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ORIGINAL ARTICLE

Biofilm formation and molecular analysis of intercellular adhesion gene cluster *(icaABCD)* among *Staphylococcus aureus* strains isolated from children with adenoiditis

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ABSTRACT

Background and Objectives: It is well known that *Staphylococcus aureus* biofilm plays an important role in adenoiditis and biofilm resistance frequently results in failure of therapy. The goal of this study was to evaluate the biofilm production of *S. aureus* isolates obtained from adenoid specimens and assess the relationship between biofilm formation ability and *ica* operon genes.

Materials and Methods: A total of 112 adenoid samples were obtained from patients under 15 years old with adenoid hypertrophy. All *S. aureus* isolates were initially identified by standard microbiological tests and amplification of *nuc* by

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polymerase chain reaction (PCR) technique. Biofilm formation of *S. aureus* isolates was evaluated and *icaADBC* genes were detected by PCR technique.

Results: There were 46 isolates (41%) identified as *S. aureus*. The ability to produce biofilm was detected among total *S. aureus* isolates. Molecular study of *ica* operon revealed that 2 (6.3%) and 19 (59.4%) isolates carried *icaA* and *icaD*, respectively. The prevalence of *icaA + icaD* was seen among 11 (34.4%) *S. aureus* isolates, while *icaC* and *icaB* were not detected.

Conclusion: Our findings indicated that *icaABCD* operon are associated with biofilm formation in *S. aureus* isolates, however the absence of these genes may not necessarily exclude this property.

Keywords: *Staphylococcus aureus*; Adenoids; Chronic infection; Biofilm; Polymerase chain reaction

INTRODUCTION

The adenoids are lobulated masses of lymphoid tissue which is located in an axial position of the upper respiratory tract (1). Adenoid hypertrophy (AH) is common in children, due to chronic inflammation that leads to the proliferation of adenoid lymphoid tissue (2, 3). Furthermore, the adenoids considered as an important bacterial reservoir in children which have a significant role in the development of infectious diseases according to the alteration of the host immune system (4). Besides, the biofilm formation in adenoid tissue may be involved in the pathogenesis of chronic adenoiditis (4). Bacterial biofilm formation is one of the most important survival mechanisms through attachment to surfaces which controlled by different genetic pathways (5). Studies show that there is a variable prevalence rate of bacterial biofilms formation in chronic adenoiditis (41%-100%), this difference can be based on the selected case series and the sampling as well as microbiological analysis methods (6-9).

Reports indicated that various bacteria including *Staphylococcus aureus (S. aureus), Streptococcus pneumoniae, Haemophilus influenza*, and *Moraxella catarrhalis* are involved in nasopharyngeal biofilm production, which are also responsible for chronic adenoiditis and middle ear infections (7-9). *S. aureus* is a common nasopharyngeal pathogen and involved in adenoiditis in children (10). The ability of *S. aureus* to produce biofilm is well considered as a virulence factor which enables this organism to withstand the host immune response (11). In addition, *S. aureus* biofilm formation enhanced resistance to the antimicrobial agents, which could become a clinical concern, particularly in children with chronic adenoiditis. According to many studies, different genes are involved in biofilm production. The intracellular adhesion *(ica)* cluster *(icaADBC),* encodes the essential proteins for the production of polysaccharide intercellular adhesion (PIA), which mediate cell to cell adhesion, thus facilitating biofilm formation of *Staphylococcus* spp. (12, 13). Among *ica* genes, *icaA* and *icaD,* have been reported to play a significant role in biofilm formation (12). Hence, the detection of the *ica* locus along with the phenotypic detection of biofilm is important in *S. aureus* isolates and it would improve the diagnostic decision for choosing the proper treatment. The main goal of the current study was to assess the biofilm production of *S. aureus* isolates obtained from adenoid specimens. In addition, the possible relationship between the biofilm formation ability and *ica* cluster genes in clinical isolates of *S. aureus* strains were evaluated.

MATERIALS AND METHODS

A total of 112 adenoid samples were obtained from patients under 15 years old with adenoid hypertrophy (AH), admitted to the department of otolaryngology at Shafa Teaching Hospitals, Kerman, Iran. The study was performed according to the ethical guidelines of the 1964 Declaration of Helsinki as reflected in a priori approval by the Kerman University of Medical Sciences's human research committee (Ethics approval code: IR.KMU.REC. IR.KMU.AH.REC.1398.035).

