Superantigen genes encoded by the egc cluster and SaPIbov are predominant among Staphylococcus aureus isolates from cows, goats, sheep, rabbits and poultry

Davida S. Smyth, 1 Patrick J. Hartigan, 2 William J. Meaney, 3 J. Ross Fitzgerald, 1 Claudia F. Deobald, 4 Gregory A. Bohach 4 and Cyril J. Smyth 1

1 Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, University of Dublin, Dublin 2, Ireland
2 Department of Physiology, Trinity College, University of Dublin, Dublin 2, Ireland
3 Teagasc, Dairy Production Research Centre, Moorepark, Fermoy, County Cork, Ireland
4 Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844, USA

In recent years several new staphylococcal enterotoxins (SEs) have been described, which currently have largely unknown frequencies of occurrence and roles in human or animal disease. One hundred and ninety-one Staphylococcus aureus isolates from cows (99), goats (39), sheep (23), rabbits (15), chickens (15) and a cat (1) were screened for SE genes sea–see, seg–seo and seq and for the tst gene encoding staphylococcal toxic shock syndrome toxin-1 using multiplex PCRs and individual PCRs for the seb and sek genes. One hundred and ten isolates tested positive for at least one of these 16 superantigen (SAg)-encoding genes. There were statistically significant differences in the frequencies of some of these SAg genes between isolates from different animals. No strain possessed either the sea or see gene. The sec gene was present in 51 isolates, the sed gene in eight and the seb gene in one. The seh gene was found in four strains and the sek and seq genes together in one isolate. The most common combinations of genes were the egc cluster, bearing the seg, sei, sem, sen and seo genes, in 47 isolates, the sec, sel and tst gene combination typical of the SaPIbov pathogenicity island in 44 isolates, the egc cluster lacking the seg gene in 11 isolates, the sed and seq genes in nine isolates, and the sec and tst genes without the sel gene in seven isolates. The higher frequencies of the sec and tst genes together and the lower frequencies of the egc gene cluster among the SAg gene-positive sheep or goat isolates compared to bovine isolates were statistically significant. Of 36 bovine isolates that were mitogenic for human T lymphocytes, four were negative for the 16 SAg genes tested for, while a further 14 gave borderline results in the mitogenicity assay, 12 of which were SAg gene-negative. Twenty-nine strains lacking all the SAg genes did not induce T-cell proliferation. This survey indicates that novel SE genes seg, sei, sel, sem, sen and seo along with the sec and tst genes predominate in S. aureus from animal hosts. The mitogenicity assays indicate that further uncharacterized SAgs may be present in bovine isolates.

INTRODUCTION

Staphylococcus aureus is a major human pathogen that causes a wide variety of diseases ranging in severity from food poisoning (McCormick et al., 2001; Le Loir et al., 2003) and life-threatening toxic shock syndrome (Llewelyn & Cohen, 2002; Proft & Fraser, 2003) to less serious infections, e.g. boils (Stulberg et al., 2002). S. aureus can also cause a number of infections in animals, such as tick-associated pyaemia in lambs (Webster & Mitchell, 1989), staphylococcosis in rabbits (Hermans et al., 2003), oedematous and necrotic dermatitis, septicemia, abcesses and chondronecrosis in chickens (McCullagh et al., 1998; McNamee et al., 1998; Takeuchi et al., 2002), and pneumonia and osteomyelitis complex in turkeys (Huff et al., 2000; Linares & Wigle, 2001).
S. aureus is the most frequent cause of bovine mastitis, a disease that is of economic importance worldwide (Beck et al., 1992; Miles et al., 1992). Typically staphylococcal mastitis is chronic in nature, with subclinical mastitis being the most common form (Gruet et al., 2001). The organism may survive for long periods of time in the host without causing overt symptoms of disease (Fitzgerald et al., 2001).

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 exoproteins (Peacock et al., 2002). The classical staphylococcal enterotoxins (SEs), SEA–SEE, cause staphylococcal food poisoning (Balaban & Rasooly, 2000; Dinges et al., 2000; Jablonski & Bohach, 2001), a food-borne intoxication characterized by a short incubation period, nausea, vomiting, abdominal pain and diarrhoea. SEA, SEB andSED are the most common enterotoxins detected in outbreaks. In a study of S. aureus from 359 UK outbreaks of food poisoning between 1969 and 1990 79% of the strains were found to produce SEA alone or together with another toxin (Wieneke et al., 1993).

