Molecular Genetic Typing of *Staphylococcus aureus* from Cows, Goats, Sheep, Rabbits and Chickens

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Summary

In this work, *S. aureus* isolates from cows, goats, sheep, rabbits, chickens and a cat were analysed by a number of different genotyping methods in an attempt to characterise the clonal relationships of animal-associated *S. aureus.*

Firstly, the prevalence of the genes encoding *S. aureus* superantigens (SAg), superantigen-like proteins (SAg-like) and the SSL toxins, modulators of the host's immune system, was determined. This was achieved by the design of PCR primers and multiplex PCR assays. The genes encoded by the *egc* locus were demonstrated to be highly prevalent in *S. aureus* strains of animal origin, being found in 42.7 % of SAg and SAg-like gene-positive isolates. Every animal strain was found to contain a locus of genes encoding the SSL proteins. Mitogenicity assays showed that strains encoding SAg and SAg-like genes could stimulate the proliferation of human T lymphocytes, a hallmark of superantigenicity. These assays also showed that, despite encoding *ssl* genes, strains that were negative for SAg and SAg-like protein encoding genes, were negative in terms of mitogenicity suggesting that the SSL proteins are not superantigens.

To further assess the role of SAg, SAg-like and SSL proteins in animal infection, *E. coli* strains expressing recombinant *S. aureus* SAg, SAg-like and SSL proteins were used in Western immunoblotting experiments. Serum samples from cows with or without mastitis were screened for antibodies to the SAg, SAg-like and SSL proteins. The SAg and SAg-like proteins did not react with any of the serum samples implying that these proteins were either not expressed *in vivo* or are expressed at very low levels. The SSL proteins, however, did react with the serum samples from cows that had mastitis implying that these proteins may play a role *in vivo*.

The techniques of Multilocus Sequence Typing (MLST), RAPD typing and *agr* typing were used to investigate the genetic relationships of 120 isolates of *S. aureus* from different animal hosts from a number of different geographical locations. MLST revealed that a limited number of types were responsible for the majority of cases of animal-associated infection. The commonest MLST type, ST133 was identified in 23 % of the animal strains tested and was only associated with strains from sheep, goats and cows.

Forty-eight animal-associated isolates that represented the major clonal types were further typed using the nucleotide sequences of seven predicted surface proteins (*sas* typing) and using the polymorphic short sequence repeat region (SSr) of the protein A-encoding gene, *spa*. The animal–associated strains generated many novel *sas* sequence types that had not been previously identified in human–associated *S. aureus*. The *spa* typing method was found to have the highest resolution of all the three sequence typing methods used. The combination of MLST and *spa* typing may prove to be a valuable alternative typing method to PFGE for the examination of animal–associated *S. aureus*.

Introduction

S. aureus can also cause a number of infections in animals such as tick-associated pyaemia in lambs, staphylococcosis in rabbits, septicaemia, abscesses and chondronecrosis in chickens and pneumonia and osteomyelitis complex in turkeys. *S. aureus* is the most frequent cause of bovine mastitis, a disease that is of economic importance worldwide (Beck *et al.,* 1992). Typically staphylococcal mastitis is chronic in nature, with subclinical mastitis being the most common form.

The ability of *S. aureus* to cause disease is thought to be due to a combination of virulence factors, namely, toxins, cell-surface-associated adhesins and secreted proteins (Peacock *et al*., 2002). Among the many known virulence factors of *S. aureus* are the staphylococcal enterotoxins and toxic shock syndrome toxin 1. The enterotoxins and TSST-1 belong to the SAg family. Superantigens (SAgs) stimulate T-cell proliferation activating as many as one in five T-cells, whereas conventional antigen presentation activates 1 in 10,000 T-cells. As a result massive amounts of cytokine are released

leading to the subsequent immunomodulative and other deleterious effects brought about by SAgs (Alouf & Müller-Alouf, 2003; Proft & Fraser, 2003).

The *S. aureus* enterotoxins (SEA–SEE) cause staphylococcal food-poisoning (Balaban & Rasooly, 2000; Jablonski & Bohach, 2001). In recent years 18 staphylococcal enterotoxins (SEs) and enterotoxin-like proteins (SEls) as well as a number of variants of some of these have been described. It is likely that additional SEs and SEls will be discovered. *S. aureus* also produces a family of exoproteins that have been shown to have sequence similarity and three-dimensional structural similarities to the SE superantigens, namely, the staphylococcal exotoxin-like proteins (SETs) that have subsequently been renamed the staphylococcal superantigen–like proteins (SSL) (Lina *et al.*, 2003). The roles of the immunomodulatory SAg and SAg-like proteins as well as the SSL proteins in animal infections have yet to be elucidated.

Various molecular typing techniques have been applied to the study of *S. aureus* from animals. Few groups have utilised novel DNA sequence typing methods such as MLST, *sas* typing and *spa* typing. No one has yet investigated strains of *S. aureus* from cows, goats, sheep, rabbits and chickens using MLST, *sas* typing and *spa* typing.

1. Novel and putative superantigen genes in strains of *Staphylococcus aureus* from cows, goats, sheep, rabbits and chickens

Little is known about the occurrence and significance of the novel SAg and SAg-like genes (*seg-sei, selj-selr, selu*) in strains of *S. aureus* from animal infection. Some of these novel SEs and SEls may contribute to the persistence of *S. aureus* in subclinical mastitis. The present study was designed to investigate the frequency of genes encoding the SEs and SEls by means of multiplex PCR in strains of *S. aureus* from cows, goats, sheep, rabbits and chickens.

- 191 animal associated isolates were tested in this study, 110 were positive for at least one SAg gene (57.6 %) (Table 1). Only seven of the 15 SE and SEl genes and the TSST-1 gene screened for were present in more than 23 % of the strains, namely, *sec*, *seg*, *sei*, *sell*, *selm*, *seln*, *selo* and *tst*. The *sea* and *see* genes were not detected in any of the strains.
- The most common combination of enterotoxin genes observed was the *egc* gene cluster that was present in 42.7 % of the SAg gene-positive strains. The combination of *sei, selm, seln* and *selo* genes (*egc* cluster lacking the *seg* gene) was found in 11 strains (10 % of the SAg gene-positive strains).
- All 51 animal–associated strains that possessed the *sec* gene also had the *tst* gene; in those lacking the *sec* gene, the *tst* gene was also absent. The *sec, sell* and *tst* genes (SaPIbov pathogenicity island combination of genes) were found in 40 % of the SAg gene-positive strains (Fitzgerald *et al*., 2001). The combination of *sec* and *tst* genes without the *sell* gene was found in a further 7 strains (6.4 % of SAg gene-positive isolates). The combination of *sed* and *selj* genes was found in 7.3 % of SAg gene-positive strains.
- Sequencing of the *sec* gene was performed for seven strains, three from goats, two from cows and two from sheep and the results could indicate the presence of novel *sec*-encoding genetic elements in these animal strains.
- Seventy-nine of the ninety-nine bovine isolates surveyed for SAg genes were screened for mitogenic activity, a hallmark of superantigen expression. Thirty-six strains induced T-cell proliferation, of which 32 had at least one or more SAg genes while the four remaining strains did not possess the SAg genes tested for. Twenty-nine isolates, that did not possess the SAg genes tested for, did not induce T-cell proliferation. The remaining fourteen isolates exhibited borderline mitogenicity; 12 of these 14 isolates did not have one of the SAg genes tested for while two possessed at least one SAg gene.