All patients gave their informed consent prior to their inclusion in the study. Fragments of adenoid referred to the microbiology laboratory for identification by standard microbiological tests. All *S. aureus* isolates were initially identified by standard microbiological tests including Gram-staining, catalase, slide and tube coagulase, DNase, and mannitol fermentation on mannitol salt agar medium (Merck, Co, Germany). Subsequently, all phenotypically characterized *S. aureus* isolates were confirmed by the amplification of *nuc* gene in polymerase chain reaction (PCR) technique (14).

Biofilm formation. Biofilm formation of *S. aureus* isolates were evaluated as described previously (15). Briefly, all *S. aureus* isolates were cultured on Trypticase Soy Agar (TSA, Merck, Co, Germany) at 37°C for 24 h, then grown colonies suspended in sterile physiological saline with turbidity adjusted to 0.5 McFarland. The 96 well microdilution plates (Cell and Tissue Culture plates, flat well bottom, Guangzhou Jet Bio-Filtration Products Co., Ltd. Guangdong, China), were filled with 180 µl Trypticase Soy Broth (TSB, Merck, Co, Germany) supplemented with 1% glucose and 20 µl of bacterial suspension added to each well. After incubation at 37°C for 24 h, broth was carefully drawn off and the plates were gently washed three times with sterile phosphate-buffered saline (PBS). For biofilm quantification, 200 µl of 2% safranin dye solution in water was added to each well and the plates were allowed to stand for 40 min at room temperature. The wells were subsequently washed thrice with sterile PBS to wash off the excess safranin. Safranin bound to the biofilm was extracted with 200 ml of 95% ethanol, and the absorbance of the extracted safranin was measured at 490 nm in an ELISA reader (BioTek, USA). TSB+1% glucose medium was used to determine background optical density (OD) as a negative control. The cut-off OD (ODc) for biofilm formation was determined as average OD of negative control +3×standard deviation (SD) of negative control. OD value was calculated for each microtiter plate separately. OD > 4×ODc indicated high biofilm formation ability; 2×ODc < OD ≤ 4×ODc indicated moderate biofilm formation ability. ODc < OD ≤ 2×ODc and OD ≤ ODc were taken as weak or none biofilm formation ability respectively.

Detection of *ica* operon genes by PCR. Genomic DNA was extracted using boiling method as previously described (16). All PCR reactions were carried out by a Gradient thermal cycler (Biometra-T300, Gottingen, Germany) in a final reaction mixture volume of 25 µl containing 1 µl of genomic DNA, 0.5 µl (10 pM) of each oligodeoxynucleotide primers, 12.5 µl of 2× Master Mix Red (Ampliqon, Co, Denmark) and 11 µl DNase and RNase free water. After amplification, the PCR products were electrophoresed on 1.5% agarose gel electrophoresis in TBE 0.5× buffer (5.4 g Tris base, 2.75 g Boric acid, 2 ml 0.5 M EDTA, in 1 L) at 100 V for 90 min. The products were detected by staining with Green Viewer Dye and then photographed. The oligonucleotide primers as well as PCR programs are presented in Table 1.

Statistical analysis. Statistical analysis of data was performed using SPSS version 23 (IBM, Armonk, NY, USA). The Chi Square test applied for the comparison of our data. A difference was considered statistically significant at a *p*-values ≤0.05.

RESULTS

There were 46 isolates (41%) identified as *S. aureus* from the adenoid tissues of, 65 (58.0%) males, and 47 (42.0%) females, with adenoid hypertrophy. Patients have revealed obstructive, infectious, and mixed symptoms. Biofilm formation was higher in children with obstructive symptoms (Table 2). Our study showed no relationship between sex and *in-vitro* biofilm production (*p*-value = 0.089). The ability to produce biofilm was detected among total *S. aureus* isolates with the severity achieved as mild, moderate, and strong for 12 (10.7%), 21 (18.8%), and 13 (11.6%) isolates, respectively. In the present study, we observed a significant difference between age as well as the adenoid size and biofilm production (*p*-value ≤ 0.05). The most biofilm production was observed in patients with moderate adenoid size and the age <=5 years old (Table 2). Fig. 1 shown the PCR result of *ica* for *S. aureus* isolates. Molecular study of these genes revealed that 2 (6.3%), and 19 (59.4%) isolates carried *icaA* and *icaD*, respectively. In addition, the prevalence of *icaA* + *icaD* was seen among 11 (34.4%) *S. aureus* isolates, while *icaC* as well as *icaB* was not detected.

