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Research Article

Molecular characterization and phylogenetic analysis of *Pseudomonas aeruginosa* isolated from active chronic otitis media

Kassim R. Dekhil

Department of Surgery, College of Medicine, University of Al-Qadisiyah, Iraq

ABSTRACT

This study had studied the prevalence of some virulence genes which were carried by *Pseudomonas aeruginosa* isolated from patients affected by active chronic otitis media (COM) in Ad-Diwaniyah teaching hospital, Iraq. Fifty samples were taken from affected patients and submitted to culture on media for enrichment then submitted to extraction of DNA by using of 16S rRNA gene in polymerase chain reaction (PCR) test. Moreover, Phylogenetic analysis tree of DNA (sequencing methods) confirmed the results. A summarized results included 38/50 (76%) of total cases caused by *Pseudomonas aeruginosa*, virulence genes included a toxic gene (84.2%), exoS gene (63.2%) and OprI gene (47.4%). The results also showed the multiple alignment analysis of sequencing similarity in 16S rRNA gene nucleotide sequences. It has been concluded that active COM may be more complicated due to virulence genes that carried by *Pseudomonas aeruginosa*; these virulence genes make the infection more difficult to be treated.

Keywords: Virulence genes; Phylogenetic analysis; Pseudomonas aeruginosa; Otitis Media.

INTRODUCTION

Pseudomonas aeruginosa is one of the most leading gram-negative pathogen implicated in a variety of infections including septicemia, chronic otitis media (COM), lower respiratory, tract infections, cystic fibrosis and pneumonia (Chaudhary et al., 2013). Chronic otitis media (COM) implies a permanent abnormality of pars tensa or flaccid most likely due to previous acute otitis media, negative middle ear cavity pressure. In the active mucosal COM, there is a permanent detect of pars tensa with an infected middle ear mucosa which produces mucous that may discharge (perforation with otorrhoea) while in active squamous subtype of COM (cholesteatoma) there is a retraction of pars flaccida or pars tensa with retained squamous epithelium debris and associated with chronic inflammation and production of scanty discharge, often from adjacent mucosa (Browning et al., 2008; LEE, 2003).

An active chronic otitis media is usually associated with discharge from the middle ear. The bacteria isolated from these cases include both aerobic and anaerobic bacteria. Among the common bacteria, Pseudomonas aeruginosa has been particularly isolated from these infections, that bacteria which secrete toxins and enzymes (Deshmukh et al., 2017).

* Corresponding Author
Email: kassimraisan2@yahoo.com
Contact: +96-47707496510
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Pseudomonas aeruginosa is a bacterium that can also infect immunocompromised persons. (Van Delden et al., 1988). It can cause acute and chronic infections in human. *P. aeruginosa* infects the urinary tract, pulmonary tract, burns, and ear (Gomez et al., 2006). Also, P. aeruginosa is considered as one of the most microorganisms that causes a big problem in the public health (Canton et al., 2005). There is an increase in the mortality and morbidity associated with *P. aeruginosa* infections due to the organism's high ability to adaption with the new environment and it can develop defense mechanisms such as virulence factors (Mitov et al., 2010; Breidenstein et al., 2011). There are many studies confirmed *that Pseudomonas aeruginosa* produce many virulence factors including biofilm formation, production of exotoxin and proteolytic enzymes (Morales et al., 2012; Holder et al., 1989). The toxA gene detected in *P. aeruginosa* isolates encoding exotoxin A protein. Exotoxin A (ETA) is a common powerful chromosomal extracellular virulence factor produced by most of the clinical *outer* membrane lipoprotein I and peptidoglycan, it was considered as an important virulence protein in *P. aeruginosa* isolates (Matthijs et al., 2013). Furthermore, exoS gene is a virulence gene; it creates the protein exodeoxyribonuclease which has a great role in the pathogenesis present in many bacteria like *P. aeruginosa* (Puyet et al., 1989; Shida et al.,1999). Recent studies had revealed that *P. aeruginosa* is one of the most frequent bacteria which forms many virulence factors like toxA, exoS, oprI genes (Vincent et al., 2000; Feinbaum et al., 2012; Zourob et al., 2008). The aim of this paper is to study the prevalence of virulence genes in *P. aeruginosa* in active chronic otitis media cases and study the

phylogenic analysis by using PCR, the importance of this subject emerges from the great distribution of this problem.

