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Isolation, Identification of Bacterial Species Causing Chronic suppurative Otitis Media and Detection Some of Their Virulence Factors

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ABSTRACT

The study is conducted to diagnose the aerobic bacterial species causing chronic suppurative otitis media (CSOM), reveal the antibiotic susceptibility pattern and detect some of their virulence factors. Samples were collected during the period from June till December 2018. From a total of eighty-two patients admitted to Samarra Hospital and outpatient clinics of both genders with different age groups, 82 bacterial culture are recovered using a cotton swab. Identification of bacterial isolates is performed depending on micro and macroscopic cultural characteristics and biochemical tests. Results of the current work show that the highest infection rates are at the age groups >1 to 5 and 11 to 20 years by (20%). Among eight bacterial species isolated in the current study (*S. aureus, P. aeruginosa, K.pneumonia, S.epidermidis, E.coli, P.vulgaris, C. freundii, E. Cloacae*), *S. aureus* had scored the highest rate (41%) of the total infections while the lowest rate was scored by *E.Cloacae*(1%). The antibiotic sensitivity test suggests that almost all isolates were sensitive to ciprofloxacin and meropenem (96% and 94% respectively) while they were resistant to Cefixime. The ability of bacteria is isolated from CSOM to produce biofilm and some virulence factors (gelatinase, hemolysin, DNase, urease) are investigated the virulence factor results revealed that. *S. aureus, P.aeruginosa, K. pneumonia* had the ability to produce biofilm and *S. aureus, P. aeruginosa* have the ability the highest production for the majority of virulence factors.

Introduction

Otitis media is an inflammation in the middle ear between the drum and the inner ear including Eustachian tube[1] When the duration of the infection lasts for two weeks to three months, it is called "chronic otitis media and usually occur after acute infection Chronic ear infections are in two forms It either occurs with a discharge "chronic suppurative otitis media "or without a discharge called "chronic non suppurative otitis media" [2] Chronic otitis inflammation gives several symptoms, including ear discharge, deafness, itching, pain and sometimes fever.Common Bacterial species that can cause chronic otitis media(*P. aeruginosa, S. aureus, K. pneumoniae, P. vulgaris, E. coli, S. pneumonia*)[3]. Some of these species of bacteria have ability to produce biofilms, which are bacterial clusters found within matrix of hydrated extracellular polymeric substances (EPS) that help the bacteria survive in extreme conditions and resist antibiotics it is also responsible for cause of chronic infection and some diseases, including otitis media[4]. Other virulence factors are secreted outside the bacterial cell, such as enzymes and various toxin that enables bacteria to penetrate host cell[5].

The aim of the study: is to isolate and diagnose the causative microorganisms of chronic suppurative otitis media, identifying their Sensitivity to antibiotics and detection on producing biofilm and some virulence factors such as hemolysin, gelatinase, urease and DNase for the bacterial isolate.

Materials and Methods

Sample Collection and Identification:

The study includes collecting 82 samples from outpatients with otitis media and patients attending ENT clinic in Samarra hospital during the period from June to December 2018. By using sterile cotton swabs, the samples were taken from the discharge

sites (right ear, left ear or both) under the direction and supervision of the specialized physician with filled in questionnaire forms. The questionnaire information included the patients names, age, place of residence, history of the disease, location of the sample, and their antibiotic intake (Patients who were taking antibiotics were excluded).

Samples are cultured on blood agar, MacConkey agar, mannitol salt agar, they are incubated at 37 Cº for 24 hours. After incubation, the colonies are identified by colony morphology, Gram stain and biochemical tests which included. oxidase, catalase, coagulase, indole, methyl red, Voges-Proskauer, citrate utilization[6], motility (hanging drop), urease, gelatinase, DNase, and hemolysin test; in addition to KIA agar[7].

Antibiotic Susceptibility Test:

Antibiotic susceptibility test is done by Kirby Bauer disk diffusion method. The isolated bacteria are cultured in Mueller Hinton agar and the antibiotic to be tested are (table 5) were put on the surface of the agar using sterile forceps after incubation at 37 °C for 24 hours the dimeter of inhibition zones is recorded and the susceptibility or resistance of the isolates are determined according to the CLSI guidelines[8].