DISCUSSION

S. aureus is one of the most common pathogen in children with adenoid hyperplasia (20). Moreover, it is well known that *S. aureus* biofilm plays an important role in adenoiditis as well as the development of antimicrobial resistance (21). According to our results, 41% of isolates identified as *S. aureus* which all strains demonstrated the ability to form biofilm. Strong ability of biofilm production was seen among 13 (11.6%) isolates. On the other hand, the majority of *S. aureus* strains in this study had the ability to produce moderate biofilm. Based on obtained results, *S. aureus* isolates showed a high ability to bio-

Table 1. Primer pairs and PCR conditions used to detection of ica operon genes in this study.

Gene target	Primer/sequence (5'-3')	PCR condition	PCR products size (bp)	Reference
icaA	F-TCTCTTGCAGGAGCAATCAA R-TCAGGCACTAACATCCAGCA	1 min 95ºC, 45 sec 60ºC, 1 min 72ºC	188	17
icaB	F-ATGGCTTAAAGCACACGACGC R-TATCGGCATCTGGTGTGACAG	1 min 95ºC, 45 sec 61ºC, 1 min 72ºC	526	18
icaC	F-ATCATCGTGACACACTTACTAACG R-CTCTCTTAACATCATTCCGACGCC	1 min 95ºC, 45 sec 63ºC, 1 min 72ºC	1013	
icaD	F-GAACCGCTTGCCATGTGTTG R-GCTTGACCATGTTGCGTAACC	1 min 95ºC, 45 sec 61ºC, 1 min 72ºC	483	19

Table 2. Comparison between age, adenoid size, symptoms, and biofilm production ability.

Factors		Biofilm		Total (n)
		Negative (n)	Positive (n)	
Age	<=5	25	31	56
	5-10	18	25	43
	>=10	3	10	13
Size	Small	10	13	23
	Moderate	23	31	54
	Large	13	22	35
Symptoms	Obstructive	26	36	62
	Infectious	6	6	12
	Mixed	14	24	38

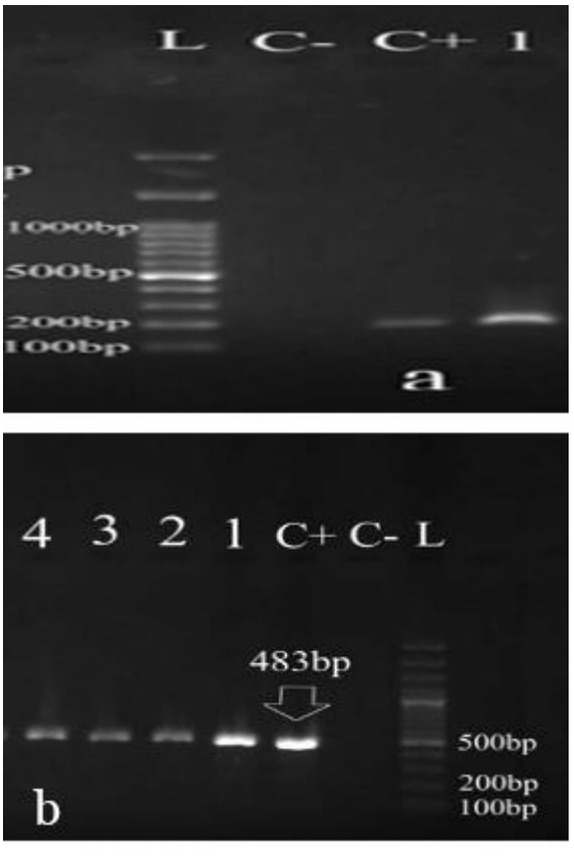

Fig. 1. PCR results of *ica* gene for *S. aureus* isolates. a; L: 100 bp DNA ladder, C-: Negative Control, C+: Positive Control, Lane 1: *icaA* (188 bp). b; L: ladder, C-: Negative Control, C+: Positive Control, Lanes: 1-4 *icaD* (483 bp).

