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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 *iacAD* 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. stuartti*, *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 recorded 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 (*icaA*,*D*,*B*,*C* and *R*) are present as a biofilm mediating operon. The *icaA* gene codes for N-acetylglucosaminyl-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 (Diemond-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 *iacA* forward 5'-TCTCTTGCAGGAGCAATCAA'-3 and *icaA* reverse 5'-TCAGGCACTAACATCCAGCA-'3 of 188 bp, whereas *icaD* forward 5'-ATGGTCAAGCCCAGACAGAG'-3 and *icaD* reverse 5'-CGTGTTTTCAACATTTAATGCAA'-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^{O}C for 5 min, followed by 50 cycles at 94^{O}C for 30 sec (denaturation) ,55.5^{O}C for 30 sec (annealing), 72^{O}C for 30 sec (extension) and finally, 72^{O}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/aligment/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. stuartti* (1), *Bacillus subtilis* (2), *Enterobacter asburiae* (2), *E. cloacae* (1), *Corynebacterium amycolatum* (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.cloacae* and *P.penneri* 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.amycolatum*,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.cloacae* 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.

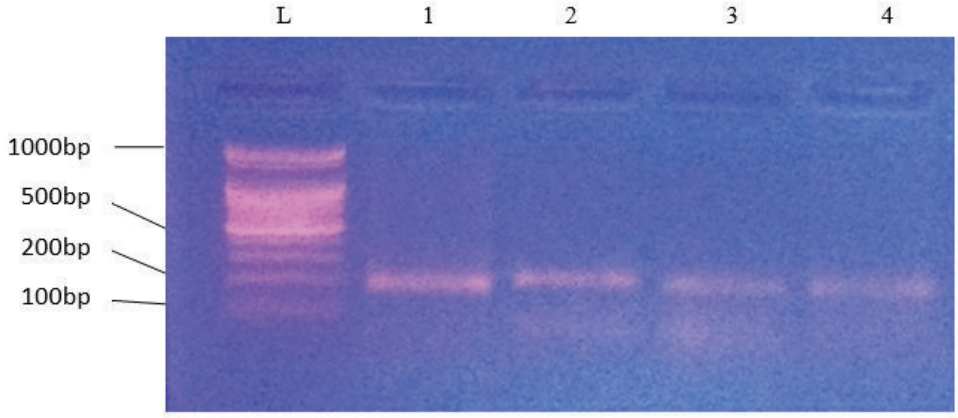


Figure 1. Electrophoresis of PCR product. Lane L: 100-1000 bp DNA ladder. Lane 1,3 and 4: *icaA* gene (188 bp). Lane 2: *icaD* gene (198 bp).

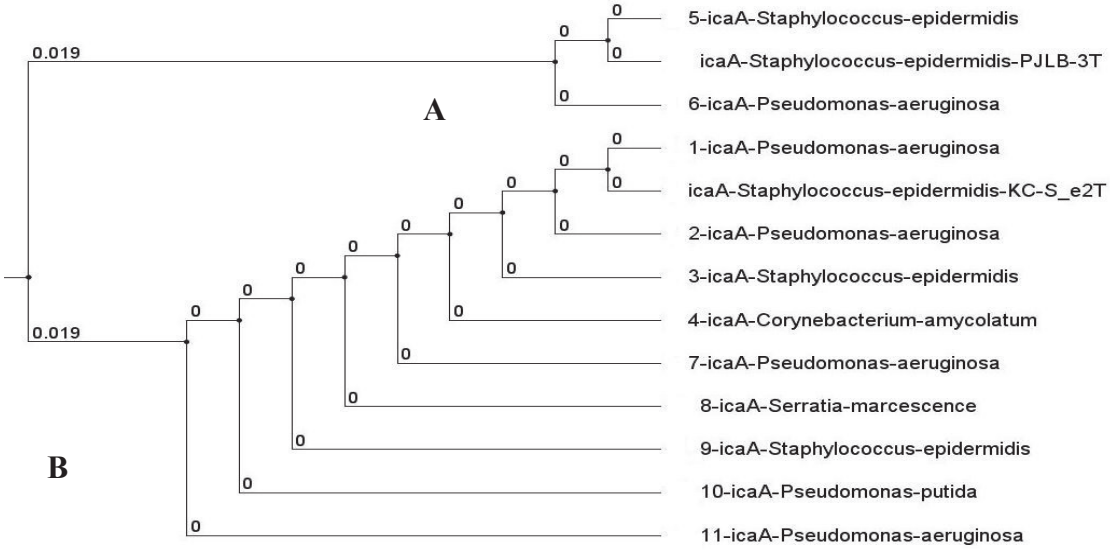
Table 1. Frequency of *icaA,D* genes in bacterial species

