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الصفحة	فهرس البحوث	ت
12 – 1	Impact of Vitamin D3 Deficiency on Liver and Adipose Tissue in Pregnant Mice Amenah Salman Mohammed	1
23 – 13	Diagnostic potential of salivary MMP-9 to differentiate between periodontal health and disease in smokers and non-smokers Tamarah Adil Mohammed Hussein Omar Husham Ali	2
35 – 24	Salivary IL-10 and TNF-α levels in Dental Caries Detection in Pediatric β-Thalassemia Major Patients Ban Hazem Hassan Zainab Abduljabbar Athab	3
46 - 36	Compare Robust Wilk's statistics Based on MM-estimator for the Multivariate Multiple Linear Regression Thamer Warda Hussein Abdullah A. Ameen	4
58 – 47	Curvature Inheritance Symmetry of C_9 –manifolds Mohammed Y. Abass Humam T. S. Al-Attwani	5
67 - 59	The issues of cultural expressions untranslatability from Iraqi Arabic into English language Ahmed Mohamed Fahid	6
80 - 68	Hematological and biochemical parameters changes associated with Coronavirus Disease (COVID-19) for some patients in Missan Province Anas, S. Abuali	7
89 - 81	Evaluation of the diagnostic efficacy of salivary malondialdehyde among smokers and nonsmokers with periodontal disease: A case-control study Haneen Fahim Abdulqader Maha Sh. Mahmood	8
104 - 90	Mapping the Slopes' Geomorphological Classification Using Geomatics Techniques: A Case Study of Zawita, Iraq Mohammed Abbas Jaber Al-humairi Elaf Amer Majeed Alyasiri	9
112 - 105	Enhancement methods of intrusion detection systems using artificial intelligence methods (TLBO)Algorithm. Mohammed Saeed Hashim Al-Hammash Haitham Maarouf	10
124 - 113	In Silico Interaction of Select Cardiovascular Drugs with the Developmental Signal Pathway Pax3 Sarah T. Al-Saray	11
135 - 125	Influence of gingivitis in preterm delivery on serum biomarkers COX-2 and PGE-2 Shaden Husham Maddah Ghada Ibrahim Taha	12
143 - 136	Detection and Identification of Chlamydia causing Ear infection by PCR. Rabab Saleh Al.sajedy Ghaida'a . J. AL.Ghizzawi	13
152 - 144	Metric areas and results of best periodic points Maytham zaki oudah Al Behadili	14
157 - 153	Structural and Optical Properties of Co doped CdS Nanoparticles Synthesised by Chemical Method Uday Ali Sabeeh Al-Jarah Hadeel Salih Mahdi	15
166 - 158	The occurrence of <i>Lactobacillus</i> and <i>Candida albicans</i> in patients with thyroid disorders Riam Hassoun Harbi Maha Adel Mahmood	16

