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Isolation and identification of types of biofilms- forming bacteria isolated from environmental samples and their production of beta- Lactamases enzymes

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Abstract
A biofilm is a community of microbes made up of microbial cells that attach to one another on living or non-living surfaces within an extracellular polymeric material matrix that the microbes themselves have generated. The process of creating a biofilm involves several steps, beginning with attachment to a surface, followed by the development of a microcolony that results in the production of a three-dimensional structure, and eventually maturation and separation. Numerous bacterial species can communicate with one another during biofilm formation thanks to a special process called quorum sensing. The QS system enables communication between intraspecies and interspecies, which involves, in terms of biofilm formation, food shortages, and environmental stress conditions, such as disinfectants, antibiotics, bacterial colonisation, the identification of irksome species, the establishment of normal intestinal flora, well as the prevention of harmful intestinal flora. The biofilm's matrix may allow for the diffusion of antibiotics. Exopolysaccharide acts as a physical barrier, which affects the diffusion or penetration of antibiotics into deeper levels of biofilm. The flow of molecules into the centre of the biofilm is slowed down when they come into direct contact with this matrix, leading to antibiotic resistance. The presence of neutralising enzymes that break down or inactivate antibiotics may be the cause of antibiotic resistance in biofilm. These enzymes are proteins that increase resistance through biochemical processes like hydrolysis and modification of antimicrobials.

Materials and Methods: Between 1/11/2021 and 1/1/2022, bacteria isolated from different environmental samples were identified in Basrah Governorate, Iraq using biochemical and Api20E tests. Biofilm phenotype formation was assessed using Congo red agar and the Tube method and drug sensitivity knowledge using the Kirby Power method. Metallo B-lactamase enzymes (MBLs) were detected by the method of combination of imipenem plus EDTA and for extended-spectrum B-lactamase enzymes (ESBLs) using HiCrome Tm Extended-spectrum B-lactamase ($ESBL_s$) Agar Base medium.

Results: The study included 140 environmental samples. Number of samples with positive growth (122) The study included samples from environments (domestic sewage, water purification plants, marine sediments, water and sediments of rivers and swamps, contaminated soil), and the bacteria were diagnosed according to traditional methods in terms of their agricultural and biochemical properties, as well as the use of APi20E. Our results showed that the number and percentage of biofilm-forming bacteria (55) samples (45.08%) were *K.pneumoniae* (41.82%), (23.64%) *P. meribilis*, (3.03%) *E.coli*, (7.27%) *Salmonella*, and (23.64%) *S.aureus*. The results of the detection of B-lactamase metallo enzymes showed that the production rates of biofilm-forming bacteria for MBLs were *K. pneumoniae* (100%), *Proteus meribilis* (92.30%), and *E. coli* (100%), *Salmonella* (100%), S. *aureus* (100%), and Extended-spectrum B-lactamase enzymes (ESBLs) were also detected, and the enzyme production rates were from bacteria forming membranes forming biofilm-forming bacteria *K. pneumoniae*. (52.17%), *Proteus meribilis* (61.53%), and *Salmonella* (76.93%). The sensitivity of the biofilm-forming bacteria to antibiotics was detected, and there were high rates of resistance.

Conclusion: During the study of environmental samples, it was found that there is a large percentage of bacteria that have the ability to form biofilms and are in direct contact with humans. Producers of B-lactamase enzymes

Keywords: Biofilms, MBLs B-lactamase ,$EBSL_s$ B-lactamase.

Introduction

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A group of microorganisms known as a biofilm produces extracellular polymeric substances (EPS) such as proteins (1-2% including enzymes), DNA (1%), polysaccharides (1-2%), and RNA (1%). In addition to these components, water (up to 97%) makes up the majority of the biofilm and is responsible for the movement of nutrients within the biofilm matrix .The architecture of a biofilm is composed of two primary elements: a water channel for the transportation of nutrients and an area of densely packed cells without obvious holes.[1]The comparison of the biofilm water channels with the system of circulation revealed that biofilms are regarded as early multicellular creatures.[2**]**

