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Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*

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Staphylococcus aureus is an important pathogen of man, but is also able to colonize and cause disease in a wide variety of mammals and birds. An extended multilocus sequencing approach, involving multilocus sequence typing (MLST), *sas* typing, *spa* typing and *agr* typing, was used to examine the molecular diversity of 118 *S. aureus* isolates recovered from a range of host species and to compare these data with the known diversity of human-derived isolates. MLST revealed that the commonest animal-associated MLST types were ST133, ST5, ST71, ST97, ST126 and ST151. ST133 appears to be an ungulate-animal-specific genotype, as no evidence of ST133 associating with humans has yet been found in the literature. Novel and unique *sas* alleles were identified in the animal-associated strains that may represent animal-associated *sas* alleles. However, *sas* typing exhibited a lower typeability than MLST for the animal strains (91.3 %). Phylogenetic analyses using neighbour-joining and maximum-parsimony trees localized ruminant-associated MLST lineages to both previously identified *S. aureus* subspecies *aureus* subgroups, thus explaining the finding of all four *agr* types within the ruminant-associated strains. *S. aureus* isolates recovered from chickens and rabbits were genotypically more similar to known human genotypes than the ruminant-associated lineages.

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INTRODUCTION

Multilocus sequence typing (MLST) has contributed greatly to the understanding of the population structure and evolution of *Staphylococcus aureus* (Cooper & Feil, 2006; Feil *et al.*, 2003; Robinson & Enright, 2003, 2004). Isolates recovered from cases of human disease, or from asymptomatic nasal carriage, correspond to a small number of widely distributed clusters of closely related genotypes called clonal complexes (CCs) (Feil *et al.*, 2003; Robinson & Enright, 2003). Further sequencing studies involving up to 30 loci and the accessory gene regulator (*agr*) locus have shown that *S. aureus* subspecies *aureus* can be divided into two subspecies groups (Cooper & Feil, 2006; Robinson *et al.*, 2005). However, these sequence-typing studies did not include animal-associated *S. aureus* strains.

S. aureus can colonize and infect a wide range of domesticated and wild animals, including sheep (Vimercati *et al.*, 2006), rabbits (Hermans *et al.*, 2003), chickens (Rodgers *et al.*, 1999) and turkeys (Linares & Wigle, 2001). *S. aureus* frequently causes mastitis, a disease that is of economic importance worldwide (Barkema *et al.*, 2006; Kapur *et al.*, 1995). Meticillin-resistant *S. aureus* strains have been isolated from cats, dogs, pigs and horses (Leonard & Markey, 2008).

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Abbreviations: CC, clonal complex; DLV, double locus variant; MLST, multilocus sequence typing; SLV, single locus variant; ST, sequence type.

Supplementary information is available with the online version of this paper.

Analyses of the MLST sequence types (STs) of animal isolates have similarly identified CCs associated with specific hosts. For example, CC97 has been associated with bovine mastitis (Aires-de-Sousa *et al.*, 2007; Monecke *et al.*, 2007; Rabello *et al.*, 2007; Smith *et al.*, 2005) and ST398 is associated with pigs and horses (van Leeuwen *et al.*, 2005). Although several studies have analysed the genetic diversity of bovine strains, only a small number of *S. aureus* strains from other animals such as sheep (Mork *et al.*, 2005; Vautor *et al.*, 2005, 2009; Ben Zakour *et al.*, 2008; Sung *et al.*, 2008), goats (Jorgensen *et al.*, 2005; Ben Zakour *et al.*, 2008; Sung *et al.*, 2008; Vautor *et al.*, 2009), horses (Sung *et al.*, 2008), chickens (Rodgers *et al.*, 1999) and rabbits (Vancraeynest *et al.*, 2006) have been characterized. More recently, a study of *S. aureus* strains from bovine mastitis has shown the association of particular STs with virulence and severity of disease (Guinane *et al.*, 2008). In addition, the ST151 genotype was shown to be hypersusceptible to the acquisition of vancomycin-resistance genes from enterococci (Sung & Lindsay, 2007). Studies such as these emphasize the importance of continued investigation of animal-associated *S. aureus*.

In the current study, molecular analysis including MLST, *sas* typing, *spa* typing and *agr* typing was carried out on 118 isolates, not only from cows, but in addition from sheep, goats, chickens and rabbits. Studies of the diversity of *sas* alleles of animal-associated *S. aureus* had not been done previously. The aims of our study were to utilize novel molecular typing methods to investigate the clonal diversity of *S. aureus* strains from animals in addition to cows and to compare them to the breadth of diversity within *S. aureus* subspecies *aureus* as a whole.

METHODS

Bacterial strains. One hundred and eighteen *S. aureus* isolates collected by various investigators in several different countries were analysed. The isolates were chosen to be diverse and, where possible, to reflect predominant animal-associated clones. Twelve isolates representing predominant *S. aureus* PFGE-defined clones from broiler chicken osteomyelitis infection in Northern Ireland were included (Rodgers *et al.*, 1999). Fifty-two bovine mastitis strains were analysed – three from Argentina, four from Spain, two from Sweden, 19 from the USA and 24 from the Republic of Ireland – of which 28 were Irish and USA mastitis isolates that had been characterized by Fitzgerald *et al.* (1997, 2000) and represented predominant electrophoretic types associated with bovine mastitis in Ireland and the USA (Fitzgerald *et al.*, 1997, 2000; Kapur *et al.*, 1995).

