

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

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

5

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22

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24 **Abstract**

25 We characterised 55 *Staphylococcus epidermidis* isolates from extraventricular devices
26 (EVD) and non-EVD cerebrospinal fluid specimens. Isolates were classified as
27 contaminants or causing device-related meningitis. We used PCR to detect the *ica* operon
28 and *aap*, known determinants of polysaccharide- and protein-mediated biofilm. 33 of 42
29 (78.6%) meningitis isolates were positive for *ica* and *aap*; five of 13 (38.5%) contaminants
30 were *ica* and *aap* negative. 71.4% of meningitis isolates and 84.6% of contaminants
31 produced biofilm. ica^+aap^+ meningitis isolates produced more biofilm than ica^+aap^-
32 isolates ($P = 0.0020$). ica^+aap^- isolates did not produce more biofilm than ica^-aap^+
33 isolates ($P = 0.4368$). *ica* and *aap* are associated with biofilm in isolates of *S.*
34 *epidermidis* causing meningitis.

35

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

40

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

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

48

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

51

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

62

63 Two methods were used to detect the biofilm; a spectrophotometric assay based on the
64 techniques of Christensen G.D *et al* (1985) [11]. Biofilm development was also examined
65 under conditions that induce biofilm production *in vitro* by supplementing the media with
66 4% ethanol and 4% salt (biofilm inducing conditions). The second method involved the
67 assessment of slime production using Congo red agar (CRA), as described previously
68 [12]. Pulsed field gel electrophoresis was used to examine the clonal variability amongst
69 the isolates according to the criteria outlined by Tenover *et al.*(1995) [13].

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

75

76 Mean biofilm ODs and standard deviations were calculated using Microsoft Office Excel
77 2003. One-tailed student t-tests were performed using GraphPad Prism 4 statistical
78 analysis programme. Biofilm production was deemed significantly different when $P \leq$
79 0.05

80

81 PCR identified 55 isolates as *S. epidermidis*: the 46 patients had a mean age of 42 years
82 (1 month-80 yr). Two patients had two episodes of infection. The underlying conditions
83 included intracranial aneurysm/haemorrhage (17 cases), tumour (13 cases) and CSF leaks
84 (5 cases).

85

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

94 contaminants was less dependent on the *ica*⁺ *aap*⁺ genotypes; four (30.7%) formed some
95 biofilm, despite being *ica*⁻ *aap*⁻.

96

97 There was good correlation between CRA colony phenotypes and biofilm production
98 under standard growth conditions, especially for *ica*⁺ *aap*⁺ isolates, which produced black
99 crusty colonies and strong biofilm. *ica*⁺ *aap*⁻ isolates were weak biofilm producers and
100 were of the red and smooth morphotype.

101

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

110

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

114

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

117 without evidence of neurosurgical meningitis. Most research to date in this area has
118 focused on central line catheter infections; little work has been done on neurosurgical
119 infections. The *ica* operon alone or with *aap* was present in the majority of *S.*
120 *epidermidis* meningitis isolates. Interestingly, a higher proportion of contaminants
121 (84.6%) produced biofilm compared with isolates causing meningitis (71.4%). When
122 biofilm production occurred, it seemed to be less dependent on *ica* and *aap* amongst
123 contaminants. Nonetheless, there was overlap between the production of biofilm and an
124 *ica*⁺ *aap*⁺ genotype in meningitis and contaminating isolates.

125

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

130

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

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

142

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

150

151 The anatomical locality and physiological milieu from which isolates are recovered may
152 affect the expression of biofilm. The *ica* gene cluster was detected in 60% of conjunctival
153 sack isolates compared with 15% of facial skin isolates [19]. Only 27% of urinary
154 isolates produced biofilm; five of seven *ica*-positive biofilm negative isolates produced
155 biofilm under external stress, indicating that *icaADBC* expression was down regulated
156 [21].

157

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

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

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169 Word count 1,358 (should be 1,000, but we would probably get away with 1,100) (is now
170 1612)

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

Isolate Group (No.)	Genotype (No of Biofilm +ve) ^a			
	<i>ica</i> ⁺ <i>aap</i> ⁺	<i>ica</i> ⁺ <i>aap</i> ⁻	<i>ica</i> ⁻ <i>aap</i> ⁺	<i>ica</i> ⁻ <i>aap</i> ⁻
Meningitis-causing ^b (42)	14 (12)	19 (13)	6(3)	3 (1)
Contaminants (13)	2 (2)	2 (2)	4 (3)	5 (4)

189 ^a isolates were deemed biofilm positive if OD value was greater than 0.12

190 ^b these isolates were considered to have caused definite, probable or possible meningitis

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

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

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

BM61	LP	1	Contaminant	<i>ica⁺aap⁻</i>	+	0.18 (0.02)	3
BM62	EVD	>3	Definite	<i>ica⁺aap⁻</i>	+	0.16 (0.02)	1
CSF41498			Control	<i>ica⁺aap⁺</i>	+++	0.75 (0.10)	3

218 ^a EVD (Extraventricular device or drain); LP (Lumbar puncture)

219 ^b Definite, probable or possible refers to the likelihood of meningitis

220 ^c On Congo red agar, + indicates red smooth circular colony phenotype, ++ indicates
221 intermediate colony phenotype, +++ indicates black crusty filamentous colony
222 phenotype.

223 ^d Biofilm OD value from growth under standard conditions.

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

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