Detection and Study *nan1* and *tox A* genes of *Pseudomonas aeruginosa* in Isolates from Otitis Media Patients Considered as Virulence Factors

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ABSTRACT

Background and Aim: Otitis media (OM) or middle ear inflammation is an inflammatory disease caused by a bacterial infection such as *Pseudomonas aeruginosa (P. aeruginosa),* provided with genetic virulence factors. This study aims to detect *nan1 and tox A* genes of *P. aeruginosa* isolated from chronic OM patients.

Materials and Methods: One hundred thirty OM patients were recruited from Al-Sader medical city and Al-Hakim general hospital in Najaf province from September to January 2021 to collect ear swaps. The Primary identification depends on Gram stain and biochemical tests. We also used Vitek 2 system to make another identification and examine resistance to antibiotics. A polymerase chain reaction (PCR) was used to amplify the required genes. Furthermore, gel electrophoresis was performed to show the amplified genes.

Results: The results have revealed that 115 samples (88.46%) give a positive result, while 15 samples (11.54%) are negative for culture bacteria. Only 49 (42.76 %) of the 115 clinical specimens showed positive tests for *P. aeruginosa*. However, the *toxA* gene is found in 67.3% of *P. aeruginosa* isolates, while the *nan1* gene is found in 38.77 % of the isolates.

Conclusion: Our findings suggest that *P. aeruginosa* is one of the most common causes of OM infection in Iraqi patients. Most bacterial isolates show the presence of *nan1* and *tox A* genes, with a high prevalence of *tox A* than *nan1*. The presence of these genes is one of the virulence factors and probably partly explains the resistance to antibiotics.

Keywords: *nan1,* otitis media, *Pseudomonas aeruginosa, tox A* genes

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1. Introduction

Otitis media (OM), often known as middle ear inflammation, is a group of inflammatory and bacterial infections that influence the middle ear **(1, 2)**. The burden of the illness is significant in resource-restricted situations, while human and physical diagnostic and treatment resources remain inadequate **(2, 3)**. Various infectious micro-organisms can cause OM, such as bacteria, viruses, and fungi **(4)**. *Pseudomonas aeruginosa* (*P. aeruginosa*) and

Staphylococcus aureus are the most common pathogens in chronic otitis media with effusion (COME), which form biofilms with other otopathogens **(4, 5)**. This biofilm may trigger innate inflammatory responses and contributes to the chronicity of OM and its progression to COME despite the proper treatment **(6, 7)**.

Pseudomonas aeruginosa is one of the opportunistic bacterium agents which is implicated in the pathogenesis of OM; these bacteria own several types of virulence factors **(8)**. In this regard, Mansoor *et al.* found that most of the isolates in the COME are *P. aeruginosa* **(9)**, and it has an inherent resistance to a broad spectrum of drugs **(9, 10)**. Enzyme synthesis, efflux pump overexpression, porin deficiencies, and target-site changes have all been reported as resistance mechanisms in *P. aeruginosa* **(10)**. Moreover, increased expression of AmpC β-lactamase is frequently linked to β-lactamase resistance in *P. aeruginosa* **(11, 12)**. In addition, cross-resistance may play a role in *P. aeruginosa's* multidrug resistance **(12)**.

Indeed, *P. aeruginosa* is intrinsically resistant to several antimicrobials and can acquire mobile genetic elements (MGEs) due to the chromosomally encoded resistance genes **(13, 14)**. This last mechanism is crucial in forming and disseminating drug resistance **(10)**. Bacteria acquire genes to survive in unfavorable environments, namely when antibiotics are present **(10)**. Plasmids and other MGEs are essential in this process since they serve as vectors for capturing and disseminating resistance genes. Exotoxin A, exoenzyme S, elastase, and sialidase are some virulence factors identified in *P. aeruginosa*. They are tightly controlled by cell-to-cell signaling networks **(15)**.

Exotoxin A is encoded by the *toxA* gene and inhibits protein biosynthesis by transferring an ADP-ribosyl moiety to elongation factor 2 of eukaryotic cells **(16)**. Moreover, neuraminidases, also known as sialidases, are encoded by the *nan1* gene, which is the main enzyme that hydrolyzes the linkage of terminal sialic acids on various sialoglycoconjugates to generate free sialic acid **(16)**. It represents the terminal sugar residue of many glycan chains on host cell surfaces involved in cell-cell recognition; thus, this enzyme has an essential role in the implantation of the bacterium **(17)**. However, no studies have investigated the existence of *nan1* and *tox A* genes in the *P. aeruginosa* isolated from OM.

