Bovine Staphylococcus aureus superantigens stimulate the entire T cell repertoire of cattle.

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Running title: Bovine T cell reactivity with Staphylococcal SAgs

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Superantigens (SAgs) represent a diverse family of bacterial toxins that induce Vβ-specific T cell proliferation associated with an array of important diseases in humans and animals, including mastitis of dairy cows. However, an understanding of the diversity and distribution of SAg genes among bovine *Staphylococcus aureus* and their role in the pathogenesis of mastitis is lacking. Population genomic analysis of 195 bovine *S. aureus* isolates representing 57 unique sequence types revealed that strains encode 2 to 12 distinct SAgs and that the majority of isolates contain 5 or more SAg genes. A genome-scale analysis of bovine reference strain RF122 revealed a complement of 11 predicted SAg genes, which were all expressed *in vitro*. Detection of specific antibodies in convalescent cows suggests expression of 7 of 11 SAgs during natural *S. aureus* infection. We determined the Vβ T cell activation profile for all functional SAgs encoded by RF122 revealing evidence for bovine host-specific activity among recently identified RF122-encoded SAgs SElY and SElZ. Remarkably, we discovered that some strains have evolved the capacity to stimulate the entire T-cell repertoire of cattle through an array of diverse SAgs suggesting a key role in bovine immune evasion.
Introduction

*Staphylococcus aureus* produces a family of at least 26 distinct superantigens (SAgs) including staphylococcal enterotoxins (SEs), SEA to E, SEG to J, SER to T, staphylococcal enterotoxin-like toxins (SEls), SEIK to Q, U, V, X, Y, Z and toxic shock syndrome toxin-1 (TSST-1) (1, 2). SAgs induce the Vβ-specific proliferation of T cells along with the release of pro-inflammatory cytokines including IL-1, IL-2, IL-6, TNF-α, IFN-γ, and chemokines CCL2 and CCL3 (3, 4). The uncontrolled release of pro-inflammatory mediators can lead to rashes, fever, multi-organ damage, coma and death from severe shock (1). The release of pro-inflammatory signals can impede the effectiveness of the immune response by creating a bias towards either the T_h1 or T_h17 response disrupting the appropriate recruitment of effector cells (2). SAgs have been implicated in a wide range of human diseases including staphylococcal food poisoning, endocarditis, necrotizing pneumonia and severe toxic shock (1, 5-7). Taken together the effects induced by SAgs are likely to cause significant deficiency in the ability of the adaptive immune response to contribute effectively to clearance during *S. aureus* infection.

*S. aureus* is a common cause of bovine mastitis, an infection of the milk-secreting tissue of the udder, which represents a huge economic problem for the dairy industry worldwide (Miles et al., 1992, Barkema et al., 2006), establishing a typically chronic infection (8). The exact role of SAgs in this disease is currently unknown, however, it has been proposed that superantigenic activity may contribute to the persistence observed (9, 10). Although Vβ specific activation of human T cells in response to staphylococcal SAgs has been well characterised (11-14) relatively little is known for Vβ-specific proliferation of bovine T cells. Previously, SEC and TSST-1 have been shown to induce Vβ-specific proliferation of bovine T cells (15-17).
However, these studies were limited by the number of TRBV gene sequences available, with only 5 subfamilies; Vβ1, 2, 4, 13, and 28 included.

The bovine genome sequencing project and cDNA analyses led to the identification of the full complement of bovine Vβ subfamilies and almost the entire repertoire of bovine TRBV genes (18, 19). This facilitated the development of a quantitative real-time PCR (qRT-PCR) assay to study the bovVβ response to stimulation with the core genome-encoded SAg SE1X (14). *S. aureus* strain RF122 belongs to the common bovine specific lineage ST151, and was the first animal-associated isolate to be fully sequenced (20). In this study we have carried out a comprehensive, genome-wide analysis of the complement of SAgs encoded by this strain and determined the capacity of each toxin to activate bovVβ specific T cells. We report host-specific functional activity for several SAgs, and reveal the remarkable capacity of bovine *S. aureus* for activation of the full bovine T cell repertoire suggesting a critical role in immune evasion. Importantly, we have also demonstrated that SAgs produced by *S. aureus* may play a role in the development of intramammary infection of dairy cows.
Results and Discussion

Population genomic analysis indicates that bovine *S. aureus* strains encode 2 to 11 intact SAg genes. We examined 195 bovine *S. aureus* genome sequences representing 57 unique STs for the presence of all 26 known members of the *S. aureus* SAg family (Table 1: Fig. 1). We employed a threshold of 90% sequence identity across the entire CDS to exclude cross matches to other members of the same SAg group (Fig. 2, Supplementary Table 1).

Consistent with previous studies, *selw* and *selx*, were found in 100% (195/195) and 79% (150/195) of isolates analysed, respectively. Previous studies identified *selw* to be inactivated in a large number of human *S. aureus* isolates examined, due to the lack of an ATG start codon (21, 22). However, the presence of alternative start codons (TTG), a continuous full-length open reading frame and the high level of sequence conservation across bovine isolates (more than 94% nucleotide sequence identity) suggests a high proportion of isolates have a functional *selw* gene. The *selx* gene was only absent in clonal complex (CC) 30 consistent with previous reports (14). The *egc* cluster was present in 21 of the 57 unique sequence types (ST) analysed and was highly prevalent within CCs 30, 151 and 45. The composition of the *egc* varied with six different gene arrangements characterised but a gene complement of *seg*, *sei*, *selm*, *seln*, *selo* and *selu/u2* was the most common observed. SaPl*bov* was less prevalent compared to the *egc* cluster, found in 10 of the 57 STs analysed, primarily in association with CC133 and CC151. Plasmid-encoded SAg* sed, sej* and *ser* were identified together in 4 strains consistent with the presence of an pIB485-like plasmid as described in human strains (23). The genes encoding *sely* and *selz* are distributed in a lineage-specific manner, (CC151 and CC9 for *sely* and CC151 for *selz*), and SAg genes; *sea, seb, seh, selk, selp* and *selq* were randomly distributed across the diversity.
of STs examined consistent with horizontal gene transfer. Genes encoding set and ses were not found in any S. aureus genomes examined suggesting they are not important in bovine pathogenesis.