Microorganism cells change from a planktonic to a sessile mode of development during the production of biofilms, which is a highly complex process. Additionally, it has been proposed that the expression of particular genes that direct biofilm construction is necessary for biofilm formation.[**3**,**4**] A set of circumstances leading to adaptation under various dietary and environmental situations results in the creation of biofilms[**5**][**6**].The microbes go through a number of steps in this multi-step process after sticking to a surface. It has been established that microorganisms that produce biofilms trigger particular processes. The following crucial processes are involved in biofilm development: (A) initial surface adhesion (B) microcolony formation (C) three-dimensional structure formation (D) biofilm formation, maturation, and removal (dispersal) .[**2]** Biofilms contain microbial cells arranged in ways that significantly differ from one another in terms of physiology and physical characteristics. Normally, both the human immune system and drugs are unable to penetrate bacterial biofilms. Biofilm-producing microorganisms have a greater ability to withstand and neutralise antimicrobial medications, which can lead to protracted therapy. Bacteria that produce biofilms activate certain genes that stimulate the expression of stress genes, which in turn switch to resistant phenotypes in response to certain changes, such as nutritional or environmental conditions, temperature, cell density, pH, and osmolarity.[**7**]The presence of neutralising enzymes that break down or inactivate antibiotics may be the cause of antibiotic resistance in biofilm. These enzymes are proteins that increase resistance through biochemical processes like hydrolysis and modification of antimicrobials. By acting on the biofilm surface, antibiotics cause these enzymes to accumulate. Slow antibiotic penetration and antibiotic breakdown in the biofilm both help to increase enzyme neutralisation. The development of resistance to various antibiotics is brought on by an overproduction of cephalosporinase AmpC enzymes. When there is an extremely high concentration of carbapenems present, this enzyme provides resistance to B -lactam.[**8**].

Materials and Methods

Sample collection

Between 1, 11, 2021, and 1, 1, 2022, 140 samples were taken from various locations, including water purification plants, schools, water tanks, and polluted soil. All of the samples were then grown in selective media.

Bacterial Identification

Samples were initially diagnosed based on phenotypic characteristics and included colony shape, colour, texture, odor, and size on agar MacConkey agar and Mannitol slate agar . Then a microscopic examination was conducted, where it was dyed with graim dye to know the type of dye, the shape of the cells and the method of their assembly. Biochemical tests were conducted. To confirm the diagnostic results, use the API20E diagnostic kit(Biomerieux, France).

Detection of biofilm production

1. **Congo Red Agar method (CRA):**

The Congo red agar (CRA) method uses 0.8 g of Congo red and 36 g of sucrose to make 37 g/L of brain heart infusion (BHI) agar, and it is a qualitative assay for finding microorganisms that generate biofilms based on the colour change of colonies inoculated on CRA medium. After a 24-hour period of incubation at 37°C, the morphology of colonies that changed colour is used to determine whether or not they produce biofilm. Biofilm producers are represented by black colonies with a dry, crystalline consistency, while colonies that have preserved their pink colour are not biofilm producers.[**9**]**.**

2. **Tube Method (TM):**

Be aware that the biofilms are visible as a violet layer on the inner walls and bottom of the tubes.[**10**,**11**] .

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Detection of beta-lactamase enzymes

1. combination of imipenem + EDTA (MBLs)

A method was adopted where two imipenem tablets with a concentration of 10 ul of EDTA solution were placed into one of the two imipenem tablets. After incubation at 37°C for 18 hours, the inhibition areas were compared to see if they were around the imipenem tablet with EDTA equal to or greater than 7 mm, indicating a positive result.[**12**].

2. **HiCrome Tm Extended- spectrum B-lactamase(ESBL) Agar Base**

20 g of HiCrome Tm Extended-spectrum B-lactamase (ESBL) Agar. The base medium was dissolved in 1 litre of distilled water, then heated to the boiling point and then sterilised by an autoclave at a temperature of 121 °C and a pressure of 15 pounds. After sterilization, the medium was left to cool at 45 °C and added to it a (supplement), which consists of five antibiotics. Then the liquid was poured into dishes and left to harden. Then we add the bacteria to the medium using the web loop and put it in the incubator. After 18–24 hours, if the bacteria appear in purple or bluish green, the result is positive.