Hence, in the current study, we aim to detect the *nan1* and *tox A* genes in *P. aeruginosa* isolates from chronic otitis media (COM) patients. The hypothesis is that these two genes are present in *P. aeruginosa* isolated from OM patients and they are part of the resistance system to antibacterial treatments.

2. Materials and Methods

Patients and Clinical Specimens' Collection

In this study, we have recruited 130 patients with COM whose range of age is (1- 85 years) from both genders and over a specific time (September 2020 to January 2021). Patients were recruited from Al-Sadder Medical City and Al- Hakeem General Hospital in Najaf city, Iraq. A well-trained otorhinolaryngologist performed the patients' diagnosis based on the ICD-10 criteria. Ear swabs were collected from all patients with a swap collection tube from JSHXRT company, China. All samples were further processed according to the standard criteria reported by Cheesbrough **(18)**. We followed the Iraqi and international ethics and privacy laws to perform the current study. Before participating in the present study, written informed consent is obtained from each individual or their parents. In the current study, the College of Medicine at the University of Kufa in Iraq has provided the IRB approval (617/2020) compliant with the Declaration of Helsinki's International Guideline for Human Research Protection.

Isolation and Identification of the Bacterial Isolates

After the samples were collected, we transferred them to the laboratory to inoculate on appropriate media, such as MacConkey's agar and blood agar plates, and incubated them at 37°C for 18-24 hours. Gram staining, colony morphology on different media, and biochemical tests were used to make the primary diagnosis of isolates. One hundred fifteen isolates were identified and validated using the Vitek-2 system, which uses a kit (GP-ID cards) for Gram-positive bacteria and 15 isolates as Gram-negative bacteria identified by (GN-ID cards) with ID message confidence levels ranging from very good to outstanding (probability percentage from 95 to 99). In addition, using the VITEK2-automated system, the final identification of bacteria isolates was confirmed biochemically. We examined the antibacterial resistance of *P. aeruginosa* isolates by VITEK 2 automated system (Table 1). Susceptibility profiles were performed with 12 antibiotics in the specific AST cards, including Amoxicillin-clavulanic acid Ceftriaxone, Cefotaxime, Cefepime, Ceftazidime, Azithromycin, Levofloxacin, Gentamycin, Amikacin, Tobramycin, Ciprofloxacin, and Imipenem.

DNA Extraction

The sample preparation of DNA extraction was done as follows: a) one ml of the overnight bacterial growth on brain heart infusion (BHI) broth was taken and placed in a 1.5 ml microcentrifuge tube and centrifuged at 10000 rpm for 1 minute, b) the supernatant was thrown away, and the bacterial cell pellets were utilized to extract genomic DNA, c) genomic DNA was extracted according to the manufacturer's procedure using a commercial extraction kit (Genomic DNA Promega Kit). UV

spectroscopy was used to assess the quality and quantity of DNA isolated from bacterial cultures. The Optical Density (OD) at 260/280 nm was used to evaluate DNA purity on a Shimadzu UV-1800 Double Beam UV/Visible Scanning Spectrophotometer.

PCR Amplification

As previously described (20), DNA samples (5 µL) were subjected to PCR apparatus in a 25µL reaction mixture using SureCycler 8800 (Agilent Technologies, Inc. USA). The following specific thermal cycling pattern was used to amplify the *nan1* gene: 35 cycles consisting of 5 min initial denaturation, 30 seconds (s) at 94°C for denaturation, 60 S at 58°C for annealing, 2 min at 72°C for extension, and 5 min for the final extension. *tox A*, gene amplification was performed following the following conditions: initial denaturation step at 94°C for 5 min; 30 cycles of denaturation at 94°C for the 30 s, annealing at 55°C for 60 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 5min. Table 2 lists the specific sequence primers used by Alpha DNA Company (Canada). Electrophoresis was used to analyze DNA fragments in a 1% agarose gel at 70 V for 1.5 h. in TBE 1X containing 4 µL of 10 mg/mL ethidium bromide using a 100-bp DNA ladder (Bioneer, Korea) as a size marker. On an ultraviolet light transilluminator (Cleaver, UK), a single band was seen at the appropriate spot; bands were photographed using a gel documentation system (Cleaver, UK).

