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ISOLATION AND IDENTIFICATION OF PSEUDOMONAS AERUGINOSA CAUSING OTITIS MEDIA AND INVESTIGATION OF SOME GENES OF VIRULENCE FACTORS OF IT IN RAMADI CITY

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ABSTRACT:

(125) samples of otitis media were collected from patients attending Al-Ramadi General Hospital and private medical clinics. 50 isolates belonging to Pseudomonas aeruginosa bacteria (40%) were isolated and diagnosed from the total samples. Microscopic and phenotypic, biochemical tests were performed, and the diagnosis was confirmed by Phytek2 2, 20 isolates were selected from among the 50 isolates with the most virulence factors for phenotypic virulence, for a PCR test. The results of the qualitative investigation of the genes (FliC, PlcH, GyrB) of virulence factors showed the presence of these genes in most of the samples in the bacteria P. aeruginosa at a percentage of 90%. The results of the sequencing of nitrogenous bases also confirmed the agreement of the diagnostic results for P. aeruginosa isolates with the isolates registered in NCBI using the MEGA7 program and the percentage of congruence was 100% For each of the gene PlcH isolate No. (8) and the gene GyrB isolate No. (20). As for the FliC gene for isolate No. (5), it showed a number. There were two transversion mutations and two nonequivalent transversion mutations, with a similarity ratio of 99%). In principle, it can be inferred that the plcH, gene and gyrBgenes were found in the same bacterial genus, but in other strains and species with the same sequence of nitrogenous bases.

Keyword: Otitis media, Pseudomonas aeruginosa, gene.

I. INTRODUCTION:

Otitis media (OM), or what is known as middle ear infection, is a group of complex inflammatory conditions associated with pathogens, whether bacterial or viral, that affect the middle ear cavity (1). Otitis media occurs in children and adults, but the rate of its incidence in children is more (2) and some studies have indicated that more than 80 % of children with this infection in the United States (Sun et al.2021). Some studies have also indicated that there are 31 million new cases of otitis media in the world annually. Otitis media is a common disease in the city of Ramadi, with a prevalence of 13.65% (3). The most common pathogens of otitis media are gram-negative bacteria such as Pseudomonas aeruginosa, followed by gram-positive bacteria such as Staphylococcus aureus, Proteuesspp, Klebsiellaspp and E. Coli (4)

otitis media can be caused by more than one bacterial type(5).Pseudomonas aeruginosa is an opportunistic pathogen of great importance, especially in cases of otitis media, due to its ability to develop resistance to many antibiotics (6).Therefore, these bacteria are considered one of the most important pathogens of otitis media, in addition to their possession of a large number of virulence factors, whether they are cell-related, such as endotoxin, cilia, flagella that cause infection, colonization of host cells, resistance to immune response, phagocytosis, or the formation of exocellular factors such as caltoxins. With low molecular weights that are secreted by bacteria outside the cell, the toxins and enzymes produced by these bacteria lead to significant tissue damage (7). This study aimed to isolate and diagnose P. aeruginosa bacteria from patients with Otitis media, and to investigate some genes of virulence factors in them using PCR, and then perform a sequence analysis of the genes using the Gene Seuecing technique.

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II. MATERIALS AND METHODS:

Samples collection:

125 samples were collected from patients suffering from chronic suppurative otitis media for both sexes and for all age groups after their diagnosis by otolaryngologists in the consulting clinics of Ramadi Teaching Hospital and private medical clinics in Ramadi for the period from 15/9/2020 to 15/3/2021.

Identification:

Bacterial colonies appeared when grown on Pale agar medium due to their inability to ferment lactose sugar (8). Then they were grown on differential media and on stermid medium to confirm the morphological and microscopic diagnosis and incubated aerobically at 37°C for 24 hours. An hour later, biochemical tests were conducted, and the diagnosis was confirmed with the Phytek2 2.

