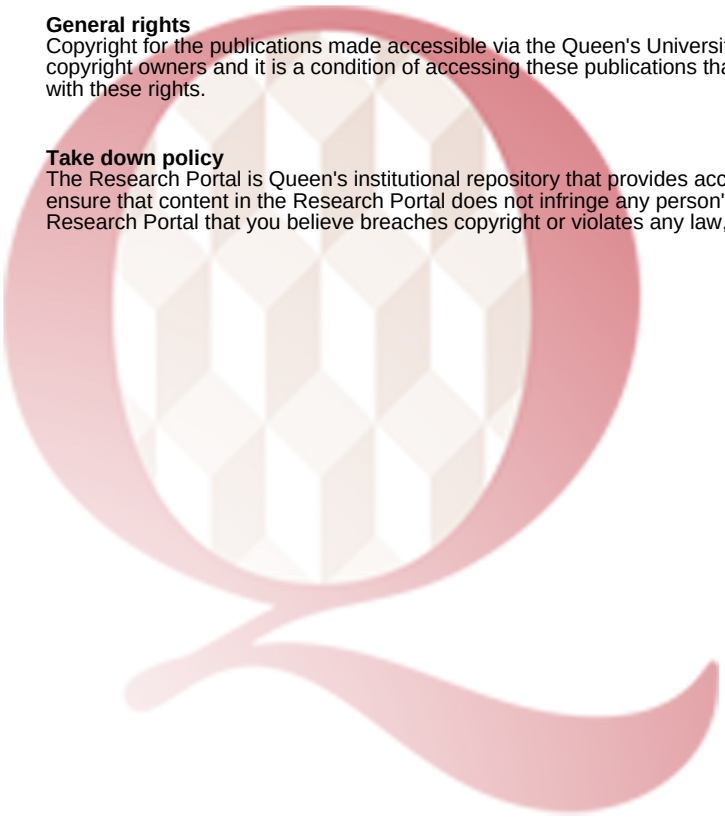
Antibiotic Susceptibility of Planktonic and Biofilm Grown Staphylococci Isolated from Implant Associated Infections: Should MBEC and Nature of Biofilm Formation Replace MIC?



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Abstract:	Purpose: The purpose of this study was to develop an alternative, more clinically relevant approach to susceptibility reporting for implant associated infections. Using 20 staphylococcal isolates, isolated from clinical implant infections, the majority (85%) demonstrated biofilm-forming capabilities. A significantly increased MBEC compared to MIC breakpoint was obtained, with MBEC values greater than 256 µg/mL for the majority of bacteria. Such a vast increase was also demonstrated for isolates defined as negligible biofilm formers via crystal violet staining, likely due to high protein content of biofilms confirmed by proteinase-K treatment. Methodology: This study employed a variety of techniques to assess minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of isolates tested. In addition, the nature of bacterial biofilm across a range of clinical isolates was investigated using crystal violet staining, sodium metaperiodate and proteinase-K treatment and PCR analysis. Results/Key findings: Infection of medical implants is associated with increased rates of infection and increased bacterial tolerance to antibiotic strategies. Clinical significance is due to the presence of pathogens attached to biomaterial surfaces, enclosed in an extracellular polymeric matrix termed the biofilm. This paper highlights the importance of defining the clinical susceptibility of implant associated infections in vitro using methods that are relevant to the biofilm phenotype in vivo, and highlights how current planktonic-based antimicrobial susceptibility tests are often misleading. Conclusion: The use of biofilm-relevant susceptibility tests would improve patient outcomes by enabling correct antimicrobial regimens to be rapidly identified, reducing treatment failure and halting the spread of antimicrobial resistant strains.

Antibiotic Susceptibility of Planktonic and Biofilm Grown Staphylococci Isolated from Implant Associated Infections: Should MBEC and Nature of Biofilm Formation Replace MIC?

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Abstract

Purpose**:** The purpose of this study was to develop an alternative, more clinically relevant approach to susceptibility reporting for implant associated infections. Using 20 staphylococcal isolates, isolated from clinical implant infections, the majority (85%) demonstrated biofilm-forming capabilities. A significantly increased MBEC compared to MIC breakpoint was obtained, with MBEC values greater than 256 µg/mL for the majority of bacteria. Such a vast increase was also demonstrated for isolates defined as negligible biofilm formers *via* crystal violet staining, likely due to high protein content of biofilms confirmed by proteinase-K treatment.

Methodology**:** This study employed a variety of techniques to assess minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of isolates tested. In addition, the nature of bacterial biofilm across a range of clinical isolates was investigated using crystal violet staining, sodium metaperiodate and proteinase-K treatment and PCR analysis.

Results/Key findings: Infection of medical implants is associated with increased rates of infection and increased bacterial tolerance to antibiotic strategies. Clinical significance is due to the presence of pathogens attached to biomaterial surfaces, enclosed in an extracellular polymeric matrix termed the biofilm. This paper highlights the importance of defining the clinical susceptibility of implant associated infections *in vitro* using methods that are relevant to the biofilm phenotype *in vivo*, and highlights how current planktonic-based antimicrobial susceptibility tests are often misleading.

Conclusion: The use of biofilm-relevant susceptibility tests would improve patient outcomes by enabling correct antimicrobial regimens to be rapidly identified, reducing treatment failure and halting the spread of antimicrobial resistant strains.