All S. aureus strains examined contained at least 2 and up to 11 SAg genes. The majority of bovine STs analysed (31/57) encode 5 or more SAgs with CC151 isolates such as RF122 generally encoding more SAgs (up to 13) than other bovine S. aureus strains. Less than half of STs (n=26) contained selw and selx only. An important example is the bovine reference strain Newbould 305, which has been the focus of a number of studies (24, 25), encodes a functional copy of selx, and a pseudogene of selw (25). The extensive variation in SAg gene complement between Newbould 305 and RF122 may have a key impact on relative pathogenesis of infections caused by these strains. Newbould 305 is associated with mild and generally subclinical infection as opposed to RF122 and other CC151 isolates which are associated with a more severe and clinical presentation of the disease (25, 26).

Analysis of the genome of the bovine S. aureus isolate RF122 (Accession: AJ938182, https://www.ncbi.nlm.nih.gov/nuccore/AJ938182.1/) revealed a complement of 11 SAg genes and 2 SAg pseudogenes (Table 1, Supplementary Table 1, Fig. 1). Namely, RF122 contains the previously characterized bovine staphylococcal pathogenicity island (SaPIbov) that contains tstbov, sellbov, secbov, the enterotoxin gene cluster (egc) in the genomic island vSaβ containing allelic variants of SAg genes seg, sei, selo, seln, selu and a pseudogene of selm. Spread out across other parts of the genome, RF122 also codes for selw (pseudogene - SAB1473c) selx, sely and selz (Fig. 1). The SAg family has been previously subdivided into phylogenetic groups I-V (group IV is composed entirely of streptococcal SAgs) (27, 28), and RF122 encodes at least 2 genes from each of the 4
staphylococcal SAg subgroups (Fig. 1). Accordingly, RF122 was selected for genome-scale analysis of the expression and function of bovine *S. aureus* SAgs.

Bovine SAg genes are expressed at different levels in a growth phase dependent manner *in vitro*. Relative transcriptional levels of RF122 SAg genes in exponential and stationary phase of growth were determined by qRT-PCR. Transcription was detected for all 11 genes and 2 pseudogenes in both growth phases, with *secbov* exhibiting the highest level of transcription and *selu*, the lowest (Fig. 3). Overall, SAg genes located on SaPIbov were transcribed at higher levels than *selx, sely, selz, egc*-encoded genes and the SAg pseudogenes. The data indicate that SaPIbov SAg genes and *selx* are up-regulated in stationary phase, consistent with regulatory control by *agr*, whereas *sely* and *selz* are transcribed maximally in mid-exponential phase suggesting *agr*-independent control. Of note, ST151 strains were previously demonstrated to have higher levels of RNAIII transcription in comparison with other ruminant clones and could provide an explanation for the high expression of some of these SAgs (26). In the current study, *egc* genes were transcribed at low levels, independent of growth phase. This finding is consistent with Derzelle *et al* who reported low *egc* transcript levels among 28 human strains (29). However, we cannot rule out the possibility that the *egc* genes are expressed at higher levels *in vivo*, as has been observed for the streptococcal SAgs SPEA and SPEC (30, 31). The differential regulation of SAg transcription *in vitro* suggests that SAgs are expressed at different stages of infection *in vivo*.

*S. aureus* SAgs are expressed during bovine infection. To determine if RF122-encoded SAgs are expressed during bovine infection, we produced recombinant
proteins for each of the encoded SAgs, and carried out western immunoblot analysis with convalescent sera from cows (Table 2). A serum sample obtained from a cow without a history of *S. aureus* mastitis, did not contain antibody reactive for any of the SAgs tested and was used as a negative control (Table 2). IgG antibodies specific for 8 of the 11 SAgs were detected in at least 1 of the 4 bovine sera samples tested whereas rSEI<sub>bov</sub>, rSEG<sub>bov</sub> and rSEI0<sub>bov</sub> were not reactive with any of the samples tested (Table 2). In a previous study by Wilson et al, rSEIX<sub>bov</sub> was demonstrated to be reactive with all bovine sera samples tested. Most human adults have antibodies specific for an array of *S. aureus* SAgs including SEA, SEB, SEC, SED, SEE, SEIX and TSST-1, as a result of exposure during colonisation or infection (14, 32, 33). The current study corroborates previous observations, which found, that despite the relatively high prevalence of the *egc* cluster in clinical isolates of *S. aureus*, neutralising antibodies are rare (34).

This suggests that either the *egc* SAgs are poorly expressed during infection or that the host is unable to generate antibodies due to low T or B-cell reactivity. Importantly in this study we have shown that SEIY, SEIZ, and to a lesser extent SEIU and SEI are expressed by *S. aureus in vivo*. Antibodies against SEIY and SEIZ have been detected in at least one serum sample of bovine and human origin, consistent with a role in pathogenesis of both host-species.

Although our data suggest low levels of expression of some SAgs, it is feasible that they can contribute to *S. aureus* immune modulation. For example, we recently demonstrated that suboptimal stimulation of human T cells with a low concentration of SAg (1 ng/ml) induced CD8+CD25+FOXP3+ regulatory T cells that strongly suppress activation of effector T cells (35). A similar phenomenon can be
observed in the bovine system as immunosuppressive CD4+CD25+FOXP3+ are activated with equivocally low concentrations of SAg (1 ng/ml) (36).

