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Coagulase gene variants associated with distinct populations of *Staphylococcus aureus*

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SUMMARY

An identifying characteristic of *Staphylococcus aureus* is the production of staphylocoagulase (coagulase). The aim of this study was to determine the clonal distribution of coagulase gene (*coa*) variants within populations of *S. aureus* defined by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and protein A variation. The N-terminal region of the *coa* gene from 43 methicillin-susceptible (MSSA) and 252 methicillin-resistant (MRSA) *S. aureus* human isolates and 9 animal *S. aureus* isolates was amplified and digested with *Hin*fI. Twelve types were identified amongst the MSSA isolates and the majority (93%) of MRSA isolates were assigned to 5 of the 12 types. MLST and PFGE analysis identified epidemic populations of MRSA and each epidemic population was characterized by a different coagulase type. Nine of the 12 MLST-defined clonal complex ancestral genotypes recently described each carried a different coagulase type suggesting that coagulase evolution and the evolution of the clonal complexes are intimately related.

INTRODUCTION

Staphylocoagulase or coagulase is an extracellular product of most strains of *Staphylococcus aureus*. It is capable of clotting plasma from a number of different species and this ability is often used for the rapid identification of potentially pathogenic staphylococci. Coagulase binds to prothrombin forming a 1:1 complex called staphylothrombin that can cleave fibrinogen giving rise to clot formation. No enzymic activity has been associated with coagulase and binding to prothrombin is sufficient for the expression of fibrinogen cleavage activity [1]. Coagulase can also bind fibrinogen and it has been suggested that this activity is responsible for the clumping of *S. aureus* in plasma [2]. However, molecular studies have indicated that coagulase is not a clumping factor [3]. The two binding activities of coagulase have been located at two distinct sites within the protein, the N-terminal region containing the prothrombin-binding site and the C-terminal region containing the fibrinogen-binding site [3].

A number of serological forms of coagulase based on cross-neutralization of clotting activity have been described. A total of eight serotypes have so far been identified and these were used to form the basis of a *S. aureus* typing scheme [4]. The DNA sequences of three coagulase serotypes have been determined [5–7]. Analysis of the *coa* gene-sequence data has shown that the N-terminal region is variable (approximately 50% identity between the three sequences) and that there is a highly conserved (>90% identity) central region. The C-terminus is composed of a tandem repeat of

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27 amino acids and each of the published sequences has a different number of repeats. Amplification of the repeats followed by restriction endonuclease digestion with *Alu*I gave banding patterns, which can be used to discriminate between isolates [8–10]. These results suggest that coagulase is a highly variable protein although the total number of different forms of the protein has yet to be established.

The population structure of *S. aureus* has been examined using a number of phenotypic and genotypic methods. Recently, multilocus sequence typing (MLST) [11] has been used to define populations of both methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolates [12–14]. Such populations can be analysed for the carriage of different genes or variants of genes. It is clear from the MLST studies that both carriage and invasive isolates can be grouped into clonal complexes, groups of isolates which differ from each other at a single locus. The ancestral genotype of each clonal complex is defined as the 'sequence type (ST) differing from the highest number of other STs within the complex at only one locus' [12]. The study found that disease isolates were more likely to have the ancestral genotype than carriage isolates and suggested that the ancestral genotype confers both fitness and virulence characteristics. The study also found that pulsed-field gel electrophoresis (PFGE) patterns from isolates with the same allele profile were very similar, although the isolates were from a restricted geographical location. The most common MRSA allele profile was identical to the epidemic MRSA (EMRSA) strain EMRSA16, one of a number of major MRSA clones prevalent around the world over recent years. These also include EMRSA15, EMRSA3 [15], the Iberian EMRSA [16], the Paediatric EMRSA [17] and the New York EMRSA [18].

A number of genotypic methods have been used to discriminate amongst *S. aureus* isolates including the *coa* gene C-terminal repeat polymorphisms described above. The most frequently used method is PFGE, which has been successful in identifying outbreaks of MRSA. Alternative typing methods for *S. aureus* include using the repeats at the 3′-end of the *spa* gene which codes for Protein A. Isolates can be distinguished by directly comparing the sequences of the repeats [19–21] and this method has been successfully applied to the identification of outbreak strains of MRSA which appear to differ by PFGE [22]. In this study we used MLST, PFGE and *spa* gene analysis to define populations of *S. aureus* and determine the carriage of different *coa* gene alleles within these populations.

Table 1. *Animal strains of* S. aureus

Strain no.	NCTC Ref. no.	Source	*Hin*fI type
AN1	1803	Sheep	1
AN2	7485	Cow	2
AN3	7718	Lamb	3
AN4	7727	Dog	4*
AN6	7972	Goat	1
AN7	9547	Cow	1
AN8	9555	Sheep	5
AN9	9556	Hen	6†
AN10	9611	Avian	6†

* Identical to *coa* type K seen in human isolates.
† Identical to *coa* type F* seen in human MRSA isolates.

METHODS

Bacterial isolates

A total of 304 *S. aureus* isolates was analysed in this study. Nine animal isolates were obtained from the National Collection of Type Cultures (PHLS, Colindale, London) (Table 1). Two hundred and fifty-two MRSA isolates were selected representing both hospital outbreak and sporadic isolates (Table 2). These were from geographically diverse sources collected over a number of years and included: 15 English isolates (11 hospital-outbreak isolates from Kettering, collected during 1991–2 plus 4 isolates from different English hospitals collected in 1989); 8 isolates from Italian hospitals; 15 isolates from Russian hospitals; 10 hospital-outbreak isolates from Malaysia, collected in June 1989; Scottish isolates (204) included 88 isolates from Aberdeen collected over 5 years (1992–7); 23 Dundee isolates collected over 2 years (1990–1); 5 Brechin isolates collected over 2 months (1996); 41 Glasgow isolates; 17 Dumfries and Galloway isolates collected over 6 months (1996); 6 Paisley isolates collected over 2 months (1996); 11 hospital-outbreak isolates from West Lothian (1996); information on the 13 Airdrie isolates was not available. Some of these isolates have been included in previous studies on typing methods for MRSA [23]. Duplicate isolates from the same patient or the same ward on the same day were excluded where the information was available. Forty-three Aberdeen MSSA isolates were collected over 2 months (1991) from different patients, different body sites and different wards.

