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Original article

Biofilm Formation and Detection of *IcaAB* Genes in Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*

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Abstract

Objective(s)
Methicillin-resistant S*taphylococcus aureus* (MRSA) is an important cause of nosocomial and community infections. Biofilm formation, mediated by a polysaccharide intercellular adhesin (PIA) and encoded by the *ica* operon, is considered to be an important virulence factor in both *S. epidermidis* and *S. aureus*. However, the clinical impact of the *ica* locus and PIA production is less well described in *S. aureus*. We studied biofilm formation in clinical isolates of MRSA in relation to the presence of the *ica* operon.

Materials and Methods
Forty five MRSA were studied for biofilm formation by colony morphology on Congo red agar (CRA) and the microtitre plate assay (MtP). Presence of the *ica* genes was detected by PCR and specific primers.

Results
The results showed that 53.3% of the isolates had the potential to form biofilm by colony morphology of which, 75% carried the *ica* operon. Weak biofilm production was observed in the MtP assay by 57.8%, of which 53.8% harbored the *ica* operon. However, about 70% of biofilm non-producers also carried the *ica* operon.

Conclusion
Overall, there was no agreement between the *icaAB* gene carriage and biofilm phenotype by either of the two phenotypic methods. However, 91% of biofilm formers on CRA also produced biofilm in the MtP assay.

Keywords: Biofilm formation, *IcaAB,* MRSA, *Staphylococcus aureus*

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Introduction

Staphylococcus aureus is an important cause of nosocomial and community acquired infections (1). Since its report in 1961, methicillin resistant *S. aureus* (MRSA) has become one of the most important pathogens that cause postoperative infections.

The ability to adhere and form biofilm on host surfaces is considered to be an important virulence factor in both *S. epidermidis* and *S. aureus* (2). Biofilm formation is influenced by a number of factors among which, the most important is synthesis of the polysaccharide intercellular adhesion (PIA) by the organism (2, 3). The enzymes required for PIA synthesis are encoded within the *icaADBC* operon, mutation of which results in a reduced capacity to form biofilm in both *S. aureus* and *S. epidermidis* (2). The *ica* operon was first identified and studied most extensively in *S. epidermidis* and was later shown to be present in *S. aureus* (3, 4). Most *S. aureus* strains appear to contain the entire *ica* operon, although there are reports to the contrary (3, 5-7). However, the clinical impact of the *ica* locus and PIA production remains less well described in *S. aureus*. The *ica* operon in both *S. epidermidis* and *S. aureus* is subject to environmental regulation but its expression is more tightly controlled in *S. aureus* (8). Low levels of biofilm expression have been observed under *in vitro* growth conditions in *S. aureus* and there are strain-dependent differences with respect to the overall capacity to form biofilm *in vitro* (3, 6, 7, 9, 10).

We studied biofilm formation in clinical isolates of methicillin resistant *S. aureus* in order to determine whether there was an association between methicillin resistance, biofilm formation and presence of the *icaADBC* operon. Potential to form biofilm was determined by colony morphology on Congo red agar (CRA) plates and the quantitative microtitre plate assay (MtP). Presence of the *ica* operon was shown by PCR and specific primers.

Materials and Methods

Bacterial isolates

Forty five isolates of MRSA were chosen from a collection of *S. aureus* isolates (Oct 2007 to Mar 2008) from two hospitals in Tehran (Taleghani and Imam Hussein). The specimens were mostly from wound [21] followed by urine [12], sputum [7] and abscesses [5]. The bacteria were maintained in Lauria Bertani broth (LB) containing 8% DMSO at –80 $^{\circ}$C. Methicillin resistance was shown by disc diffusion and measurement of minimum inhibitory concentrations (MIC) was carried out by broth microdilution as recommended by the NCCLS (11, 12). Biofilm positive *S. epidermidis* strain RP62A and its isogenic biofilm negative mutant RP62NA were kindly provided by Dr. Gerald Pier (Harvard Medical School, Boston, MA).

Phenotype analysis of biofilm production on CRA

Biofilm forming colony morphology was detected for MRSA isolates on CRA plates containing 21 g Mueller–Hinton broth, 15 g granulated agar; 36 g sucrose and 0.8 g Congo red per liter of distilled water (13). Bacteria were grown in 10 ml tryptic soy broth (TSB) containing 0.25% glucose at 37 $^{\circ}$C for 24 hr without shaking, plated onto CRA plates and were incubated at 37 $^{\circ}$C for 24 hr. An additional 24 hr incubation period was allowed at room temperature before recording the colony morphology. Crusty black colonies with dry filamentous appearance were recorded as biofilm producers and smooth pink colonies as non-producers (14).