Detection of Virulence Factors Production:

1- Biofilm Production

Biofilm production is detected by the Congo red agar method the medium consists of heart infusion broth 37 gm/L, sucrose (50 gm/L), agar no.1 (10 gm/L) and the Congo red stain (0.8 gm/L). Congo red stain was prepared as an aqueous solution and sterilized by autoclaving at 121°C for 15 minutes, then it was mixed with medium at 55°C. The mixture is poured into Petri dishes, inoculated and incubated aerobically at 37°C for 24-48 h black colonies positive result, pink colonies negative result as shown in Fig (1)[9].

2- Urase test

900 ml of distilled water is added to the urea agar and sterilized by autoclaving at 121°C for 15 minutes, cooled to 50°C. Then a urea solution (40 g of urea was added to 100ml of distilled water, mixed thoroughly and filter sterilized) was distributed into sterile tubes by streaking it on the surface of the urea agar slants that the tubes contained. Then, the tubes were incubated at 37°C for up to 2 to 7 days yellow color indicates negative result , pink color indicates positive result as shown in Fig (2)[6,10].

3- Gelatin hydrolysis test

The Gelatin medium is prepared by adding: 15g Agar, 1g Yeast extract, 4g Peptone, and 15g Gelatin into 1000 ml of distilled water. The components were dissolved by heating then the medium was distributed into test tubes. The test tubes are sterilized by autoclaving at 121°C for 15 minutes. They are left to cool and they are inoculated and incubated at 37°C for up to 14 days; then they were placed in the refrigerator at 4°C to check for any liquefaction the result as shown in Fig (3)[6,10].

4- Hemolysin Production Test

Blood agar media were inoculated with the isolated bacteria, incubated for 24 hours at 37°C and observed for beta hemolysis as shown in Fig (4), the media were prepared by adding sterile blood to Nutrient agar[11].

5- Deoxyribonuclease (DNase) Test

DNase agar is prepared according to the manufacturer's directions, it was poured in Petri dishes and inoculated with heavy inoculum of bacteria. After inoculation and incubation for 18-24 hours at 35 °C; 1N HCl is added to the surface of the plates. hydrolyzed DNA is insoluble in HCl and will form a precipitate (negative result) while, oligonucleotides formed from the action of DNase will dissolve in HCl forming a clear zone around the inoculum (Positive result) as shown in Fig (5)[6].

Results and Discussion

The current study is involved in collecting of eighty-two cotton swab samples (82) from patients with chronic ear infection. The collected bacterial isolates were identified at the level of Species depending on microscopic exam, cultural characteristics and biochemical tests. 71 (87%) samples had recovered bacterial growth, 11(13%) samples no bacterial growth as shown in table (1) This finding was agreed with study of [12,13], where they found that 13.2% and 7.15% did not show growth, which may be more than a reason for this negative result, including viral or fungal infections.

Table 1: Positive culture and negative culture

Bacterial growth	number	Percentages
Positive Culture	71	13
Negative culture	11	87
Total	82	100

In this study, the rate of infections in females is higher comparing to that in males as shown in table (2). This study agreed with the findings of two other studies where the rate of infections in females was also higher (55%) [14] (45%) [15].

Table 2: Patients distribution according to gender

Gender	Number	Percentage
Male	32	45
Female	39	55
Total	71	100

The patients' ages ranged from six months to 75 years. The highest rates of infections (20%) were among patients aging 1_5 and at an average age of 11 to 20 years, followed by 17% at age group 6 to10 and 14% at age group 21 to 30, 8% at age group 41 to 50 were. The lowest percentage of all infection, 10% was at age group 31 to 40 years. As shown in table (3).

