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Research article

Comparison of *Staphylococcus aureus* surface protein extraction methods and immunogenicity

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ABSTRACT

Staphylococcus aureus is the major contagious bovine mastitis pathogen and has no effective vaccine. Strain variation and limited knowledge of common immunogenic antigen/s are among major constraints for developing effective vaccines. *S. aureus* cell surface proteins that are exposed to the host immune system constitute good vaccine candidates. The objective of this study was to compare two novel *S. aureus* surface protein extraction methods with biotinylation method and evaluate immune-reactivity of extracted proteins. Surface proteins were extracted from nine genetically distinct *S. aureus* strains from cases of bovine mastitis. After extraction, bacterial cell integrity was examined by Gram staining and electron microscopy to determine if extraction methods caused damage to cells that may release non-surface proteins. The extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and evaluated for immune-reactivity using western blot. Results showed that all three extraction methods provided multiple protein bands on SDS-PAGE. Western blot result showed several immunoreactive surface proteins, in which some proteins strongly (well-resolved, thick, dark, and intense band) reacted across the nine strains tested. The three methods are valid for the extraction of surface proteins and hexadecane, and cholic acid methods are more feasible than biotinylation since both are easier, cheaper, and have minor effects on the bacterial cell. Strongly immune-reactive surface proteins may serve as potential candidates for a vaccine to control *S. aureus* mastitis in dairy cows.

1. Introduction

Bovine *Staphylococcus aureus* (*S. aureus*) mastitis is a major problem of the dairy industry. It results in the massive use of antimicrobials as dry cow therapy (DCT) for prophylactic control as well as for treatment of cases of mastitis (Barkema et al., 2006; Brady et al., 2006; Sanchez et al., 1994). Currently, available bacterin-based vaccines (Lysigin® and Startvac®) are ineffective for the prevention of mastitis caused by *S. aureus* (Bradley et al., 2015; Luby et al., 2007; Middleton et al., 2009; Middleton et al., 2006; Schukken et al., 2014). In the USA, Lysigin®, a Bacterin made of five strains is used as a vaccine against *S. aureus* intramammary infection (IMI) (Ma et al., 2004). Lysigin® is claimed for reducing new IMI caused by *S. aureus,* and coagulase-negative *Staphylococcus* species (CNS) provided that vaccination program starts on heifers at an early age of 6 months with booster vaccination every six months until calving (Nickerson et al., 1999). However, in other studies it was shown that Lysigin® vaccinated cows were not protected from new IMI, had no significant increase in antibody titers in milk and had no significant reduction in somatic cell count (SCC) as compared to non-vaccinated control cows (Luby et al., 2007; Middleton et al., 2009; Middleton et al., 2006). *S. aureus* is highly adaptive and often resistant to antimicrobial treatment and host defense systems (Lozano et al., 2016; Monecke et al., 2016; Proctor et al., 2014), and thus threatens human and animal health by turning into a multidrug-resistant pathogen (Fitzgerald, 2012a; 2012b; Holmes and Zadoks, 2011). Literature review (Erskine et al., 2004; Oliver et al., 2011) of previous studies did not support widespread antimicrobial resistance among mastitis