film formation meaning that adenoids may be proper settings for biofilm production. In accordance with our results, Torretta et al. reported *S. aureus* was the most frequent pathogen in the adenoid biopsy specimens which 58.3% of isolates were more frequently weak biofilm producers (22). These findings suggested the importance of removing the adenoids completely in order to ensure the total eradication of biofilm-producing bacteria. However, our finding showed no significant relationship between sex and *in-vitro* biofilm production. These results may be due to our relatively small sample size which needs further investigation in larger case series. We also showed there was a significant correlation between age and biofilm production. The most biofilm production was observed at the age of ≤ 5 years old. Since the adenoidectomy is one of the most prevalent surgeries in children, rapid and accurate detection of bacterial biofilm formation in adenoids is potentially important to eradicate the patient's various symptoms and complications. On the other hand, according to the high prevalence of biofilm formation, surgery of children with small adenoid size at an early age, despite mechanical obstruction, has high efficacy particularly in patients who have moderate and severe symptoms. In this study, the ability of biofilm

production was evaluated targeting the *icaADBC*. As mentioned in the results, 2 (6.3%) and 19 (59.4%) isolates carried *icaA* and *icaD*, respectively. Besides, amplification of these genes revealed 11 *S. aureus* isolates possessing both *icaA* and *icaD*. Similar results were seen in other reports which indicated that there is a relationship between the presence of *ica* operon and biofilm formation (12). In the study by Omidi et al. among 136 of 146 (93.1%) *S. aureus* isolates that produced biofilm phenotypically, 18 methicillin-resistant *S. aureus* (MRSA) isolates carried *icaA* while *icaD* was not detected in all strains (23). Namvar et al. reported that *S. aureus* isolates had no ability to form biofilm unless they were positive for *icaD* gene (19). In contrast, some studies reported the presence of *icaA/D* genes was not always associated with biofilm formation (24). Although different genes are involved in biofilm production, in contradiction to other studies, *icaC* and *icaB* were not detected in our biofilm producer isolates. In a study by Azmi et al. on 248 MRSA biofilm producer isolates, all of them were positive for one of *icaD/icaA* (25). Indeed, these findings demonstrate that *icaABCD* operon are associated with biofilm formation, but the absence of these genes may not necessarily exclude this property. In conclusion, the data reported here represent the major role of *S. aureus* in adenoiditis in childhood. *Staphylococcal* biofilm formation is an important virulence factor which biofilm resistance frequently results in failure of therapy. This suggests that the clinical treatment of adenoid hyperplasia patients requires more extensive consideration of bacterial biofilm-forming activity. In this study, all *S. aureus* isolates revealed the ability in biofilm production and a remarkable percentage of isolates carried *icaD* gene. Variations in the ability of biofilm production as well as the presence of *icaADBC* genes from studies might be related to selected case series, epidemiological varieties and the methods used in studies also contribute to these differences.