S. aureus is estimated by the CDC to cause 185 000 cases of food poisoning in the USA annually (Mead et al., 1999). Although humans are the main reservoir of S. aureus associated with food poisoning, animals are often heavily colonized, leading to contamination of foodstuffs during processing of domestic animals and their products. Due to its osmotolerance S. aureus can be isolated from many types of food products, e.g. bakery products, beef, pork sausages, poultry, cooked meats, dairy products, egg products and seafood (Jablonski & Bohach, 2001). SEC or SED is produced by 10–30% of bovine isolates (Kenny et al., 1993; Matsuenga et al., 1993, Fitzgerald et al., 2000; Cenci-Goga et al., 2003) and SED by 35% and SEC by 22% of isolates from sheep milk (Bautista et al., 1988). In the study of Foshino et al. (2002) the incidence of enterotoxin-C producers among caprine S. aureus strains was 23%.

The SEs belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins. They are involved in other types of toxigenic illnesses with symptoms of shock in humans and animals (Michie & Cohen, 1998; McCormick et al., 2001; Llewelyn & Cohen, 2002). These proteins share common structural features, sequence similarity and phylogenetic relationships. The SE genes are often associated with mobile genetic elements such as bacteriophage, transposons and plasmids (Lindsay et al., 1998; Hentschel & Hacker, 2001; Novick et al., 2001). One such element is the bovine pathogenicity island that encodes the three superantigens (SAgs) SEC–bovine, TSST-1 and SEL (Fitzgerald et al., 2001).

The enterotoxin gene cluster (egc) that was identified by Jarraud et al. (2001) comprises the five enterotoxin genes seg, sei, sem, sen and seo.

These enterotoxins and TSST-1 can act as SAgs, which stimulate T-cell proliferation (Alouf & Müller-Alouf, 2003; Proft & Fraser, 2003; Persson et al., 2004). SAgs bypass conventional antigen presentation. They interact simultaneously with the major histocompatibility complex class II molecule of the antigen-presenting cells and the Vβ domain of the lymphocyte T-cell receptor, forming trimolecular complexes (Petersson et al., 2004). The interaction activates as many as one in five T cells, whereas conventional antigen presentation activates one in 10 000 T cells. This explains the release of massive amounts of cytokine and the subsequent immunomodulative and other deleterious effects brought about by SAgs (Alouf & Müller-Alouf, 2003; Proft & Fraser, 2003).

Eighteen SE genes have been identified to date, namely sea–see, seg–ser and seu (Fitzgerald et al., 2001; Jarraud et al., 2001; Kuroda et al., 2001; Orwin et al., 2001, 2002; Yarwood et al., 2002; Letertre et al., 2003a; Omoe et al., 2003). The enterotoxicity (emetic effect) and superantigenicity of SEs appear to be distinct properties of these molecules (Schlievert et al., 2000; Proft & Fraser, 2003). Not all of the currently identified SEs are emetic, although all exhibit lymphocyte mitogenic activity towards human and/or other mammalian lymphocytes. SEI, SEK and SEQ have been shown to lack the cysteine loop structure important for emetic activity, resulting in reduced activity in SEI and a lack of activity in SEK and SEQ (Orwin et al., 2003).

At present little is known about the occurrence and significance of the novel enterotoxins (SEG–SER, SEU) in strains of S. aureus from animal infection (Akineden et al., 2001; Larsen et al., 2002). Some of these novel SEs may contribute to the persistence of S. aureus in subclinical mastitis. Because of the putative significance of these enterotoxins for public health and food safety, greater knowledge of their occurrence and an efficient means of screening for their genes is needed (Wieneke et al., 1993; Mead et al., 1999; Shimizu et al., 2000; Chen et al., 2004). Changes in food processing and retailing over recent decades have the potential to introduce toxigenic strains of S. aureus of animal origin into the food chain (Roscic & Gigaud, 2002; Woteki & Kineman, 2003; Chen et al., 2004). Since several of these SEs have been discovered in last 5 years, there is reason to believe that, as research on pathogenic S. aureus isolates continues, additional SAgs will be described. This present study was designed to investigate the frequency of genes encoding the classical and novel SEs by means of multiplex PCR in strains of S. aureus from cows, goats, sheep, rabbits and chickens.

METHODS

Bacterial strains and DNA isolation. A total of 191 S. aureus isolates were analysed – 99 from cows (five from Argentina, one from Denmark, four from Spain, two from Sweden, 37 from the USA and 50 from the Republic of Ireland – 12 RAPD type 4, 15 RAPD type 5, 10 RAPD type 7 and 13 RAPD type not determined), 38 from goats (four from Austria, 25 from Italy and nine from Norway), 23 from sheep (three from Denmark, three from Finland, three from Iceland, 12 from Norway and two from Sweden), 15 from chickens (all from Northern Ireland) and 15 from rabbits (11 from Belgium and four from Spain) (Fitzgerald et al., 1997, 2000; Rodgers et al., 1999; Hermans et al., 2000; Larsen et al., 2000; Foshino et al., 2002). One Swedish cat isolate was also included. S. aureus strains were routinely grown in tryptic soy broth or on tryptic soy...
PCR primer design and amplification of bacterial DNA. Primers for SE genes seq, sec–see and seq–sej and tst were designed in a previous study (Monday & Bohach, 1999). The sec primers used in sequencing analysis could amplify variants of the sec gene including the bovine variant (Marr et al., 1993; Fitzgerald et al., 2001). The sec primers used would amplify known variants of the sec gene (Blaiotta et al., 2004). Nucleotide sequences for each of the novel enterotoxin genes sek, sel, sem, sen, seo and seq were obtained from GenBank by using their specific accession numbers (Table 1). The sequences were aligned and compared using CLUSTAL_W (http://www.ebi.ac.uk/clustalw) (Thompson et al., 1994). All primer sets were designed to anneal to unique internal regions of these genes and generated amplification products that allowed identification of each enterotoxin gene based on the size of its PCR product (Table 1).

