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Abstract

Staphylococci have been confirmed to form biofilms on various biomaterials. The purpose of this study was to investigate biofilm formation among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients with urinary tract infection (UTI) and to assess the relationship between biofilm-forming capacities and virulence determinants/clinical background. Over a 12-year period from 1990 through 2001, a total of 109 MRSA isolates were collected from patients (one isolate per patient) with UTI at the urology ward of Okayama University Hospital. We used the *in vitro* microtiter plate assay to quantify biofilm formation. We then investigated the presence of several virulence determinants by polymerase chain reaction assay and found eight determinants (*tst*, *sec*, *hla*, *hly*, *fnbA*, *clfA*, *icaA*, and *agrII*) to be predominant among these isolates. Enhanced biofilm formation was confirmed in *hla*-, *hly*-, and *fnbA*-positive MRSA isolates, both individually and in combination. Upon review of the associated medical records, we concluded that the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases. The percentage of *hla*-, *hly*-, and *fnbA*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases. Our studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla*, *hly*, and *fnbA* gene products.

KEYWORDS: methicillin-resistant *Staphylococcus aureus*, urinary tract infection, biofilm formation

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Original Article

**Biofilm Formation among Methicillin-Resistant
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Staphylococci have been confirmed to form biofilms on various biomaterials. The purpose of this study was to investigate biofilm formation among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients with urinary tract infection (UTI) and to assess the relationship between biofilm-forming capacities and virulence determinants/clinical background. Over a 12-year period from 1990 through 2001, a total of 109 MRSA isolates were collected from patients (one isolate per patient) with UTI at the urology ward of Okayama University Hospital. We used the *in vitro* microtiter plate assay to quantify biofilm formation. We then investigated the presence of several virulence determinants by polymerase chain reaction assay and found eight determinants (*tst*, *sec*, *hla*, *hly*, *fnaA*, *clfA*, *icaA*, and *agrII*) to be predominant among these isolates. Enhanced biofilm formation was confirmed in *hla*-, *hly*-, and *fnaA*-positive MRSA isolates, both individually and in combination. Upon review of the associated medical records, we concluded that the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases. The percentage of *hla*-, *hly*-, and *fnaA*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases. Our studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla*, *hly*, and *fnaA* gene products.

Key words: methicillin-resistant *Staphylococcus aureus*, urinary tract infection, biofilm formation

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been identified as a major pathogen in nosocomial infections [1, 2]. The percentage of MRSA among nosocomial *S. aureus* isolates in Japan is estimated to be 50% to 70% [3]. The incidence of urinary tract infection (UTI) caused by MRSA is increasing because patients are more frequently fitted with various

urinary stents and catheters as endourology progresses technologically [4].

Staphylococci, including *S. aureus*, are known to form biofilms on various biomaterials [5]. These organisms can persist in clinical settings and gain increased resistance to antimicrobial agents through biofilm formation that appears to be a bacterial survival strategy [6, 7]. Therefore, biofilms formed by MRSA have become resistant to most available antimicrobial agents. The polysaccharide intracellular adhesin (PIA), encoded by *ica* genes, has been shown to be required for biofilm

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formation by staphylococci [5]. More recently, α -toxin (Hla) has also been shown to play an integral role in biofilm formation [8]. The pathogenesis of *S. aureus* is attributed to the combined effects of extracellular factors and toxins, together with invasive properties such as adherence, biofilm formation, and resistance to phagocytosis.

S. aureus secretes a plethora of virulence factors such as toxins and enzymes [9], some of which cause particular diseases. For example, toxic shock syndrome toxin-1 (TSST-1) causes toxic shock syndrome (TSS) and staphylococcal enterotoxins (SEA, SEB, SEC, etc.) cause food poisoning. TSST-1 and SEs are known as superantigens. *S. aureus* also produces a number of cytotoxic molecules that include four hemolysins (α - [Hla], β - [Hlb], δ - [Hld], and γ - [Hlg] toxins). Production of these virulence factors in *S. aureus* is carefully controlled in response to cell density (quorum sensing), energy availability, and environmental signals by accessory gene regulators including Agr, Sar, Sae, and others [10]. These global regulators also control surface proteins (adhesins), such as 2 fibronectin-binding proteins A and B (FnBPA and FnBPB), 2 fibrinogen-binding proteins known as clumping factors A and B (ClfA and ClfB), and a collagen-binding protein (Cna), which are responsible for the adherence, colonization, and biofilm formation of MRSA isolates [5, 7, 10, 11]. However, no clear mechanism has been elucidated for biofilm formation and pathogenicity of *S. aureus* infections of the urinary tract.

