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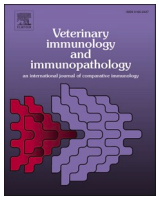

Immunoproteomic analysis of the secretome of bovine-adapted strains of *Staphylococcus aureus* demonstrates a strain-specific humoral response

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ABSTRACT

Staphylococcus aureus is a common pathogen associated with bovine intramammary infection. A number of distinct *S. aureus* lineages are associated with such infections although there is a dearth of knowledge regarding the major immunogenic antigens associated with each lineage and whether these antigens provide protection against heterologous strains. Identification of the major immunogenic antigens of the predominant bovine-adapted *S. aureus* lineages would assist in the design of effective vaccines and diagnostic tests to control intramammary infections caused by *S. aureus*. The aim of this study was to characterise the serum IgG response to *S. aureus* extracellular proteins in cows infected with strains from different lineages, as well as to identify antigenic proteins produced by these strains. Genotypic characterisation found that strain MOK124 (CC151) encoded more toxins, including the ruminant-specific leukocidin LukMF, compared to strain MOK023 (CC97). In addition, MOK124 secreted more toxins *in vitro*, compared to MOK023. Immunoproteomic analysis was performed using sera from cows infected with either MOK023 or MOK124. One-dimensional serum blotting revealed that cows infected with MOK023 predominantly generated a humoral response against high molecular weight proteins while cows infected with MOK124 primarily generated a humoral response against low molecular weight proteins. Two-dimensional serum blotting demonstrated that antibodies produced by an MOK023 infected cow could cross react with some of the extracellular proteins produced by MOK124 and *vice versa*. Mass spectrometry analysis of immunoreactive proteins identified common candidate immunogens produced by both strains, including α-hemolysin and β-hemolysin. In addition, strain-specific candidate immunogens were also identified. This study demonstrates that genes encoding important *S. aureus* secreted virulence factors, the production of cognate gene products, and the humoral immune response to infection is, to an extent, strain-dependent. However, the identification of some common candidate immunogens suggests that there are proteins that can be exploited for further vaccine or diagnostic research that targets *S. aureus* strains from a variety of lineages.

1. Introduction

Bovine mastitis is of significant economic consequence in the global dairy industry, with bacterial intramammary infection (IMI) being the main cause. *Staphylococcus aureus* is a common pathogen associated with bovine IMI (Persson et al., 2011, Katholm et al., 2012, Keane et al., 2013, Fesseha et al., 2021). *S. aureus* has been associated with both clinical and subclinical infections; however, *S. aureus* IMI are often chronic and recurrent in nature (Bradley, 2002, Barkema et al., 2009). The success of *S. aureus* as a major pathogen is facilitated by the extensive repertoire of virulence factors encoded within its genome. These virulence factors are commonly surface-exposed; associated with the bacterial cell membrane, covalently attached to the cell wall, non-covalently associated with the cell surface or secreted (Heilmann, 2011). They have many roles in facilitating bacterial persistence and survival including nutrient acquisition, host colonisation or adherence and internalisation, immune

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regulatory functions and immune evasion functions. Among the important secreted factors produced by *S. aureus* are the hemolysins α-hemolysin (Hla), β-hemolysin (Hlb) and the bi-component γ-hemolysin (Hlg) and the bicomponent leukotoxins (Luk) as well as an extensive repertoire of superantigens (SAg) and staphylococcal superantigen-like (SSL) toxins (Otto, 2014, Tam and Torres, 2019). The SAgs produced by *S. aureus* include the staphylococcal enterotoxins (SEs), the staphylococcal enterotoxin-like toxins (SEls) and toxic shock syndrome toxin-1 (TSST-1) (Wilson et al., 2018). *S. aureus* also encodes many different exoenzymes, including aureolysin and phosphatid ylinositol-specific phospholipase C (PI-PLC).

Given the status of *S. aureus* as an important pathogen within the dairy industry, there is a clear need for improved diagnostic and prevention methods. However, the genetic variability among bovine-adapted *S. aureus* strains could account for some of the difficulties to-date in generating new tools. The *S. aureus* lineages CC151 and CC97 are two of the most common lineages associated with bovine mastitis (Smyth et al., 2009, Bergonier et al., 2014, Budd et al., 2015, Thomas et al., 2021). Previous studies have demonstrated differences among strains of these lineages in virulence traits *in vitro* (Budd et al., 2016, Murphy et al., 2019). Additionally, challenge of cows with strains belonging to CC97 predominately results in subclinical infections (Hensen et al., 2000, Niedziela et al., 2020) while challenge of cows with strains belonging to CC151 mostly results in clinical infections (Wilson et al., 2018, Niedziela et al., 2020). These studies suggest that the production of virulence factors *in vivo* differs between strains belonging to different lineages of *S. aureus*. Elucidation of the repertoire of virulence factors produced *in vivo* by different *S. aureus* strains will inform diagnostic and vaccine research. In this study, an immunoproteomic approach was used to characterise the serum IgG response to *S. aureus* surface-associated and secreted proteins in cows infected with CC97 or CC151 strains. Antigenic surface-associated and secreted proteins produced by these strains were identified and validated.

2. Material and methods

2.1. S. aureus strains and culture conditions

S. aureus strains MOK023 and MOK124 used in this study were recovered from milk of cows presenting with mastitis as described previously (Keane et al., 2013). Multi-locus sequence typing demonstrated that MOK023 belonged to ST3170 (CC97) while MOK124 belonged to ST151 (CC151) (Budd et al., 2015). *S. aureus* strains were stored in Trypticase Soy Broth (TSB) (LabM) supplemented with 15% (v/v) glycerol at − 80 °C until required. Strains were recovered on Trypticase Soy Agar (TSA) (LabM) at 37 ºC overnight. Strains were single colony purified every 7 days onto fresh TSA. For liquid cultures, strains were grown in Trypticase Soy Broth (TSB) at 37 ºC with 200 rpm orbital shaking.

2.2. Cow sera collection

Bovine sera came from a *S. aureus* intramammary infection trial by Niedziela et al. (2020). In this trial, two groups of disease-free, first--lactation Holstein-Friesian cows (n = 7 per group) were infected by inoculation of approximately 500 colony forming units of *S. aureus* into the teat sinus of the left hind quarter of each cow; one group was infected with MOK023 and the other with MOK124. Infection was monitored for 30 days post-challenge. Infection was not established in 3 cows (2 and 1 from the MOK023 and MOK124 groups respectively). Cows infected with MOK023 developed subclinical mastitis, while MOK124-infected cows generally developed clinical mastitis and experienced a greater drop in milk yield and higher somatic cell count than MOK023 infected cows. Two MOK124-infected cows that developed severe clinical mastitis were removed from the study on animal welfare grounds. Therefore, sera from 5 and 4 cows from the MOK023 and MOK124 infected groups respectively were available for analysis. Serum samples were from 5 times points over the infection trial: pre-infection (day 0) and 7, 14, 21 and 29 days post-infection. Serum from day 29 post-infection was missing for one cow (582) from the MOK124 group.

2.3. Genome assembly and annotation

Whole genome sequence data for MOK023 and MOK124 were available (Budd et al., 2015; Niedziela et al., 2020), accession numbers SRS775827 and SRS2841713 respectively. Prior to genome assembly, quality control checks were carried out on both forward and reverse read FASTQ files using the FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were subsequently trimmed of low quality and adapter sequences and a draft genome assembled *de novo,* using the automated A5-miseq pipeline (Coil et al., 2015). The Perl script for this pipeline is available at https://sourceforge.net/p/ngopt/wiki/A5PipelineREADME/. Draft genomes were annotated using the RAST server (Aziz et al., 2008), set to the following parameters; domain 'bacteria', genus 'staphylococcus', species 'aureus' and genetic code option 11 (for bacteria). The pipeline options were set to; ClassicRAST annotation scheme, RAST gene caller, and the FIGfam version Release70. The server was also set to allow to backfill gaps and automatically fix errors. For each putative protein, subcellular location was predicted using PSORTb v3.0 (Yu et al., 2010).