The primers were combined in three multiplex PCR reactions to ensure reliability and reproducibility. Multiplex PCR 1 contained primers for the sea, sec, sed, see and 16S rRNA genes, multiplex PCR 2 contained primers for the seq, seh, sei, sej, tst and 16S rRNA genes and multiplex PCR 3 contained primers for the sel, sem, sen, seo, seq and 16S rRNA genes. The sec primer set from a previous study (Mehrotra et al., 2000) and the sek primer set described in this study were used individually. To ensure that gene-negative samples were interpreted correctly and that a sufficient quantity of template DNA was present, each PCR included a primer set that annealed to the S. aureus 16S rRNA gene generating a 228 bp amplicon. Each set of PCR reactions also included appropriate SE gene-positive and -negative controls.

The multiplex PCR conditions were as previously described (Monday & Bohach, 1999) with the following cycling parameters: 95 °C for 10 min, 15 cycles of 95 °C for 1 min, 58 °C for 45 s and 72 °C for 1 min and 16 cycles of 95 °C for 1 min, 54 °C for 45 s and 72 °C for 1 min. The reaction was terminated with a 10 min incubation at 72 °C. PCR products were resolved by electrophoresis in 1.5 % (w/v) agarose gels (0.5 × Tris/boric acid-EDTA) (Sambrook & Russell, 2001) at 90 V (constant voltage), stained with ethidium bromide and visualized using UV light. Product sizes were determined by comparison with a 100 bp ladder (Promega).

Sequencing of sec PCR products. Single locus PCR was performed for the sec gene using four strains, namely two goat isolates (one Italian and one Austrian) and two sheep isolates (one Norwegian and one Icelandic). The same conditions as for the multiplex PCR reactions were used with the sec primers (Marr et al., 1993). The PCR products were ethanol precipitated and sequenced by MWG-Biotech. The resulting sequences were aligned with those of known sec variants using CLUSTAL_W.

Southern hybridization analysis. PCR results using novel SE primers designed in this study were confirmed using genomic DNA from a number of representative Irish bovine isolates and control strains FR913, FR472, FR1569, COL and RF122. Genomic DNA was digested with restriction endonuclease HindIII and resolved by electrophoresis in 0.8 % agarose. Southern hybridization analysis was performed using standard methods (Sambrook & Russell, 2001) and the digoxigenin (DIG)-labelling system (Roche).

Crude toxin isolation. Seventy-nine of the bovine isolates were tested (29 from the USA and 50 from Ireland). One millilitre of overnight culture was precipitated with 5 vols of ethanol, then placed at −20 °C for 1 h. Samples were centrifuged and supernatant fractions were discarded. The pellets were air-dried, resuspended in 50 μl of sterile distilled water.
and clarified by centrifugation at maximum speed in a bench-top centrifuge (1–2 min). Supernatant fractions were removed and tested for their ability to induce lymphocyte proliferation.

**Mitogenicity.** Crude toxin preparations from the bovine isolates were diluted 10-, 100- and 1000-fold in sterile distilled water and were assayed for their ability to induce proliferation of human T lymphocytes by a standard 4 day mitogenicity assay (PoinDEXter & Schlievert, 1985). Background levels of proliferation were determined by testing identically prepared samples from the non-toxigenic strain *S. aureus* RN4220 (Monday & Bohach, 1999).

Lymphocytes were isolated from heparinized human blood that was fractionated by centrifugation through a Ficoll-Paque PLUS gradient (Amersham Biosciences). Lymphocytes were washed in RPMI 1640 medium ( Gibco/Invitrogen) and suspended to a concentration of 1·0 × 10^6 cells ml⁻¹ in RPMI 1640 medium supplemented with 2% (v/v) fetal bovine serum, 2 mM glutamine, 200 U sodium penicillin G ml⁻¹ and 200 μg streptomycin sulphate ml⁻¹. The T lymphocytes were distributed into 96-well plates (200 μl per well) and exposed to diluted crude toxin preparations (25 μl per well). Plates were incubated at 37 °C, 6% CO₂, for 72 h and then 3·7 × 10^6 Bq [³H]thymidine (Moravek) was added to each well. After incubation for a further 18–24 h, DNA was harvested with a Packard BioScience Filtermate Harvester (Meriden). The amount of [³H]-radiolabel incorporated into cellular DNA was measured using a Packard BioScience TopCount NXT scintillation and luminescence counter.

