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MOLECULAR GENETIC STUDY CONFIRMING THE TRANSMISSION OF NASOPHARYNGEAL BACTERIA TO MIDDLE EAR IN PATIENTS WITH CHRONIC SUPPURATIVE OTITIS MEDIA, INCLUDING NEW GLOBAL STRAINS IN GENBANK: MUNAALA1, MUNAALA2, IRQBAS5 AND IRQBAS6

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Abstract: One hundred samples were collected randomly from the middle ear (ME) of 50 Chronic Suppurative Otitis Media (CSOM) patients and 50 from their nasopharynx (NP). Ninety six bacterial isolates (66 from ME and 30 from NP) were successful to identify by 16S rDNA sequencing. Thirty three different species were obtained from both sources (22 from ME and 11 from NP). The most common bacterial species in ME were *Pseudomonas aeruginosa* 17 (25.75%), *Staphylococcus aureus* 13 (19.69%) and *Staphylococcus epidermidis* 12 (18.18%). However, in the present study, five different species: *Pseudomonas putida, Pseudomonas stutzeri, Providencia vermicola, Enterobacter asburiae* and *Enterobacter cloacae* were isolated from ME in the first time. Rooted phylogenetic tree showed 8 (16%) of 50 patients have the same bacterial species in their ME and NP. Moreover, 5 (62%) of these 8 patients have either *S. aureus* or *S. epidermidis* in these two sources. Furthermore, the UPGMA program for those 5 patients (depended on RAPD) appeared three (60%) patients, each has a strain (*S. aureus* or *S. epidermidis*) in the ME identical with that from his NP. These results confirm the hypothesis of transmission of bacteria from NP to the ME. Four isolates (45-*Bordetella trematum* and 78-*Pseudomonas Putida* from the ME, and 65-*Klebsiella pneumonia* and 21G-*Providencia stuartii* from NP) were reported as new global separated strains in the GenBank as MunAala2 (HG427201), IRQBAS5 (HG416957.1), MunAala1 (HG416956.1) and IRQBAS6 (HG427202) respectively.

Keywords: Transmission, Nasopharynx, Bacteria, Patient, CSOM

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INTRODUCTION

Otitis media (OM) in general, is an inflammation of the middle ear. The most common bacterial pathogens in OM are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis,* and the other pathogens responsible for OM are *Staphylococcus aureus*, *Escherichia coli, Klebsiella spp, Pseudomonas aeruginosa*, and *Proteus* spp [1,2]. Literature reported that the geographical area and respiratory infections may affect the type of OM pathogens [2]. Chronic Supportive Otitis Media (CSOM) is defined as persistent or intermittent infected discharge of more than three months duration through the perforated or non intact tympanic membrane that often results in partial or total loss of the tympanic membrane [3], caused by dysfunction of the Eustachian tube and bacterial infections [4]. There are many reports describing bacteriologic distributions of the middle ear (ME), mastoid and external auditory canal in normal and CSOM patients [4-6] Since, CSOM is a disease causing significant morbidity, thus, the identification of pathogen is responsible for CSOM [7]. Some studies have acknowledged the value of the nasopharyngeal bacterial reservoir as the etiology of acute otitis media[8], but there are few bacteriology comparisons between the nasopharynx (NP) and middle ear in CSOM patients [4]. Extensive bacterial colonization is proven in the nasopharynx [9]. But, there is a little evidence of bacterial colonization in the healthy middle ear, therefore, it is perceived to be sterile [10]. 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections [11]. By 16S rDNA sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible and phylogenetic relationships have been determined [12,13]. Moreover, randomly amplified polymorphic DNA (RAPD) is a PCR-based technique, using arbitrary primers to detect changes in the DNA sequence at sites in the genome, which is used for molecular epidemiological typing as it is relatively fast and easy [14,15]. The polymorphism within the set of DNA generated fragments has been used in discriminating microorganisms both at the interspecies and intraspecies level [15].

The aim of the present study was to determine the types of bacterial species in ME of patients with CSOM, and if there was a rule for nasopharyngeal bacteria in this disease.

MATERIALS AND METHODS:

1-Source of isolates

From Al-Basrah general hospital, Al-Sadr teaching hospital and medical clinics (from August 28 to November 30 of 2012), 100 samples were collected: middle ear (ME) swabs ($n_{=}50$) and nasopharynx (NP) swabs ($n_{=}50$) of CSOM patients aged between 1-60 years old using sterile cotton-swabs. A sterile cotton swab was inserted by the physician through the nose towards the posterior NP until there was a reflection of the patient, and other swabs from the ME was

inserted by the physician through the auditory canal until arriving at the tympanic membrane. Swabs were placed in sterile tubes and transferred to the laboratory [16].

All the samples collection and experiments in the original research and clinical trial articles were accomplished to the relevant regulatory standards with the agreement of the ministry of health and ministry of higher education in Iraq.

2-Genomic DNA extraction

In the middle ear, all colonies were extracted. But, in NP, only these colonies similar (Gram's stained and microscopic shapes) to ME colonies from the same patient were extracted. Five ml of Brain Heart Infusion (BHIB) was inoculated with tested bacterial colonies and incubated at 37^{O}C for 18h [17]. The grown bacteria were washed three times by distilled water with centrifuging for 5 minutes at 13,000 x g and discarded the supernatant. The procedure was performed according to the Genomic DNA Mini Kit (Blood\Culture Cell) from Geneaid (Korea). To check for DNA, the samples were loaded in 0.8% agarose gel 1 × TBE (54 g Tris-base, 0.5 M EDTA, 1- L distilled water, PH = 8, then diluted with 400 ml of distilled water) and electrophoresed at 60V for 30 min.