2.4. Extracellular protein purification

S. aureus strains were grown overnight in 5 ml of TSB at 37 ℃ with 200 rpm orbital shaking. The following morning, the overnight cultures were diluted into 60 ml fresh TSB to an $OD_{600\ nm}$ of 0.01 and incubated at 37 °C, 200 rpm for 9 h. Cultures were then centrifuged at 1000 x g for 30 min, the supernatant removed and filter sterilised using a 0.22 μm PVDF filter (Millipore). The supernatant was subsequently nuclease digested by adding 100 X Protease Inhibitor Mix (GE Healthcare) and 100 X Nuclease Mix (GE Healthcare) to a final concentration of 1 X and allowing digestion for 50 min at room temperature with frequent mixing. Desalting was then carried out using the PD-10 Desalting Column (GE Healthcare), following the gravity flow protocol, as per the manufacturer's instructions. The elution step was repeated twice. For protein concentration and buffer exchange, 15 ml of digested, desalted supernatant was added to a 3 kDa molecular weight cutoff filter (Amicon, Millipore) and centrifuged at 4000 x g until 1 ml of supernatant remained in the unit. The flow through was discarded, and PBS added to bring the sample back to 15 ml. The filter device was again centrifuged at 4000 x g until 1 ml of supernatant remained in the unit and flow through discarded. The PBS wash was repeated once and Isoelectric Focusing (IEF) Rehydration buffer (10 mM Tris, 8 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 1% (v/v) TritonX-100) or Storage buffer (6 M Urea, 2 M Thiourea, 0.1 M Tris-HCL pH 8.6, filter sterilized) was added to bring the sample back to 15 ml. The filter device was centrifuged at 4000 x g until no more flow through passed and the concentrated supernatant removed to a fresh 1.5 ml Eppendorf tube. Protein quantification was performed using the 2-D Quant kit (GE Healthcare), as per the manufacturer's instructions.

2.5. One-dimensional (1D) SDS-PAGE

Protein samples, as well as purified recombinant α-hemolysin (~ 33 kDa; Abcam) and LukM (~ 32 kDa: kindly gifted by Dr. Manouk Vrieling, Dr. Kok van Kessel and Dr. Lindert Benedictus (Utrecht University)), were prepared for electrophoresis by adding LDS Sample Buffer (NuPAGE, Thermo Scientific) to a final concentration of 1X. Samples were boiled for 10 min and allowed to cool before protein separation on 10 or 12% SDS-PAGE gels using the Mini-PROTEAN® Tetra Cell (Bio-Rad). Following electrophoresis, proteins were either stained by colloidal coomassie staining or immobilised onto PVDF membrane.

Colloidal coomassie staining was performed as previously described by Dyballa and Metzger (2009) with one modification; $(NH_4)_2SO_4$ was used in place of $Al_2(SO_4)_314–18\ H_2O$. Protein transfer onto PVDF membrane was performed at 20 V for 1.5 h using the Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell.

2.6. Two-dimensional (2D) SDS-PAGE

Non-linear gradient strips (Immobiline® Drystrip pH 3–10 NL; GE Healthcare) were used for separation of proteins according to their isoelectric point (p*I*). Protein samples were adjusted to a total concentration of 60 μg in 125 μl of IEF Rehydration Buffer containing 0.8% (w/v) IPG pH 3–10 NL buffer (GE Healthcare), 15 mg/ml DeStreak Reagent (GE Healthcare) and a trace amount of bromophenol. IPG strips were rehydrated with the protein sample overnight at room temperature. Following rehydration, strips were focused using the Agilent 3100 OFFGEL Fractionator until a kVh of 8 was reached. Focused strips were reduced for 10 min with agitation in Equilibration buffer (30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea, 50 mM Tris-HCL pH 8.8) containing 2% (w/v) dithiothreitol (DTT) followed by alkylation for 10 min with agitation in Equilibration buffer containing 2.5% (w/v) iodoacetamide (IAA). After protein alkylation, strips were briefly washed in 1X SDS running buffer (25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS) and placed on top of a 10% SDS-PAGE gel with protein ladder (10–250 kDa PageRuler™, Thermo Scientific), soaked filter paper, placed beside the IPG strip. The strips and filter paper were overlaid with liquid Agarose Sealing Solution (0.7% (w/v) Agarose in 1X SDS running buffer) which was allowed to solidify at room temperature for 30 min. Gels were electrophoresed using the Mini-PROTEAN Tetra cell (Bio-Rad) at 5 mA per gel until the dye front entered the gel, increased to 15 mA per gel until the dye front reached the end of the gel. Following electrophoresis, gels were either stained by colloidal coomassie staining or immobilised onto PVDF membrane, as described above for 1D SDS-PAGE.

2.7. Serum blotting

Membranes were blocked in 10% non-fat, dried milk in PBS for 1 h at room temperature followed by three 10 min washes in PBS. Membranes were then incubated overnight at 4 °C with cow sera diluted 1:2000 in PBS. Membranes were washed again in PBS before probing for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-cow IgG (Abcam), diluted 1:5000 in 5% BSA in PBS. The membranes were subsequently washed 4 times in PBS for 10 min each. The membranes were incubated with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and imaged using the Omega Lum™ C Imaging System (Aplegen).

2.8. Supernatant protein preparation for mass spectrometry

Protein samples in storage buffer, were adjusted to the same concentration and prepared for mass spectrometry analysis as previously described (Owens et al., 2014, Owens et al., 2015), with some modifications. Adjusted protein samples (15 μl) were mixed with 5.2 vol 50 mM NH_4HCO_3, reduced with 1 μl 0.5 M DTT for 20 min at 56 °C and alkylated with 2.7 μl 0.55 M IAA for 15 min in the dark. Samples were digested overnight at 37 °C with 1.8 μl of sequencing-grade trypsin (1 μg/μl in 50 mM acetic acid) (Promega), in the presence of 1 μl ProteaseMAX (1% (w/v) in 50 mM NH_4HCO_3) (Promega). Digestion was halted by addition of 1 μl neat trifluoroacetic acid (TFA), the supernatant was harvested and peptides dried in a new sterile tube.

2.9. Identification of immunogenic proteins

Serum blotted membranes were manually aligned to the corresponding colloidal coomassie stained SDS-PAGE gel. Spots identified as immunoreactive were excised from the corresponding gel and each placed into a separate sterile eppendorf tube. Excised spots were prepared for mass spectrometry, with overnight digestion at 37 °C, as described by Shevchenko et al. (2006). Following digestion, the supernatant was harvested and peptides dried in a new sterile tube.

2.10. Mass spectrometry-based identification of proteins

Dried digested samples were resuspended in 0.5% (v/v) TFA before sample desalting using C18 ziptips (Pierce). Desalted peptides were resuspended in Loading Solution (0.05% (v/v) TFA, 2% (v/v) acetonitrile) and analysed on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer coupled to an UltiMate™ 3000 RSLCnano System (Thermo Scientific), according to the method of Owens et al. (2015). Mass spectrometry data were analysed using Proteome Discoverer software (v1.4; Thermo Scientific™) against protein databases created for each strain using the SEQUEST algorithm with the following settings (i) trypsin was selected as the cleavage enzyme with up to 2 missed cleavages allowed (ii) oxidation of methionine was set as a variable modification and (iii) carbamidomethylation of cysteine was set as a fixed modification. Results were filtered using the Percolator module and only medium confidence peptides (False Discovery Rate (FDR) < 0.05) were retained. Further filtering was performed to remove proteins only identified by a single peptide. To identify candidate immunogenic proteins where more than one protein was detected in a given excised spot, proteins were considered as candidates based on (i) predicted subcellular location, (ii) predicted molecular weight, (iii) predicted p*I* and (iv) the number of unique peptides.