In the present study, we investigated the relationship between biofilm-forming capacities and virulence determinants/clinical background of 109 MRSA isolates collected from patients with UTI over a 12-year period from 1990 to 2001 at the Department of Urology, Okayama University Hospital. We analyzed the presence of genes encoding superantigens (*tst*, *sea*, *seb*, *sec*), hemolysins (*hla*, *hlb*), surface proteins (*fnbA*, *fnbB*, *clfA*, *cna*), PIA (*icaA*), and global regulators (*agrI*, *agrII*, *agrIII*, and *agrIV* subgroup) in the MRSA isolates and retrospectively reviewed the associated medical records.

Materials and Methods

Bacterial isolates. The bacterial isolates used in this study were MRSA isolated from patients with UTI at the Department of Urology, Okayama University Hospital, over a 12-year period from 1990 through 2001. A total of 109 isolates that grew to $> 10^4$ CFU/ml in

urinary culture were selected for this study. All 109 patients (one isolate per patient) had documented pyuria (WBC > 5 /hpf). MRSA was defined as an *S. aureus* isolate possessing the *mecA* gene [4].

Biofilm formation assay. MRSA isolates were grown overnight at 37 °C in brain heart infusion broth supplemented with 2% glucose and 2% sucrose [12]. The culture was diluted 1:100 in medium, and 150 μ l of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 48 h at 37 °C without shaking, wells were gently washed three times with 300 μ l of distilled water, dried in an inverted position, and stained with 300 μ l of 2% crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 200 μ l of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD₅₇₀ value from the control wells was subtracted from the mean OD₅₇₀ value of tested wells.

Polymerase chain reaction (PCR) assay. PCR assays were performed to detect various genes in the MRSA isolates. The primers and PCR conditions used in this study are summarized in Table 1. Total cellular DNA was prepared as follows: 0.5 ml of MRSA culture, grown overnight in brain heart infusion broth (Nissui, Tokyo, Japan), was centrifuged, and the pellet was resuspended in 50 μ l of InstaGene (Bio-Rad Laboratories, Hercules, CA, USA). After the suspension was heated for 10 min at 100 °C, 2.5 μ l (or 5 μ l for detection of *agrI*, *agrII*, *agrIII*, and *agrIV*) of the supernatant was mixed with 22.5 μ l (or 20 μ l for detection of *agrI*, *agrII*, *agrIII*, and *agrIV*) of premade reaction mixture to start the reaction. The primer pairs (2.5 pmol) for *tst*, *sea*, *seb*, *sec*, *hla*, *hlb*, *fnbA*, *fnbB*, *clfA*, *cna*, and *icaA*, and those (5 pmol) for *agrI*, *agrII*, *agrIII*, and *agrIV* were added to the respective reaction mixtures. The 25- μ l reaction volume contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, MgCl₂ (concentrations shown in Table 1), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of

Taq DNA polymerase (Takara Shuzo, Shiga, Japan). DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 94 °C for 5 min, the denaturation, annealing, and extension reactions shown in Table 1 for 30 cycles (35 cycles for detection of *agrI*, *agrII*, *agrIII*, and *agrIV*), respectively, followed by final extension at 72 °C for 7 min. PCR products were then analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, gels were stained with ethidium bromide (1 mg/l) and photographed under a UV transilluminator. A 100-bp DNA Ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker. The fragment sizes of each PCR product are shown in Table 1.

Retrospective clinical study. We retrospectively reviewed the medical records of the 109 patients and classified their UTI as catheter-related or catheter-

unrelated, polymicrobial or monomicrobial, and febrile or non-febrile cases. Febrile UTI was defined as UTI in a patient with a body temperature of ≥ 38.0 °C.