**RF122-encoded SAg**s are mitogenic for bovine T cells. In order to examine the mitogenicity of each of the 11 identified SAg, we constructed a SAg-deficient mutant of *S. aureus* strain RF122 to facilitate plasmid-mediated expression of each SAg in isolation by its native *S. aureus* strain. *S. aureus* RF122-1, a TSST-1-deficient derivative of RF122 had been constructed previously by allele replacement of the *tst* gene with a tetracycline resistance cassette (17). In turn, we sequentially deleted the *sec, sel, egc* genes, *selx, sely and selz* by allele replacement (Fig S1) resulting in the sequential mutants RF122-2 to RF122-8 and the final SAg-deficient derivative RF122-8 (Table S3 and Fig S1). Finally, to limit the Hla-mediated toxicity for T cells, we constructed *hla* mutants in the parent RF122 and SAg-deficient derivatives resulting in strains RF122t-α and RF122-8α, respectively (Supplementary Table 3). The mutants were validated to rule out spurious mutations accrued during *in vitro* passage that impact on secreted virulence proteins (Fig S2). Analysis of the mitogenicity of stationary and mid-exponential phase culture supernatants of RF122 and RF122-8 confirmed loss of all detectable mitogenic activity (Fig S2).

Previously, superantigenic activity of RF122-encoded SEC<sub>bov</sub>, TSST-1<sub>bov</sub> and SEIX<sub>bov</sub> has been described (14-17). In order to examine the mitogenic potential of all SAg encoded by RF122 expressed in a native *S. aureus* background, SAg genes were cloned into the inducible expression plasmid pALC2073. This allowed controlled expression in the SAg-deficient RF122-8α, facilitating analysis of the effect of individual SAgS produced in their native strain context on bovine T cells *in vitro*. Proteins of the predicted molecular weight were detected in supernatants of induced
RF122-8α cultures for each SAg plasmid construct with the exceptions of SEGbov, SEINbov, SELbov and SELObov. (Fig. S3). To examine the mitogenicity of RF122-encoded SAgs for bovine T cells, culture supernatants of RF122-8α containing pALC2073::SAg constructs and recombinant SAg proteins were used to stimulate bovine PBMC, and proliferation was measured using a thymidine incorporation assay (Fig. 4). Mitogenic activity for bovine T cells was detected for 7 of the 11 SAgs expressed in the SAg-free RF122-8α including TSST-1, SECbov, SELbov, SELObov, SEINbov, SELX, and SEIZbov at total protein concentrations ranging from 10 pg/µl to 10 ng/ml, but there was no detectable mitogenic activity for SELObov, SEGbov, and SELUbov (Fig 3a). However, recombinant proteins rSEGbov and rSEIUbov and rSEIX expressed in E. coli could stimulate T cell proliferation at higher concentrations (Fig. 3b). Accordingly, of the 11 SAgs encoded by RF122, only SELObov did not exhibit any capacity for stimulation of bovine T cells. Taken together, these data indicate that RF122 encodes an array of SAgs that are potent bovine T cell mitogens.

RF122-encoded SAgs have the capacity to stimulate the entire bovine Vβ repertoire. Most previous studies of the bovine Vβ-dependent T cell activation capacity of staphylococcal SAgs have been limited by the number of identified bovine Vβ subfamilies (15, 17). Recently, we developed a novel qRT-PCR assay which is representative of the full complement of bovine Vβ subfamilies (14). Supernatants from tetracycline induced cultures of RF122-8α containing pALC2073::SAg constructs were used to stimulate bovine T cells. If the supernatant was unable to induce proliferation at a total protein concentration of 0.01µg/ml then purified recombinant protein was used as an alternative to determine the boVβ profile (Fig.
5). Accordingly, in the current study we were able to comprehensively evaluate the response of 18 bovVβ subfamilies to stimulation with all RF122-encoded SAgs by qRT-PCR (Table 3 and Fig. 5). In order to examine the host-specificity of bovine SAgs, we also examined the capacity of recently characterized SAgs SElY and SElZ to stimulate Vβ-dependent activation of human T cells (Fig. 6 and Table 3). We found that all SAgs encoded by RF122 with the exception of SElObov induced Vβ-specific stimulation of bovine T cells (Fig. 5) with a unique bovVβ activation profile similar to humVβ activation profiles T cell (37). Of note, the data indicate that each of the 18 bovVβ subfamilies tested are activated by at least one RF122-encoded SAg, such that RF122 has the potential to stimulate the entire bovVβ repertoire (Fig. 5). Remarkably, the 3 SAgs encoded by SaPlbov alone activate 13 of 18 bovVβ subfamilies highlighting the potential importance of SaPlbov in bovine immune evasion. In comparison, despite being twice in number, the egc SAgs activate only 11 of 18 subfamilies. Extensive duplication within the bovVβ repertoire has resulted in 9 multimember subgroups, the largest of which, bovVβ 1, 10 and 13, contain 23, 9 and 20 functional TRBV genes, respectively (18, 19). Each of the SaPlbov encoded SAgs, SECbov, SEILbov and TSST-1bov, and egc encoded SElbov can activate at least one of these large subfamilies each (Fig. 5). SEILbov activates both bovVβ 1 and 10 which is consistent with the large proportion of T cells which are induced in response to stimulation with this SAg (Fig. 4 and 5). It has been shown previously that all humVβ subfamilies (with the exception of humVβ 4 and 11) are activated by at least one SAg (2). Our data also indicates that some bovVβ subfamilies can be activated by multiple SAgs, for example, Vβ 16 and X are activated by 6 RF122-encoded SAgs, and Vβ 24 and 17 are activated by 5 of them. This apparent functional redundancy implies that activation of these Vβ subfamilies is of critical importance in S. aureus infection.
similar redundancy has been observed in the humVβ response to S Ags, with Vβ1, 3, 5, 6, 9, 12, 18 and 21 targeted by at least 5 or more different S Ags (2).