Table 1. PCR analysis for SAg genes in animal-associated strains of *S. aureus*.

SAg	Cow (n=99)		Goat (n=39)		Sheep (n=23)		Rabbit (n=15)		Chicken (n=15)		Total (n=191)	
gene	n*	%	n	%	n	%	n	%	n	%	n	%
SE genes	45	45.5%	22	56.4%	20	87%	8	53.3%	14	93.3%	110	57.6%
sea	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
seb	0	0%	0	0%	1	4.3%	0	0%	0	0%	1	0.5%
sec	19	19.2%	18	46.2%	14	60.9%	0	0%	0	0%	51	26.7%
sed	6	6.10%	0	0%	1	4.3%	1	6.7%	0	0%	8	4.2%
see	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
seg	22	22.2%	4	10.3%	3	13%	5	33.3%	13	86.7%	47	24.6%
seh	0	0%	0	0%	1	4.3%	2	13.3%	1	6.7%	4	2.1%
sei	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%
sej	6	6.1%	0	0%	1	4.3%	1	6.7%	0	0%	8	4.2%
sek	0	0%	0	0%	0	0%	0	0%	1	6.7%	1	0.5%
sel	18	18.2%	16	41%	10	43.5%	0	0%	0	0%	44	23%
sem	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%
sen†	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%
seo	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%
seq	0	0%	0	0%	0	0%	0	0%	1	6.7%	1	0.5%
TSST-1 gene‡	19	19.2%	18	46.2%	14	60.9%	0	0%	0	0%	51	26.7

* Total number of animal-associated strains positive for any SE gene.

† 7 of the bovine strains and 1 of the sheep strains did not generate an amplicon for *sen* in multiplex PCR but were found to produce amplicons in single locus PCR.

‡ Animal specific variants of the gene encoding TSST-1 exist, for example TSST-ovine (Ho *et al*., 1989). In the current study the *tst* amplicons were not sequenced.

The present study is the most comprehensive survey for SAg genes in *S. aureus* strains of animal origin to date. The *sec* gene sequencing data suggest that *sec*-ovine maybe present on a pathogenicity island with the *tst* gene in the presence or absence of the *sell* gene. The mitogenicity findings suggest that a number of the strains may harbour genes for as yet unidentified SAgs or *selp*, *selr* and *selu* genes, the latter two of which were identified during the present study (Kuroda *et al*., 2001; Letertre *et al*, 2003; Omoe *et al*, 2003).

The demonstration herein that the *egc* cluster and the bovine pathogenicity island SaPIbov are the commonest in clinical *S. aureus* isolates of animal origin raises questions about the potential roles of these SAgs in animal infection. One or more of these SAgs may confer a survival advantage in the udder through modulation of the immune response.

2. An investigation into the presence and composition of the staphylococcal exotoxin-like gene locus (*ssl*) in strains of *S. aureus* of animal origin.

Members of the SSL family of exoproteins can interact with components of the immune system such as immunoglobulin and complement and can interact with human antigen-presenting cells (monocytes and dendritic cells) (Al-Shangiti *et al*., 2005). This activity could potentially facilitate bacterial colonisation rather than contribute to host protection. Thus, like SAgs, the SSL exoproteins may distract the host's immune system, but do so via entirely different molecular mechanisms to SAgs. At the start of this study little data were available on the frequency of the *ssl* genes in strains of *S. aureus* from human and animal sources. The aims of this study were to develop a PCR-based screening method for *ssl* genes and to investigate the frequency of these genes in strains of *S. aureus* of animal origin. 52 animal–associated strains tested that represented major clonal types associated with animal *S. aureus* infection were analysed. Quarter milk and serum samples from cows with and without mastitis were obtained from Teagasc Dairy Production Centre in Moorepark, Fermoy, Co. Cork. The milk samples were analysed for the presence of *S. aureus* strains, that were subsequently RAPD typed. The RAPD profiles obtained for the new isolates were compared to the RAPD profiles previously obtained for Irish bovine isolates (Fitzgerald *et al*., 1997). These isolates were also tested for the presence of the *ssl* genes as described herein and for the SAg and SAg-like genes as described in the previous section.

- All 52 strains contained at least one *ssl* gene and there was variation in the number of *ssl* genes encoded by the animal–associated strains (including those from recent mastitis infection). Clonally related strains had the same complement of *ssl* genes.
- The *ssl10, ssl5, ssl8* and *ssl7* genes were present in 100% of the animal-associated strains. The *ssl9* gene was present in all strains but one bovine isolate, namely, MSA1455. The *ssl3* gene was present in 50 of the 52 animal-associated strains tested (except for one bovine and one caprine isolate).

In the current study, the *ssl* locus was found to be present in every animal-associated strain, to show minor variation in gene content by PCR, and to show variation in RFLP patterns in Southern blots. The number and combination of *ssl* genes in strains of animal origin seems to reflect clonal relationships.

Table 2. Combinations of *ssl* genes in 52 isolates of *S. aureus* of animal origin and 6 control human strains.

Strain *	Origin	Host	RAPD type†	*ssl* genes ‡							Mitogenicity §	SAg and SAg-like genes#
NCTC 8325-4	USA	Human	ND¶	3	4	5	7	8	9	10	ND	
MSSA-476	UK	Human	ND	3	4	5	7	8	9	10	ND	
MRSA252	UK	Human	ND	3	4	5	7	8	9	10	ND	
COL	USA	Human	ND	3	4				9	10	ND	
NCTC 6571	USA	Human	D	3	4	5	7		9	10	ND	
MSA2335	USA	Human	D		4	5	7	8	9	10	ND	
RF101	IRL	Cow	11	3		5	7	8	9	10	–	Negative
RF102	IRL	Cow	33	3		5	7	8	9	10	+	SaPIbov, *egc*
RF103	IRL	Cow	11	3		5	7	8	9	10	+	Negative
RF107	IRL	Cow	11	3		5	7	8	9	10	–	Negative
RF108	IRL	Cow	33	3		5	7	8	9	10	+	SaPIbov, *egc*
RF110	IRL	Cow	33	3		5	7	8	9	10	+	SaPIbov, *egc*
RF111	IRL	Cow	16	3		5	7	8	9	10	–	Negative
RF115	IRL	Cow	16	3		5	7	8	9	10	–	Negative
RF116	IRL	Cow	16	3		5	7	8	9	10	–	Negative
RF117	IRL	Cow	16	3		5	7	8	9	10	–	Negative
RF120	IRL	Cow	33	3		5	7	8	9	10	+	*egc*
RF121	IRL	Cow	33	3		5	7	8	9	10	+	*egc*
RF122	IRL	Cow	33	3		5	7	8	9	10	+	SaPIbov, *egc*
RF123	IRL	Cow	11	3		5	7	8	9	10	–	Negative
RF285	IRL	Cow	23	3		5	7	8	9	10	+	SaPIbov
RF286	IRL	Cow	23	3		5	7	8	9	10	+	SaPIbov
2 (2521-3rd)	IRL	Cow	ND	3		5	7	8	9	10	ND	*seh*
6 (2242-3rd)	IRL	Cow	ND	3		5	7	8	9	10	ND	*seh*
7 (2242-3rd)	IRL	Cow	ND	3		5	7	8	9	10	ND	*seh*
10 (2242-4th)	IRL	Cow	ND	3		5	7	8	9	10	ND	*seh*
12 (2242-4th)	IRL	Cow	ND	3		5	7	8	9	10	ND	*seh*
14 (2480-4th)	IRL	Cow	ND	3		5	7	8	9	10	ND	*egc*
21 (2487-3rd)	IRL	Cow	ND	3		5	7	8	9	10	ND	Negative