3. 3. Results

3.1. S. aureus extracellular protein profile

MOK023 and MOK124 were predicted by PSORT to encode 94 and 111 extracellular proteins respectively. Of these, 78 were predicted to be extracellular in both strains, including well-known secreted virulence factors such as α-hemolysin, LukED, coagulase, von Willebrand factor-binding protein, Aureolysin, Spl serine protease-like proteins and SSL proteins. The predicted extracellular proteins unique to each strain included a number of hypothetical and phage proteins; however, MOK124 was predicted to encode a number of toxins not found in MOK023 including the ruminant-specific leukocidin, LukMF, and the enterotoxin gene cluster (*egc*).

Mass spectrometry analysis of the supernatant of early stationary phase cultures of MOK023 and MOK124 identified 16 and 18 of the PSORT-predicted extracellular proteins respectively (Table 1). Eleven proteins were common to the supernatant of both strains with Atl being the most abundant in supernatant of both strains. Differences were seen in the toxin profile of the two strains. Both strains produced α-hemolysin and β-hemolysin while MOK023 produced γ-hemolysin (B subunit) and SSL11 and MOK124 produced both subunits of LukMF and LukED. Supplementary file 1 lists all proteins detected by mass spectrometry in the supernatants of MOK023 and MOK124.

3.2. Characterisation of the humoral response to S. aureus supernatant proteins from MOK023 and MOK124

Supernatant extracts from MOK023 and MOK124 early stationary phase cultures were separated by 1D SDS-PAGE and immobilised onto PVDF membrane. The supernatant proteins were probed with serum from each cow infected with that strain. Fig. 1A shows the results of the MOK023 proteins probed with serum from cows infected with this strain. Immunoreactive bands were predominantly high molecular weight, with major bands identified at ~70, ~130 and ~180 kDa. For two cows (506 and 565) low molecular weight bands were also identified (~25 and ~35 kDa). Notably, pre-challenge sera from all cows showed evidence of immunoreactivity. Fig. 1B shows the results of the

Table 1
All predicted extracellular proteins detected in early stationary phase supernatant extracts of *S. aureus* strains MOK023 (CC97) and MOK124 (CC151).

Protein Name	MOK023			MOK124		
	#Unique Peptides	tMr (kDa)*	tpl^{+}	#Unique Peptides	tMr (kDa)	tpI
Bifunctional autolysin (Atl)	49	137.4	9.61	51	136.7	9.58
Alpha-hemolysin (Hla)	18	35.9	8.73	17	35.9	8.73
Phage-encoded chromosome degrading nuclease (YokF)	14	25.1	9.25	11	25.1	9.41
Beta-hemolysin (Hlb)	11	37.2	8.7	18	37.2	8.7
Phosphatidylinositol-specific phospholipase C (PI-PLC)	10	37.1	8	12	37	8
Staphylococcal superantigen-like protein 11 (SSL11)	10	26.2	8.75	–	–	–
Manganese superoxide dismutase (SodM)	7	22.7	5.19	5	22.7	5.19
N-acetylmuramoyl-L-alanine amidase (AmiA) family 4	7	69.3	6.3	2	69.3	6.24
Staphopain B (SspB)	5	44.6	5.97	15	44.6	5.81
Gamma-hemolysin component B (HlgB)	4	36.7	9.35	–	–	–
Glutamyl endopeptidase (GluV8)	4	36.3	5.19	9	35	5.19
Extracellular fibrinogen-binding protein (Efb)	3	18.8	9.85	2	18.8	9.92
Serine protease (Spl)	3	26.1	9.22	–	–	–
Aureolysin (Aur)	2	56.3	5.27	9	56.3	5.22
FIG01107880: hypothetical protein	2	27.3	9.35	–	–	–
Immunodominant staphylococcal antigen A (IsaA)	2	24.2	6.62	–	–	–
Staphopain A (ScpA)	–	–	–	14	44.2	9.64
Leukocidin F subunit (LukF)	–	–	–	9	36.5	9.23
Leukocidin M subunit (LukM)	–	–	–	6	35	9.42
Hyaluronate lyase (HysA)	–	–	–	5	92	8.06
Leukotoxin D (LukD)	–	–	–	5	36.9	9.14
Leukotoxin E (LukE)	–	–	–	5	34.7	9.54
Triacylglycerol lipase (Lip2)	–	–	–	2	76.6	8.44

*tMr = theoretical molecular weight (kDa) calculated from amino acid sequence
+tpI =theoretical isoelectric point calculated from amino acid sequence