Keywords: Antibiotic susceptibility; Antibiotic resistance; Biofilms; Biomaterials; Indwelling devices

Introduction

Infection of indwelling medical devices are commonly caused by multi-drug resistant pathogens. The implant surface provides an optimum environment for microbial attachment and growth, with numerous benefits to microorganisms including increased availability of nutrients; likelihood of survival, maturation and potential for symbiotic relationships (1). This results in failure of treatment, increased spread of resistant pathogens, device removal, morbidity and increased mortality (2). A recent UK government report outlined that without significant investment in new therapies, deaths due to infection are predicted to rise to more than 10 million deaths by 2050, a figure greater than cancer (3). Pathogen susceptibility is typically determined by the minimum inhibitory concentration (MIC) as recommended either by the British Society for Antimicrobial Chemotherapy (BSAC) or the Clinical and Laboratory Standards Institute (CLSI) guidelines (4). However, successful treatment of indwelling devices usually requires eradication of the bacterial pathogens growing in a biofilm. Biofilm associated infection is extremely difficult to eradicate and as a result, treatment commonly fails. Therefore, despite their high cost to healthcare and the economy, infections of implants remain unsolved and an ongoing burden (5).

The fundamental function of *in vitro* antimicrobial susceptibility tests (AST) in clinical laboratories is to provide the prescriber with accurate information on appropriate antimicrobial therapy (6). However, although effective against bacteria *in vitro,* it is well established that concentrations of antibiotics used in standard AST are not predictive of the concentrations required to eradicate infections associated with indwelling devices (7,8). In addition, studies have shown that sub-inhibitory concentration of antibiotics can both stimulate and impede biofilm formation further confusing the issue of appropriate treatment (9-11). Therefore, certain studies have suggested that a minimum biofilm eradication concentration (MBEC) would be more indicative of the antibiotic concentration required to eliminate bacteria in biofilm (12).

In addition to determining the MBEC, it would be clinically useful to characterise the degree and nature of the bacterial biofilm and the total biomass present by e.g. crystal violet (CV) staining (13) and treatment with sodium metaperiodate and proteinase-K respectively (14,15). This could, in turn, be used to inform the clinician treating the biofilm infection. For example, protease-directed therapy could be used as a means of eradicating those isolates which have been shown to possess considerable protein-mediated biofilm. Furthermore, using PCR it is also possible to identify those isolates which possess the *ica* operon which codes for production of enzymes required for bacterial adhesion by means of polysaccharide intracellular adhesin (PIA) synthesis.

This study aimed to determine the susceptibility of 20 clinical staphylococcal isolates, cultured from indwelling human devices, to a range of antibacterial agents when grown planktonically and in biofilm. In addition, we sought to determine the degree and nature of biofilm formation of the retrieved isolates and investigate the relationship between degree and mechanism of biofilm formation, possession of the *ica* operon and antibiotic susceptibility with a view to an alternative method of susceptibility reporting.

Materials and Method

Reagents

CV powder, glacial acetic acid, JumpStart® Taq Polymerase, methanol, sodium metaperiodate, proteinase-K, oxacillin, tetracycline and vancomycin were obtained from Sigma-Aldrich (Gillingham, Dorset, United Kingdom). *Ica* primers (16) were obtained from Operon Biotechnologies (Cologne, Germany). DEPC-treated water was obtained from Ambion (Warrington, UK). Müeller Hinton Agar (MHA), Müeller Hinton Broth (MHB) and Tryptone Soya Broth (TSB) were obtained from Oxoid (Basingstoke, UK). E-tests® were obtained from Bio-Stat (Stockport, UK). Benzylpenicillin as Crystapen® was obtained from Britannia Pharmaceuticals Ltd

(Redhill, Surrey, UK). Clindamycin as clindamycin hydrochloride was obtained from Taresh Ltd (Banbridge, Northern Ireland).

Bacterial Isolates

Nine coagulase-negative staphylococci (CoNS) and three meticillin-sensitive *Staphylococcus aureus* (MSSA) clinical isolates (B1-B12) obrained from patient samples were provided by the Microbiology Department, Belfast City Hospital, Belfast Health and Social Care Trust. A further eight clinical CoNS isolates (B24-B71) were cultured following surgical removal of catheters as previously described (17). Details of site of culture are provided in Table 1. Eleven of the twenty total isolates were chosen to determine susceptibility of both CoNS and *S. aureus* isolates growing as biofilm, and isolates cultured from a range of indwelling clinical devices growing as biofilm (Tables 1 and 4). For all isolates tested, a negative control of sterile TSB was included.

Planktonic Susceptibility Testing

Antimicrobial susceptibility of all isolates was determined using the CLSI disk diffusion (DD) method and E-test® strips (18).

Quantification of Biofilm Formation

Bacterial biofilms were grown and classified in sterile Nunc™ 96-well microtitre plates (VWR International, Leicestershire, UK) as previously described (13), using TSB as a growth medium. Sterile TSB was also used as a negative control. Absorbance at 590nm was then measured using a Tecan Sunrise® plate reader (Tecan, Theale, Reading, United Kingdom). ODc was defined as three standard deviations above the mean optical density of the negative control (13).