Bacterial resistance

We evaluated these bacteria's susceptibilities to antibiotics using the disc diffusion method on Mueller-Hinton agar. Antibiotic use is advised by the Clinical and Laboratory Standards Institute to determine how resistant certain bacteria are[**13**] (CLSI,2020).

Statistical analysis / Statistic evaluation

The statistical programme was utilised to ascertain the statistically significant variations among the various variables. The 25th edition of the statistical programme for social science The homogeneity test of the samples was run in this statistical analysis using Chi-Square Tests at a probability level of 0.05.

Results

A total of 140 environmental samples, including soil, sewage from homes, sewage from schools, and sewage from colleges, were gathered. The samples were then classified based on colony colour and morphology. As determined by API20E, biochemical tests were carried out, and the materials and working techniques employed were in accordance with what was indicated in the internationally recognised diagnostic systems. The percentage of positive growth showed 122 samples, or 86.14%, where the percentage of bacteria forming biofilms was 55 samples, or 45.08%. As in the following table(1)and (2).

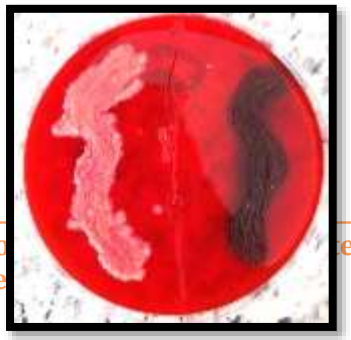
Table1: The number and percentage of biofilm-forming bacteria

Bacteria	*K.pneumonia*	*p.mirabilis*	*Salmonella spp*	*E.coli*	*S.aureus*
percentage	% 41.82	% 23.64	% 7.27	% 3.63	% 23.64
A number of samples	23	13	4	2	13

biofilm formation

Table 2: Proportions and numbers of biofilm-forming bacteria by the two methods of Congo red and tube

Bacteria	*K.pneumonia*	*p.mirabilis*	*Salmonella spp*	*E.coli*	*S.aureus*
CRA	(78.26%)18	(76.92%)10	(75%)3	(100%)2	(84.61%)11
TM	(21.73%)5	(23.07%)3	(25%)1	0	(15.38%)2
A number of samples	23	13	4	2	13



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A **B** **C**

Fige 1:

A. Formation of biofilms using the CRA method.
B. No biofilm production.
C. Formation of biofilms using the TM method.

B-lactamase formation

Table 3: The ratios of MBLs enzyme and ESBLs enzyme from biofilm-forming bacteria

Bacteria	*K.pneumonia*	*p.mirabilis*	*Salmonella spp*	*E.coli*	*S.aureus*
MBLs enzyme	(100%)23	(92.30%)12	(100%)4	(100%)2	(%100)13
ESBLs enzyme	(52.17%)12	(61.53%)8	(50%)2	(100%)2	(%76.93)10

A B

Fig2: Detection of enzymes
A. ESBL$_s$ enzyme by HiCrome Tm Extended-spectrum B-lactamase (ESBL) Agar
B. MBL$_s$ enzyme by EDTA – imp method.

Sensitivity tests for biofilm-forming bacteria

The Kirby-Bauer procedure, which uses sixteen antibiotics, was applied to all bacterial isolates under study. Although they come from many biological groups, most are common forms that are used to diagnose and treat various diseases. According to the information provided, the diameter of the inhibition zone surrounding the anticonvulsant was measured, and the results were compared. (CLSI, 2020).

1.*P.mirabilis*

From Table 4, we find the value of Chi-Square Tests is 129.310 with a significance level of 0.000 > 0.05, that is, there are significant differences in the level of effect of antibiotics on *P. mirabilis* bacteria. The results of the study showed that these isolates were highly resistant to antibiotics such as AX, OX, AMC, S and VA, respectively, with percentages of 92.30%, 92.30%, 92.30%, 76.92% and 69.23%. . The isolates also showed great sensitivity to Ak,AT,Gen,TOB and C, respectively, 100%, 100%, 92.30%, 92.30%, and 92.30%, respectively, as shown in Figure (3).