Statistical methods. Data are expressed as mean values \pm standard deviation (SD). Comparison of OD₅₇₀ values between groups was carried out using Fisher's exact test or Mann-Whitney's *U* test. All results were considered statistically significant at the $P < 0.05$ level.

Results

Biofilm formation. Of the 109 MRSA isolates, 10 (9.2%), 31 (28.4%), 63 (57.8%), and 5 (4.6%) isolates exhibited strong (OD₅₇₀ ≥ 0.5), medium (OD₅₇₀ ≥ 0.2 to < 0.5), weak (OD₅₇₀ 0 to < 0.2), and no biofilm formation, respectively. The mean OD₅₇₀ of the 109

Table 1 PCR primers and conditions used in this study

Primer specificity	Primer sequences	Product length [bp]	PCR conditions cycling	MgCl ₂ (mM)	Reference
<i>tst</i>	F: 5'-ATGGCAGCATCAGCTTGATA-3' R: 5'-TTTCCAATAACCCCGTTT-3'	350	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
<i>sea</i>	F: 5'-TTGGAACGGTTAAAAACGAA-3' R: 5'-GAACCTTCCCATCAAAAACA-3'	120	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
<i>seb</i>	F: 5'-TCGCATCAAACGACAAACG-3' R: 5'-GCAGGTACTCTATAAGTGCC-3'	478	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
<i>sec</i>	F: 5'-GACATAAAAGCTAGGAATTT-3' R: 5'-AAATCGGATTAACATTATCC-3'	257	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
<i>hla</i>	F: 5'-CTGGCCTTCAGCCTTTAAGG-3' R: 5'-CTGTAGCGAAGTCTGGTGAAA-3'	455	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	this study
<i>hlb</i>	F: 5'-GCCAAAGCCGAATCTAAG-3' R: 5'-CGCATATACATCCCATGGC-3'	845	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
<i>fnbA</i>	F: 5'-GCGGAGATCAAAGACAA-3' R: 5'-CCATCTATAGCTGTGTGG-3'	1278	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
<i>fnbB</i>	F: 5'-GGAGAAGGAATTAAGGCG-3' R: 5'-GCCGTCGCCTTGAGCGT-3'	811	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
<i>clfA</i>	F: 5'-CGATTGGCGTGGCTTCAG-3' R: 5'-GCCAGTAGCCAATGTACAC-3'	1004	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
<i>cna</i>	F: 5'-AGGATCAGATTCAGGTGGACAGCA-3' R: 5'-GAGTGCCTTCCCAAACCTTTTGG-3'	711	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	this study
<i>icaA</i>	F: 5'-GATTATGTAATGTGCTTGG-3' R: 5'-ACTACTGCTGGTTAATAAT-3'	770	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	4	15
<i>agr</i> subgroup I	F: 5'-ATCGCAGCTTATAGTACTTTGT-3' R: 5'-CTTGATTACGTTTATATTTTCATC-3'	739	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
<i>agr</i> subgroup II	F: 5'-AACGCTTGACGAGTTTATTT-3' R: 5'-CGACATTATAAGTATTACAACA-3'	691	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
<i>agr</i> subgroup III	F: 5'-TATATAAATTTGTGATTTTTATTG-3' R: 5'-TTCTTTAAGAGTAAATTGAGAA-3'	712	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
<i>agr</i> subgroup IV	F: 5'-GTTGCTCTTATAGTACATGTT-3' R: 5'-CTTAAAAATATAGTGATCCAATA-3'	683	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15

isolates was 0.24 ± 0.18 (mean \pm SD).

Presence of various genes in MRSA isolates. Of the 109 MRSA isolates, 79 (72.5%), 8 (7.3%), 21 (19.3%), 78 (71.6%), 89 (81.7%), 73 (67.0%), 79 (72.5%), 3 (2.8%), 84 (77.1%), 6 (5.5%), 108 (99.1%), 1 (0.9%), 99 (90.8%), 2 (1.8%), and 0 (0%) isolates possessed *tst*, *sea*, *seb*, *sec*, *hla*, *hnb*, *fnbA*, *fnbB*, *clfA*, *cna*, *icaA*, *agrI*, *agrII*, *agrIII*, and *agrIV*,