Bacterial species*	n=	*icaA* n=	*icaD* n=
Pseudomonas aeruginosa	12	12	11
Staphylococcus epidermidis	7	7	6
Staphylococcus aureus	6	5	6
Serratia marcescens	2	1	2
Providencia vermicola	2	1	2
Bacillus subtilis	2	2	1
Enterobacter asburiae	2	2	0
Enterobacter cloacae	1	0	1
Corynebacterium amycolatum	1	1	1
Staphylococcus hominis	1	1	1
Bordetella trematum	1	1	1
Providencia stuartti	1	1	1
Proteus mirabilis	1	1	1
Proteus penneri	1	0	1
Lysinibacillus fusiformis	1	1	1
Pseudomonas putida	1	1	1
Enterococcus faecalis	1	1	1
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; 21Rohde *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



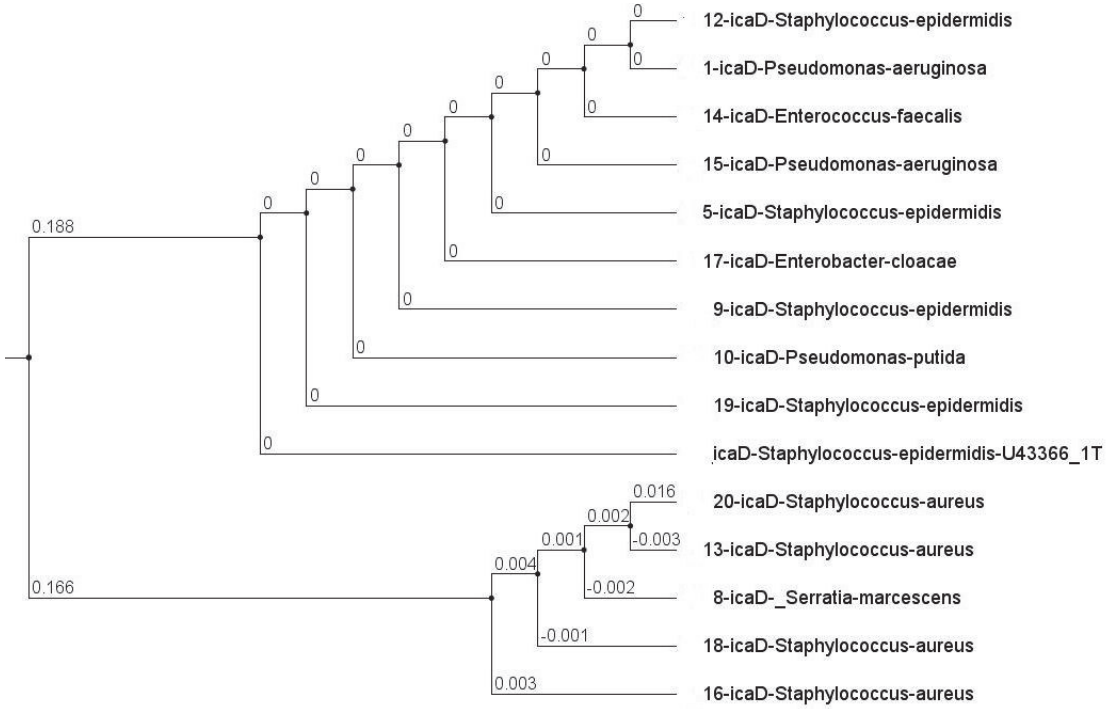
T: GenBank strain

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.

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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

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* unconcealed 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.