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Table 2. *Distribution of* S. aureus coa *gene HinfI types of MRSA and MSSA isolates from different geographical sources*

		*Hin*fI Types												
Origin*	No.	A	B	C	D	E	F	G	H	I	J	K	L	Other
Aberdeen	88	16			15	3					19	18	13	4
Glasgow	41										7	20	14	
D&G†	17	1			16									
Dundee	23	2	1		9						2	7	2	
West Lothian	11				11									
Brechin	5				1							4		
Airdrie	13				2						11			
Paisley	6				3						3			
Kettering	11										11			
Italy	8										1		7	
England	4		4											
Russia	15								6		9			
Malaysia	10										10			
Total	252	19	5		57	3			6		73	49	36	4
		7·5%	1·9%		22·6%	1·2%			2·4%		29·0%	19·4%	14·3%	1·6%
Aberdeen MSSA	43	7	4	1	1	1	7	2	1	5	6	5	3	
Animal MSSA	9											1		8
Total	52	7	4	1	1	1	7	2	1	5	6	6	3	8
		13·5%	7·7%	1·9%	1·9%	1·9%	13·5%	3·8%	1·9%	9·6%	11·5%	11·5%	5·8%	15·4%

* Unless otherwise indicated isolates are methicillin resistant.
† D&G, Dumfries and Galloway.

DNA purification

DNA from all isolates was purified by a modification of the method of Pitcher et al. [24]. Cells from an overnight nutrient agar plate were resuspended in 0·15 ml of 1 mM EDTA, 10 mM Tris–HCl buffer, pH 8·0 (TE buffer), and to this was added 0·15 ml of 150 mM NaCl, 10 mM EDTA containing 20 μg lysostaphin (Sigma, Poole, UK) and 1 mg lysozyme (Sigma). The cells were incubated for 1–2 h at 37 °C. Complete lysis of the cells was achieved by the addition of 0·5 ml GES reagent (5 M guanidium thiocyanate (Sigma), 0·1 M EDTA and 0·5% v/v Sarkosyl) which was held at room temperature until clearing of the cell suspension was observed. To this 0·27 ml of 7·5 M ammonium acetate was added and kept on ice for 10 min, 0·25 ml chloroform/isoamylalcohol (24:1 v/v) was added and the mixture vortexed vigorously for 5 min before centrifugation (13 000 ***g***) for 15 min. DNA was recovered from the aqueous phase by precipitation with 0·54 vols of isopropanol and centrifugation (13 000 ***g***) for 5 min. The pellet was resuspended in TE buffer, and then phenol- and chloroform-extracted, ethanol precipitated and finally resuspended in TE buffer and stored at 4 °C.

PCR amplification

Primers for amplification of the 5′ and 3′ regions of the *coa* gene were synthesized on an ABI 891 DNA synthesizer. Primer sequences were based on regions of identity between the three published *coa* gene sequences [5–7]. COAG-1 (5′-CAG ATG CGA TAG TAA CAA AGG ATT, positions 71–94 in the DNA sequence numbering of strain 8325-4 [7]) and COAG-4 (5′-TGT TCC ATC GTT GTA TTC AC, positions 1476–1457) amplified a 1400 bp region covering the variable N-terminal region and the conserved central region of the *coa* gene. COAG-5 (5′-TAT GAA GCG AGA CCA AGA TT, positions 1483–1502) and COAG-6 (5′-TGT CGC AGT ACC ATC TGC, positions 1887–1870) amplified the tandem repeat region located at the 3′ end of the gene. The size of the amplified product varied among the isolates, and was dependent on the number of repeats present in the *coa* gene.

Amplification was carried out as described previously [25]. The final concentration of reagents in the amplification mixture was: DNA 2 ng/μl, deoxynucleoside triphosphates (dNTPs) 200 μM (each), primers 0·25 μM (each), *Taq* polymerase (Bioline, London, UK) 0·025 U/μl, 1 × reaction buffer (supplied

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with *Taq* polymerase). Final volumes of 20 μl and 50 μl were used routinely. Samples were heated to 94 °C for 4 min and then subjected to 30 cycles of 94 °C for 1 min (denaturation), 51 °C for 1 min (annealing), 72 °C for 2 min (extension). PCR was performed on a PerkinElmer Cetus DNA Thermal Cycler Model 9700 (Applied Biosystems, Warrington, UK). PCR products were analysed on 2% agarose (Sigma) gels, stained with ethidium bromide and photographed under UV.

Amplification of the repeat region of the *S. aureus* Protein A (*spa*) gene and the methicillin resistance (*mec*) gene was carried out as described previously [26, 27]. Amplification products were analysed as described for the *coa* gene.

Restriction endonuclease digestion

A total of 10 μl of PCR product were digested for 4 h at 37 °C with *Hin*fI (10 U, Roche Diagnostics Ltd, Lewes, UK) or *Alu*I (10 U, Roche) according to the manufacturer's instructions. The products of digestion were analysed on 2% (w/v) agarose gels. Gels were stained as described above.