3-16S rDNA gene amplification (PCR)

The 16S rDNA was detected by thermo cycler apparatus (Thermo) according to Miyoshi *et al* [18] by the primers: 27 Forward 5'-AGAGTTTGATCCTGGC-3' and 1492 Reverese 5'-GGTTACCTTGTTACGACTT-3'. The polymerase chain reaction (PCR) is a mixture of the final volume of 50 µl containing 10 µl (30 ng), DNA template 2 µl of each primer (10 pmol), 25 µl Go Taq Green Master Mix (Promega) 2x and 11 µl Nuclease-free water. The PCR program involved initial denaturation at 92°C for 2 min, 30 cycles (denaturation at 94°C for 30 Sec, annealing at 51.8°C for 45 Sec and extension at 72°C for 1.5 min) and final extension at 72°C for 5 min. The amplified PCR mixtures were resolved by electrophoresis through 1% agarose gel at 60V for 1.5 h.

4-16S rDNA gene sequences

The sequencing of 16S rDNA gene and its preparation was according to MWG Operon Co. and BIONEER Co. with the procedure of Barker *et al*. [19]. For the purification of PCR product, 60 µl of 20% polyethylene glycol applied Biosystem (ABI) was added to 30 µl of PCR product (*16S rDNA* gene) and mixed by vortex (Whirlimixer) and incubated at 4°C overnight after centrifugation (Scotlab) at 1200 rpm for 20 min (twice). The pelleted DNA was mixed with 0.5 ml of 70% chilled ethanol and the recentrifuged product was dried in a vacuum drier (Thermo) for 30 min. The pellet was resuspended in 20µl (or 30µl for sending to MWG Co.) of free nuclease water, then left overnight at 4^{O}C. then checked by electrophoresis through 1% agarose gel at 60V for 1.5h.

5-Identification of bacteria

All bacterial species were identified (using the PCR sequencing products) in "BLAST" provided by the National Center for Biotechnology Information Service (NCBI) "http://www.ncbi.nlm.nih.gov" [20].

6-Phylogenetic tree

All the corrected sequences of 16S rDNA data from the present study were aligned for the concatenated of 1327 bp. and compared to assign the differences using "CLUSTALW" http://www .ebi. ac.uk / clustalw/ [20], then a phylogenetic tree by Neighbour Joining method construction using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) Version 7 http://mafft.cbrc.jp/alignment/server was appeared. The MAFFT website also provides a tree file, with a DNA extension, which can be opened by forester-1027 [21].

7- Random Amplified Polymorphic DNA (RAPD) –PCR

RAPD-PCR protocol and primers were according to Olorunfemi *et al* [22]; Abd Al-Abbas *et al* [23] for *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively. PCR primers (5'-TCGCCAGCCA-3') and (5'-GACACGGACC-3') tested in the present study were purchased from Promega Co. and each of 10 nucleotides long. Amplifications were performed by thermocycler apparatus (Thermo Co.) in 40µl reaction mixture consisting of 10 µl (30 ng) genomic DNA and 12.5µl of PCR PreMix (Go Taq Green Master Mix. 2x from Promega) covered by the USA the two primers were used together in a reaction as 3µl (100pmol) for each followed by 11.5 µl of free nuclease water. The reaction mixture was overlaid with 25µl of mineral oil to prevent evaporation. The cyclic program was (i) 1 cycle of 94^{o}C for 3 min., (ii) 45 cycles of 94^{o}C for 1 min. (denaturation), 36^{o}C for 1 min. (annealing) and 72^{o}C for 2 min. (extension), and (iii) a final extension at 72^{o}C for 7 min. The reaction products were resolved by electrophoresis in a 2% agarose gel at 60V for 1.5h. prepared in 1×TBE as described above (DNA extraction). A 100bp. ladder (Promega) was inoculated as molecular size marker. The gel was viewed under ultraviolet light, and the banding patterns were photographed by a digital camera (Apple).

8-Data Analysis

The RAPD bands of each individual strain were calculated for their distances based on the ladder's bands. The data of the RAPD patterns of all strains according to Olorunfemi *et al* [22] were transformed to the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm program creating and modifying by Garcia-Vallve and Puigbo [24]. RAPD patterns of individual strains were compared based on the index of similarity between samples [25], providing a mathematical model by calculating a similarity matrix and transforms similarity coefficients into a distance matrix (Distance Matrix = "0.000" indicating identical strains) and

makes a clustering to construct a dendrogram from a set of variables, to study genetic variation especially with difficult or closely related RAPD patterns.

RESULTS:

PCR amplification with 16S rDNA primers (27 Forward and 1492 Reverse) showed the gene bands at 1500 bp. for all bacterial isolates. The identification of ME isolates (in the present study) comparison with their reference strains by phylogenetic tree at 1327 bp. (except the species of *Bordetella trematum* because of its 1005 bp. only) as Figure (1) showed the *Pseudomonas aeruginosa* 17 (25.75%), *Staphylococcus aureus* 13 (19.69%) and *Staphylococcus epidermidis* 12 (18.18%) were the predominant isolates, but there was also a frequency of *Providencia stuartii* 3 (4.54%), *Klebsiella pneumoniae* , *Proteus mirabilis* and *Proteus penneri* 2 (3.03% for each), moreover, *Corynebacterium amycolatum*, *Alcaligenes faecalis, Serratia marcescens*, *Bacillus subtilis*, *Enterobacter hormaechei*, *Enterococcus faecium*, *Corynebacterium striatum*, *Bordetella trematum*, *Staphylococcus hominis* and