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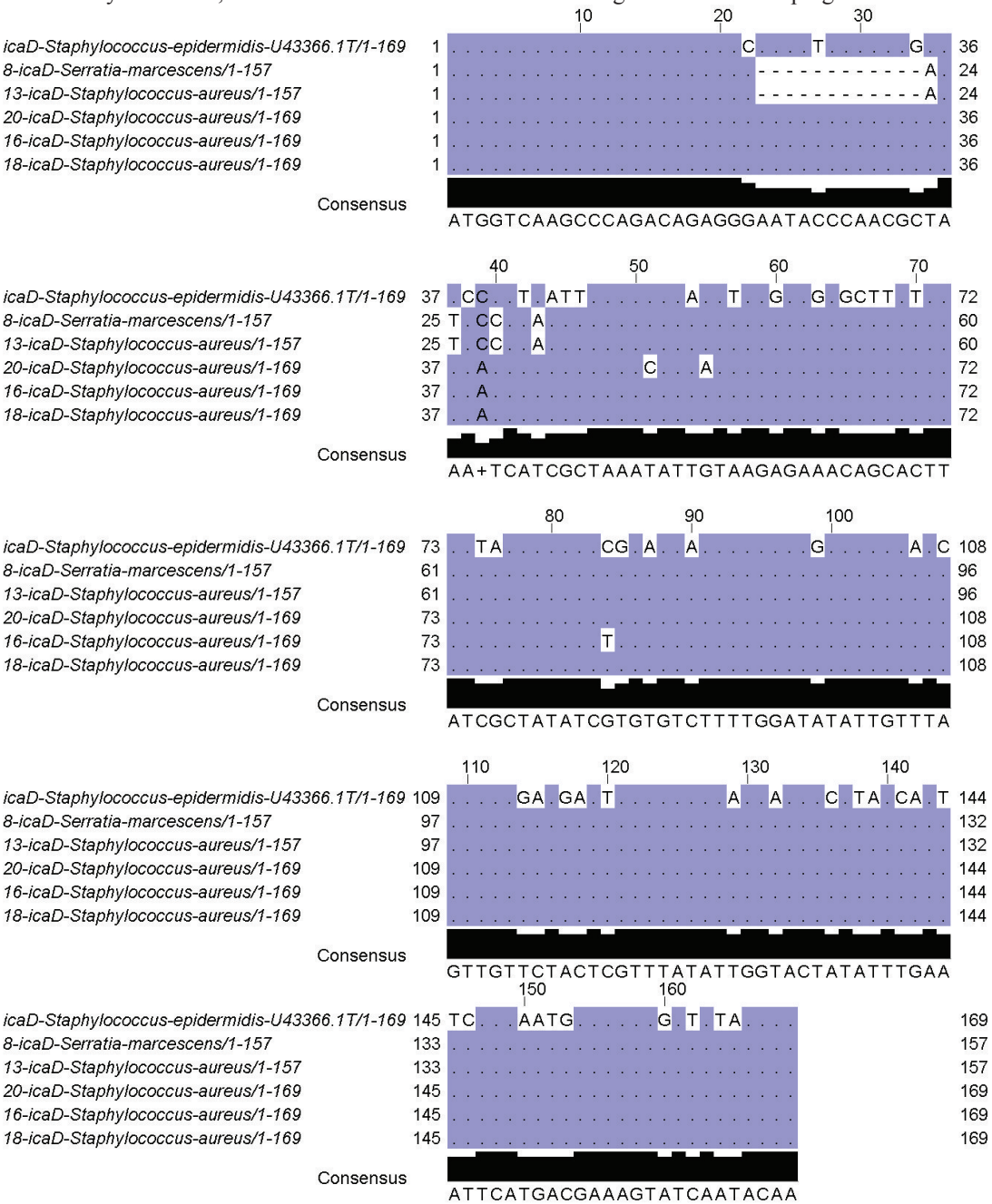

Figure 5. Comparison by "CLUSTAL omega" program among the *icaD* gene nucleotide sequences of strains No. 8 = IRQBAS21 (LT840188), 13 = IRQBAS22 (LT840189), 16 = IRQBAS23 (LT840190), 18 = IRQBAS24 (LT840191) and 20 = IRQBAS25 (LT840192) from otitis patients, and U43366 from GenBank. Different

mutations appeared between 22 bp to 165 bp.