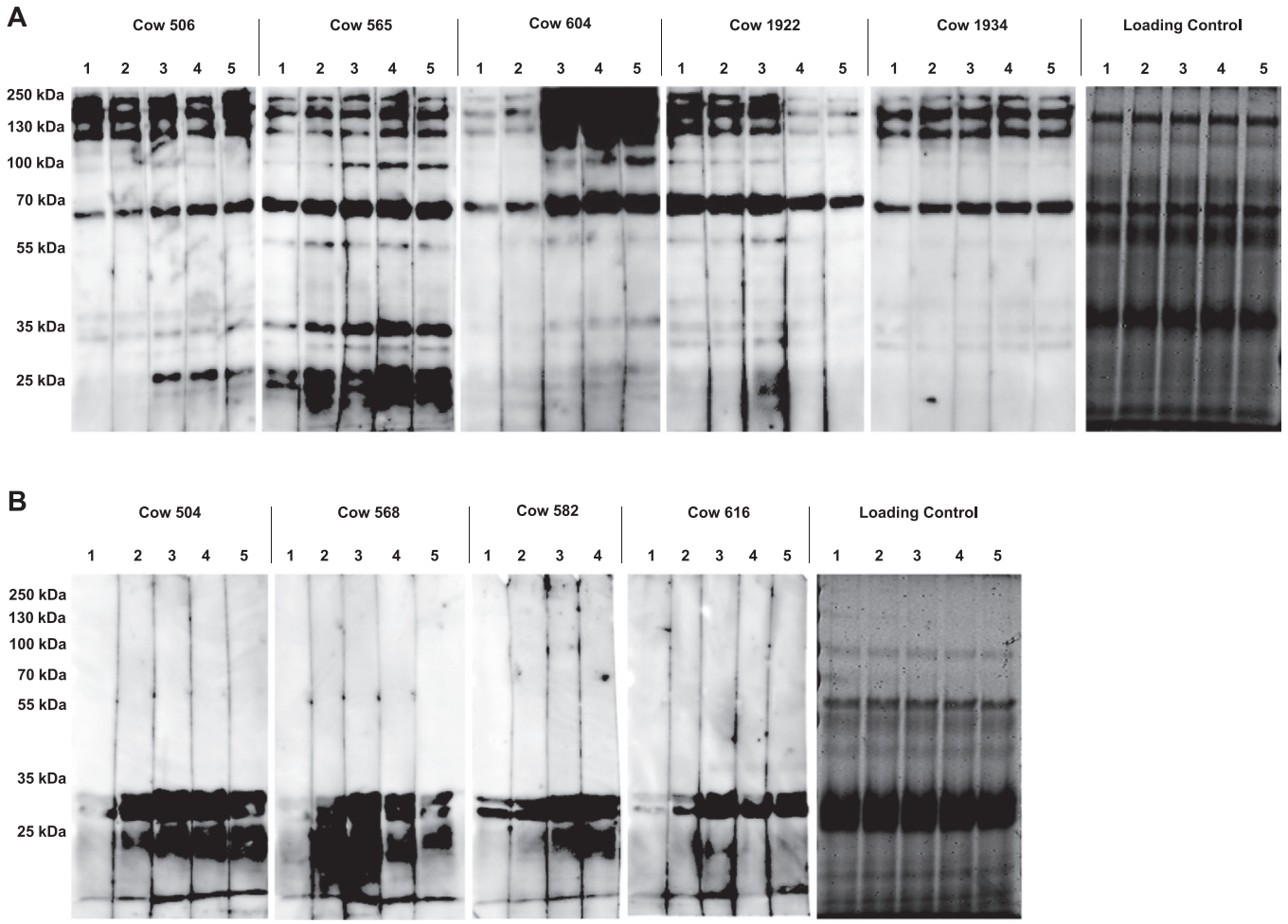

Fig. 1. One-dimensional serum blots of *S. aureus* supernatant proteins. **(A)** MOK023 supernatant proteins probed with sera from MOK023 infected cows and **(B)** MOK124 supernatant proteins probed with sera from MOK124 infected cows. Lane 1 = Day 0 pre-infection, Lane 2 = Day 7 post infection, Lane 3 = Day 14 post infection, Lane 4 = Day 21 post infection and Lane 5 = Day 29 post infection.

MOK124 proteins probed with serum from cows infected with this strain. Immunoreactive proteins were of low molecular weight, with major bands at ~22 and ~30 kDa (Fig. 1B). Immunoreactive bands could be identified in only one cow (582) prior to intramammary challenge.

3.3. Identification of immunoreactive supernatant proteins from S. aureus MOK023 and MOK124

The supernatant extracts of MOK023 and MOK124 were separated by 2D electrophoresis and immobilised onto PVDF membrane. Proteins from both strains were probed with serum collected pre-infection and 14 days post-infection from cows 604 (MOK023 infected) and 504 (MOK124 infected) to identify both strain-specific and common immunoreactive proteins. Spots that increased in intensity post-infection were considered immunoreactive. For each comparison, ten spots were selected for mass spectrometry analysis and included spots from a range of molecular weight and p*I* regions.

The results of 2D coomassie stained gels and serum blots of MOK023 supernatant proteins probed with sera from cow 604 (MOK023 infected) and cow 504 (MOK124 infected) are shown in Fig. 2. As seen in the 1D gels, antibodies raised in cow 604 against MOK023 supernatant proteins primarily targeted high molecular weight (> 55 kDa) proteins (Fig. 2C); however for cow 504, antibodies against MOK023 proteins reacted against both high and low molecular weight proteins (Fig. 2F). In addition, antibodies raised in cow 504 primarily targeted low p*I* proteins (Fig. 2F) compared to the antibodies raised in cow 604 (Fig. 2C). More immunoreactivity was evident for the cow infected with the homologous strain compared to the heterologous strain. Candidate immunogenic proteins from MOK023 were identified by mass spectrometry (Table 2). Of the twenty excised spots chosen for analysis, proteins were identified for 18 spots and more than one candidate protein was detected in 11 spots. Table 2 lists the top two candidate proteins identified in each spot. Supplementary file 1 lists all detected proteins in each of the spots.

The results of 2D coomassie stained gels and serum blots of MOK124 supernatant proteins probed with sera from cow 504 (MOK124 infected) and cow 604 (MOK023 infected) are shown in Fig. 3. In agreement with the 1D analysis, antibodies generated by both cows primarily targeted low molecular weight (< 45 kDa) proteins (Fig. 3C and F). The humoral immune response was largely strain-specific with few common immunoreactive spots. Overall, more immunoreactive spots were identified when the MOK124 proteins were probed with serum from the cow infected with the homologous strain compared to the heterologous strain. Candidate immunogenic proteins from MOK124 were identified by mass spectrometry. Of the twenty excised spots chosen for analysis, proteins were identified in only 13 spots and more than one candidate protein was detected in five spots. Table 3 lists the top two candidate proteins identified in each excised spot. Supplementary file 1 lists all detected proteins in each of the excised spots.

3.4. Validation of identified immunoreactive proteins

The 2D serum blotting identified candidate immunogenic proteins in strains MOK124 and MOK023. However, in many cases immunoreactive spots contained more than one protein and in order to confirm immune reactivity, validation of candidate proteins was performed. Proteins α-hemolysin and LukM were chosen for validation. Purified recombinant proteins were separated by 1D electrophoresis, immobilised onto PVDF membrane and probed with serum collected pre and post-infection from all cows.

The results of the 1D serum blots of α-hemolysin are shown in Fig. 4. For the MOK023 infected cows (Fig. 4A), cow 604 raised the strongest humoral response to α-hemolysin, with weak reactions for cows 506 and 565. Cow 604 also had some antibodies to α-hemolysin pre-challenge indicating prior exposure to *S. aureus*. In contrast, all the MOK124 infected cows induced a humoral response against α-hemolysin post-infection (Fig. 4B). In addition, all cows had antibodies to α-hemolysin pre-challenge indicating prior exposure to *S. aureus*.

Fig. 5 shows the results of the 1D serum blots of LukM. For MOK023 infected cows (Fig. 5A), 506, 565 and 604 showed some serum reactivity to LukM while for cow 1922 reactivity was evident 7 days post-infection only. In addition, cows 506 and 565 had prior exposure to potentially