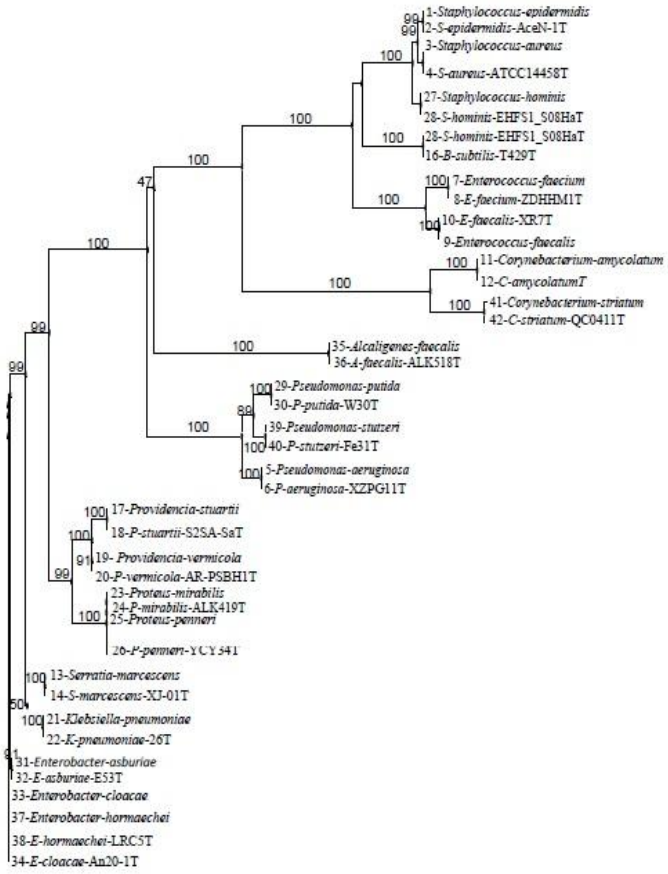

Figure (1): Rooted Neighbour Joining tree constructed from concatenated sequences of 1327 bp. for each strain (derived from an alignment of 16SrDNA gene sequences) then produced from a MAFFT alignment and visualized using forester version 1027. This N-J tree showing the

distribution and phylogenetic relationships of 21 different species were isolated from middle ear in this study and 22 reference strains (T). All horizontal branch lengths were drawn to scale. Bootstrap values after 1000 repetitions are indicated.

Enterococcus faecalis 1 (1.51% for each). While, the following five species were isolated from ME as the first time, including: *Providencia vermicola, Pseudomonas putida*, *Enterobacter asburiae, Enterobacter cloacae* and *Pseudomonas stutzeri* 1 (1.51% for each).

Out of 50 patients only 10 have the same bacterial species in the ME and their NP, including in each patient: 39-*Pseudomonas aeruginosa* and 38- *P. aeruginosa*, 14G-*Enterococcus faecium* and 7- *E. faecium,* 18-*Staphylococcus aureus* and 20-*S. aureus*, 134G-*S. aureus* and 70-*S.aureus*, 87-*S. aureus* and 42-*S. aureus*, 1-*Staphylococcus epidermidis* and 31-*S. epidermidis*, 18G- *S. epidermidis* and 29-*S.epidermidis*, or 22-*S. epidermidis* and 19-*S. epidermidis*, respectively. But, when depended on the similarity of 16S rDNA sequencing among them, they become only 8 (16%) patients because 66-*Klebsiella pneumonia* and 65-*K. pneumonia*, 27-*K. pneumonia* and 12-*K. pneumonia* were non identical in ME and NP (respectively) as Figure (2). For strains detection, the RAPD reaction (Figure 3) was performed with only *Staphylococcus aureus* or *Staphylococcus epidermidis* from 5 of 8 (62%) patients. The dendrogram (Figure 4) showed three of five patients (60%) each has strain 87-*S. aureus*, 18-*S. aureus* or 22-*S. epidermidis* in his ME identical to strain 42-*S. aureus*, 20-*S. aureus* or 19-*S. epidermidis* in his NP (respectively) with a Distance Matrix of 0.000 for each strains pair (Table 1). However, the *16S rDNA* gene sequencing with 'Blast' revealed that four bacterial isolates (No.45 and 87 from the ME, and No.65 and 21G from NP) were different from their reference strains in several positions of nucleotide sequences (Figures 5-11). Therefore, these isolates' database were published in European Nucleotide Archive (ENA) as new globally strains followed in GenBank which is a part of the National Center for Biotechnology Information (NCBI). The first isolate No.78-*Pseudomonas putida* as strain IRQBAS5 [GenBank: HG416957.1] was closely related (99%) to *Pseudomonas putida* strain W30, but with a Gene or Point mutation type Transition (C instead T) at the position 422 bp. The second isolate No.65-*Klebsiella pneumonia* as strain MunAala1 [GenBank: HG416956.1] was closely related (99%) with *Klebsiella pneumonia* strain HM2, but with seven Gene or