PFGE

PFGE was carried out as described by Bannerman et al. [28]. *S. aureus* chromosomal DNA in agarose plugs was digested with 20 U *Sma*I at 25 °C for 4 h. Fragments were separated on 1% (w/v) agarose gels run in 0·5 × TBE (44·5 mM Tris, 44·5 mM borate 1 mM EDTA) on a CHEF DRII PFGE system (BioRad, Hemel Hempstead, UK) run at 6 V/cm for 24 h with an initial switch time of 10 s linearly ramped to a final switch time of 25 s. Samples were run with lambda concatenated markers (BioRad) and *S. aureus* 8325 chromosomal DNA digested as above.

MLST and sequencing

MLST was carried out following the protocol of Enright et al. [13]. Each product was sequenced with the forward and reverse primer. Sequence data were analysed using the Seqed sequence analysis program (Applied Biosystems). MLST sequence data were compared to the *S. aureus* MLST database and allele numbers obtained (http://mlst.zoo.ox.ac.uk). Other amplification products were purified prior to sequencing using Centricon C-100 columns (Millipore, Watford, UK) to remove excess primers and dNTPs. All products were sequenced using an Applied Biosystems BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI 377 automated DNA sequencer. For sequencing the *coa* gene amplification product, COAG-1 and COAG-4 and a number of internal primers designed from the sequence data obtained with COAG-1 and COAG-4, were used to complete the sequence of each fragment and aligned using ClustalV. The *spa* amplification product was sequenced with the primers used in the PCR.

Nucleotide sequence accession numbers

The DNA sequence of each *coa* allele has been deposited in EMBL with accession numbers AJ309178–AJ309189 for types A–L, AJ309190 for type I* (A48) and AJ309191 for type A* (A87), and AJ311979 for type F* (A56). Coagulase alleles from the animal isolates have the accession numbers AJ311974 (type 1), AJ311975 (type 2), AJ311976 (type 3), AJ311977 (type 5), AJ311978 (type 6).

RESULTS

coa gene amplification and sequencing

Amplification of the *coa* gene using COAG-1 and COAG-4 gave a single band of approximately 1400 bp on agarose gels with all isolates. Digestion of the amplified product from the 43 MSSA clinical isolates with *Hin*fI identified 12 different restriction enzyme patterns (Types A–L) (Fig. 1). One of the 12 patterns, type C, was observed in a single isolate only. Three additional types, A*, F* and I* were identified among the 252 MRSA isolates which had similar *Hin*fI patterns to A, F and I respectively but had different sequences. Sequencing of the amplification product showed that identical *Hin*fI patterns gave identical sequences. Digestion of the PCR product from the 9 animal isolates gave six different patterns (Table 1) (*Hin*fI patterns not shown). The type 1 *Hin*fI pattern was identical to that expected for the BB strain (a sheep strain of *S. aureus*), type 4 was identical to type K, and type 6 was identical to type F*, the pattern seen in one Aberdeen MRSA isolate, A56, although sequence analysis showed them to be slightly different. The other three *Hin*fI patterns had not been observed previously. Amplification and digestion of the *coa* gene was repeated for all isolates and identical results were obtained.

The majority (234/252) of MRSA isolates were assigned to five major types, A, D, J, K and L (Table 2) which accounted for 93% of all MRSA isolates. The

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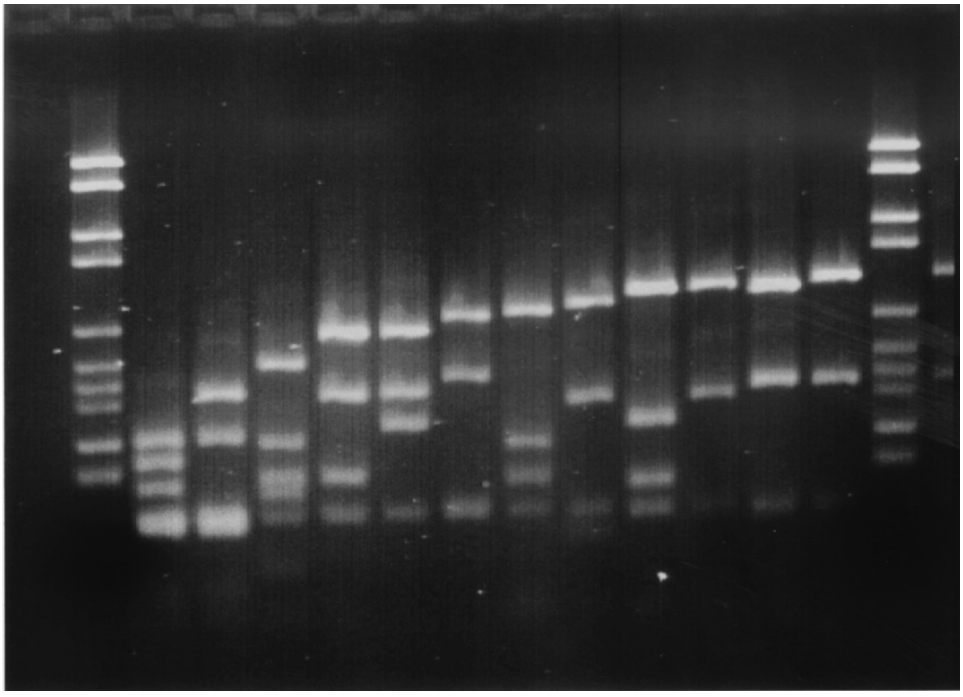

Fig. 1. Agarose gel (4%) showing the 12 *Hin*fI patterns (A–L) observed amongst the *S. aureus* and MRSA isolates. DNA standards (Roche) in first and last lanes ranged from 2176-154 bp.

*Hin*fI digest patterns of strains 213 and 8325-4 predicted from the published sequence data suggest that these correspond to coagulase types K and L respectively. *Hin*fI digestion of the COAG-1 and COAG-4 amplification product of strain 8325 (related to strain 8325-4) was consistent with coagulase type L. This was confirmed by sequence analysis.