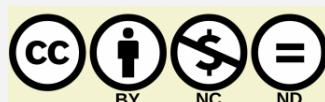
173 - 167	An overview of the loquat's (Eriobotrya japonica) active components Shahad Basheer Bahedh Dina Yousif Mohammed	17
183 - 174	Study the mineralogy of Al-Faw soil in southern Iraq and determine swelling properties by indirect methods Haneen.N. Abdalamer Huda.A.Daham	18
192 - 184	The Role of pknF and fbpA as a virulence genes with Interleukin4-and 6, in the Pathogenesis of Tuberculosis Samih Riyadh Faisal	19
203 - 193	لغة الانفعال في النص الشعري التسعيني أحمد عبد الكريم ياسين العزاوي	20
218 - 204	الحماية الدستورية لحقوق الأطفال عديمي الجنسية في التعليم في التشريعات العراقية (دراسة مقارنة) الباحث كامل خالد فهد هند علي محمد	21
230 - 219	التنبؤ بالطلب على الخزين باستعمال الشبكات العصبية الاصطناعية مع تطبيق عملي أيمن خليل اسماعيل لمياء محمد علي حميد	22
240 - 231	بعض التقديرات المعلمية واللامعلمية لأنموذج الانحدار الدائري بالحاكاة رنا صادق نزر عمر عبد المحسن علي	23
258 - 241	القتل في القران والسنة (دراسة في الاسباب والاثار والوقاية) جاسب غازي رشك	24
271 - 259	الطريقة الصوفية البكتاشية دراسة تحليلية جبار ناصر يوسف	25
286 - 272	السياسات التعليمية في الفكر الإسلامي مدخل لتعزيز البناء الاجتماعي حامد هادي بدن	26
306 - 287	دراسة سندية لحديث: (أهل بيتي أمان لأمتي...) وفق المنهج الحديث عند أهل السنة حكمت جراح صبر	27
321 - 307	القياس والافصاح المحاسبي عن الانتاج المرئي وفق معايير المحاسبة الدولية رائد حازم جودة خوله حسين حمدان	28
332 - 322	اسس تطبيق فن الايكيبانا في دروس الإشغال الفنية بقسم التربية الفنية سهاد جواد فرج الساكني	29
353 - 333	تنبؤ العلاقات العامة بالآزمات عبر تطبيقات الذكاء الاصطناعي ليث صبار جابر	30
374 - 354	روايات أهل البيت (ع) في مدح وذم أهل الكوفة دراسة تحليلية محمد جبار جاسم	31
385 - 375	تجليات الصراع الوجودي في لامية اوس بن حجر مشتاق طالب منعم	32
392 - 386	ازدواجية الهوية الدينية وفهم الذات في رواية (عازف الغيوم) لعلي بدر أنموذجا نور خليل علي	33
402 - 393	مشروع الحلف الاسلامي السعودي وموقف الكيان الصهيوني (دراسة تحليلية في الوثائق الامريكية) سعد مهدي جعفر	34



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Detection and Identification of *Chlamydia* causing Ear infection by PCR.

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ABSTRACT:

Chlamydia bacteria were isolated and identified from One hundred ear swab are collected from patients had otitis externa and otitis media. Ear swabs are taken from patients attending the ENT consulting clinics at Basra General Hospital and Abi Al-Khasib Hospital, of both sexes and of different ages, from November 2022 to May 2023. Polymerase chain reaction (PCR) is used to diagnose 19 cases of chlamydia, including 11 cases of chlamydia trachomatis and 8 cases of chlamydia pneumonia, There are 14 co-infections with other bacteria and 5 single cases of *chlamydia*. The results of the current study shows the registration of 19 cases of *C.trachomatis*, *C.pneumoniae* and more injuries within the age group (1monthes-10) years, by 7 cases of infection and by 36.8%, while 5 cases of infection are recorde in the age group (11-20) years and by 26.3%, 4(21.05%) cases of infection in the age group (21-30) years ,Two cases of infection in the age group (31-40) rate of (10.5%) and One case (5.2%) in the age group (>60) .These pathogens are find inside cells that are difficult to identify, and they are the cause of pneumonia, ear effusion, inflammation of the auditory canal, and perforation of the eardrum, which affects the hearing mechanism.

Keywords: Chlamydia trachomatis, Chlamydia pneumonia, Otitis Externa, Otitis Media , Hearing Loss ,PCR reaction.

1. Introduction:

Ear infection represented by otitis externa(OE) and otitis media(OM), OE is a group of acute or chronic infections or inflammation with effusion in the external auditory canal or the pinna of the ear swimmer's ear.(Hateet *et al* .,2022).

It occurs as a result of disruption of the protective squamous epithelial layer of the ear canal through infection with chlamydia or other bacteria or as a result Several risk factors that predispose the individual to develop otitis externa, including water trapping in the ear canal due to sweating, dampness and swimming.(Wiegand *et al* .,2019) .Either epithelial disintegration occurs as a result of an increase in the pH or removal of serum as a result of negative cleaning of the auditory canal. Acute otitis externa causes auditory fullness and itching. Trachomatis (Pantazidou, *et al*.,2022).