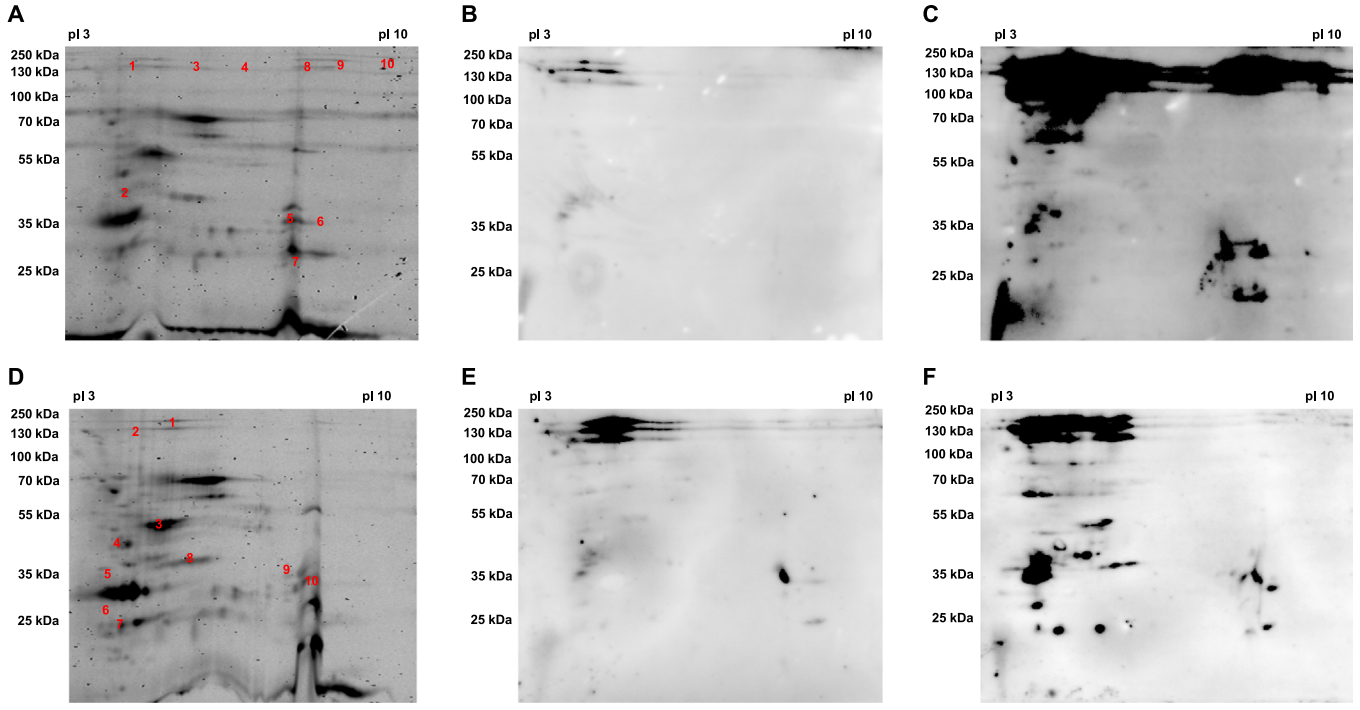

Fig. 2. Two-dimensional colloidal coomassie stained gel and serum blots of MOK023 supernatant proteins. (A & D) Colloidal coomassie stained gel of MOK023 supernatant protein extract. Numbers in red indicate the spots excised for mass spectrometry analysis. Membranes were probed using day 0 (B & E) and day 14 (C & F) serum from cow 604 (MOK023 infected) (A-C) and cow 504 (MOK124 infected) (D-F).

Table 2
List of MOK023 (CC97) candidate immunoreactive proteins detected by mass spectrometry.

Serum Source	Spot Number	Protein Name	# Unique Peptides	Predicted Localisation	Theoretical Mr (kDa) / p*I**	Observed Mr (kDa) / p*I*$^{+}$
Cow 604 (MOK023 infected)	1	Serine-aspartate repeat-containing protein E (SdrE)	19	Cellwall	126.2 / 4.46	~ 180 / 4.5 – 4.7
		ATP synthase beta chain (AtpD)	2	Cytoplasmic Membrane	51.4 / 4.82	
	2	Pyruvate dehydrogenase E1 component beta subunit (PdhB)	4	Cytoplasmic	35.2 / 4.73	~ 39 / 4 – 4.2
	3	Serine-aspartate repeat-containing protein E (SdrE)	8	Cellwall	126.2 / 4.46	~ 180/ 5.4 – 5.5
		ATP synthase beta chain (AtpD)	2	Cytoplasmic Membrane	51.4 / 4.82	
	5	Alpha-hemolysin precursor (Hla)	14	Extracellular	35.9 / 8.73	~ 32 / 6.3 – 6.6
		Manganese ABC transporter, periplasmic-binding protein (SitA)	11	Cytoplasmic Membrane	34.7 / 8.66	
	6	Alpha-hemolysin precursor (Hla)	6	Extracellular	35.9 / 8.73	~ 32 / 6.7 – 7.1
		Manganese ABC transporter, periplasmic-binding protein (SitA)	2	Cytoplasmic Membrane	34.7 / 8.66	
	7	Staphylococcal superantigen-like protein 7 (SSL7)	4	Extracellular	26.0 / 8.54	~ 23 / 6.35 – 6.4
		Staphylococcal superantigen-like protein 11 (SSL11)	2	Extracellular	26.2 / 8.75	
	8	Serine-aspartate repeat-containing protein E (SdrE)	6	Cellwall	126.2 / 4.46	~ 180 / 6.45 – 6.5
		ATP synthase beta chain (AtpD)	2	Cytoplasmic Membrane	51.4 / 4.82	
	9	Serine-aspartate repeat-containing protein E (SdrE)	6	Cellwall	126.2 / 4.46	~ 200 / 7.4 – 7.7
		ATP synthase beta chain (AtpD)	2	Cytoplasmic Membrane	51.4 / 4.82	
	10	Serine-aspartate repeat-containing protein E (SdrE)	2	Cellwall	126.2 / 4.46	~ 200 / 9.3 – 9.6
Cow 504 (MOK124 infected)	1	Serine-aspartate repeat-containing protein E (SdrE)	26	Cellwall	126.2 / 4.46	~ 200 / 5.35 – 5.4
	2	Serine-aspartate repeat-containing protein E (SdrE)	26	Cellwall	126.2 / 4.46	~ 150 / 4.7 – 4.8
	3	Bifunctional autolysin (Atl)	6	Unknown	4136.4 / 9.61	~ 66 / 4.98 – 5.2
	4	Serine-aspartate repeat-containing protein E (SdrE)	13	Cellwall	126.2 / 4.46	~ 62 / 4.5 – 4.7
		Staphopain B precursor (SspB)	2	Extracellular	44.6 / 5.97	
	5	Alpha-hemolysin precursor (Hla)	3	Extracellular	35.9 / 8.73	~ 35 / 3.98 – 4.1
	6	Gamma-hemolysin component A (HlgA)	4	Extracellular	36.4 / 9.72	~ 27 / 3.98 – 4.2
	7	Immunodominant staphylococcal antigen A (IsaA)	4	Extracellular	24.2 / 6.62	~ 25 / 4.5 – 4.7
		Staphylococcal superantigen-like protein 11 (SSL11)	3	Extracellular	26.2 / 8.75	
	9	Alpha-hemolysin precursor (Hla)	19	Extracellular	35.9 / 8.73	~ 35 / 6.6 – 7.1
		Beta-hemolysin (Hlb)	16	Extracellular	37.2 / 8.70	
	10	Alpha-hemolysin precursor (Hla)	26	Extracellular	35.9 / 8.73	~ 34 / 7.1 – 7.4
		Manganese ABC transporter, periplasmic-binding protein (SitA)	12	Cytoplasmic Membrane	34.7 / 8.66	

*Theoretical Mr / p*I* = theoretical molecular weight / isoelectric point, calculated from the amino acid sequence
$^{+}$Observed Mr / p*I* = observed molecular weight / isoelectric point, based on location following 2D electrophoresis

LukM-positive *S. aureus*. All MOK124 infected cows (Fig. 5B) induced a humoral immune response against LukM. In addition, cows 504 and 582 showed evidence of prior exposure to LukM-positive *S. aureus*.

4. Discussion

Herd vaccination strategies, in conjunction with effective diagnostic testing and good farm management practices, would be of benefit for the control of bovine IMI. There are a number of distinct *S. aureus* lineages associated with bovine IMI (Zadoks et al., 2011, Bergonier et al., 2014, Budd et al., 2015, Haag et al., 2019) and there is a dearth of knowledge regarding the major immunogenic antigens associated with each lineage and whether these antigens could provide protection against heterologous strains. Identification of the major immunogenic antigens of the predominant bovine-adapted *S. aureus* lineages would assist in the design of more effective vaccines and diagnostic targets to control bovine IMI caused by this organism.

Genotypic characterisation of the CC97 strain MOK023 and the CC151 strain MOK124 demonstrated that MOK124 encoded more toxins, in particular SAgs along with the ruminant-specific leukocidin, LukMF. This agrees with previous studies that determined that strains belonging to CC151 encode more toxins compared to strains belonging to CC97 (Guinane et al., 2008, Collery et al., 2009, Jamrozy et al., 2012, Vrieling et al., 2016, Wilson et al., 2018). In addition, MOK124 produced more secreted proteins *in vitro* than MOK023. Mass spectrometry data from supernatant extracts from both strains also showed the presence of a number of predicted cytoplasmic proteins (Supplementary file 1). Whether such proteins are found due to cell lysis or are truly extracellular remains difficult to determine. Some cytoplasmic proteins have alternative functions resulting in surface exposure (Singh et al., 2012, Monteiro et al., 2015, Tartaglia et al., 2020, Wolden et al., 2020). Therefore, the detection of these proteins in the supernatants of MOK023 and MOK124 could be due to non-classical secretion, their presence in extracellular vesicles or their release during cell wall turnover or cell division. However, these proteins were not considered candidates for this study.

