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PCR DETECTION OF BIOFILM GENE'S IN ANTIBIOTIC RESISTANCE *PSEUDOMONAS AERUGINOSA* ISOLATES FROM OTITIS MEDIA

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Abstract

The study aimed to investigating the bacterial from patients suffering otitis media infection. The study included two main parts, the first was the bacterial diagnosis based on relied diagnostic procedures. Second part were genetic study to virulence factors (biofilm form) to common bacteria that causes otitis media infection. A total of 130 specimens were referred to Al-Sader medical city and Al-Hakim general hospital in Al-Najaf province (Ear swab), within period from September to December 2019. The results indicated 115 samples (88.46%) gave positive result, while 15 sample (11.54%) was negative for culture bacteria, Primary identification was depended on Gram stain and biochemical tests. Finally identification with vitek 2 system was done. The results demonstrate the 115 clinical specimens only 48(41.7%) isolates were belonged to *Pseudomonas aeruginosa*, 20(17.39%) isolates were *Staphylococcus aureus*. Out of the 115 specimens, only 22(19.1%) *Proteus mirabilis*, 21(18.2%) isolates were *Klebsiella pneumonia* and 4(3.4%) isolates belong to *Burkholderia cepacia*. Genetically, study for some genes encoding virulence factors that associated with pathogenicity of *P. aeruginosa* by using PCR technique. The results indicated, *P. aeruginosa* isolate contain 45(94%) *AlgD* gene that responsible for polysaccharide (alginate) and alpha factor in RNA polysaccharide. *PelA* gene present with percentage 44(91.6%) and *PslA* gene present with percentage A 27(56.25%) from *P. aeruginosa* isolate that encoding to biofilm formation, which is one of the most important virulence factor of these bacteria.

Key word: *P. aeruginosa*, *PslA* gene, *AlgD* gene, *pelA* gene.

Introduction

Otitis media (OM) is a collective term to describe a group of inflammatory and infective conditions affecting the middle ear. Its involves pathology of the middle ear and middle ear mucosa. Otitis media is one of the leading causes of healthcare visits and the complications of OM are important causes of preventable hearing loss (Sign, 2003). Otitis media have many types which include Acute Otitis media (AOM), otitis media effusion (OME) and chronic suppurative Otitis media (CSOM).

A subtype of AOM is acute suppurative OM, characterised by the presence of pus in the middle ear, otitis media with effusion (OME) is a chronic condition without acute incidence, which follows a slowly resolving AOM, there is an effusion of glue like fluid in the absence of signs and symptoms of acute inflammation. Chronic suppurative Otitis media (CSOM) is a chronic suppurative middle ear inflammation, usually with a perforated tympanic membrane. Acute Otitis media (AOM)is acute inflammation of the middle ear which may be caused by various types of infectious microorganisms such as bacteria, viruses and fungi. (Hall Stoodley *et al*., 2004).

Pseudomonas aeruginosa is one of bacterial agent that causing otitis media infection have various types of virulence factor enable the bacteria to cause infection. A biofilm is one of important virulence factor, its a structured community of bacteria encased in a thick, protective and structural matrix, the biofilm matrix is composed of extracellular polymeric substance (EPS), which consists of secreted exopolysaccharides, nucleic acids, proteins and other molecules (Mann and Wozniak, 2012). Though initially believed to be simple clumps of bacterial cells, biofilm structures are actually quite complex; composed of microcolonies, they contain channels for wastes, water and nutrient distribution, leading to a variety of microenvironments with different oxygen, nutrient and waste exposures (Hall Stoodley *et al*., 2004). *Pseudomonas aeruginosa* displays resistance to a variety of antibiotics, including aminoglycosides, quinolones and β-lactams (Hancock and Speert, 2000).

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Table 1: Primers used in this study.

Primer Type	Primer sequence (5'-3')	Amplicon size (bp)	Reference
Alg- D	F:ATCCGAATCAGCATCTTTGGT R:CTACCAGCAGTGCCCTCGGC	1210	Carmen *et al.*, (2013)
Pel- A	F: CCTTCAGCCATCCGTTCTTCT R:TCGCGTACGAAGTCGACCTT	118	Colvin *et al.*, (2011)
Psl-A	F:CACTGGACGTCTACTCCGACGATAT R:GTTTCTTGATCTTGTGCAGGGTGTC	1119	Hou *et al.*, (2012)