Biofilm forming ability of the strains was classified as follows:

$OD \leq ODc$	=	non adherent (0)
$ODc < OD \leq 2 \times ODc$	=	weakly adherent (+)
$2 \times ODc < OD \leq 4 \times ODc$	=	moderately adherent (++)
$4 \times ODc < OD$	=	strongly adherent (+++)

Antimicrobial Susceptibility of Bacterial Isolates in Biofilm

Bacterial biofilms were grown in 96-well trays using TSB as previously described (13). Following overnight incubation to allow biofilm growth, the 96-well trays were washed gently with sterile PBS to remove any non-adherent bacteria. Each isolate was then exposed to five antibiotics (penicillin, oxacillin, clindamycin, tetracycline and vancomycin) ranging in doubling concentrations from 4 µg/mL – 256 µg/mL. The specific isolates chosen were to allow comparison of both CoNS and *S. aureus* isolates and isolates cultured from a range of indwelling clinical devices. For all isolates tested, positive and negative controls were included.

Detection of the Mechanism of Biofilm Formation

Bacterial biofilms were grown in 96-well trays using TSB as previously described (13).

Sodium metaperiodate (NaIO4) and proteinase-K have previously been shown to degrade polysaccharide and protein-mediated biofilms respectively (19,20). Specifically, as demonstrated by Wang *et al.* (15), if the polysaccharideb-1, 6-N-acetyl-D-glucosamine mediates biofilm formation, treatment with metaperiodate will result in biofilm dispersal. In contrast, if biofilm formation is protein-mediated, treatment with metaperiodate will have no effect, whereas treatment with proteinase-K will result in biofilm disruption and dispersal.

Following overnight incubation the plates were washed twice with 150µL sterile PBS. Plates were then simultaneously treated as follows:

Plate 1: Following discarding of the supernatant and washing, each well was filled with 200µL 40mM $NaIO_4$ solution and incubated for a further 24 hours at 4°C. After incubation, the $NaIO_4$ solution was discarded and the wells washed thoroughly with PBS. The 96-well plates were dried and stained with CV as described above. Absorbance was then measured at 590nm.

Plate 2: Following discarding of supernatant and washing, each well was filled with 200µL of a proteinase-K solution (1mg/mL in 100 mM tris(hydroxymethyl)aminomethane) and incubated for 4 hours at 37°C. After incubation, the proteinase-K solution was discarded and the wells washed thoroughly with PBS. The 96-well plates were dried and stained with CV as described above. Absorbance was then measured at 590nm.

Detection of *ica* Gene

A polymerase chain reaction (PCR) assay to detect the gene products of the *ica* operon was carried out using conditions as previously described (16) . PCR products of the expected size were visualised using gel electrophoresis and a UV-transilluminator (Gel-Doc, BioRad, Hertfordshire, UK). In addition to the test isolates, the RP62A (ATCC 35984) *S. epidermidis* isolate (known to form biofilms) was used as a reference biofilm-forming organism.

Statistical analysis

Statistical analysis was conducted where appropriate using a one-way analysis of variance (ANOVA). In all analyses, a p value < 0.05 denoted statistical significance. Statistical analysis was performed using the SPSS® software package.

Results

Planktonic Susceptibility Testing

The antibacterial susceptibility of each isolate to the 10 antibiotics routinely used in the Belfast City Hospital Microbiology Department is outlined in the antibiogram (Table 2). The number of isolates sensitive to each antibiotic is also shown in Table 2. Of the 20 isolates tested, two (B1 and B24) were susceptible to all 10 antibiotics according to CLSI breakpoint guidelines. A further nine isolates showed resistance to only one antibiotic and in all of these, the antibiotic was either penicillin or erythromycin. In addition, two of the tested isolates (B37 and B5) were resistant to two and three antibiotics, respectively. Two isolates (B3 and B9) were resistant to five antibiotics while a further five isolates were resistant to six of the 10 tested antibiotics. Of the three *S. aureus* isolates tested, each was susceptible to all antibiotics with the exception of penicillin. As shown in Table 2, vancomycin and teicoplanin proved to be most effective with all isolates demonstrating susceptibility to both antibiotics. Conversely, penicillin was the least effective antibiotic with only six of the 20 isolates (30%) demonstrating susceptibility. In addition, erythromycin also demonstrated limited efficacy with only 8 of the 20 isolates (40%) reported as susceptible.

Quantification of Biofilm Formation

Results of biofilm detection are shown in Table 3. Sixteen (80%) of the isolates tested were biofilm formers. Of these 16, 8 isolates proved to be weakly (+) adherent, two isolates moderately (++) adherent and a further six strongly (+++) adherent. Of the six isolates that demonstrated strongly adherent biofilm formation all possessed the *icaA*, *icaB* and *icaC* operons (Table 5).

Antimicrobial Susceptibility of Bacterial Isolates in Biofilm

The antibacterial susceptibility of 11 of the clinical isolates growing in biofilm to five antibiotics is shown in Table 4. Of 11 isolates tested, 9 were resistant to all five antibiotics at the highest

concentration tested (256 µg/mL). Only isolate B1, with MBEC values of 32 µg/mL and 256 µg/mL for clindamycin and vancomycin respectively, and isolate B11 with an MBEC value of 32 µg/mL for vancomycin displayed any sensitivity when grown in biofilm.