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pathogens; however, recent findings (Abdi et al., 2018) showed increased resistance to some antimicrobials. In light of increased antimicrobial resistance to some antimicrobials in dairy farms (Abdi et al., 2018), alternative control measures such as vaccines (Daum and Spellberg, 2012; Lee, 1996), probiotics, prebiotics, selection for mastitis resistance traits (Wall et al., 2005) and improved husbandry practices (management and nutrition) are required. *Staphylococcus aureus* is equipped with several cell-surface proteins that are promising antigen candidates (Foster, 2005; Foster et al., 2014; Foster and Hook, 1998). However, the number of these proteins vary with strains and growth conditions of strains (Foster et al., 2014; Wadstrom et al., 1974). Therefore, targeting only a single protein as a vaccine candidate might not protect against *S. aureus* since this pathogen has several gene repertoires that perform a similar/redundant function (Daum and Spellberg, 2012). Thus, combining multiple surface proteins into a single vaccine should be a feasible approach, since they provide a high level of protection against experimental challenges of *S. aureus* in mouse models (Gaudreau et al., 2007; Mazmanian et al., 2000; Stranger-Jones et al., 2006). We also found that vaccination of dairy cows with multiple immune-reactive staphylococcal surface proteins as vaccine antigens induced partial protection against *S. aureus* mastitis (Merrill et al., 2019). However, there is limited information on how to extract staphylococcal surface proteins that are accessible to the host immune system. Therefore, this study aimed to compare two novel *S. aureus* surface protein extraction methods with biotinylation method (de Boer et al., 2003; Hempel et al., 2010) and evaluate immune-reactivity of extracted proteins. Following extraction, we combined proteomics (SDS-PAGE) with immunoblotting (western blot) to identify surface proteins that react to immune serum (hyperimmune serum) from a cow previously vaccinated with *S. aureus* surface proteins (SASP) and protected from mastitis upon subsequent challenge with heterologous strain of *S. aureus* (Merrill et al., 2019). We also cut bands containing immune-reactive surface proteins and sequenced peptides to determine the identity of the protein in the band.

2. Materials and methods

2.1. Bacteria

Staphylococcus aureus strains used in this study were obtained from a collection of *S. aureus* isolates from cases of mastitis in Tennessee by the Tennessee Quality Milk Laboratory. *S. aureus* isolation and identification was conducted following the National Mastitis Council (NMC) guidelines (Oliver et al., 2004). Briefly, 100 μL of milk sample was inoculated on blood agar plates and incubated at 37 °C overnight. The identity of the bacterium was confirmed by a series of tests including hemolysis, Gram stain, Staph API strip, and coagulase test. Nine genetically.

distinct *S. aureus* strains were used in this study. Overall, 111 *S aureus* isolates were collected from 49 cows with mastitis from 35 farms. *Staphylococcus aureus* isolates were genotyped by pulsed-field gel electrophoresis (PFGE) (Supplementary Fig. 1, MSc thesis, data unpublished) as described by Abdi et al. (2018). Nine genetically representative *S. aureus* strains were determined by PFGE technique.

2.2. Extraction of S. aureus surface proteins

One pure colony of *S. aureus* grown on a blood agar plate was inoculated into 500 mL Tryptic soy broth (TSB) (Becton, Dickinson and Company, Sparks, MD) and incubated at 37 °C with shaking at 180 rpm. The culture was grown to mid-log phase to OD600 of 0.5, followed by centrifugation at 500 x g for 5 min at 4 °C. The pellet was re-suspended in 30 mL TSB and aliquoted into three tubes, each containing 10 mL. The aliquots were centrifuged at 500 x g for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in cholic acid, hexadecane or biotinylation and proteins extracted as described below.

2.3. Extraction of S. aureus surface proteins using cholic acid

The cell pellets from 10 mL volume as described above, were resuspended in 30 mL of 1% cholic acid (1% in water, sodium cholate hydrate, Sigma Aldrich Co. St. Louis, MO) and incubated at room temperature with shaking at 80 rpm for 2 h. After incubation, the suspension was centrifuged at 1250 x g for 30 min at 4 °C, and the resulting supernatant was collected and filter sterilized using 0.2-μm membrane (Thermo Fisher Scientific, Waltham, MA). The proteins in the supernatant were then concentrated using a centrifugal filter membrane with minimum molecular weight cut-off of 10 kDa (EMD Millipore Corporation, Billerica, MA).

