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Molecular Study of Biofilm Production by Methicillin Resistant *Staphylococcus aureus*

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Abstract

Background *Staphylococci* are a group of bacteria that cause diseases ranging from minor skin infections to life-threatening bacteremia. Biofilm formation was determined by a number of methods and is available to detect the capability of staphylococci to colonize the biomedical devices. The *icaA* and *icaD* have been reported to play a significant role in biofilm formation.

Objective To achieve and detect the molecular basis of adhesion properties in respect to methicillin resistant *Staphylococcus aureus*.

Methods Clinical samples were taken from Burn patients; identified and Methicillin susceptibility was tested. The genes *icaA* and *icaD* were amplified in methicillin resistant *Staphylococcus aureus* and the polymerase chain reaction products were sequenced and aligned with the previous recorded sequences online.

Results There was a great correlation between the presence of *icaD* genes and the slime production. Methicillin resistant *Staphylococcus aureus* did not reveal any correlation between *icaA* and *icaD* and slime layer production; nonetheless, a correlation was noticed between *icaD* alone and a biofilm production

Conclusion The present findings indicated that methicillin resistant *Staphylococcus aureus* was able to form biofilm. None of the methicillin resistant *Staphylococcus aureus* isolates harboured *icaA*; while 100% of them contained *icaD*.

Keywords Methicillin resistant *Staphylococcus aureus*, *icaA*, *icaD* gene

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List of abbreviations: CRA = Congo Red Agar, DNA = Deoxy nucleic acid, MRSA = Methicillin resistance Staphylococcus aureus, MRSE = Methicillin resistance staphylococcus epidermidis, MtP = Microtiter plate method, OD = Optical density

Introduction

Staphylococci are a diverse group of bacteria that cause diseases ranging from minor skin infections to life-threatening bacteremia. In spite of large-scale efforts to control their spread, they persist as a major cause of both hospital and community acquired infections worldwide. The two major opportunistic pathogens of this genus are *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) [(1)]. The widespread use of Methicillin and other semisynthetic penicillin in the late 1960s led to the emergence of Methicillin resistance *S. aureus* (MRSA) and *S. epidermidis* (MRSE), which continue to persist in both the healthcare and community environments. Biofilm formation may be determined by a number of available methods determine the capability of *staphylococci* to colonize the biomedical catheters. The Congo red agar (CRA) assay described by Freeman et al. [(2)] and/or the microtiter plate (MtP) test devised

by Christensen et al. [3] were the most commonly used as the phenotypic methods for the detection of biofilm production. The *icaA* and *icaD* have been reported to a play a significant role in biofilm formation. The *icaA* gene encodes N acetyl glucosaminyl transferase, the enzyme involved in Polysaccharide intercellular adhesion (PIA) synthesis. On the other hand, *icaD* has been reported to a play a critical role in the maximal expression of N-acetylglucosaminyl transferase, leading to the full phenotypic expression of the capsular polysaccharide [4]. Wide controversial aspects were emerged about the nature of MRSA and MRSE biofilms, the basis of adhesion and best method for detection. From this perspective, the present study was designed and aimed to achieve to achieve and detect the molecular basis of adhesion properties in respect to methicillin resistant *S. aureus* by evaluating the most frequent methods (CRA and MtP) employed for the detection of adhesion properties in respect to MRSA and MRSE, detecting the presence of the icaA and icaD in MRSA and MRSE isolates and finally determination of the nature of biofilm adhesion via treatment with proteinase K and $NaIO_4$.

Methods

Specimen

Fifty clinical specimens referring to burn were collected from patients attending Sulaimani Teaching Hospital, Emergency Hospital, and Child Teaching Hospital; for the period from November 2018 to March 2019. The specimens were collected by the attending physician and health officer using sterile applicator stick with cotton swabs moistened with normal saline and test tubes were used to collect the sample. Bacteria were stored for more than three months in nutrient broth containing 20% glycerol at (-20 ˚C) without significant loss of viability.

Isolation of *staphylococci*

All specimens were streaked on mannitol salt agar and blood agar. Thereafter, all plates were incubated aerobically for 24 h at 37 °C. Isolates were identified by the Vitek system.