Amplification of the 3′ end of the *coa* gene with COAG-5 and COAG-6 gave a fragment whose size was a multiple of 81 bp, the length of the repeat sequence. Among the 304 isolates, 6 different fragment sizes were observed, equivalent to the presence of 3, 4, 5, 6, 7 or 8 repeats in the *coa* gene. All isolates gave a single amplification product. The majority of the MRSA isolates (65%) had 6 repeats and none had 8 repeats. To ensure that the single product observed was not due to preferential amplification of one of a number of *coa* genes in an isolate, a mixture of DNA from two isolates with a different number of repeats in the *coa* gene was amplified with COAG-5 and COAG-6. Both amplification products were observed (results not shown). In an attempt to discriminate further between the *S. aureus* isolates the COAG-5 and COAG-6 product from 27 MSSA and 23 MRSA isolates was digested with *Alu*I and the fragments separated on agarose gels [8]. All fragments were either 81 bp or multiples thereof. The banding patterns obtained from some isolates with 5 and 6 *coa* gene repeats were indistinguishable (results not shown) and the method was not used routinely.

spa and *mec* gene amplification

Amplification of the *spa* gene repeat region gave different sized products ranging from 2 to 18 repeats. Only two isolates gave no amplification product and were deemed to lack the *spa* gene. All MRSA isolates were positive for the *mec* gene and all MSSA were negative for this gene.

PFGE

The *Sma*I restriction patterns of all isolates were compared. A number of isolates from temporally and geographically diverse sources were indistinguishable or had patterns that differed in up to six bands suggesting that they were related to each other [29, 30]. Epidemic strains of MRSA were identified by comparison with epidemic reference strains obtained from the Scottish MRSA Reference Laboratory and patterns published elsewhere [31, 32]. The patterns relating to EMRSA 3, 15 and 16 accounted for 57% of all MRSA isolates studied but the Iberian EMRSA pattern (7/252) was uncommon in our collection of isolates. Isolates with

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Table 3. *Association of genetic groups defined by different techniques with the Hin*fI *type*

*Hin*fI Type	Strain no.	MEC†	MLST Alleles‡							ST	CC§	Origin‖	Group¶	PFGE#
A	A65	R	10	14	8	6	10	3	2	ST45	CC45	ABN	6A10	A1
A	D1	R	10	14	8	6	10	3	2	ST45	CC45	DUN	6A9	A1
A	RS06	S	10	14	8	6	10	3	2	ST45	CC45	ABN	6A10	A1
A*	A87	R	19	23	15	2	19	20	15			ABN	7A*6	
B	C02	R	1	3	1	8	11	5	11	ST12	CC12	ENG	6B7	B1
B	RS14	S	1	1	1	1	1	1	1	ST1	CC1	ABN	5B10	B2
B	D5	R	14	6	11	2	13	12	4			DUN	7B12	B3
C	RS67	S	N8	7	6	2	9	9	7			ABN	8C8	
D	DG11	R	7	6	1	5	8	8	6	ST22	CC22	D&G	6D16	D1
D	N1	R	7	6	1	5	8	8	6	ST22	CC22	DUN	6D18	D1
D	P6	R	7	6	1	5	8	8	6	ST22	CC22	PAIS	6D16	D1
D	RS54	S	7	6	1	5	8	8	6	ST22	CC22	ABN	6D12	D2
D	EMRSA15	R	7	6	1	5	8	8	6	ST22	CC22	REF	6D16	D1
E	A10	R	1	N3	1	14	11	N27	10			ABN	5E7	E1
E	RS61	S	3	3	1	1	1	22	10			ABN	5E8	E2
F	RS08	S	13	13	1	1	12	11	13	ST15	CC15	ABN	4F11	F1
F	RS62	S	13	13	1	1	12	11	13	ST15	CC15	ABN	5F9	F1
F*	A56	R	3	1	1	8	1	1	1	ST188		ABN	6F*6	F*1
G	RS27	S	6	5	6	2	7	14	34			ABN	8G2	G1
G	RS44	S	6	5	6	2	7	14	5	ST121	CC121	ABN	8G8	G2
H	C17	R	12	1	1	15	11	1	N1			RUS	6H4	H1
H	RS15	S	1	1	1	1	22	1	1			ABN	6H10	H2
I	RS57	S	4	1	4	1	5	5	4	ST25	CC25	ABN	5I10	
I*	A47	R	10	14	8	6	10	3	2	ST45	CC45	ABN	6I*7	I*1
J	RS46	S	2	2	2	2	2	2	2	ST39	CC30	ABN	5J8	J1
J	N5	R	2	2	2	2	3	3	2	ST36	CC30	DUN	3J12	J1
J	EMRSA16	R	2	2	2	2	3	3	2	ST36	CC30	REF	4J11	J1
J	A62	R	2	2	2	2	6	2	2	ST40	CC30	ABN	6J8	J1
J	RS19	S	2	2	2	2	6	3	2	ST30	CC30	ABN	6J7	J1
J	RS29	S	2	2	2	2	6	3	2	ST30	CC30	ABN	6J10	J1
J	RS40	S	2	2	2	2	6	3	2	ST30	CC30	ABN	4J9	J1
J	RS43	S	2	2	2	2	6	3	2	ST30	CC30	ABN	4J9	J1
J	B06	R	2	3	1	1	4	4	3			GLAS	6J7	J2
J	B07	R	2	3	1	1	4	4	3			GLAS	6J7	J3
J	B24	R	2	3	1	1	4	4	3			GLAS	6J7	J4
J	B33	R	2	3	1	1	4	4	3			GLAS	6J7	J5
J	C08	R	2	3	1	1	4	4	3			RUS	6J5	J6
J	C11	R	2	3	1	1	4	4	3			RUS	6J6	J4
J	IT C	R	2	3	1	1	4	4	3			Italy	5J6	J7
J	M3	R	2	3	1	1	4	4	3			MALAY	6J7	J9
J	D9	R	2	3	1	1	21	4	3			DUN	6J8	J8
K	A18	R	1	4	1	4	12	1	10	ST5	CC5	ABN	6K10	K1
K	A67	R	1	4	1	4	12	1	10	ST5	CC5	ABN	6K10	K1
K	B03	R	1	4	1	4	12	1	10	ST5	CC5	GLAS	6K10	K1
K	D7	R	1	4	1	4	12	1	10	ST5	CC5	DUN	6K10	K1
K	N8	R	1	4	1	4	12	1	10	ST5	CC5	DUN	6K11	K2
K	RS07	S	1	4	1	4	12	1	10	ST5	CC5	ABN	6K9	K1
K	S2	R	1	4	1	4	12	1	10	ST5	CC5	BRECH	6K10	K1
K	B40	R	2	2	2	2	2	2	2	ST39	CC30	GLAS	6K10	J1
L	A34	R	3	3	1	1	4	4	3	ST8	CC8	ABN	5L10	L2
L	B01	R	3	3	1	1	4	4	3	ST8	CC8	GLAS	4L7	L2
L	B25	R	3	3	1	1	4	4	3	ST8	CC8	GLAS	4L7	L2
L	B42	R	3	3	1	1	4	4	3	ST8	CC8	GLAS	4L6	L3
L	IT B	R	3	3	1	1	4	4	3	ST8	CC8	Italy	5L10	L4
L	RS24	S	3	3	1	1	4	4	3	ST8	CC8	ABN	5L7	L2

