1-7-2008

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Intended category Research note

Biofilm and the role of the *ica* operon and *aap* in *Staphylococcus epidermidis* isolates causing neurosurgical meningitis.

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Key words: meningitis; neurosurgery; *Staphylococcus epidermidis*; biofilm; *ica* operon

Running title "Biofilm and *Staphylococcus epidermidis* meningitis"

Abstract

We characterised 55 *Staphylococcus epidermidis* isolates from extraventircular devices (EVD) and non-EVD cerebrospinal fluid specimens. Isolates were classified as contaminants or causing device-related meningitis. We used PCR to detect the *ica* operon and *aap,* known determinants of polysaccharide- and protein-mediated biofilm. 33 of 42 (78.6%) meningitis isolates were positive for *ica* and *aap*;five of 13(38.5%) contaminants were *ica* and *aap* negative. 71.4% of meningitis isolates and 84.6% of contaminants produced biofilm. $ica^{+}aap^{+}$ meningitis isolates produced more biofilm than $ica^{+}aap^{-}$ isolates (P =0.0020). $ica^{+}aap^{-}$ isolates did not produce more biofilm than $ica^{-}aap^{+}$ isolates (P =0.4368). *ica* and *aap* are associated with biofilm in isolates of *S. epidermidis* causing meningitis.

Recent decades have seen advances in our understanding of the mechanisms of adhesion and biofilm production in staphylococcal device-associated infection [1, 2]. In *Staphylococcus epidermidis* the *icaADBC* operon encodes polysaccharide intercellular adhesin (PIA) and represents an important biofilm determinant [3].

Devices are commonly used in neurosurgical procedures, e.g. to drain excess cerebrospinal fluid (CSF) and *S. epidermidis* cause infections associated with neurosurgical devices [4]. Recent studies have illustrated the complex nature of the biofilm on CSF shunts [4, 5]. The genetic determinants of biofilm formation include the presence of the *icaADBC* operon, which codes for polysaccharide intercellular adhesion

(PIA). Other factors such as autolysins, accumulation-associated protein (e.g. AAP) encoded by *aap* and teichoic acids, are also important [6, 7, 8].

We assessed biofilm formation and the presence of the *ica* and *aap* loci in *S. epidermidis* isolates from CSF causing meningitis, and we compared these with CSF contaminants.

From 46 patients, 62 Coagulase-negative staphylococcus (CoNS) isolates were collected from CSF specimens between February 2004 and May 2006. Isolates were grouped in to two categories; (1) isolates from patients with an extraventricular device or drain (EVD), (2) non-EVD isolates e.g. isolates from CSF taken *via* lumbar puncture. Strain CSF41498 was used as an *ica* PCR and biofilm assay positive control. *S. epidermidis* ATCC 35984(RP62A) was used as an *aap* PCR positive control. All isolates were identified as *S. epidermidis* biochemically, using the API® Staph identification system, and by PCR as described previously [9]. The primer set ICAR1 and ICAC1 was used in a PCR assay to screen isolates for the presence of the *ica* operon, while the methods of Vandecasteele *et al*. (2003) [10] were used to screen isolates for the presence of *aap*.

Two methods were used to detect the biofilm; a spectrophotometric assay based on the techniques of Christensen G.D *et al* (1985) [11]. Biofilm development was also examined under conditions that induce biofilm production *in vitro* by supplementing the media with 4% ethanol and 4% salt (biofilm inducing conditions). The second method involved the assessment of slime production using Congo red agar (CRA), as described previously [12]. Pulsed field gel electrophoresis was used to examine the clonal variability amongst the isolates according to the criteria outlined by Tenover *et al*.(1995) [13].

Definite CoNS meningitis was defined according to the criteria outlined by Huang C.-R *et al* (2005) [14]. Contamination was defined as the isolation of CoNS but in the absence of symptoms or evidence of inflammation on biochemical and cellular analysis. Probable and possible meningitis were defined by a combination of the criteria used to determine definite meningitis and contamination [14].

Mean biofilm ODs and standard deviations were calculated using Microsoft Office Excel 2003. One-tailed student t-tests were preformed using GraphPad Prism 4 statistical analysis programme. Biofilm production was deemed significantly different when $P \leq 0.05$

PCR identified 55 isolates as *S. epidermidis*: the 46 patients had a mean age of 42 years (1 month-80 yr). Two patients had two episodes of infection. The underlying conditions included intracranial aneurysm/haemorrhage (17 cases), tumour (13 cases) and CSF leaks (5 cases).