Biofilm formation by microtiter plate method (MtP)

A suspension of bacterial isolate that equivalent to the McFarland No. 0.5 turbidity standard were inoculated in Nutrient broth and incubated for 18-24 h at 37 ˚C in individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plate stationary phase. Nutrient broth culture supplemented with glucose (0.5%) or NaCl (1%). After that, 200 µl of the inoculum were transferred to the assay wells, which corresponds to an inoculum of approximately 5×10^6 cells/well. Subsequently, inoculated assay plates were transferred to an incubator set at 37 °C for 18-24 h without shaking. Negative and positive control wells were included in the test. After incubation, the optical density (OD) was measured by spectrophotometer at OD 570 nm of each well using a multi-well plate reader to quantify overall growth (Table 1).

Genomic DNA extraction and amplification of icaA and icaD genes

Genomic DNA from all biofilm producer isolates (37 MRSA) was extracted using Genomic DNA Extraction kit (Promega, USA), then the presence of the *icaA* and *icaD* genes these isolates were detected as described by Arciola et al. [5], with two sets of primers for *icaA* F5'-TCTCTTGCAGGAGCAATCAA-'3 and *icaA* R5'TCAGGCACTAACATCCAGCA-'3, for *icaD* detection F5'-ATGGTCAAGCCCAGACAGAG-'3 and *icaD* R5'-CGTGTTTTCAACATTTAATGCAA-'3. Reaction conditions were 94 °C for 5 min initial incubation, 94 °C for 30 sec denaturation, 55.5 °C for 30 sec annealing, 72 °C for 30 sec extension and final extension for 1 min at 72 °C.

DNA Sequencing

Purified PCR products were sent to Macrogen Company, Korea for the DNA sequencing and analyzed by NCBI Blast tools.

Results

Isolation and Identification

Of *Staphylococci* from collected samples, only 50 isolates (91%) have grown on Mannitol salt agar [6]. Taking together, the results were revealed that all 37 isolates were diagnosed as *S. aureus*; whereas the other 13 were comprised as *S. epidermidis*.

Biofilm detection by microtiter plate method (MtP):

The present findings indicated that MRSA was able to form biofilm, and the (OD) value ranged between 0.147-0.315. Using MtP method for the detection of biofilm formation *S. aureus* isolates, when grown in nutrient broth without any supplementation, 100% MRSA isolates were able to form weak biofilm (Table 1).

Table 1. Classification of bacterial adherence by micro titer plate method

Mean OD750	Adherence Biofilm Formation
OD ≤ ODc	Non-adherent
ODc < OD ≤ 2*ODc	Weakly adherent
2*ODc < OD ≤ 4*ODc	Moderately adherent
4*ODc < OD	Strongly adherent

Amplification of *icaA* and *icaD* genes

PCR amplicons obtained from genomic DNA extracted from Positive control MRSA isolate yielded a 188-bp band for *icaA*, and a 198-bp band for *icaD* genes (figure 1). Results of PCR study for 37 genomic DNA extracted from MRSA isolates revealed that 0/37 (0%) MRSA isolates had *icaA* gene, while 37/37 (100%) harbored *icaD*. The current results, suggests that all MRSA isolated from burn specimens were *icaD* positive (figure 1).

DNA sequencing

In order to confirm the results of *icaA* and *icaD* amplification, PCR products were sequenced, analyzed by Bio-Edit software and similarity searches were carried out using with the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Results revealed that GenBank accession numbers for the nucleotide sequences of the *icaA* gene fragments were reference isolates DQ846812, DQ846811, and DQ836167 whereas those of *icaD* gene fragments were AY138959 and FN433596. However, some deletions and insertions of nucleotides were noticed (Figure 2).