Evidence for host-adaptation by bovine S. aureus S Ags. For the recently characterized S Ags SEIY and SEIZ, we examined the Vβ-dependent activation of human and bovine T cells. We utilised protein variants for both SEIY and SEIZ derived from human and bovine isolates to investigate the possibility of host-adaptation. Both human and bovine variants of SEIY and SEIZ induced similar levels of expansion of human T cells (Fig. 6a). SEIY induced expansion of a broad number of human Vβ subfamilies (Fig. 6b), while SEIZ induced expansion of a single human Vβ subfamily (13.2). In contrast, the bovine Vβ expansion profile, both human and bovine variants of SEIY and SEIZ activated different bov Vβ subfamilies. SEIYbov activated bov Vβ subfamilies 1, 3, 7, 11, 16, 17, 24, 28, and X, while the SEIZhum activated bov Vβ subfamilies 24, 28, and X (Fig. 6c). It is also noteworthy that SEIYbov exhibited a 10-fold greater potency than SEIYhum for stimulating bovine T cell proliferation (Fig. 6a). This could be explained by activation of a broader number of bov Vβ subfamilies by SEIYbov compared to the human variant (Fig. 6c). Strikingly, SEIYbov induced expansion of a broad array of bov Vβ subfamilies, while SEIYhum was unable to induce activation of bovine T-cells (Fig. 6a, c). Combined, these results suggest adaptive evolution of SEIY and SEIZ to the bovine host.

Analysis of the protein variants SEIY and SEIZ revealed a number of unique residues which may be responsible for the difference in phenotype observed between the human and bovine variants (Fig. S4). For SEIY, three positions varied between the bovine allele from RF122 (ST151) and the human allele from MSA2020 (ST121) (E19G, T67A and I183V). In particular the glutamic acid residue at position 19 was
identified in SElY allele of ST151 and other cattle isolates (STs 3140, 504, 706 and 3099), but not in any of the SElY variants of human origin. For SElZ, four positions varied between the bovine allele from RF122 (ST151) and the human allele from MSA1695 (P6L, N55S, D75N and G106A). Of note, the glycine residue at position 106 of RF122 SElZ was found in all but one of the bovine SElZ variants analysed and was absent among the majority of human variants (6/8).

Some of the differences between human and bovine Vβ activation profiles are due to absence of an orthologous subgroup, such as the activation of humVβ 12, 14, 20, 22, and 23 (absent in bovine), and bovVβ 10, 28 and X (absent in human) (18). However, there are cases where Vβ subfamilies from one host are activated, but the orthologous subgroup from the other is not (Table 3). For example, SElLbov activates bovVβ 6 and 24 but not humVβ 6 and 24, TSST-1bov activates only bovine Vβ 4, and 24, SEIbov activates bovVβ 16 and humVβ 5 and 6, but not the equivocal variants in the opposite species, and SEINbov activates bovVβ 3, 16 and 24 and humVβ 7 and 8, but not the equivalent human or bovine subgroups. It is important to note that with the exception of SECbov and SEIXbov, the human Vβ profiles described here were determined in previous reports in response to stimulation with SAgS derived from human S. aureus strains (11, 12). It is feasible that distinct human Vβ profiles could be stimulated by bovine SAg variants. Our analysis of SElY and SElZ, and previous analysis of SElX (14) support the notion that allelic variants of SAgS made by S. aureus from different host species have evolved to preferentially activate the Vβ repertoire of the strains target host. Together, these data indicate that some SAgS encoded by bovine S. aureus have undergone host-adaptation associated with broader stimulation of Vβ–subfamilies, and increased potency of bovine T cell activation. Furthermore, we report that SElY and SElZ are classical SAgS in that they have a
unique Vβ activation profiles with capacity to mediate immune modulation in both humans and cattle.

**Preliminary examination of the role of SAgs in the pathogenesis of bovine mastitis.** The functional analysis of bovine SAgs made by a single strain in the current study suggests a profound role in host-pathogen interactions and pathogenesis. In order to examine the role of SAgs in *S. aureus* bovine mastitis, preliminary experimental infections of bovine mammary glands were carried out using RF122 and RF122-8 over a course of 21 days. Seven healthy dairy cows in their 1st to 4th lactation were enrolled in two groups of 4 and 3 cows, challenged with wild type RF122 or the SAg-deficient RF122-8, respectively. There were no differences observed between the groups in terms of somatic cell count, milk yields and core body temperatures (Fig. S5). *S. aureus* was isolated from the mammary gland of all animals during the trial, taken together with the milk quality and somatic cell counts, these data indicate that SAg are not required to establish sub-clinical mastitis. The group infected with wild type RF122 exhibited clinical mastitis at least once, in three out of the four animals infected during the course of the study (Fig. 7). In contrast, clinical mastitis was not observed in the animals infected with the SAg-deficient mutant. Although the study was not powered for statistical significance, the data are suggestive of a role for bovine SAgs in the development of staphylococcal clinical mastitis. Further experimentation would be required to confirm this preliminary observation.

We speculate that SAgs may contribute to pathogenesis through expression of some SAgs such as SEC1 and TSST-1 at high concentrations to promote the release of proinflammatory cytokines which in turn induce tissue damage, inflammation, and
Furthermore, some SAgs such as *egc* SAgs expressed at low concentrations may induce immunosuppressive regulatory T cells to promote colonisation of the host.

**Concluding comments.** In conclusion, the role of SAgs during pathogenesis is very complex. The array of identifiable staphylococcal SAgs is expanding and has been expedited with advances in genomic analyses. The extensive diversity is potentially driven by the need to activate a large number of T cells, and bind to MHC class II molecules in multiple ways, contributing to immune evasion. Our findings contribute to the understanding of staphylococcal SAg diversity and provide a comprehensive analysis of the bovine T cell response to SAgs. In addition, we report examples of toxins that contribute to the capacity of *S. aureus* to adapt to different host species.
Methods

Ethics Statement

All in vivo work was done after local ethical review and under the oversight of the Kalamazoo IACUC, and in accordance with local, state, and federal animal welfare regulations. Bovine venous blood was taken under the authority of a UK Home Office Project License (PPL 604394) within the terms and conditions of the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986 and the code of practice for the housing and care of animals bred, supplied, or used for scientific purposes. Human venous blood was taken from healthy donors in accordance with a human subject protocol approved by the national research ethics service (NRES) committee South East Scotland under the research ethics committee reference 11/AL/0168. Volunteers were recruited by a passive advertising campaign within the Roslin Institute (University of Edinburgh) and written consent was given by each volunteer before each sample was taken.