Quantitative determination of biofilm production

Biofilm production was determined by the MtP method as described previously (15). Briefly, overnight grown bacteria in trypticase soy broth (TSB) containing 0.25% glucose were diluted (1:100) and 200 µl portions were inoculated into 96-well flat bottom polystyrene microtiter plates (Cellstar, greiner bio-one). Incubation was carried out at 37 $^{\circ}$C for 22-24 hr before removal of the cultures. The wells were washed 3 times with phosphate buffered saline (PBS, pH, 7.2), air dried and stained with 0.1% safranin. The optical density of the wells was measured at 490 nm using micro Elisa auto reader (Stat Fax 2100, Awareness Technol. Inc). An optical density of 0.12 was chosen to distinguish biofilm producers

from those that did not form biofilm. Biofilm-positive and negative strains of *S. epidermidis* were included in each plate as was a negative control of medium without bacteria. The tests were carried out in quadruplicate and all strains were tested on at least two different days.

Detection of icaADB operon

Genomic DNA was extracted by boiling (16). Briefly, several colonies from an overnight grown culture on nutrient agar were resuspended in 250 µl ddH$_2$O and placed in a boiling water bath for 20 min before centrifugation at 12000 ×g for 5 min. PCR detection of the *ica* cluster was performed by amplification of a DNA region partially covering the *icaA* and *icaB* genes. The primers were selected based on the sequence available in the National Center for Biotechnology Information gene bank (locus AF086783) and were: *icaAB* forward (5'-AAA CTT GGT GCG GTT ACA GG-3') and *icaAB* reverse (5'-TCT GGG CCT GAC CAT GTT G-3') amplifying a 750 bp fragment (17). Reaction mixtures (25 µl) contained 10 µl genomic DNA, 40 pM of each oligonucleotide primer, 0.2 mM dNTP mix, 1.25 u Taq polymerase (Cinnagen, Iran) and 1.5 mM MgCl$_2$ in 10mM Tris-HCl (pH, 8.3). Amplifications were performed using a Bioer Little Genius thermal cycler with the following thermal cycling profile: initial denaturation at 94 ^{o}C for 2 min followed by 30 cycles of amplification (denaturation at 94 ^{o}C for 1 min, annealing at 55 ^{o}C for 1 min and extension at 72 ^{o}C for 2 min) and a final extension period of 7 min at 72 ^{o}C.

Results

Results of colony phenotype on CRA plates showed that 24 isolates (53.3%) formed black colonies and were potential biofilm producers of which, 18 (75%) carried the ica operon (Figure 1). Of the organisms which formed pink colonies on CRA plates, 14 (70%) harbored the *icaAB* genes suggesting no relation between the *icaAB* carriage and colony morphology. The quantitative MtP assay results showed biofilm formation in 26 isolates (57.8%) of which, 14 (53.8%) harbored the *ica* operon (Figure 2). All biofilm formers produced weak biofilms with optical densities ranging from 0.12 to a
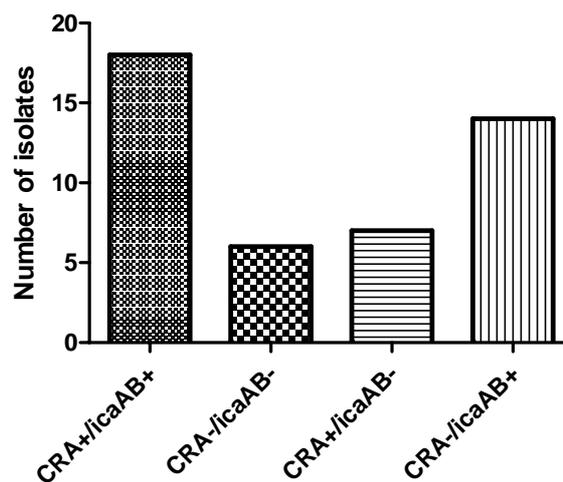