S. aureus strains from goats, rabbits and sheep had been originally obtained on the basis that they were typical of predominant genotypes/phenotypes in the collections of the donors in different countries. Of the 32 isolates from goats, four were mastitis isolates from Austria, 20 were from milk samples from Italy (Foschino *et al.*, 2002) and eight were diverse PFGE types from Norway. Of the 12 rabbit-associated strains, four were representative isolates from Spain and eight were rabbit staphylococcosis strains from Belgium representing both high- and low-virulence strains (Hermans *et al.*, 2000). Of the 10 sheep mastitis isolates, two were from Iceland, two were from Denmark, one was from Sweden and five were from Norway and represented diverse PFGE types. The strains used are detailed in Supplementary Table S1 in JMM Online. Their geographical origins are shown in Table 1.

S. aureus isolates were verified by Gram stain and testing of their ability to grow and produce acid on mannitol salt agar, a positive coagulase test, their ability to produce acetoin, their ability to grow on TSA supplemented with 7 μg acriflavine ml^{-1} (Devriese, 1981), and a negative PCR for the *Staphylococcus intermedius* 16S rRNA gene.

DNA extraction and MLST. Bacterial genomic DNA was extracted using the DNeasy Genomic DNA Extraction kit (Qiagen) or as previously described (Smyth *et al.*, 2007). MLST was carried out using the primers of Enright *et al.* (2000) as previously described. Briefly, the method involves the PCR of several housekeeping genes followed by cycle sequencing using purified PCR products, 1 pmol primers and the *Taq* FS-Big Dye polymerase system (Applied Biosystems). DNA sequences were read on both strands using an ABI 3700 Prism Sequencer. Following sequencing, the data were assembled using the SeqMan program (DNASTAR). The *S. aureus* MLST website (http://saureus.mlst.net/) was used to verify whether an allelic sequence was already in the database or differed by one base pair or more to a known allele. In the latter instance, a new allele number was assigned to the sequence. The combined allele numbers for all seven housekeeping genes corresponded to the allelic profile of the isolate. Allelic profiles for each isolate were submitted to the database to obtain a ST number.

The clustering of animal-associated STs was analysed using the eBURST (Based Upon Related Sequence Types) algorithm (Feil *et al.*, 2004) (www.eburst.mlst.net). CCs were composed of STs that shared at least six out of the seven alleles in common and a predicted ancestral ST and its associated single locus variants (SLVs; variants that differ at one of the seven MLST alleles from the ancestor) and double locus variants (DLVs; variants that differ at two of the seven MLST alleles from the ancestor).

***agr* typing.** A multiplex PCR scheme was used to amplify across the variable region of the accessory gene regulator (*agr*) locus (Gilot *et al.*, 2002). This method utilizes a universal forward primer (*agr*1-4_{sa}-1) and four *agr* type-specific primers (*agr*1_{sa}-2, *agr*2_{sa}-2, *agr*3_{sa}-2 and *agr*4_{sa}-2). Each *agr* type generates a different-sized, type-specific PCR product that can be distinguished from other types upon gel electrophoresis.

Table 1. Countries of origin of the animal strains studied

	Numbers of strains of indicated animal origin				
	Cow	Sheep	Goat	Rabbit	Chicken
Argentina	3				
Austria			3		
Belgium				9	
Denmark		2			
Iceland		2			
Ireland	24				
Italy			21		
Northern Ireland					12
Norway		5	8		
Spain	4			3	
Sweden	2	1			
USA	19				
Total	52	10	32	12	12

***sas* and *spa* typing.** Both *sas* and *spa* typing were performed as previously described (Robinson & Enright, 2003). *sas* typing involves the amplification of approximately 450 bp internal regions of seven genes encoding putative surface-associated proteins (*sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH* and *sasI*). *spa* typing involves the amplification of the short-sequence repeats of the Protein A (*spa*) gene. In both cases, the PCR products were purified and subsequently sequenced on both strands by MWG-Biotech. The *sas* sequences obtained were checked against the *sas* database (available upon request) and known alleles and types were identified. Novel *sas* alleles were added to the database. *sas* types were assigned numbers arbitrarily. The Ridom database was used to assign *spa* types (http://www.spaserver.ridom.de). The SNAP program (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) was used to calculate d_S/d_N ratios by the modified Nei and Gojobori method (Korber, 2000). The d_S/d_N ratio is the number of synonymous or silent nucleotide changes per synonymous site (d_S) to the number of non-synonymous or amino-acid-replacing nucleotide changes per non-synonymous site (d_N).

Levels of discrimination. To determine the level of discrimination achieved by MLST, *sas* typing and *spa* typing, Simpson's index of diversity (*D*) (Grundmann *et al.*, 2001) was calculated using the equation $D=1-[\{1/N(N-1)\}\times\Sigma n_i(n_i-1)]$, where D=index of discrimination, N=number of isolates in the sample and n_i=number of isolates in group i. This method calculates the probability that two random isolates will be of different types by considering both the number of clusters (groups) and the number of isolates within each cluster. The index ranges from 0 to 1, with a value close to 0 indicating low genetic diversity and a value close to 1 indicating high genetic diversity.