A study by Niedziela et al. (2020) reported that infection with MOK124 led to severe clinical IMI compared to infection with MOK023, which caused subclinical infection. Cows infected with MOK124 had lower milk yield, a higher number of somatic cells recruited to the mammary gland, higher concentration of IL-8 in milk, higher anti-*S. aureus* IgG in serum and milk and higher anti-*S. aureus* IgA in milk, compared to MOK023 infected cows. Analysis of the total serum IgG response by 1D serum blotting demonstrated that the humoral

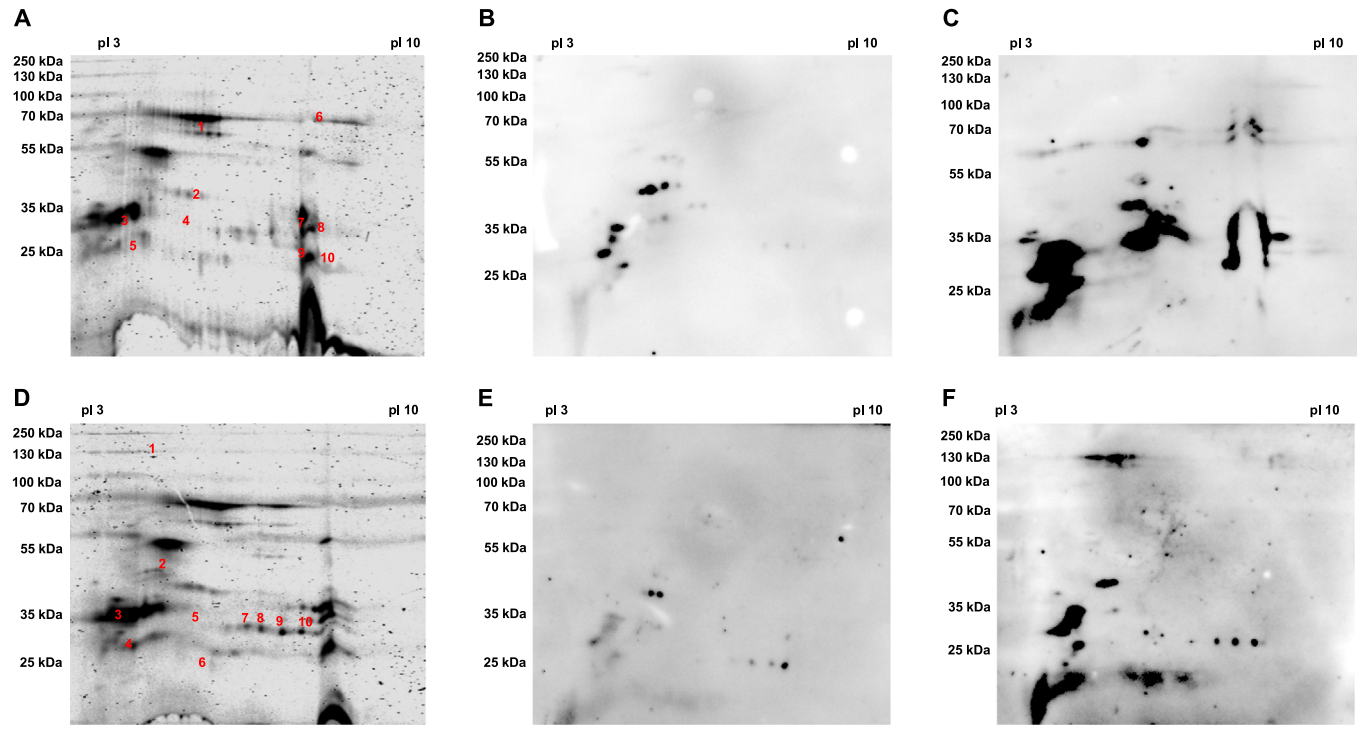

Fig. 3. Two-dimensional colloidal coomassie stained gel and serum blots of MOK124 supernatant proteins. (A & D) Colloidal coomassie stained gel of MOK124 supernatant protein extract. Numbers in red indicate the spots excised for mass spectrometry analysis. Membranes were probed using day 0 (B & E) and day 14 (C & F) serum from cow 504 (MOK124 infected) (A-C) and cow 604 (MOK023 infected) (D-F).

Table 3
List of MOK124 (CC151) candidate immunoreactive proteins detected by mass spectrometry.

Serum Source	Spot Number	Protein Name	# Unique Peptides	Predicted Localisation	Theoretical Mr (kDa) / p*I**	Observed Mr (kDa) / p*I*$^{+}$
Cow 504 (MOK124 infected)	3	Leukocidin F subunit (LukF)	7	Extracellular	36.5 / 9.23	~ 34 / 4.2 – 4.5
	4	Alpha-hemolysin precursor (Hla)	9	Extracellular	35.9 / 8.73	~ 35 / 5.3 – 5.4
		Phosphatidylinositol-specific phospholipase C (PI-PLC)	4	Extracellular	37.0 / 8.00	
	7	Beta-hemolysin (Hlb)	15	Extracellular	37.2 / 8.70	~ 35 / 6.6 – 6.7
		Leukocidin M subunit (LukM)	11	Extracellular	35.0 / 9.42	
	8	Alpha-hemolysin precursor (Hla)	29	Extracellular	35.9 / 8.73	~ 34 / 6.4 – 6.7
		Leukocidin M subunit (LukM)	12	Extracellular	35.0 / 9.42	
	9	Phosphatidylinositol-specific phospholipase C (PI-PLC)	8	Extracellular	37.0 / 8.00	~ 28 / 6.4 – 6.5
		Beta-hemolysin (Hlb)	2	Extracellular	37.2 / 8.70	
	10	Bifunctional autolysin (Atl)	4	Extracellular	136.7 / 9.58	~ 29 / 6.5 – 6.95
		Autolysin precursor (Aaa)	3	Cellwall	35.9 / 9.67	
Cow 604 (MOK023 infected)	2	Staphopain B precursor (SspB)	18	Extracellular	44.6 / 5.81	~ 47 / 4.6 – 4.79
	3	Leukocidin F subunit (LukF)	12	Extracellular	36.5 / 9.23	~ 33 / 3.8 – 3.98
	5	Phosphatidylinositol-specific phospholipase C (PI-PLC)	5	Extracellular	37.0 / 8.00	~ 32 / 5.29 – 5.35
	7	Phosphatidylinositol-specific phospholipase C (PI-PLC)	3	Extracellular	37.0 / 8.00	~ 31 / 5.6 – 5.65
	8	Phosphatidylinositol-specific phospholipase C (PI-PLC)	9	Extracellular	37.0 / 8.00	~ 30 / 5.8 – 5.9
	9	Phosphatidylinositol-specific phospholipase C (PI-PLC)	10	Extracellular	37.0 / 8.00	~ 29 / 6 – 6.1
	10	Phosphatidylinositol-specific phospholipase C (PI-PLC)	6	Extracellular	37.0 / 8.00	~ 28 / 6.3 – 6.45