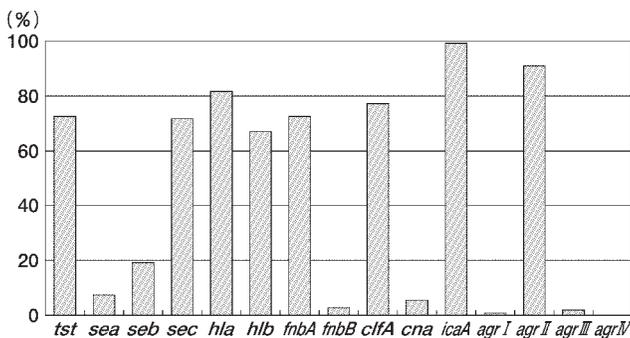


Fig. 1 Percentage of *tst*-, *sea*-, *seb*-, *sec*-, *hla*-, *hnb*-, *fnbA*-, *fnbB*-, *clfA*-, *cna*-, *icaA*-, *agrI*-, *agrII*-, *agrIII*-, and *agrIV*-positive isolates among MRSA isolates.

respectively (Fig. 1). Eight determinants (*tst*, *sec*, *hla*, *hnb*, *fnbA*, *clfA*, *icaA*, and *agrII*) were found to be predominant among these isolates.

Relationship between biofilm formation and several virulence determinants. We evaluated the relationship between biofilm formation and the 6 predominant genes of the MRSA isolates (Table 2). The 6 determinants were as follows: *tst*, encoding the toxic shock syndrome toxin 1; *sec*, encoding the staphylococcal enterotoxin C; *hla*, encoding the α -toxin; *hnb*, encoding the β -toxin; *fnbA*, encoding the fibronectin-binding protein A; and *clfA*, encoding the fibrinogen-binding protein A. The other predominant genes, *icaA* of the intercellular adhesin locus and *agrII* of the accessory gene regulator, were excluded from the evaluation, since more than 90% of the MRSA isolates possessed these genes (Fig. 1). As shown in Table 2, the mean OD₅₇₀ value (mean \pm SD) was significantly higher in *hnb*- and *fnbA*-positive isolates than in *hnb*- and *fnbA*-negative isolates ($P = 0.0034$ and $P = 0.0052$, respectively). The value was also higher in *hla*-positive isolates than *hla*-negative isolates ($P = 0.0836$). The percentage of *hla*-, *hnb*-, and *fnbA*-positive isolates was

Table 2 Relationship between biofilm-forming capacities and several virulence determinants/clinical background

	No. of isolates	OD ₅₇₀ (mean \pm SD)	<i>P</i> value (Mann-Whitney's <i>U</i> test)
Total isolates tested	109	0.24 ± 0.18	
Virulence determinants			
<i>tst</i> -positive	79	0.21 ± 0.29	0.7783
<i>tst</i> -negative	30	0.30 ± 0.46	
<i>sec</i> -positive	78	0.21 ± 0.29	0.6503
<i>sec</i> -negative	31	0.29 ± 0.45	
<i>hla</i> -positive	89	0.26 ± 0.38	0.0836
<i>hla</i> -negative	20	0.12 ± 0.08	
<i>hnb</i> -positive	73	0.29 ± 0.40	0.0034
<i>hnb</i> -negative	36	0.12 ± 0.11	
<i>fnbA</i> -positive	79	0.28 ± 0.39	0.0052
<i>fnbA</i> -negative	30	0.12 ± 0.12	
<i>clfA</i> -positive	84	0.23 ± 0.32	0.8996
<i>clfA</i> -negative	25	0.25 ± 0.43	
Clinical background			
catheter-related	51	0.29 ± 0.39	0.0162
catheter-unrelated	58	0.19 ± 0.30	
polymicrobial	50	0.25 ± 0.40	no significance
monomicrobial	59	0.22 ± 0.30	
febrile	21	0.18 ± 0.15	no significance
non-febrile	88	0.25 ± 0.38	