Bacterial culture conditions

*S. aureus* strains were grown in tryptone soya broth (TSB) or brain heart infusion broth (BHI) (Oxoid, UK) shaken at 200 rpm, or on tryptone soya agar (TSA) (Oxoid, UK) at 37 °C for 16 h unless otherwise stated. *E. coli* strains were grown in Luria-
Bertani Broth (LB) (Melford Laboratories, UK) shaken at 200rpm, or on LB-agar (Melford Laboratories, UK) at 37 °C for 16 h unless otherwise stated. Media were supplemented where appropriate with 150 μg/ml X-gal, 50 μg/ml ampicillin, 10 μg/ml erythromycin or chloramphenicol (Sigma-Aldrich, Dorset, UK). For growth curve analysis of *S. aureus*, strains were cultured overnight in 5 ml Brain–Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) in triplicate. After 12 h strains were sub-cultured at a dilution of 1 in 100 into 30 ml fresh BHI broth in 250 ml Erlenmeyer flasks and placed in a shaking incubator at 37 °C and 200 rpm. Absorbance readings were measured at 600 nm (OD_{600}) using a spectrophotometer (Cecil Aurius CE2021, Thistle Scientific Ltd., Glasgow, UK) over a period of 12 h and a growth curve was determined.

Sequence analysis of staphylococcal SAg genes

The sequences of characterised staphylococcal SAg genes were obtained from the NCBI Genbank database (Supplementary Table 1). SAg homologs were identified in publicly available whole genome sequences of bovine and representative human *S. aureus* genomes using BLASTn with a minimum alignment of 90% nucleotide identity averaged across the entire gene sequence using the Blastable script (github.com/bawee/blastable). Representative genomes with unique sequence types and SAg content were selected and a core genome alignment was built using Parsnp (38). The association between SAg content and phylogeny was visualised using iTol (39). Nucleotide sequences corresponding to each reference SAg were aligned at the codon level using translatorx and mafft (40, 41). A Maximum-Likelihood tree was constructed from the translated amino acid alignment using RAxML (v8.2.10) with the following settings: -m PROTCATAUTO -f a -N 1000 -x 123 -p 123 (42).
(43) was used to construct the circular genome representation and GC content plot with *S. aureus* RF122 genome sequence (Accession number: AJ938182, https://www.ncbi.nlm.nih.gov/nuccore/AJ938182.1/) as a reference.

**Transcriptional analysis of SAg genes**

Total RNA was extracted from *S. aureus* strains RF122 exponential (OD<sub>600</sub> = 0.6) and stationary phase (12 h) cultures using the RNeasy miniprep kit (QIAgen, UK) as described in the manufacturer's instructions except for an added lysis step with re-suspension of the bacterial pellet in TE buffer with 100 µg/ml Lysostaphin and incubation at 37°C for 20 min. RNA was treated with Turbo DNase (Thermo Fisher, UK). 0.5 µg mRNA was analysed for gene transcription using the same protocol outlined in Wilson *et al* (2011), SAg primers are listed in Supplementary Table 2.

**Allelic replacement of SAg genes**

Gene deletion constructs of SAg genes in RF122 were performed using constructs prepared in the plasmid pMAD (44) (Supplementary Table 3). Plasmid construction and allelic replacement were performed as described elsewhere (14, 44). The resulting mutant strain which had lost the gene of interest (GOI) was analysed by PCR for no amplification with primers within the deleted region or with pMAD MCS primers (Supplementary table 2). The mutant strains were also sequenced using primers upstream (E) and downstream (Z) of the GOI to confirm the predicted deletion event. Sequencing reactions were carried out by Edinburgh Genomics (King’s Buildings, University of Edinburgh, UK). To investigate the possibility that deletion of the genes could have pleiotropic effects, the phenotype of WT and mutant strains was compared. Firstly, a growth curve was determined for RF122, RF122t and RF122-8,
grown in BHI liquid culture for 10 h at 37 °C, which revealed growth rates and yields were similar for each strain (Fig S2). In addition, the haemolysis of rabbit erythrocytes incubated with culture supernatants of RF122 and SAg-deficient derivative strains was investigated. In each case the haemolytic titre of RF122 and SAg-deficient derivatives was 1022, indicating that the deletion of SAg genes had no effect on haemolytic activity and that the agr locus was functional (Fig S2). Deletion of the hla gene in RF122 resulted in a reduction in haemolytic titre indicating these strains are less toxic than the wild type. Analysis of the profile of secreted and CWA proteins of WT and mutant strains revealed no unexpected differences (Fig S2).

Analysis S. aureus secreted and CWA proteins

Secreted and cell-wall associated (CWA) proteins were extracted from S. aureus mid-exponential (OD600=0.6) and stationary phase (12 h) cultures grown in BHI. Cells were centrifuged at 4000 x g and supernatant fractions containing secreted proteins were removed and concentrated with Amicon Ultra-15 Centrifugal Filter units with a 10 kDa MWCO as described in the manufacturer’s instructions (Merk Millipore, UK). To extract CWA proteins, pelleted cells were washed with 1 ml PBS (Oxoid, Cambridge, UK), re-suspended in 1 ml lysis buffer (50 mM TrisHCl, 20 mM MgCl2, 30% Raffinose (Fluka, UK), adjusted to pH 7.5) containing 200 μg/ml Lysostaphin (AMBI products LLC, NY, USA) and protease inhibitors (Roche, UK) and incubated at 37 °C for 20 min. Samples were centrifuged at 6000 x g for 20 min and CWA proteins were recovered from the supernatant fraction. Protein preparations were separated on 10% SDS-PAGE gels, stained overnight at room temperature with Coomassie Blue (Severn Biotech), or transferred to Nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western blot.
analysis. The membrane was incubated with primary antibody for 1 h with 1:2500 dilution of anti-SEC (Santa Cruz Biotechnology, Heidelberg, Germany), or 2 h with a 1:2000 dilution of rat antisera specific for rTSSST-1, rSEIL, or rSEIXbov. Membrane was incubated with secondary antibody for 1 h at dilutions; 1:2500 (Rabbit anti-mouse IgG, Zymed, Invitrogen, UK), or 1:1500 Goat polyclonal antibody to rat IgG/HRP, (Abcam, Cambridge, UK), and visualised by ECL.