22 (2487-3rd)	IRL	Cow	ND	3		5	7	8	9	10	ND	Negative
MSA12.1	USA	Cow	ND	3		5	7	8	9	10	+	*egc* without *seg*
MSA13.1	USA	Cow	31	3		5	7	8	9	10	ND	ND
MSA17.1	USA	Cow	35	3		5	7	8	9	10	±	*egc*
MSA103.14	USA	Cow	ND	3		5	7	8	9	10	+	*egc*
MSA148	USA	Cow	ND	3		5	7	8	9	10	+	*egc* without *seg*
MSA915	USA	Cow	16	3		5	7	8	9	10	ND	*egc*
MSA916	USA	Cow	24	3		5	7	8	9	10	–	Negative
MSA927	USA	Cow	ND	3		5	7	8	9	10	–	Negative
MSA930	USA	Cow	ND	3		5	7	8	9	10	+	*egc* without *seg*
MSA948	USA	Cow	19	3		5	7	8	9	10	ND	Negative
MSA1003	USA	Cow	16			5	7	8	9	10	–	Negative
MSA1006	USA	Cow	3	3		5	7	8	9	10	ND	*sed, selj*
MSA1058	USA	Cow	ND	3		5	7	8	9	10	–	Negative
MSA1363	USA	Cow	6	3		5	7	8	9	10	+	*egc*
MSA1369	USA	Cow	11	3		5	7	8	9	10	ND	ND
MSA1455	USA	Cow	ND	3		5	7	8		10	–	Negative
MSA1460	USA	Cow	ND	3		5	7	8	9	10	+	*egc*
MSA1468	USA	Cow	21	3		5	7	8	9	10	±	Negative
St65	ITA	Goat	42	3		5	7	8	9	10	ND	Negative
St66	ITA	Goat	42	3		5	7	8	9	10	ND	Negative
St24	ITA	Goat	7		4	5	7	8	9	10	ND	*sec, tst*
1563-4	NOR	Goat	26	3		5	7	8	9	10	ND	ND
895-1	NOR	Sheep	29	3		5	7	8	9	10	ND	SaPIbov, *egc*
KH454	BEL	Rabbit	18	3		5	7	8	9	10	ND	*egc*
KH275	BEL	Rabbit	3	3		5	7	8	9	10	ND	Negative
KH365	BEL	Rabbit	1	3		5	7	8	9	10	ND	*seh*
39	NIR	Chicken	2	3		5	7	8	9	10	ND	*egc*
72	NIR	Chicken	2	3		5	7	8	9	10	ND	*egc*

* Strains 2, 6, 7, 10, 12, 14, 21 and 22 were isolated from milk samples from cows with recent mastitis and were not included in Smyth *et al.* (2005); the numbers in brackets refer to the cow and the udder quarter from which the milk sample was obtained.

† RAPD – a number of the bovine strains included herein had been previously RAPD typed by Fitzgerald *et al*. (1997, 2000). For the purposes of the present study, all RAPD types were numbered or renumbered to provide a simple designation of RAPD genotype for in-house isolate comparison.

‡ Not all of the genes encoded by the *ssl* locus were investigated; seven of the genes that lie in the variable region of the *ssl* locus were tested for.

§ Mitogenicity assay (ability to induce proliferation of human T-lymphocytes) results for 29 strains (Smyth *et al.,* 2005) are indicated; + = positive, – = negative, ± = borderline mitogenicity

se = staphylococcal enterotoxin; *sel* = staphylococcal enterotoxin-like; *egc* = enterotoxin gene cluster comprising the *seg*, *sei*, *selm*, *seln*, *selo* genes, with either the *selu* gene or two pseudogenes *ψent1* and *ψent2* located between the *sei* and *seln* genes; SaPIbov = *S. aureus* pathogenicity island bovine possessing the *sec*-bovine, *sell* and *tst* genes (Fitzgerald *et al.*, 2001; Jarraud *et al*., 2001; Letertre *et al.*, 2003)

¶ ND = not done

3. Analysis of the *in vivo* expression of the SSL proteins and of the enterotoxin and enterotoxin-like proteins encoded by the *egc* locus.

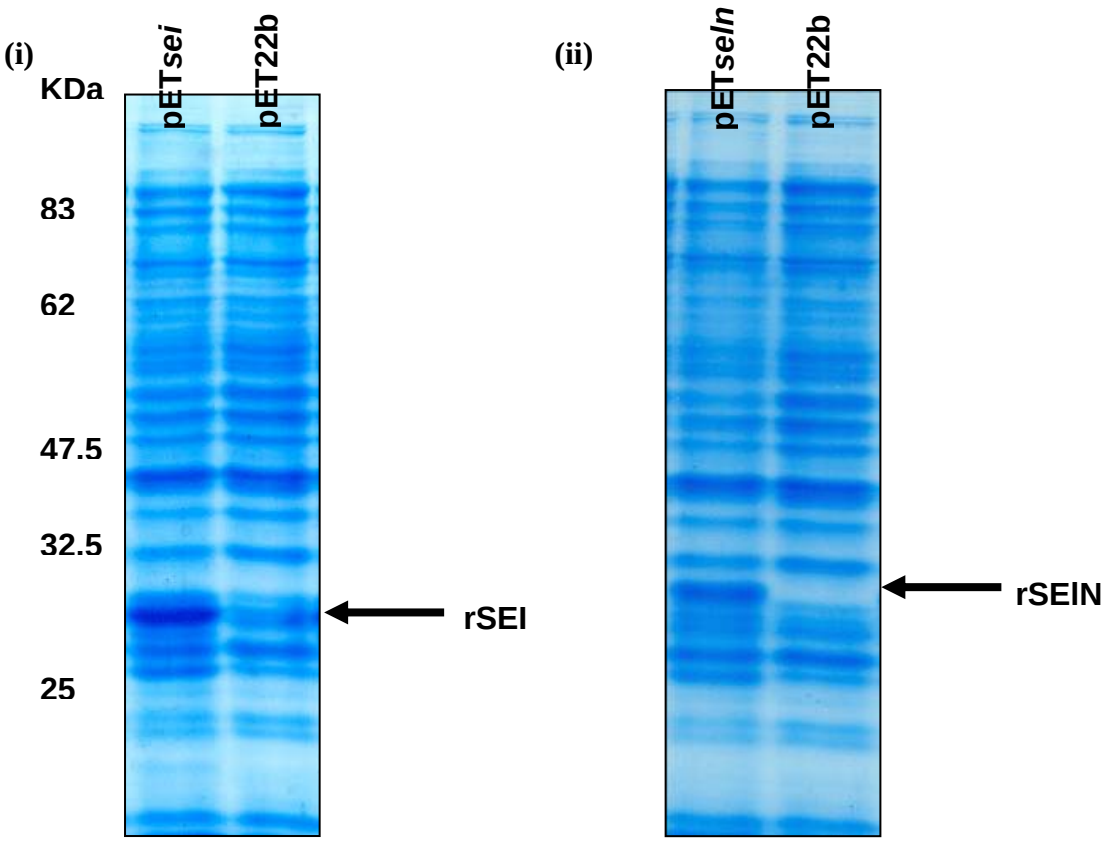
The aim of this study was to investigate if the SAg and SAg-like genes associated with the *egc* cluster (*seg*, *sei*, *selm*, *seln* and *selo*) and the *ssl* genes were expressed *in vivo* during bovine mastitis. This work also aimed to reveal if these proteins were potentially important virulence factors during infection and accordingly if they might have potential as vaccine candidates.