45.6%, 57.4%, and 50% among those with OD₅₇₀ values of 0 to <0.2, 90.3%, 80.7%, and 83.9% among those with OD₅₇₀ values of ≥0.2 to <0.5, and 100%, 90.3%, and 90% among those with OD₅₇₀ values of ≥0.5, respectively (Fig. 2). The percentage of *hla*- and *fnbA*-positive isolates was significantly higher in the strong biofilm-forming group than in the weak group ($P = 0.012$, $P = 0.020$), and the percentage of *hnb*-positive isolates was higher in the strong biofilm-forming group than in the weak group ($P = 0.079$). We also evaluated the biofilm-forming capacities of MRSA isolates in seven groups based on the presence/absence of *hla*, *hnb*, and *fnbA* genes (Fig. 3). As shown with a box and whisker plot, the MRSA isolates with 3 or 2 combinations of *hla*, *hnb*, and *fnbA* genes had greater capacities for biofilm formation than did those lacking these three genes. The Mann-Whitney's *U* test of 2 mean OD₅₇₀ values (mean ± SD), 0.31 ± 0.43 and 0.15 ± 0.01 , in 62 *hla*-, *hnb*-, *fnbA*-positive and 13 *hla*-, *hnb*-, *fnbA*-negative isolates, respectively, confirmed that MRSA isolates possessing *hla*, *hnb*, and *fnbA* genes together had significantly greater capacities for biofilm formation than did those lacking these 3 genes ($P = 0.0186$).

Relationship between biofilm formation and clinical background. The 109 cases of UTI caused by MRSA consisted of 51 catheter-related (46.8%) and 58 catheter-unrelated cases (53.2%), 50 polymicrobial (45.9%) and 59 monomicrobial cases (54.1%), and 21 febrile (19.3%) and 88 non-febrile cases (80.7%). The biofilm-forming capacities of MRSA isolates from

catheter-related cases were significantly greater than those from catheter-unrelated cases ($P = 0.0162$) (Table 2). As shown in Fig. 4, the percentage of *hla*-, *hnb*-, and *fnbA*-positive isolates was 88.2%, 72.5%, and 76.5%, respectively, among MRSA isolates from catheter-related cases ($n = 51$) and 75.9%, 60.3%, and 70.7%, respectively, among those from catheter-unrelated cases ($n = 58$).

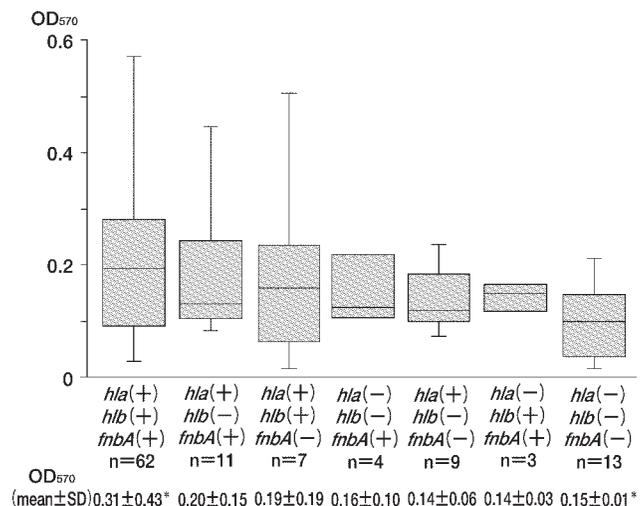


Fig. 3 Biofilm-forming capacities of MRSA isolates in seven groups based on the presence/absence of *hla*, *hnb*, and *fnbA* genes. OD₅₇₀ values of the isolates in seven groups are shown by the box and whiskers plot that is a five-number summary (upper extreme, upper quartile, median, lower quartile, and lower extreme). The mean OD₅₇₀ values (mean ± SD) in seven groups are also shown. * $P = 0.0186$ (Mann-Whitney's *U* test)

