Frequency and Comparison of Each icaA and icaD gene Sequences in Bacteria isolated from Otitis Patients

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Abstract
Biofilm formation is a part of the pathogenicity by allowing the bacteria to colonize and multiply within the host. Different bacterial species (n=43) of otitis were tested for icaAD genes, then compared by sequencing these genes. All isolates showed positive to icaA gene and / or icaD gene with the frequency of 88.4% for each gene. Serratia marcescens, Providencia vermicola, P. stuartii, Bacillus subtilis, Enterobacter asburiae, E. cloacae, Corynebacterium amycolatum, Bordetella trematum, Proteus mirabilis, P. penneri, Lysinibacillus fusiformis, Pseudomonas putida and Enterococcus faecalis were appeared to have ica gene for the first time. Therefore, the investigation to own ica gene should be in regardless of a selected bacteria. icaD gene showed many mutations in its sequence among five isolates changing a number of amino acids. So, four strains of S. aureus and one Serratia marcescens were appeared as new within the GenBank. ica gene is not confined to certain genera of bacteria, however is diffused among strains, particularly pathogens. Generally, ica genes have many various alleles among the bacteria revealing to give new serious of illness strains.

Keywords: icaAD, sequence, otitis, mutation, alleles

1. Introduction
Bacterial biofilms have a major role for 80% of diseases like urinary tract infections, dental plaque, gingivitis, wounds infection and otitis (O’Gara and Humphreys 2000; 2Percival and Knottenbelt 2011; 3Ploneczka-Janeczko 2014). Over 22 bacterial species were accountable for chronic suppurative otitis media (Pinar et al., 2008; 5Abd Al-Abbas and Chmag 2014). In bound cases, the Ostaki channel is obstructed allowing the bacteria to aggregate and blockade the channel, so the biofilm forming bacteria can adhere to the surface mediated by its capsular polysaccharide consisting of glycosaminoglycans then multiply to create a multilayered biofilm that permits a cell to cell adhesion (Yazdani et al., 2006). The high numbers of bacteria and their fluids within the cavity causing acute otitis forming an inside pressure which can cause ear drum perforated (Howard 2007).

The polysaccharide intercellular adhesion (PIA) genes (icaAD,B,C and R) are present as a biofilm mediating operon. The icaA gene codes for N-acetylgulosaminyl-transferase concerned in PIA synthesis, icaD is enjoying a task within the full expression of N-acetylglucosaminyl-transferase forming the entire phenotype expression of the capsule (Gad et al., 2012; 9Namvar et al., 2013). Further, icaB gene codes for an enzyme responsible for deacetylation of mature PIA, icaC gene is involved in the externalization and elongation of the growing capsular polysaccharide (Dimond-Hernandez et al., 2010). In addition, icaR gene is seemed to has the regulatory function (Terki et al., 2013). However, the present of icaA and icaD genes together leads to a significant increase in the effectiveness and complete phenotypic expression of the PIA (Oliveira et al., 2010; 8Gad et al., 2012; 9Namvar et al., 2013). Several bacterial species were reported to have icaA and icaD genes such as Staphylococcus epidermidis, S.aureus, S.saprophyticus, S.hominis and Pseudomonas aeruginosa (Hou et al., 2012; 11Terki et al., 2013; 14El-Amin et al., 2015; Gowrishankar et al., 2016).

This work is for detecting the frequency of icaA gene and/or icaD gene in the 43 different bacterial isolates of otitis patients, and to compare the nucleotides sequence of each gene followed by a comparison of their amino acids sequence.

2. Material and Methods
The present study is focused on 43 bacterial isolates DNA of chronic suppurative otitis media obtained from a previous study of Abd Al-Abbas and Chmag (2014) identified by 16SrDNA gene sequencing.