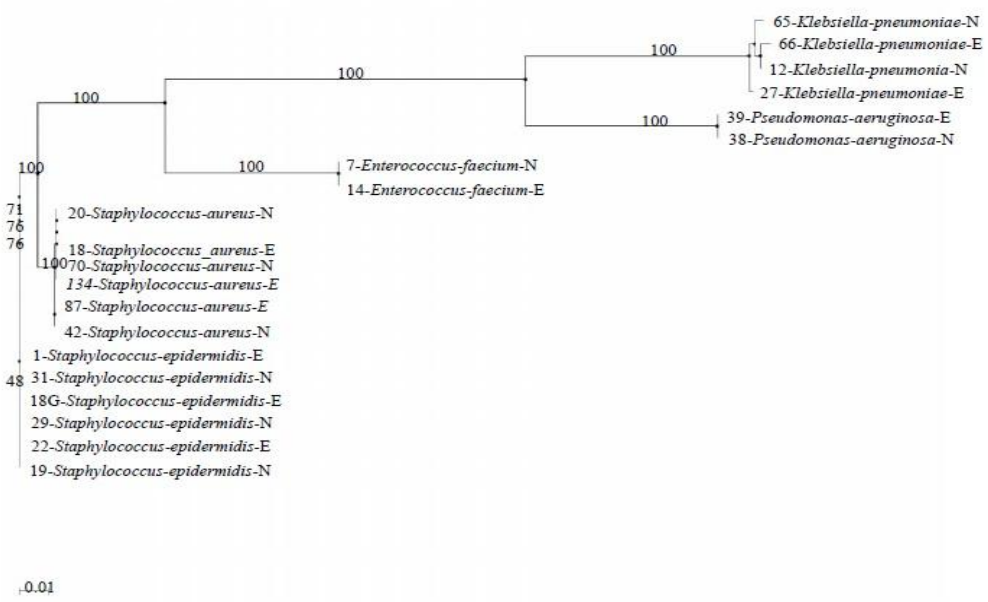

Figure (2): Rooted Neighbour Joining tree constructed from concatenated sequences of 1327 bp. for each strain (derived from an alignment of 16SrDNA gene sequences) then produced from a MAFFT alignment and visualized using forester version 1027. This N-J tree showing the similarity and identical phylogenetic relationships brtween each two species isolated from middle ear (E) and nasopharynx (N) of the same patient. All horizontal branch lengths were drawn to scale. Bootstrap values after 1000 repetitions are indicated.

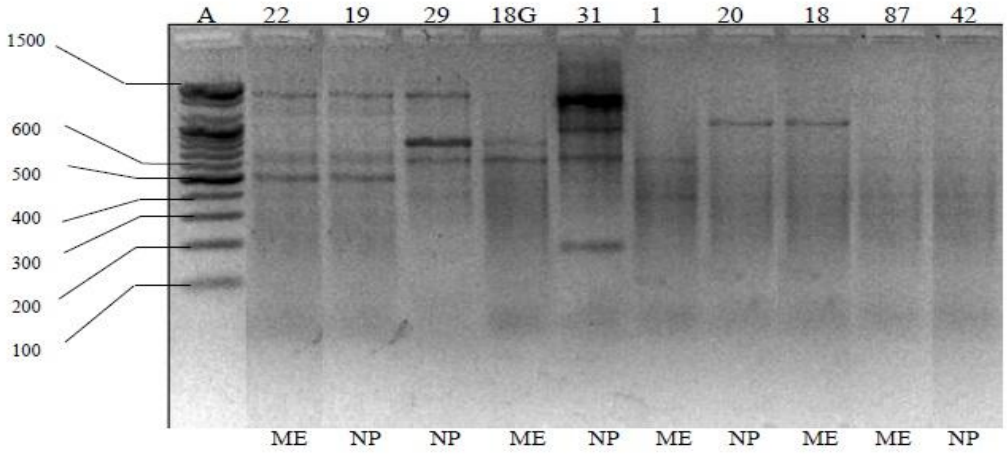

Figure (3): Agarose gel electrophoresis shows RAPD pattern of *Staphylococcus epidermidis* (No. 22, 19, 29 and 18G) and *Staphylococcus aureus* (No. 31, 1, 20, 18, 87 and 42) strains. ME: middle ear and NP: nasopharynx. Lane A: 100bp. ladder.

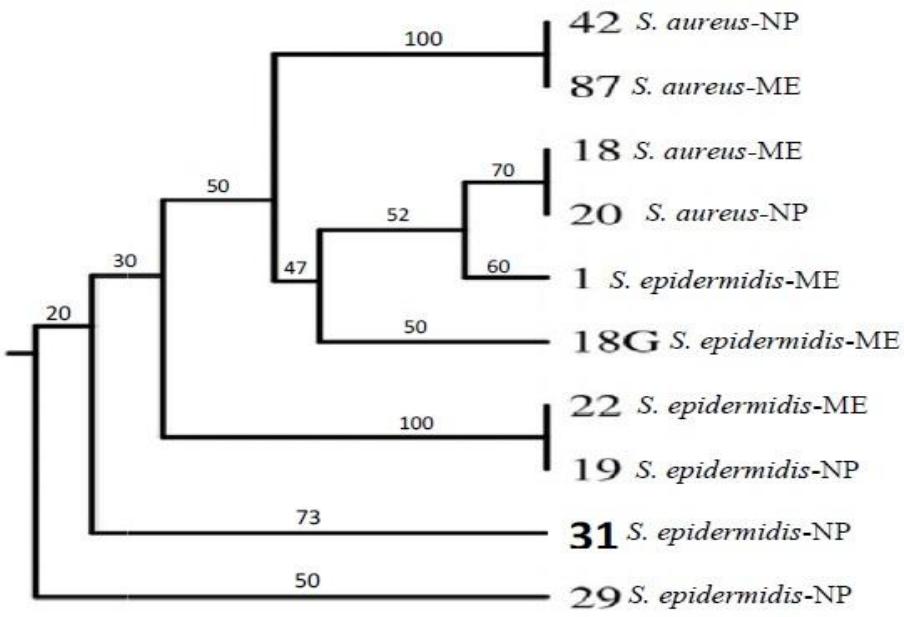

Figure (4): Dendrogram of *Staphylococcus epidermidis* and *Staphylococcus aureus* strains from ME (1, 18G, 22, 87 and 18) and NP (19, 31, 29, 42, and 20) of 5 patients, constructed by a set of variables RAPD bands using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Bootstrap values after 100 repetitions are indicated.