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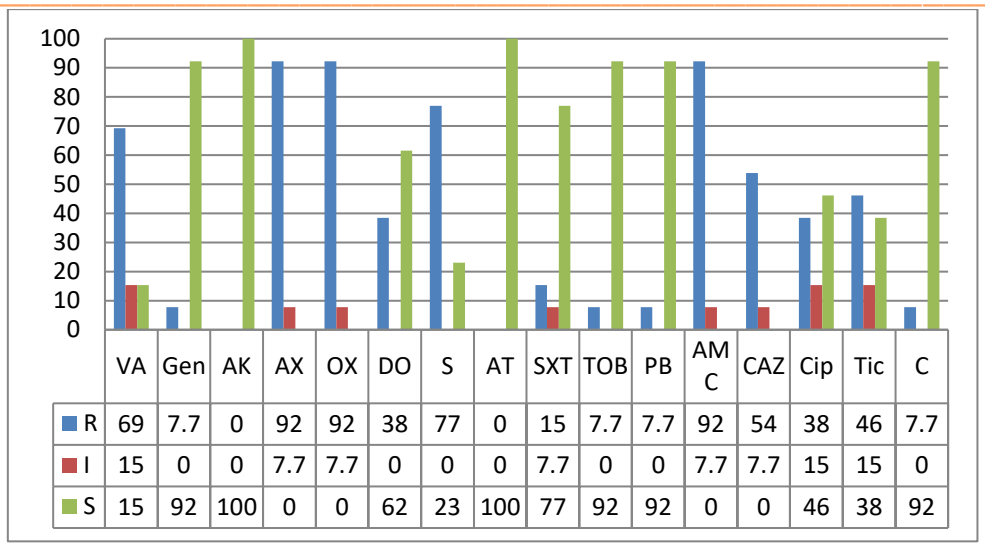
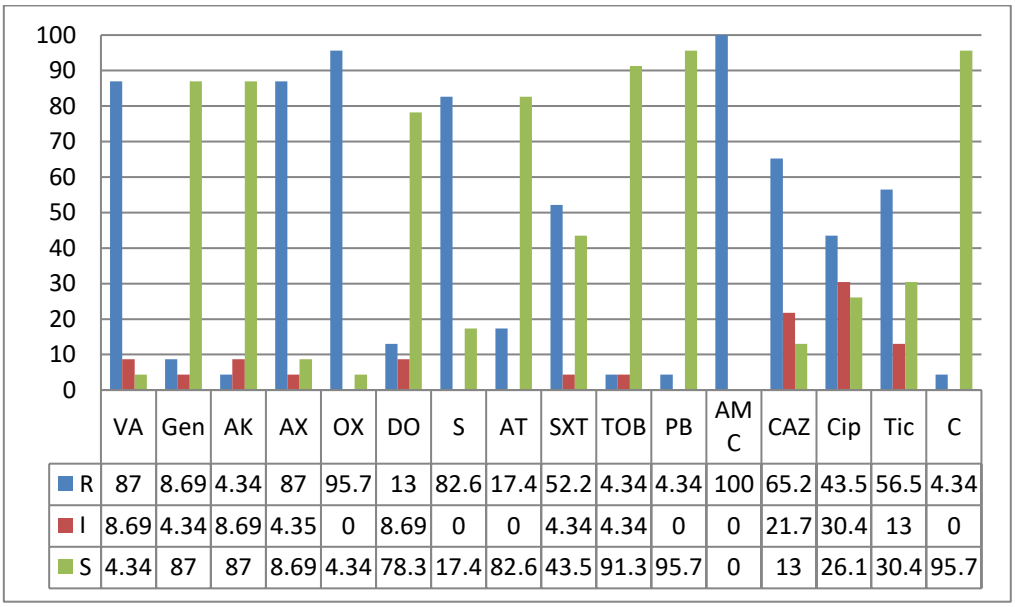


	VA	Gen	AK	AX	OX	DO	S	AT	SXT	TOB	PB	AMC	CAZ	Cip	Tic	C
R	69	7.7	0	92	92	38	77	0	15	7.7	7.7	92	54	38	46	7.7
I	15	0	0	7.7	7.7	0	0	0	7.7	0	0	7.7	7.7	15	15	0
S	15	92	100	0	0	62	23	100	77	92	92	0	0	46	38	92

Figure 3: Diagram showing the preparation of resistant antibiotics by *P. mirabilis* isolates.