PATIENTS & METHODS

Sample collection

Fifty swabs were taken from active COM infection patients in Ad- Diwaniya teaching hospital, Diwaniyah City-Iraq from June 2015 to July 2016. The samples placed in sterile transport media then send to Microbiology Lab. of Medical College and kept in the refrigerator for bacterial identification.

Bacterial agent isolation

Pseudomonas aeruginosa was cultured on Brain Heart Infusion Broth at (37°C) and kept for 12 hours in rich culture, and then the bacterial culture on chrome agar at (37°C) for 12-24 hours for selective growth of a pure culture of *Pseudomonas aeruginosa*.

Bacterial genomic DNA extraction

(Preston Mini gDNA Bacteria Kit. Geneaid. Made in the USA) was the kit which was used for the extraction of Genomic DNA. (1ml) of bacterial growth solution cultured on BHI broth, then the tube submitted to centrifuge at (10.000) cycle for a minute. Discard the supernatant (high layer), pellets of the bacterial cells used for genomic DNA, the extraction process was done depending on company directions. Finally, Nanodrop used to test the genomic DNA then kept in the refrigerator for PCR technique.

Polymerase chain reaction (PCR)

PCR assay was used for detection and identification of some virulence factor genes in pathogenic Ps. aeruginosa isolated from active chronic otitis media samples and method described by Nikbin et al (2012). 16s rRNA gene primers and virulence factor genes primers; exotoxin A (toxA), exoenzyme S (exoS), outer membrane lipoprotein I (oprI) were designed in this study to depend on NCBI- Genbank and primer 3 plus website for design online, as in table (1).

The primers used in our study taken from (Bioneer Company. Made in Korea). The PCR master mix was prepared by using a kit called (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix consists of the following materials (Taq DNA polymerase enzyme, MgCl2 1.5mM, KCl 30mM, Tris-HCl 10mM, dNTPs 250µM, dye, and stabilizer). The directions of the company are used for the preparation of the PCR master mix as in the table (2). thermocycler apparatus used for reaction making (this apparatus is Mygene Bioneer. Made in Korea) according to the table (3). The PCR final products were tested by electrophoresis at a concentration (1.5%) agarose gel, and the used stain called ethidium bromide and the band watched under UV reader.

DNA sequencing method

Phylogenic tree analysis or DNA sequencing method was done for 16S rRNA gene in local pathogenic *P. aeruginosa* isolates and compared with NCBI-Genbank global *Ps. aeruginosa* isolates and finally submitting our isolates to NCBI-Genbank database. The 16S rRNA gene 569bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). Product Samples of the purified

16S rRNA gene PCR were sent to Macrogen Company in Korea for performing the DNA sequencing using (AB DNA sequencing system). The phylogenetic tree analyzed in NCBI-Blast alignment identification system and (Mega version 6 edition software).

RESULTS

Bacterial isolation results for Ps. aeruginosa from active chronic otitis media infection were shown in the table (4). All *P. aeruginosa* isolates were positive PCR detection based on 16S rRNA gene.

Table 1: Size and gene bank code in the 16SrRNA and virulence gene toxA, exoS, oprI

Primer	Sequence		Size	GenBank code
16SrRNA	F	TCAACCTGGGAACTGCATCC	697bp	KF483133.1
	R	CAGACTGCGATCCGGACTAC		
toxA	F	CATGTCGCCGATCTACACCA	351bp	JX026663.1
	R	CTGATGACCGTGGGCTTGAT		
exoS	F	TTGAAGGGACTCGACAAGGC	569	AY029246.1
	R	GCGGACATACCTTGGTCGAT		
April	F	GTCTGACCGCTACCGAAGAC	150bp	JN628629.1
	R	CTTTTTCCAGCATGCGCAGG		

Table 2: Preparation of the PCR master mix

The composition	Size
Genomic DNA	5µl
Forwarding primer	1.5µl 10pmole
Reverse primer	1.5µl 10pmole
PCR water	12 µl
Total	20 µl

Table 3: Reaction making thermocycler apparatus

The stage	Temperature	Time	No of cycles
Initial denaturation	95 °C	5 min.	1
denaturation	95 °C	30 second	30 cycles
annealing	58 °C	30 s	--
extension	72 °C	1min	--
final extension	72 °C	10 min	--

Table 4: Number and percentage of total and positive samples were taken from Swab samples of active chronic otitis media

Sample	Total samples	Positive isolates	Percent
Swab samples of otitis media	50	38	76%

Table 5: Number and Percentage of virulence genes in P. aeruginosa that isolated from active COM