DNA genome extraction:

Genomic DNA was extracted from the selected samples (20) of P. aeruginosa isolates using a DNA extraction kit produced by Promega Genomic Extraction Kit. Specific primers for genes, fliC, PlcH, and GyrB were prepared by (Alpha DNA Canada).

DNA Primer

The specific prefixes for thEfliC,PlcH,GyrB gene were provided by Alpha DNA Canadacompany as shown in Table (1)

Table 1: Primers and size products

No.	اسم البحث	الجين والحجم الجزيئي (bp)	5-3→ (Primer) البادىء
1	Aghamoliaei et al,2015	*gyrB* 222	F:CCTGACCATCCGTCGCCACAAC R: CGCAGCAGGATGCCGACGCC
2	Sabharwal et al.,2014	*fliC* 1.02 kb (type a) 1.25 kb (type b)	F: GGCAGCTGGTTNGCCTG R: GGCCTGCAGATCNCCAA
3	Sabharwal et al.,2014	*plcH* 307	F: GAAGCCATGGGCTACTTCAA R: AGAGTGACGAGGAGCGGTAG

Table(2) Programs of PCR Thermocycling Conditions of gene.

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	*55 OR 57	00:30	
Extension	72	00:45	
Final extension	72	07:00	1
Hold	10	10:00	

Flic, and PlcH 55°C while gyrB57°C

DNA Sequencing :

After conducting a polymerase chain reaction (PCR) to amplify DNA, the results were sent to the Korean company Macrogen to analyze the genetic sequence of the isolates and compare them with the information available at the National Center for Bioinformatics (Gene Bank).

III. RESULTS AND DISCUSSION:

125 samples were collected from patients with chronic suppurative otitis media, and 110 samples (88%) of the total samples showed positive growth and 15 samples 12% gave negative growth to P. aeroginosa.

Agricultural characteristics:

It appeared when cultivated on the McCongy agar medium, where it appeared in a pale due to its inability to ferment the sugar lactose (8).The bacterial isolates under study were grown on sterimide agar 0.03%, which is selective, and all isolates showed their ability to grow on this medium and gave a positive result and the ability to resist steramide, one of the components of this medium, which is toxic to the vast majority of bacterial species, except for P. aeruginosa.All isolates were grown on Muller-Hinton agar medium, and they produced pigments that varied from yellowish-green to bluish-green, and characterized by a fetid smell similar to the smell of ripe fruits, and this was agreed with (9).

Microscopic diagnosis:

Bacteria that do not form spores appeared singly or in the form of chains of red to pink color resulting from staining of bacterial cells with safranin dye (10).

all the DNA bundles appeared clearly and with high accuracy and in all the selected bacterial isolates that were given the numbers (1-20), and at the same level as in Figure (1), meaning that they belong to the same bacterial type P aeruginosa, which indicates a high concentration.

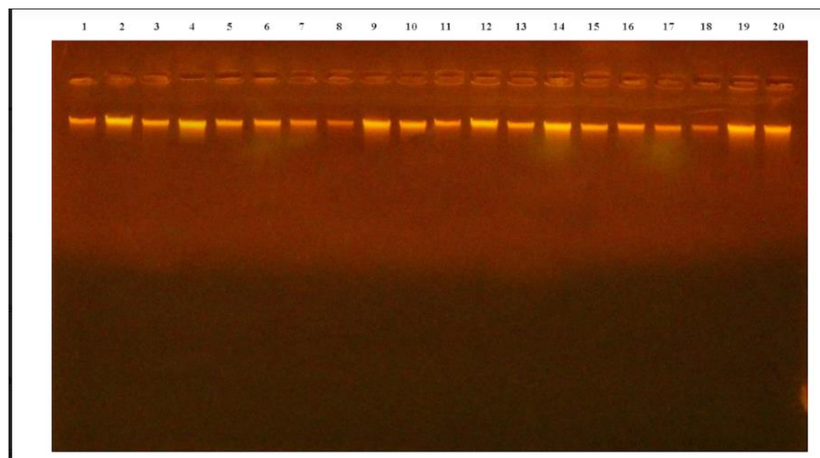

Figure (1) shows the migration of genomic DNA for 20 isolates of P. eruginosa.