Table 3 (*cont.*)

*Hin*fI Type	Strain no.	MEC†	MLST Alleles‡							ST	CC§	Origin‖	Group¶	PFGE#
L	RS26	S	12	3	1	1	4	4	3			ABN	5L8	L5
L	RS33	S	3	3	1	1	4	4	3	ST8	CC8	ABN	5L7	L7
L	A73	R	3	3	1	12	4	4	16			ABN	5L10	L1
L	D3	R	3	3	1	12	4	4	16			DUN	5L12	L1
L	IT D	R	3	3	1	12	4	4	16			Italy	5L11	L6
L	IT E	R	3	3	1	12	4	4	16			Italy	5L12	L1
L	Iberian	R	3	3	1	12	4	4	16			REF	5L11	L1

† R, MRSA; S, MSSA.
‡ All sequence types were determined by reference to the MLST *S. aureus* database and are ordered *arc*, *aro glp*, *gmk*, *pta*, *tpi*, *yquil*. A new allele not present in the MLST database is indicated by N with the closest allele in the database indicated.
§ CC, clonal complex [12].
‖ ABN, Aberdeen; BRECH, Brechin; DUN, Dundee; D&G, Dumfries and Galloway; ENG, England; GLAS, Glasgow; MALAY, Malaysia; PAIS, Paisley; REF, Scottish MRSA Reference Laboratory; RUS, Russia.
¶ Group refers to the number of coagulase repeats, the coagulase *Hin*fI pattern and the number of Protein A repeats. Hence 6A10 has 6 coagulase repeats, coagulase type A and 10 Protein A repeats.
# PFGE patterns differing by seven or more bands have separate codes.

the *coa* type A (Table 3) gave indistinguishable or closely related patterns suggesting this group may represent a local (Scottish) strain.

Each of the epidemic strains identified by their PFGE patterns was associated with a single *coa* gene type. For example, all 58 isolates with *coa* type D gave PFGE patterns related to EMRSA15, and two-thirds of the *coa* type J-containing isolates were related to EMRSA16. However, isolates unrelated by their PFGE pattern were found to have the same coagulase type (Table 3). Animal isolates AN9 and AN10 had related PFGE patterns but AN1, AN6 and AN7 were unrelated.

MLST

MLST was performed to determine the genetic relationship amongst isolates with the same coagulase type. Isolates with the same *coa* type but different PFGE patterns, number of coagulase repeats, Protein A repeats or from different geographical regions were analysed. Identical sequence types (STs) were found within isolates having *coa* types A (ST45), D (ST22) and K (ST5, except B40 which was ST39) (Table 3). Results from the MLST analysis were in agreement with the PFGE data for EMRSA. From the *S. aureus* MLST database *coa* type K isolates were ST5, a sequence type associated with EMRSA3 strains, *coa* type D (ST22) were associated with EMRSA15, *coa* type J (ST36) associated with EMRSA16 and *coa* type L with the Iberian EMRSA [14]. Although the majority of the type A isolates were collected from the Aberdeen area the common ST and PFGE pattern associated with these isolates suggests it may also represent a regularly occurring strain. One isolate, B40, showed a PFGE pattern and ST profile closely related to the epidemic strain EMRSA16. However, the isolate carried coagulase type K, suggesting a recombinational event replacing type J which is normally associated with EMRSA16.

Only two of the animal isolates, AN4 and AN8 gave previously recognized STs, ST5 and ST97 respectively. Both of these STs had previously been seen among human isolates of *S. aureus*. All the other animal isolates produced novel alleles at a number of different loci. This suggests that very different populations of *S. aureus* are present in animals and humans. The PFGE pattern of AN4 was related to that of other type K isolates (K1) that were also ST5 (Table 3).