Mechanism of Biofilm Formation

Results of the CV staining after treatment with $NaIO_4$ and proteinase-K are shown in Fig. 1. Statistical analysis of the results using a one-way ANOVA test revealed a significant reduction ($p < 0.05$) in absorbance after treatment with both NaIO4 and proteinase-K, in comparison with untreated biofilm. Of the 20 isolates tested, 11 showed a decrease in absorbance in comparison with untreated biofilm, after treatment with sodium metaperiodate. In addition, 15 of the 20 isolates showed a decrease in absorbance in comparison with untreated biofilm after treatment with proteinase-K.

Detection of *ica* Genes

Following PCR, the resulting amplicons were observed using UV trans-illumination. Bands corresponding to the expected size of *icaA*, *icaB* and *icaC* gene products were observed at 814, 526 and 989 base pairs [Fig. 2(a)-2(c)]. Upon analysis of PCR products, 9 out of 20 isolates tested displayed the *ica*A gene, 9 out of 20 isolates tested displayed the *ica*B gene and 9 out of 20 isolates tested displayed the *icaC* gene. Notably, the *ica*ABC genes detected were all in the same isolates.

Discussion

The colonisation of indwelling medical devices by bacteria growing in biofilm is thought to be the major contributing factor in the pathogenesis of device-related infections (21). This is in part due to the high innate resistance of these organisms to antimicrobial therapy and also due to the further increased resistance to even the highest concentrations of antibiotics when growing as a biofilm,

notably when the organism possesses an *ica* operon (22-26). Given this relationship between bacteria in biofilm and increased resistance to antibiotic therapy, the ability of 20 isolates retrieved from indwelling devices to form biofilms was investigated, together with their resistance to conventional antimicrobial therapy.

It is important to provide information on the MBEC to clinicians for several reasons. Currently lab reports only show antibiotic sensitivity to planktonic bacteria. Therefore to be accurate they could include a caveat indicating that this may not be a true reflection of the situation in the biofilm. Otherwise the lab report is not providing valid information. It could also be a means of educating the clinical team on interpreting lab results, as this should be done with due consideration of the clinical situation.

As expected, the 20 isolates tested demonstrated a scope of planktonic susceptibility to the 10 antibiotics tested; all isolates were susceptible to both glycopeptide antibiotics (vancomycin and teicoplanin). This is similar to previous studies which have also reported 100% susceptibility of Gram-positive isolates to vancomycin when tested planktonically (27). Of the remaining antibiotics, the variation in susceptibility can be explained by inter-species variation. Similar to the findings in this study, erythromycin resistance in staphylococcal species has previously been reported as varying from 0% in *S. lugdunensis* to almost 90% in *S. haemolyticus* (28). In addition to planktonic susceptibility as determined by the DD method, 11 of the retrieved isolates were selected to determine their antimicrobial susceptibility when grown in biofilm. As with other studies, the results reported here confirm that when in biofilm, staphylococcal isolates display resistance to antimicrobial concentrations greater than 10-1000 times greater than that of MIC breakpoints (29,30). It is of clinical significance that the isolates tested in this study were chosen to reflect typical bacterial isolates from different sites and a range of implanted devices. Therefore,

the MBEC results reported in this study demonstrate that this greatly elevated MBEC value, in comparison with established MIC breakpoints, is completely independent of any specific implant or location. Furthermore, the 11 isolates tested also displayed a variation in degree of biofilm formation from non-adherent (-) to strongly adherent (+++). As virtually all the isolates in this study displayed an MBEC of >256 μg/mL, this suggests that, although the previously described CV method (13) classifies four isolates (B2,B4, B10 and B37) as non-biofilm forming, they are able to adhere to an implant surface to some extent and subsequently form a biofilm structure. A possible explanation for this could be that charged CV stain is retained following interaction with negatively charged teichoic acids in polysaccharide-mediated biofilm but to a much lesser extent in protein-mediated biofilm. Furthermore, studies have reported that protein synthesis plays a role in early biofilm formation as well as having a role in interactions with an abiotic surface (31). Therefore, those isolates which reported negligible biofilm could in fact possess a protein-based biofilm structure which displays minimal CV absorbance. A further explanation could be simply due to phenotypic variation of different bacteria in biofilm. Previous studies have reported that the amount of biofilm produced by individual *S. epidermidis* isolates displayed considerable phenotypical variation and that this biofilm was regulated by several factors (19,32-34). Therefore, it is possible that, depending on the degree and constitution of the biofilm, the CV could be physically or chemically prevented from giving an accurate indication of the true extent of biofilm present. In addition, the biofilm could become more saccharide (sugary) in nature in the presence of antibiotics versus a more proteinaceous one when unchallenged.

Several studies have reported that sub-optimal use of certain drugs, such as tetracycline and nafcillin may lead to increased biofilm formation by means of upregulation of certain genes responsible for intracellular adhesion (11,29). As the ability to form a biofilm in microtiter plates has a strong correlation with the ability of *S. epidermidis* to cause disease in a clinical setting it also