2.*k.pneumoniae*

From Table 5, we find the value of Chi-Square Tests at 527.427 with a significance level of 0.000 > 0.05. That is, there are significant differences in the level of the effect of antibiotics, as the results showed a high resistance of *K. pneumoniae* isolates isolated from different environments. The results showed a high resistance of *K. pneumonia*e bacteria to AMC, OX, AX, VA, S, and CAZ, and the resistance percentages were 100%, 95.65%, 86.95%, 86.95%, 82.60%, and 68%, respectively. The isolates also showed great sensitivity to C antibiotics. AK, AT and DO, respectively, 95.65%,, 86.95%, 82.60 and 78.26%. As shown in Fig(4).



	VA	Gen	AK	AX	OX	DO	S	AT	SXT	TOB	PB	AMC	CAZ	Cip	Tic	C
R	87	8.69	4.34	87	95.7	13	82.6	17.4	52.2	4.34	4.34	100	65.2	43.5	56.5	4.34
I	8.69	4.34	8.69	4.35	0	8.69	0	0	4.34	4.34	0	0	21.7	30.4	13	0
S	4.34	87	87	8.69	4.34	78.3	17.4	82.6	43.5	91.3	95.7	0	13	26.1	30.4	95.7

Figure 4: Diagram showing the preparation of resistant antibiotics by *K.pneumoniae*

3.*Salmonella spp*

From Table 6, we find the value of Chi-Square Tests is 57.231 with a significance level of 0.000 > 0.05, meaning there are significant differences in the level of the effect of antibiotics on *Salmonella spp* bacteria. The results showed that the isolates were 100% resistant to the following antibiotics: OX, S, and TIC, as well as showing a high sensitivity to the following antibiotics: Gen, C, and TOB, respectively: 100%, 100%, and 75%, as shown in the tables (6) and Fig(5).

