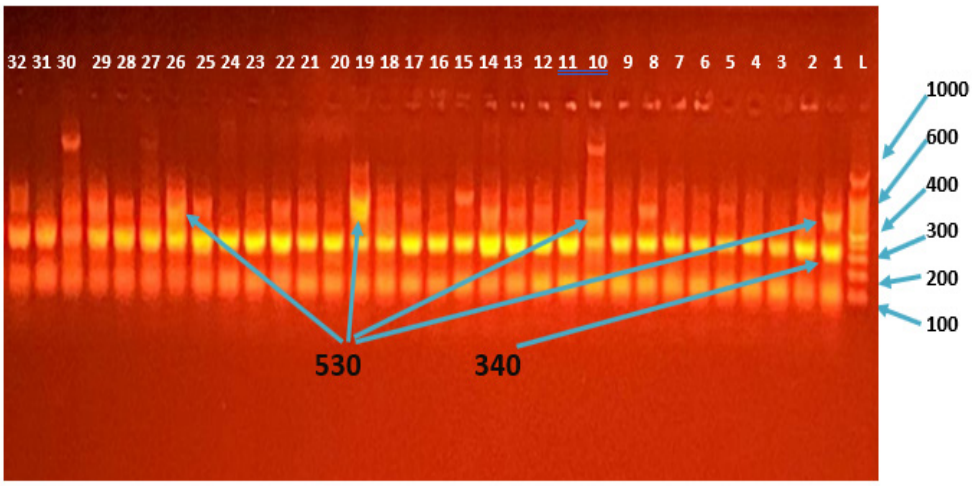

Figure. (1) shows the molecular diagnosis of *Klebsiella pneumoniae* (16SRNA)

The results shown in Figure (1) show the effect of the resistance of bacterial isolates to various antibiotics, as the diameter of the inhibition zone was measured in millimeters around the used tablets, and the results were compared with modern standard tables in the Clinical Laboratory Standards Institute (CLSI) for the year 2023. As the highest rate of inhibition of *K. pneumoniae* bacteria (23.4 mm) using the Levofloxacin antibiotic tablet compared with other antibiotics. The two antibiotics, Apicillin and Cefepime, recorded the lowest rate of inhibition among the different antibiotics, with an inhibition rate of (1.81mm and 5.2mm), with a resistance of 90.0% and 75% to *K. pneumoniae*, respectively, and a medium sensitivity to Apicillin of 5%, respectively, while The sensitivity of bacteria to Cefepime was shown to be 25%, and no intermediate percentage was shown by the isolates of this antigen. On the other hand, the least resistance to Levofloxacin was obtained by 25%, with a sensitivity of 45%, and intermediate of 30%.

And the resistance of bacteria to the antibiotic Cefoxitin reached 60%, with a sensitivity rate of 20%, with an intermediate of 20%. The two antibiotic tablets, Trimethoprim and Tetracycline, recorded a resistance rate of 50% for each of them, and a sensitivity rate to the antibiotic Trimethoprim amounted to 40%, with an intermediate of 10%. (Sensitivity) to bacteria is 45%, with an intermediate of 5%. The bacteria's susceptibility to Azithromycin and Tobramycin antibiotics was 30% and 35%, respectively, with a resistance rate of 70% and 65%, respectively, without any intermediate percentage. The current study recorded a difference from study **(13),** where the rate of resistance of bacterial isolates to each of the standard antibiotics Azithromycin and Tobramycin reached 20%. On the other hand, this study closely agreed with **(14),** as the results showed that the bacterial isolates were resistant to Cefepime and Ampicillin with a high rate of 85%. It differed with the Trimethoprim tablet, where its resistance rate was 75%. On the other hand, the current study recorded **(15)** a difference in the rates of resistance with the antibiotics Levofloxacin and Cefoxitin, as the resistance rate for the Levofloxacin tablet was 15% and for the Cefoxitin tablet 40%. The results, in comparison with previous studies, indicate that there is a fluctuation in the percentage

rate with the increase in antibiotic resistance, as there is a development and change in the virulence factors of *K. pneumoniae.*

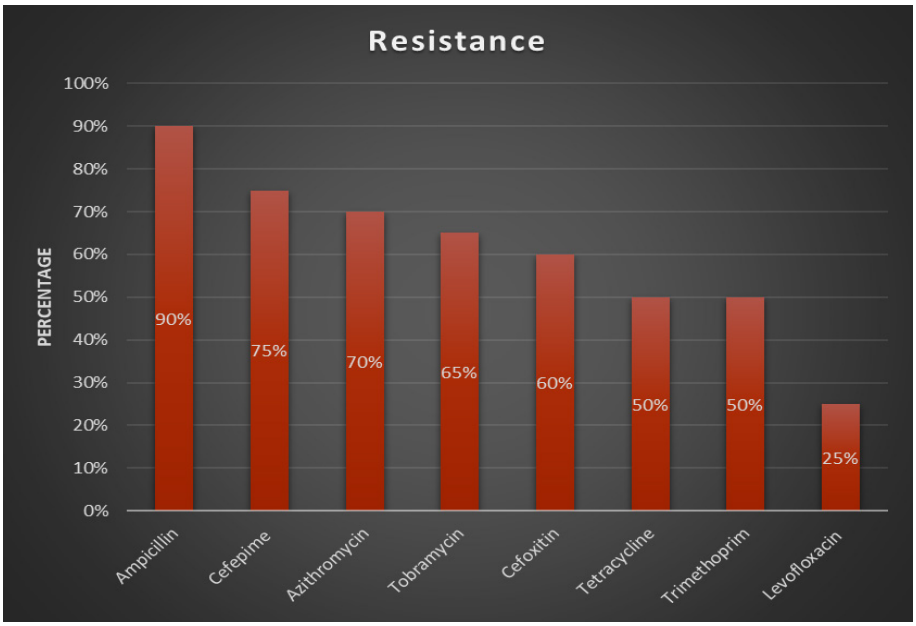

Figure (1) shows the percentages of the rate of resistance of bacterial isolates to different types of antibiotics

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