is reasonable to assume that these findings would be similar to results encountered *in vivo* and be of clinical significance (35,36). Despite the 20 isolates showing variation in biofilm formation, as determined by CV staining, the MBEC values showed similar levels of resistance. To further investigate this, the mechanism of biofilm formation was investigated. Numerous studies have reported that staphylococcal biofilm formation occurs *via* a cell-surface interaction that is mediated by a number of factors such as surface proteins (37), extracellular proteins (38), PIA (39) and an autolysin encoded by the *atlE* gene (40). Of these factors, it is recognised that, in the majority of isolates, biofilm formation is mediated by production of PIA, synthesised by enzymes encoded by the *ica* operon (41). It is clear from the results of the current study that PIA does play a substantial role in biofilm formation. However, it is equally apparent that a proteinaceous mechanism also plays a fundamental role in biofilm formation of certain isolates. These findings are in agreement with previous studies for both *S. epidermidis* (42,43) and *S. aureus* (44-46). Furthermore, it has also been reported that protein factors are sufficient for biofilm formation in *S. epidermidis* isolates (20). In addition, studies have demonstrated that certain clinical staphylococcal isolates are biofilm positive and *ica* negative (47) while previous studies have reported the presence of extracellular DNA as an important factor for biofilm formation in *P. aeruginosa*, *Streptococcus intermedius* and *Streptococcus mutans* (48,49). In those isolates (e.g. B51 and B71) that displayed absorption (A_{590}) after both proteinase-K and $NaIO_4$ treatment it may be the case that, as previously reported, extracellular DNA may contribute to the biofilm formation (47). To date, numerous studies have reported the importance of PIA, synthesised by *icaADBC*-encoded proteins, in staphylococcal biofilm formation. Furthermore, recent studies have found a strong correlation between presence of the *ica* operon and strong to intermediate biofilm formation (26). It is therefore unsurprising that of the six isolates that demonstrated strongly adherent biofilm formation all possessed the *icaA*, *icaB* and *icaC* operons, confirming the suggestion that *icaADBC* is widespread in clinically significant *S. epidermidis* isolates (16,20,29). In addition to those isolates which were positive for

the *icaA*, *icaB* and *icaC* operons there are also isolates present which, although negative for these operons did produce a detectable biofilm. Although unusual, this phenotype has been previously reported, notably in a nosocomial setting (47). Under antibiotic selective pressure, normal biofilm negative/*ica* negative isolates are able to develop the biofilm positive/*ica* negative phenotype which subsequently is more resistant to vancomycin than biofilm positive/*ica* positive isolates. Conversely, isolates B9 and B11 are *ica* positive, yet only form weak biofilms. In these instances it is possible that, although the *ica* gene is present, for some unknown reason it is not fully expressed.

The highly resistant nature of the isolates in biofilm tested in this study indicate that all clinical isolates associated with indwelling devices, even in the absence of the *ica* gene, are still able to form some sort of adherent structure which resists conventional antimicrobial therapy.

Conclusion

Although limited to 20 clinical staphylococcal isolates, the results of this study clearly indicate that although these isolates demonstrated MIC susceptibility when exposed to antibiotic therapy, they displayed a much higher MBEC when grown in biofilm. As biofilms are related to the majority of infectious diseases (50) and are recognised as playing a fundamental role in infections associated with indwelling devices, it may be necessary to employ the MBEC as a clinical breakpoint when treating certain biofilm infections as opposed to current MIC breakpoints.

Although MBEC tests would incur additional laboratory costs and would be prohibitive on processing all central venous catheter (CVC) samples it could be offered on a basis of clinical need where it was imperative to keep the CVC *in situ*. This may occur when a patient is seriously ill and

there is no other means of vascular access. It may also aid clinical decision making to either stop toxic antibiotics or extend the range of antibiotics tested for the MBEC. In addition, biofilm may be present while the patient is asymptotic. However, the patient may be starting chemotherapy which will compromise their immunity and if the MBEC is known it could direct the empirical antibiotic therapy when infection occurs in the agranulocytosis phase.

By developing a technique to include degree of biofilm formation in conjunction with mechanism of biofilm formation, it may be possible to tailor regimens, such as protease therapy, for difficult to eradicate biofilm-mediated infections. This would serve to more closely replicate clinical infection *in vitro*, improving: the ability to diagnose the presence and nature of biofilm infection; the validity of antibiotic(s) prescribed; clinical outcomes and reducing the threat of antimicrobial resistance.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Table 1 Sites from which clinical isolates were cultured, including abbreviations.

Isolate	Abbreviation	Location of isolation
B1, B24, B37, B48, B49, B51, B63, B64, B71	CAPD	Continuous Ambulatory Peritoneal Dialysis Catheter or Fluid
B2, B3, B5, B6, B8, B9	CLT	Central Line Tip
B4	PCT	PermCath Tip
B7	ELT	Epidural Line Tip
B10	JLT	Jugular Line Tip
B11	VT	Venflon Tip
B12	FLT	Femoral Line Tip

Table 2 Antibiogram showing antibiotic susceptibility data for all isolates

Isolate	Organism	Penicillin	Oxacillin	Erythromycin	Clindamycin	Fusidic Acid	Tetracycline	Gentamicin	Vancomycin	Teicoplanin	Ciprofloxacin
B1	CoNS	S	S	S	S	S	S	S	S	S	S
B2	*S.aureus*	**R**	S	S	S	S	S	S	S	S	S
B3	CoNS	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S
B4	*S.aureus*	**R**	S	S	S	S	S	S	S	S	S
B5	CoNS	**R**	**R**	**R**	S	S	S	S	S	S	S
B6	CoNS	**R**	**R**	**R**	**R**	**R**	S	S	S	S	**R**
B7	CoNS	**R**	**R**	**R**	**R**	**R**	S	S	S	S	**R**
B8	CoNS	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S
B9	CoNS	**R**	**R**	**R**	**R**	S	S	**R**	S	S	S
B10	*S.aureus*	**R**	S	S	S	S	S	S	S	S	S
B11	CoNS	**R**	**R**	**R**	**R**	**R**	S	S	S	S	**R**
B12	CoNS	**R**	**R**	**R**	**R**	S	S	**R**	S	S	**R**
B24	CoNS	S	S	S	S	S	S	S	S	S	S
B37	CoNS	**R**	S	S	S	**R**	S	S	S	S	S
B48	CoNS	**R**	S	S	S	S	S	S	S	S	S
B49	CoNS	S	S	**R**	S	S	S	S	S	S	S
B51	CoNS	S	S	**R**	S	S	S	S	S	S	S
B63	CoNS	**R**	S	S	S	S	S	S	S	S	S
B64	CoNS	S	S	**R**	S	S	S	S	S	S	S
B71	CoNS	S	S	**R**	S	S	S	S	S	S	S