Phylogenetic analysis. In order to identify the relationships of the animal-associated STs to the human-associated STs, a phylogenetic approach was used. Each animal-derived ST noted in the current study was compared to a selection of major human-associated STs, not found in the current study, from Cooper & Feil (2006) (STs 7, 9, 10, 13, 15, 17, 30, 36, 45, 49, 50, 55, 59, 182, 207, 239 and 240) and Robinson *et al.* (2005) (STs 12, 27, 80, 88, 93, 101 and 188) as well as the emerging pig- and horse-associated clone ST398 (van Leeuwen *et al.*, 2005). The concatenated sequences of all seven MLST alleles for each ST were aligned using the CLUSTAL W program with default parameters followed by manual inspection. Insertion and deletion polymorphisms were ignored during phylogenetic analyses. MEGA (v. 4) was used to construct neighbour-joining and maximum-parsimony trees (Tamura *et al.*, 2007). Neighbour-joining trees were constructed using the absolute number of nucleotide differences between STs. Maximum-parsimony trees were constructed using a heuristic search and random addition of taxa (STs). Bootstrapping was performed with 1000 replicates.

RESULTS

MLST-defined diversity

The distribution of the MLST STs among the animal-associated strains is shown in Table 2. The 118 animal-associated isolates generated 37 STs, of which 15 were novel types not present in the MLST database (STs 692–701, 703, 705, 706, 708 and 709). Of the 15 new STs, 14 are each accounted for by one strain only, the exception being ST703, which was observed in four isolates recovered from goats in Italy. MLST type ST133 was found only in strains from sheep, goats and cows and in several different countries (Table 2). ST151 was only found in Irish cows, but the closely related ST705 was found in a Swedish sheep.

Recent patterns of evolutionary descent within CCs were reconstructed using eBURST (http://eburst.mlst.net) by comparing the present dataset to that of the MLST database of 1227 STs as of December 2008. eBURST generated 57 groups and 189 singletons (STs with no SLVs in the database). Of the 57 groups, 21 contained animal-associated STs (CCs 1, 5, 8, 20, 22, 25, 30, 50, 96, 97, 101, 121, 126, 130, 133, 151, 350, 479 and 703 and a further two CCs with no predicted ancestor containing ST350 and ST692). ST699 was a singleton. Eight CCs contained more than one animal-associated ST (CCs 5, 8, 97, 126, 130, 133, 151 and 703) (Supplementary Fig. S1 in JMM Online). The most widely distributed ST identified in the current study (ST133) was predicted to be the primary founder of a CC containing 20 SLVs and 2 DLVs (Fig. 1). CC133 contained 17 STs that were confirmed to be associated with intramammary infections of cows, sheep and goats, along with six STs of unknown origin (no host origin stated in the database), constituting the largest animal-associated CC within *S. aureus* and comprising strains from several different geographical locations (Norway, France, UK, Portugal, Brazil, Ireland, Sweden, USA, Italy, Iceland and Austria).

agr diversity

The distribution of *agr* types among the animal-associated strains is presented in Table 2. As expected, strains sharing the same ST shared *agr* types and were distributed as follows: *agr* type I (STs 8, 22, 20, 25, 71, 97, 101, 115, 133, 352, 407, 480, 522, 692, 693, 695, 696, 697, 701, 703 and 709), *agr* type II (STs 5, 126, 151, 350, 479, 694, 705, 706 and 708), *agr* type III (STs 1, 39, 96, 699 and 700) and *agr* type IV (STs 121 and 698). The majority of the animal isolates were of *agr* type I [72 strains (61.0 %)] and *agr* type II [32 strains (27.1 %)], but several strains of *agr* type III [10 strains (8.5 %)] and of *agr* type IV [four strains (3.4 %)] were also identified. Only the bovine-associated strains had representative strains of each *agr* type. However, this difference could have been due to the larger number of bovine strains analysed (44.1 % of animal isolates) relative to other hosts.

sas typing and *spa* typing

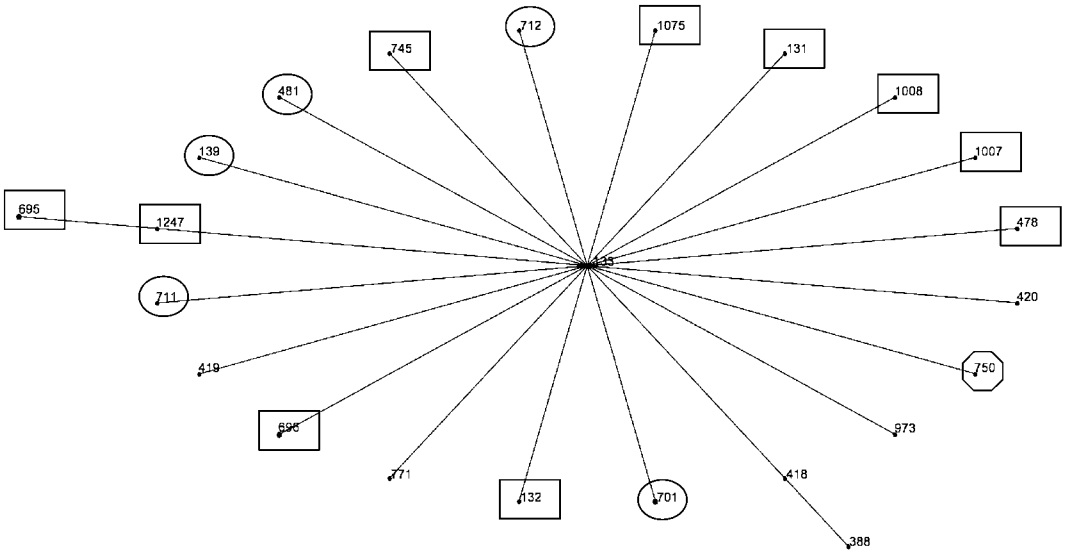
Previously, Robinson & Enright (2003) used an additional set of seven highly variable, putative surface-protein-encoding genes, designated *sas* genes, to augment the MLST scheme. Inclusion of the *sas* genes allowed these authors to resolve a more complete evolutionary history of meticillin-resistant *S. aureus*. As studies of the variability of the *sas* and *spa* genes in strains from various animal hosts had not been done previously, 46 strains representing predominant MLST clones identified herein (31 STs belonged to 15 CCs, two STs had no predicted CC and one ST was a singleton) as well as a number of randomly