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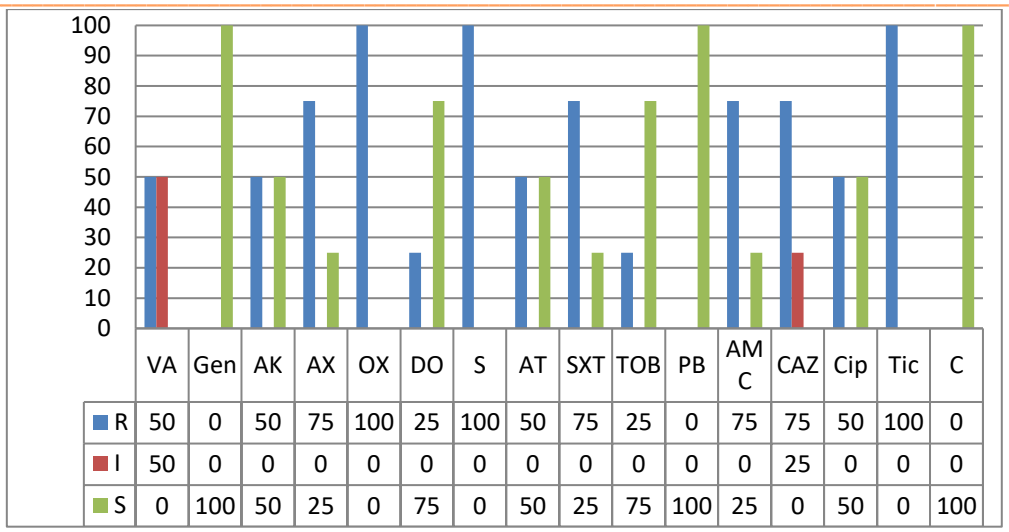
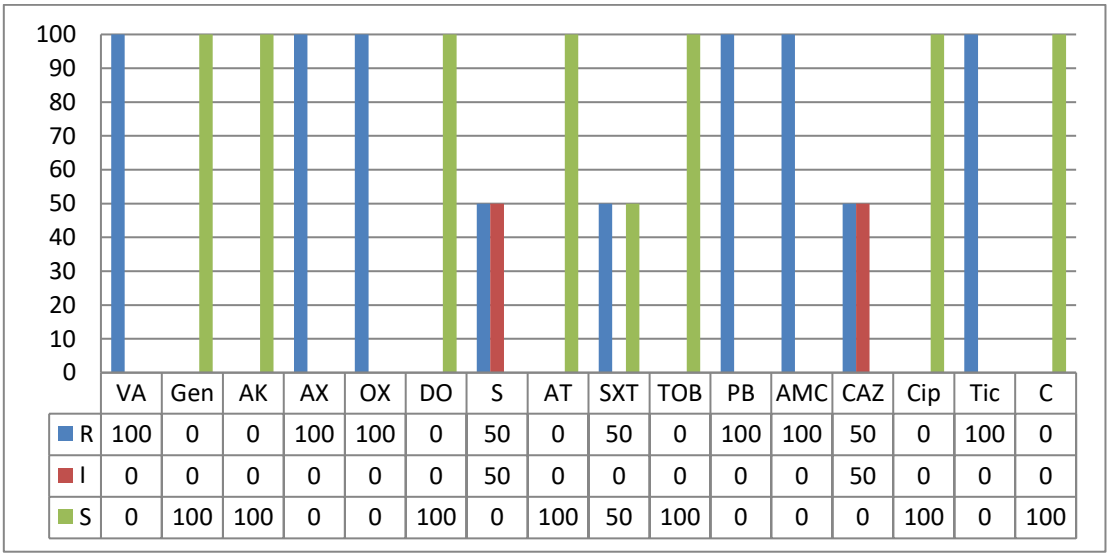


	VA	Gen	AK	AX	OX	DO	S	AT	SXT	TOB	PB	AMC	CAZ	Cip	Tic	C
R	50	0	50	75	100	25	100	50	75	25	0	75	75	50	100	0
I	50	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0
S	0	100	50	25	0	75	0	50	25	75	100	25	0	50	0	100

Figure 5: Diagram showing the preparation of resistant antibiotics by *Salmonella spp*

4.*E.coli*

From Table 7, we find the value of Chi-Square Tests is 30.345 with a significance level of 0.448 > 0.05. That is, there are no significant differences in the level of the effect of antibiotics on *E. coli* bacteria. The results of the study showed that there is a high resistance of *E. coli* isolates with a percentage of 100% VA, AX, OX, AMC, and Tic. The study also showed that there is a sensitivity of 100% for these isolates to Gen, AK, DO, AT, TOB, and C, as shown in the following Fig(6).



	VA	Gen	AK	AX	OX	DO	S	AT	SXT	TOB	PB	AMC	CAZ	Cip	Tic	C
R	100	0	0	100	100	0	50	0	50	0	100	100	50	0	100	0
I	0	0	0	0	0	0	50	0	0	0	0	0	50	0	0	0
S	0	100	100	0	0	100	0	100	50	100	0	0	0	100	0	100

Figure 6: Diagram showing the preparation of resistant antibiotics by *E.coli*

5. ***S. aureus***

From Table 8, we find the value of Chi-Square Tests is 149,537 with a significance level of 0.000 > 0.05, that is, there are significant differences in the level of effect of antibiotics on *S. aureus* bacteria. The results of the study showed that the isolates have high resistance to both AX and OX antibiotics. The percentage was 100%, respectively, and for VA, AZ, and AMC, the percentages were 92.30%, 92.30%, and 84.62%, respectively. The results also showed that there was a high sensitivity for each of the antigens Gen, TOB, and C by 100%, as shown in Figure (7).