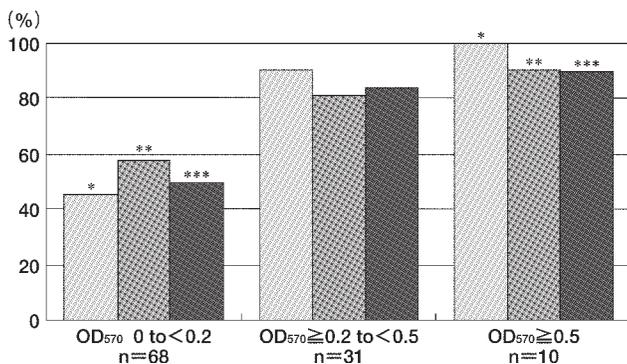


Fig. 2 Percentage of *hla*-, *hnb*-, and *fnbA*-positive isolates among MRSA isolates that belong to the three biofilm-forming groups: OD₅₇₀ 0 to <0.2 (weak biofilm former), OD₅₇₀ ≥0.2 to <0.5 (medium biofilm former), and OD₅₇₀ ≥0.5 (strong biofilm former). Bars: □, *hla*; ▒, *hnb*; ■, *fnbA*. * $P = 0.012$, ** $P = 0.079$, *** $P = 0.020$ (Fisher's exact test)

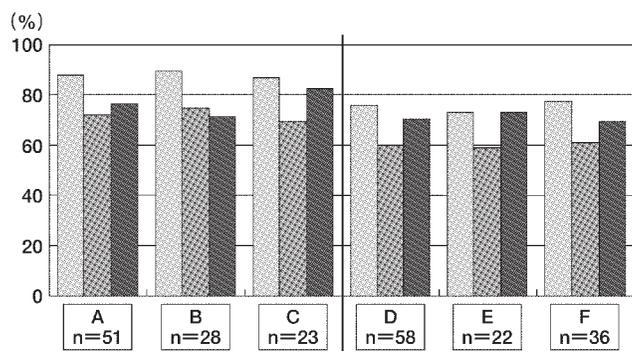


Fig. 4 Percentage of *hla*-, *hnb*-, and *fnbA*-positive isolates among MRSA isolates, from catheter-related cases (A), catheter-related polymicrobial cases (B), catheter-related monomicrobial cases (C), catheter-unrelated cases (D), catheter-unrelated polymicrobial cases (E), and catheter-unrelated monomicrobial cases (F). Bars: □, *hla*; ▒, *hnb*; ■, *fnbA*.

Discussion

On our urology ward, 20% of UTI caused by MRSA are febrile and patients who are asymptomatic are often observed without any intervention [4]. We previously reported that the presence of both the *tst* and *sec* genes may be associated with the incidence of febrile cases of UTI caused by MRSA [4]. MRSA isolated from urine rarely causes serious infectious symptoms, but once this occurs, therapy is difficult. One reason for this is that MRSA forms biofilms in the urinary tract [16]. It is also difficult to eradicate bacteria completely in patients with an indwelling urinary catheter and/or stent. Therefore, it is important to understand biofilm formation and the pathogenicity of MRSA infections in the urinary tract.

Biofilms are surface-associated, sessile bacterial communities. A mature biofilm is formed when planktonic cells initially colonize a surface, aggregate and/or grow into multicellular colonies, and embed themselves in an exopolysaccharide matrix [6, 7]. In general, staphylococcal cells embedded in a biofilm or in microcolonies are much more resistant to antimicrobial agents than are planktonic cells [5]. Many patients with a chronic staphylococcal infection have been treated with various antimicrobial agents, mostly without much success. Genetic analyses of staphylococci have shown that the progression of biofilm development consists of 2 steps: initial cell-to-surface interactions followed by cell-to-cell interactions [5, 11, 17].

S. aureus is especially capable of adhering to a large variety of matrix components to initiate colonization [5]. This adherence is frequently mediated by protein adhesins of the family known as MSCRAMM (microbial surface components recognizing adhesive matrix molecules). The collagen-binding protein, fibronectin-binding proteins, and fibrinogen-binding proteins belong to this family. In this study, we analyzed the presence of four determinants (*fnbA*, *fnbB*, *clfA*, and *cna*) encoding surface proteins FnBPA, FnBPB, ClfA, and Cna, respectively. Of 109 MRSA isolates, 79 (72.5%), 3 (2.8%), 84 (77.1%), and 6 (5.5%) isolates possessed *fnbA*, *fnbB*, *clfA*, and *cna*, respectively. Of the four determinants, *fnbA* and *clfA* genes were predominant in the isolates, and the *fnbA*-positive isolates had significantly greater capacities for biofilm formation than did the *fnbA*-negative isolates ($P = 0.0052$) (Table 2). The percentage of *fnbA*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases

(Fig. 4).