Otitis media is one of the most common diseases in children and is considered the second most important cause of hearing loss and decreased ability to learn and academic achievement represented

by acute and chronic inflammation or otitis media with effusion or inflammation of the tympanic

membrane (Tesfa *et al.* ,2020) This occurs as a result of viral upper respiratory tract infection that disrupts the mucosal system and weakens the mechanical defense. The primary infection of the host is caused by bacterial invasion, chlamydia, or a defect in the Eustachian tube, or as a result of genetic and environmental factors. (Bhatia *et al.*,2022).

Among the most important and dangerous pathogens of upper respiratory tract infection and otitis media in children are *Chlamydia trachomatis* and *Chlamydia pneumoniae*, as the ear is considered the appropriate environment for its reproduction (Daloglu ,2023). *Chlamydia pneumoniae* is a common pathogen of upper respiratory tract infection, bronchitis, sinusitis, otitis media, and pneumonia due to the high affinity of the epithelial lining of the airways.(Daloglu ,2023)

This study aims to isolation and identification of *Chlamydia trachomatic* and *chlamydia pneumoniae* associated with otitis externa and otitis media. It is estimated that *Chlamydia pneumoniae* infection causes about 70 asymptomatic or very few respiratory infections, 20 symptomatic upper respiratory tract infections and 10 cases of pneumonia. (Alves *et al.* ,2020).

Chlamydia trachomatis is also considered a pathogen causing ear infections since 1960 and has been isolated from patients with acute ear infections or otitis media with effusion. (WHO ,2022) In addition , It causes eye infections (Thajeel,2019)and injuries to premature babies.(Jomaa,2022).

Polymerase chain reaction(PCR) is a technique which allows for the amplification of limited part of the bacterial DNA present in the middle ear fluid, (Sawada *et al.*, 2019) also indicated that *C. pneumoniae* is the main cause of pneumonia and otitis media and was diagnosed with PCR technology. In addition to the health and economic situation, it has a significant impact on the occurrence of ear infection and is considered one of the obstacles affecting the health of the ear. The study of (Block *et al.* ,1997) indicated that *C. pneumoniae* is a common cause of pneumonia and otitis media in children and found within his study that 8 children with acute ear infections and discovered five infections were positive *C. pneumoniae*. A study Hammerschlag(2000), isolated *C. pneumoniae* from middle ear fluid in children with ear infections, and indicated that pneumonia may play a role in causing acute middle ear infections.

2- Materials and Methods :

Samples were collected through the use of special sterile cotton (Swaps) for patients with otitis externa and otitis middle , The swap was placed in a special sterile and refrigerated container by an ice bag refrigerated box according to the method (Furr and Taylor-Robinson, 1987), and transferred directly to the laboratory to detect the presence of *chlamydia* through DNA extraction using Presto Buccal Swab g DNA extraction kit and amplified by (PCR) Polymerase chain Reaction to detect it moleculely. (Sawada *et al.*, 2019)

2-1 DNA extraction of Chlamydia:

A complete kit prepared by Geneaid is used to extract DNA from the study samples according to the manufacturer's steps as follows:

- Sample preparation: removing the lumbar part of the swaps by sterile tweezers and placed in a centrifugal tube (Eppendorf tube) with a size of 1.5 ml, added to it (500) microns of S1 Buffer solution, and (20) microns of Proteinase K and mixed by the mixer Vortex for 10 seconds, and then incubated with a water bath at a temperature of 60 m ° C for 10 minutes, After the tubes came out of the water bath, the cotton part and the solution from it were transferred to the filter column tubes after placing them in the collection tubes with a size of 2 ml using sterile forceps for each sample, while keeping the centrifuge tubes aside.