Gene detection:

To detect the gyrB gene in P. aeruginosa, a specialized primer was used that targets the specific sequence of the specific gyrB gene of P. aeruginosa with a molecular size (222bp). The program for this gene was installed in the thermocyclic PCR machine and the reaction was performed and then the product was migrated to Acarose gel at a concentration of 1.5% and for a period of 90 minutes, it was observed that bands with a molecular size of 222 base pairs appeared at the same level for most of the isolates and 90%)) of the total selected isolates under study (Fig. 1).This study converged with the findings of the researcher (11), where she indicated in her study the presence of the gyrB gene in most of the bacterial isolates she obtained, and the current results indicate the great benefit of sequencing analysis of the gyrB gene as it can be used as a guide Excellent along with 16SrRNA gene analysis in detecting pathological samples and bacterial species whose type cannot be ascertained using 16SrRNA alone (12).

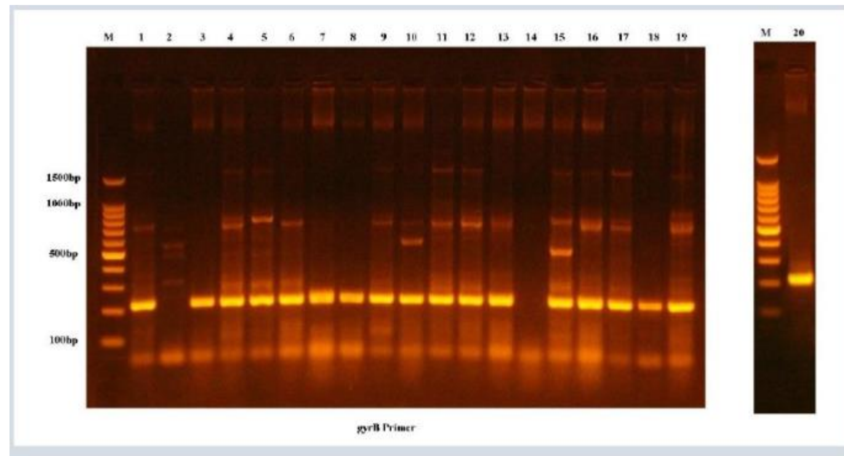

Figure (2) Picture No. (2-3) Electrophoresis of the product of the DNA polymerase chain reaction using the initiator of the gyrB gene on the medium of agarose gel at a concentration of 1.5%

As for the fliC gene, a polymerase chain reaction (PCR) test was conducted for the DNA chain, and for that, the kit for the reaction and the primer for thefliC gene was used with the reaction mixture to detect the presence of this gene, which has a molecular weight of 380bp, a pair of bases, according to the program designated for this. Then, the reaction product was carried out by electrophoresis on an agarose gel at a concentration of 1.5%) and it was examined by ultraviolet rays. The results of the electrophoresis showed that all selected bacterial isolates amounting to 20) isolates of P. aeruginosapossess the flagellum genefliC Figure (5). The fliC gene consists of two types: type A, which has a molecular weight of 1020 base pairs, and type B, which has a molecular weight of 1250 base pairs. And by type a, b) it means flagellin, which is the main component of the flagellum (13).They can be distinguished on the basis of molecular size and interaction with a specific type of polyclonal with antibodies (14), and this is what was obtained in the results of our study, as the bundles at the top were of type b with a molecular weight of 1050bp) and their number was 12 isolates out of the total isolates (20), as for the bundles below, they are of the type A FliC gene with a molecular weight of 1020bp, and their number is 8 out of a total of (20) isolates.

