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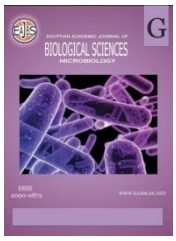

Isolation and Identification of Bacteria from The Middle Ear, Nose, Pharynx, Phenotypic and Investigation of Biofilm Formation in Isolated Bacteria

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ABSTRACT

The study included the collection of 120 clinical samples from the middle ear, nose and pharynx regions, 101 showed positive growth, while 19 isolates did not show growth, and for the period between 1-11-2021 to 1-3-2022, from both sexes in different age groups from hospitals (Samarra General - Dhuluiya year), after conducting microscopic and biochemical tests and using the Vitek compact system 2 device, 32 (31.6%) isolates of *S. aureus* bacteria 19 (18.8%) isolates of *P. aeruginosa* 15 (14.8%) isolates of *K. pneumonia* bacteria 13 (12.8%) isolates of *S. pyogenes* 7 (6.9%) isolates of *E. faecalis* 6 (5.9%) isolates of *M. catarrhalis* 5 (4.9%) isolates of *E. coli* 4 (3.9%) isolates of *P. mirabilis* bacteria, the resistance of the diagnosed bacterial isolates to antibiotics was tested by using 10 antibiotics, Azethromycin, Vancomycin, Chloramphenicol, Gentamicin, Penicillin G , Erythromycin, Ceftriaxone, Cephalothin, Amoxcillin clavulinic acid, Cefotaxime , the results showed that most of the isolates had great resistance to these antibiotics, As for the production of biofilm, the micro-standard plate method has been shown .Micro titer Plates Most of the bacterial isolates came to form the biofilm in different proportions. The results showed that the bacteria differ in the rate of biofilm formation and in different proportions. The strongest bacteria in the biofilm formation were *P. aeruginosa* by 88% and in varying degrees, followed by *M. catarrhalis* bacteria by 83% and to varying degrees. , followed by E. coli bacteria with a percentage of 80% and to varying degrees, followed by *K. pneumoniae* bacteria with a percentage of 78% and to varying degrees, followed by *P. mirabilis* bacteria with a percentage of 75% and to varying degrees, followed by a bacterial *S. aureus* with a percentage of 71% and with varying degrees.Varying degrees, followed by *E. faecalis* with a percentage of 70% and in varying degrees. Finally, the least biofilm formation rate came with bacteria *S. pyogenes* by 67% and to varying degrees. Through our observation of the results, there is a significant relationship between biofilm formation and antibiotic resistance. The strongest antibiotic-resistant bacteria was *P. aeruginosa*, as it resisted most antibiotics, and the strongest biofilm-forming bacteria also came, as well as *S. pyogenes* came as the least antibiotic-resistant bacteria and also the weakest biofilm-forming bacteria, as well as the rest of Bacterial species resisted most types of antibiotics and came as a good component of biofilms. We conclude that the greater the value of biofilm formation, the greater the ability of bacteria to resist antibiotics. Providing protection for bacteria against host immune response and antibiotics, thus it is an important virulence factor to promote bacterial colonization of host cells and is important in bacterial survival.

INTRODUCTION

The main function of the respiratory system is to obtain oxygen and excrete carbon dioxide resulting from the metabolic processes carried out by the various cells in the body (Wakim and Grwel, 2020) and its functions are not limited to gas exchange only, but it is of great importance in the secretion of thick mucus, which in turn works To prevent the entry of pathogenic microorganisms such as bacteria into the respiratory system (Gachanja *et al*., 2021) the respiratory system has secondary functions of humidifying, warming and filtering air, and this includes the lungs to control pH levels in the body and the vocal cords in the larynx to produce sound (Tu *et al* ., 2013) It occurs to deliver air to the lungs, and the second occurs in which gas exchange occurs, as carbon dioxide gas spreads from the blood to the air and oxygen between the air and the blood (Wakim and Grwel, 2020) Pneumonia infection is of varying impact and sometimes it is asymptomatic and leads to severe pneumonia and this leads to Respiratory failure (Li *et al*., 2020) The bacterial species responsible for respiratory infections and opportunistic infections were identified by isolating them from people suffering from respiratory disorders. These isolated bacterial species are (*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis*) (**Swain *et al.*, 2019**). It is defined as inflammation or injury to the middle ear space (Mittle *et al*., 2018) and it is one of the common diseases that include acute otitis media (AOM) Acute otitis media (Atkinson *et al*., 2015), and otitis media effusion (OME). Otitis media with effusion and chronic suppurative otitis media (CSOM), although the infection is more common in children, but it occurs in other age groups (Meherali *et al*., 2019). The age group of children from three to seven years is the most susceptible to infection with otitis media (OM) (Al-Johani *et al*., 2018), and the frequent infection of children due to the shortness of their Eustachian tube being at a more horizontal angle than it is in adults, and this causes the transmission of pathogens from the nasopharyngeal region to the middle ear region (Sakulchita and Goldenman, 2017).Biofilm They are cellular aggregates that are stuck together by a group of extracellular polymeric compounds, which include proteins, exogenous polysaccharides, and nucleic acids in some cases. Its importance lies in providing protection for bacteria against the host's immune response and antibiotics, thus it is an important virulence factor to promote bacterial colonization of host cells and is important in the survival of bacteria (Jasim *et al*., 2021).

