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Isolation and identification of bacteria from Respiratory tract and Phenotypic and molecular investigation of *Hla* gene bacteria in *Staphylococcus aureus* isolated

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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 (13.13%) 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 and Cefotaxime. The results of the molecular examination of the gene (*Hla*) in *S. aureus* bacteria, which is responsible for hemolysin, showed that 20 isolates of these bacteria were selected because they were clearer and more effective in hemolysis for the purpose of investigating the *Hla* gene encoded for the production of hemolysin using PCR technique. Responsible for the production of hemolysin, which is one of the virulence factors in S. *aureus* bacteria, Primer amplification products showed that 19 out of 20 samples possessed this gene (95%), where the bands appeared after being transferred on the agarose gel within the expected region of this gene (535 base pairs).

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 (1) 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 (2) 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 (3) 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 (1)

Pneumonia infection is of varying impact and sometimes it is asymptomatic and leads to severe pneumonia and this leads to Respiratory failure (4) 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 and Proteus mirabilis*) (5). It is defined as inflammation or injury to the middle ear space (6) and it is one of the common diseases that include acute otitis media (AOM) (7), 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 (8). The age group of children from three to seven years is the most susceptible to infection with otitis media (OM) (9), 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, as well as children are less immune compared to adults (10) otitis media (OM) can have multiple sources of infection, including viral, bacterial or fungal (11) or it may be a joint infection of the bacterial species that cause otitis media, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp, *Streptococcus* spp *Klebsiella pnuomonia*, *Escherichia coli*, *Protus* spp (12). The *Hla* gene is the most prominent cytotoxin in Staphylococcus bacteria that can act against a wide range of host cells including red blood cells, epithelial cells, endothelial cells, T cells, monocytes and macrophages (13), Hemolysin plays an important role in all diseases caused by *S. aureus* bacteria, as it helps in decomposing the host cell membrane, distorting or evading the immune system, Pore formation is a multi-step process that involves the secretion of alpha-hemolysin monomers that bind to the target membranes. The receptive cells contain specific receptors that allow alpha-toxin to attach, causing the appearance of small pores and this leads to the leakage of small cytoplasm components less than 2 daltons, such as potassium and calcium ions, which leads to the change of ionic degrees and the occurrence of necrosis and cell death as a result of the exit of its contents (13).

Materials and methods

Sample collection

A 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(14), 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°C, 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 (15).

Bacterial DNA extraction

DNA was extracted from bacteria using ready-made (Bacterial kit) equipment from a company Geneaid (USA), and according to the company's instructions, the extracted DNA was detected using a special device Nano drop spectrophotometer to detect and measure the concentration of nucleic acids. ranges between (280-260) nanometers.

Primer preparation

The *Hla* gene primers prepared from mecrogen company shown in table 1, were Dried freezing in Nuclease free water to give a final concentration of 100 picomoles as a stock solution, Store at 20° C, In order to avoid repeated freezing and thawing, the working solution was prepared by adding 10 µl of storage solution to 90 µl of Nuclease free water to obtain a solution with a concentration of 10 picomoles/µl and stored at 20° C.

Table 1: The prefixes used in the study

Gene name	Sequence	package size (bp)	temperature (°C)	Refrence
Hla-F	5`-GGT TTA GCC TGG CCT TC -3`	535	50	(16)
Hla-R	5`-CAT CAC GAA CTC GTT CG-3`			

PCR Reaction Mixture

The main reaction mixture for the extracted DNA samples was prepared for the PCR polymerase chain reaction by mixing the reaction components in tubes to obtain a final volume of 20 µl as shown in table 2, the reaction solution drops on the wall of the tube and then the tubes were placed in the thermocyer to start the reaction according to the specified program steps.

Table 2: The main reaction components of the PCR technique**.**

N	Component	Volume (µl)
1	water Nuclease-free	6
2	Master mix (2x)	10
3	DNA DNA sample (25-50 ng)	2
4	Forward primer (10 Pmol/µl)	1
5	Reverse primer (10 Pmol/µl)	1
	Total volume	20

Result and discussion

A 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 3 the results from the study are close to those of (17).

Table 3: 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 in table 4. These results did not agree with the researcher's findings (Sura, 2019).