**RESULTS**

**PCR detection of SE genes and the tst gene**

PCR analysis using control strains showed that the primers produced amplicons consistent with their predicted sizes (Fig. 1). PCR products of SE genes were used as DNA probes in Southern blot experiments with control strain genomic DNA and representative Irish bovine strain genomic DNA. The Southern blots confirmed the multiplex PCR results (data not shown).

Of the 191 animal isolates tested in this study, 110 were positive for at least one SAg gene (57·6 %) (Table 2). Only eight of the 16 SAg genes screened for were individually present in more than 23% of the strains (40% of the SAg gene-positive isolates), namely sec, seg, sei, sel, sem, sen, seo and tst (Table 2). 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, which was present in 42·7 % of the SAg gene-positive strains (Table 2). The sec, sel and tst genes (the SaPIbov pathogenicity island combination of genes) were found in 40% of the SAg gene-positive strains. The combination of sec and tst without the sel gene was found in a further seven strains (6·4% of SAg gene-positive). The combination of sei, sem, sen and seo (ec gene cluster lacking seg) was found in 10% of the SAg gene-positive strains. The combination of sed and sej was found in 8·2% of SAg gene-positive strains.

The 99 bovine isolates included the main RAPD (random amplified polymorphic DNA) types associated with mastitis in the USA (RAPD types 1–4 and 9–12) and the Republic of Ireland (RAPD types 4, 5 and 7) that had previously been analysed for the presence of the classical enterotoxins and TSST-1 by RPLA (reversed passive latex agglutination) (Fitzgerald et al., 2000). In the latter study 87·5% (21 of 24) of Irish RAPD type 7 isolates were found to produce SEC and TSST-1.

In the current study 45 bovine isolates (45·5%) were positive for at least one SAg gene (Table 2). Of these 45 strains, 24 (three American, two Argentinian, one Danish, 15 Irish, one Spanish and two Swedish; 53·3%) were positive for one or more of the classical SE genes (sec, sel; 5; sec and sed, 1; Table 3). The genes encoded by the SaPIbov pathogenicity island (Fitzgerald et al., 2001), namely sec-bovine, sel and tst, were present in 18 of the SAg gene-positive strains [15 Irish (including seven known RAPD type 7 strains), one American, one Spanish, one Argentinian; 40%] and one Danish strain had the sec and tst genes only. Six of the SAg gene-positive strains (three American, one Argentinian, two Swedish; 13·3%) harboured the sed and sej genes. The genes encoded by the egc gene cluster were found in 22 of the SAg gene-positive strains (eight American, two Argentinian, one Danish, 10 Irish, one Spanish; 48·9%), with a further 10 American strains containing the sei, sem, sen and seo genes only (22·2%; Table 3). RAPD type 4 and 5 isolates (31 strains) that had previously been analysed for the production of classical enterotoxins and TSST-1 by RPLA (Fitzgerald et al., 2000) were negative for the presence of all 16 SAg genes tested for.

Of the 39 caprine strains analysed, 22 (three Austrian, 11 Italian, eight Norwegian; 56·4%) were positive for at least one SAg gene, with 18 of these being positive for one of the classical SE genes (sec, 18; 81·8%: Table 2). Sixteen of the 22 SAg gene-positive strains (three Austrian, five Italian, eight Norwegian, 72·7%) contained the three SAg genes associated with the SaPIbov pathogenicity island, with a further two Italian strains possessing the sec and tst genes only (9·1%; Table 3). Four Italian strains (18·2% of SAg gene-positive isolates) were positive for the entire egc cluster.
Table 2. PCR analysis for SAg genes in animal-associated strains of S. aureus

<table>
<thead>
<tr>
<th>SAg gene</th>
<th>Cow (n = 99)</th>
<th>Goat (n = 39)</th>
<th>Sheep (n = 23)</th>
<th>Rabbit (n = 15)</th>
<th>Chicken (n = 15)</th>
<th>Total (n = 191)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. *</td>
<td>%</td>
<td>No. *</td>
<td>%</td>
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<td>%</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>4</td>
<td>10.3</td>
<td>3</td>
<td>13.0</td>
</tr>
<tr>
<td>seh</td>
<td>19</td>
<td>19.2</td>
<td>18</td>
<td>46.2</td>
<td>14</td>
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</tr>
<tr>
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<td>1</td>
<td>4.3</td>
</tr>
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<td>0</td>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sec†</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>seq†</td>
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<td>0</td>
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<td>18</td>
<td>46.2</td>
<td>14</td>
<td>60.9</td>
</tr>
</tbody>
</table>

*Number of positive strains.
†Seven of the bovine strains and one 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.