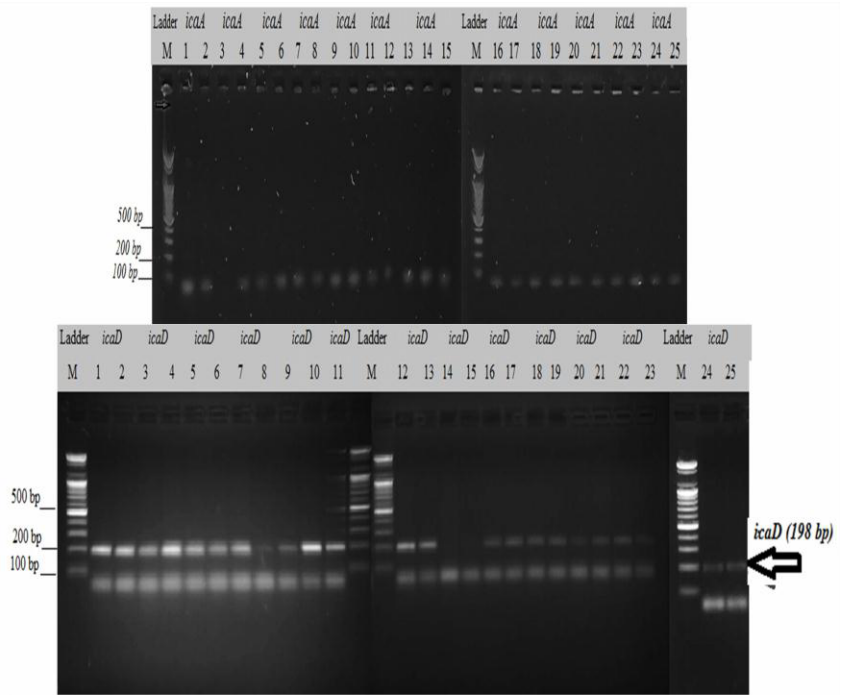
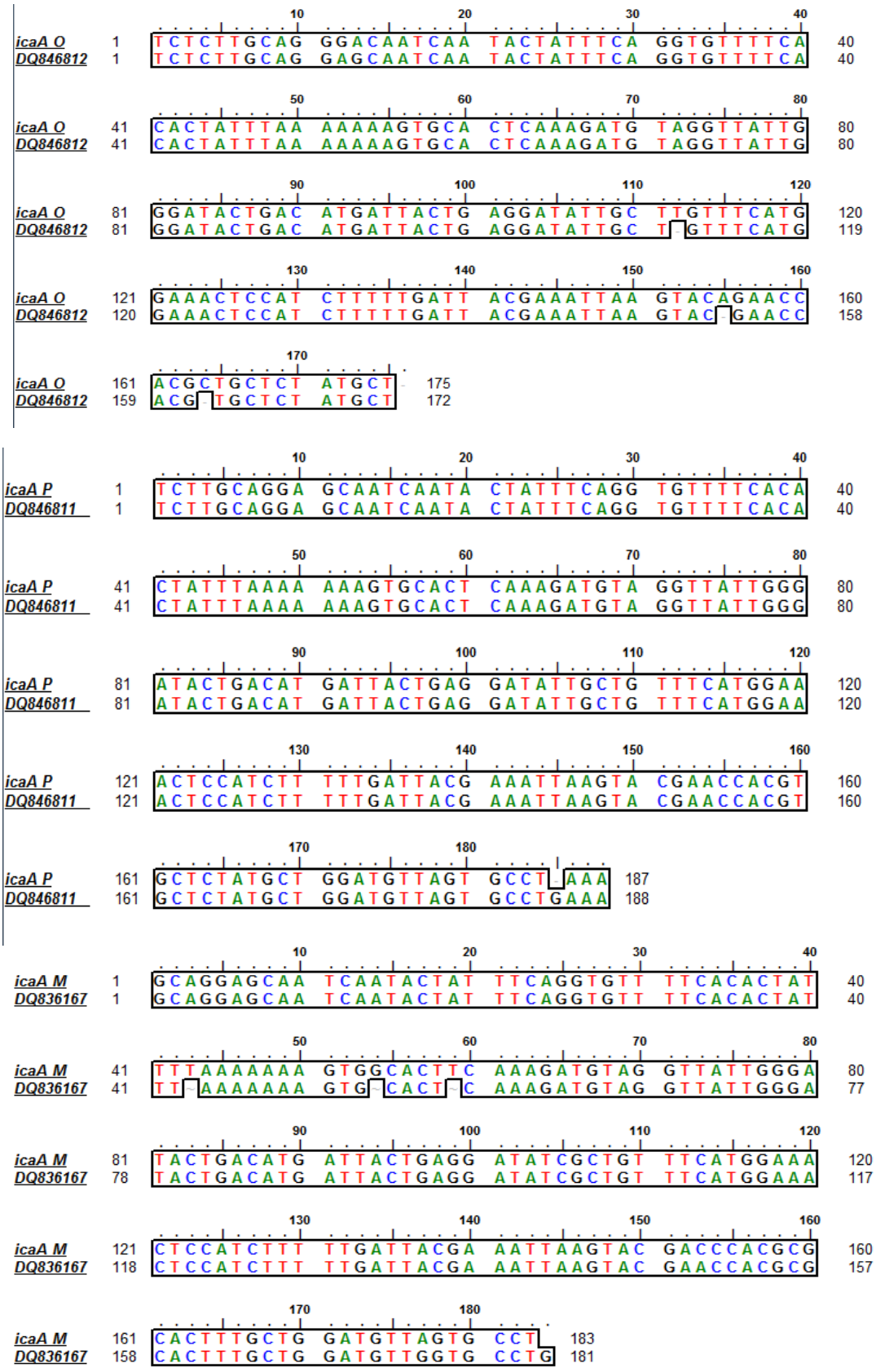