Table 1. Percent of resistance of *P. aeruginosa* isolates against tested antibiotics

No.	Type of Antibiotic	Percent of total isolates
1	Amoxicllin-clavulinc acid	93.75
2	Ceftriaxone	81.25
3	Cefotaxime	70.83
4	Cefepime	52.08
5	Ceftazidime	52.08
6	Azithromycin	52.08
7	Levofloxacin	18.75
8	Gentamycin	18.75
9	Amikacin	18.75
10	Tobramycin	29.16
11	Ciprofloxacin	16.66
12	Imipenem	8.33

Table 2. Primers used in this study

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
nan1	F: AGGATGAATACTTATTTTGAT R: TCACTAAATCCATCTCTGACCCGATA	1316	(20)
ToxA	F: GGTAACCAGCTCAGCCACAT 3' R: TGATGTCCAGGTCATGCTTC 3	352	(20)

3. Results

Distribution and Characteristics of Patients

Table 3 shows the patients' distribution and demographic characteristics across two hospitals that provided the samples. Most of the patients were recruited from AL-Sadar Medical City in Najaf province. We found that 74 (57%) specimens were acquired from males and 56 (43%) from females. Based on the residency, 79.6% of specimens were taken from urban patients, whereas 51.9% of from rural. Table 4 shows the patient distribution by age and gender. We found the OM most prevalent among adults aged 21-30 and the least prevalent among older adults 51-80 from both genders. Table 5 revealed that most bacterial isolates were gram-positive 115, taken from urban people, while only 15 isolates showed a Gram-negative test.

Table 3. Distribution and characteristics of patients recruited in the presented study

Variables	AL-Hakeim Teaching Hospital	AL-Sadar Medical City
No.	50	80
Gender (M/F)	28/22	46/34
Residency (R/U)	19/31	32/48
Diagnosis (GP/GM)	45/5	70/10

No.: Number, M: Male, F: Female, R: Rural, U: Urban, GP: Gram-Positive, GM:
Gram Negative.

Table 4. Age-Gender distribution of the patients.

Age(year)	Male	Female	Total %
1-10	13	11	24(18.4%)
11-20	12	8	20(15.3%)
21-30	22	18	٤٠(30.7 %)
31-40	10	7	17(13%)
41-50	6	10	١٦(12.3%)
51-80	11	2	1٣(10%)
Total	74 (57%)	56 (43%)	130 (100%)

Table 5. Distribution of type of bacteria with the residence of patients

Residence	Positive	Negative	Total %
Urban	71(54.6%)	8(6.1%)	79(60.7%)
Rural	44(33.8%)	7(5.٤%)	51(39.2%)
Total	115(88.4%)	15(11.5%)	130(100%)

Detection of the *nan1* Gene

The *nan1* gene (1316 bp) of *P. aeruginosa* was detected in the isolates using the PCR technique; the findings indicate that 19 (38.77%) of the isolates tested positive for the gene, as shown in Figure 1.
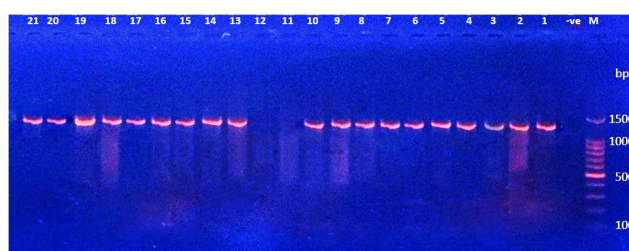

Figure 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *P. aeruginosa* using primer nan1 gene with product 1316bp. The electrophoresis was performed at 70 volts for 1.5-2hr. lane (M), DNA molecular size marker (1500 bp ladder). Lane (-ve) shows negative controls. Lanes (1 to 2 except 11, 12) show positive results with gene nan1.

Detection of *toxA* Gene

The presence of the *toxA* gene (352 Bp) in *P. aeruginosa* isolates was determined by PCR amplification. The data in Figure 2A, 2B, 2C showed that 33 (67.3%) of the bacterial isolates tested positive for the *toxA* gene.