MATERIALS AND METHODS

Sample Collection:

120 samples were collected from patients coming to Samarra General Hospital and Dhuluiya General Hospital, Ear, Nose and Throat Department (ENT Section) with the help of specialized doctors for the period between 1-11-2021 to 1-3-2022, from male and female in different age groups, gave 101 positive growth samples, 84.17% after development on Nutrient agar medium, while 19 samples with a rate of 15.83% did not have any growth, the reason for this is due to the fact that the pathogen is not bacteria (fungi, viruses, anaerobic bacterial types, or others), which can be diagnosed by other methods because the media used It does not meet the requirements of its growth After the isolates grew on culture media, they were diagnosed through phenotypic and microscopic diagnosis and biochemical tests (Mahon *et al*., 2014), then a group of antibiotics was tested against bacteria, where ten antibiotics were used, which are Azethromycin (AZM), Vancomycin (VA), Chloramphenicol (C), Gentamicin (CN), Penicillin G (P), Erythromycin (E), Ceftriaxone (CRO), Cephalothin (KF), Amoxcillin clavulinic acid (AMC), Cefotaxime (CAZ). (Bioanalyase /Turkey). The medium of the blood agar base was prepared and sterilized, then sterilized with an autoclave, then cooled to a

temperature of 5%, then the mixture was mixed and poured into Petri dishes and left to solidify, Decomposition, whether alpha or beta, appears in the form of a transparent halo around the colony, and this indicates a positive test, and when the halo does not appear, this indicates the inability to decompose (Cappuccino *et al.,* 2018).

Qualitative Examination of The Ability of Bacterial Isolates to Form the Biofilm Using Microtiter Plate:

The susceptibility of the isolated bacteria to biofilm formation was tested (Babapour *et al.,* 2016), where the isolates were activated on solid brain-Heart infusion agar), and incubated for 18-24 at a temperature of 37 °C, and after completion from the incubation process

(1) 1-3 pure and young colonies were transferred to test tubes containing 5 ml of physiological solution, to form a bacterial suspension, and the growth was compared with the standard tube (Maker Fland) after the turbidity of the formed growth was compared with the turbidity of a standard turbidity solution (Maker Fland).
(2) 20 microliters of the bacterial suspension were transferred to the micro titer pits with 3 replicates for each isolate.
(3) Add 180 microliters of sterile liquid heart and brain infusion to which 2% sucrose is added.
(4) 180 µl of sucrose + (BHI) medium without bacterial suspension was added at 3 holes as control points.
(5) Micro titer plate was incubated at 37°C for 24 hours.
(6) After completion of the incubation period, the pits were washed three times with (Pbs) Phosphate buffer Saline PH (7.2) to remove non-adherent cells.
(7) 200 µl of Crystal violet 1% dye was added after leaving the pits to dry at a temperature of 25° C for 15 minutes.
(8) The plate was left for 15 minutes at a temperature of 25 to fix the dye in the cells. After the fixation process was completed, the dye was removed by washing it three times with Pbs Phosphat buffer Saline PH (7.2) after which the plate was left to dry at a temperature of 25 ° C.
(9) Using ethyl alcohol at a concentration of 95% clean to the pits and leave it for ten minutes and then read the absorbance of alcohol at a wavelength ((630 nm) using an ELISA device, as the percentage of biofilm formation was calculated compared to the control point The results were recorded according to the absorbance of optical density O.D.