- DNA suspension: (500) ml of absolute ethanol and cooled are added and mixed well, transfer (750) ml of the mixture to GD Column and centrifuge him (14-16000) g for one minute, then get rid of the filtrate and tube and transfer GD Column filter to a new collection tube (2) ml.
- Wash: Add (400) ml of W1 Buffer solution to GD Column and make a centrifuge for (30) seconds and then put the GD column tube in a new collection tube and get rid of the filtrate and was placed in the centrifuge for (3) seconds for drying.
- Elution: Transfer GD Column to the eppendorf tube clean and sterile centrifuge tube and add (100) ml of warm dissolving solution Elution Buffer (withdraw a quantity of dissolving solution directly from the can and put in the eppendorf tube tubes and placed in the water bath for heating, the required amount is taken and added to the sample for the purpose of dissolving DNA) and leave for (3) minutes and make a centrifuge for a minute and repeat it again and then keep the DNA extract in freezing in -20°C.

2-2 PCR test: Four specific primers are designed for 16 s ribosomal gene, two primers of *Chlamydia trachomatis* genome and two primers of *Chlamydia pneumoniae* . The primer sequences to *C.trachomatis* are as: Forward: 5'-TGG CGG CGT GGA TGA GGC AT-3' and Reverse: 5'-CTC AGT CCC AGT GTT GGC GG-3' and the length of PCR target is 300 bp (12). The primer sequences to *Chlamydia pneumoniae* are as: Forward: 5' – AAGGGCTATAAAGGCGTTGCT-3 and Reverse: 5' –TGGTGCGCAGACTTTGTTCCA- 3' and the length of PCR target is 474bp . PCR reaction was done in a total volume of 20 µl PCR master mix (13).As in the table (1).

Table 1.PCR solution reaction mixture.

Chemicals	Volume
Master mix	5 ml
Premier Forward	1 ml
Premier Reverse	1 ml
DNA	7 ml
DdH2O	6 ml
Total Volume	20 ml

2-3 PCR amplification program: The Spring thermal cycle device is used to amplify the DNA to *C.trachomatis* after all additions are made and according to the PCR program (Hameed and Al – Farttoosy,2022) . Initial denaturation at 94°C for 3 min, followed by 30 cycles of denatuation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 1min; and final extension at 72°C for 5 min. The DNA of *C. pneumoniae* was amplified after completing the additions according to the PCR program, Initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, extension at 72 °C for 1min; and final extension at 72°C for 5 min. . PCR products were separated by electrophoresis in 2% gel agarose, stained with ethidium bromide and visualized by UV light. (Keles ,2005)

2-4 The statistical program SPSS (Statistical package for Social Science) Ver.11 was adopted to determine the statistical significant differences between the different variables in the statistical analysis using the sample homogeneity test (chi-square).

3-Results and Discussion :

100 samples are collected from several hospitals in the city of Basra, The results of the study shows that chlamydia trachomatis, chlamydia pneumonia and other bacteria can be isolated from patients with otitis media and otitis externa (acute,chronic and with efussion). Polymerase chain reaction (PCR) is used to detect chlamydial infection in people with Otitis media and otitis externa because this reaction proved to be more sensitive and to identify the microbial type quickly and inexpensively than traditional microbiology methods to check for the presence of infection-causing microbes in the middle and outer ear. These molecular methods provide More positive results than traditional methods. (Sawada *et al.*, 2019).