Figure 2 (A). Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *P. aeruginosa* using primer toxA gene with product 352bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (1500 bp ladder).). Lane (-ve) show negative controls**.** Lanes (1 to 18 except 7,10,14,16 and 19) show positive results with gene toxA gene.
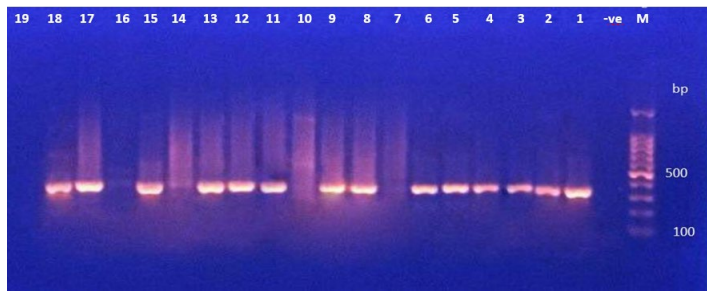

Figure 2 (B). Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *P. aeruginosa* using primer toxA gene with product 352bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (1500 bp ladder).). Lane (-ve) show negative controls without DNA. Lanes (21 to 39 except 30 and 36) show positive results with gene toxA gene.
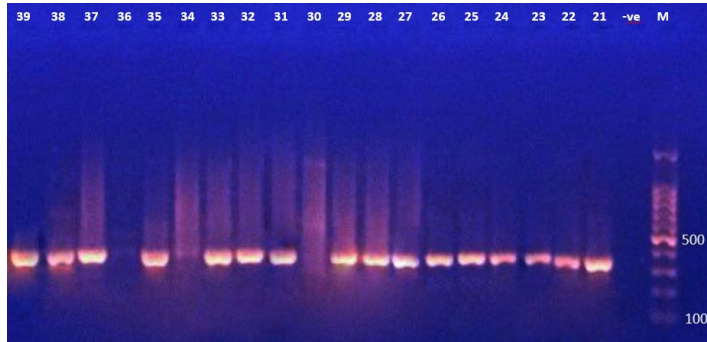

Figure 2 (C). Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *P. aeruginosa* using primer toxA gene with product 352bp. The electrophoresis was performed at 70 volt for 1.5-2hr. lane (L), DNA molecular size marker (1500 bp ladder).). Lane (-ve) show negative control witout DNA. Lanes 40,45 and 46 show positive results with gene toxA gene. Lanes41,42,43,44,47 and 48 were negative

4. Discussion

The major finding in the presented study was that most isolates of *P. aeruginosa* from OM patients showed the presence of *nan1* and *toxA* genes. We found that 19 (38.77%) isolates carried the *nan1* gene, as shown in Figure 1. Khattab *et al.* also found this gene in four out of ten (40%) pulmonary tract isolates and likewise (40%) burn isolates, but not in blood infection isolates [19]. These results confirmed the presence of this gene in *P. aeruginosa*, which is considered one of the virulence factors [19]. *P. aeruginosa* has been reported as the most prevalent cause of OM, followed by *Staphylococcus aureus*, and infection rates for the first two types are the greatest among all instances of infection [20]. However, other studies have found that *S. aureus* is the most common cause of OM [21, 22]. The pattern of microbiological distribution differs in OM due to geographical variance, local antibiogram pattern, and differences in climate, community, and patient characteristics [23].

All protein virulence factors in the *P. aeruginosa* PAO1 strain are chromosomally encoded, and their gene sequences and locations are well known (earlier called "*P. aeruginosa* strain 1") in the genome's

sequence [24]. The coexistence of antimicrobial resistance determinants, which virulence factors may partially explain in *P. aeruginosa* isolates, is a serious issue in treating this bacterial infection [25]. It is essential to detect all mediators in multidrug-resistant organisms to determine the optimal treatment options for such infections [26].

The pathogenicity of *P. aeruginosa* was measured using a variety of cell-associated chemicals, including (e.g., flagella, pili, alginate capsule, and lipopolysaccharide) [27]. *P. aeruginosa*, on the other hand, can secrete substantial amounts of extracellular components (mainly toxins like exotoxin A and exoenzymes) as well as enzymes that may contribute to pathogenicity (e.g., proteases, elastases, phospholipase C, and neuraminidases) [28]. The *nan1* gene encodes a critical cell-surviving protein called sialidase or neuraminidase enzyme [29]. This enzyme is essential for adhesion to the respiratory tract [29]. Extracellular neuraminidase aids bacterial implantation, albeit the genetic basis of this action is unknown [29]. Some studies found that the *nan1* gene was found in bacterial isolates from the ear and burnt swabs than in blood isolates [20,

30, 31). Differences in virulence factor gene distributions in populations increase the likelihood that some *P. aeruginosa* strains are more adapted to the unique conditions encountered in specific infectious locations. Hence, virulence gene expression varies according to the infection site and severity **(14, 32)**.