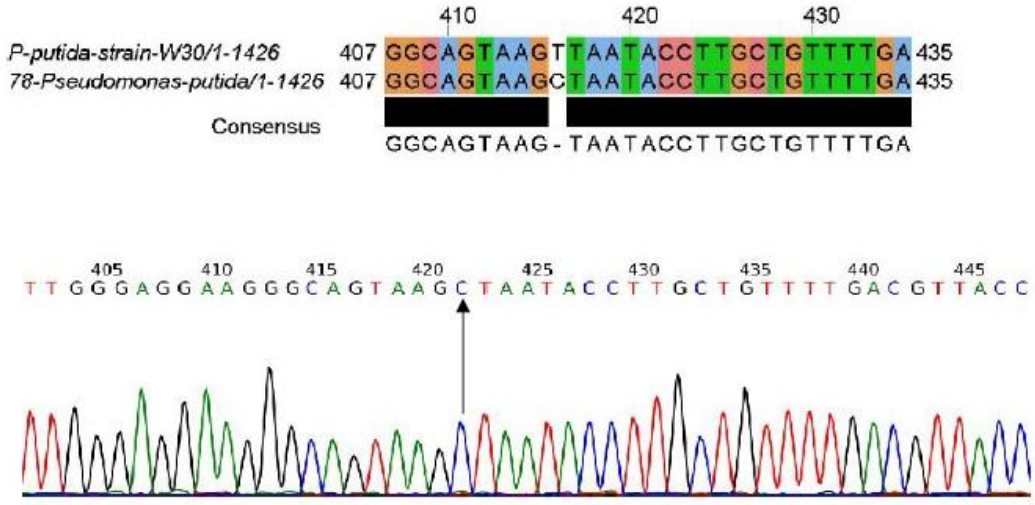

Figure (5): Comparison of 16S rDNA nucleotide sequences (1426bp.) for the isolate 87-*Pseudomonas putida* "IRQBAS5" (with peaks) and its reference strain W30. A Gene or Point mutation type Transition (C instead T) at the position 422bp.

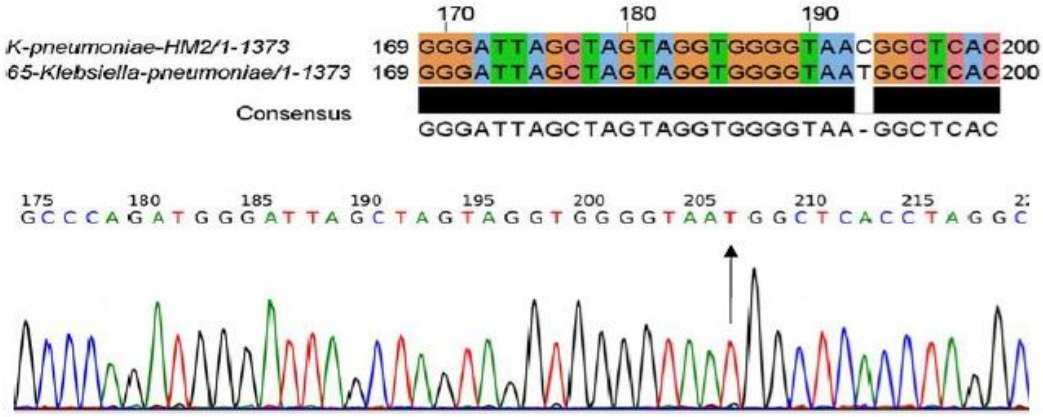

Figure (6): Comparison of 16S rDNA nucleotide sequences (1373bp.) for the isolate 65-*Klebsiella pneumoniae* "MunAala1" (with peaks) and its reference strain HM2. A Gene or Point mutation type Transition (T instead C) at the position 207 bp.