To this end the SAg-like genes associated with the *egc* cluster (*seg*, *sei*, *selm*, *seln* and *selo*) were cloned into plasmids in *E. coli* that facilitate the expression of non-*E. coli* proteins. In section 2 quarter milk and serum samples from four cows with mastitis had been obtained from Teagasc Dairy Production Centre, Moorepark, Fermoy, Co. Cork along with four samples of serum from cows with low somatic cell counts and considered to be free of mastitis infection. The serum samples were used in the present investigation in Western immunoblotting experiments using whole cell lysates of *E. coli* strains bearing expression plasmids with cloned SAg, SAg-like encoding genes and *ssl* genes.

- Lysates of *E. coli* strains containing recombinant SEI, SElM, SElN and SEO proteins failed to react with the bovine sera from all eight cows (Figure 1). Cow 2480 that was infected by a strain encoding the *egc* locus (*seg*, *sei*, *selm*, *seln* and *selo*) did not react. This implies that either these proteins are not immunogenic in the cow or that they are expressed at very low levels or not expressed at all *in vivo*. This was unexpected based on the results of the mitogenicity assays in Section 1 which suggested that the culture supernatants of animal–associated strains encoding the *egc* locus (*seg*, *sei*, *selm*, *seln* and *selo*) did cause the proliferation of T-cells suggesting that these proteins were expressed.
- Lysates of *E. coli* strains containing recombinant SSL proteins on the other hand were found to react with antibodies in the sera of the cows with mastitis (Figure 2). These were obtained from a previous study (Fitzgerald *et al.* 2003). This is the first study to investigate the expression of these proteins by *S. aureus* in cows. The SSL6 protein was the most reactive. In comparison, this protein only reacted with 4 of 19 human samples tested in the study of Fitzgerald *et al.* (2003).

The SSL proteins may represent novel targets in vaccine development as they do not exhibit the hallmarks of Sags and are expressed in the host. Although more studies are needed to evaluate the role and function of these proteins *in vivo,* it is clear that they represent a family of immuno-modulatory proteins, the genes of which are present in every strain regardless of the host it is derived from. Already applications of these proteins are being developed such as their use as chaperones in staphylococcal vaccine development.

(A)


(i)
KDa
pETsei
pET22b
83
62
47.5
32.5
25
rSEI
(ii)
pETseln
pET22b
rSEIN

(B)

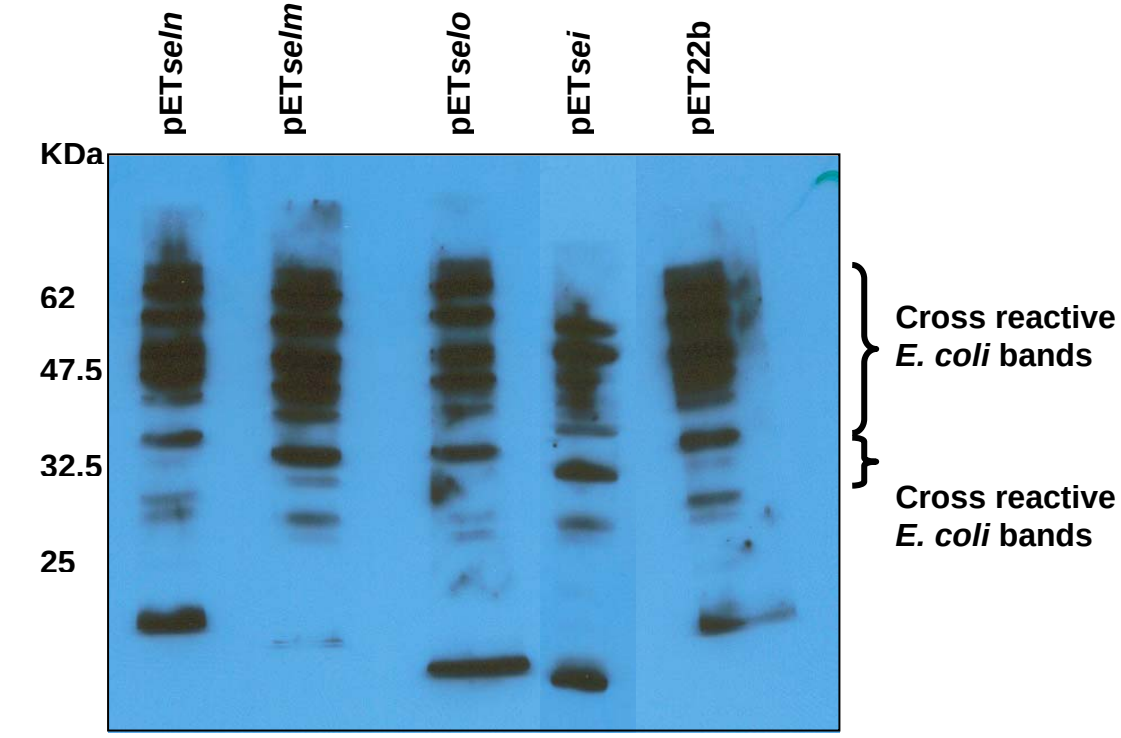
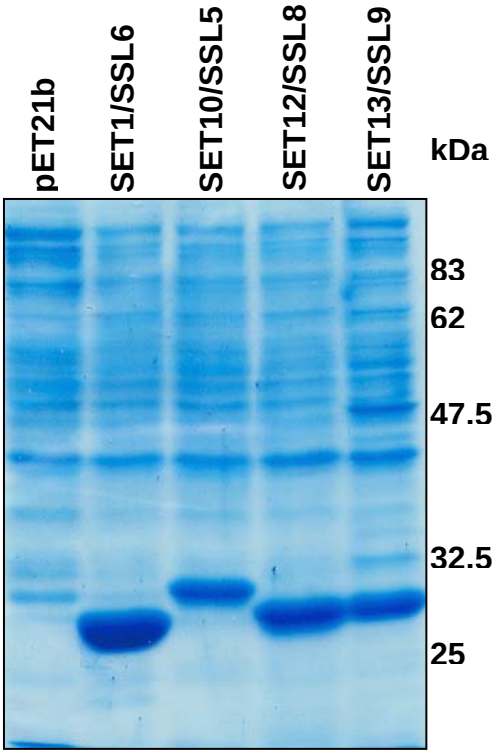

KDa
pETseln
pETselm
pETselo
pETsei
pET22b
62
47.5
32.5
25
Cross reactive
E. coli bands
Cross reactive
E. coli bands

Figure 1: Western immunoblot analysis of *E. coli* lysates expressing recombinant SAg and SAg-like proteins

Panel (A) (i) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SEI protein, and (ii) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SElN protein. In the case of the SAg and SAg-like proteins, the PVDF filters were strained with Ponceau S and the lanes of the gels were cut into strips to facilitate easier processing of multiple samples and different sera samples.

Panel (B) shows X-ray films of strips of PVDF membrane to which the *E. coli* lysates proteins were transferred. The strips of membrane was subjected to western immunoblotting using serum from cow 2242 that had active mastitis infection when this serum sample was taken. Cross-reactive bands were seen in every strip implying that the serum was reacting to *E. coli* proteins in the lysates. No reactive band to the SAg and SAg-like proteins was observed.