Figure 1. Agarose gel electrophoresis of polymerase chain reaction amplification of *icaA* and *icaD* genes in methicillin resistant *S. aureus* (numerals). M represents 100 bp DNA molecular size marker, in 1.6 % Agarose gel on (85 V for 90 minute). Visualized under U.V light after staining with Ethidium bromide dye

Discussion

Babakir-Mina et al. [7] stated that *S. aureus* accounted for 22% of all patients in Sulaymaniyah Burn Hospital, and constituted 36% from burn specimens. Resistance to methicillin in *Staphylococcus spp*. is primarily mediated by the presence of penicillin-binding protein 2a, encoded by the *mecA* gene. In certain MRSA strains, the *mecA* gene is heterogeneously expressed in vitro [8]. Locally, according to the results of Al-Dahbi [9], the incidence of MRSA among *S. aureus* was 94.3%, Babakir-Mina [7] observed that among *S. aureus* positive cases, 88% were MRSA. Bacteria isolates from burn infection seems to be more resistant to most other antibiotics compared to other sites. Sputum seemed to have the lowest Methicillin resistance percentage in comparison to other specimens. Cefoxitin is a cephamycin antibiotic and has been described as an inducer of methicillin resistance [10]. The performance of cefoxitin either as a disc or as a supplement in agar medium for the detection of MRSA has been confirmed extensively [11]. According to the literature, the quantitative MtP assay eliminates subjectivity in reading of obtained results and predicts clinical