*Theoretical Mr / p*I* = theoretical molecular weight / isoelectric point, calculated from the amino acid sequence
$^{+}$Observed Mr / p*I* = observed molecular weight / isoelectric point, based on location following 2D electrophoresis

responses against these two *S. aureus* strains were quite distinct and largely strain-specific. All cows infected with MOK023 displayed a similar immunoreactive profile and primarily generated antibodies against high molecular weight proteins while all cows infected with MOK124 also had a similar profile and primarily generated antibodies against low molecular weight proteins. As toxins and cytotoxic enzymes are generally small in size (Otto, 2014, Tam and Torres, 2019), the serum blot results suggest the antibody responses against MOK124 are primarily against these cytotoxic factors. As cows infected with MOK023 primarily generated antibodies against high molecular weight proteins, this suggests that the immunogens produced by MOK023 *in vivo* are, to at least some extent, distinct from those produced by MOK124. As the

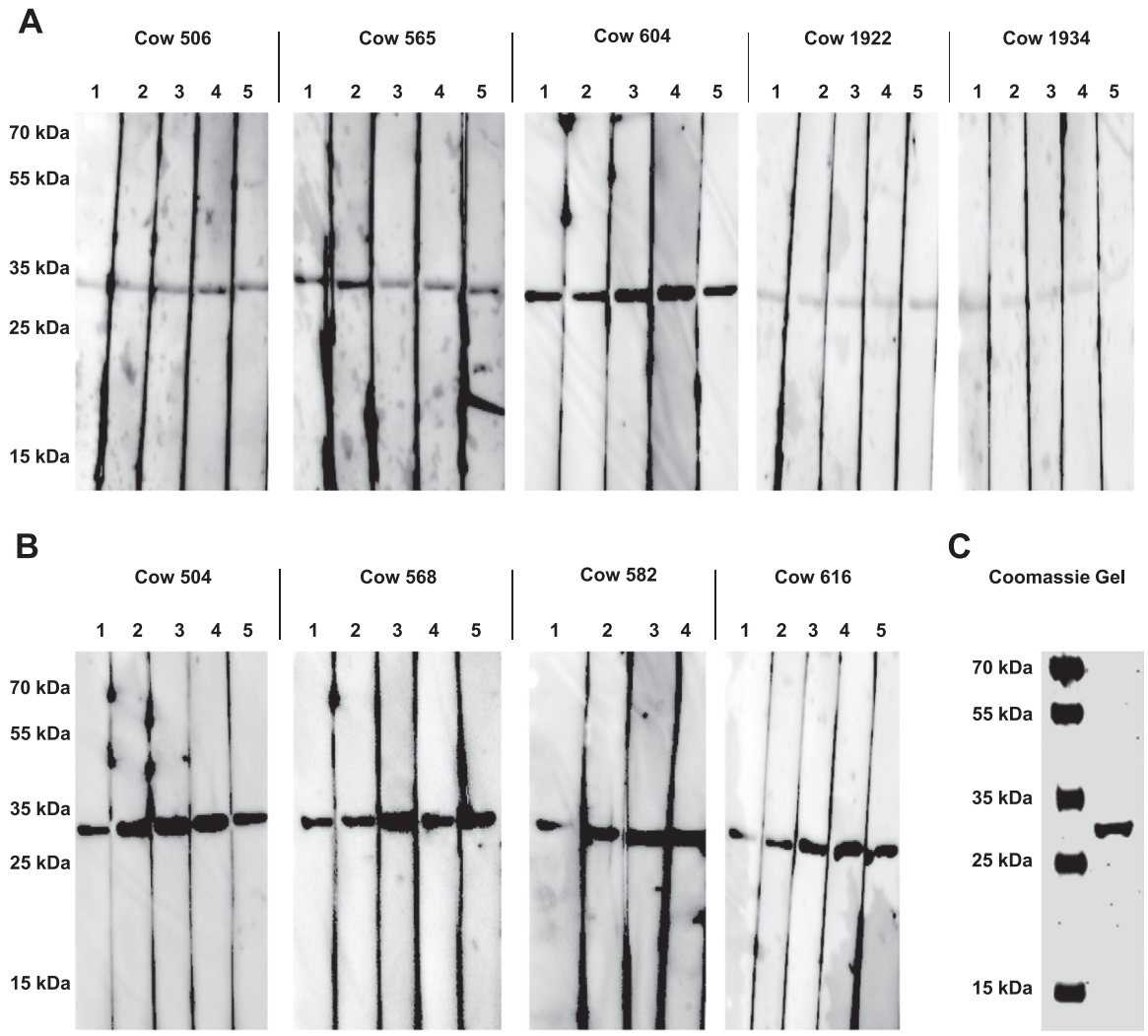

Fig. 4. One-dimensional serum blots of recombinant α-hemolysin. Membranes were probed using sera from (A) MOK023 (CC97) and (B) MOK124 (CC151) infected cows. (C) Coomassie stained gel of the purified α-hemolysin. Lane 1 = Day 0 pre-infection, Lane 2 = Day 7 post infection, Lane 3 = Day 14 post infection, Lane 4 = Day 21 post infection and Lane 5 = Day 29 post infection.

1D serum blots demonstrated that the humoral response among cows within a group was similar, only a single representative cow from each group was used for 2D immunoproteomic analysis to identify antigenic proteins produced by MOK124 and MOK023. The 2D serum blots showed agreement with the 1D blots and identified a number of strain-specific proteins to which a humoral response was induced post-intramammary challenge. However, antibodies produced by cow 604 (MOK023 infected) could cross react with secreted proteins produced by MOK124 and *vice versa*, indicating some common immunoreactive proteins. Common candidate immunogens included α-hemolysin, β-hemolysin, Atl and PI-PLC. MOK124-specific candidate antigens included LukM and LukF while MOK023-specific candidate antigens included SdrE, SSL11 and SSL7.

Purified recombinant α-hemolysin and LukM were validated as candidate immunogens. Each protein was probed with sera (Day 0, 7 14, 21 and 29) from all infected cows. All MOK124 infected cows had a strong humoral response to α-haemolysin indicating this toxin was likely produced at high levels *in vivo* by this strain. However, only cow 604 from the MOK023 infected group showed a notable humoral response to α-haemolysin, indicating that MOK023 may not have widely produced this toxin *in vivo*. Niedziela et al. (2020) showed that cow 604 had a higher total anti-*S. aureus* IgG response in serum compared to other MOK023 infected cows. This cow therefore appears to have generated a strong IgG response, which could account for why there was a response to α-haemolysin by this cow only. All MOK124 infected cows generated a strong humoral immune response to LukM. In addition, three of the MOK023 infected cows generated antibodies that reacted with LukM. This was surprising as MOK023 does not encode *lukM*. Pairwise sequence alignment shows that LukE from MOK023 has moderate sequence identity to LukM from MOK124 (72.8%). Other reports have also shown sequence similarity between these two proteins (Yoong and Torres, 2013) and both can interact with host receptor CCR5 (Alonzo et al., 2013, Vrieling et al., 2015). Therefore, there may be common immunoreactive epitopes in both proteins.