Table 2. Distribution of MLST and *agr* types amongst the 118 animal isolates

MLST	Allelic profile	CC*	*agr* type	Country†	No. of isolates from indicated animal				
					Cows	Goats	Rabbits	Chickens	Sheep
1	1,1,1,1,1,1,1	1	3	NIR				1	
				ARG	1				
				BEL			1		
5	1,4,1,4,12,1,10	5	2	NIR				10	
8	3,3,1,1,4,4,3	8	1	USA	1				
				BEL			1		
20	4,9,1,8,1,10,8	20	1	ESP	1				
22	7,6,1,5,8,8,6	22	1	ITA		1			
25	4,1,4,1,5,5,4	25	1	USA	1				
				ITA		1			
39	2,2,2,2,2,2,2	30	3	ITA		1			
71	18,1,1,1,1,5,3	97	1	IRL (6), ESP (1), USA (1)	8				
				AUS		1			
				DEN					1
96	12,1,1,15,11,1,40	96	3	BEL (2), ESP (2)			4		
97	3,1,1,1,7,5,3	97	1	IRL	6				
				BEL			2		
101	3,1,14,15,11,19,3	101	1	ITA		1			
115	3,1,1,1,1,5,53	97	1	USA	1				
121	6,5,6,2,7,14,5	121	4	BEL (2), ESP (1)			3		
126	3,68,1,4,1,5,40	126	2	ESP (1), USA (4)	5				
				ITA		5			
133	6,66,46,2,7,50,18	133	1	IRL (6), USA (1), SWE (1)	8				
				ITA (5), NOR (7), AUS (1)		13			
				NOR (5), ISL (2)					7
151	6,72,12,43,49,67,59	151	2	IRL	6				
350	6,79,51,47,7,70,61	–	2	USA	1				
352	3,78,1,1,1,5,3	97	1	USA	3				
407	3,3,57,1,4,4,67	8	1	ESP			1		
479	52,79,54,18,56,32,65	479	2	ARG	1				
480	6,57,63,2,7,58,52	130	1	NOR		1			
522	18,95,45,2,7,15,5	703	1	AUS (1), ITA (1)		2			
692	12,89,1,1,4,5,90	–	1	NIR				1	
693	3,126,1,37,1,5,3	97	1	USA	1				
694	3,68,1,4,1,5,89	126	2	USA	1				
695	6,66,46,69,91,50,18	133	1	USA	1				
696	6,66,46,19,7,50,18	133	1	ESP	1				
697	3,78,1,1,1,5,91	97	1	SWE	1				
698	16,127,12,2,13,13,15	50	4	USA	1				
699	1,89,1,15,1,4,3	Singleton	3	USA	1				
700	6,57,45,2,7,95,52	130	3	ITA		1			
701	6,129,46,2,7,50,18	133	1	ITA		1			
703	18,128,45,2,7,15,5	703	1	ITA		4			
705	6,72,50,43,49,67,59	151	2	SWE					1
706	86,3,1,1,1,1,10	5	2	DEN					1
708	1,4,99,4,12,1,10	5	2	USA	1				
709	3,1,1,1,95,5,92	97	1	ARG	1				
Total					52	32	12	12	10

*–, The ancestral type of the CC of these strains could not be identified.

†ARG, Argentina; AUS, Austria; BEL, Belgium; DEN, Denmark; ESP, Spain; IRL, Ireland; ISL, Iceland; ITA, Italy; NIR, Northern Ireland; NOR, Norway; SWE, Sweden; USA, United States of America. The numbers of strains from each country are listed in parentheses.



	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
ST133	6	66	46	2	7	50	18
ST696	6	66	46	**19**	7	50	18
ST701	6	**129**	46	2	7	50	18
ST711	6	66	46	2	7	**97**	18
ST481	6	66	46	2	**54**	50	18

Fig. 1. eBURST-generated CC containing the major animal-associated ST133. MLST type ST133 was the most widely distributed ST identified, accounting for 23 % of the strains, and forms the primary founder of this CC comprising 23 STs. It was associated with cows, goats and sheep. The table shows the allelic profiles of a number of the STs in the CC with deviations from the allelic profile of ST133 in bold, including two novel STs identified herein (ST696 and ST701). Many of the STs in this CC have previously been shown to be associated with cows, goats and sheep from Norway, France, Spain, Italy, UK, Portugal and Brazil (according to the MLST database). The remaining STs have no known source listed in the MLST database. Circles indicate goat, squares indicate bovine and octagons indicate sheep origins.

selected STs only represented once (n=15) were *sas*-typed (Table 3).