Virulence gene	*P. aeruginosa* isolates of chronic otitis media infection. total percentage %
toxA gene	32/38 (84.2%)
exoS gene	24/38 (63.2%)
oprl gene	18/38 (47.4%)

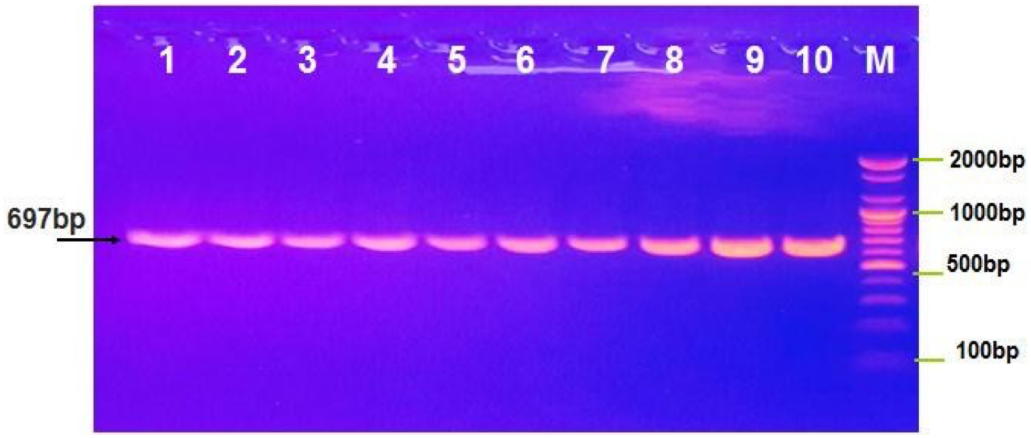

Figure 1: The PCR assay shows positive amplification of 16S rRNA gene on electrophoresis that used for detection of *P. aeruginosa* isolates, (M) represents DNA marker (100-2000bp), the band (1-10) represents *P. aeruginosa* isolates at 697bp

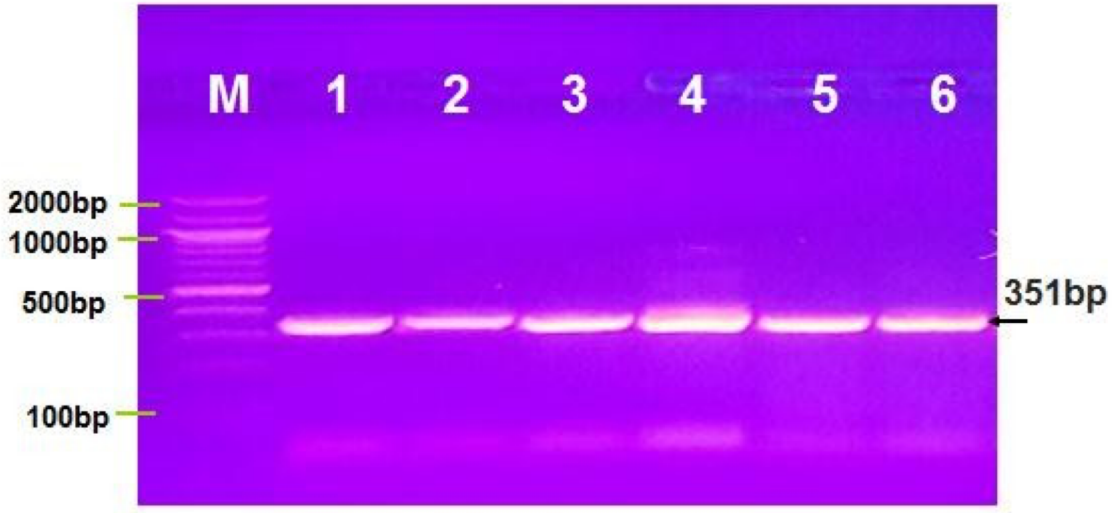

Figure 2: The PCR assay shows positive amplification of virulence factor toxA gene in Agarose gel electrophoresis of *P. aeruginosa* isolates. (M) represents DNA marker (100-2000bp), the band (1-6) *P. aeruginosa* isolates at 351bp