PCR for the presence of the *icaADBC* and *aap* genes placed each isolate in to four genotypic groups; $ica^{+}aap^{+}$; $ica^{+}aap^{-}$; $ica^{-}\ aap^{+}$; $ica^{-}\ aap^{-}$(Table 1). The *ica* operon alone or with *aap* was common in the majority of meningitis isolates, 33/42 (78.6%), *aap* alone or with *ica* was less common, 20/42 (47.6%). Five of 13 (38.5%) contaminants were $ica^{-}\ aap^{-}$ but *ica* and *aap* either alone or together was nearly as common amongst these strains. $ica^{+}aap^{+}$ meningitis isolates produced more biofilm than $ica^{+}aap^{-}$ isolates under standard growth conditions ($P = 0.0020$). Interestingly, $ica^{+}aap^{-}$ isolates did not produce higher levels of biofilm than $ica^{-}aap^{+}$ isolatese ($P = 0.4368$). Biofilm amongst the

contaminants was less dependent on the *ica*$^{+}$ *aap*$^{+}$ genotypes; four (30.7%) formed some biofilm, despite being *ica*$^{-}$*aap*$^{-}$.

There was good correlation between CRA colony phenotypes and biofilm production under standard growth conditions, especially for *ica*$^{+}$*aap*$^{+}$ isolates, which produced black crusty colonies and strong biofilm. *ica*$^{+}$*aap*$^{-}$ isolates were weak biofilm producers and were of the red and smooth morphotype.

Forty-two isolates were associated with meningitis (Table 2). Under normal laboratory conditions, 13 (30.9%) meningitis isolates formed a strong biofilm. The dominant genotype of meningitis isolates was *ica*$^{+}$*aap*$^{+}$; two isolates were *ica*$^{-}$*aap*$^{+}$. Biofilm formation was absent in the remaining 11 (26.2%) meningitis isolates. The majority of the remaining biofilm-negative isolates were either *ica*$^{+}$*aap*$^{-}$ (six isolates) or *ica*$^{-}$*aap*$^{-}$ (three isolates), but there were two *ica*$^{+}$*aap*$^{+}$ isolates were detected (Isolates BM30 and BM43 Table 2). When re-examined under inducing conditions, these two isolates produced biofilm.

Of the contaminating isolates, six formed a strong biofilm, five formed a weak biofilm, and two were biofilm negative; ten (76.9%) contaminants were obtained from non-EVD CSF samples, suggesting the skin as a likely source.

We attempted to correlate the presence biofilm, the genes known to code for biofilm and clinical parameters in isolates from patients with and without devices *in-situ* and with and

without evidence of neurosurgical meningitis. Most research to date in this area has focused on central line catheter infections; little work has been done on neurosurgical infections. The *ica* operon alone or with *aap* was present in the majority of *S. epidermidis* meningitis isolates. Interestingly, a higher proportion of contaminants (84.6%) produced biofilm compared with isolates causing meningitis (71.4%). When biofilm production occurred, it seemed to be less dependent on *ica* and *aap* amongst contaminats. Nonetheless, there was overlap between the production of biofilm and an *ica*$^+$ *aap*$^+$ genotype in meningitis and contaminating isolates.

To exclude the possibility that we were investigating a few clonal strains, PFGE was performed. Nineteen (34.5%) isolates belonged to a single clonal group (group 1), and six (10.9%) belonged to a second clonal group (group 2), the remaining 30 (54.5%) isolates were not clonal.

Limitations in our study included the somewhat arbitrary categorisation of isolates as meningitis-causing or contaminants, the absence of a control group of isolates, e.g. carriage isolates from neurosurgical patients or from a healthy population, and also the failure to consider other potential mechanisms for biofilm formation. We used CRA as an indicator of PIA production and correlated this with *ica*-dependent biofilm production. Ziebuhr *et al*. (1997) [15] in a study of *ica* and phase variation in *S. epidermidis* blood culture and mucosal isolates associated the presence of the *ica* with the formation of black-crusty colonies on CRA, while adhesin-negative isolates had red-smooth colonies. Although several other studies [16, 17, 18, 19] have used CRA as a marker of slime

production and *ica*-dependent biofilm formation, direct detection of PIA would be a better method of correlating adhesin production and biofilm formation.

Stress-inducing growth conditions and sub-inhibitory tetracycline increased the number of *ica* positive, biofilm positive, isolates in a previous study of isolates from our intensive care unit [20]. Biofilm formation may be variably expressed amongst clinical isolates due to phase variation. Two *S. aureus* and one *S. epidermidis* isolates, from intravenous catheters (*icaA* and *icaD* positive), exhibited variance on CRA [17]. Vandecasteele and colleagues assessed the presence of *ica*, *aap* and *atlE* genes and found that the prevalence of *atlE* was similar between colonizing and contaminaning isolates [10].