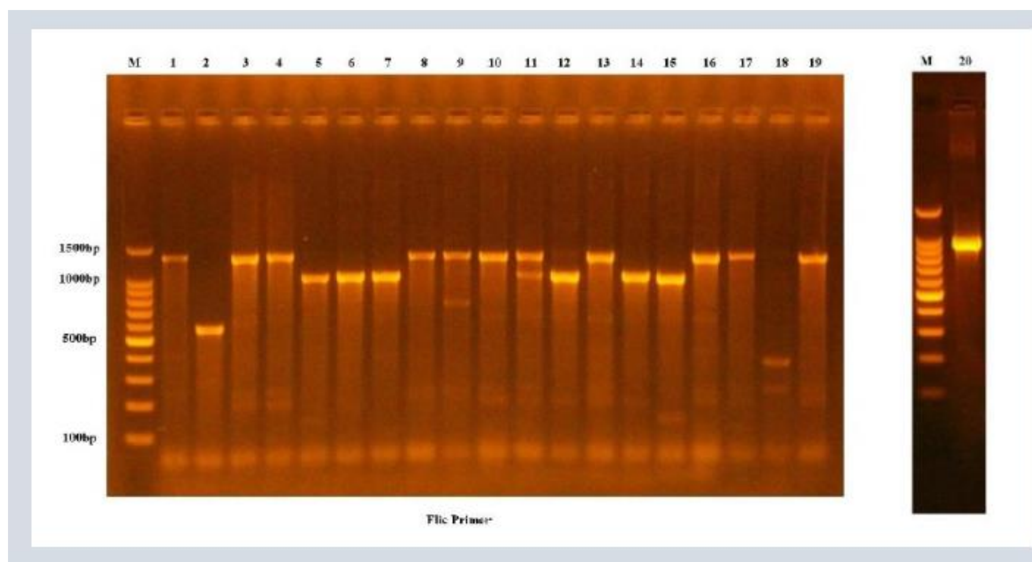

Figure (5) Electrophoresis of the product of the DNA polymerase chain reaction using the initiator of the FliC gene on the medium of agarose gel at a concentration of 1.5%.

This study agreed with the findings of the researcher (15) in his study. Where he indicated its presence in all isolates obtained from different sources. This study also agreed with the findings of the researcher (16), which showed the presence of this gene in all isolates obtained in his study. In addition, the researcher (17) found in a study he conducted on P.aeruginosa bacteria isolated from Diabetic foot infections (DFIs) that all isolates contain the fliC gene and this gene had an important role in the tissue penetration by pathogenic bacterial cells. It survives under anaerobic conditions. . And as it is known that flagella in the bacteria that contain them are used for movement and locomotion.Among the types of bacteria that possess flagella is Pseudomonas aeruginosa, which contains a single

polar flagellum that is one of the factors of virulence and movement and is an initial stage of infection with this bacteria.

The polymerase chain reaction (PCR) was carried out to detect theplcH gene using the reaction mixture prepared with the kit for this gene and according to the manufacturer's instructions and the primer for this gene was used. Isolation and dependence of volume index (100-1500bp), DNA bundles were examined by UV rays, and the results of the examination indicated the presence of theplcH gene, which has a molecular weight (307bp) in most of the bacterial isolates under study Picture No. (6).Our study agreed with the study conducted by (18), in which they indicated that the percentage of the presence of the plcH gene in the isolates they obtained from burn wound infections was 96%)), and also these results were close to what was reached (19) was found to spread theplcH gene in 75% of the isolates of Pseudomonas aeruginosa obtained in his study. It also differed with the results of (20), where they found that the percentage of pseudo isolates in their study that possessed the plcH gene was 66%)), on the other hand, other researchers found in 2017 that the percentage of isolates that possessed the plcH gene in their study was 18% (21).

The plcH gene, as it is known, encodes the phospholipase enzyme, which is one of the most important virulence factors of Pseudomonas aeruginosa because it helps pathogens invade the host's cell. Helping bacteria in the mechanism of evading host immunity(22), as well as this gene encodes a hemolytic toxin that destroys cell membranes and degrades phospholipids, especially phosphatidylcholine, and thus leads to the degradation of erythrocytes and surface al-lipids (23). The proof of its presence in the bacterial isolates under study is evidence of the high resistance possessed by these isolates, and this is what was reached, as the multi-resistant isolates were selected, which possessed most of the phenotypic virulence factors in our study.