The *ica* locus, which is required for the synthesis of the polysaccharide intracellular adhesin (PIA) of staphylococci, plays a role in cell-to-cell interactions during biofilm formation and is predominantly present in clinical isolates [5]. Our data showed that 108 of 109 (99.1%) of MRSA isolates possessed *icaA*. Only one isolate without the *icaA* gene possessed *tst*, *sec*, *hla*, *hnb*, *fnbA*, and *clfA*, and the biofilm-forming capacity of the *icaA*-negative isolate was weak (OD_{570} value: 0.02). Even though the isolate does not produce PIA, other surface-associated virulence factors may be overexpressed, functionally compensating for the lack of PIA. Other factors, such as the autolysin, the D-alanine esterification of teichoic acids, the accumulation-associated protein, and the like that contribute to biofilm formation were described in a review article [5]. Previously unknown factors, in particular adhesins, which have been identified by whole genome sequencing of MRSA, may also contribute to biofilm formation [18].

Caiazza *et al.* [8] showed that Hla, a 34-kDa protein that causes host cell lysis by heptamerizing upon insertion into eukaryotic cell membranes, plays a role primarily in cell-to-cell interactions during biofilm formation. The level of Hla correlates with the level of biofilm formation. Caiazza *et al.* [8] were initially surprised to find that a secreted toxin had such a dramatic impact on biofilm formation. In this study, we showed that the biofilm-forming capacities of MRSA isolates were higher in *hla*- and *hnb*-positive isolates than in *hla*- and *hnb*-negative isolates, respectively ($P = 0.0034$, $P = 0.0836$). Other examples exist in which secreted toxins and enzymes may play a role in biofilm formation [19, 20, 21, 22]. These toxins encoded by *hla* and *hnb* genes may be bifunctional enzymes and cause tissue damage of urinary epithelium. The percentage of *hla*- and *hnb*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases (Fig. 4).

Quorum sensing via the accessory gene regulator (*agr*) system has been assigned a central role in the pathogenesis of staphylococci, particularly *S. aureus* [10, 11]. The *agr* system regulates a wide array of virulence factors, including those involved in surface-associated virulence and biofilm formation [5, 7, 10, 11]. *S. aureus* strains can be divided into 4 major groups based on *agr* variations [10]. The relationship between *agr* groups and clinical features has been reported [10, 23]; for instance, most menstrual TSS strains

belong to *agr* group III [24], all the strains causing leucocidin-induced necrotizing pneumonia belong to *agr* group III [25], most intermediate-level glycopeptide resistance strains belong to *agr* group II [26], and most exfoliatin-producing strains belong to *agr* group IV [27]. Our data showed that 99 of 109 (90.8%) of the MRSA isolates belonged to *agr* group II. We are unable to assess the relationship between *agr* group II and UTI, since *agr* group II may be predominant in MRSA isolated in Japan, based on a database search [18].

The epidemic of UTI caused by MRSA at the Okayama University Hospital appears to be representative of the changing epidemiology of *S. aureus* throughout Japan [4]. Molecular typing of MRSA isolates by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses revealed no apparent clonality of these isolates in the urology ward over a 10-year period. It is possible that the MRSA in the urology ward originate from other wards in the hospital, other hospitals, or other communities. MRSA isolates that had previously been largely confined to hospitals have recently started emerging in the outside community [28, 29, 30].

In this study, the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases ($P = 0.0162$). Biofilm formation by staphylococci occurs not only with indwelling devices but also in response to any bacterial factor that mediates adherence to components of the extracellular matrix of the host [5]. Peacock *et al.* [15] reported that seven determinants (*fnbA*, *cna*, *sdrE*, *sej*, *eta*, *hlg*, and *ica*) of *S. aureus* were significantly more common in invasive isolates. No single factor predominated as the major predictor of virulence, and their effects appeared to be cumulative. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown. Host factors for *S. aureus* disease are likely to include a genetic predisposition via one or more susceptibility genes and acquired factors such as the presence of intravenous devices, surgical wounds, and other events that perturb normal host defenses.

Taken together, our studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla*, *hly*, and *fnbA* gene products.

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