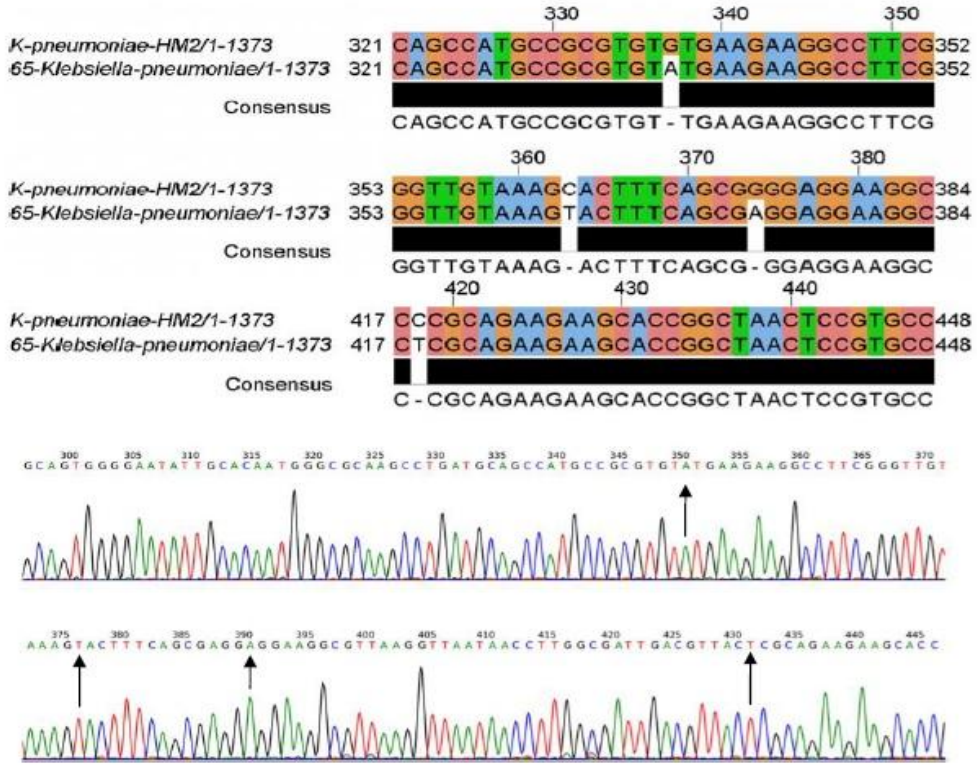

Figure (7): Comparison of 16S rDNA nucleotide sequences (1373 bp.) for the isolate 65-*Klebsiella pneumoniae* "MunAala1" (with peaks) and its reference strain HM2. Gene or Point mutations type Transition (A, T, A, and T instead G, C, G and C, respectively) at the positions 351, 377, 391 and 432 bp., respectively.

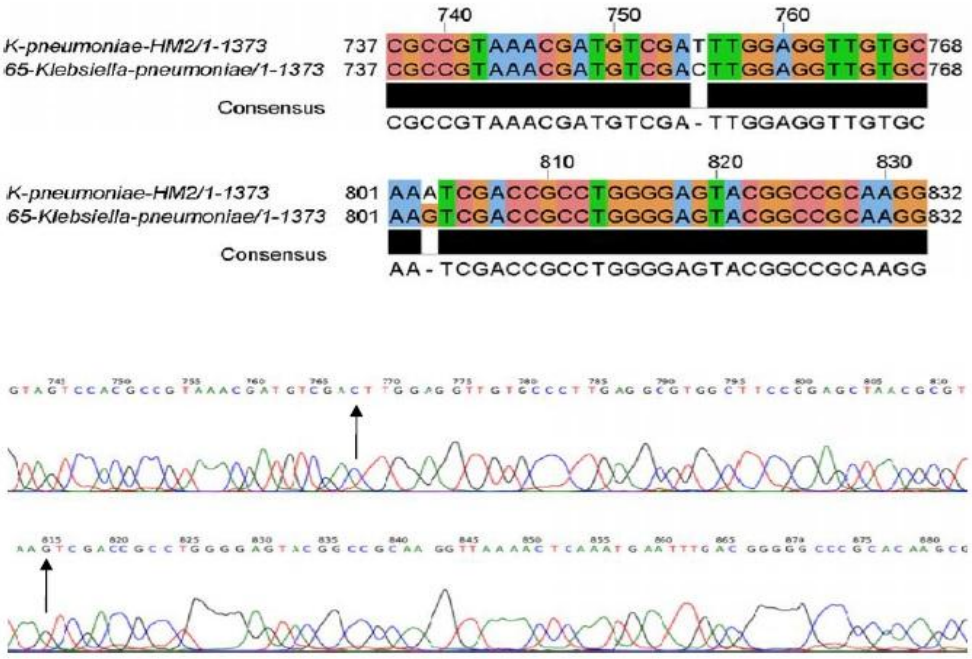

Figure (8): Comparison of 16S rDNA nucleotide sequences (1373bp.) for the isolate 65-*Klebsiella pneumoniae* "MunAala1" (with peaks) and its reference strain HM2. Gene or Point mutations type Transition (C and G instead T and A, respectively) at the position 768bp. and 815 bp., respectively.

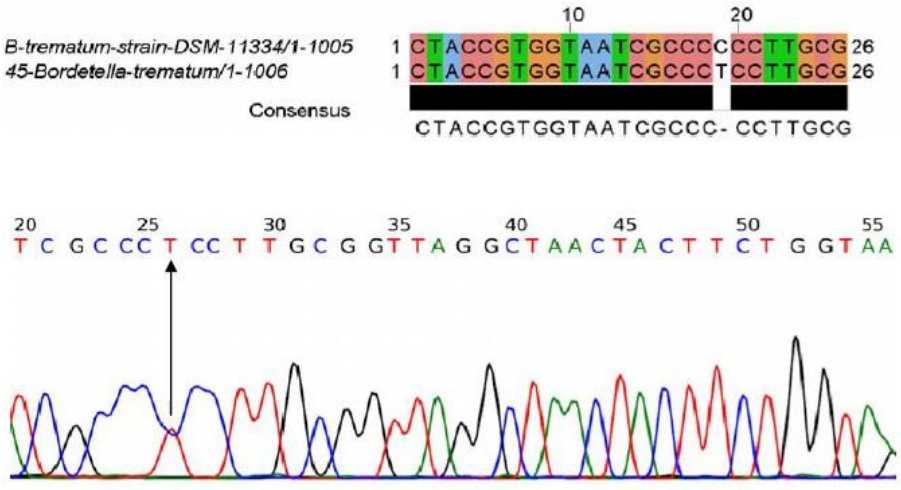

Figure (9): Comparison of 16S rDNA nucleotide sequences (1005bp.) for the isolate 45-*Bordetella trematum* "MunAala2" (with peaks) and its reference strain DSM 11334. Gene or Point Mutation type Transition (T instead C) at the position 26 bp.