19 cases of *chlamydia* bacteria were diagnosed , including 11 cases of *chlamydia trachomatis* and 8 cases of *chlamydia pneumoniae* as in Table (1). There are 14 cases of co-infection with other bacteria and 5 single cases of *chlamydia*. The special gene is amplified by the forward and reverse primers of the two types of *chlamydia* and gave a positive result after comparing it with the ladder size (100-2000)pb. as shown in fig.(1) and (2)

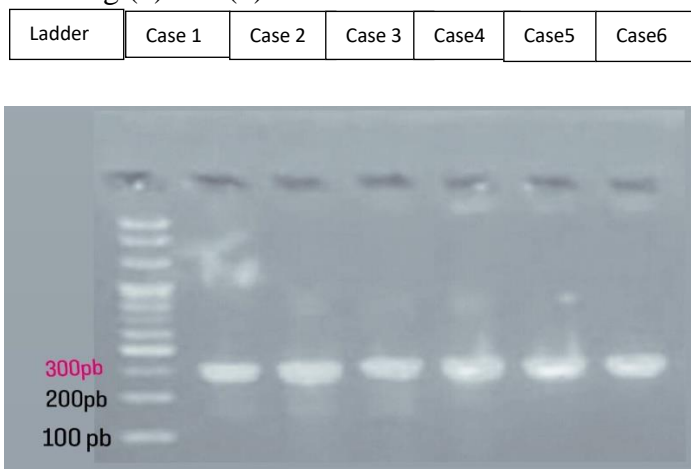


Fig. (1) Electrophoresis of *C.trachomatis* products (300bp) on agarose gel at a concentration of 2% and dye with ethidium bromide dye 0.5 microns with a voltage difference of 90 volts for (45-30) minutes.

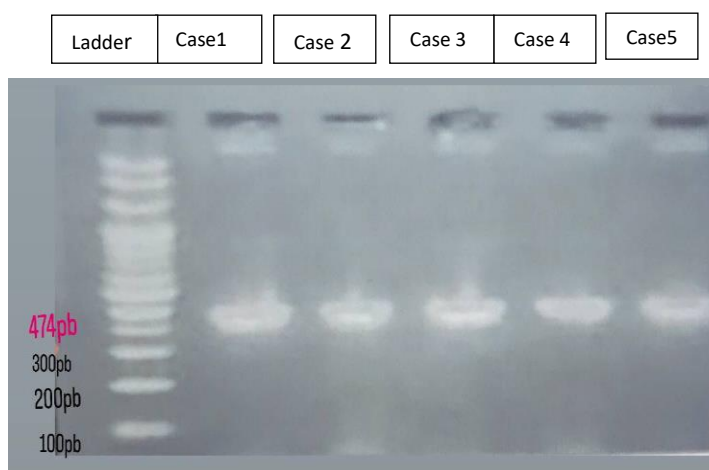


Fig.(2)Electrophoresis of *C.pneumoniae* products (474bp) on agarose gel at a concentration of 2% and dye with ethidium bromide dye 0.5 microns with a voltage difference of 90 volts for (45-30) minutes.

The results of the current study shows the presence3(15.7%) of single infections with *Chlamydia trachomatis*, two single infections with *Chlamydia pneumoniae* (10.5%), and the presence of combined infections with *Chlamydia trachomatis* and *Chlamydia pneumoniae* with other bacteria 8(42.1%), 6(31.5%) respectively. As it mentioned in the Table (2).

Table2 . Distribution of single and co-infections of *C.trachomatis* and *C.pneumoniae* in middle and outer ear infections.

Chlamydia species	Single injuries	Common injuries	The total number
<i>C.trachomatis</i>	3(15.7%)	8(42.1%)	11(57%)
<i>C.pneumoniae</i>	2(10.5%)	6(31.5%)	8(42.1%)
Total	5	14	19

The results of the current study shows the registration of 19 cases of *C.trachomatis*, *C.pneumoniae* and more injuries the age group (1mon-10) years by 7 cases of injury and by 36.8%, It is considered the most affected age group , while 5 cases of infection were recorded in the age group (11-20) years and by 26.3%, 4 cases of infection in the age group (21-30) years by 21.05% and two cases of infections in the age group (31-40) years by 10.5% and one case of infection in the group (>60) years by 5.2% , as shown in Table (3). The results of the statistical analysis of the percentages showed that there are not significant differences below the level of significant ($P \leq 0.05$).