The anatomical locality and physiological milieu from which isolates are recovered may affect the expression of biofilm. The *ica* gene cluster was detected in 60% of conjunctival sack isolates compared with 15% of facial skin isolates [19]. Only 27% of urinary isolates produced biofilm; five of seven *ica*-positive biofilm negative isolates produced biofilm under external stress, indicating that *icaADBC* expression was down regulated [21].

Bateman *et al* [22] described the ability of a conserved protein domain consisting of five repeating glycine residues, termed the G5 domain, to bind *N*-acetylglucosamine. *S. epidermidis* has proteins that have G5 domains, one of which is AAP [6, 22]. The presence of AAP may enhance biofilm production by binding PIA, increasing intercellular adhesion at the cell surface, facilitating the accumulation of mature biofilm.

Although biofilm is relatively common amongst isolates causing device-associated meningitis, genes other than *ica* and *aap* are likely to be involved. Changes in the physiology of the CSF together with cellular factors may be significanct in biofilm production. Further research on the mechanism of device-associated meningitis involving biofilm, may indicate new approaches by prevention and treatment.

Word count 1,358 (should be1,000, but we would probably get away with 1,100) (is now 1612)

This work was funded by a Health Research Board (Ireland) Project Grant. We are grateful to Dr Eoghan O' Neill and the Microbiology diagnostic laboratory at Beaumont hospital for their medical and scientific advice during this research. Hilary Humphreys has received research support from 3M and Steris.

Table 1. *ica* and *aap* genotypes of CSF *S. epidermidis* isolates

Isolate Group (No.)	Genotype (No of Biofilm +ve)[a]			
	ica^{+}*aap*$^{+}$	*ica*$^{+}$*aap*$^{-}$	*ica*$^{-}$*aap*$^{+}$	*ica*$^{-}$*aap*$^{-}$
Meningitis-causing[b] (42)	14 (12)	19 (13)	6(3)	3 (1)
Contaminants (13)	2 (2)	2 (2)	4 (3)	5 (4)

[a] isolates were deemed biofilm positive if OD value was greater than 0.12

[b] these isolates were considered to have caused definite, probable or possible meningitis

Table 2. Relationship between clinical significance and biofilm phenotype of 55 *S. epidermidis* isolates recovered from the CSF of neurosurgical patients

S. epidermidis isolate	Source[a]	No. of Positive cultures	Clinical significance[b]	Genotype	Phenotype		Clonal group[e]
					CRA[c]	Biofilm OD492nm[d](±SD)	1
BM2	EVD	2	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.13 (0.01)	3
BM3	EVD	3	Definite	*ica*$^{-}$*aap*$^{+}$	+	0.30 (0.04)	3
BM4	EVD	1	Probable	*ica*$^{-}$*aap*$^{+}$	+	0.10 (0.03)	3
BM5	EVD	2	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.15 (0.02)	1
BM6	EVD	1	Possible	*ica*$^{+}$*aap*$^{+}$	+++	0.70 (0.37)	3
BM7	EVD	2	Probable	*ica*$^{-}$*aap*$^{+}$	+	0.19 (0.08)	3
BM8	EVD	1	Probable	*ica*$^{-}$*aap*$^{+}$	+	0.09 (0.01)	3
BM10	EVD	3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.12 (0.02)	1
BM11	EVD	3	Definite	*ica*$^{+}$*aap*$^{+}$	++	0.28 (0.16)	2
BM12	EVD	1	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.14 (0.01)	1
BM13	EVD	2	Probable	*ica*$^{+}$*aap*$^{+}$	+++	1.83 (0.49)	3
BM14	Other	2	Probable	*ica*$^{+}$*aap*$^{+}$	++	0.31 (0.06)	3
BM15	Other	2	Probable	*ica*$^{+}$*aap*$^{+}$	++	0.34 (0.34)	3
BM16	EVD	1	Contaminant	*ica*$^{+}$*aap*$^{-}$	+	0.26 (0.05)	3
BM17	Other	1	Contaminant	*ica*$^{-}$*aap*$^{+}$	+	0.27 (0.02)	3
BM18	LP	1	Contaminant	*ica*$^{-}$*aap*$^{-}$	+	0.15 (0.02)	3
BM21	EVD	1	Contaminant	*ica*$^{-}$*aap*$^{+}$	+	0.15 (0.26)	3
BM22	Other	1	Contaminant	*ica*$^{+}$*aap*$^{+}$	+++	1.26 (0.21)	3
BM25	Other	1	Contaminant	*ica*$^{-}$*aap*$^{-}$	+	0.17 (0.03)	3
BM26	Other	1	Probable	*ica*$^{-}$*aap*$^{+}$	+	0.31 (0.03)	3
BM27	EVD	2	Probable	*ica*$^{+}$*aap*$^{+}$	+	0.16 (0.03)	2
BM28	EVD	2	Probable	*ica*$^{+}$*aap*$^{+}$	+++	0.31 (0.06)	2
BM29	Other	1	Contaminant	*ica*$^{-}$*aap*$^{+}$	+	0.33 (0.04)	3
BM30	EVD	>3	Definite	*ica*$^{+}$*aap*$^{+}$	+	0.04 (0.06)	3