relevance more reliably than the tube test [12]. This method has been reported to be the most sensitive, accurate and reproducible screening method for determination of biofilm production by clinical isolates of staphylococci and has the advantage of being a quantitative tool for comparing the adherence of different strains [13]. The *icaA* operon genes have been widely described in *S. epidermidis* and *S. aureus*, several authors have found similarity in other coagulase negative staphylococci species. Nevertheless, results cannot be extended to all pathogenic species [12]. As it is reported by these authors, the genes of *ica* operon frequently appeared in strains of *S. aureus* [14].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DQ846812 1 TCTCTTGCAG GAGCAATCAA TACTATTTCA GGTGTTTTCA 40
icaA O 41 CACTATTTAA AAAAAGTGCA CTCAAAGATG TAGGTTATTG 80
DQ846812 41 CACTATTTAA AAAAAGTGCA CTCAAAGATG TAGGTTATTG 80
icaA O 81 GGATACTGAC ATGATTACTG AGGATATTGC TTGTTTCATG 120
DQ846812 81 GGATACTGAC ATGATTACTG AGGATATTGC TGTTTCATG 119
icaA O 121 GAAACTCCAT CTTTTTGATT ACGAAATTAA GTACAGAACC 160
DQ846812 120 GAAACTCCAT CTTTTTGATT ACGAAATTAA GTACGAACC 158
icaA O 161 ACGCTGCTCT ATGCT 175
DQ846812 159 ACGTGCTCT ATGCT 172
icaA P 1 TCTTGCAGGA GCAATCAATA CTATTTCAGG TGTTTTCACA 40
DQ846811 1 TCTTGCAGGA GCAATCAATA CTATTTCAGG TGTTTTCACA 40
icaA P 41 CTATTTAAAA AAAGTGCACT CAAAGATGTA GGTTATTGGG 80
DQ846811 41 CTATTTAAAA AAAGTGCACT CAAAGATGTA GGTTATTGGG 80
icaA P 81 ATACTGACAT GATTACTGAG GATATTGCTG TTTCATGGAA 120