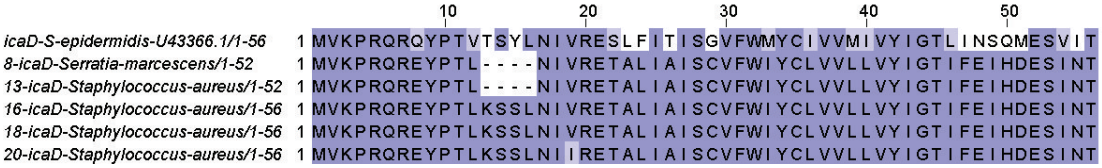

Figure 6. Comparison by "CLUSTAL omega" program among amino 56 acids sequence of *icaD* gene (New alleles) of otitis bacterial strains No: 8, 13, 16, 18 and 20, and U43366 of GenBank.

REFERENCES

Abd Al-Abbas M. J. and Chmag, A. A. (2014). Molecular genetic study confirming the transmission of nasopharyngeal bacteria to middle ear in patients with chronic suppuratice otitis media, including newglobal strains in genbank:MUNAALA1, MUNAALA2, IRQBAS5 and IRQBAS6. *Bio-Sci*. 3 (5): 379-397.

Arciola, C. R., Baldassarri, L. and Montanaro, L. (2006). Presence of *icaA* and *icaD* Genes and Slime Production in a Collection of Staphylococcal Strains from Catheter-Associated Infections. *J. Clin. Microbiol*. 39 (6): 2151-2156

Arciola, C. R., Lucilla Baldassarri, L. and Montanaro, L. (2001). Presence of *icaA* and *icaD* Genes and Slime Production in a Collection of Staphylococcal Strains from Catheter-Associated Infections**.** ***J. Clin. Microbiol***. 39 (6): 2151–2156.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W. and Gotz, F. (1999). The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun*. 67 (10): 5427-33.

Diemond-Hernandez, B., Solorzano-Santos, F., Leanos-Miranda, B., Peregrino-Bejarano, L. and Miranda-Novales, G. (2010). Production of icaADBC-encoded polysaccharide intercellular adheson and therapeutic failure in pediatric patients with *Staphylococcus* device-related infections. *BMC. Infec. Dis*. 10:68.

El-Amin, M. M., Mohamed, H. E., Abd-Elrazek, G. and Amer, N. G. (2015). Comparison of different methods for detection of biofilm formation in *Staphylococcus aureus* and *epidermidis* isolates from central venous catheters. *Int. J. Advan. Res*. 3 (7): 93-101.

Fletcher, M. and Marshall, K. C. (1982). Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Appl. Environ. Microbiol*. 44 (1): 84-192.

Gad, G. F., Abdel Aziz, A. A. and Alylbrahim, R. (2012). *In-vivo* adhesion of *Staphylococcus* spp. To certain orthopedic biomaterials and expression of adhesion genes. *JAPS*. 2 (6): 145-149.

Gowrishankar, S., Kamaladevi, A., Balamurugan, K. and Pandian, S. K. (2016). *In vitro* and *In vivo* biofilm characterization of methicillin-resistant *Staphylococcus aureus* patients associated with pharyngitis infection. *Bio. Res. Int*. 2016: 1-14.

Heilmann, C., Gerke, C., Perdreau-Remington, F. and Gotz, F. (1996). Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect. Immun*. 64 (1): 277-282.

Hennig, S., Nyunt Wai, S. and Ziebuhr, W. (2007). Spontaneous switch to PIA-independent biofilm formation in an ica-positive *Staphylococcus epidermidis* isolate. ***Int. J. Med. Microbiol***. 297 (2): 117-**1**22.

Hou, W., Sun, X., Wang, Z. and Zhang, Y. (2012). Biofilm-forming capacity of *Staphylococcus epidermidis*, *Staphylococcus aureus*, and Pseudomonas aeruginosa from ocular infection. *ARVO*. 54 (9): 5624-5631.

Howard, D. (2007). Intercultural cumunication and conductive hearing loss. *J. Fi. Peop. Ch. Fam. Rev*. 3 (4): 96-105.