BM31	EVD	>3	Definite	*ica*$^{+}$*aap*$^{+}$	++	0.41 (0.09)	2
BM32	EVD	>3	Definite	*ica*$^{+}$*aap*$^{+}$	+	0.15 (0.07)	3
BM33	Other	1	Definite	*ica*$^{+}$*aap*$^{+}$	+++	0.85 (0.11)	3
BM34	EVD	2	Definite	*ica*$^{+}$*aap*$^{+}$	+++	0.52 (0.08)	3
BM35	EVD	1	Contaminant	*ica*$^{-}$*aap*$^{+}$	+	0.31 (0.04)	3
BM36	EVD	2	Probable	*ica*$^{-}$*aap*$^{+}$	+	0.07 (0.01)	3
BM37	EVD	1	Probable	*ica*$^{+}$*aap*$^{+}$	+++	0.76 (0.17)	3
BM39	LP	1	Contaminant	*ica*$^{+}$*aap*$^{+}$	+	0.17 (0.04)	3
BM40	EVD	2	Possible	*ica*$^{+}$*aap*$^{-}$	+	0.23 (0.07)	2
BM41	Other	2	Possible	*ica*$^{-}$*aap*$^{-}$	+	0.10 (0.03)	3
BM42	EVD	3	Probable	*ica*$^{+}$*aap*$^{-}$	+	0.15 (0.04)	1
BM43	EVD	3	Probable	*ica*$^{+}$*aap*$^{+}$	+	0.10 (0.02)	3
BM44	Other	1	Contaminant	*ica*$^{-}$*aap*$^{-}$	+	0.06 (0.01)	3
BM45	EVD	3	Probable	*ica*$^{+}$*aap*$^{-}$	+	0.08 (0.02)	1
BM46	LP	1	Probable	*ica*$^{+}$*aap*$^{-}$	++	0.62 (0.16)	3
BM47	EVD	1	Possible	*ica*$^{-}$*aap*$^{-}$	+++	0.13 (0.03)	3
BM48	Other	1	Contaminant	*ica*$^{-}$*aap*$^{-}$	+	0.06 (0.01)	3
BM49	EVD	2	Possible	*ica*$^{-}$*aap*$^{-}$	+	0.19 (0.02)	3
BM50	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.19 (0.03)	1
BM51	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.12 (0.05)	1
BM52	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.19 (0.08)	1
BM53	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.12 (0.01)	1
BM54	Other	1	Contaminant	*ica*$^{-}$*aap*$^{-}$	+	0.27 (0.09)	3
BM55	EVD	1	Possible	*ica*$^{+}$*aap*$^{-}$	+	0.11 (0.01)	1
BM56	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.11 (0.01)	1
BM57	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.13 (0.01)	1
BM58	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.14 (0.02)	1
BM59	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.14 (0.02)	1
BM60	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.13 (0.02)	1

BM61	LP	1	Contaminant	*ica*$^{+}$*aap*$^{-}$	+	0.18 (0.02)	3
BM62	EVD	>3	Definite	*ica*$^{+}$*aap*$^{-}$	+	0.16 (0.02)	1
CSF41498			Control	*ica*$^{+}$*aap*$^{+}$	+++	0.75 (0.10)	3

[a] EVD (Extraventricular device or drain); LP (Lumbar puncture)

[b] Definite, probable or possible refers to the likelihood of meningitis

[c] On Congo red agar, + indicates red smooth circular colony phenotype, ++ indicates intermediate colony phenotype, +++ indicates black crusty filamentous colony phenotype.

[d] Biofilm OD value from growth under standard conditions.

[e] isolates were placed into the following groups on the basis of PFGE banding patterns; (1) major clonal group 1, (2) major clonal group 2, (3) variant clonal group were isolates could not be placed in either major clonal group 1 or 2.

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CMI-HHChanges(14-11-07)