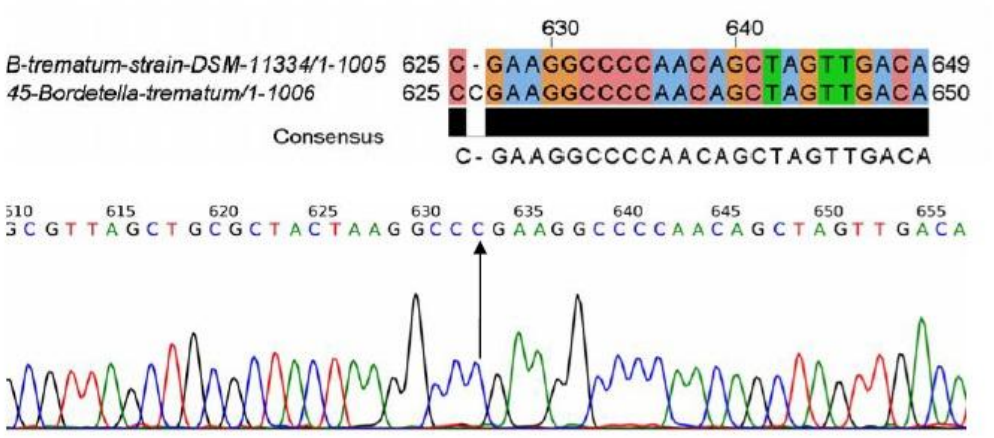

Figure (10): Comparison of 16S rDNA nucleotide sequences (1005bp.) for the isolate 45-*Bordetella trematum* "MunAala2" (with peaks) and its reference strain DSM 11334. Frame Shift mutation (insertion C) at the position 633 bp.

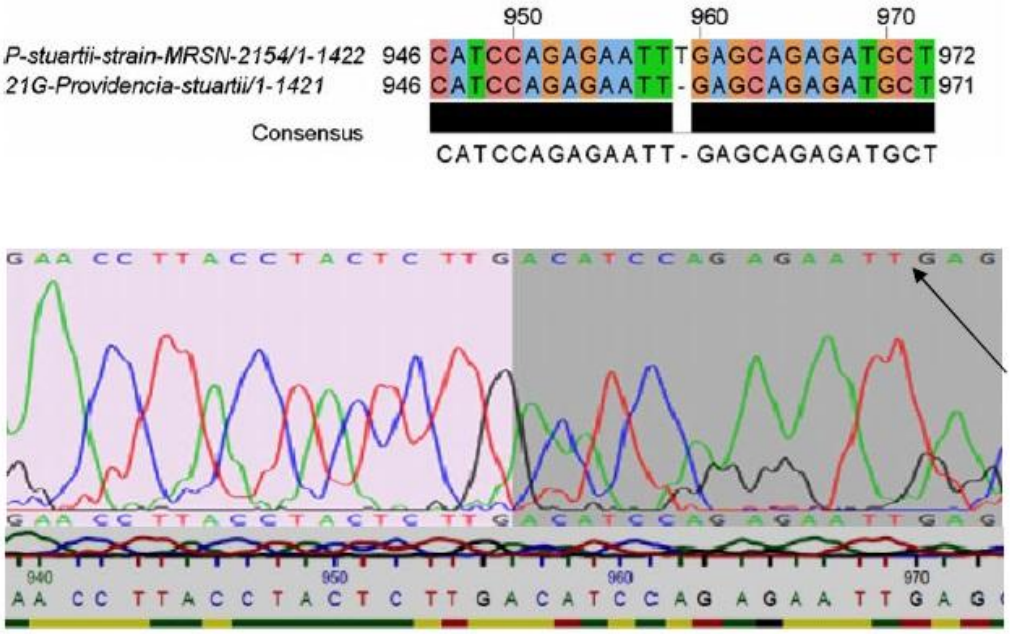

Figure (11): Comparison of 16S rDNA nucleotide sequences (1421bp.) for the isolate 21G-*Providencia stuartii* "IRQBAS6" (with peaks) and its reference strain MRSN. Frame Shift mutation (Deletion T) at the position 969bp.

Point mutations type Transition, these are: T, A, T, A, T, C and G instead of C, G, C, G, C, T and A at the positions 207, 351, 377, 391, 432, 768 and 815 (respectively). The third isolate No.45-*Bordetella trematum* as strain MunAala2 [GenBank: HG427201] was closely related (99%) to *Bordetella trematum* strain DSM 11334, but with Gene or Point mutation type Transition (T instead C) at the position 26, also it has a frame shift mutation (insertion of base C) at the sequence position 633 bp. The fourth isolate No.21G-*Providencia stuartii* as strain IRQBAS6 [GenBank: HG427202] was closely related (99%) to *Providencia stuartii* strain MRSN 2154, but with a Frame Shift mutation (Deletion of T) at the position 969bp.

DISCUSSION:

In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and in the discovery of novel bacteria in clinical microbiology laboratories [13]. The universal primers F27 and R1492 used in the present study to amplify the *16S rDNA* gene for all DNAs of bacteria isolated from ME and NP (n=96) were to prevent the loss of any species. Sixty six bacterial isolates from ME and 30 from NP were identified. The most common organism in ME of CSOM was *Pseudomonas aeruginosa* 17 (25.75%) which is an agreement with studies of Aslam *et al* [26], Verhoeff [27] and Alsaimary *et al* [28]. The second causative agent was 13 isolates of *Staphylococcus aureus* (19.69%) nearly with the result of Aslam *et al* [26]. Moreover, 12 isolates of *S. epidermidis* (18.18%) showed the third causative agent approving with the results of Feigin *et al* [29] , furthermore, it develops to consider as a common cause of infection [30] . Decreasingly, *Providencia stuartii* was 4.54%, *Klebsiella pneumoniae, Proteus mirabilis* and *Proteus penneri* had 3.03% for each, while each of the remaining other species had the least percentage (1.51% for each). However, the previous studies [28, 31, 32] showed variable domains of these species. Since, the reason of this variability may be due to the microbiological cultures which are shown multiple organisms, variety of climates, patient population and the use of antibiotics leading to a shift in the etiology of the disease [4]. Therefore, all these factors have a wide variation to affect on the results of these different studies [3, 33].