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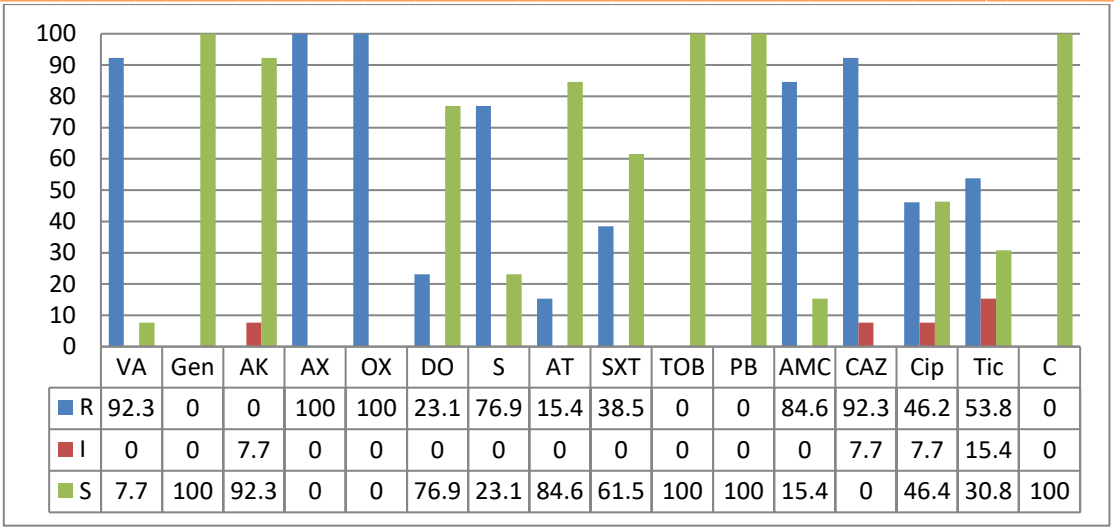

	VA	Gen	AK	AX	OX	DO	S	AT	SXT	TOB	PB	AMC	CAZ	Cip	Tic	C
R	92.3	0	0	100	100	23.1	76.9	15.4	38.5	0	0	84.6	92.3	46.2	53.8	0
I	0	0	7.7	0	0	0	0	0	0	0	0	0	7.7	7.7	15.4	0
S	7.7	100	92.3	0	0	76.9	23.1	84.6	61.5	100	100	15.4	0	46.4	30.8	100

Figure 7: Diagram showing the preparation of resistant antibiotics by *S.aureus*

Discussion

Biofilms in bacteria are one of the most difficult challenges that researchers have taken care of in the most important areas of life, such as the industrial and environmental fields, because they cause many big and dangerous problems in industry, shipping, and public health, such as increasing resistance to antibiotics and chemical pesticides, in addition to increasing rates of gene exchange that assist in the transfer of genes resistant to antibacterial agents between species that are located within the biofilm.

1.*S.aureus*

The results of the study showed that the percentage of biofilm-forming bacteria *S.aureus* was 23.64%, which did not agree with[**14**]. It also did not agree with the study of [**15**] where it reached 90%. It also did not agree with the results of [**16**]As it reached 81.81%. The process of biofilm formation is affected by various factors, including the level of nutrients. According to the results of the study, *S. aureus* produced 100% of the MBLs enzyme, which did not agree with the results of the researcher [**17**]which amounted to 85%.The results of the study showed the production rate of EBSLs enzymes reached 100%, which did not agree with the results. [**17**]As it reached 35%. The results of the current study showed that the currencies under study are resistant to anti-VA, as it reached 92.30%.It differed with the results of the study by [**18**] where it was 100% sensitive to the antibody. It also did not agree with what the researchers found [**19**].where the antibody was sensitive to isolates. and It did not agree with what [15]. Reached at 56.50%. It also showed the sensitivity of the isolates under study to anti-TOB by 100%, as it did not agree with what [**15**].Reached. The resistance ratio was 56.5. It is under study and the results of the isolates showed that they are resistant to the anti-Oxacillin (OX), where the percentage of resistance reached 100%. The findings of [15].Are close to the results of the current study as it reached 86.6.