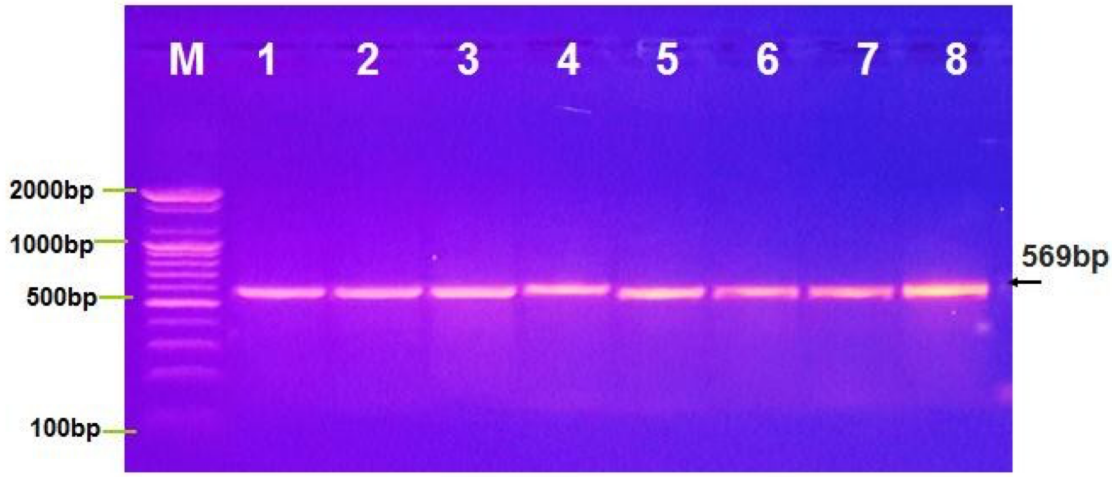

Figure 3: The PCR assay shows positive amplification of virulence factor exoS gene in *P. aeruginosa* isolates in electrophoresis apparatus. (M) represents DNA marker (100-2000bp), the band (1-8) *P. aeruginosa* isolates at 569bp

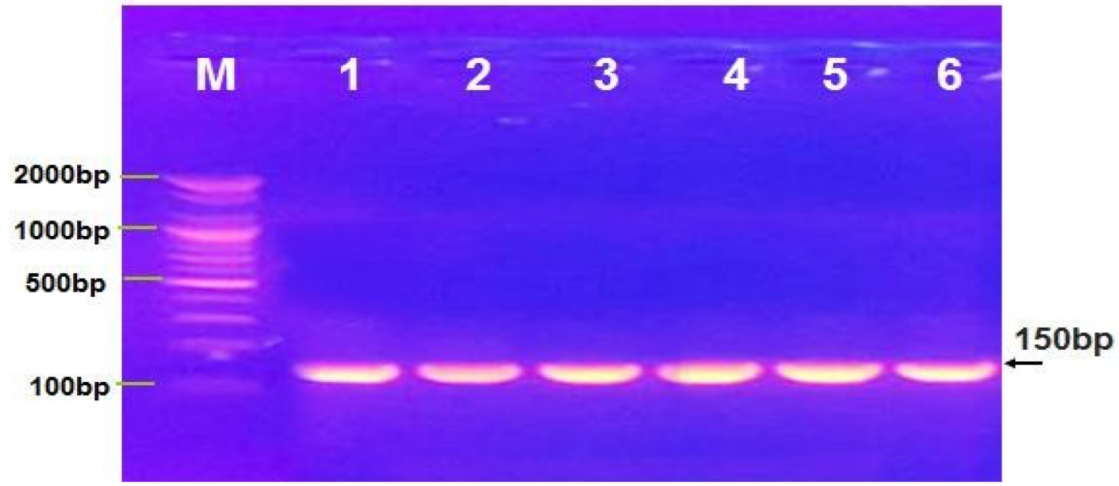

Figure 4: The PCR assay shows positive amplification of virulence factor or gene in *P. aeruginosa* isolates in electrophoresis assay. (M) represents DNA marker (100-2000 bp), the band (1-6) *P. aeruginosa* isolates at 150bp