DQ846811 81 ATACTGACAT GATTACTGAG GATATTGCTG TTTCATGGAA 120
icaA P 121 ACTCCATCTT TTTGATTACG AAATTAAGTA CGAACCACGT 160
DQ846811 121 ACTCCATCTT TTTGATTACG AAATTAAGTA CGAACCACGT 160
icaA P 161 GCTCTATGCT GGATGTTAGT GCCTAAA 187
DQ846811 161 GCTCTATGCT GGATGTTAGT GCCTGAAA 188
icaA M 1 GCAGGAGCAA TCAATACTAT TTCAGGTGTT TTCACACTAT 40
DQ836167 1 GCAGGAGCAA TCAATACTAT TTCAGGTGTT TTCACACTAT 40
icaA M 41 TTTAAAAAAA GTGGCACTTC AAAGATGTAG GTTATTGGGA 80
DQ836167 41 TTAAAAAAAA GTGCACTC AAAGATGTAG GTTATTGGGA 77
icaA M 81 TACTGACATG ATTACTGAGG ATATCGCTGT TTCATGGAAA 120
DQ836167 78 TACTGACATG ATTACTGAGG ATATCGCTGT TTCATGGAAA 117
icaA M 121 CTCCATCTTT TTGATTACGA AATTAAGTAC GACCCACGCG 160
DQ836167 118 CTCCATCTTT TTGATTACGA AATTAAGTAC GAACCACGCG 157
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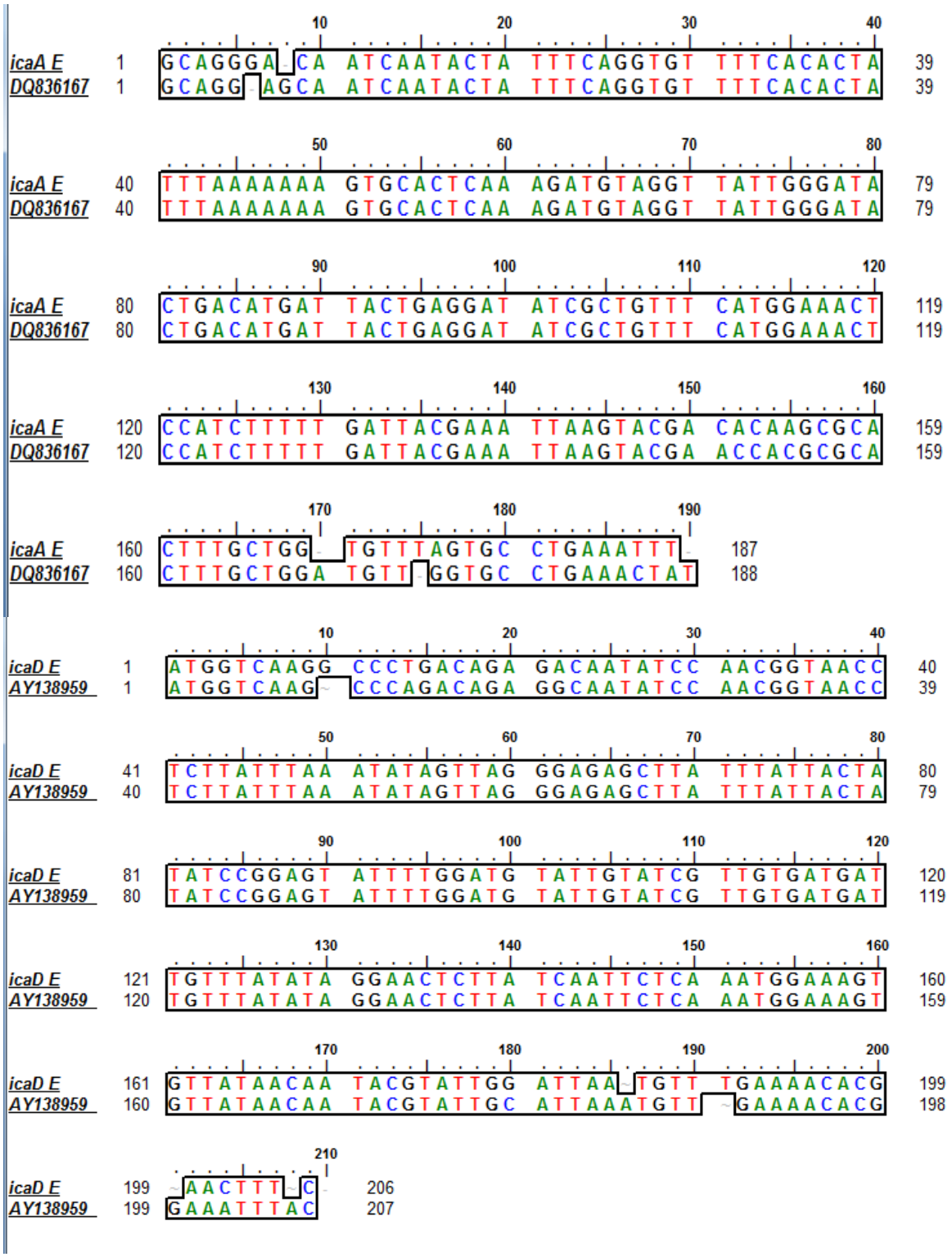
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DQ836167 1 GCAGG-AGCA ATCAATACTA TTTCAGGTGT TTTCACACTA 39
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DQ836167 40 TTTAAAAAAA GTGCACTCAA AGATGTAGGT TATTGGGATA 79
icaA E 80 CTGACATGAT TACTGAGGAT ATCGCTGTTT CATGGAAACT 119
DQ836167 80 CTGACATGAT TACTGAGGAT ATCGCTGTTT CATGGAAACT 119
icaA E 120 CCATCTTTTT GATTACGAAA TTAAGTACGA CACAAGCGCA 159
DQ836167 120 CCATCTTTTT GATTACGAAA TTAAGTACGA ACCACGCGCA 159
icaA E 160 CTTTGCTGG-TGTTTAGTGC CTGAAATTT- 187
DQ836167 160 CTTTGCTGGA TGTT-GGTGC CTGAAACTAT 188
icaD E 1 ATGGTCAAGG CCCTGACAGA GACAATATCC AACGGTAACC 40
AY138959 1 ATGGTCAAG- CCCAGACAGA GGCAATATCC AACGGTAACC 39