The 46 animal-associated *S. aureus* strains generated 25 *sas* types, 18 of which were novel (numbered 8–25). Four of the strains failed to generate a PCR product for one of the *sas* alleles, namely bovine strains RF122 and RF80 (negative for *sasB*) and goat strains St11 and St66 (negative for *sasF*), and as a result were non-typable. Analysis of the recently sequenced genome of bovine strain RF122 (accession no. NC_007622; Herron-Olson *et al.*, 2007) showed that this strain lacks the region amplified by the *sasB* primers. Strain RF80 possessed the identical ST, *agr* type, *spa* type and superantigen gene profile of strain RF122 [ST151; *agr* type 2; Ridom *spa* type t529; SaPIbov (*sec*, *sell*, *tst*), *egc* (*selo*, *selm*, *sei*, *selu*, *seln*, *seg*)] (Smyth *et al.*, 2005). Goat strains St11 and St66 possessed different MLST types, ST522 and ST703. These non-typeable isolates resulted in a typeability of 91.3 % for *sas* typing.

Table 3 shows the nucleotide sequence variation among alleles of housekeeping and *sas* loci in the 46 animal-associated strains typed. The number of alleles identified in the animal strains ranged from 8 (*gmk*) to 19 (*sasF* and *sasI*). The number of variable sites ranged from 10 (*gmk*) to 76 (*sasA*) and the number of parsimony informative sites ranged from 2 (*sasD*) to 51 (*sasA*). Knowing that variation in the genes encoding surface-exposed proteins could be due to the selective pressure of the host immune system, the ratio of synonymous to non-synonymous mutations was examined. However, no evidence of diversifying selection was identified in any of the *sas* gene fragments sequenced as the proportion of synonymous nucleotide changes was greater than the proportion of non-synonymous nucleotide changes (Table 3).

The distribution of the *sas* types among the animal strains is shown in Table 4. Isolates that were identical by MLST STs shared identical *sas* types, with the exception of six MLST

Table 3. Nucleotide sequence variation among alleles of housekeeping and *sas* loci in the 46 animal-associated strains

Gene	Sequence length (bp)	No. of alleles	No. of polymorphic sites*	No. of informative sites*	d_S/d_N†
arcC	456	9	13	5	8.4033
aroE	456	16	32	7	5.8950
glpF	465	12	15	3	7.3194
gmk	429	8	10	5	14.2635
pta	474	10	13	5	6.2209
tpi	402	15	20	10	7.2258
yqiL	516	17	30	17	10.0331
sasA	462	18	76	51	8.8984
sasB	462	14	50	21	12.7283
*sasD**	453	13	16	2	
sasE	450	15	52	22	9.1203
*sasF**	439	19	31	11	
sasH	467	17	46	30	10.0685
sasI	467	19	70	45	6.1120

*Excludes insertions and deletions at *sasD* and *sasF*.
†The d_S/d_N ratio is the number of synonymous or silent nucleotide changes (d_S) to the number of non-synonymous or amino-acid-replacing nucleotide changes (d_N).

ST133 isolates which generated two *sas* types – *sas* type 9 (n=4) and *sas* type 10 (n=2). MLST ST696, an SLV of ST133, shared *sas* type with ST133, namely *sas* type 9. Strains that did not share MLST STs but were found in the same CC often shared *sas* types. MLST ST352 and ST697 (CC97) were both of *sas* type 11. Two of the animal-associated strains that shared MLST STs with human isolates were found to have differing *sas* alleles from the human-associated STs, namely strain 1006 (MLST ST8) and DS46 (MLST ST121), which differed at the *sasF* allele (allele 39, unique to the animal strains) and at the *sasA* (allele 25, unique to the animal strains), *sasB* (allele 30, unique to the animal strains) and *sasH* (allele 15, found in MLST ST51, an SLV of ST121) alleles, respectively (Table 4).

Thirty of the *sas*-typed isolates were also *spa*-typed. The Ridom *spa* types are listed in Table 4. STs from the same CC shared similar or identical *spa* repeat profiles. However, *spa* typing was slightly more discriminatory on the basis of its index of discrimination (D 0.9609, CI 0.9261–0.9958) than either *sas* typing (D 0.9494, CI 0.9124–0.9865) or MLST typing (D 0.9379, CI 0.8859–0.9899) or *sas* and MLST typing combined (D 0.9540, CI 0.9200–0.9881). However, the confidence intervals did overlap. One rabbit strain (ST97) generated a *spa* type with a single repeat (KH17, strain code DS50).

Estimates of recombination using MLST

Using eBURST, SLVs of ancestral STs were identified. Ten of the SLVs contained alleles that were found to differ at one nucleotide position from their predicted ancestral allele (Table 5). Nine substitutions were found to be non-synonymous. Two STs (ST71 and ST696) were found to contain alleles that differed at two nucleotides from their ancestors (ST97 and ST133). Both alleles were found in several CCs, indicating possible recombination events.

Phylogenetic analysis of MLST types

Neighbour-joining and maximum-parsimony trees were generated to compare each ST in the current study with the major human-associated STs from Cooper & Feil (2006) and Robinson *et al.* (2005) (Fig. 2). The previously identified division of the *S. aureus* subspecies *aureus* population into two subgroups is indicated on the tree, namely subspecies groups 1 and 2, indicated by an arrow (subspecies group 1 is further divided into subgroups 1a and 1b in the study of Cooper & Feil, 2006).

These phylogenetic analyses assigned the animal-associated STs to both subgroups. Ruminant-animal-associated lineages, with high bootstrap support, were identified: one in subspecies group 2 and three in subspecies group 1. Lineage L1 is composed of STs 71, 97, 115, 352, 693 and 697, and is the same cluster as CC97. Lineage L2 is predominantly composed of ruminant-animal-associated isolates corresponding to STs 133, 695, 696 and 701, and this cluster is also the same as CC133. It is interesting to note that ST696 comes out basal to ST133 on the neighbour-joining tree (Fig. 2) owing to its imported *gmk* allele, which is present in unrelated CCs. Lineage L3 consists of two pairs of goat-associated STs 480, 522, 700, and 703, while lineage L4 contains ST151, the bovine-associated clone (Guinane *et al.*, 2008), along with STs 350, 479 and 705.