Katoh, K., Misawa, K., Kuma, K. and Myata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Ac. Res*. 30 (14): 3059-3066.

Mckenney, D., Hubner, J., Muller, E., Wang, Y., Goldmann, D. A. and Pier, G. B. (1998). The ica locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide / adhesion. *Infect. Immun*. 66 (10): 4711-4720.

Melo, P. D., Ferreira, L. M., Filho, A. N., Zafalon, L. F., Vicente, H. I. G. and Souza. V. D. (2013). Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Brezil. J. Microbiol*. 44 (1): 119-124.

Montanaro, L., Arciola, C. R., Borsetti, E., Brigotti, M. and Baldassarri, L. (1998). A polymerase chain reaction (PCR) methods for the identification of collagen adhesion gene (*can*) in *Staphylococcus*-induced prosthesis infections. *New microbial*. 21(4): 359-363.

Namvar, A. E., Asghari, B. and Lari, A. R. (2013). Detection of the intercellular adhesion gene cluster (ica) in clinical *Staphylococcus aureus* isolates. *GMS. Hyg. Infec. Control*. 8 (1): 2196-5226.

Nasr, R. A., AbuShady, H. M. and Hussein, H. S. (2012). Biofilm formation and presence of *icaAD* gene in clinical isolates of staphylococci. Egypt. *J. Medi. Hu. Gen*. 13 (3): 269-274.

O'Gara, J.P., Humphreys, H. (2001). *Staphylococcus epidermidis* biofilms:importance and implications. *J. Med. Microbiol*. 50 (7): 582-587.

Oliveira, A., de Lourdes, R. S. and Cunha, M. (2010). Comparison of methods for the detection of biofilm production in ciagulase-negative staphylococci. *BMC. Res. Notes*. 3: 260.

Percival, S.L. and Knottenbelt, D. C. and Cochrane, C.A. (2011). Biofilms and veterinary medicine. *Springer, Berlin*. 257p.

Ploneczka-Janeczko, K., Lis, P., Bierowiec, K., Rypula, Krzysztof. And Chorbinski, P. (2014). Identification of *bap* and *icaA* genes involved in biofilm formation in coagulase negative staphylococci isolated from feline conjunctiva. *Vet. Res. Commun*. 38: 337-346.

Rohde, H., Frankenberger, S., Zahringer, U. and Mack, D. (2010). Structure, function and contribution of polysaccharide intercellular adhesion (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infection. *Eur. J. Cell. Biol*. 89 (1): 103-111.

Terki, I. K., Hassaine, H., Oufrid, S., Bellifa, S., Mhamedi, I., Merium, L. and Timinouni, M. (2013). Detection of *icaA* and *icaD* genes and biofilm formation in *Staphylococcus* spp. Isolated from urinary catheters at the university hospital of tiemcen (Algeria). *Afri. J. Microbiol. Res*. 7 (47): 5350-5357.

Vandecasteele, S. J., Peetermans, W. E., Merckx, R. and Eldere, J. V. (2003). Expression of biofilm-associated genes in *Staphylococcus epidermidis* during *In vitro* and *In vivo* foreign body infection. *J. infect. Dis*. 188 (5): 730-737.

Yazdani, R., Oshaghi, M., Havayi, A., Pishva, E., Salehi, R. and Sadeghi-zadeh, M, *et al*. (2006). Detection of *icaAD* gene and biofilm formation in *Staphylococcus aureus* isolates from wound infection. *Iran. J. Pulp. Health*. 35 (2): 25-28.

Zhou, S., Chao, X., Fei, M. Dai, Y. and Liu, Bao. (2013). Analysis of *S.epidermidis icaA* and *icaD* genes by polymerase chain reaction and slime production: a case control study. *BMC inf. Dis*. 13: 242.

Ziebuhr W, Krimmer V, Rachid S, Lossner I, Gotz F, Hacker J. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS*256*. *Mol. Microbiol*. 32 (6):345–356.

Zmasek, C. M. and Eddy, S. R. (2001). ATV: display and manipulation of annotated phylogenetic trees. *Bioinformatics*. 17: 383-384.