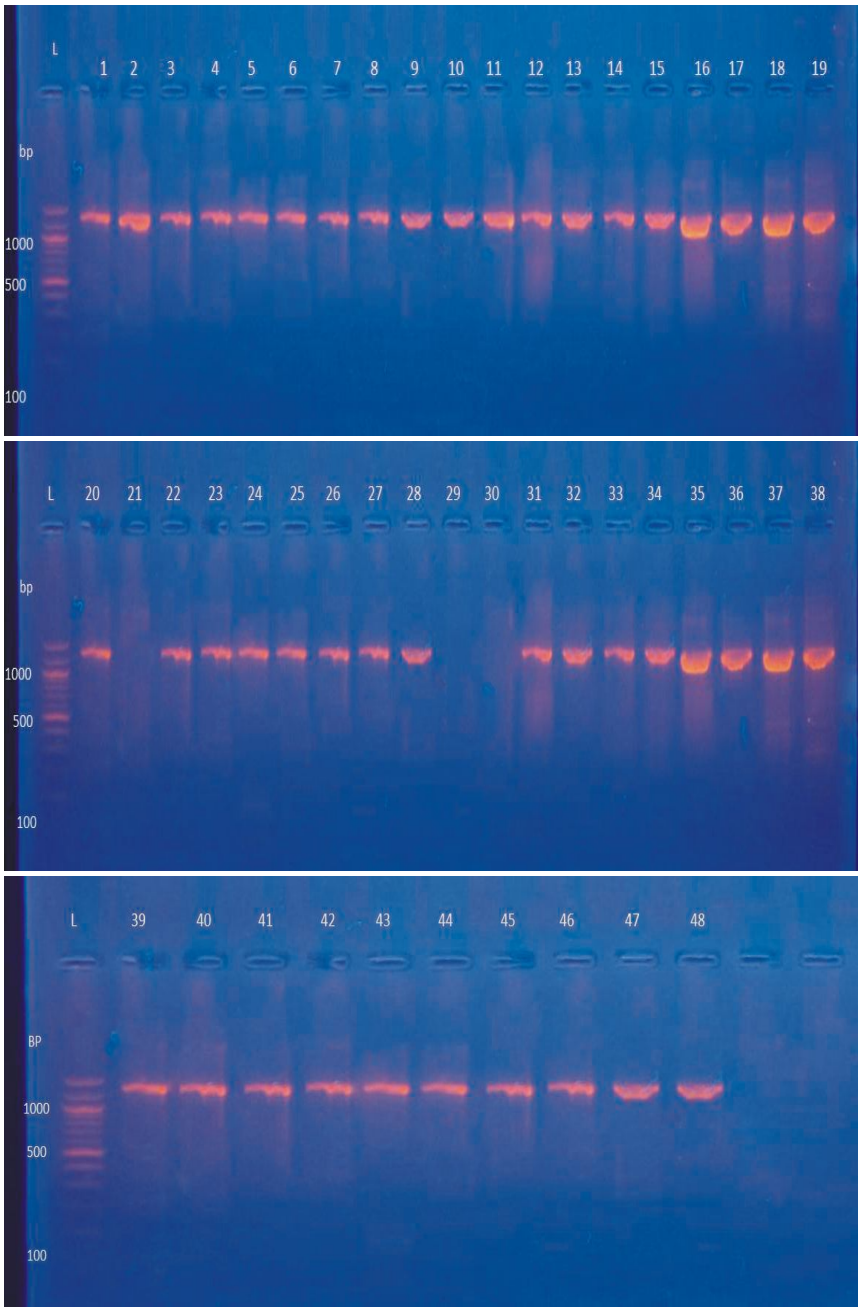

Fig. 1: Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *Pseudomonas aeruginosa* using primer *AlgD* gene with product 1210bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (100 bp ladder). Lanes (1 to 48 except 21, 29, 30) show positive results with gene *Alg-D gene*.

Generally, the major mechanisms of *P. aeruginosa* used to counter antibiotic attack can be classified into intrinsic acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic inactivating enzymes, the acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes (Breidenstein *et al.*, 2011). The adaptive resistance of *P. aeruginosa* involves formation of biofilm in middle area of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells (Drenkard, 2003).

Materials and Methods

Patients and clinical specimens collection

A total of 130 clinical specimens (ear swab) were collected from patient suffering from otitis media infection attended to (Al-Sadder Medical City and Al- Hakeem General Hospital) in Al- Najaf province from September to December 2019. The specimens were collected from both gender and (1-80) years age. The clinical samples were processed according to standard techniques (Cheesbrough, 2000).