Table 4: 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 5 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 these bacteria. As for Table6), 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 5: 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 6: 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	Γ	ND	-

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

Relationship of some predisposing factors with Otitis Media infection

It was found that the infection is more common in males than females, as the infection was in males 58.82%, and females 41.18%. The reason why males are infected more than females is due to the fact that they are exposed to risk factors more than females, in addition to their practice of outdoor work and swimming in pools and pools. Which contains pathogenic bacteria, which makes them more susceptible to infection than females, and this is consistent with many studies. It was noted through the current study that the most common age groups

affected by otitis media are the age groups from 16 to 30 years with a percentage of 41.20%. to 15 years at a rate of 23.52%, and this is due to the fact that they have a horizontal Eustachian tube, in addition to a weak immune system and more infection, and poor hygiene plays a major role in causing injury and the use of contaminated tools and their introduction into the ear Then the age group from 31 to 45 years with a percentage of 20.58%, and as shown in Table 7, followed by the group from 46 to 60 years with a rate of 8.80%, and finally 61 years and over, which was less affected by 5.90% due to the presence of chronic diseases in addition to weakness immune system(18).

Table 7: Distribution of OM by gender and age of the patient.

Age	Male	Female	Total	Percentage
1-15	5	3	8	23.52%
16-30	8	6	14	41.20%
31-45	4	3	7	20.58%
46-60	2	1	3	8.80%
61and above	1	1	2	5.90%
Total	20	14	34	100%

Relationship of some predisposing factors with Pharynx and Nose infection

The results of the current study showed that the prevalence of respiratory infections in females is lower than in males, as shown in Table 8, as the number of patients was 67 patients, 28 female patients, with rates of 41.79%, and the number of male patients with a percentage of 39, with a rate of 58.21%, and the reason is attributed that most males occupy different fields of work and are more in contact with society than females, and this makes them more susceptible to infection with infectious agents, and that the reason for this difference may be due to some factors that qualify for infection, which spread among males to a large extent, such as alcohol consumption and smoking, (20).

Table 8: The relationship between sex, age and Nose and pharynx infections.

Age	Male	Female	Total	Percentage
15-24	9	3	12	%17.91
25-34	10	7	17	%25.37
35-44	6	5	11	%16.42
45-54	5	6	11	%16.42
55-64	4	4	8	%11.94
65-74	3	2	5	%7.46
84-75	2	1	3	%4.48

Phenotypic and molecular investigation of Hla gene using Polymerase Chain Reaction (PCR)

The results showed that most of the *S. aureus* isolates analyzed the blood phenotypically with a percentage of (95%), the results of this study did not agree with what was reached by the researcher (Al-Amri, 2005), where he found that *S. aureus* bacteria are phenotypically hemolytic with a percentage of (19%). In terms of genetics, 20 isolates of these bacteria were selected because they were more visible and effective in hemolysis. The *Hla* gene encoded for the production of alpha-hemolysin was investigated using PCR technique. The *Hla* gene responsible for the production of hemolysin, which is one of the virulence factors in *S. aureus* bacteria, and after taking PCR results and placing them on agarose gel at a concentration of 1.5%, electrophoresis was performed, gene bundles appeared by 95%, meaning 19 out of 20 bacterial isolates of *S. aureus* had this gene within the expected region for this gene (535 base pairs). as shown in Figure 1. The results of the current study came close to what was reached (19), and it was found that all isolates (100%) contain this gene encoded for the production of hemolysin. These bacteria because of their possession of the enzyme hemolysin, where they found that the percentage of genes encoding the production of *Hla* (81.18%).