Of the 23 isolates of ovine origin tested, 20 (two Danish, two Finnish, three Icelandic, 12 Norwegian, one Swedish; 87 %) were positive for at least one SAg gene and sixteen were positive for one of the classical SE genes (sea, 1; sec, 14; sed, 1; 80 % of SAg gene-positive isolates, Table 2). Ten Norwegian strains (50 % of SAg gene-positive) harboured the SaPlbov pathogenicity island-associated genes, with an additional four strains (two Italian, two Norwegian; 20 %) containing the sec and tst genes only (Table 3). One Finnish strain possessed the seq gene, one Icelandic strain contained the seh gene and one Finnish strain had the sed and seq genes in combination. Three Scandinavian strains (15 % of SAg gene-positive strains) contained the egc cluster in its entirety, with one Danish strain containing the sei, sem, sen and seq genes only.

Of the 15 strains from rabbits analysed, eight (six Belgian, two Spanish, 53.3 %) were positive for at least one SAg gene (Table 2). Only one Belgian strain contained a classical SE gene, sed, in combination with sej. Two strains (one Belgian, one Spanish) possessed the seh gene, while five (four Belgian, one Spanish; 62.5 % of SAg gene-positive isolates) strains harboured the egc locus in its entirety (Table 3).

Of the 15 poultry strains tested, none contained a classical SE gene. Fourteen (93.3 %) were positive for one or more of the novel and newly described SE genes (Table 2). One strain from poultry was positive for the seh gene, together with the sek and seq genes, while 13 of the SAg gene-positive strains (92.9 %) contained the egc locus (Table 3).

One Swedish cat isolate was tested. This strain was positive for the sed and sej genes.

The differences in the frequencies of SAg genes between bovine isolates and those from sheep or chickens were statistically significant (χ²-test, both P < 0.001). The differences in the frequencies of SAg genes between goat isolates and those from sheep or chickens are also both statistically significant (χ²-test, P < 0.025 and P < 0.01, respectively) and between rabbit and sheep or chicken isolates (χ²-test, both P < 0.025).

The percentages of SAg gene-positive isolates from sheep or goats harbouring at least one SAg gene or a classical SE gene (sea–sec), or the sec and tst genes together, or the complete egc cluster of genes were quite similar and not statistically different. The differences in the frequencies of genes encoding the classical SEs in SAg gene-positive strains between the bovine isolates and the sheep or goat isolates were statistically significant (χ²-test, P < 0.025 and P < 0.01, respectively). The higher occurrences of the sec and tst genes among the SAg gene-positive sheep or goat isolates compared to bovine isolates were statistically significant (χ²-test, P < 0.05 and P < 0.01). The lower occurrences of the egc gene cluster
among the SAg gene-positive sheep or goat isolates compared to bovine isolates were also statistically significant ($\chi^2$-test, $P < 0.01$ and $P < 0.025$, respectively). When complete and incomplete egc gene clusters were considered together, the differences between SAg gene-positive sheep or goat isolates and bovine isolates attained higher statistical significance ($\chi^2$-test, both $P < 0.001$).

**sec sequencing**

Several SEC variants have been described (Marr et al., 1993), including SEC-bovine, which is associated with the SaPIbov pathogenicity island (Fitzgerald et al., 2001). Sequencing of the sec gene was performed using four representative non-bovine strains, two from goats and two from sheep. One goat isolate (Italian) and one sheep isolate (Icelandic) had the sec and tst genes only; the other goat isolate (Austrian) and the sheep isolate (Norwegian) had the sec, tst and sel genes. The sequencing data were aligned with the sequences of known sec alleles, namely sec1, sec2, sec3, sec-MNCopeland, sec-4446, sec-bovine and sec-ovine (Marr et al., 1993, Fitzgerald et al., 2001), using CLUSTAL_W. Three of the ovine and caprine sequences had 100% identity to sec-ovine. The sec sequence of the remaining goat isolate (Italian) was identical to the sec3 variant of the human-associated strain Mu50 genome sequence but exhibited a single base pair deletion at position

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**Table 3. Combinations of SAg genes in 110 SE gene-positive and tst-positive strains of *S. aureus* of animal origin**