Table 3. Distribution of Study Samples by Age Groups of Patients with Chlamydia.

Age group	Number of infection cases	Percentage
1 month- 10	7	36.8%
11-20	5	26.3%
21-30	4	21.05%
31-40	2	10.5 %
41-50	-	-
51-60	-	-
>60	1	5.2%
Total	19	-

($P \leq 0.05$, $\chi^2=6.000$)

There were also 7 cases of infection with the highest infection rate (36.8%) in the age group (1mon -10 years), due to the widening and short of the Eustachian tube in children, especially infants, which leads to milk coming down through the Eustachian tube to settle in the middle ear cavity. As the European authors noted in Their studies that children are more susceptible to developing purulent otitis media , which is the main cause of hearing loss in children aged (2-7) years, at a rate of 30.2%. This is what paves the way for opportunistic bacteria to cause infection, and continuous swimming leads to entering water, especially polluted water. This causes the growth of fungi, which in turn affects the lining membranes and gives an opportunity to Chlamydia and some types of bacteria to cause infection or exposure to viruses (especially the common cold).(Kim *et al.*,2019).

As indicated Keles *et al.*,(2005) age group (2-14) is more infected with *C. trachomatis*. Sawada *et al.*,(2019) also showed in his study that *C. pneumoniae* and other bacteria are present in the fluids of the middle ear and nasopharynx of pediatric patients with acute otitis media.

Table 4. Distribution of Study Samples by Sex of Patients with *C. trachomatis* and *C. pneumoniae*.

Gander	Number	Percentage
Male	7	36.8%
Female	12	63.1%

($P \leq 0.05$, $\chi^2=1.316$)

The results of the current study shows females were infected with *Chlamydia* bacteria that cause ear infections 12 (63.1%) more 7 (36.8%) males infections. This indicates that females are more injured than males. The results of the statistical analysis showed that there were no significant differences in terms of sex for patients infected with Chlamydia bacteria that cause ear infections below a significant level ($P \leq 0.05$). As show in Table (4).

This is due to increased ear moisture in women and their exposure to upper respiratory infections directly or indirectly through taking care of children with colds or touching their

belongings. This study is consistent with what was found. To it(Ogawa, 1990) he found 2 cases of C.trachomatis infection out of 6 patients with otitis media, 3 cases out of 13 patients with otitis media with infiltrates, and 9 cases out of 31 patients with suppurative otitis media

The results of the current study shows that among 100 patients, 51(62.1%) cases have otitis media and 40 patients with otitis externa. Of these 12 cases of *chlamydia* infection, which causes OM, and 7 cases of *chlamydia* infection of OE. Table (5).

which indicates that most cases of ear infection are otitis media as a result of continuous exposure to viral upper respiratory tract infections , which sets the stage for infection. Bacteria can disrupt the mucous system ,which weakens immunity or the basic mechanical defense of the host. . They are transmitted through the Eustachian tube, which is responsible for ventilation of the middle ear and drainage of fluids formed in the middle ear. The channel opens in the pharynx near the nose. It is natural for microbes to move from the pharynx and nose into the tube and then reach the middle ear(Coronado-Cerda.*et al* .,2020)

Table 5. shows the types of infection for patients with ear infections

Injury cases	Number of infection	Percentage %
Otitis Media(OM)	51	62.1 %
Otitis Externa(OE)	40	48.7 %
Chlamydia cases OM	12	23.5%
Chlamydia cases OE	7	17.5%

($P \leq 0.05$, $\chi^2=1.330$)

This study is similar to what Pontefract *et al* .,(2019) indicated that acute otitis media and otitis media with infiltrates (suppuration) occur primarily in children, while otitis externa occurs in children and adults. The study Mehel *et al* .,(2022)that external otitis occurs at any age and is diagnosed through symptoms and examination results and often occurs in the summer. The study McBride *et al* .,(2020) proved that quarantine reduced the incidence of ear infections due to the decrease in the incidence of ear infections. Respiratory.