Cloning of SAg genes into pALC2073

5’ oligos to amplify RF122-encoded SAg genes for cloning into the expression plasmid pALC2073 were designed to prime upstream of the predicted ribosome binding site (RBS) with a KpnI site incorporated to facilitate cloning (Supplementary table 2). The 3’ primer was designed to include the stop codon of the gene with a SacI site incorporated (Supplementary table 2). PCR reactions were carried out with 10 ng RF122 gDNA, 100 nmol forward and reverse primers, as listed in Supplementary Table 2, using 1 U Vent polymerase (New England Biolabs, Herts, UK) as described in the manufacturer’s instructions. PCR products were cloned into the Strataclone pSC-B plasmid (Agilent, Cheshire, UK), inserts were released by digestion with SacI and KpnI for 3 h at 37 °C, purified by gel extraction, ligated with digested pALC2073 plasmid DNA using T4 DNA ligase, and transformed into E. coli DH5α. The resulting pALC2073::SAg plasmids were isolated from DH5α and transformed by electroporation into an intermediate electro-competent strain of S. aureus, RN4220. Subsequently the plasmids were re-isolated and transformed into the SAg deficient strain RF122-8. S. aureus strains were made competent as described previously (14). RF122-8 strains containing each of the pALC2073::SAg constructs were induced with a sub-inhibitory concentration of tetracycline (50 ng/ml) (Sigma-Aldrich, Dorset, UK)
when cultures reached mid-exponential phase, and grown for a further 4 h (Bateman B.T et al, 2001).

Recombinant expression of SAg genes

5′ primers for cloning into the pET15b (Merk Millipore, UK) or pQE30-Xa (QIAgen, UK) plasmids, were designed to anneal immediately after the signal peptide coding region, as predicted by Signal P 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), and 3′ primers were designed to include the stop codon of the gene (Supplementary Table 2). The cloning procedure was performed as outlined for pALC2073 and ligated constructs were transformed into E. coli DH5α or XL-1 blue (for pQE30-Xa constructs) cells. pET constructs were isolated from DH5α using the QIAprep Spin Miniprep kit (QIAgen, UK), and transformed into E. coli BL21 (DE3). BL21 or XL-1 blues cells containing expression constructs were cultured in Luria-broth containing 50 µg/ml ampicillin (Sigma-Aldrich, Dorset, UK) and induced in mid-exponential phase of growth (OD600=0.6), with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (ForMedium Ltd., Norfolk, UK) for 4 h. Cells were recovered by centrifugation at 8000 xg, disrupted using a French Press, and His-tagged recombinant proteins were purified by affinity chromatography on a Ni-NTA nickel affinity column (GE healthcare, UK). Proteins were dialysed using Spectra/Por Float-A-Lyzer tubing with a 8000 to 10000 molecular weight cut off (MWCO) (Spectrum Laboratories, California, USA).

Immunoblot analysis of convalescent bovine serum

SDS-PAGE and western blotting was carried out on SAgs overexpressed in E. coli. The nitrocellulose membrane (Amersham Hybond™ ECL™, GE Healthcare, Slough,
UK) was incubated with 10ml of blocking buffer containing 5% (w/v) skimmed milk powder (Sigma Aldrich, UK) in PBST (PBS with 0.05% Tween 20 (Sigma Aldrich, UK)) overnight at 4°C. The membrane was then incubated for 2 hours with a 1:1000 dilution of pooled bovine convalescent serum in PBST with 1% (w/v) skimmed milk and washed three times with PBST. Secondary antibody (goat anti-bovine IgG-HRP (Santa Cruz Biotechnology, Heidelberg, Germany)) was added at a concentration of … for 1 h at room temperature. The blot was washed again as. Immunoreactivity was visualised by chemiluminescence from ECL.

T cell proliferation assays

Blood was obtained from Holstein-Friesian cattle aged 18 to 36 months via jugular vein puncture. Animals were reared indoors and maintained on a ration of hay and concentrates. PBMC were isolated by density gradient centrifugation using Ficoll Paque PLUS (GE Healthcare, UK) as described previously (45). Human PBMC were isolated from venous blood, drawn from healthy human volunteers and mixed with acid-citrate-dextran (ACD) (25 g D-glucose (Sigma-Aldrich, UK) and 20.5 g trisodium citrate (Sigma-Aldrich, UK) added to 1 L of ddH2O). The buffy coat was isolated by spinning the blood at 1500 x g for 15 min with no break and then PBMC were isolated using Ficoll Paque PLUS (GE Healthcare, UK) according to the manufactures specification. PBMCs were adjusted to a concentration of 1 × 10⁶ cells/ml in complete cell culture medium (RPMI 1640 (Sigma Aldrich, UK)) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Gibco, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine. (PSG) (Gibco, UK), were stimulated in triplicate at least with concentrated total protein S. aureus supernatant fraction or recombinant protein. Culture media and 50 μg/ml
Concanavalin A were used as negative and positive controls respectively. Proliferation of bovine and human PBMCs were assessed by \[^{3}H\]-thymidine incorporation assay as described previously (14). Total RNA was extracted from bovine PBMC (4× 10^6 cells) using Tri-reagent (Sigma–Aldrich, Dorset, UK) as described in the suppliers instructions or using the RNeasy plus kit (Qiagen, UK) according to the manufactures instructions. First-strand cDNA was generated from 0.5 μg of RNA using Power SYBR® Green RNA-to-CT 2-Step Kit or High Capacity RNA-to-cDNA Kit and Power SYBR® Green PCR Master Mix (Thermo Fisher, UK). The reverse transcription reaction was performed in a 20 μl volume according to the manufacturer’s specifications. Bovine Vβ subfamily specific qRT-PCR reactions were carried out as described previously (14). Human Vβ activation analysis was performed as described previously (12, 46).