Table 3: Age distribution of patients with chronic suppurative otitis media

Age groups	Number	Percentage
0-5	14	20
6-10	11	17
11-20	14	20
21-30	11	14
31-40	7	10
41-50	6	8
50<	8	11
Total	71	100

As for ages, most of the affected age groups are less than 1 to5 and 11 to 20 years old this result was similar to most studies[16],This can be caused by poor hygiene and the introduction of contaminated tools in the ear and playing with soil lead to the entry of the pathogen from the external environment to the ear, or occur as a result of transmission among students[17],in children incomplete immune system contributes to increased infection rate, and to the eustachian tubes shortness making them more susceptible to infection lead to the transmission of pathogens from the respiratory system to the ear[18].

This study views that the most common isolated species causing bacterial infections was *S. aureus* (41%), followed by *P. aeruginosa* (27%), *K. pneumonia* (11%), *S. epidermidis* (10%), and *E. coli* (4%). 3% of the isolated species were *P. vulgaris* and *C. freundii*. *E. cloacae* represented the least common isolated bacterial species (1%), as table (4) shows.

Table 4: Bacterial species isolated of chronic suppurative otitis media

Bacterial isolates	number	Percentage
staphylococcus aureus	29	41%
pseudomonas aeruginosa	19	27%
klebsiella pneumonia	8	11%
staphylococcus epidermidis	7	10%
Escherichia coli	3	4%
proteus vulgaris	2	3%
Citrobacter freundii	2	3%
Enterobacter cloacae	1	1%
Total	71	100%

Similar Species of bacteria organisms were also isolated in various studies to CSOM[19,20] The results agree with the study of [3,21] where they point out that *S. aureus* and *P. aeruginosa* are the most common bacterial species in CSOM.

Antibiotic susceptibility test for bacteria isolated from CSOM the results as shown in table (5) found *S. aureus* showed 52% sensitivity with chloramphenicol, 72% with rifampicin, 93%with trimethoprim and meropenem,97% with tetracycline, and the highest sensitivity to ciprofloxacin and ampicillin it was 100% sensitivity. The lowest effect was 10% with cefixime.

In the current study *S. epidermidis* presents 100% sensitivity with rifampicin, trimethoprim, ampicillin, meropenem, tetracycline, chloramphenicol, ciprofloxacin and While the resistance was 100 % with cefixime.

Results illustrate the sensitivity of *P. aeruginosa* to antibiotics where the highest effect was for both meropenem and ciprofloxacin89% and 84% respectively. And she was 100% resistance with tetracycline, observed sensitivity26% with rifampicin, 21% with trimethoprim, 47% with gentamicin, 16% with cefixime, 68% with chloramphenicol, 58% with ampicillin.

K. pneumonia 100% were resistant rifampicin and tetracycline and showed 100% sensitivity with cefixime, meropenem and ciprofloxacin, found sensitivity of 38 % with ampicillin 13% with trimethoprim, 50% with chloramphenicol.

E. coli appears 33% sensitivity with cefixime and trimethoprim, 100% with meropenem and ciprofloxacin, and showed 100% resistant with tetracycline, chloramphenicol, ampicillin, rifampicin.

Throughout investigation *P. vulgaris* present 100% sensitivity with trimethoprim, meropenem, ciprofloxacin and tetracycline. Resistance100% with and rifampicin, ampicillin, chloramphenicol, cefixime.

C. freundii it was sensitivity 100% with trimethoprim, chloramphenicol, tetracycline, ciprofloxacin and found resistant 100% meropenem, cefixime, ampicillin and rifampicin.

E. cloacae illustrates resistant with rifampicin, ampicillin, cefixime, it was sensitivity with trimethoprim, meropenem, ciprofloxacin, tetracycline and chloramphenicol. for the sensitivity test.

Table 5: Antibiotics Susceptibility test of Bacteria Isolated from Chronic suppurative Otitis Media