4-CONCLUSION :

Based on the results of the current study, the following conclusions can be reached:

- 1 - *Chlamydia* has emerged as the second cause of ear infections after *S.aureus* bacteria.
- 2- Two types of *chlamydia* appeared in this study: *C. pneumoniae* and *C.trachomatis*.
- 3 - It is possible for the patient to become infected with *Chlamydia pneumoniae*, which causes upper respiratory tract infection and for the infection to spread to the middle ear through the Eustachian tube.

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Detection and Identification of *Chlamydia* causing Ear infection by PCR.

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Summary:

The current study aimed to isolate and diagnose the bacteria *Chlamydia* causing otitis from infected patients in Basra city. 100 swabs were collected from patients from government hospital consultants in Basra city for the limited period from 1 - November -2022 to 31 - May -2023.

Chlamydia bacteria were isolated and diagnosed through DNA extraction by . Buccal swab gDNA extraction kit.

The current study showed that the number of cases of otitis media 51 (62.1%) is more than the cases of otitis externa 40 (48.7%), and showed 20 (24.3%) cases of perforation of the eardrum membrane and 27 (32.9) cases of ear suppuration.

Using molecular diagnostic methods, 11 (57%) cases of *Chlamydia trachomatis* and 8 (42.1%) cases of *Chlamydia pneumoniae* were isolated , The effect of infection on patients was studied in terms of age groups and sex, the age group (1 month - 10 years) years appeared to be the most exposed to infection with *Chlamydia* bacteria and causing ear infection, the study found that the rate of infection of females with *Chlamydia* bacteria 12 (63.1%) is more than the rate of male infection 7 (36.8%), where it showed 5 cases of single infection *Chlamydia* and 14 cases of infection combined with other bacteria.

تحديد وتشخيص *Chlamydia* المسببة لعدوى الأذن باستخدام PCR.

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الخلاصة :

هدفت الدراسة الحالية الى عزل وتشخيص بكتريا *Chlamydia* المسببة لالتهاب الاذن من المرضى المصابين في مدينة البصرة . جمعت 100 مسحة من المرضى من استشارات المستشفيات الحكومية في مدينة البصرة للفترة المحدودة من 1 - تشرين الثاني -2022 الى 31 - ايار -2023 .

تم عزل وتشخيص بكتريا *Chlamydia* من خلال استخلاص الحامض النووي DNA بواسطة Buccal swab . gDNA extraction kit .

اوضحت الدراسة الحالية ان عدد حالات الاصابة بالتهاب الاذن الوسطى Otitis Media 51 (62.1%) اكثر من حالات الاصابة بالتهاب الاذن الخارجية Otitis Externa 40 (48.7%) . واطهرت 20 (24.3%) حالات اصابة بتقرب غشاء طبلة الاذن و27 (32.9) حالة اصابة بتقيح الاذن (الخارجية والوسطى) .

باستخدام طرق التشخيص الجزيئي تم عزل 11 (57%) حالة اصابة *Chlamydia trachomatis* و8 (42.1%) حالات اصابة *Chlamydia pneumoniae* . دُرُس تأثير الاصابة على المرضى من ناحيتي الفئات العمرية والجنس , ظهرت الفئة العمرية (1 شهر – 10 عاماً) سنة هي الاكثر تعرضاً للإصابة ببكتريا *Chlamydia* والمسببة عدوى الاذن, وجدت الدراسة ان نسبة اصابة الاناث ببكتريا *Chlamydia* 12 (63.1%) اكثر من نسبة اصابة الذكور 7 (36.8%). حيث اظهرت 5 حالات اصابة مفردة *Chlamydia* و14 حالة اصابة مشتركة مع البكتريا الاخرى.