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>No. of strains</th>
<th>Animal source* (No.)</th>
<th>Gene combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>1</td>
<td>B</td>
<td>(sec, sel, tst)†, (seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Argentina</td>
<td>1</td>
<td>B</td>
<td>sed, sej, (seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Austria</td>
<td>3</td>
<td>G</td>
<td>(sec, sel, tst)†</td>
</tr>
<tr>
<td>Belgium</td>
<td>1</td>
<td>R</td>
<td>seh</td>
</tr>
<tr>
<td>Belgium</td>
<td>1</td>
<td>R</td>
<td>sed, sej</td>
</tr>
<tr>
<td>Belgium</td>
<td>4</td>
<td>R</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Denmark</td>
<td>1</td>
<td>S</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Denmark</td>
<td>1</td>
<td>S</td>
<td>(sei, sem, sen, seo)§</td>
</tr>
<tr>
<td>Denmark</td>
<td>1</td>
<td>B</td>
<td>sec, tst, (seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Finland</td>
<td>1</td>
<td>S</td>
<td>seb</td>
</tr>
<tr>
<td>Finland</td>
<td>1</td>
<td>S</td>
<td>sed, sej</td>
</tr>
<tr>
<td>Iceland</td>
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<td>S</td>
<td>seh</td>
</tr>
<tr>
<td>Iceland</td>
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<td>S</td>
<td>sec, tst</td>
</tr>
<tr>
<td>Italy</td>
<td>5</td>
<td>G</td>
<td>(sec, sel, tst)†</td>
</tr>
<tr>
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<td>G</td>
<td>sec, tst</td>
</tr>
<tr>
<td>Italy</td>
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<td>G</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Northern Ireland</td>
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<td>C</td>
<td>(seg, sei, sem, sen, seo)‡</td>
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<tr>
<td>Northern Ireland</td>
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<td>C</td>
<td>seh, sek, sec</td>
</tr>
<tr>
<td>Norway</td>
<td>17</td>
<td>G (8), S (9)</td>
<td>(sec, sel, tst)†</td>
</tr>
<tr>
<td>Norway</td>
<td>1</td>
<td>S</td>
<td>(sec, sel, tst)†, (seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Norway</td>
<td>2</td>
<td>S</td>
<td>sec, tst</td>
</tr>
<tr>
<td>Republic of Ireland</td>
<td>8</td>
<td>B</td>
<td>(sec, sel, tst)†</td>
</tr>
<tr>
<td>Republic of Ireland</td>
<td>7</td>
<td>B</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Republic of Ireland</td>
<td>3</td>
<td>B</td>
<td>(seg, sei, sen, sen, seo)‡</td>
</tr>
<tr>
<td>Spain</td>
<td>1</td>
<td>R</td>
<td>seh</td>
</tr>
<tr>
<td>Spain</td>
<td>1</td>
<td>B</td>
<td>(sec, sel, tst)†</td>
</tr>
<tr>
<td>Spain</td>
<td>2</td>
<td>B (1), R(1)</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>Sweden</td>
<td>3</td>
<td>B (2), Cat (1)</td>
<td>sed, sej</td>
</tr>
<tr>
<td>Sweden</td>
<td>1</td>
<td>S</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>USA</td>
<td>1</td>
<td>B</td>
<td>(sec, sel, tst)†, sed, sej</td>
</tr>
<tr>
<td>USA</td>
<td>1</td>
<td>B</td>
<td>sed, sej</td>
</tr>
<tr>
<td>USA</td>
<td>1</td>
<td>B</td>
<td>sed, sej, (seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>USA</td>
<td>7</td>
<td>B</td>
<td>(seg, sei, sem, sen, seo)‡</td>
</tr>
<tr>
<td>USA</td>
<td>10</td>
<td>B</td>
<td>(sei, sem, sen, seo)§</td>
</tr>
</tbody>
</table>

*Animal source: B, bovine; G, goat; S, sheep; R, rabbit; C, chicken.
†SaPIbov pathogenicity island combination of genes.
‡Complete egc cluster.
§egc cluster gene combination lacking the seg gene.
596 in the alignment based on duplicate sequencing of both strands of PCR products (accession no. NC_002758).

**Geographical distribution of SE and TSST-1 genes in animal isolates**

Ninety-five percent of the strains from Norway, 93% of the Northern Ireland strains, 100% of the strains from Iceland, 80% of the strains from Sweden and 75% of the strains from Denmark were positive for SAg genes, while only 36% of strains from the Republic of Ireland and 54.1% of strains from the USA were positive for enterotoxin genes. However, the isolates from Norway were from sheep and goats that had a high frequency of SAg genes (Tables 2 and 3). Likewise the Northern Ireland strains were from chickens that also had a high frequency of SAg genes. The isolates with a low frequency of SAg genes were from the Republic of Ireland and the USA, in both cases from cows. However, it is important to note that 54% of the Irish isolates tested in this study were of RAPD types 4 and 5 that were negative for all 16 enterotoxin genes. Thus these differences in the frequencies of SAg genes on a geographical basis probably reflect the clonality of strains infecting cows, sheep and goats in these countries.