Isolation and Identification of bacterial isolate

Following collection, clinical specimens were transported to the laboratory without delay, ear swabs were inoculated on suitable media, which included MacConkey's agar, blood agar and Mannitol salt agar plates and incubated at 37°C for 18-24 hours. Primary diagnosis of isolates was made on the basis of Gram's staining, colonial morphology on different media and biochemical test. In addition the final identification of bacteria isolates confirmed biochemically with VITEK2-automated system.

Table 2: PCR program of *intI* primer that apply in the thermocycler.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
Alg- D	95°C for 2 min		95°C for 30 sec	55°C for 30 sec	72°C for 30 sec	72°C for 5 min
Pel- A	94°C for 5 min		94°C for 30 sec	52°C for 40 sec	72°C for 50 sec	72°C for 50 sec
Psl-A	94°C for 10 min		94°C for 1min	55°C for 30 sec	72°C for 1 min	72°C for 10 min

DNA Extraction

Genomic DNA was extracted by using a commercial extraction system (Genomic DNA promega Kit).

Molecular Identification

Gel electrophoresis was used for detection of DNA by UV transilluminator. The PCR assay was performed to detect biofilm Gene's for *P. aeruginosa* shown in table 2. This primer was designed by Alpha DNA company, Canada as in table 1. Amplified products were confirmed using 1% agarose gel electrophoresis to estimate the PCR products size. The gel was stained with 4 µLof 10mg/mL ethidium bromide (Sigma, USA) and it run at 80v for 1.5h. A single band was observed at the desired position on ultraviolet light transillumintor (Cleaver, UK); bands were photographed using gel documentation system (Cleaver, UK). A 100bp ladder (Bioneer, Korea) was used to measure the molecular weights of amplified products.

Results and Discussion

A total of (130) clinical specimens (swabs) were collected from patients suffering from otitis infection,who attending to Al-Sader medical city and Hakeem teaching hospitals in Al-Najaf Province, during a period extended from September to December, 2019

Identification of bacteria

The initially identification of bacterial specimens depended on some criteria which included cultural, morphology and biochemical tests. The final identification was performed with the automated vitek-2 compact system using GP, GN-ID cards which contained 64 biochemical tests and one negative control.Exactly 115 isolates performed identification and confirmed by vitek-2 system by using kit (GP-ID cards) to Gram positive bacteria and (GN-ID cards) to Gram negative bacteria (appendix1), with ID message confidence level ranging between very good to excellent (Probability percentage from 95 to 99) (Appendix2).

The initially identification of gram negative rod, *Pseudomonas* spp. was depended on the colonial morphology by produce non-lactose fermented colony on Maconky agar. production of pyocyanin pigment and giving strongly oxidase and motility test positive and give K/K on TS (Koneman *et al*., 2006).

The results of present study revealed that *P. aeruginosa* was the most common isolate followed by *P. mirabilis and K. pneumoniae*. Such result was similar to result study of Deshmukh *et al.,* (2017), some studies have shown that *P. aeruginosa* are the most common cases of otitis media, followed by *S. aureus* and the infection rates for the first types are the highest among the total cases of infection, other studies have reported that *S. aureus* was the most common agent. This difference might be due to geographical variation and local anti-biogram pattern and because of variation in climate, community and patient characteristics, the pattern of microbiological distribution varies in Otitis Media (Shyamla and Reddy, 2012).

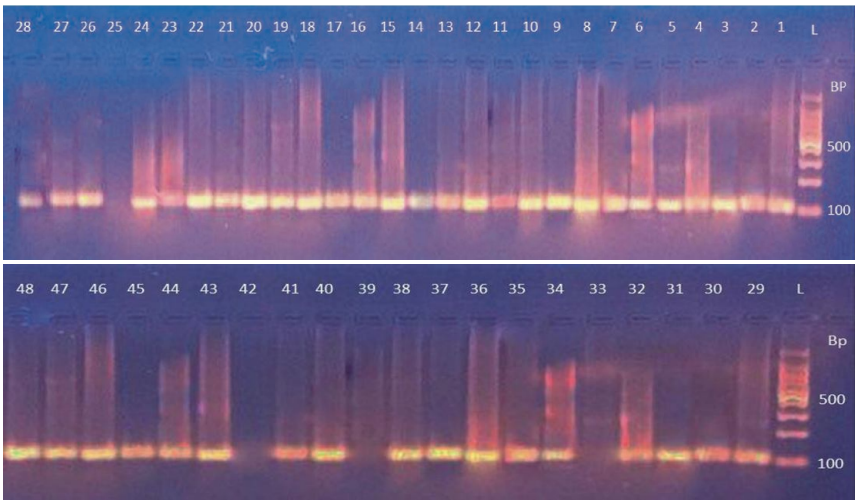

Fig. 2: Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *Pseudomonas aeruginosa* using primer *Pel-A* gene with product 118bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (100 bp ladder). Lanes (1 to 48 except 25, 33, 39, 42) show positive results with gene *Pel-A*.