2.4. Extraction of S. aureus surface proteins using hexadecane

Hexadecane was used for extraction of surface proteins of *S. aureus* as following protocols described elsewhere (Caldwell and Lattemann, 2004), with modifications. Briefly, the bacterial cell pellet was re-suspended in 30 mL of PBS (pH7.2), followed by addition of a 5 mL of pre-warmed (37 °C) 99 % N-hexadecane solution (Thermo Fisher Scientific) into the bacterial suspension. The suspension was then vortexed for 30 s, centrifuged at 6,000 x g for 15 min at 4 °C, and placed on ice until the N-hexadecane solidified. After that, the liquid phase of the sample was collected, and filter sterilized using a 0.20-μm membrane (Thermo Fisher Scientific). Proteins were then concentrated as described.

2.5. Extraction of S. aureus surface proteins using biotinylation

The biotinylation-based extraction of *S. aureus* surface proteins was conducted using the Pierce®cell surface protein isolation kit (Pierce, Rockford, IL) following the manufacturer's protocol with modifications. Briefly, the cell pellet was re-suspended in 2 mL of PBS containing 1 mM protease inhibitor (Roche, Indianapolis, IN) and the biotinylation reaction was performed by adding 500 μL of a 1.5 mM fresh Sulfo–NHS–SS-Biotin solution (Pierce) to the bacterial suspension and incubated on ice for 2 h with gentle shaking (80 rpm). After incubation, bacterial cells were centrifuged at 4000 x g for 5 min at 4 °C, and the cell pellet was washed 3X by centrifugation (1500 x g, 3 min) with 2 mL of ice-cold PBS (pH 8.0) containing 500 mM glycine. Bacterial cells were re-suspended in 500 μL of PBS (pH 8.0) containing 1 mM protease inhibitors (Roche) and lysed by sonication of 15 s bursts 6 times at lower pitch of 2.5 on ice. The lysate was centrifuged at 20,000 x *g* for 30 min at 4 °C, and the biotinylated proteins were isolated and purified using the NeutrAvidin agarose (Pierce) affinity-purification system. 500-μL NeutrAvidin agarose was loaded to the column, and the agarose was washed 3x with 500 μL of 1% Triton X-100 in PBS by centrifugation at 1,000 x *g* for 1 min. The cell lysate was then added to the agarose and incubated for 90 min with gentle shaking on ice. The NeutrAvidin agarose was washed 6x as mentioned above. Finally, the proteins from NeutrAvidin agarose were eluted by adding 40 μL of an elution buffer containing 20 μL of 1 M DTT in 400 μL SDS-PAGE sample buffer and subsequently incubated for 1 h with gentle shaking at room temperature.

2.6. Evaluation of bacterial cell wall integrity after surface proteins extraction

After extraction, we examined the integrity of the bacterial cell wall by Gram staining, transmission (TEM), and scanning electron microscopy (SEM).

2.7. Gram staining

After incubation with cholic acid, the supernatant from the remaining pellet was collected.

The pellet was sampled using a sterile wire loop, and thin smear was prepared and gram stained (Hardy Diagnostics, Santa Maria, CA).

Similarly, after the liquid phase of the sample with hexadecane was collected, the remaining solid phase was placed at room temperature until it melted and thin smear was made and Gram-stained (Hardy Diagnostics) from the melted pellet. The smear was air-dried, fixed by heat, and Gram stained. The bacteria were visualized for appropriate Gram-positive staining using an Olympus BX41 microscope with a DP27 camera (Olympus Corporation of America, Center Valley, PA). An aliquot of *S. aureus* cells suspension taken from the same culture before cholic acid or hexadecane treatment was used as a control for Gram staining.