The distribution of bovine STs across the two subspecies groups would also explain the presence of all four *agr* types among bovine-associated strains. In the study of Robinson *et al.* (2005), STs in subspecies group 2 were of *agr* types I–III and STs in subspecies group 1 were of *agr* types I–IV.

DISCUSSION

In the current study, molecular typing methods have been applied to the characterization of genotypically/phenotypically predominant strains of *S. aureus* that were representative of those in diverse geographical origins obtained from animals in addition to cows. This has allowed their relationships to human isolates to be analysed at the molecular genetic level.

We initially characterized all the isolates by MLST and *agr* typing and on these molecular criteria chose representatives for *sas* and *spa* typing. To our knowledge, this study represents the first application of *sas* typing to the epidemiology of animal-associated *S. aureus*. Whilst *sas* typing showed a similar discriminatory ability to MLST, in some cases the animal-associated strains exhibited *sas*

Table 4. Distribution of *sas* and *spa* types among the 46 animal-associated strains that were typed by MLST, *sas* typing and *spa* typing

sas type*	*sas* allelic profile *A,B,D,E,F,H,I*	MLST ST	CC	*spa* repeat profile†	Ridom *spa* type	Country‡	No. of isolates from indicated animal				
							Cows	Goats	Rabbits	Chickens	Sheep
1	2,3,2,1,6,5,5	5	5	26-23-17-34-17-20-17-12-17-16 (n=5)	t002	NIR				7	
2	5,5,4,7,2,4,8	39	30	–	–	ITA		1			
3	3,7,3,4,5,1,2	22	22	26-23-13-23-31-05-17-25-17-25-16-28	t005	ITA		1			
4	7,15,9,8,28,22,12	25	25	04-21-12-41-20-17-12-17	t081	USA	1				
4	7,15,9,8,28,22,12	25	25	04-21-12-41-20-17-12-12-17	t078	ITA		1			
5	2,10,9,8,27,19,10	97	97	26-23-12-21-17-34-34-34-33-34	t1236	IRL	1				
5	2,10,9,8,27,19,10	97	97	07-23-34-34-33-34	t1247	IRL	2				
5	2,10,9,8,27,19,10	97	97	07	–	BEL			1		
6	11,3,8,1,18,12,17	1	1	07-23-21-16-34-33-13	t127	BEL			1		
7	19,3,9,12,7,23,6	101	101	04-20-12-17-20-17-12-17-17	t056	ITA		1			
8	13,10,3,8,16,15,10	71	97	04-17	t524	IRL	2				
9	23,29,3,27,34,15,30	133	133	03-16-12-21-17-23-13-17-17-17-23-24 (n=1)	t2678	IRL	2				
9	23,29,3,27,34,15,30	133	133	–	–	ITA		1			
9	23,29,3,27,34,15,30	133	133	03-16-12-21-17-23-13-17-17-17-23-24	t2678	NOR					1
9	23,29,3,27,34,15,30	696	133	03-16-12-21-17-23-13-17-17-17-23-24	t2678	ESP	1				
10	30,29,3,27,34,15,30	133	133	03-23-24	t1403	SWE	1				
10	30,29,3,27,34,15,30	133	133	–	–	NOR		1			
11	2,10,22,8,27,19,10	352	97	07-16-34-34-33-34	t1201	USA	1				
11	2,10,22,8,27,19,10	697	97	–	–	SWE	1				
12	32,10,9,8,27,19,10	115	97	07-23-12-21-17-34-34-34-33-34	t267	USA	1				
13	15,18,12,15,40,18,9	698	50	04-20-17-25	t519	USA	1				
14	2,3,2,1,6,5,37	708	5	–	–	USA	1				
15	19,3,21,32,18,39,5	699	Singleton	–	–	USA	1				
16	31,21,21,31,38,40,10	692	None	–	–	NIR				1	
17	2,2,3,1,new,6,3	407	8	11-12-12-21-17-34-24-34-22-25	t656	ESP			1		
18	7,10,14,33,36,38,10	694	126	07-23	t605	USA	1				
19	2,2,3,1,39,6,3	8	8	–	–	USA	1				
20	27,27,24,26,33,23,28	700	130	–	–	ITA		1			
21	26,28,26,26,33,36,33	480	130	–	–	NOR		1			
22	25,30,3,11,21,15,11	121	121	14-44-13-12-17-23-18-17	t645	BEL			1		
23	7,10,14,33,36,38,36	126	126	07-23	t605	USA	1				
24	29,26,25,30,37,41,34	350	None	–	–	USA	1				
25	19,32,9,1,10,42,35	96	96	–	–	BEL			1		
NT	34,27,3,28, ,15,31	703	703	04-31-17-25-17-17	t1534	ITA		1			
NT	34,27,3,28, ,15,31	522	703	–	–	ITA		1			
NT	32, ,19,31,34,55,54	151	151	04-34	t529	IRL	2				
Total							22	10	5	8	1

*NT, Non-typeable.

†–, Not done.