(A)


pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
kDa
83
62
47.5
32.5
25

(B)

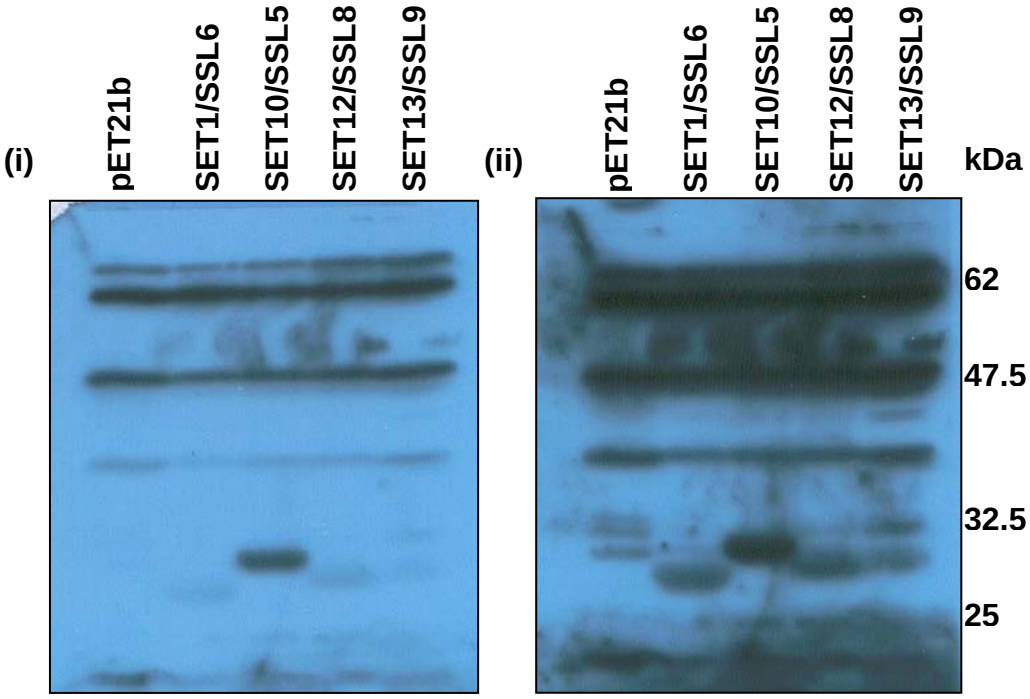

(i)
pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
(ii)
pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
kDa
62
47.5
32.5
25

Fig. 2 : Western immunoblot analysis of lysates of *E. coli* expressing recombinant SET (SSL) proteins

Panel (A) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SET (SSL) proteins that were subjected to wet-transfer and stained post-transfer with Coomassie brilliant blue. It is interesting to note the high amounts of recombinant SET (SSL) proteins that remained in the SDS-PAGE gel.

Panel (B) shows X-ray films of a PVDF membrane to which the *E. coli* lysate proteins were transferred. The membrane was subjected to Western immunoblotting using serum from cow 2487 that had active mastitis infection when this serum sample was taken. Film (i) was exposed for 2 min and film (ii) for 3 min. The longer the exposure, the higher the background. The reactive band corresponding to the SET10 (SSL5) protein was clearly observed at both exposure times; however, reactive bands corresponding to the other proteins were weak at 2 min became clearer after a 3-min exposure.

4. Multi-locus Sequence Typing (MLST), Random Amplified Polymorphic DNA Typing, and *agr* typing of *S. aureus* of animal origin.

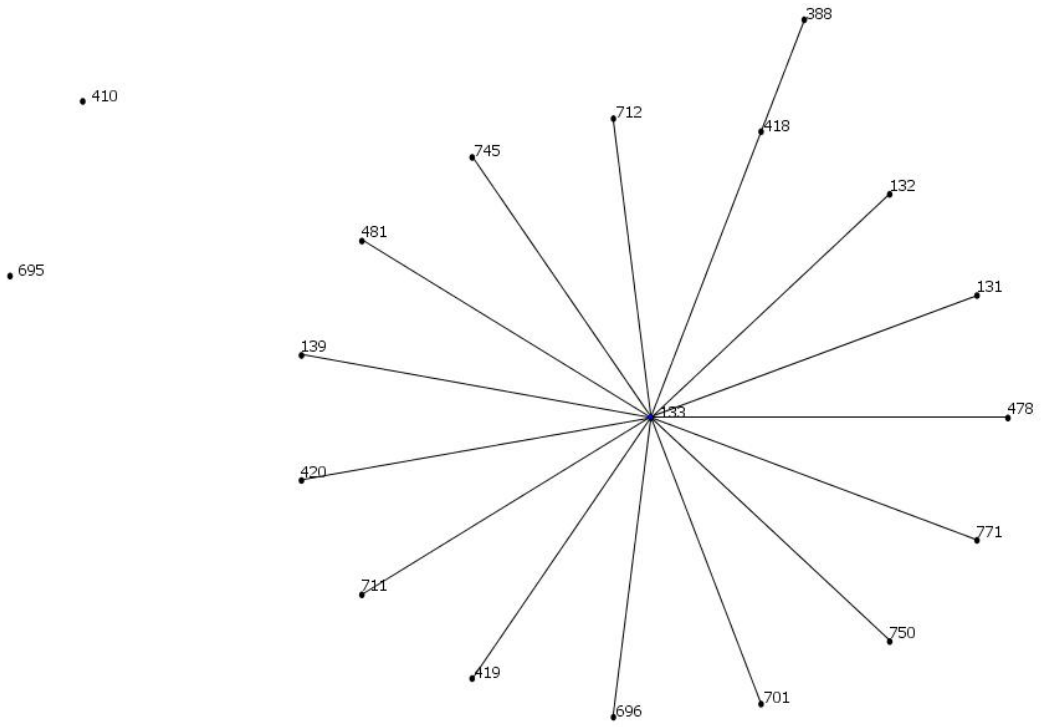
Animal-associated *S. aureus* populations are still an important area of *S. aureus* research. Due to the recent emergence of methicillin-resistant strains of *S. aureus* in the animal population (Lee *et al*., 2003, Weese *et al*., 2005), the population dynamics of *S. aureus* from animals continues to warrant investigation.

To this end the techniques of Multi-locus sequence typing (MLST), RAPD typing and *agr* typing were used to investigate the evolutionary relatedness of 119 isolates of *S. aureus* from animal hosts (52 from cows, 32 from goats, 12 from chickens, 10 from sheep, 12 from rabbits and 1 from a cat) and 3 strains from human infection from a number of geographic locations. These isolates represented the predominant clones associated with animal infection as determined by one or more of PFGE, RAPD and MLEE. MLST performed as previously described (Robinson and Enright, 2003). eBurst analysis was used on the allelic profiles observed to assemble clonal complexes (Feil *et al*., 2004). RAPD and *agr* typing were done as previously described (Fitzgerald *et al.*, 1997; Lina *et al*., 2004).