Detection of mosaic structures within coagulase

Representative isolates from each pattern were amplified using COAG-1 and COAG-4 and the amplification products sequenced. Analysis of the data showed that the sequence of type L was identical to the published sequence of strain 8325-4; the sequence of type K was identical to strain 213 except at position 73 (8325-4 numbering [7]); the sequences of three animal strains (AN1, AN6 and AN7) were identical to strain BB except for an insertion of three bases around position 126 in the BB sequence and Met in the animal

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sequences at position 128. The sizes of *Hin*fI fragments predicted from the sequence data concurred with the actual sizes observed from agarose gel electrophoresis. In order to identify mosaic structures within the *coa* gene the DNA sequences were aligned using ClustalV and analysed using Sawyer's method [33]. This test allows an overall assessment of the recombination within a group of sequences based on a pairwise examination of the silent polymorphic sites. Application of the test to all the sequences gave a P value of $<10^{-4}$ confirming the occurrence of recombination within the group of sequences. The maximum χ^2 test [34] was used to determine the location of the cross-over points. Significance is determined by comparison of the data with T trial pairs, the observed mosaic structure being significant at $P<1/T$. Pairwise comparisons confirmed significant cross-over points within types D and E ($P<10^{-3}$), E and I ($P<10^{-4}$), and A and B ($P<10^{-3}$), all cross-overs occurring around the region of residue 300. Although the Sawyer's test identified a significant mosaic between type I and strain 213 ($P<10^{-4}$) analysis, the maximum χ^2 test failed to identify a cross-over point at $P<10^{-2}$.

DISCUSSION

The aim of this study was to determine the distribution of *coa* gene alleles amongst well-defined *S. aureus* populations. The variability of the 3′-end repeat region has been well documented and is used primarily for sub-typing staphylococcal isolates. A total of 18 different coagulase types were observed at the 5′-end of the gene. Only one type was found to be present in each isolate and this was confirmed by the occurrence of a single species of repeat unit at the 3′-end of the *coa* gene. The presence of more than one allele of coagulase in a single isolate has been reported based on serological studies and also PCR amplification of the repeat region [8] but this was not observed here.

In this study, we have identified discrete populations of *S. aureus* each carrying different *coa* types. The majority of MRSA isolates were epidemic strains identified by both PFGE and MLST and each epidemic strain was associated with a different *coa* gene. A recent study [14] showed that acquisition of the *mec* determinant occurred in a population of MSSA isolates that was dominant when methicillin was first used in the early 1960s. The allele profile of these early MSSA and MSRA isolates, 3 3 1 1 4 4 16 [14], is closely related to that of the *coa* type L population (Table 3). Although this population was the original recipient of the *mec* determinant it has since been acquired by a number of different populations as shown both in this and previous studies using MLST [13, 16], multilocus enzyme electrophoresis [35] and gene array technology [36].

The MLST allele profile of *coa* type J-containing isolates identified two distinct and unrelated populations of *S. aureus* carrying this particular *coa* type (Table 3). One population consisted of isolates belonging to clonal complex 30 [12] including EMRSA16 isolates (ST36). The second, non-EMRSA16 population has an allele profile previously identified in the Brazilian/Hungarian EMRSA [37]. It is closely related to isolates carrying *coa* type L and includes isolates from Russia, Italy and Malaysia. Two closely related MLST populations were also apparent amongst isolates carrying the type L *coa* gene (Table 3). Their allele profiles, 3 3 1 12 4 4 16 (Iberian EMRSA) and 3 3 1 1 4 4 3 (ST8) are single locus variants of MRSAs isolated in the United Kingdom and Denmark in the early 1960s (allele profile 3 3 1 1 4 4 16 [14]). The ST8 population is also closely related to the non-EMRSA16 type J population (2 3 1 1 4 4 3). The recombination event replacing *coa* type L with type J must have occurred prior to the dissemination of the clones since both types are distributed around the world. Whether this event occurred prior to the acquisition of the *mec* determinant is not clear. Recent analysis on the make-up of the *mec* determinant [37] suggests that the *coa* type J non-EMRSA16 population and the *coa* type L ST8 have different *mec* types indicating two independent insertion events. If this is the case then acquisition of the *mec* determinant must have occurred after the recombination event changing the *coa* type. The presence of both MSSA and MRSA isolates within the populations of epidemic strains as defined by MLST attests to the success of these particular strains in maintaining and spreading through the human population.

Analysis of the *S. aureus* MLST data identified 12 clonal complexes among a collection of carriage, community-acquired and hospital-acquired invasive *S. aureus* isolates [12]. Each clonal complex (CC) consists of an ancestral genotype, representing the largest clone in the complex, linked to the highest number of other STs differing at a single locus. The ancestral genotype occurs in a significantly higher proportion of disease isolates compared with carriage isolates suggesting whatever helps maintain this genotype within the host may also be responsible for increasing its frequency in disease [12]. A comparison of the

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                                                         1 111 111 111 111
       11 122 222 334 444 444 555 555 555 667 778 888 999 990 000 011 111 222
   578 906 714 578 092 356 789 123 456 789 030 481 235 012 360 235 913 489 036
D  AGG GAC ATA AAG GTG AAT TAA AGA TAG CAG ATG AAA GCC AAA GGT AAT TGC AAC ATG
E  ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• •••
I  GAA AGA GGT GGA CGA GTA CTG TTC AGA TTA TCA TTT AGT GGC AAA TGA AAA CTT GAT

   111 111 111 111 111 111 222 222 222 222 222 222 222 222 223 333 333 333 333
   334 444 445 567 778 899 112 333 455 555 566 777 778 888 990 133 344 445 566
   080 235 676 971 560 158 698 123 334 568 918 034 693 468 174 036 912 581 703
D  CTG AAG AAG AAA GAG ATA AAC TTA AGC AAT TCG TTT AAT ATA TGG TTA CCA TAA CTG
E  ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• •••
I  AAA CCA TCA GGG ACA GCG GTT GCT GAG TTA ATA GCA TTC TAT CAC ACT TTT CTG TAT