**Mitogenicity assays**

Mitogenicity assays on a sec/tst knockout mutant of a bovine strain previously aided the discovery of the SaPibov pathogenicity island (Fitzgerald et al., 2001). Therefore 79 of the 99 bovine isolates surveyed for SAg genes were screened to determine whether the presence of SAg genes correlated with the expression of biological activity and whether the SAg gene-negative isolates were mitogenic. 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 14 isolates exhibited borderline mitogenicity; 12 of these 14 isolates did not have any of the SAg genes tested for, while two possessed at least one SAg gene.

**DISCUSSION**

Most previous studies have indicated that in animal-associated isolates of **S. aureus** the sea, seb, sec and seh genes are absent or occur rarely – < 5% of tested strains (Larsen et al., 2000, 2002; Akineden et al., 2001; Hazariwala et al., 2002) – or that animal-associated strains are rarely producers of the SEA, SEB, SEE and SEH toxins (Kenny et al., 1993; Fitzgerald et al., 2000; Foschino et al., 2002; Cenci-Goga et al., 2003). Likewise in this study only 4-5% of strains possessed these genes. These general findings contrast with the recent Swiss study of Scherrer et al. (2004) in which 10-9% of isolates from bulk-tank milk samples from sheep and goats possessed either the sea or seb gene (1-6% in the present study).

Moreover, a Polish study (Kuźma et al., 2003) demonstrated the presence of the sea and seh genes in 18-8% and of the seh gene in 34-9% of tested bovine strains.

Of the genes encoding the classical SEs, the sec gene has been reported to have the highest frequency in strains from ungulates. In the case of bovine strains Larsen et al. (2002) reported a frequency of 26-6% and Akineden et al. (2001) of 15-5% (19-2% in the present study). However, in a Danish study the sec gene was detected at a frequency of 0-25% (Larsen et al., 2000). Scherrer et al. (2004) detected the sec gene in 42% of sheep and goat isolates compared to 51-6% reported herein. The frequency in poultry isolates is lower, 2-9% in Hazariwala et al. (2002) and 0% reported herein.

The frequencies of the sed gene among isolates from sheep, goats and poultry reported previously (< 2%) agree with our findings (Hazariwala et al., 2002; Scherrer et al., 2004). The frequencies of occurrence of the sed gene vary greatly among bovine isolates from different countries (Larsen et al., 2002). Low frequencies (< 6%) have been reported for Scandinavian countries, Germany and Ireland versus 17-35% in Finland, Iceland, Switzerland and the USA. These differences probably reflect the principal clonal types causing bovine mastitis.

The co-occurrence of the sec and tst genes in bovine isolates from 10 countries has been noted (Larsen et al., 2002, 95-6%) and also from Germany (Akineden et al., 2001, 100%; Zschöck et al., 2004, 100%) or co-production of SEC and TSST-1 (Orden et al., 1992, ~90%; Kenny et al., 1993, 97-7%; Fitzgerald et al., 2000, 100% in Irish RAPD type 7 strains). This contrasts with findings of rare co-occurrence of the sec and tst genes in human isolates of blood and nasal origin (Peacock et al., 2002; Becker et al., 2003; Nashev et al., 2004). Fitzgerald et al. (2001) discovered the SaPibov pathogenicity island harbouring the sec-bovine and tst genes along with a novel enterotoxin gene sel. The present study extends the co-occurrence of these genes to isolates from goats and sheep and concurs with the recent report of Scherrer et al. (2004) in which 95-9% co-occurrence of the sec and tst genes in sheep and goat isolates was observed. It is noteworthy that Orden et al. (1992) observed 95-3% co-production of SEC and TSST-1 in sheep and goat strains. Kuźma et al. (2003) noted that all isolates containing the tst gene were simultaneously positive for the sec gene.

While it is almost certain that the sec-bovine variant occurs in the majority of the bovine isolates containing the sec and tst genes, the primers used in the present study and by Scherrer et al. (2004) would detect all the known sec variants (Marr et al., 1993; Fitzgerald et al., 2001). In the case of three of the sheep and goat isolates investigated in this study the sec-ovine variant was confirmed (Marr et al., 1993) irrespective of the presence of the sel gene. These limited data suggest that sec-ovine may be present on a pathogenicity island with the tst gene in the presence or absence of the sel gene. In the remaining goat isolate examined, a sec3 variant gene was found to have a single base-pair-deletion mutation. Apart from the sec-bovine gene, the sec1 gene has been shown to be
present on a pathogenicity island termed SaPI4 along with genes encoding three other novel enterotoxins (Novick, 2003). In the genome sequence of S. aureus strain Mu50, the genes sel and sec3 are contiguous, with the sec3 gene separated from the tst gene by one ORF encoding a hypothetical protein (Kuroda et al., 2001; accession no. NC_002758). The close proximity of these genes suggests their co-occurrence on a chromosomal genetic element.