Antimicrobial agent	*Staphylococcus aureus* N=29		*Staphylococcus epidermidis* N=7		*Pseudomonas aeruginosa* N=19		*Klebsiella pneumonia* N=8		*Escherichia coli* N=3		*Proteus vulgaris* N=2		*Citrobacter freundii* N=2		*Enterobacter cloacae* N=1		Total
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	%
RIF	21(72)		7(100)		5(26)		0(0.0)		0(0.0)		0(0.0)		0(0.0)		0(0.0)		46%
TMP	27(93)		7(100)		4 (21)		1(13)		1(33)		2(100)		2(100)		1(100)		63%
CN	NT		NT		9 (47)		NT		NT		NT		NT		NT		47%
AMP	29(100)		7(100)		11 (58)		3(38)		0(0.0)		0(0.0)		0(0.0)		0(0.0)		70%
CFM	3(10)		0(0.0)		3 (16)		8(100)		1(33)		0(0.0)		0(0.0)		0(0.0)		21%
MEM	27(93)		7(100)		17 (89)		8(100)		3(100)		2(100)		0(0.0)		1(100)		94%
C	15(52)		7(100)		13 (68)		4(50)		0(0.0)		0(0.0)		2(100)		1(100)		62%
TE	28(97)		7(100)		0 (0.0)		0(0.0)		0(0.0)		2(100)		2(100)		1(100)		40%
CIP	29(100)		7(100)		16(84)		8(100)		3(100)		2(100)		2(100)		1(100)		96%

RIF:rifampicin, TMP: trimethoprim CN: gentamicin, AM: ampicillin, CFM: Cefixime, MEM: meropenem, C: chloramphenicol, TE: tetracycline, CIP: ciprofloxacin, NT: Not test, N: number

It has that the most effective drug for the treatment of ear infection is ciprofloxacin, meropenem and this was results agree based on a number has been by a number of researchers[22,23]. The study the remarks that the majority of isolates possessed resistance against Cefixime can be due to several reasons, most importantly the bacteria have enzyme production AmpC βlactamases that also hydrolyze especially 3rd generation this enzyme leads to the hydrolysis of the beta-lactamase ring of the antibiotic which makes the antibiotic ineffective[24].

The results reveals the detection of virulence factors as shown in the table (6) that the *S. aureus*, *P. aeruginosa*, *K. pneumonia*, and *C. freundii* have the ability to produce a biofilm this is agreed with several studies by different researchers who found that both *P. aeruginosa* and *S. aureus* and *K. pneumonia* were also biofilm producers.[4,25,26] The results of the current study agrees with[27], where they view that the isolates of the *Citrobacter* they ability product biofilm by the Congo red method.

The ability of bacteria to produce extracellular enzymes as shown in table 3 that the isolates of *S. aureus*, *P. aeruginosa* and *K. pneumonia* were shown to produce urease and *P. aeruginosa* and *E. cloacae* were able to produce gelatin enzymes , The results also affirm that the isolates of *S. aureus* and *P. aeruginosa* were able to hemolysin on blood agar , The DNase test was performed on *S. aureus* isolates where it produces these enzymes extracellular enzyme is similar to most of the findings in the research[28-30].

Table 6: Detection results of virulence factors

Bacteria	Urease	Gelatinase	Hemolysin	DNase	Biofilm
Staphylococcus aureus	+	_	+	+	**V**
Staphylococcus epidermidis	_	_	_	NT	_
Pseudomonas aeruginosa	V	+	+	NT	**V**
Klebsiella pneumonia	+	_	_	NT	+
Escherichia coli	_	_	_	NT	_
Proteus vulgaris	_	_	_	NT	_
Citrobacter freundii	V	_	_	NT	+
Enterobacter cloacae	_	+	_	NT	_

+:Positive - : Negative V: Variable N T: Not test

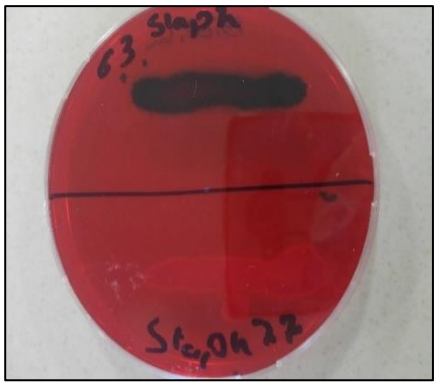

Fig. 1: Biofilm detection by Congo red agar Black colonies show biofilm formation, Red colonies show non biofilm formation