- The 119 animal isolates generated 39 types of which 18 were newly described types. A number of STs had more than one representative isolate: ST1, 3 (2.5%); ST5, 10 (8.2%); ST8, 2 (1.6%); ST20, 2 (1.6%); ST25, 2 (1.6%); ST71, 10 (8.2%); ST96, 4 (3.3%); ST97, 8 (6.6%); ST121, 3 (2.5%); ST126, 12 (8.2%); ST133, 28 (22.9%); ST151, 6 (4.9%); ST352, 3 (2.5%); ST522, 2 (1.6%), and ST703, 4 (3.3%).
- The remaining STs, including novel STs, had only one representative strain in the collection of strains examined. MLST type ST133, the commonest type, was only found in strains from sheep [7 (5.7%)], goats [13 (10.7%)] and cows [8 (6.6%)].
- MLST revealed that a limited number of clones are responsible for many cases of animal–associated staphylococcal infection with certain clones predominating among different animal hosts.
- A number of animal–associated sequence types clustered with human associated sequence types, suggesting an evolutionary relationship between certain animal and human clones.

- eBurst analysis has revealed that there appears to be an animal specific clonal complex centering on ST133 (goat, sheep and cow isolates were of this and related sequence types) (Figure 3). This would suggest that these isolates may have evolved preferentially to infect and colonise the ruminant animal over a long period of time.
- The chicken isolates were all sequence type 5 which is a predominant human–associated sequence type that is a highly prevalent nosocomial MRSA ST. This would seem to suggest that strains of this sequence type have recently been introduced to chickens by humans and have not yet evolved host specific traits.
- RAPD typing revealed 44 RAPD types amongst the 123 isolates examined.
- The isolates were predominately of *agr* type 1 [74 strains (60.2%)] and *agr* type 2 [33 strains (26.8%)], but several strains of *agr* type 3 [12 strains (9.8%)] and *agr* type 4 [4 strains (3.3%)] were identified. All clonally related strains appeared to be the same *agr* type.
- There was a strong correlation of MLST and *agr* typing with all strains of the same MLST ST being the same *agr* type.
- Two of the six major MLST types showed 100 % correlation between all typing methods. The remaining three types showed variation in their RAPD types and also in terms of SAg genes being present. SAg genes are often horizontally acquired, so variation in SAg gene content in strains of the same MLST type is also not unprecedented.

Previous data had indicted the correlation of *agr* type with infection type for example strains involved with toxic shock syndrome were often *agr* type 3. In this study no correlation was observed between *agr* type and infection type as TSST-1 containing isolates were often *agr* type 1 and 2.



	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
ST133	6	66	46	2	7	50	18
ST131	40	66	46	2	7	50	18
ST132	6	67	46	2	7	50	18
ST410	6	66	46	43	7	14	18
ST672	6	66	46	67	90	50	18

Figure 3: eBURST-generated clonal complex containing the major animal-associated ST 133

MLST type ST133 was the most prevalent sequence type (ST) identified in this work accounting for 23% of the strains and forms the primary founder of this group. It was associated with cows, goats and sheep. The group contains novel STs identified herein, ST ST695 (from a cow), ST696 (from a cow) and ST701 (from a goat). The table shows the allelic profiles of some of the STs in the complex. This group also contains STs 139, 481, 711, 712 which have previously been shown to be associated with goats, STs 132, 131, 478, and 745 which have previously been shown to be associated with cows and a sheep-associated ST, 750 (www.mlst.net). ST410 and ST695 are DLVs of ST133.

5. Typing of *S. aureus* strains of animal origin using the nucleotide sequences of putative surface proteins (*sas* typing) and the short sequence repeat region (*ssr*) of the protein A gene (*spa* typing).

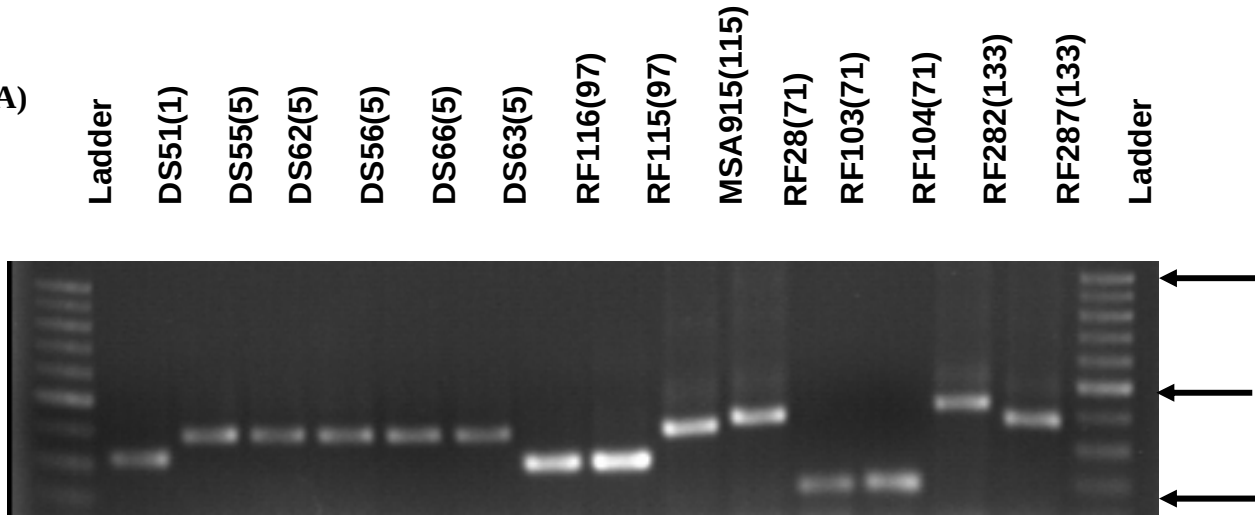
DNA sequencing of the short sequence repeat region (SSr) of the protein A gene (*spa*) has been used as an alternative to current techniques for the typing of *S. aureus* (Shopsin *et al.*, 1999; Koreen *et al.*, 2004). The method involves PCR using primers that anneal to conserved regions of the gene which traverse the repeat region and the subsequent sequencing of the PCR product. Few have applied this method to the study of animal–associated *S. aureus*. Another novel sequence typing method that has been recently developed is *sas* typing (Robinson & Enright, 2003). The principle is similar to MLST in that the DNA sequences of seven genes are analysed *sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH*, and *sasI* and a database of *sas* types is used to determine the *sas* type. This technique has yet to be applied to animal–associated *S. aureus.* Little data are available on the possible host specificity and variability of the *sas* and *spa* genes in *S. aureus* strains from cows, goats, sheep, rabbits and chickens. The present study was undertaken to evaluate *spa* typing and *sas* typing based on discriminatory power, reproducibility, and ease of interpretation using a small number of representative isolates from different animal hosts.

48 *S. aureus* isolates from animal infection, previously subjected to MLST, were analysed. Both *sas* and *spa* typing were carried out as previously described (Robinson and Enright, 2003).