‡See Table 2 for three-letter country codes.

alleles that could serve to discriminate animal-associated STs from human-associated STs. No evidence was found of selection acting upon the *sas* alleles. However, two instances of gene loss were identified in the animal-associated STs, namely the loss of the *sasB* gene from ST151 (lineage L4) and the loss of the *sasF* gene from ST522 and

Table 5. Allelic variation in SLVs

SLV ST	Ancestral ST	Variable locus	Ancestral allele	SLV allele	No. of nucleotide differences	Other clones containing SLV allele	Amino acid change
71	97	*arcC*	3	18	2	Multiple	Non-synonymous
115	97	*yqiL*	3	53	1	115, 347, 349	Non-synonymous
352	97	*aroE*	1	78	1	352 only	Non-synonymous
480	130	*glpF*	45	63	1	480 only	Synonymous
522	703	*aroE*	128	95	1	522 only	Non-synonymous
694	126	*yqiL*	40	89	1	694 only	Non-synonymous
696	133	*gmk*	2	19	2	Multiple	Synonymous
698	50	*aroE*	16	127	1	698 only	Non-synonymous
700	130	*tpi*	58	95	1	700 only	Non-synonymous
701	133	*aroE*	66	129	1	701 only	Non-synonymous
705	151	*glpF*	12	50	1	351, 356, 705	Non-synonymous
708	5	*glpF*	1	99	1	708 only	Synonymous

ST703 (lineage L3). The recent analysis of the genome sequence of a bovine strain, RF122 (ST151), revealed evidence for extensive loss of gene function, a common characteristic of bacteria undergoing adaptation to a novel niche (Herron-Olson *et al.*, 2007). The loss of *sas* genes in these STs may serve as a host adaptation. The loss of the *sasF* gene is of interest as this gene was found to be present in all strains in the study of Cooper & Feil (2006) and showed the closest agreement with the consensus *S. aureus* species tree. However, their study did not include isolates of animal origin.

ST151 has yet to be isolated from humans. This lineage appears to be at present restricted to Ireland and the UK, as we did not find animal-associated isolates of ST151 from the other countries sampled. ST151 has been shown to be hypersusceptible to the acquisition of resistance genes from enterococci (Sung & Lindsay, 2007). No investigation of the susceptibility to gene acquisition from enterococci by the other members of the L4 lineage, namely ST359, ST479 and ST705, has been done to date. The occurrence of additional STs that may have the potential to acquire resistance to vancomycin warrants further investigation.

The ruminant-associated lineage L1 was more closely related to other human *S. aureus* lineages than to lineages L2, L3 and L4. The L2 lineage consisted only of strains infecting cows, sheep and goats. No evidence of CC133 causing human infection has been found to date. We note that ST133 has been found herein in ruminant animals in several European countries as well as the USA (www.mlst.net). This would appear to indicate that this clone is particularly well adapted to its host.

In some cases, animal-associated STs were shared with or were closely related to STs of human origin, indicating a sporadic anthropozoonotic infection or a very recent host adaptation. Unlike the ruminant-associated clones identified here, which correspond to many novel MLST STs and *sas* types and show evidence of gene loss, the chicken-associated clone shared MLST alleles, *sas* alleles and the *spa*-repeat profile of human-associated ST5 strains, providing evidence for a recent transfer event (Robinson & Enright, 2003). The chicken isolates had been found to be susceptible to penicillin and shown not to produce Protein A as part of another study (Smyth *et al.*, 2006), indicating that these strains were poultry biotypes. It is possible that this avian clone represents a recent transfer and adaptation to a new host, although it cannot be discounted that humans were colonized by chicken-associated, penicillin-susceptible strains that subsequently acquired β-lactam antibiotic resistance and became globally successful. The differences in phenotype, i.e. penicillin susceptibility and lack of Protein A expression, may allow these particular isolates to colonize and infect different hosts.

CC121, whilst predominantly associated with serious skin infections in humans (Melles *et al.*, 2004), was associated with rabbits in the current study and previously with sheep-associated clones from France (Vautor *et al.*, 2005). The rabbit strains of ST121 had previously been identified as being highly virulent in European rabbitries and being capable of causing epidemics characterized by subcutaneous abscesses, mastitis, pododermatitis and septicaemia (Hermans *et al.*, 2000, 2003; Vancraeynest *et al.*, 2006). Like the chicken-associated clone, rabbit-associated ST121 shared the MLST and *spa*-repeat profile of human skin-infection-associated ST121 (Melles *et al.*, 2004), but differed at three *sas* loci, one allele of which was found in human isolates that had been *sas*-typed.

The current study used sequence-typing methods to investigate the diversity of a relatively large number of *S. aureus* isolates from five different animal species. Only a small number of sequence-typing studies describing MLST types of animal-associated *S. aureus* have been presented, and none has included chicken-associated *S. aureus*. ST97 was found herein to also be associated with cows and rabbits and has previously been shown to be associated with humans (Melles *et al.*, 2004). In a molecular-typing study of 77 animal-associated strains of *S. aureus* using AFLP, van Leeuwen *et al.* (2005) showed that MLST types