Pseudomonas putida, Pseudomonas stutzeri, Providencia vermicola, Enterobacter asburiae and *Enterobacter cloacae* were isolated in the present study as the first time from discharge of the middle ear. Since, the geographical area and respiratory infections may affect on the type of otitis media pathogens [2]. Moreover, the development and widespread of antibiotics could change the pathogenic microorganisms resistant to antibiotics [7]. However, these five of Gram negative isolates could be found as a result of contaminated water where people bath [3]. In contrast, the absence of *Streptococcus* sp., *Haemophilus influenzae* or *Moraxella catarrhalis* in the present study as a common causative agent like other studies supports the findings that these pathogens are not important in chronic suppurative otitis media (CSOM) as in acute otitis media [34].

Many studies explained the relationship between NP bacteria and the ME infected [35-38]. But, these studies compared the bacterial species from the ME of patient and NP of other patients, while the present study had been compared bacterial species from the two sources of each patient severally. It has been appeared 16%, of patients, each had the same bacterial species in their ME and NP, this result gives a green light of the role of nasopharyngeal bacteria in middle ear infection of the same patient. Chang *et al* [4] found that out of 68 bacteria identified in the middle ear were 18 bacteria (26.52%) correspond to those of the nasopharynx, but these bacteria were identified by Gram staining and biochemical tests only.

Out of six patients, 3 have identical 16S rDNA sequencing of *S. epidermidis* in their ME and NP, and 3 have identical 16S rDNA sequencing of *S. aureus* in their ME and NP. But, one patient of them had 134G-*S. aureus* and 70- *S. aureus* in their ME and NP, (respectively) have been already different strains as a result to present *tst* gene in one strain (another study). Nevertheless, the explanation of genetic relationships of the strain level between these two sources is of importance in epidemiology and ecology. However, closely related isolates are difficult to identify and differentiate using the biochemical methods [22]. The identical of sequences of 16S rDNA for species from ME and NP is not sufficient to confirm identity between bacterial strains from two sources. Therefore, the aim of the present study is to detect the genetic differences of different strains of *S. epidermidis* and *S. aureus* only (because these species are common in NP while the other species *Pseudomonas aeruginosa* and *Enterococcus faecium* are not) by RAPD-PCR. Moreover, the use of more than one RAPD primers improves the differentiating power of RAPD process [23, 39].

Three patients of 5 (60%) having the same strain in their ME and NP confirm that the nasopharyngeal bacteria could be the source of CSOM, and this confirmation was performed by RAPD-PCR as the first time, but similar to Tonnaer *et al* [40] suggesting that the molecular fingerprints (AFLP) from *Streptococcus pneumoniae* derived from two different anatomic sites within patients were very similar in 80% of OME patients and in 90% of acute otitis medium patients, indicating their genetic relatedness. Interestingly, since an opportunistic pathogen causes infection in immunocompromized individuals [41], such as those caused by contraction of a viral agent, so the impaired functioning of the immune response in the Eustachian tubes, which would allow bacteria from the NP to ascend into the middle ear cavity [42]. This assumption is reinforced, especially by the observation of the identical close relation between bacterial strains present in the nasopharynx and the middle ear [40].

On the other hand, the four novel isolates (65-*Klebsiella pneumonia* "MunAala1", 78-*Pseudomonas putida* "IRQBAS5", 21G-*Providencia stuartii* "IRQBAS6" and 45-*Bordetella trematum* "MunAala2") which were reported as new separated strains in GenBank, each had 1% difference with the sequence of 16S rDNA from its reference strain. According to some guidelines, a range of about a 0.5% to 1% difference (99.5 to 99% similarity) is often used for

classification [43]. Bosshard *et al* [44] used ≥ 99% similarity to define species and ≥ 95 to < 99% to define a genus whereas, Hall *et al* [45] adopted a distance score of 0.00 to less than 1% as the criterion for species identity. While, Tang *et al* [46, 47], suggested a 0.5% difference as the limit for species designation. Furthermore, a strain with a small genotypic difference (less than 0.5%) has been considered as subspecies [48]. In contrast, Abd Al-Abbas [49] used ≥ 99% similarity to define a strain level. When there is a clear phenotypic uniqueness, genogroups with less than 1% differences in sequence have been named as a new species [50]. However, a comparison of sequences for several subspecies shows differences from 1 to 14 bp. [51]. Some of these variations among the researchers could be due to the fact that the percent difference can vary if it is calculated using only the first 500 bp. or all 1500 bp. of 16S rDNA sequences, and can also vary with the program used for calculations [52].

CONCLUSION:

Pseudomonas aeruginosa, Staphylococcus aureus and *Staphylococcus epidermidis* were the prevalent types in CSOM, in addition to other 19 different bacterial species but with less percentage. Moreover, three cases have the same bacterial strains in their ME and nasopharynx confirming the probability that the bacteria have the ability for transmission between the two sources. Furthermore, the study was recorded four bacterial strains as new globally.

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Table (1): Distance Matrix among *Staphylococcus epidermidis* and *Staphylococcus aureus* strains from ME and NP sources.

Strains	42	87	18	20	31	1	18G	29	22	19
42	0	0.000	0.831	0.831	1.776	0.884	1.170	1.572	1.284	1.284
87		0	0.831	0.831	1.776	0.884	1.170	1.572	1.284	1.284
18			0	0.000	1.832	0.301	0.824	1.335	1.472	1.472
20				0	1.832	0.301	0.824	1.335	1.472	1.472
31					0	1.880	1.965	2.096	1.529	1.529
1						0	0.870	1.493	1.520	1.520
18G							0	1.465	1.668	1.668
29								0	1.896	1.896
22									0	0.000
19										0

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