The primers (BIONEER, Korea) for amplifying were according to Arciola et al. (2001) including icaA forward 5’-TCTCTTGCAGAGCAATCAA-3 and icaA reverse 5’-TCAGGCACATACATCCAGCA-3 of 188 bp, whereas icaD forward 5’-ATGGTCAAGCCCAGACAGAG-3 and icaD reverse 5’-CGTGTTTCACATTTATAAGCA-3 of 198 bp. The whole volume (25 µl) of PCR reaction for each separated gene was 1 µl (10 pmol) of each primer with 5 µl DNA, and 12.5 µl GoTag Green Mastermix with 5.5 µl Nuclease free water (Promega, USA). The thermocycler (Thermo, USA) program steps for each gene was 94°C for 5 min, followed by 50 cycles at 94°C for 30 sec (denaturation), 55.5°C for 30 sec (annealing), 72°C for 30 sec (extension) and finally, 72°C for 1 min. Agarose gel electrophoresis (2% agarose in TBE with 1 µl ethidium bromide) with 100 bp DNA ladder (Promega, USA) were used to detect the gene bands, then visualized by UV transillumination system (Velber Lourmat).
All bacterial genes were sent to SangonBiotech, China for sequencing, only 20 isolates were success for icaA and / or icaD gene sequencing. The nucleotide sequences were identified by BLAST program "http://www.ncbi.nlm.nih.gov". The nucleotides or amino acids sequence for icaA or icaD gene were compared using Clustal omega program "www.ebi.ac.uk/Tools/msa/Clustal/". The rooted phylogenetic tree of each gene was constructed using MAFFT program version7 "http://mafft.cbrc.jp/alignment/server/" as katoh et al. (2002), then viewed by forester-1027 (Zmasek and Eddy, 2001). The gene sequencing of < 99% in similarity was published in European Nucleotide Archive (ENA) and GenBank as a new allele.

3. Results
According to the genes amplification (Figure 1), all the 43 bacterial species had icaA and / or icaD gene with the frequency of 38 (88.4%) for each (Table 1). Although this is the first study detected icaA and / or icaD gene in Serratia marcescens (2), Providencia vermicola (2), P. stuartii (1), Bacillus subtilis (2), Enterobacter asburiae (2), E. cloaceae (1), Corynebacterium mycolatum (1), Bordetella trematum (1), Proteus mirabilis (1), P. penneri (1), Lysinibacillus fusiformis (1), Pseudomonas putida (1) and Enterococcus faecalis (1), however there have been 9 isolates of P.aeruginosa, S.epidermidis, S.aureus, S.marcescens, P.vermicola, B.subtilis, E.asburiae, E.cloaceae and E.faecalis showed losing in one between the two genes.

The phylogenetic tree of 11 icaA genes sequence (159 bp in concatenation) of the various isolates (Figure 2) showed that group A consisting of strains 5-S.epidermidis, 6-P.aeruginosa and S.epidermidis (PJLB-3) from GenBank were identical. Similarly, group B is consisting of other identical icaA genes sequence of strains 1,2,7,11-P.aeruginosa,3,9,S.epidermidis,4-C.mycolatum,8-S.marcescens,10-P.putida and S.epidermidis (KC-S_e2) from GenBank. The variations between the two groups were appeared within the amino acids Y (tyrosine) and E (glutamic acid) in group A comparing with T (threonine) and L (leucine) in group B at the position 51 and 52 respectively (Figure 3).

On the other hand, the phylogenetic tree of 14 icaD genes sequence (198 bp in concatenation) of the various isolates (Figure 4) showed that the strains 1,15-P.aeruginosa,5,9,12,19-S.epidermidis,10-P.putida,14-E.faecalis,17-E.cloaceae and S.epidermidis (U43366-1) from GenBank were identical. Oppositely, several differences (99% similarity) were appeared in the first time for icaD gene sequences of 5 strains (Figure 5), thus they recorded in European Nucleotide Archive (ENA) and GenBank as new strains, these were named IRQBAS21 (LT840188), IRQBAS22 (LT840189), IRQBAS23 (LT840190), IRQBAS24 (LT840191) and IRQBAS25 (LT840192) for strains 8-S.marcescens,13,16,18 and 20-S.aureus (respectively). Moreover, strain No. 8 and 13, both suffered mutations involving deletion of 12 nucleotides between the positions 23 and 34 bp, transversion (A,T and A instead T,A and T) at the positions 35, 37 and 43 bp (respectively), transition (C instead T) at the position 40 bp as compared with the other strains. Strain No. 16 has only one transversion mutation (T instead G) at the position 84 bp to show discrepancy different from strain No.18. Strain No.20 showed two transition mutations (C and A instead T and G) at the position 51 and 55 bp, respectively.