Genetic detection of biofilm formation

- **Detection of the *Alg-D* gene:**

Polymerase chain reaction amplification were used to detected the presence of *AlgD* gene (1210bp) among *Pseudomonas aeruginosa*, the results revealed that 45 (94%) of isolates gave positive result predominance of *AlgD* among Pseudomonal isolates fig. 1. The same results was appear in AL-luhabi study, (2015). He found that (97%) *P.*

aeruginosa isolates were possess this gene.

Pseudomonas aeruginosa possesses a large number of cell-associated and extracellular virulence factors, which are tightly regulated by cell-to-cell signalling systems (Van Delden and Iglewski, 1998). The involving *of AlgD* genes in formation of mucoid colonies of *P. aeruginosa* composed from alginates, protects the bacterium from the host's immune response such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement and from antibiotics and thus contributes to chronic pulmonary inflammation (Govan and Deretic, 1996).

• Detection of the *Pel-A* gene:

The results of PCR analysis to detected the presence of *Pel-A* gene (118bp) among Pseudomonal isolates revealed that 44(91.6%) of isolates were contain *Pel-A* gene fig. 2. *P. aeruginosa* can use either *Psl* or *Pel* as the primary biofilm matrix polysaccharide (Overhage *et al*., 2005). The *PslA* and *PelA* genes play an important role in formation of carbohydrate-rich structure of biofilm matrix. Therefore, the mutations in *PslA* and *PelA* genes cause deficiency in the biofilm formation ability (Colvin *et al*., 2011).

Pel gene is involved in the production of glucose and pellicle formation. *in vitro* production of biofilm may be due to presence of *PelA* gene in the clinical isolates which did not allow the formation of biofilm in *Pel* mutant strains; whereas biofilms were observed in wild type strains and alginate mutant strains thus revealing that *Pel* is an essential exopolysaccharide which is often required in biofilm matrix formation (Friedman and Kolter, 2004).