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REFERENCES

1. Mnatsakanian A, Heil JR, Sharma S. Anatomy, head and neck, adenoids. StatPearls Publishing, Treasure Island (FL). 2020.
2. Cassano P, Gelardi M, Cassano M, Fiorella ML, Fiorella R. Adenoid tissue rhinopharyngeal obstruction grading based on fiberendoscopic findings: a novel approach to therapeutic management. *Int J Pediatr Otorhinolaryngol* 2003;67: 1303-1309.
3. Bulfamante AM, Saibene AM, Felisati G, Rosso C, Pipolo C. Adenoidal disease and chronic rhinosinusitis in children—is there a Link? *J Clin Med* 2019;8: 1528.
4. Post JC, Stoodley P, Hall–Stoodley L, Ehrlich GD. The role of biofilms in otolaryngologic infections. *Curr Opin Otolaryngol Head Neck Surg* 2004;12: 185-190.
5. O'Gara JP. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus. FEMS Microbiol Lett* 2007;270: 179-188.
6. Torretta S, Drago L, Marchisio P, Ibba T, Pignataro L. Role of biofilms in children with chronic adenoiditis and middle ear disease. *J Clin Med* 2019;8: 671.
7. Rajeshwary A, Rai S, Somayaji G, Pai V. Bacteriology of symptomatic adenoids in children. *N Am J Med Sci* 2013;5: 113-118.
8. Torretta S, Drago L, Marchisio P, Mattina R, Clemente IA, Pignataro L. Diagnostic accuracy of nasopharyngeal swabs in detecting biofilm-producing bacteria in chronic adenoiditis: a preliminary study. *Otolaryngol Head Neck Surg* 2011;144: 784-788.
9. Kania RE, Lamers GE, Vonk MJ, Dorpmans E, Struik J, Tran Ba Huy P, et al. Characterization of mucosal biofilms on human adenoid tissues. *Laryngoscope* 2008;118: 128-134.
10. Lin CD, Tsai MH, Lin CW, Ho MW, Wang CY, Tsou YA, et al. Association of adenoid hyperplasia and bacterial biofilm formation in children with adenoiditis in Taiwan. *Eur Arch Otorhinolaryngol* 2012;269: 503-511.
11. Foster TJ. Immune evasion by Staphylococci. *Nat Rev Microbiol* 2005;3: 948-958.
12. Cue D, Lei MG, Lee CY. Genetic regulation of the intercellular adhesion locus in staphylococci. *Front Cell Infect Microbiol* 2012;2: 38.
13. Rohde H, Frankenberger S, Zähringer U, Mack D. Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. *Eur J Cell Biol* 2010;89: 103-111.
14. Ziasistani M, Dabiri S, Fekri Soofi Abadi M, Afshari SAK, Ghaioumy R, Morones-Ramírez JR, et al. Determination of antibiotic resistance genes, immune

evasion cluster and *agr* types among *Staphylococcus aureus* strains isolated from children with adenoiditis. *Gene Rep* 2020;21: 100875.

15. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. *APMIS* 2007;115: 891-899.
16. Adwan K. Fast DNA isolation and PCR protocols for detection of methicillin-resistant staphylococci. *Folia Microbiol (Praha)* 2014;59 :5-8.
17. Arciola CR, Baldassarri L, Montanaro L. Presence of *icaA* and *icaD* genes and slime production in a collection of Staphylococcal strains from catheter-associated infections. *J Clin Microbiol* 2001;39: 2151-2156.
18. Cafiso V, Bertuccio T, Santagati M, Campanile F, Amicosante G, Perilli MG, et al. Presence of the *ica* operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. *Clin Microbiol Infect* 2004;10: 1081-1088.
19. Namvar AE, Asghari B, Ezzatifar F, Azizi G, Lari AR. Detection of the intercellular adhesion gene cluster *(ica)* in clinical *Staphylococcus aureus* isolates. *GMS Hyg Infect Control* 2013;8: Doc03.
20. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial interactions during upper respiratory tract infections. *Emerg Infect Dis* 2008;14: 1584-1591.
21. Hamilos DL. Biofilm formations in pediatric respiratory tract infection: part 1: biofilm structure, role of innate immunity in protection against and response to biofilm, methods of biofilm detection, pediatric respiratory tract diseases associated with mucosal biofilm formation. *Curr Infect Dis Rep* 2019;21: 6.
22. Torretta S, Drago L, Marchisio P, Gaffuri M, Clemente IA, Pignataro L. Topographic distribution of biofilm-producing bacteria in adenoid subsites of children with chronic or recurrent middle ear infections. *Ann Otol Rhinol Laryngol* 2013;122: 109-113.
23. Omidi M, Firoozeh F, Saffari M, Sedaghat H, Zibaei M, Khaledi A. Ability of biofilm production and molecular analysis of spa and ica genes among clinical isolates of methicillin-resistant *Staphylococcus aureus. BMC Res Notes* 2020;13: 19.
24. Vancraeynest D, Hermans K, Haesebrouck F. Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. *Vet Microbiol* 2004;103: 241-247.
25. Azmi K, Qrei W, Abdeen Z. Screening of genes encoding adhesion factors and biofilm production in methicillin resistant strains of *Staphylococcus aureus* isolated from Palestinian patients. *BMC Genomics* 2019;20: 578.