In general, out of 56 amino acids of icaD gene, the five new isolates showed 11 new types of amino acids including Q-glutamine, V-valine, T-threonine, Y-tyrosine, S-serine, L-leucine, F-phenylalanine, G-glycine, M-methionine, I-isoleucine and N-asparagine as compared with strain U43366.1 (Figure 6). According to the genetic standard code in "Table 11" of NCBI that initiated with AUG , strains No.8 and No.13 showed uncommon amino acids sequence as a result of they lost four amino acids between the position 13 and 16 by a deletion mutation, whereas the other mutations were silent. Similarly, the amino acids sequence were the same in strains No.16 and No.18. On the other hand, only single isoleucine amino acid at position 19 of strain No.20 was present comparing with valine of the other strains.

Figure 1. Electrophoresis of PCR product. Lane L: 100-1000 bp DNA ladder. Lane 1,3 and 4: icaA gene (188 bp). Lane 2: icaD gene (198 bp).
Table 1. Frequency of icaA,D genes in bacterial species

<table>
<thead>
<tr>
<th>Bacterial species*</th>
<th>n=</th>
<th>icaA n=</th>
<th>icaD n=</th>
</tr>
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<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Providencia vermicola</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Enterobacter asburiae</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter cloaceae</td>
<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>Corynebacterium amylolatum</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus hominis</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bordetella trematurn</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Providencia stuartti</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Proteus penneri</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lysinibacillus fusiformis</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

n (%) 43 38(88.4) 38(88.4)

*from previous study (Abd Al-Abbas and Chmag 2014)
P< 0.05

4. Discussion

Several studies had been proven that there was no statistical correlations between the phenotypic methods such as congo red agar and microtiter plate, and the ica-PCR for detecting the biofilm producing bacteria. Thus, Hou et al. (2002) advised that the phenotypic assay can be used as a putative screening manner for early designation of biofilm. Therefore, the current study was rely on both icaA and icaD genes (Figure 1), despite the fact that some studies were relied on either icaA gene (Vandecasteele et al., 2003; 3Ploneczka-Janeczko et al., 2014) or icaD genes (Hennig et al., 2007; Rohde et al., 2010; Zhou et al., 2013). In general, most of studies were dogmalised to use icaAD genes together (Nasr et al., 2012; Terki et al., 2013; Melo et al., 2013). Though all the 43 bacterial isolates have ica gene (100%), but the whole share of icaA gene and icaD gene were 88.4% for each (Table 1). This a high percentage is thanks to the otitis as a source where the pathogenic bacteria are isolated from. Since, the foremost vital step for otitis infection is the ability of bacteria to mend and

![Phylogenetic tree](image)

**Figure 2.** Rooted neighbour joining phylogenetic tree of icaA gene sequences (159 bp concatenation). Constructed by "MAFFT" and visualized using "forester". This tree showed the phylogenetic relationships between group A including the identical strains No. 5,6 of otitis and PJLB-3 of GenBank (T), and group B including the identical strains No. 1,2,3,4,7,8,9,10,11 of otitis and KC-S_e2 of GenBank (T). Bootstrap of 1000 value.
Figure 3. Comparison by "CLUSTAL omega" program between 53 amino acids sequence of icaA gene of otitis bacterial strains. Group A has Y (tyrosine) and E (glutamic acid) while group B has T (threonine) and L (leucine) at the position 51 and 52 respectively.