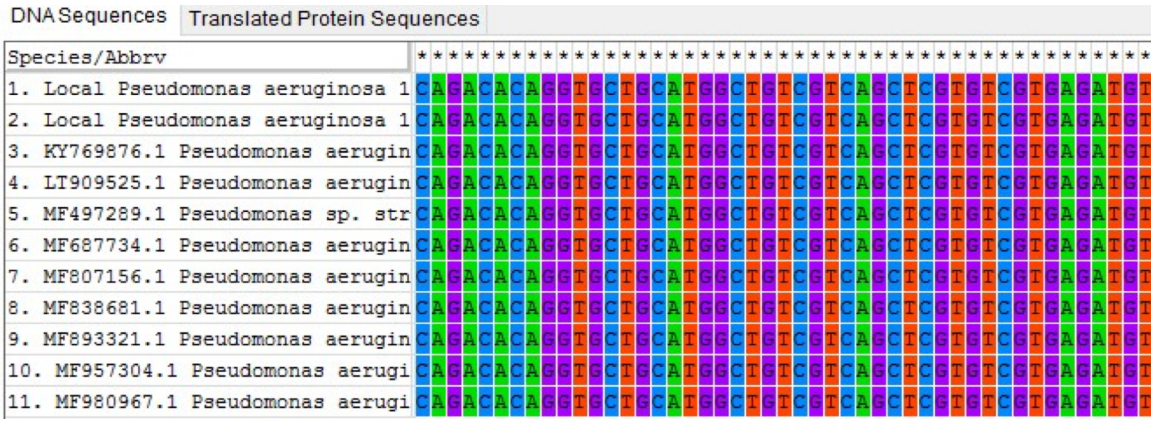

Figure 5: Multiple sequence alignment analysis of the partial 16S rRNA gene sequence in local *P. aeruginosa isolates of* active chronic Otitis Media (OM- IQD.No.1 & OM-IQD.No.2) depends on analysis of ClustalW alignment technique. The software called (MEGA 6.0), MEGA consists of multiple alignment analysis tools. The figure shows similarities and differences in sequences of 16S rRNA gene

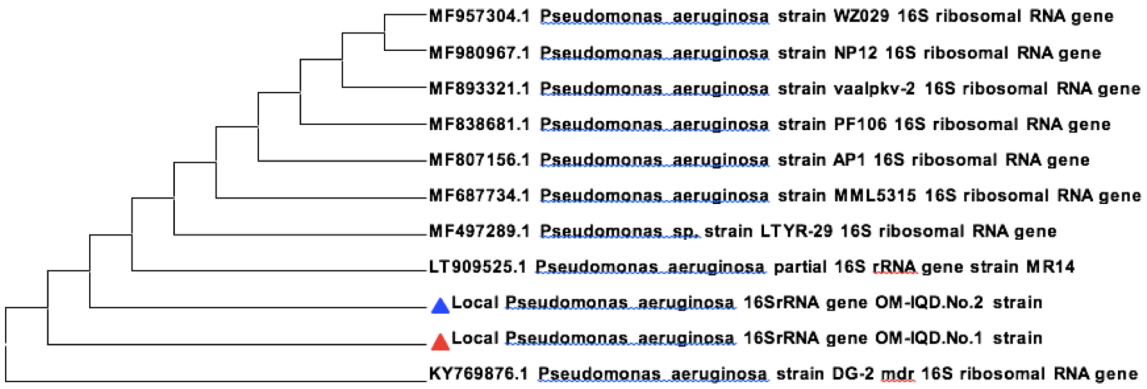

Figure 6: Phylogenetic tree analysis based on the 16S rRNA gene partial sequence that used for local P. aeruginosa isolates of active chronic Otitis Media (OM- IQD.No.1 & OM-IQD.No.2) strains. The phylogenetic tree was constructed using maximum likelihood tree method in (MEGA 6.0 version). The local P. aeruginosa isolates of active COM (OM-IQD.No.1 & OM-IQD.No.2) shows close relation to NCBI-Blast P. aeruginosa strain with identity range from 99-100

Whereas, these *P. aeruginosa* isolates appeared vibration in the detection of virulence factors genes (toxA, exoS, and orpI) as in the table (5) and figures (1) (2) (3) (4).

DNA sequencing results

The DNA sequencing of 697bp 16S rRNA gene that amplified by PCR from two local *P. aeruginosa* isolates of active COM (OM-IQD.No.1 & OM-IQD.No.2) strains, which was submitted to GenBank (accession No. MG062746 & MG062747) respectively. BLAST analysis showed that the 16S rRNA gene shared more than 99% homology with the sequences of P. aeruginosa. Furthermore, the phylogenetic tree analysis of 16S rRNA gene indicated that (OM-IQD.No.1 & OM-IQD.No.2) 16S rRNA gene shared higher homology with other 9 P. aeruginosa strains available in GenBank. The homologies of nucleotide were from 99.9% to 100%.