Table 3 Source of isolate tested and degree of biofilm formation based on classification described by Stepanovic *et al.* (2000). $OD_c = 0.255$

Isolate	Organism	Source	Biofilm Formation	Absorbance (A590)
B1	CoNS	CAPD	+	0.340
B2	*S. aureus*	CLT	-	0.220
B3	CoNS	CLT	++	0.775
B4	*S. aureus*	PCT	-	0.211
B5	CoNS	CLT	+	0.453
B6	CoNS	CLT	+++	2.762
B7	CoNS	ELT	+	0.284
B8	CoNS	CLT	+	0.308
B9	CoNS	CLT	+	0.272
B10	*S. aureus*	JLT	-	0.164
B11	CoNS	VT	+	0.261
B12	CoNS	FLT	+	0.269
B24	CoNS	CAPD	+++	1.956
B37	CoNS	CAPD	-	0.230
B48	CoNS	CAPD	+	0.344
B49	CoNS	CAPD	+++	2.169
B51	CoNS	CAPD	+++	2.440
B63	CoNS	CAPD	++	0.659
B64	CoNS	CAPD	+++	1.242
B71	CoNS	CAPD	+++	3.720

Table 4 MBEC (µg/mL) of bacterial isolates in biofilm

Isolate	Antibiotic				
	Penicillin	**Oxacillin**	**Clindamycin**	**Tetracycline**	**Vancomycin**
B1	>256	>256	32	>256	256
B2	>256	>256	>256	>256	>256
B4	>256	>256	>256	>256	>256
B6	>256	>256	>256	>256	>256
B10	>256	>256	>256	>256	>256
B11	>256	>256	>256	>256	32
B24	>256	>256	>256	>256	>256
B37	>256	>256	>256	>256	>256
B48	>256	>256	>256	>256	>256
B51	>256	>256	>256	>256	>256
B71	>256	>256	>256	>256	>256

Table 5 Key of isolates in Figures 4a-4c

Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Isolate	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
Ica operon	✗	✗	✗	✗	✗	✓	✗	✗	✓	✗	✓
Number	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Isolate	B12	B24	B37	B48	B49	B51	B63	B64	B71	+ve	-ve
Ica operon	✗	✓	✗	✗	✓	✓	✓	✓	✓	✓	✗

Figure 1

Click here to download Figure Mechanism of biofilm formation as determined by CV staining.docx