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AY138959 40 TCTTATTTAA ATATAGTTAG GGAGAGCTTA TTTATTACTA 79
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AY138959 120 TGTTTATATA GGAACTCTTA TCAATTCTCA AATGGAAAGT 159
icaD E 161 GTTATAACAA TACGTATTGG ATTAA-TGTT TGAAAACACG 199
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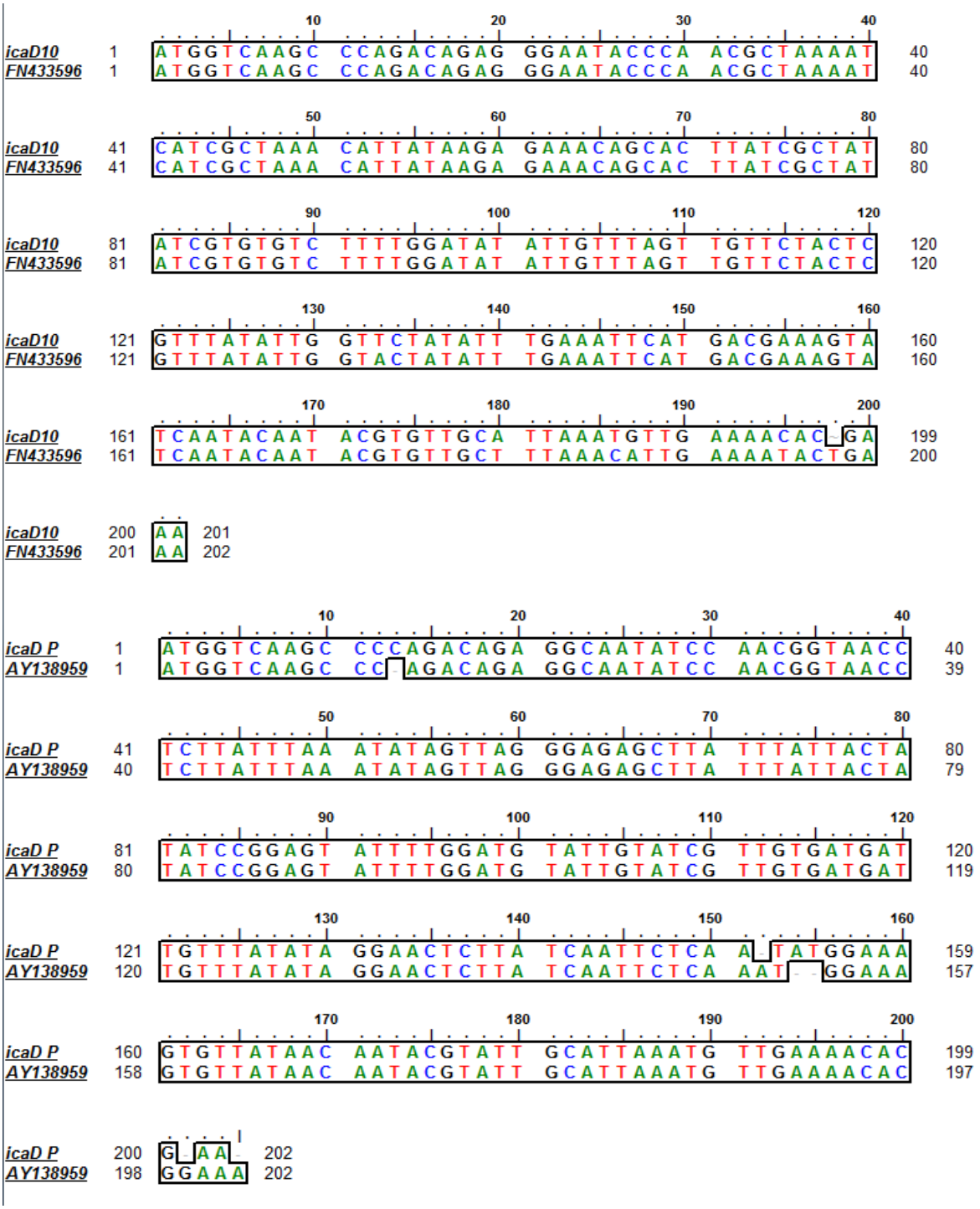
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FN433596 41 CATCGCTAAA CATTATAAGA GAAACAGCAC TTATCGCTAT 80
icaD10 81 ATCGTGTGTC TTTTGGATAT ATTGTTTAGT TGTTCTACTC 120
FN433596 81 ATCGTGTGTC TTTTGGATAT ATTGTTTAGT TGTTCTACTC 120
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FN433596 121 GTTTATATTG GTACTATATT TGAAATTCAT GACGAAAGTA 160
icaD10 161 TCAATACAAT ACGTGTTGCA TTAAATGTTG AAAACACGA 199
FN433596 161 TCAATACAAT ACGTGTTGCT TTAAACATTG AAAATACTGA 200
icaD10 200 AA 201
FN433596 201 AA 202
icaD P 1 ATGGTCAAGC CCCAGACAGA GGCAATATCC AACGGTAACC 40
AY138959 1 ATGGTCAAGC CC-AGACAGA GGCAATATCC AACGGTAACC 39
icaD P 41 TCTTATTTAA ATATAGTTAG GGAGAGCTTA TTTATTACTA 80
AY138959 40 TCTTATTTAA ATATAGTTAG GGAGAGCTTA TTTATTACTA 79
icaD P 81 TATCCGGAGT ATTTTGGATG TATTGTATCG TTGTGATGAT 120
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icaD P 121 TGTTTATATA GGAACTCTTA TCAATTCTCA A-TATGGAAA 159
AY138959 120 TGTTTATATA GGAACTCTTA TCAATTCTCA AAT--GGAAA 157
icaD P 160 GTGTTATAAC AATACGTATT GCATTAAAATG TTGAAAACAC 199
AY138959 158 GTGTTATAAC AATACGTATT GCATTAAAATG TTGAAAACAC 197
icaD P 200 G-AA 202
AY138959 198 GGAAA 202