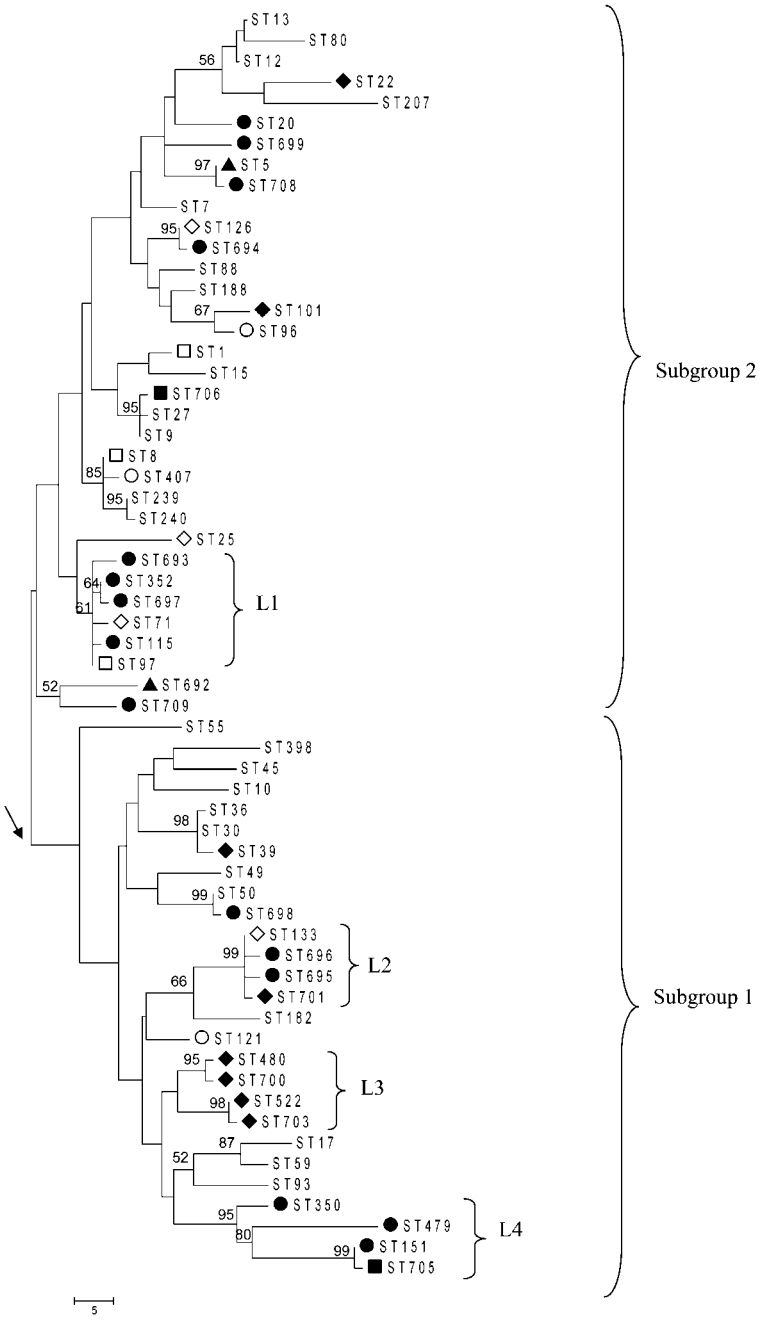


Fig. 2. MEGA-generated maximum-parsimony tree using the concatenated nucleotide sequences of the seven MLST genes. One of the 32 most parsimonious trees is shown. One representative of each animal-associated ST was used along with representative human-associated STs. Animal-associated STs found to be only associated with cows are indicated by filled circles, with goats by filled diamonds, with chickens by filled triangles, with sheep by filled squares and with rabbits by unfilled circles. Ruminant-associated STs are indicated by an unfilled diamond and non-host-specific STs, i.e. those associated with various animals, are indicated by unfilled squares. *S. aureus* subspecies *aureus* subgroups 1 and 2 are those described by Cooper & Feil (2006) and Robinson *et al.* (2005). This division is indicated by an arrow. Subgroup 1 was further subdivided into subgroups 1a (STs 10, 22, 30, 36, 45 and 207) and 1b (ST17, 50, 49, 59, 121 and 182) (Cooper & Feil, 2006). Lineages L1, L2 and L4 are well supported by bootstrapping (bootstrap support is indicated on the tree if >50). Lineage L3 consists of two pairs of well-supported STs.

ST1, ST7, ST8, ST9, ST15, ST22, ST30 and ST45 were associated with animals and were divided into four AFLP clusters. STs 1, 8 and 33 were identified in the current work.

The majority of the animal-associated isolates herein were of *agr* types I and II, which agrees with previous studies using animal-associated and human-associated *S. aureus* (Gilot & van Leeuwen, 2004; Gilot *et al.*, 2002; Goerke *et al.*, 2003; Lina *et al.*, 2003; Monecke *et al.*, 2007), although there have been rare occurrences of strains of *agr* types III and IV. The most prevalent ST (ST133) was of *agr* type I. Type ST151 isolates were *agr* type II (Fitzgerald *et al.*, 2001). The ST121 isolates from rabbits herein were *agr* type IV. In terms of subspecies subgroups (Fig. 2), all four *agr* types were distributed in subgroup 1 but only *agr* types I, II and IV were in subgroup 2. This likely reflects the distribution of animal-associated STs and hence their *agr* types in the two subgroups (Robinson *et al.*, 2005).

The present molecular genetic typing analysis provides further vistas into the diversity of genotypes found in animal-associated *S. aureus* and the relationships of these genotypes to those found in the human population. In addition, our genetic evidence would seem to suggest that whilst certain ruminant-associated lineages have diverged

from human-associated lineages (animal-specific MLST STs, *sas* types) as evidenced by gene loss, and distinct animal-associated *sas* alleles, avian-associated and rabbit-associated clones may have been recently transmitted from humans or to humans owing to the sharing of genotypes across these hosts. Taken together, these data provide new insights into the ruminant and avian and rabbit host adaptation of *S. aureus*.

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