   333 333 333 333 444 444 444 444 444 444 444 444 444 444 444 555 555 555 555
   666 788 999 999 001 122 223 333 344 445 555 566 778 889 999 000 111 112 222
   467 846 034 678 784 902 670 345 912 461 347 956 230 790 468 568 134 682 345
D  GAA TAG TTC ATC AAA CTA CGA ACT AAC AAG TAA AAT AGA GTT CGG AGC GAA ATT ATA
E  ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• •••
I  TTC ATA AAG GCA TTG AGT TAG GGA GCA GCA AGG TCC TCT TAG TAT GCG AGG TAG GAG

   555 555 555 555 555 555 556 666 666 666 666 666 667 777 777 777 777 777 777
   335 556 667 777 777 888 990 223 446 666 788 888 990 000 011 112 222 334 456
   170 261 470 123 469 237 140 149 020 369 801 249 092 457 812 691 689 290 130
D  TAT AAA ATC GTG CTC GAG CTA GAA GCA ATG TCA AAA GCC ATT TTG ACA AAA GGA AGA
E  ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• ••• •••
I  GGC GTC TAT ACA GCT ACA TAG CTT AAG TAA GAT TTC ATA CAA ACA TAG GGT ACC TAG

                                    11 111 111 111 111 111 11
   777 777 777 888 888 888 899 999 999 900 000 111 111 223 334 44
   777 888 899 022 344 559 900 177 899 900 889 447 789 220 071 12
   027 124 624 778 669 025 689 556 413 634 032 023 651 788 973 62
D  GGA GAA ACA TAG TTT TTT TGG GAT ACG CAC ACT CCC TTG GAA CTT AT
E  ••G •C• TTC GGA AGA GAG CAT ATC GGA TCA GAG TTT CAA ACG A•A GG
I  AA. A.G ••• ••• ••• G•G CAT ATC GGA TCA GAG TTT CAA ACG AGA GG
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Fig. 2. Positions in the DNA sequences of coagulase types D, E and I, which are polymorphic. The translation products of coagulases D, E and I were aligned using ClustalV and the alignment used in the χ^2 analysis. Bases that differ between three sequences are shown and their positions in the alignment are numbered vertically. ●, Sequence identical to coagulase type D.

ancestral genotypes and coagulase types shows that 9 of the 12 ancestral genotypes are associated with a different form of the *coa* gene, for example, type A with CC45, type D with CC22 (Table 3) although recombination has obscured some of this association. The epidemic strains EMRSA3, -15, -16 and the Iberian epidemic strain belong to different clonal complexes and each carries a different *coa* type. This distinct demarcation of ancestral genotypes by *coa* type suggests that generation of novel forms of coagulase may be related to the emergence of new clonal complexes although linkage of the *coa* gene to other genes cannot be ruled out.

Analysis of the coagulase sequence data using Sawyer's test [33] and χ^2 test identified significant recombination among the genes. This is most clearly demonstrated when comparing types D, E and I (Fig. 2). The sequence of types D and E are identical up to position 781 after which they differ, whereas E and I differ up to position 861 after which they are identical. This suggests that recombination between types D and I gave rise to type E. However, this event is not recent as the MLST allele profiles of isolates carrying *coa* types D (ST22, CC22), I (ST25, CC25) and E (a SLV of ST9, the ancestral genotype of CC9) (Table 3) are very different. Interestingly, all three coagulase alleles are associated with different ancestral genotypes although they are clearly related by recombination, again suggesting a role for novel *coa* types in the emergence of new clonal complexes. Evidence for recombination also comes from identification of the same *coa* allele in very different populations of *S. aureus*. For example *coa* type B is found in three STs, and type F in two. Transfer of DNA between *S. aureus* isolates may occur by transformation, although the species is not thought to be naturally competent for DNA uptake, or transduction via specific bacteriophages. Alternatively, isolates which contain two or more *coa* alleles may

Table 4. *Sequence of* spa *repeats from MRSA isolates with identical coagulase genes and PFGE patterns*

Strain no.	Date isolated	PFGE	Coagulase repeat no. and *Hin*fI pattern	*spa* Repeat sequence*
A5	04/1994	D1	6D	T1-J1----E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A11	06/1994	D1	6D	T1-J1----E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A30	02/1995	D1	6D	T1-J1----E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A64	01/1996	D1	6D	T1-J1----E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A36	04/1995	D1	6D	T1-J1-J1-E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A63	09/1996	D1	6D	T1-J1-J1-E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A85	NA†	D1	6D	T1-J1-J1-E1-J1-N1-I2-M1-N1-I2-M1-O1-M1-O1-K1-R1
A54	11/1995	D1	6D	T1-J1-J1----------I2-M1-O1-M1-O1-K1-R1
B5	NA	K1	6K	T1-I1-M1-B1-M1-D1-M1-G1-M1-K1
B21	NA	K1	6K	T1-I1-M1-G1-M1-D1-M1-G1-M1-K1
B28	NA	K1	6K	T1-I1-M1-G1-M1-D1-M1-G1-M1-K1
B45	NA	K1	6K	T1-I1-M1-G1-M1-D1-M1-G1-M1-K1
B34	NA	K1	6K	T1-I1-M1-B1-M1-D1-M1-G1----K1
B39	NA	K1	6K	T1----M1-B1-M1-D1-M1-G1-M1-K1
B8	NA	K1	6K	T1-I1----------M1-G1-M1-K1
B35	NA	K1	6K	T1-I1----------M1-G1-M1-K1

* Identification code for each *spa* repeat unit taken from reference [20].
† NA, Not available.

undergo rearrangement to produce the different coagulase forms.