RESULTS AND DISCUSSION

120 samples were collected from patients coming to Samarra General Hospital and Dhuluiya General Hospital, Ear, Nose and Throat Department (ENT Section) with the help of specialized doctors for the period between 1-11-2021 to 1-3-2022, from Male and Female in different age groups, gave 101 positive growth samples, 84.17% after development on Nutrient agar medium, while 19 samples with a rate of 15.83% did not have any growth, the reason for this is due to the fact that the pathogen is not bacteria (fungi, viruses, anaerobic bacterial types, or others), which can be diagnosed by other methods because the media used It does not meet the requirements of its growth After the isolates grew on culture media.

Microscopic Identification:

Based on Gram staining results, 22 isolates were obtained with a percentage of 64.70% negative and 12 isolates positive for Gram staining with a percentage of 35.30%. As in Table (1) the results from the study are close to those of (Tadesse *et al.,* 2019).

Table 1: Proportions and numbers of positive and negative isolates of gram stain for patients with middle ear OM**.**

Bacteria isolates	Number	Percentage
Gram-negative bacteria	22	64.70%
Gram positive bacteria	12	35.30%
Total	34	100%

While the results of Gram staining for bacteria isolated from the nose and pharynx, 42 isolates were obtained positive for Gram stain with a percentage of 62.69%, and 25 negative isolates were obtained for Gram stain with a percentage of %37.31 as Table (2). These results did not agree with the researcher's findings (Al-Tikriti, 2019).

Table 2: Proportions and numbers of positive and negative isolates of gram stain for patients with rhinopharyngitis.

Bacteria isolates	Number	Percentage
Gram-negative bacteria	25	37.31%
Gram positive bacteria	42	62.69%
Total	67	100%

Biochemical Tests:

Table (3) shows the biochemical tests for gram-negative bacteria, which included Enterobacteriaceae, *P. aeruginosa* and *M. catarrhalis*. *E. coli* bacteria producing catalase enzyme but not producing oxidase enzyme were motile and lactose-fermenting positive for indole and methyl red assay due to production of acid negative for urease and Fuchs Proscurrency and not consuming citrate in the middle of Simon Street. Either *K. pneumoniae* were positive for urease, catalase, and fuchsproskauer assay and consumed citrate as the only non-motile, non-oxidase-producing carbon source and negative for methyl red and indole assay. As for P. mirabilis, it was negative for the oxidase assay, positive for the catalase and urease assay, positive for the indole and methyl red test, and negative for the test for citrate consumption and Fuchs Proscure. Either *P. aeruginosa* was a producer of the enzymes catalase and oxidase and it grows on steramide agar. It consumes citrates, not producing indole, not fermenting lactose, and heterogeneous to produce urease. As for *M. catarrhalis* bacteria, it was not a fermenter of carbohydrates, and these tests are essential for the diagnosis of this bacteria. As for Table (4), it showed the biochemical tests for positive bacteria *S. aureus*, they were positive for catalase test positive for coagulase test, positive for mannitol test and negative for oxidase selection and complete hemolysis from beta type As for *S. pyogenes*, it was negative for catalase, oxidase, and, mannitol and the hemolysis was complete, beta-lysis. As for *E. faecalis*, it was negative for oxidase, catalase, a mannitol and an incomplete analysis of blood, gamma type.

Table 3: Results of biochemical tests for Gram-negative bacteria.

Test / Bacteria	Cat	Oxi	Ur	IMViC				TSI				Sugar fermentation				
				Ind	MR	VP	Ci	Gas	H_2S	Butt	Slope	Lac	Suc	Glu	Xyl	Man
E. coli	+	-	-	+	+	-	-	+	-	Y	Y	+	V	+	+	+
P. aeruginosa	+	+	V	-	-	-	+	-	-	R	R	-	-	+	+	V
K. pneumonia	+	-	+	-	-	+	+	+	-	Y	Y	+	+	+	+	+
P. mirabilis	+	-	+	+	+	-	-	+	+	Y	Y	+	+	+	+	+
M. catarrhalis	+	+	-	-	N	N	N	N	N	N	N	-	-	-	-	-

Cat: catalase, oxi: oxidase, ur: urease, I: indol, M-R: methyl red, V-P: voges proskaure, C:citrate, man: mannitol, xy: xylose, gl: glucose, su: sucrose, la: lactose.