 shield itself within the waxy material of the ear. Relatively, the ica gene are twice more frequent in bacteria isolated from infection than from alternative sources (Yazdani et al., 2006). However, with exception of P. aeruginosa, S. epidermidis, S. aureus and S. hominis, all other 13 species were positive for icaA and / or icaD genes in the first time revealing that additional studies ought to have an interest to analyze concerning ica gene in alternative bacterial isolates as a vulnerable role of biofilm in the pathogenicity, especially once Mckenney et al. (1998) proven that ica gene is carried on a plasmid. Therefore, several genera and species will acquire the adhesion gene throughout conjugation. In several bacterial species, the adhesion mechanism were either by producing polysaccharide slime or the host includes proteins that adsorbate onto bacterial surface (Montanaro et al., 1998). Fletcher and Marshall (1982) found that Pseudomonas strains may will be separated from polystyrene surface but not from glass. During this case, the ica gene at sequencing level is extremely helpful to avoid the confusion. Moreover, the gene sequences coding for biofilm can refer to the prevalent adhesion mechanism (Arciola et al., 2001).

Figure (2) appeared two groups of icaA gene counting on sequences, however there was no new nucleotides mutations. Whereas there were five new bacterial strains (No.20,13,8,18 and 16) have several new mutations in their icaD gene sequences (Figure 5). Although, the strains No. 8 and 13 have the same icaD gene sequences, but they are completely from different genera. However, Heilmann (1996) discovered the mutations within the ica locus were impact on cell-cell adhesion but not for cell-solid. Significantly, some mutations are cause amino acids change but other not. Ziebuhr et al. (1999)

T: GenBank strain

Figure 4. Rooted neighbour joining phylogenetic tree of icaD gene sequences (169 bp concatenation). Constructed by "MAFFT" and visualized using "forester". This tree showed the phylogenetic relationships among the identical icaD gene sequences of 9 different bacterial strains No. 1,5,9,10,12,14,15,17,19 of otitis and U43366 of GenBank (T) while strains No. (8 and 13),16,18 and 20 were different in each other. Bootstrap of 1000 value.

Figure 4. Rooted neighbour joining phylogenetic tree of icaD gene sequences (169 bp concatenation). Constructed by "MAFFT" and visualized using "forester". This tree showed the phylogenetic relationships among the identical icaD gene sequences of 9 different bacterial strains No. 1,5,9,10,12,14,15,17,19 of otitis and U43366 of GenBank (T) while strains No. (8 and 13),16,18 and 20 were different in each other. Bootstrap of 1000 value.

found tha
t a mutation just like the insertion of IS256 within the icaA gene inflecting non-slime forming bacteria, but no insertion was described in icaD. Oppositely, the present study appeared there was no nucleotides inserted in icaA gene, whereas the icaD gene suffered by deleting 12 nucleotides in strains No.18 and 13 at position 23-35 bp. Therefore, four amino acids had been losted. Moreover, icaD gene showed many alternative alleles coding
Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.59, 2017

for the same gene by using the same couple primers. Withal, the comparison between the ica gene amino acids sequence of S.epidermidis and S.aureus unconfessed that just 59 to 78% of amino acids were identity (Cramton et al., 1999).

5. conclusion
All otitis bacteria had icaA gene and / or icaD gene regardless of the bacteria type, that helped to record new species having these genes. icaD gene showed new varieties in their nucleotides sequence of some strains inflecting to seem new amino acids then new alleles for this gene. However, there was no pre-known alignment for icaD bellow this study.

Acknowledgement
The author is grateful to the laboratory staff of the Cell and Biotechnology Researches Unit / College of Science / University of Basrah, furthermore to the Ph. D. student Chmag A. A. for her helping.

Figure 5. Comparison by "CLUSTAL omega" program among the icaD gene nucleotide sequences of strains No. 8 = IRQBAS21 (LT840188), 13 = IRQBAS22 (LT840189), 16 = IRQBAS23 (LT840190), 18 = IRQBAS24 (LT840191) and 20 = IRQBAS25 (LT840192) from otitis patients, and U43366 from GenBank. Different
mutations appeared between 22 bp to 165 bp.

**Figure 6.** Comparison by “CLUSTAL omega” program among amino 56 acids sequence of icaD gene (New alleles) of otitis bacterial strains No: 8, 13, 16, 18 and 20, and U43366 of GenBank.

**REFERENCES**


Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.59, 2017