Although MLST can identify genetically related isolates it is apparent from this study that it does not adequately distinguish between isolates for epidemiological purposes. Isolates that have identical MLST allele profiles have clearly distinguishable PFGE patterns, *spa* repeat numbers and *coa* repeat numbers. Previous work using *spa* gene typing identified 37 different types of individual repeat sequences [20]. Sequencing of *spa* repeats from isolates with the same coagulase type and indistinguishable PFGE patterns but with a different number of *spa* repeats showed that these closely related isolates contained repeat sequences differing only by the deletion/insertion of individual repeats or the alteration of one of the repeats (Table 4). The *spa* repeat sequence observed for the *coa* type K isolates (ST5, EMRSA3) is similar to the *spa* repeat sequence observed in the 'Paediatric' [17] and 'New York' [18] epidemic strains, both of which are also ST5 [14]. This suggests that these epidemic strains are in fact part of the EMRSA3 epidemic strain and as such should also carry *coa* type K. The longest *spa* repeat sequence from the *coa* type D-containing EMRSA15 isolates (Table 4) have been identified in EMRSA15 isolates from New Zealand (P. Carter, unpublished observations). This *spa* repeat sequence may be characteristic of the EMRSA15 clone. Changes in the number of repeats within the *spa* gene occur more frequently than changes in the number of *coa* gene repeats, the type of coagulase or the alterations of the PFGE patterns, thus allowing *spa* gene amplification and sequencing to be used for epidemiological investigations of outbreaks of MRSAs [19, 21, 22]. However the close similarity of the repeat pattern in epidemic strains may limit its usefulness. A previous study has shown that epidemiologically unrelated MRSA isolates which had different PFGE and *coa* repeat patterns could have identical *spa* gene repeat sequences [22]. This emphasizes the importance of using a number of typing methods to determine the relationship amongst isolates. Although variation at the 5′-end of the *coa* gene may not be adequate for epidemiological purposes it may provide a useful method for the rapid identification of epidemic strains of MRSA.

Previous population genetic studies have shown that strains of *S. aureus* remain closely associated with their animal hosts with little transfer between species [38]. We have also found that the animal isolates had STs very different from each other and the human isolates although two STs from AN4 and AN8 have been identified among human isolates. Coagulase activity may have been selected for species-specificity. Previous studies of coagulase activity from *S. aureus* strains isolated from animals showed that different strains could coagulate different animal sera with varying efficiencies [39]. In this study, however, the same coagulase type was identified in three different animals. Conversely, three different coagulase types were detected in the same species of animal (Table 1). This

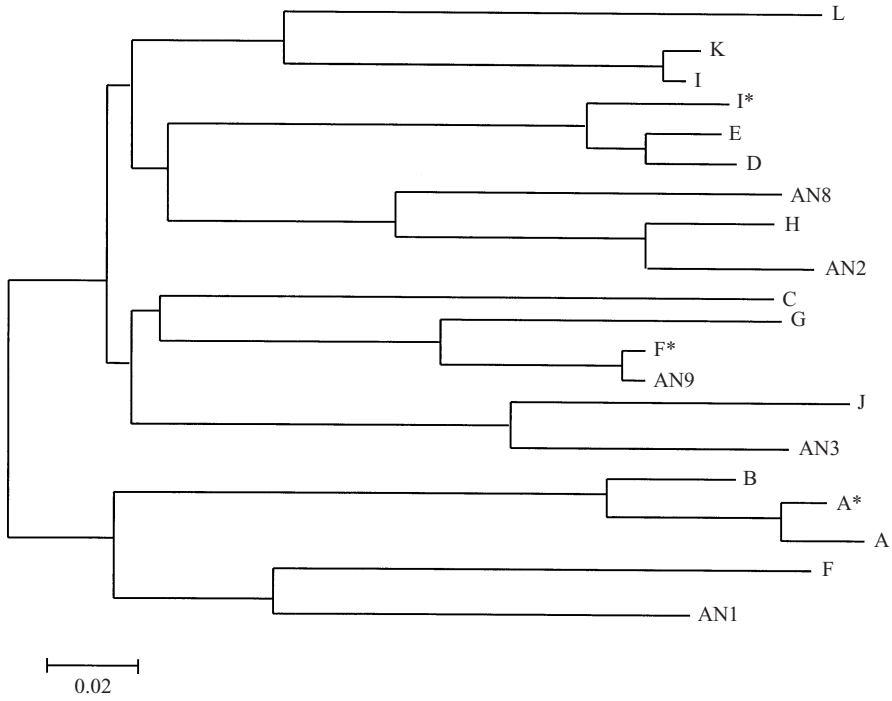

Fig. 3. Dendrogram of the different types of coagulase found in this study. Sequences were aligned and the tree calculated using ClustalV.

suggests that there is no selection of particular alleles of coagulase by individual species. A dendrogram of the genetic relationship of all the coagulase types (Fig. 3) shows that animal isolates are as variable as human isolates and are closely related to them. Only one of the animal isolate *coa* genes (AN4) gave a sequence identical to one of the clinical isolate forms (type K). Thus the inherent variability of coagulase apparent from our study does not appear to be due to selection of different forms capable of activating prothrombin from particular species.

It is apparent that the variation seen in the *coa* genes arises due to a number of different mechanisms: recombination among the different forms resulting in mosaic structures; slipped-strand mispairing generating insertions/deletions; variation in the number of repeats at the 3′-end; point mutations within the gene. The potential role of the observed variation of coagulase in the pathogenicity of *S. aureus* is not clear. The use of the coagulase test to distinguish the more pathogenic *S. aureus* from other staphylococci initially suggested coagulase may be involved in virulence mechanisms although removal of the gene does not impair the ability of *S. aureus* to cause infection in mice [7, 40]. The association of different forms of coagulase with the ancestral genotype of clonal complexes suggests it may play a role in the evolution of virulent or selectively advantaged strains. Further work on the interactions of the different forms of coagulase with the host are required to determine their biological significance and its implication for the success of the pathogen.

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