2.*Salmonella spp*

The results of the current study showed that the percentage of biofilm formation by the CRA method reached 75%, which did not agree with what was reached by the researcher[**20**].where the percentage of membrane formation reached 50%. Although the CRA test is quick and easy to perform, its sensitivity, specificity, and accuracy are low[**21**]. The results of the study showed that the percentage of biofilm formation by the TM method reached 25%, which agrees with what the researcher[**20**].Reached, which amounted to 22.2%, and did not agree with the results of researcher [**22**], where the percentage of biofilm formation reached 68%. The results of the current study showed that the percentage of resistance to the antagonist Amoxillin (AX) reached 75%, as it did not agree with what was reached by the researcher [**23**] As the resistance rate reached 100%. It also did not agree with a study in Bangladesh by researchers[**24**,**25**] where the percentage of resistance was 100%, respectively. The current study found that the percentage of resistance to anti-S reached 100%, as it did not agree with the results of researcher [**23**] which amounted to 34.61%. According to [**26**]. Research, the antibody is sensitive. The current study showed that the percentage of resistance to Ceflazidim (CAZ) was 75%, and it did not agree with the results of the study by [27]. which amounted to 37.5%. The current study also showed that the antagonist Amikcin (AK) was 100%

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sensitive, as it agrees with the study of [**25**] . The sensitivity rate was 100%. The results of the study showed that the percentage of resistance to DO antagonist reached 25%, which was close to the results of the researcher [27] which amounted to 37.5%, and did not agree with the results of the researcher [24]. which amounted to 50%. The results of the current study also showed that the resistance to (AT) was 50%, as it did not agree with what was reached by[28] where the resistance ratio reached 27.27%.

3.*K.pneumoniae*

The results of the current study confirmed that the percentage of *K.pneumoniae* isolates producing biofilms was 23, with a rate of (41.81%), which did not agree with the study[29] which amounted to 83%. There are several environmental factors that may affect the process of producing the mucous layer of bacteria, including oxygen and temperature, and there are other conditions that may give different results.[30] . The percentage of ESBL enzyme production reached 52.17, which agreed with the results of the researchers [31].which amounted to 42.3%, and it did not agree with the study of the researchers[32].which amounted to 27.5%, and it did not agree with [17]. which reached 75%. It did not agree with the [33]. study, which amounted to 40%, and[34] .Did not reach the researcher, which amounted to 71.9%.%. The rate of production of MBLs enzyme reached 100%, [17] did not reach, as it reached 40%. The current study revealed that *K.pneumoniae* isolates have a high resistance to anti-AX with a rate of 86.95, which was somewhat close to what was reached by the researcher [35]. where the resistance rate reached 100%. The sensitivity of anti-C was 92.30%, and it is in agreement with what was found by[36], where the sensitivity rate was 96.25%. As for the sensitivity of the AK antagonist, it reached 86.95%, which did not agree with the results of the researchers[37] .which amounted to 56%.

4.*P.mirabilis*

The results of the current study showed that the number of *p.mirabilis* bacteria that produce biofilms is 13 isolates, i.e., 23.63%. The percentage of MBLs enzyme production was 92.30% and EBSLs enzyme 61.53%, which did not agree with[17]. study, which amounted to 65% and 90%, respectively. The percentage of Cip resistance reached 38.46%, as it differed with the results of [38]. The percentage of resistance was 15%. The results of the study showed that the sensitivity of *p.mirabilis* was 92.30%, which is consistent with what was reached by the researcher [39], who revealed that *p.mirabilis* isolates are sensitive to anti-C.

5.*E.coli*

The results of the current study showed that the number of isolates of *E. coli* bacteria producing biofilms by the CRA method was 3.63%. The results of the current study showed that the number of isolates of E. coli bacteria producing biofilms by the CRA method was 3.63%, and the researcher [40] confirmed the ability of bacteria to produce biofilms as a result of having curli fimbriae on the outer surface of the cell.[**41**]. The results of the current study showed that the percentage of *E. coli* isolates that produced ESBLs was 50. Where it does not agree with the study of[**42**] when it reached 12%, as it differed from the study of [**17**]. which amounted to 75%, The percentage of MBLs enzymes reached (100%), as it did not agree with what reached[**17**]. which amounted to 60%.

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