- The animal-associated strains generated 35 *spa* repeat profiles. Six *spa* repeat profiles accounted for 19 strains, namely: D2KGFMJEMMMJQ (3 strains), TJMBMDMGMK (7 strains), UJ (3 strains), UJBBPB (2 strains), ZB (2 strains) and ZM (2 strains) (Figure 4).
- The smallest number of repeats was 1 (strain DS74, *spa* repeat profile D2) and the largest number of repeats was 12 (strain RF282, *spa* repeat profile, D2KGFMJEMMMJQ).
- The 22 cow-associated strains generated 18 *spa* repeat profiles with five profiles accounting for 10 strains. The eight chicken-associated strains generated only 2 *spa* repeat profiles, 1 of which – TJMBMDMGMK– accounted for 7 of the strains (all MLST ST5). The 11 goat-associated strains generated 11 different *spa* repeat profiles. The 5 rabbit-associated strains generated 5 different *spa* repeat profiles. The single sheep-associated strain generated the D2KGFMJEMMMJQ *spa* repeat profile. The cat isolate, DS71, generated a unique *spa* repeat profile.
- Analysis of the nucleotide sequences of the *sas* genes of the 48 animal-associated *S. aureus* generated 28 *sas* ST types for the 48 strains, 21 of which were novel *sas* types. Four of the strains failed to generate a PCR product for a single *sas* allele, namely, strains RF122 and RF80 did not generate a *sasB* gene PCR product and strains DS17 and DS20 failed to generate a PCR product for the *sasF* gene.
- Ten *sas* ST types accounted for 30 of the strains, namely: *sas* ST1 (7 strains), *sas* ST38 (2 strains), *sas* ST60 (4 strains), *sas* ST100 (2 strains), *sas* ST101 (5 strains), *sas* ST102 (2 strains), *sas* ST103 (2 strains), *sas* ST104 (2 strains), *sas* ST107 (2 strains), and *sas* ST115 (2 strains). Many new *sas* gene alleles were identified.
- Using Simpsons index of diversity (Grundmann *et al*., 2001, 2002), it was found that the single method with the highest resolution was *spa* typing (Index of diversity, 0.9725), followed by *sas* typing (Index of diversity, 0.9654), and then MLST (Index of diversity, 0.9627). The combination of *spa* typing and MLST and the combination of *spa* typing and *sas* typing had higher resolutions than either *sas* typing or MLST alone. The combination of MLST and *spa* typing had a higher resolution power than *spa* typing alone and the same resolution power as using all three methods (Table 3).

These data, along with the results of the study of Robinson & Enright (2003), suggest that MLST and/or *sas* typing along with *spa* typing could serve as an alternative to gel based methods such as PFGE for investigating genetically similar strains. These methods would be of use in analysing large collections for population-based studies.

(A)



(B)

Strain	*spa* repeats	Number of repeats
DS51	UJFKBPE	7
DS55	TJMBMDMGMK	10
DS62	TJMBMDMGMK	10
DS56	TJMBMDMGMK	10
DS66	TJMBMDMGMK	10
DS63	TJMBMDMGMK	10
RF116	UJBBPB	6
RF115	UJBBPB	6
RF915	UJGFMBBBPB	10
RF28	TJGFMBBBBPB	11
RF103	ZM	2
RF104	ZM	2
RF282	D2KGFMJEMMMJQ	12
RF287	D2KFMJEMMJQ	10

Figure 4. *spa* tying of animal-associated *S. aureus* strains.
Panel (A) shows the results of *spa* gene PCR. The generated PCR products vary in size according to the numbers of repeats present in the *spa* gene. The MLST types of the strains are indicated in parentheses. The 1-kb, 500-bp and 200-bp bands in the ladder (top to bottom) are indicated with arrows.
Panel (B) tabulates the sequences and numbers of repeats present in each strain in the above gel.

Table 3. Resolution of sequence based typing methods.

Typing method	No. of types	Index of diversity
spa typing	35	0.9725
sas typing	28	0.9654
MLST	31	0.9627
MLST + *sas*	32	0.9671
MLST + *spa*	38	0.9778
sas + *spa* typing	36	0.9751
sas + *spa* typing + MLST	38	0.9778

The closer the index of diversity is to 1, the higher the resolution power of the method.

Conclusions

The main conclusions of this work can be summarised as follows:

- The *egc* cluster and the SaPIbov combination of genes were found to be highly prevalent in strains of animal origin.
- Every animal strain tested was found to contain an *ssl* locus of genes.
- The animal strains may contain a novel pathogenicity island containing the *sec*-ovine gene in place of the *sec*-bovine gene along with the *tst* gene and with or without the *sell* gene.
- The *egc* cluster-encoded genes that were found to be highly prevalent in strains of animal origin, were not found to react with the bovine serum samples, implying a lack of detectable antibodies. This could mean that the proteins are not expressed *in vivo* or are expressed at very low levels or that antibodies are not produced to these proteins. It might be concluded that the lack of an immune response to these proteins suggests that these proteins are not important in terms of virulence, since they do not appear to be expressed at high enough levels to elicit an immune response. However, these SAgs may still exert biological effects at very low levels that might contribute to bacterial persistence within the udder.
- The SSL proteins reacted with antibodies in the bovine sera samples, implying the *in vivo* expression of the proteins. Although a role for these proteins has not been identified to date, the current PCR and Western immunoblot data suggest that the *ssl* genes present in animal *S. aureus* strains are expressed in the cow. These proteins warrant further investigation to see if they are virulence factors and/or immunogens that could be utilised in anti-staphylococcal therapy.
- Animal–associated *S. aureus* were found to have a clonal population structure, with a small number of clonal types causing the majority of infection. The major MLST ST, ST133, was found in 28 of the 123 strains analysed, and this MLST ST was only found in strains from cows, goats and sheep. ST133 had not been found in the human *S. aureus*

population. Many novel MLST STs were identified that may represent animal-specific clonal types. Also some of the predominant MLST STs had previously been identified in the human *S. aureus* population. In particular, the chicken strains were found to be of ST5, a highly prevalent MRSA–associated ST.

- *spa* typing was the method with the highest resolution. The *sas* typing method had not been previously applied to *S. aureus* from cows, goats, sheep, rabbits and chickens and as a result many novel *sas* alleles and *sas* STs were identified. The *spa* typing method also revealed the presence of novel *spa* short sequence repeats and *spa* repeat profiles in animal-associated *S. aureus*.
- The combination of either MLST or *sas* typing with *spa* typing has great potential for the analysis of *S. aureus* from animals. In future studies of animal-associated *S. aureus*, these methods would be a suitable alternative to gel-based methods such as PFGE and RAPD typing.
- The use of these techniques would facilitate the generation of databases of the *S. aureus* genetic types involved in animal infections. Database formation allows the easy comparison of studies between laboratories allowing investigators to share and combine their results.

The results of the molecular genetic typing of *S. aureus* from cows, goats sheep, rabbits and chickens completed herein should be of value to researchers involved in animal *S. aureus* epidemiology.

References

- **Alouf, J. E. & Müller-Alouf, H. (2003).** Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *Int J Med Microbiol* **292**, 429–440.
- **Al-Shangiti, A. M., Nair, S. P. & Chain, B. M. (2005).** The interaction between staphylococcal superantigen-like proteins and human dendritic cells. *Clin Exp Immunol* **140**, 461–469.
- **Balaban, N. & Rasooly, A. (2000).** Staphylococcal enterotoxins. *Int J Food Microbiol* **61**, 1–10.
- **Beck, H. S., Wise, W. S. & Dodd, F. H. (1992).** Cost benefit analysis of bovine mastitis in the UK. *J Dairy Res* **59**, 449–460.
- **Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. & Spratt, B. G. (2004).** eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* **186**, 1518–1530.
- **Fitzgerald, J. R., Meaney, W. J., Hartigan, P. J., Smyth, C. J. & Kapur, V. (1997).** Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. *Epidemiol Infect* **119**, 261–269.
- **Fitzgerald, J. R., Monday, S. R., Foster, T. J., Bohach, G. A., Hartigan, P. J., Meaney, W. J. & Smyth, C. J. (2001a).** Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J Bacteriol* **183**, 63–70.
- **Fitzgerald, J. R., Reid, S. D., Ruotsalainen, E., Tripp, T. J., Liu, MY., Cole, R., Kuusela, P., Schlievert, P. M., Järvinen, A. & Musser, J. M. (2003).** Genome diversification in *Staphylococcus aureus*: Molecular evolution of a highly variable chromosomal region encoding the Staphylococcal exotoxin-like family of proteins. *Infect Immun* **71**, 2827–2838.
- **Jablonski, L. M. and Bohach, G. A**. (**2001**). *Staphylococcus aureus*. In *Fundamentals of Food Microbiology*, pp. 410–434. Edited by L. Beuchat, M. Doyle, and T. Montville. ASM Press, Washington, D.C.
- **Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M. & Kreiswirth, B. N. (2004).** *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *J Clin Microbiol* **42**, 792–799.
- **Kuroda, M., Ohta, T., Uchiyama, I., & 34 other authors (2001).** Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240.
- **Lee, J. H. (2003).** Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl Environ Microbiol* **69**, 6489–6494.
- **Letertre, C., Perelle, S., Dilasser, F. & Fach, P. (2003c).** Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J Appl Microbiol* **95**, 38–43.
- **Omoe K., Imanishi, K., Hu, D.–L., Kato, H., Fugane, Y., Abe, Y., Hamaoka, S., Watanabe, Y., Nakane, A., Uchiyama, T. & Shinagawa, K. (2005).** Characterization of novel staphylococcal enterotoxin-like toxin type P. *Infect Immun* **73,** 5540–5546.