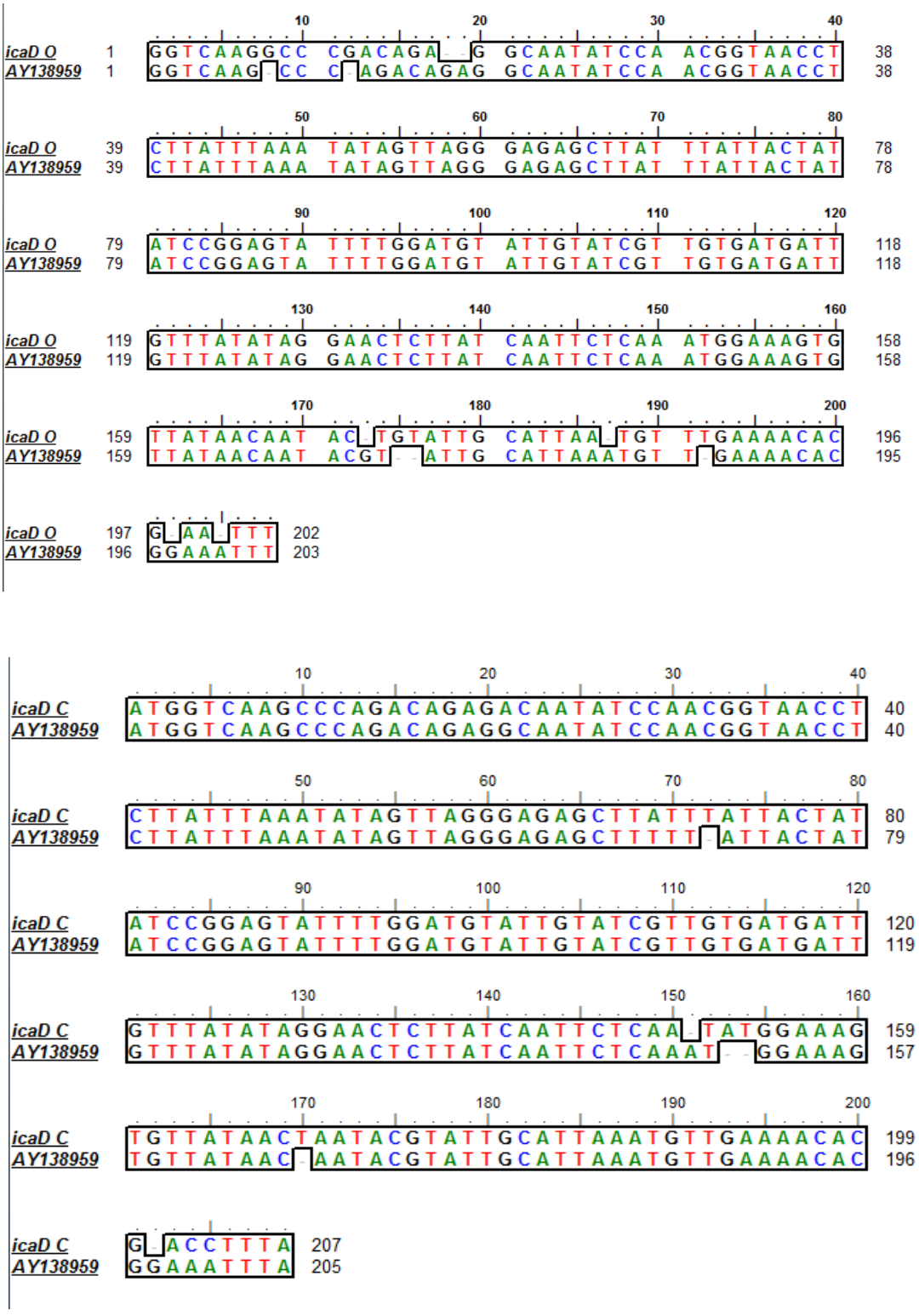

Figure 2. Multiple sequence alignment of nucleotide sequencing *Staphylococcus aureus* clinical isolates in Sulaymaniyah hospitals. Numbers beside the gene names represent MRSA. The codes below the gene name signify the accession number

The obtained results have an agreement with those of Petrelli et al. *(15)* as they recorded the existence of the *icaA* and *icaD* genes in about 94.6% contained both *icaA* and *icaD*. In contrast to the current results, when as the finding in the current study that all MRSA isolated from burn specimens were icaD positive. Diemond-Hernandez et al. [16] reported that *icaA* genes were present in 27.8%, of coagulase negative staphylococci isolates and only (10%) of *S. aureus* isolates were positive for *icaA* + *icaD* genes. Zhou et al. [17] demonstrated that *icaD* had higher positive rate than *icaA* in all *S. aureus* isolates. Other findings pointed to an important role of the *icaA* and *icaD* due to their ability to produce slime strongly in a high percentage of clinical isolates collected from patients with catheters associated infection [18]. Zhou et al. [17] reported that the co-expression of *icaA* with *icaD* can increase slime production remarkably.

From the present study it can be concluded that all MRSA isolates have the ability to produce a slime layer in different amounts of production. This study indicates the absence of *icaA* from the genome of MRSA isolates; whereas, most of MRSA harbored *icaD* gene.

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Conflict of interest

The author has no conflict of interst.

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