Fig. 2: Urease test Pink color positive result, yellow negative result

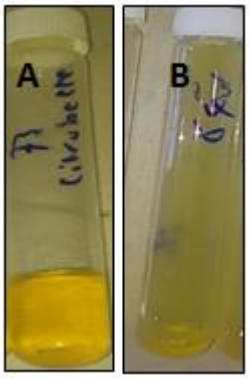

Fig. 3: Gelatin test A: positive Result B: negative Result

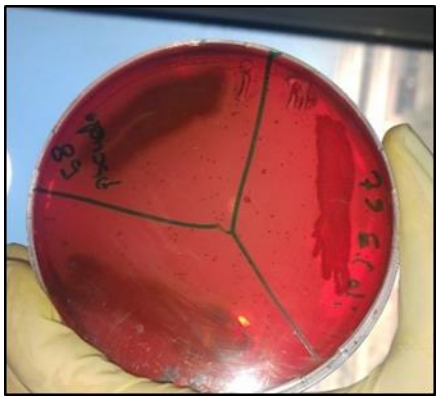

Fig. 4: Hemolysin test: Green colonies positive result , Red colonies negative result

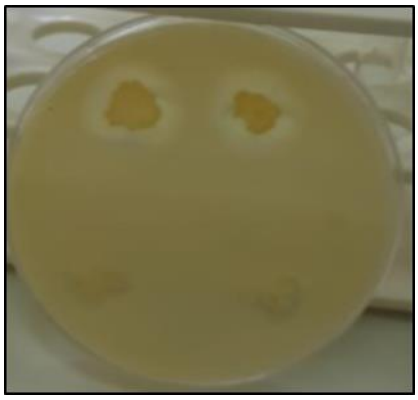

Fig. 5: DNase test positive result show clear zone around colony

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عزل وتشخيص الأنواع البكتيرية المسببة لالتهاب الاذن الوسطى القيحي المزمن والتحري عن بعض عوامل الضراوة

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[2]كلية الطب ، جامعة تكريت ، تكريت ، العراق

الملخص

أجريت هذه الدراسة لتشخيص الأنواع البكتيرية الهوائية المسببة الالتهاب الاذن الوسطى القيحي المزمن ولإجراء اختبار الحساسية للمضادات الحيوية والكشف عن قابلية البكتيرية لتكوين عوامل الضراوة، تم جمع 82 عينة لكلا الجنسين ومن مختلف للمرضى الوافدين الى مستشفى سامراء العام والعيادات الخارجية خلال الفترة (شهر تموز الى كانون الأول 2018) جمعت العينات باستخدام ماسح قطني وتم تشخيص العزلات بالاعتماد على الفحوصات المجهرية والبكتيرلوجية والاختبارات الكيموحيوية، أظهرت نتائج الاختبارات أن معدلات الإصابة كانت اعلى في الفئات العمرية اقل من سنة الى 5 ومن 6 الى 11كانت بنسبة 20%.وتم الكشف على ثمانية أنواع بكتيرية (*Staphylococci aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococci epidermidis, Escherichia coli, Proteus vulgaris, Citrobacter freundii, Enterobacter Cloacae*) وكانت اعلى معدل للإصابة هي *Staphylococci aureus*بنسبة 41% واقل نسبة للإصابة هي *Enterobacter Cloacae* بنسبة 1%. فيما أظهر اختبار الحساسية للمضادات الحيوية ان جميع العزلات تقريباً كانت حساسة لكل من ciprofloxacin و meropenem (96% و 94% على التوالي) في حين ان الغالبية كانت مقاومة للمضاد الحيوي Cefixime 21%. وتم التحري على قدرة البكتريا المعزولة من التهاب الاذن القيحي المزمن (CSOM) على انتاج الغشاء الحيوي وبعض عوامل الضراوة مثل(gelatinase , hemolysin, DNase , urease) وأوضحت النتائج ان كل من *S. aureus, P. aeruginosa, K .pneumonia* كان لها القدرة علة انتاج الغشاء الحيوي كما ظهرت ان كل من *S. aureus* و *P.aeruginosa* لديها اعلى قدرة اعلى انتاج غالبية عوامل الضراوة.