Another virulence factor recruited by *P. aeruginosa* is endotoxin A, which is considered the pathogenic bacterium's most toxic virulence factor **(33)**. This protein is encoded by the *toxA* gene of *P. aeruginosa* (Figure 2), and many isolates exhibit the presence of this gene. Like other extracellular virulence agents, including diphtheria, cholera, and pertussis toxin, Exotoxin A inhibits protein synthesis **(34)**. An ADP-ribosyl transferase coats the host elongation factor-2, halting protein synthesis and resulting in cell death and hearing loss **(34)**. Exotoxin can infiltrate the inner ear within hours, enhancing round window membrane (RWM) permeability during acute inflammation. But it decreases during persistent infections or the post-infectious phase **(34, 35)**, which may lead to hearing loss with the onset of deterioration at 24-48 h after instillation, indicating rapid incursion **(34)**. Therefore, treating the infection at the beginning of the illness protects against hearing damage.

The regulation of *toxA* expression is a complicated process involving numerous gene products. RegA, Vfr, Fur, PvdS, PtxR, and PtxS are transcriptional regulators suggested playing a role in *toxA* production, among other proteins **(36)**. PtxR and PtxS are the most perplexing regulators **(37)**. There has been no direct interaction between these proteins and the *toxA* promoter, and the molecular mechanism by which PtxS regulates *toxA* expression is unknown **(36)**. *P. aeruginosa* is antibiotic-resistant due to various mechanisms, including a low-permeability membrane, efflux pumps, and a variety of beta-lactamases **(38)**. There is also a possible dependency of antibiotic resistance to the existence of *toxA* and *nan1* gene **(37)**. A higher frequency of these genes in isolates resistant to treatment indicates the involvement of these genes in drug resistance **(38)**.

In particular, *P. aeruginosa* was implicated in the progressive destruction of middle ear tissues through its toxins and enzymes **(39)**. *P. aeruginosa* is a gram-negative bacterium with high adaptability and intrinsic resistance to antibiotics, enabling it to colonize a variety of settings **(40)**. It has been proposed that during OM, the ear environment became suitable for the colonization of *P. aeruginosa,* and that difficult to eradicate or remove its toxins and enzymes, which damage the ear tissue. *P. aeruginosa* can avoid the host immune response

by minimizing blood flow and forming a shell that protects itself **(41)**. It has several exotoxins and enzymes that are secreted passively or through the secretion system; most importantly, exotoxin A (toxA), protease (lasA), and elastase (lasB), all these virulence factors secreted via secretion system type II **(42)**. In Najaf city of Iraq, we found a high prevalence of OM patients due to infection with *P. aeruginosa*, and that probably resulting from a) increased antibiotic resistance as the people can get any antibiotic over their counter, b) lack of hygiene, especially in government hospitals that enhance the prevalence of many opportunistic bacteria such as *P. aeruginosa* c) the potentials of bacteria to adapt with various environments.

There are some limitations in the current study that should be considered when interpreting the present findings. First, we could not examine the association of *nan1* and *toxA* genes with different antibiotic resistance. However, many of the isolates were resistant to some antibiotics. Further studies should examine only resistant bacteria. Moreover, real-time polymerase chain reaction (RT-PCR) should recruit to determine whether these genes are translated and produce proteins.

5. Conclusion

Our findings reveal that the bacterial isolates of P. aeruginosa from OM patients have the *nan1* and *tox A* genes, with a high incidence of tox A among *nan1*. Probably these genes partly explain the resistance of the bacteria to the treatment. Furthermore, *P. aeruginosa* is the most cause of OM in Iraqi patients.

Conflict of Interest

We declared no conflicts of interest.

Authors' Contributions

The isolates have been gathered from two hospitals by Maryam R. Mansor and Zainab Sabah AL-Khalidi. Estabraq Hassan Almuhanna has tested the isolates for antibiotic resistance and for identifying each species of bacteria. Haider Ali Alnaji, Maryam R. Masor, and Hiba Ridha Hussein have managed to do the PCR and Abbas F. Almulla has written Gel

Electrophoresis and the article with contributions of Maryam R. Finally, Mansor and Haider Ali Alnaji. Abbas F. Almulla, Haider Ali Alnaji, and Maryam R. Mansor have updated the article.

Ethical Approval

The College of Medicine at the University of Kufa in Iraq provided the IRB approval (617/2020) compliant with the Declaration of Helsinki's International Guideline for Human Research Protection.

Availability of Data and Materials

All the relevant data is present within the current version of the study; however, upon request, any extra information will be provided by the corresponding author (AA).

Ethics Approval and Consent to Participate

We followed the Iraqi and international ethics and privacy laws to perform the current study. Before participating in the present study, written informed consent was obtained from each individual or their parents.

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