**Experimental infection of dairy cattle**

Adult cows (Holstein) in their 1st to 4th lactation at 92 to 174 Days in Milk (DIM) were used in this study. Overnight cultures of *S. aureus* were inoculated 1:50 into fresh TSB and grown until an OD_{600} of 1.1 was reached. Staphylococci were diluted in TSB to obtain an inoculum of 5x10^7 CFU/ml. Inocula were determined by CFU enumeration following serial dilution, plating on TSA, and growth at 37°C. Animals were challenged via teat dip immersion twice daily (22mm immersion) until a score of 1 or greater for milk appearance or udder evaluation was observed and the animal developed an intra-mammary infection twice within a five day period. Following infection animals were observed for a total of three weeks. Somatic cell counts (SCC) and cultures were taken twice a week. Udder and milk clinical scores, milk yield and
Statistical analysis

All statistical analysis was performed in Graphpad Prism 7. Fold change enrichment data was analysed using student t-test with Welch's correction if required. Tests were unpaired and two-tailed and significant differences were considered when the p-value was <0.05.

Acknowledgements

The authors are grateful to Dr Gregory Bohach for sharing his expertise and contributing to this project. The authors would like to thank Dr Sara Clohisey and Dr Kenneth Baillie for their assistance with organising the blood donation study and the volunteers from the Roslin Institute who provided human blood samples. We would like to extend our thanks to Sarah Salmon and Dennis Peterson of Zoetis Animal health for their assistance with the animal infection study. J.R.F. was supported by grants BB/K00638X/1, BB/I013873/1 and institute strategic grant funding (BBS/E/D/20002173 from the Biotechnology and Biological Sciences Research Council (UK), a Medical Research Council (UK) doctoral training grant, and Zoetis Animal Health. This work was also partially supported by grants from Center for Biomedical Research Excellence in Pathogen-Host interactions, National Institute of General Medical Sciences, NIH (1P20GM103646-01A1) and Animal and Plant Quarantine Agency, South Korea (I-1543081-2015-17-01) to K.S.S.
Table 1: SAgs encoded by *S. aureus* strain RF122

<table>
<thead>
<tr>
<th>Gene</th>
<th>Toxin</th>
<th>Size (kDa)*</th>
<th>Locus tag</th>
<th>Homology with characterised SAg gene (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tstbov</em></td>
<td>Toxic shock syndrome toxin-1 (TSST-1bov)</td>
<td>22</td>
<td>SAB_RS01910</td>
<td>98% (<em>tst</em>)</td>
</tr>
<tr>
<td><em>secbov</em></td>
<td>Staphylococcal enterotoxin C-bovine (SECbov)</td>
<td>27.6</td>
<td>SAB_RS01930</td>
<td>99% (<em>sec1</em>)</td>
</tr>
<tr>
<td><em>sellbov</em></td>
<td>Staphylococcal enterotoxin like toxin L-bovine (SElLbov)</td>
<td>24.7</td>
<td>SAB_RS01935</td>
<td>99% (<em>sel1</em>)</td>
</tr>
<tr>
<td><em>segbov</em></td>
<td>Staphylococcal enterotoxin G-bovine (SEGbov)</td>
<td>20.6</td>
<td>SAB1696c</td>
<td>77% (<em>seg1</em>)</td>
</tr>
<tr>
<td><em>sei bov</em></td>
<td>Staphylococcal enterotoxin I-bovine (SEIbov)</td>
<td>24.9</td>
<td>SAB_RS09045</td>
<td>97% (<em>sei1</em>)</td>
</tr>
<tr>
<td><em>selnbov</em></td>
<td>Staphylococcal enterotoxin like toxin N-bovine (SElNbov)</td>
<td>26.1</td>
<td>SAB_RS09035</td>
<td>95% (<em>sen1</em>)</td>
</tr>
<tr>
<td>selubov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>27.2</td>
<td>SAB_RS09040</td>
<td>97% (selu1)</td>
</tr>
<tr>
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</tr>
<tr>
<td>selmbov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>N/A</td>
<td>SAB1700c</td>
<td>87% (selm1)</td>
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<tr>
<td>selobov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>27.1</td>
<td>SAB_RS09055</td>
<td>98% (selo2)</td>
</tr>
<tr>
<td>selw</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>N/A</td>
<td>SAB1473c</td>
<td>54% (sea1)</td>
</tr>
<tr>
<td>selxbov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>19.5</td>
<td>SAB_RS01710</td>
<td>45% (tst)</td>
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<tr>
<td>selybov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>22.5</td>
<td>SAB_RS13070</td>
<td>58% (set)</td>
</tr>
<tr>
<td>selzbov</td>
<td>Staphylococcal enterotoxin like toxin</td>
<td>27.1</td>
<td>SAB_RS00140</td>
<td>57% (seg1)</td>
</tr>
</tbody>
</table>

* Predicted size of the mature protein based on amino acid sequence. Pseudogenes not included.

Table 2: Immunogenicity of recombinant SAg proteins from RF122 with sera from bovine and human *S. aureus* infections.

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>SEIZ</th>
<th>SEIV</th>
<th>SEG</th>
<th>SEI</th>
<th>SEO</th>
<th>SEU</th>
<th>SEIN</th>
<th>SEC</th>
<th>SEIL</th>
<th>TSST-1</th>
<th>SEIX</th>
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<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IE19</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IE37</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>2521</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
+ or – indicate whether or not serum samples are reactive with SAg proteins.

Human serum samples were obtained from infective endocarditis patients between 2006-2009, New Royal Infirmary of Edinburgh. Bovine samples obtained from bovine mastitis cases, and from an animal (cow 2211) without a history of *S. aureus* infection were provided by C. Smyth, originally obtained from Teagasc Dairy Production Centre in Moorepark, Fermoy, Co. Cork.

Data previously reported (14)

Table 3: Activation of Vβ subfamilies in response to RF122-encoded SAg.