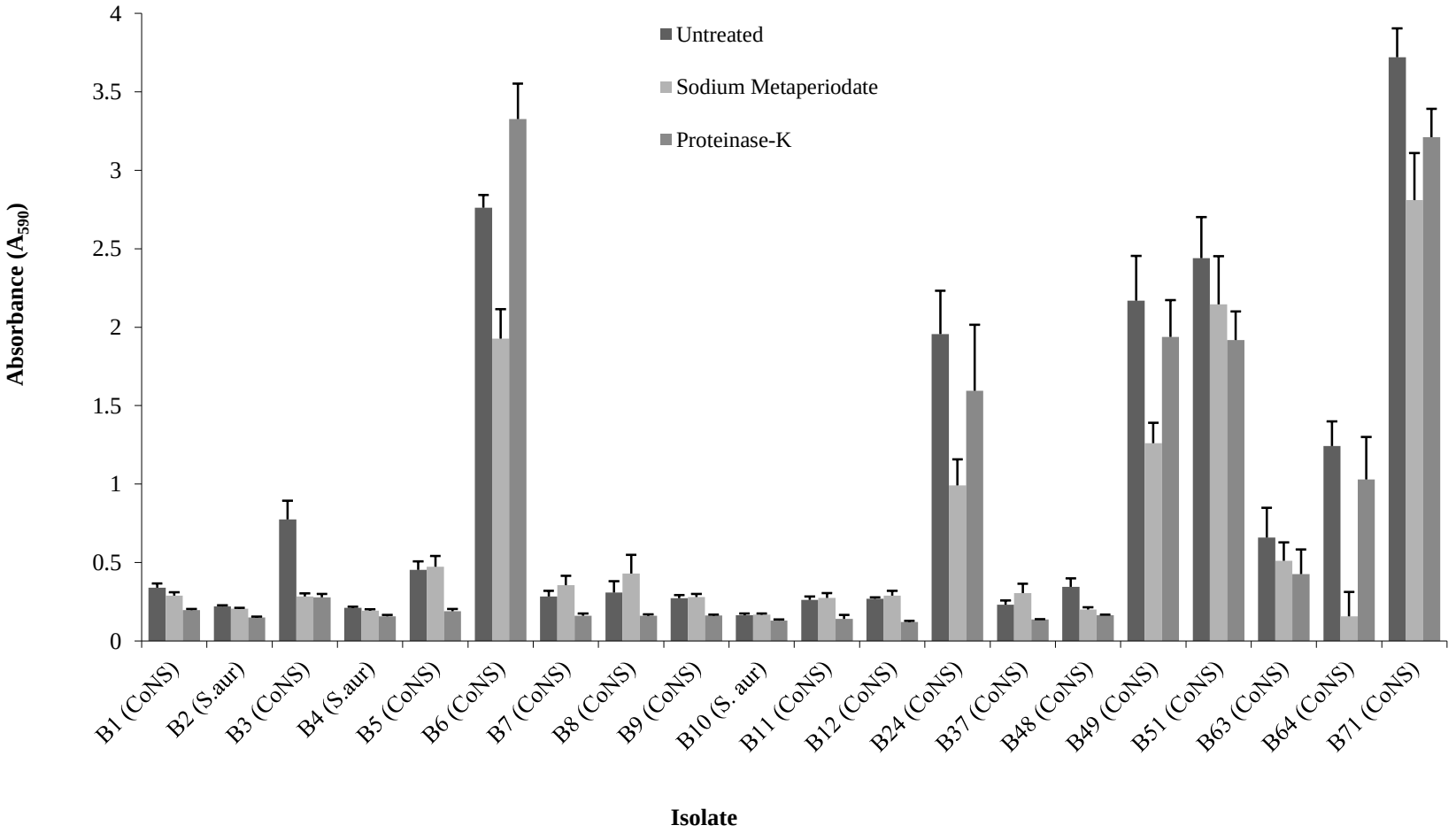

Figure 2

Click here to download Figure (a) icaA, (b) icaB and (c) icaC genes in isolates B1-B71.docx

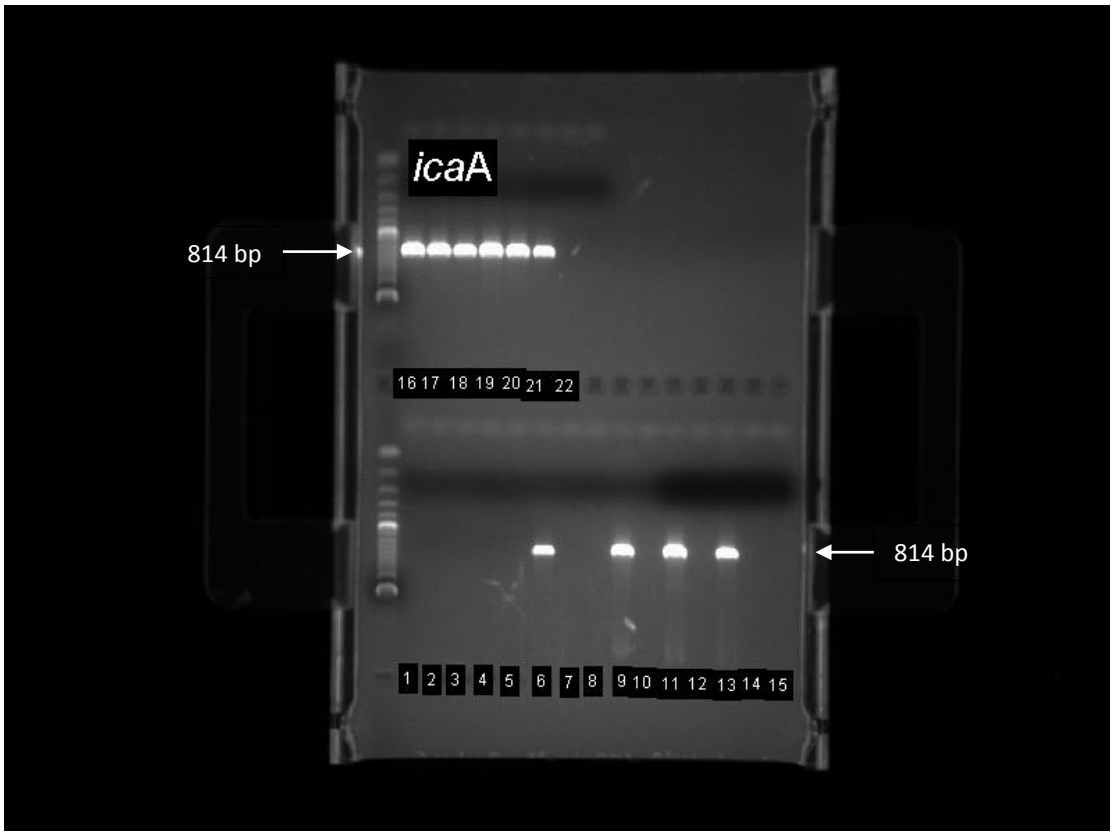

Figure 2 (a)