Figure 1. Comparison of the number of potential biofilm formers on CRA plates and *icaAB* gene carriage among 45 clinical isolates of methicillin resistant *Staphylococcus aureus*.
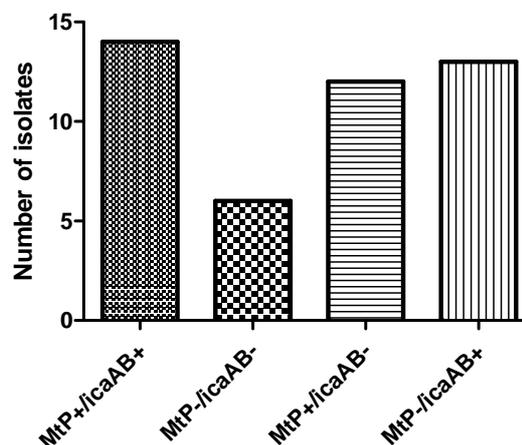

Figure 2. Comparison of biofilm formation by the MtP assay and *icaAB* gene carriage among 45 clinical isolates of methicillin resistant *Staphylococcus aureus*.
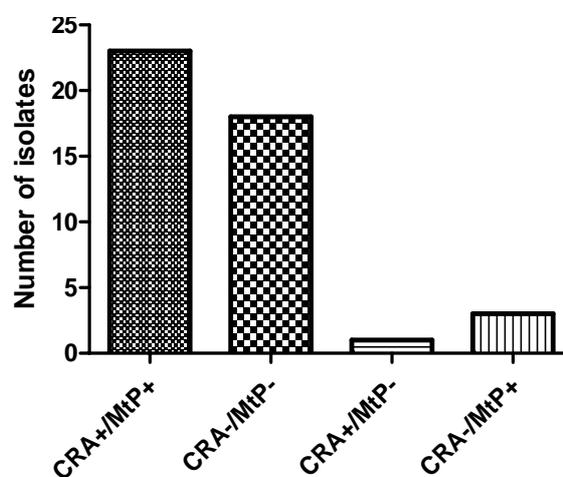

Figure 3. Comparison of biofilm formation by the MtP and CRA plate assays among 45 clinical isolates of methicillin resistant *Staphylococcus aureus*.

maximum of 0.4. Among the 19 organisms that did not produce biofilm by the MtP method, 13 (68.42%) carried the *ica* operon (Figure 2). No relation was observed between the *icaAB* gene carriage and biofilm phenotype by either of the two methods. In addition, there was no connection between the type of infection (source of the organisms) with biofilm formation, nor presence of the *ica* operon. Interestingly, as shown in Figure 3, there was 91% agreement between potential biofim formers on CRA plates and biofilm formation in microtitre plates (MtP +/ CRA+ or MtP -/ CRA -).

Discussion

Pathogenesis of *S. aureus* is attributed to a number of factors among which, adherence and biofilm formation on wounds and implant surfaces is thought to be important. Biofilms help bacteria to survive hostile environments such as the host immune response and antibiotics. To measure the ability of biofilm formation *in vitro*, detection of the *ica* operon has been studied along with phenotypic methods such as colony morphology on CRA plates as well as the MtP assay. A number of studies have shown that majority of *S. aureus* isolates including MRSA, carry the *ica* operon or part of it regardless of being capable of producing biofilms *in vitro* (6, 18, 19).

We found that 57.8% of the MRSA isolates were capable of producing weak biofilms. Low levels of biofilm expression have been reported *in vitro* compared to the *in vivo*

growth in *S. aureus* (9). There is also evidence presented for environmentally regulated *icaADBC* independent biofilm development mechanism (s) in MRSA (20). Rashid *et al* demonstrated that expression of *ica* is at least partially controlled by the stress response transcription factor σ^B (21). In addition, anaerobic growth was found to induce expression of the *ica* operon and PIA production in both *S. epidermidis* and *S. aureus* (22). O'Neill *et al.* showed that biofilm development in MRSA is regulated by SarA and Agr, independent of the *ica* operon (23). We have also shown that biofilm formation is independent of the *icaADBC* carriage in clinical and skin isolates of *S. epidermidis* (15, 24).

The results presented here suggest that *in vitro* biofilm formation process is complex and independent of *ica* gene carriage in MRSA isolates. In our opinion, detection of the *ica* operon as a molecular standard for biofilm formation is misleading and does not reflect the actual biofilm phenotype under *in vitro* or *in vivo* conditions. The agreement between the two phenotypic methods (91%) in this study, also shown by Grinholc *et al*, suggests that environmental conditions independent of the *ica* operon are responsible for the outcome of biofilm phenotype (18).

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