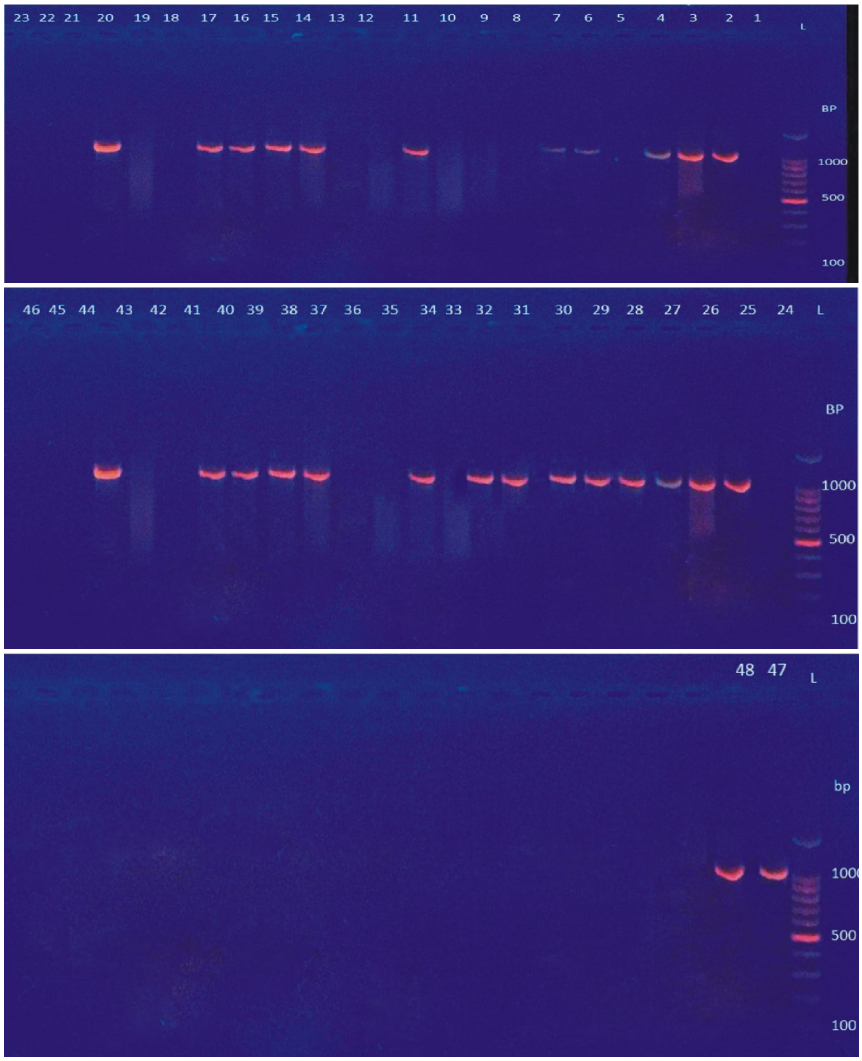

Fig. 3: Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *Pseudomonas aeruginosa* using primer *Psl-A* gene with product 1119bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (1000 bp ladder). Lanes (1 to 48 except 1, 5, 8, 9, 10, 12, 13, 18, 19, 21, 22, 23, 24, 33, 35, 36, 41, 42, 44, 45, 46) show positive results with gene *PsI-A*.

Hypothesized that *Pel* is capable of providing protection to planktonic cells when artificially overexpressed, thus suggesting that *Pel* plays an important protective role in biofilms of pseudomonas strains. Fux *et al*., (2005) showed the occurrence of biofilm formation in *Psl* mutation isolates, because the lack of *Psl* was compensated with the high transcription of *Pel* gene, therefore, *Pel* gene was considered as a biofilm-formation marker for *P. aeruginosa* isolates. Biofilm bacteria exhibit reduced susceptibility to antimicrobials, detergents and the host immune system compared to planktonic cells (Chang *et al*., 2007).

• Detection of the *Psl-A* gene:

The PCR amplification were used to detected the presence of *Psl-A* gene (1119bp) in bacterial isolates. The result appear that 27(56.25%) of *Pseudomonas aeruginosa* gave positive to *Psl-A* gene detection, fig. 3. The polysaccharide synthesis locus *(Psl)* contains 12 genes, 11 of which are necessary for synthesis and export of the *Psl* polysaccharide (Byrd *et al*., 2009). *Psl* gene contribute to biofilm formation in *P. aeruginosa* by encoding for specific exopolysaccharides that make up part of the EPS. The *Psl* cluster contains

15 co-transcribed genes (*PslA* to *PslO*) encoding proteins predicted to be involved in polysaccharide biosynthesis, *Psl* (polysaccharide synthesis locus) is involved in mannose production (Vasseur *et al*., 2005).

The study of Ma *et al*., (2009) looked at the distribution of *Psl* exopolysaccharide during the attachment stage, this polysaccharide was noted primarily on the bacterial cell surface in a helical pattern, it is hypothesized that this helical nature may contribute to cell-to-cell interactions with adjacent bacteria, which will begin to establish a matrix between the two individual cells. Another theory is that other proteins or lipids may have a similar shape and so insertion of cells is easier. This finding is further substantiated by studies documenting that *PslA B* mutants are unable to initiate biofilm formation; the A and B are different genes within the *Psl* cluster of genes (Jackson *et al*., 2004).

Depending on environmental conditions and nutrient cues, biofilms can form flat structures or microcolonies with a three-dimensional arrangement. Conditions that are high in nutrients favor production of flat biofilms whereas in low nutrient environments, the "classical" three-dimensional mushroom shaped biofilm will form,a pellicle may form which is a part of the biofilm that forms at the air-liquid interface (Kirisits *et al*., 2005).

Regulation of *Pel and Psl* expression is complex, with multiple levels of intricate control, many studies have demonstrated multiple pathways of transcriptional control for both *Pel* and *Psl*. FleQ represses transcription of the *Pel* and *Psl* operons. *RpoS* acts as a positive transcriptional regulator of *Psl* gene expression and quorum sensing has been suggested to positively regulate *Pel* and *Psl* expression as well (Agladze *et al*., 2005). Another regulatory system controlling *Pel* and *Psl* gene expression is the Gac-Rsm signal transduction pathway (Ventre *et al*., 2006).

Conclusions

1. Bacteria have a big role in infected ear and causes otitis media.
2. *Pseudomonas aeruginosa* been recognized as a major public health problem, especially among otitis media infection.
3. Appearance of *P. aeruginosa* isolates high resistance to antibiotic.
4. *AlgD* (formation alginate) displayed in all *P. aeruginosa* isolates,it elucidates pathogenesis. While *PslA* and *PelA* gene (responsible for biofilm formation) present high percentage in *P. aeruginosa*.

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