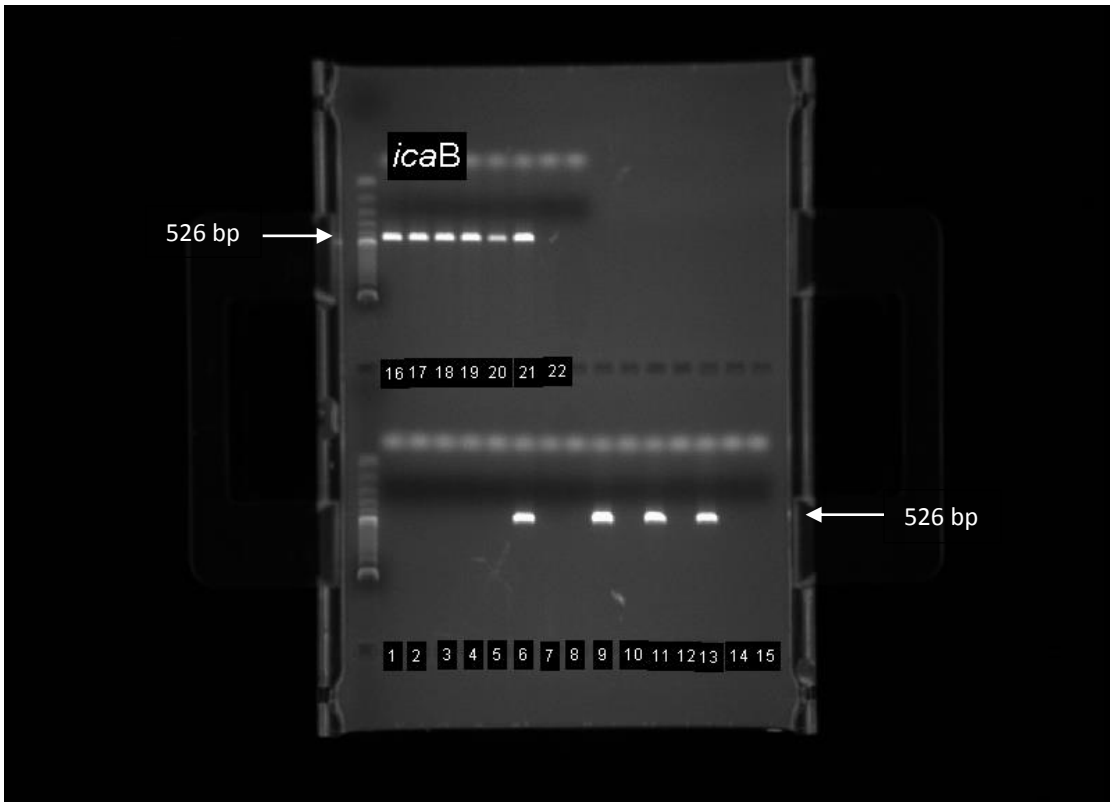

Figure 2b

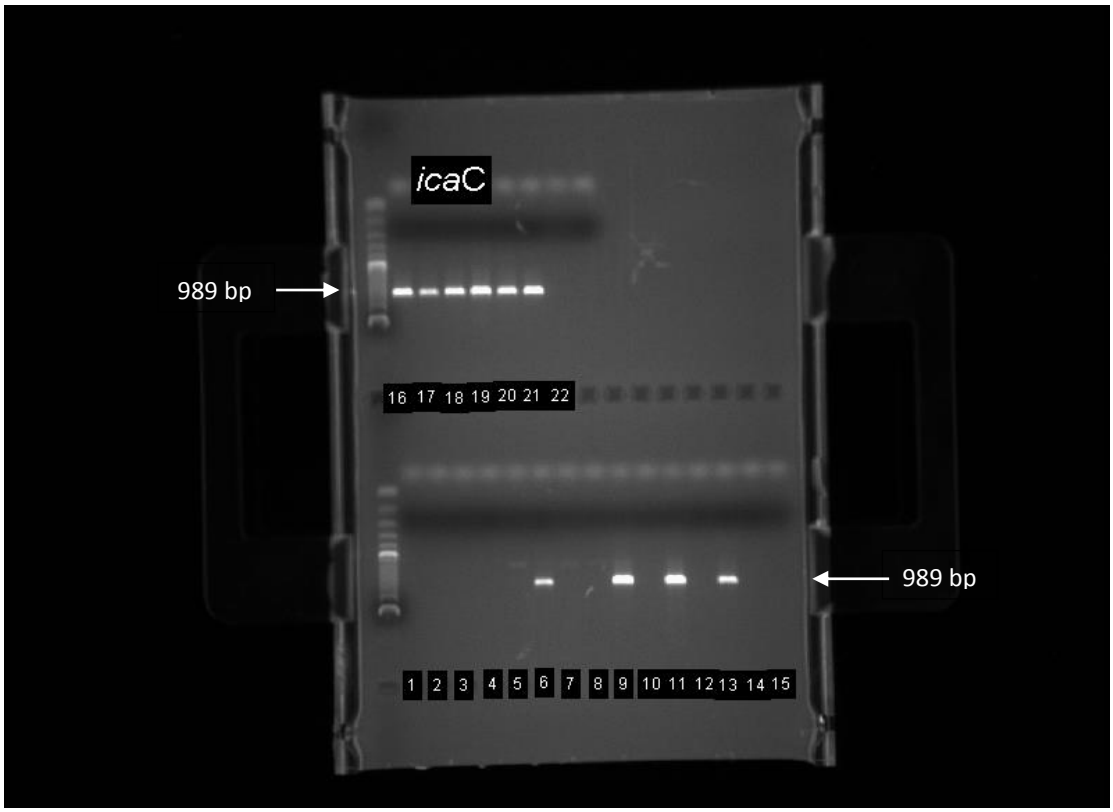

Figure 2c