DISCUSSION

Pseudomonas aeruginosa is a gram-negative and rod-shaped pathogenic bacterium associated with hospital-acquired infections and many other infections. It is also one of the most common microorganisms of ear infections in all age groups (Yeo et al., 2007; Hancock, 1998). The results of this study had shown that active chronic otitis media (COM) could be caused by *P. aeruginosa*, where it is isolated from these cases. This agrees with results of Song et al., (2000). Also, the detection and genotyping of the bacteria are very important for successful treatment. PCR assay is a molecular technique used for detection of bacteria, it needs a short time for doing the process (Xu et al., 2004). Based on the results of present study, the percentage of active chronic otitis media infection cases that caused by *P. aeruginosa* is 38/5 (76%). (79.5%) was recorded by Al-Shammary et al., (2014), this percentage is very close to current results, while Neamah (2017) who took samples from different infected locations including chronic otitis media had found a percentage of (68.7%), and this represents slightly lower than our rate. While other studies recorded a percentage of *pseudomonas aeurogenosa* in otitis media cases less than current results, where Auda et al (2015) recorded a percentage of (59%), Lee et al., (2010) recorded (25.8%) and Al-Saadi (2012) recorded (11%). On the other hand, Deshmukh et al., (2017) had found (84.6%) as a percentage of COM cases positive for *P. aeruginosa* which represents more than our rate. There is a difference in the percentage of occurrence of infections due to P. aeruginosa between our results and those of other studies. There are many reasons which can explain this variation between the reports, may be due to nutrition requirements, collection place of clinical samples and environmental factors (Ogunseilan et al., 2005). Furthermore, the prevalence and susceptibility depend on time and geographical area, and the use of antibiotics (Hassan et al., 2007). *Pseudomonas aeruginosa* has several virulence genes which will increase the pathogenesis in the active COM, some genes responsible for the production of a protein that has a great role in the pathogenesis and causes extensive tissue damage (Gaines et al., 2007; Hirakata et al., 2002). According to this study survey, the percentage of tox A gene is 32/38(84.2%), a study done by Al-Kaaby (2005) had found different values ranged between (72.2%-94%) and this percentage set at our range that detected from Pseudomonas aeruginosa isolated from active COM cases, also near to the results of Al-Shammary et al., (2014) where the percentage was (84%) Nikbin et al (2012) recorded a result less than current study results, while the percentage was (89.4%) by Rawya et al (2008). The percentage of tox A gene was (33.3%) by Thamir et al., (2014) in chronic otitis media cases, that represents very low rate as compared with our study. The present study had found that exo A gene, and OprI gene were 24/38(63.2%) and 18/38(47.4%) respectively, which it's encoded as a protein; it has a great role and importance in the pathogenesis of the disease. Neamah (2017) had

found a percentage of exoA gene as that found by AL-Zubaidi et al., (2014). It was considered less than our results. Neamah (2017) found (45.4%) of exo A gene in *Pseudomonas aeruginosa* isolates, and the percentage of our gene is (54.5%) in otitis media cases. Al-Kaaby (2005) recorded a prevalence as (50%-56%) of our gene in Pseudomonas aeruginosa that isolated from different cases. Virulence genes of *P. aeruginosa* isolates have a great role in the pathogenicity of strains as seen by Nibkin et al., (2012). The difference in the values, the prevalence of Pseudomonas *aeruginosa* and percentage of virulence factor genes depend on several causes including nature of places, immune status of patients, the degree of contamination and virulence of the strain as seen by Khan et al., (1994). Biofilm formation of *P. aeruginosa* may vary among the strains. Genetic variation and genomic exchange may also responsible for increasing the virulence genes and factor. Further gene sequencing used for local strains helps successful treatment and reduce the mortality rate among patients. Variation in results of these reports related to the number of clinical isolates from different sites (Lomholt et al, 2001; Feltman et al., 2001). In addition, the difference in the distributions of virulence genes in the population depends on infection site and type (Cotar et al., 2013; Schmidtchen et al., 2001; Lee et al., 2010). 16S rRNA is the gene that used to study phylogenic tree analysis in *P. aeruginosa* strains. The strains recorded under code (OM-IQD.No.1 & OM- IQD.No.2) by using software called (MEGA 6.0 version). The results had shown that *P. aeruginosa* strains closely related to database of NCBI-Blast of *P. aeruginosa* strains with identity range from 99-100%.

CONCLUSION

It has been concluded the active chronic otitis media caused by *Pseudomonas aeruginosa* which has many virulence genes such as exoA, tox A and opal gene that have an important role in disease developing, should take attention and beware about the distribution of this dangerous organism.

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