- **Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. & Day, N. P. (2002).** Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect Immun* **70**, 4987–4996.
- **Proft, T. & Fraser, J. D. (2003).** Bacterial superantigens. *Clin Exp Immunol* **133**, 299–306.
- **Robinson, D. A. & Enright, M. C. (2004).** Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J Bacteriol* **186**, 1060–1064.
- **Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S. & Kreiswirth, B. N. (1999).** Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* **37**, 3556–3563.
- **Weese, J. S., Rousseau, J., Traub-Dargatz, J. L., Willey, B. M., McGeer, A. J. & Low, D. E. (2005).** Community-associated methicillin-resistant *Staphylococcus aureus* in horses and humans who work with horses. *J Am Vet Med Assoc* **226**, 580–583.

Publications

Conference Abstracts

Smyth, D., Hartigan, P.J., Meaney, W.J., and Smyth, C.J. (2002). Novel and putative exotoxin genes in strains of *Staphylococcus aureus* from bovine mastitis, *Proceedings of the 32nd Annual Food Science and Technology Conference*, Cork, Ireland, September 2002, 114 (Abstract).

Smyth, D., Hartigan, P.J., Meaney, W.J. and Smyth, C.J**.** (2002). Frequencies of novel and putative exotoxin genes in strains of *Staphylococcus aureus* from bovine mastitis, *Proceedings of the 151st Ordinary Meeting of the Society for General Microbiology*, Loughborough, England, September 2002, 44 (Abstract MI 05).

Smyth, D., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2002). Frequencies of novel and putative exotoxin genes in strains of *Staphylococcus aureus*, *Proceedings of the 10th International Symposium on Staphylococci and Staphylococcal Infections*, Tsukuba, Japan, October 2002, 129 (Abstract ISSI-259-Abstract-01).

Smyth, D., Feil, E.J., Meaney, W.J., Hartigan, P.J., Fitzgerald, J.R., Robinson, D.A., Smyth, C.J. and Enright, M.C. (2004). Genotypic variation of animal-associated *Staphylococcus aureus* as determined using molecular typing techniques, *Proceedings of the Society for General Microbiology Irish Branch Meeting "An Update on Bacterial Pathogenicity",* Galway, Ireland, April 2004, Abstract P83.

Smyth, D., Feil, E.J., Meaney, W.J., Hartigan, P.J., Fitzgerald, J.R., Robinson, D.A., Smyth, C.J. and Enright, M.C. (2004). Clonal relationships of animal-associated *Staphylococcus aureus* revealed by molecular typing methods, *Abstracts of the 104th General Meeting of the American Society for Microbiology*, New Orleans, U.S.A., May 2004, 164 (Abstract C-227).

Smyth, D., Kennedy, J., Twohig, J, Bolton, D.J. and Smyth, C.J. (2004). *Staphylococcus aureus* isolates from Irish domestic fridges possess novel superantigen genes and are clonal in nature, *Proceedings of Society for Applied Microbiology Summer Conference "Dairy and Food Microbiology: Challenges and Opportunities"*, Cork, Ireland, July 2004, 35 (Abstract P33).

Smyth, D.S., Collery, M. and Smyth, C.J. (2004). Finger lickin' good? – Genotypes of *Staphylococcus aureus* associated with broiler chickens. **In** *Food Pathogen Epidemiology: Microbes, Maladies and Methods*, Maunsell, B., Sheridan J., and Bolton, D.J. (eds.), European Union Risk Analysis Information Network (EU-RAIN) International Conference, Padua, Italy, December 2004, 148, (Abstract P6).

Oral Presentations

Smyth, D., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2003). Application of multiplex PCR to determine the frequencies of classical and novel staphylococcal enterotoxins in strains associated with mastitis, *Proceedings of the 57th Conference of the Association of Veterinary Teachers and Research Workers,* Scarborough, England, April 2003, *Research in Veterinary Science*, 74, Suppl. A, 8 (Abstract).

Smyth, D., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2003). Application of multiplex PCR to detect novel enterotoxin genes in strains of *Staphylococcus aureus* from goats, sheep, pigs and rabbits, *Proceedings of 1st FEMS Congress of European Microbiologists,* Ljubljana, Slovenia, June-July, 2003, 35 (Abstract A3–5).

Smyth, D., Feil, E.J., Meaney, W.J., Hartigan, P.J., Fitzgerald, J.R., Robinson, D.A., Smyth, C.J. and Enright, M.C. (2004). *Staphylococcus aureus* isolated from cows, goats, sheep, rabbits and chickens: Genotypic variation as revealed using molecular typing techniques, *Abstracts of the 5th World Congress on Foodborne Infections and Intoxications,* Berlin, Germany, June 2004, 38 (Abstract S-A15).

Papers

Smyth, D. S., P. J. Hartigan, W. J. Meaney, J. R. Fitzgerald, C. F. Deobald, G. A. Bohach, and C. J. Smyth. (2005). Superantigen genes encoded by the *egc* cluster and SaPIbov are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. *Journal of Medical Microbiology* **54:** 401-411.

Smyth, C. J., **D. S. Smyth**, J. Kennedy, J. Twohig, and D. Bolton. (2004). *Staphylococcus aureus*: from man or animals – an enterotoxin iceberg?, p. 85-102. **In** B. Maunsell, J. Sheridan, and D. J. Bolton (eds.), *Food pathogen epidemiology: microbes, maladies and methods*, Proceedings of the European Union Risk Analysis Information Network (EU-RAIN) conference, December 2004, Padua, Italy. Teagasc–National Food Centre, Dublin, Ireland.

Smyth, D. S., E. J. Feil, W. J. Meaney, P. J. Hartigan, J. R. Fitzgerald, D. A. Robinson, C. J. Smyth and M. C. Enright. (2006). *Staphylococcus aureus* isolated from cows, goats, sheep,

rabbits and chickens: Genotypic variation as revealed using molecular typing techniques. (In preparation).

Smyth, D. S., P. J. Hartigan, W. J. Meaney and C. J. Smyth. (2006)**.** Occurrence of *ssl* genes in isolates of *Staphylococcus aureus* from animal infection. (Accepted for publication to the Journal of Medical Microbiology).