<table>
<thead>
<tr>
<th>SAg</th>
<th>Bovine Vβ&lt;sup&gt;a,b,c&lt;/sup&gt;</th>
<th>SAg</th>
<th>Human Vβ&lt;sup&gt;a,b,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>15, 17, 28</td>
<td>SEC&lt;sub&gt;bov&lt;/sub&gt;,</td>
<td>12, 13, 14, 15, 17, 20</td>
</tr>
<tr>
<td>SEI&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>1, 10, 16</td>
<td>SEI</td>
<td>1, 5, 6, 23</td>
</tr>
<tr>
<td>SEI&lt;sub&gt;Lbov&lt;/sub&gt;</td>
<td>1, 5, 6, 7, 10, 16, 24</td>
<td>SEIL</td>
<td>1, 5, 7, 16, 22, 23</td>
</tr>
<tr>
<td>SEI&lt;sub&gt;Nbov&lt;/sub&gt;</td>
<td>3, 9, 16, 24, X</td>
<td>SEIN</td>
<td>7, 8, 9, 17</td>
</tr>
<tr>
<td>SEI&lt;sub&gt;Xbov&lt;/sub&gt;</td>
<td>3, 5, 8, 11, 16, 17, 24, X</td>
<td>SEIX&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>1, 6, 18, 21</td>
</tr>
<tr>
<td>TSST-1&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>2, 4, 24, X</td>
<td>TSST-1</td>
<td>2</td>
</tr>
<tr>
<td>SEG&lt;sub&gt;bov&lt;/sub&gt;</td>
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<td>SEG</td>
<td>3, 12, 13, 14, 15</td>
</tr>
<tr>
<td>SEIU&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>17</td>
<td>SEIU</td>
<td>13, 14</td>
</tr>
<tr>
<td>SEIY&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>4, 7, 10, 16, 24, 28, X</td>
<td>SEIY&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>1, 3, 5, 6, 7, 21, 22, 23, 24</td>
</tr>
<tr>
<td>SEIZ&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>1, 3, 7, 11, 16, 17, 24, 28, X</td>
<td>SEIZ&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>13, 2</td>
</tr>
<tr>
<td>SEIO&lt;sub&gt;bov&lt;/sub&gt;</td>
<td>N/A</td>
<td>SEIO</td>
<td>5, 7, 22</td>
</tr>
</tbody>
</table>

<sup>a</sup> Vβ subfamilies were named according to the classification of Arden et al (47).
Bovine and human Vβ subfamilies activated in response to the same SAg are highlighted in bold.

N/A, not applicable.

HumVβ activation data was compiled from the references or this study for SEIY and SEIZ (11, 12, 14, 15)

References


Figure Captions

**Figure 1:** RF122 encodes SAg from all four phylogenetic groups. **a**) Maximum-Likelihood phylogenetic tree of 26 superantigen protein sequences showing clustering of SAg into 4 general groups (1). Branches with more than 80% bootstrap support are marked with black or grey circles. SAg present in the RF122 strain are indicated by asterisks. **b**) A circular representation of the genome of reference strain RF122 showing the location of SAg genes.

**Figure 2:** Bovine isolates of *S. aureus* typically encode 5 or more SAg genes. Distribution analysis of SAs in bovine *S. aureus* isolates showing the repertoire of SAs that are encoded. Phylogeny is based on a core genome alignment and major clonal complexes are noted. Coloured boxes indicate the presence of the SAg gene and are sorted according to association with mobile genetic elements.
Figure 3: RF122 SAgs are expressed in vitro and exhibit growth phase dependent expression. Transcription levels of RF122-encoded SAg genes from exponential and stationary cultures, relative to 16S rRNA. Relative quantities of RF122 reverse-transcribed mRNA normalized to the internal control 16SrRNA, determined by qRT-PCR. Results shown are the means of triplicate experiments and error bars indicate ± S.D.

Figure 4: Proliferation of bovine T cell populations in response to stimulation with RF122-encoded SAgs. PBMC proliferation after 4 d exposure to a) RF122-8 supernatants containing SAgs, and b) recombinant SAg proteins as indicated by the incorporation of [³H] thymidine. Results shown are the means of at least triplicate measurements from 2 animals ± S.E.M.

Figure 5: RF122-encoded SAgs are able to stimulate all Vβ subsets of the bovine T cell population. Relative fold change in bovine Vβ expression after stimulation with RF122 SAgs. Bovine Vβ subfamilies were named according to the classification of Arden et al (1995). The bTRBV analysed are functional genes tested previously (14). Bovine T cells were stimulated with supernates from induced RF122-8α containing pALC2073::SAg constructs (a-i) or purified recombinant proteins (g-j). Relative fold change in bovine Vβ expression after stimulation with human and bovine alleles of SEiY and SEiZ. Results given as mean fold change in expression ± SEM of 6 measurements, 3 each from two animals. * Indicates expansion of a subfamily based on a significant increase from the baseline (p <0.05). Expansion profiles of all 11 SAgs from RF122 are summarised (k).

Figure 6: SAgs exhibit host-dependent functional activity. a) PBMC proliferation after 4 d exposure to bovine and human alleles of SEiY and SEiZ as indicated by the incorporation of [³H] thymidine. Results shown are the means of at least triplicate measurements from 3 donors ± SEM. Differences between proliferation induced by human and bovine variants of these SAgs was assessed using Two-way ANOVA with Holm-Sidak's multiple comparisons.
test, asterisk denotes curves are significantly different (* p < 0.05, ** p < 0.01). b) Expansion index of Vβ human CD3+ cells after stimulation with human and bovine alleles of SEiY and SEiZ. Expansion index was determined from the mean of three measures from 2 donors ± SEM. * Indicates expansion of a subfamily based on a significant increase from the baseline (p < 0.05) and an expansion index > 1.

c) Relative fold change in bovine Vβ expression after stimulation with human and bovine alleles of SEiY and SEiZ. Results given as mean fold change in expression ± SEM of 9 measurements, 3 each from three animals. * Indicates expansion of a subfamily based on a significant increase from the baseline (p < 0.05).

Figure 7: SAgS promote clinical bovine mastitis. Number of animals infected with RF122 or RF122-8, which exhibited evidence of clinical mastitis at any point during the 21 days of the trial. Clinical mastitis in this experiment was defined as observable inflammation in any of the four quarters of the cow’s udder during the study.