We believe the present study is the most comprehensive survey of SAg genes in S. aureus strains of animal origin to date. Examining animal-associated strains for the novel SE genes in addition to the classical SE genes and the tst gene increased the number of SAg gene-positive isolates from 60 strains (31.4 %) to 110 strains (57.6 %). The commonest SE gene combination was the egc cluster, namely sei, sem, sen and seo with or without the seg gene (52.7 % of SAg gene-positive isolates). Akinaden et al. (2001) screened bovine mastitis isolates for the sea–seg genes only. Strains possessing the seg and sei genes, i.e. the egc locus, accounted for 48 % of SAg gene-positive strains (48.9 % of bovine SAg gene-positive strains reported herein). Strains that were sei gene-positive but lacked the seg gene, i.e. possessed the egc locus minus the seg gene, accounted for 22.7 % of SAg gene-positive isolates (22.2 % of bovine isolates reported herein). The seg primers used in the current study would amplify the known variants of the seg gene and should not account for the seg-negative results. In two studies on human wound and nasal isolates, approximately 75 % of SAg gene-positive strains possessed both the seg and sei genes (Becker et al., 2003, Nashev et al., 2004). In a recent survey of 55 human isolates of S. aureus from food-poisoning outbreaks that were negative for classical SEs, only eight strains possessed the seg, seh or sei genes (Chen et al., 2004). Of 139 isolates from food samples 13 possessed the seg, seh or sei genes without classical SE genes, 15 strains possessed one or more of those genes in combination with the sea gene and one strain in combination with the sed gene (Chen et al., 2004). A food-poisoning outbreak in Norway has been linked to seh gene-positive isolates of S. aureus (Loncarevic & Mathisen, 2004).

The sed and seg genes have been localized to a plasmid (Zhang et al., 1998). The sed and seg genes in combination were present in nine animal isolates, with the egc locus in two instances and with the SaPIbov pathogenicity island in one. Six of these nine strains were of bovine origin. Akinaden et al. (2001) reported the sed/seg gene combination in 28 % of SAg gene-positive bovine strains (13-3 % in this study). Scherr et al. (2004) reported this combination in four sheep or goat isolates (2-1 % versus 1-6 % reported herein).

Of the 34 bovine isolates harbouring one or more SAg genes, 94.1 % were unequivocally mitogenic for human T cells. The other two isolates possessing SAg genes may express SAgS at low levels, leading to the borderline findings. Of the 45 SAg gene-negative isolates four were mitogenic and 12 potentially mitogenic. The latter findings suggest that these strains may harbour genes for as-yet-unidentified SAgS or the sep, ser and seu genes, the latter two of which were identified during the course of the present study (Kuroda et al., 2001; Letertre et al., 2003a; Omo et al., 2003). The ser gene localizes to a plasmid that encodes the sed and seg genes. Accordingly sed-seg-positive isolates would be anticipated to also harbour the ser gene. The seu gene has only been reported in some strains that contain the egc cluster. Thus, it is likely that some of the animal strains analysed in the present study may contain the seu, ser and sep genes.

This work has extended a previously existing multiplex PCR to screen for novel SAg genes. Sergeev et al. (2004) have developed an assay that involves PCR amplification of SE genes using degenerate primers, followed by characterization of the amplicons by microchip hybridization with oligonucleotide probes specific for each SE gene. This assay has the advantage that it can detect previously unidentified SE genes. Letertre et al. (2003b) have developed a real-time PCR that can detect and toxin-type strains for the sea to seg genes. Letertre et al. (2003c) have also developed a 5′-nuclease assay that comprises three triplex PCRs for the amplification of the sea–seg genes. Martin et al. (2004) have used molecular typing methods along with multiplex PCR for SE genes to investigate the epidemiology of food-borne outbreaks of S. aureus. All of these newly developed or extended methods provide a range of strategies to allow different laboratories to rapidly screen S. aureus strains of animal and human origin for SE genes. The present multiplex assays could be readily adapted to screen for additional genes including the newly described sep, ser and seu as well as others that may be described in the future.

The demonstration in this study that the egc cluster of SE genes and the SaPIbov SE genes are the commonest in clinical S. aureus isolates of animal origin raises questions about the potential roles of these SAgS in the variety of infections caused by S. aureus in their host animal species. The statistically significant higher frequency of the egc locus with or without the seg gene and the lower frequency of the sec and tst genes in bovine isolates compared to those from sheep or goats suggest that egc-associated SAgS may be more important as putative virulence factors in bovine mastitis. One or more of these egc-associated SAgS may confer a survival advantage in the udder through modulation of the immune response. The ability of S. aureus to persist in the mammary gland in the chronic disease state is a conundrum that needs to be resolved for the development of an effective vaccine.

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