Previous studies have identified a number of antigenic proteins from ruminant *S. aureus* isolates using a immunoproteomic approach similar to that used here (Misra et al., 2018, Cunha et al., 2020, Longheu et al., 2020). This study expands these findings by demonstrating that, for at least some proteins, the systemic IgG response to *S. aureus* is strain-specific. The number of candidate antigenic proteins identified in this study was somewhat limited. In addition, only one representative cow from each group was used for antigen identification; therefore no statistical analysis of the 2D immunoproteomic data could be undertaken. To gain further insights into the important virulence factors produced *in vivo* by these genetically distinct *S. aureus* strains, all immunoreactive proteins could be identified as well as detecting immunoreactive proteins using serum from all cows from both infected cow groups. Ultimately, the ability of the candidate proteins identified

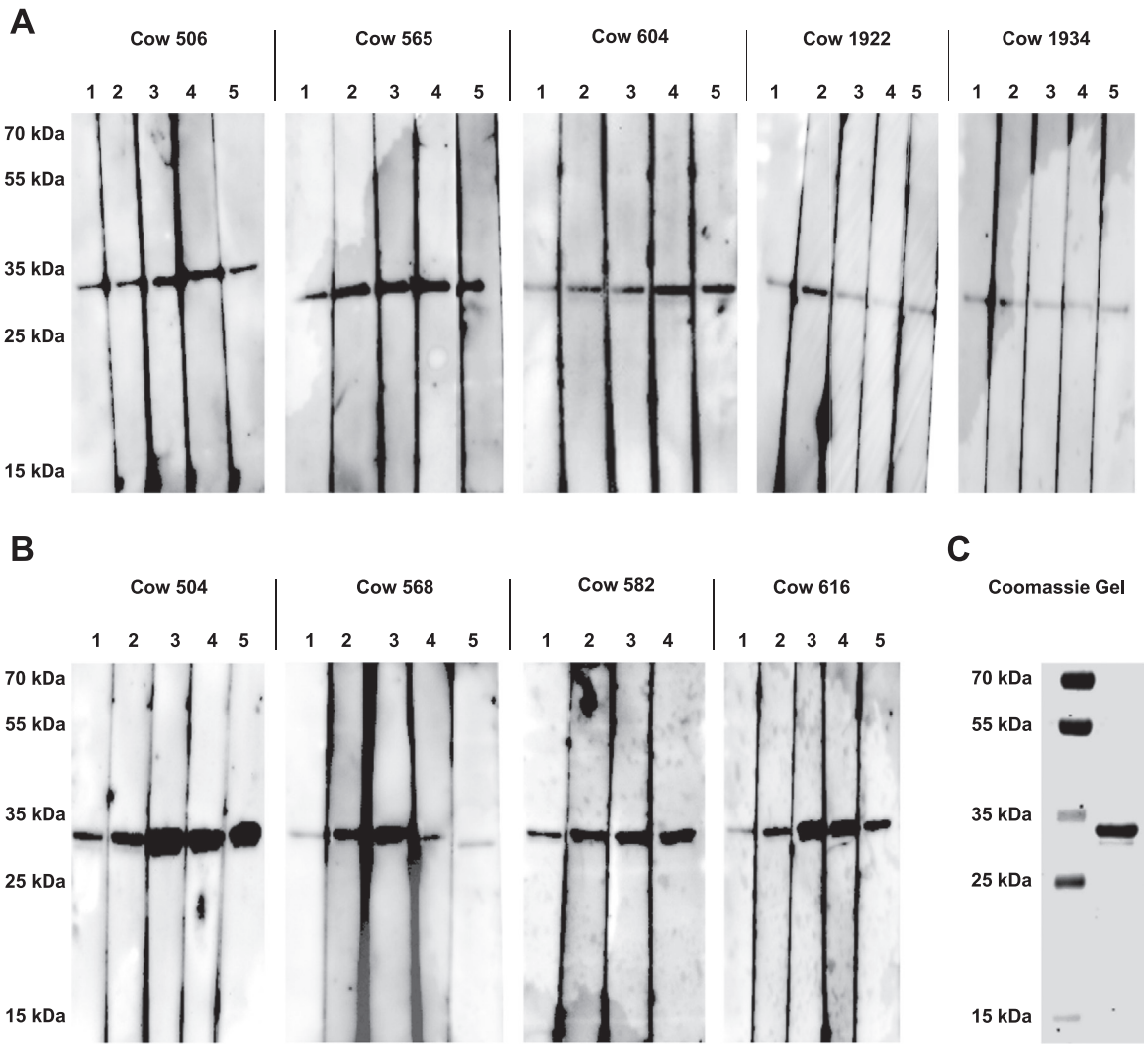

Fig. 5. One-dimensional serum blots of recombinant LukM. Membranes were probed using sera from (A) MOK023 (CC97) and (B) MOK124 (CC151) infected cows. (C) Coomassie stained gel of purified LukM. Lane 1 = Day 0 pre-infection, Lane 2 = Day 7 post infection, Lane 3 = Day 14 post infection, Lane 4 = Day 21 post infection and Lane 5 = Day 29 post infection.

in this study to elicit a protective immune response would need to be evaluated by vaccination and subsequent intramammary challenge of the vaccinated cows. However, this present study shows that immunoproteomics can successfully characterise the humoral responses in infected cows, as well as provide information on the proteins produced *in vivo* by these two *S. aureus* strains.

The serum blots indicated that the cows used in the infection trial by Niedziela et al. (2020) had prior exposure to *S. aureus* as there was immunoreactivity pre-infection. Calves are commonly naturally exposed to *S. aureus* (Benedictus et al., 2019) and prior to the infection trial the nose, vulva and groin of the cows were swabbed and *S. aureus* was detected in at least one of these anatomical sites in all cows (Niedziela, pers. comm.). However, results from previous studies looking at the role of *S. aureus* exposure or colonisation on disease in cattle is conflicting. Studies by Da Costa et al., (2014) and Svennesen et al. (2019) suggested that teat skin colonization with *S. aureus* was associated with an increased risk of *S. aureus* IMI in cows; however, a study by Zadoks et al. (2002) did not demonstrate this correlation between *S. aureus* colonisation and infection. The effect of *S. aureus* exposure or colonisation on infection outcome may also be modulated by different factors such as the health status of the host, the genetic background of the *S. aureus* strains or the specific host immune response against the strains. Additionally, the contribution of prior *S. aureus* exposure or indeed prior exposure to other microorganisms, to immune priming and innate immune memory remains to be determined. Therefore, further investigation is warranted to understand the role of *S. aureus* exposure and colonisation on the subsequent immune responses of cattle, and how this may affect the development of an efficacious vaccine.

This study has identified a number of *S. aureus* antigenic proteins and demonstrated that the systemic IgG response is, to some degree, strain-specific. However, it should be noted that the experimental method only detects immunogenic proteins that are produced under the *in vitro* culture conditions used. Previous studies have demonstrated the effect of the growth environment on variation in gene and protein expression *in vitro* and *in vivo* (Ferreira et al., 2012, Sadaka et al., 2014, Jenkins et al., 2015). Therefore, the growth conditions used may have limited the number of immunogens detected. Future research could examine the production of extracellular virulence factors under *in vitro* growth conditions that better represent the mammary gland environment. Investigation into specific IgA responses in milk to identify antigenic proteins that stimulate a local mucosal response is also warranted, along with confirmation of the protective nature of these antibodies. Lastly, confirmation that the antigenic proteins identified in this study can be detected in milk would allow for development of these antigens for use in rapid on-site *S. aureus* diagnostic tests.

5. Conclusions

This present study has demonstrated that genes encoding important *S. aureus* secreted virulence factors, as well as their production *in vitro*, is strain dependent. In addition, the humoral IgG response of cows infected with MOK124 (CC151) was distinct from that of cows infected with MOK023 (CC97), at least for extracellular proteins. In addition, this study demonstrated that more toxins were produced *in vitro* by MOK124 compared to MOK023 and these were also likely highly produced *in vivo*. This suggests that the mechanism of infection between these strains could be distinct from one another. MOK124 may have a more toxic virulence mode of infection compared to MOK023. This agrees with the infection trial by Niedziela et al. (2020) that reported cows infected with MOK023 experienced subclinical infection while cows infected with MOK124 presented with severe clinical IMI, which became gangrenous in one case. However, some common immunogenic proteins were produced by both *S. aureus* strains suggesting that developing a vaccine or a diagnostic test that targets genetically distinct strains of *S. aureus* is possible and warrants further investigation.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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