Table 4: Results of biochemical tests for Gram-positive bacteria.

Bacterial type	Cat.	Oxi.	Coa.	Hem.	Bac.	Man.
S. aureus	+	-	+	B	ND	+
S. pyogenes	-	-	ND	B	S	-
E. faecalis	-	-	ND		ND	-

No data available :ND ,cat: Catalase ‹oxi :Oxidase ‹coa: Coagulase hem: Hemolysis‹bac: Bacitracin ‹man : mannitol.

Antibiotic Resistance:

Isolated and identifiable bacteria in this study have determined their resistance to these antibiotics based on measuring the diameter of the inhibition zone (in millimeters) and comparing it with the standard ratios in the Laboratory Standards Institute Clinical and (CLSI, 2018). Rhinopharyngitis and middle ear infections were resistant to many antibiotics The results showed that the isolated bacteria possessed absolute resistance to two antibiotics and an absolute sensitivity to the antibiotic Ceftriaxone (CRO), Cephalothin (KF), and an absolute sensitivity to the antibiotic Azethromycin (AZM) as in the Table (5).

Table 5: The resistance of bacterial isolates to antibuotics.

bacterial isolates	AZM	VA	C	CN	P	E	CRO	KF	AMC	CAZ
S. aureus	S	R	S	R	R	S	R	R	S	R
S. pyogenes	S	R	S	R	R	R	R	R	S	R
P. aeruginosa	S	R	S	R	R	S	R	R	R	R
E. faecalis	S	S	S	R	R	R	R	R	S	R
K. pneumonia	S	S	R	S	R	R	R	R	S	R
E. coli	S	S	S	R	R	S	R	R	R	R
M. Catarrhalis	S	R	R	R	R	R	R	R	S	S
P. mirabilis	S	R	S	S	R	R	R	R	S	R

Biofilm Formation Results:

The composition of the biofilm of bacteria isolated from the middle ear, nose and pharynx was investigated, and after reading the micro titer plate containing the biofilm-forming bacteria on the ELISA device, we got the results shown in Table (6), as the results showed that bacteria differ in the rate of formation The biofilm and in different proportions, as the strongest bacteria in the biofilm formation were *P. aeruginosa* by 88% and in varying degrees, followed by *M. catarrhalis* bacteria by 83% and in varying degrees, followed by P. mirabilis bacteria with a percentage of 75% and to varying degrees, followed by *S. aureus* bacteria 71% and to varying degrees, then *E. faecalis* bacteria by 70% and to varying degrees, and finally and the least biofilm formation rate came bacteria *S. pyogenes* by 67% and in varying degrees, through our observation of the results we found There is a great relationship between biofilm formation and antibiotic resistance, as the strongest bacteria resistant to antibiotics was *P. aeruginosa*, it resisted most of the antibiotics and the strongest biofilm-forming bacteria also came, as well as *S. pyogenes* bacteria came the least antibiotic resistant bacteria and also the weakest bacteria Component of the biofilm, as well as the rest of the bacterial species resisted most types of antibiotics and came as a good component of biofilms, from here we conclude that the greater the value of biofilm formation, the greater the ability of bacteria to resist The greater the antibiotic resistance and the less the ability of bacteria to resist antibiotics whenever the formation of the biofilm is weak, because the biofilm is important in providing protection for bacteria against the immune response of the host and antibiotics. (Jasim *et al*., 2021). This study came close to the study conducted by the researcher Al-Janabi (2010), where all

samples of P. *aeruginosa* bacteria were biofilm-forming and in different proportions. mirabilis (95.31%) made up the biofilm. The results of this study were also in agreement with (Wakimoto *et al*., 2004), as they found that E. coli bacteria are biofilm-forming with a percentage of (77.4%).

Table 6: Rate of biofilm formation in varying proportions by bacteria isolated from the middle ear, nose and pharynx.

Type of bacteria	Prepare It	Biofilm formation rate (%)
S. aureus	28(%30.11)	20(%71)
P. aeruginosa	17(%18.27)	15(%88)
K. pneumonia	14(%15.05)	11(%78)
S. pyogenes	12(%12.90)	8(%67)
E. faecalis	7(%7.53)	5(%70)
M.catarrhalis	6(%6.45)	5(%83)
E. coli	5(%5.38)	4(%80)
P. mirabilis	4(%4.30)	3(%75)

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