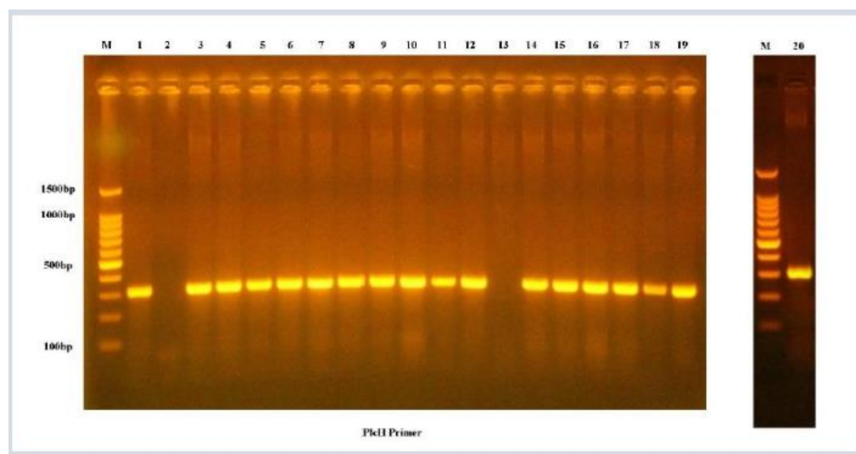

Figure (6) Electrophoresis of the product of the DNA polymerase chain reaction using the initiator of theplcH gene on the medium of agarose gel at a concentration of 1.5%.

50 (microliters) of PCR products of five isolates of P. aeruginosa with the primers of the genes ,(fliC, PlcH, GyrB) were sent to Macrogen Company in South Korea for the purpose of determining the sequence of nitrogenous bases as well as And then compare it with the sequences recorded in the National Center for Informatics (NCBI) using blastn2.6.4 to get the serial number ID that pertains to each of the isolatesPaeruginosa bacteria from the global site of genes, these isolates were selected based on their high resistance to antibiotics and their possession of most virulence factors.

DNA Sequencing:

The results of this study indicated that isolate No. (5) belonging to theFliC gene showed the results of comparison with the first sequence that carries the symbol (CP032569.2) due to P. aeruginosa bacteria and the presence of changes in the sequence of nitrogenous bases, where four substitution, two mutations were found. There are two transition mutations in which T>C is transformed at position 4277166 and C>T is transformed at position 4277313, in addition to two non-equivalent transvrsion mutations, one in which G>C is transformed at position 4277199 and the other transforms G>C at position 4277550. The concordance rate was 98%)), Figure No. (1)according to the study(24) .There were also no variations in isolate No. (8) of the gene (PlcH), and the homology was 100%) as in Figure (4) according to the study (25), as for isolate No. (20) of thegyrB gene)) No variations appeared, and the percentage of congruence was 100%)), according to(26).

Table (3) Type of substitution:

No. Of sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID
5_flic-F	Transition	4277166	T>C	4276886 to 4277827	CP032569.2
	Transversion	4277199	G>C		
	Transition	4277313	C>T		
	Transversion	4277550	G>C		
8_plcH-F	----------------			266 to 529	MN066138.1
20_gyrB-F	----------------			84 to 266	MK625175.1

IV. CONCLUSIONS:

P.aeruginosa bacteria is one of the most importantcauses of Otitis media because it has manyvirulence factors, including phospholipase, protease andenzymes, which increase its pathogenicity, and this iswhat was shown by the investigation of the genes ofthe aforementioned bacterium virulence factors that itpossesses these genes in high proportions as well as thecomparison of the(fliC,PlcH,GyrB)gene sequence. Isa suitable method for inferring the genetic relationshipsof P. aeruginosaisolates on a molecular basis and in thefuture. By studying the genetic map, it is possible tolearn more about P. aeurginosa.

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