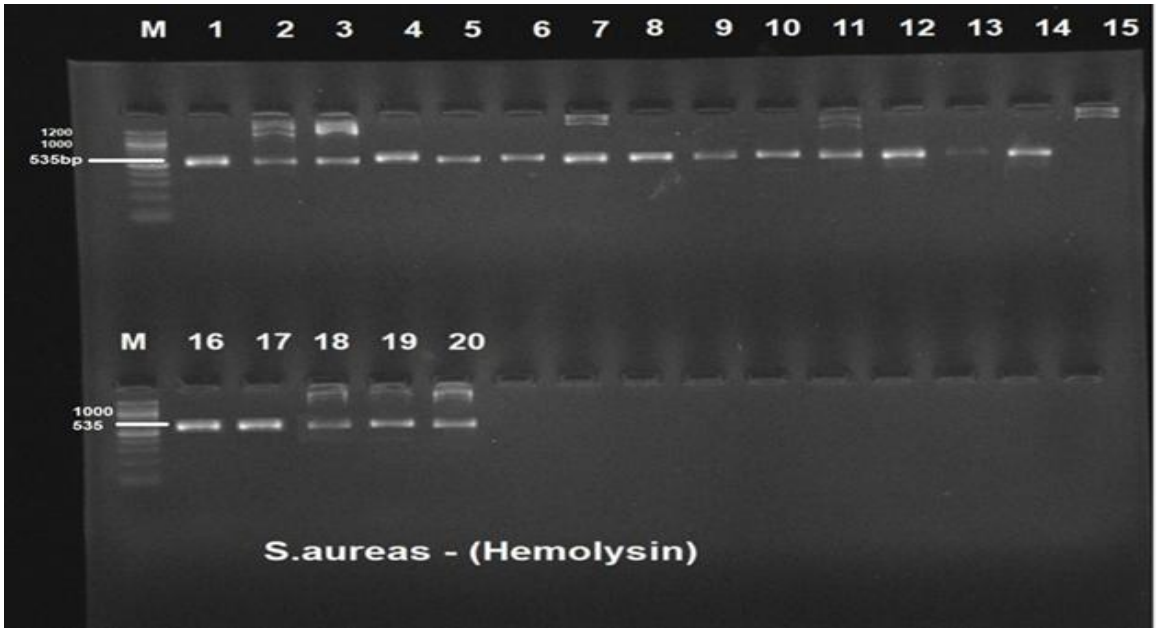

Figure 1: Electrophoresis results of the PCR product for the Hla gene of S. aureus isolates and bundles of 535bp size at a voltage of 100 v/cm for one hour, M: Volumetric index, 20-1: Isolate numbers of S. aureus.

Antibiotic resistance

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 9.

Table 9: 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

Conclusions

The study concluded that S. aureus was the most common bacterial isolate from systemic infections, with high antibiotic resistance. The Hungarian tissue results also revealed the presence of the HLA gene, responsible for hemolysin production, in 95% of S. aureus isolates, suggesting its secondary role as a virulence factor.

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عزل وتشخيص بكتيريا المكورات العنقودية الذهبية *Staphylococcus aureus* من الجهاز التنفسي مع التحري المظهري والجزيئي لجين الضراوة *Hla*

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الخلاصة:

شملت الدراسة جمع 120 عينة سريرية من مناطق الأذن الوسطى والأنف والبلعوم، أظهرت 101 عينة نموًا إيجابيًا، بينما لم تظهر 19 عينة نموًا، وللفترة من 1-11-2021 إلى 1-3-2022، من كلا الجنسين في فئات عمرية مختلفة من مستشفيات (سامراء العام - الضلوعية العام)، وبعد إجراء الفحوصات المجهرية والكيميائية الحيوية وباستخدام جهاز Vitek compact system 2، تم الحصول على 32 (31.6%) عزلة من بكتيريا المكورات العنقودية الذهبية، 19 (18.8%) عزلة من الزائفة الزنجارية، 15 (14.8%) عزلة من بكتيريا كلسية الرئة، 13 (13.13%) عزلة من المكورات العنقودية الذهبية، 7 (6.9%) عزلة من الإشريكية القولونية، 6 (5.9%) عزلة من الماكروبيوتك كاتاراليس، 5 (4.9%) عزلة من الإشريكية القولونية. 4 (3.9%) عزلات من بكتيريا P. mirabilis، تم اختبار مقاومة العزلات البكتيرية المشخصة للمضادات الحيوية باستخدام 10 مضادات حيوية وهي: أزيثروميسين، فانكومايسين، كلورامفينيكول، جنتاميسين، بنسلين ج، إريثروميسين، سيفترياكسون، سيفالوثين، أموكسيسيلين حمض الكلافولينيك وسيفوتاكسيم. أظهرت نتائج الفحص الجزيئي لجين (Hla) في بكتيريا المكورات العنقودية الذهبية، المسؤول عن الهيموليزين، اختيار 20 عزلة من هذه البكتيريا نظرًا لوضوحها وفعاليتها في انحلال الدم، وذلك بغرض دراسة جين Hla المُشفر لإنتاج الهيموليزين باستخدام تقنية تفاعل البوليميراز المتسلسل (PCR). وقد أظهرت نواتج تضخيم البادئ أن 19 عينة من أصل 20 عينة تحتوي على هذا الجين (95%)، حيث ظهرت الأشرطة بعد نقلها على هلام الأجاروز ضمن المنطقة المتوقعة لهذا الجين (535 زوجًا قاعديًا).