2.8. Scanning and transmission electron microscopy

Bacterial cell wall structure was evaluated using SEM and TEM electron microscopy using a protocol described by (Graham and Orenstein, 2007). Samples for TEM were prepared using bacterial suspensions grown to mid-log phase (OD_{600} of 0.5) at 10^7 CFU/ml, as follows. The *S. aureus* cells pellet from a 10 ml TSB culture (16 h, 37 °C) was re-suspended in 10 ml PBS (pH7.2, Control), cholic acid or hexadecane as described under surface protein extraction. From each treatment, 1 mL was washed once in PBS (pH 7.2) and re-suspended in 0.1 M sodium phosphate buffer (PB) dissolved in a distilled water with the final volume adjusted to 1L (pH7.4) with glutaraldehyde (3%) for 90 min. Samples were washed 3X in PB and re-suspended in PB (0.1 M) with osmium tetroxide (2%). After 90 min, samples were washed 2X in water and serially dehydrated in ethanol, starting with 25% and followed by 50%, 75%, 95%, and 100% for 30 min each.

For SEM, after dehydration samples were allowed to settle onto silicon chips, then critical point dried in liquid carbon dioxide in a Ladd Research Industries critical point dryer (Williston, VT). Once dried, samples were imaged with a Zeiss Auriga 40 Dual Beam SEM (Zeiss Microscopy, Thornwood, NY).

For TEM, after dehydration in ethanol, samples were further dehydrated in propylene oxide, then embedded in EM bed 812 (Electron Microscopy Sciences, Hatfield, PA). Thin sections of the epoxy-embedded samples were cut using a Leica EM UC7 ultra-microtome (Leica Microsystems, Buffalo Grove, IL). Thin sections were post-stained with uranyl acetate followed by Reynolds lead citrate (Zeiss Microscopy) before examination in a Zeiss Libra 200MC (Zeiss Microscopy) (Graham and Orenstein, 2007).

2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein concentration was measured by Pierce 660nm protein assay (Pierce). A total of 20 μg surface proteins in a total volume of 10 μL of PBS was mixed with an equal volume (10 μL) of 2x Laemmli buffer with 5% β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA). The mixture was heated at 95 °C for 5 min. The protein samples were run in a 12% pre-casted stain-free protein gel or 12% pre-casted regular protein gel (Bio-Rad) (Laemmli, 1970; Schagger and von Jagow, 1987) in a mini protein electrophoresis tank (Bio-Rad) at 110 V with a constant current for 90 min. Protein bands in stain-free gel were scanned with ChemiDoc® Touch Imaging System (Bio-Rad). Protein band images in the SDS-PAGE were captured using a ChemiDoc™ Touch Imaging System (Bio-Rad) and analyzed using Image Lab Software version 5.2.1 (Bio-Rad).

2.10. Western blot

The surface proteins of *S. aureus* separated by SDS-PAGE were transferred to nitrocellulose membrane (Bio-Rad) at a constant voltage of 100 V with 400 mA for 60 min using the wet transfer method (Bio-Rad). The membrane was blocked overnight at 4 °C in phosphate-buffered saline with 0.05% Tween 20 (v/v) (PBS-T) and 1% gelatin (w/v) (PBS-TG, pH 7.3).

After overnight blocking, membranes were washed 3X in PBS-T at room temperature. We incubated the membrane with known positive convalescent serum (diluted 1:500 dilution in PBS-TG for 1h) from a cow previously vaccinated with *S. aureus* surface proteins (SASP) and protected from clinical mastitis upon challenge (infection) in our previous study (Merrill et al., 2019).

We used the cholic acid extraction method for bacterial surface proteins based vaccine trials in our previous study (Merrill et al., 2019). Serum of a cow with high antibody titer that was protected from mastitis upon challenge was identified from our previous vaccine trials (Merrill et al., 2019). We used the serum collected at peak antibody titer following three series of vaccination at 2 weeks interval. The serum was collected on the challenge day, but immediately before the challenge.

The membrane was washed 3X with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG (H + L) secondary antibody (Bethyl Lab. Montgomery, TX) at the dilution of 1:10,000 in PBS-TG for 1 h. The Precision Protein® Strep Tactin-HRP conjugate (Bio-Rad) was used for detection of Precision Plus Protein® unstained standards (molecular weight markers) at a dilution of 1:5,000. Finally, the membrane was washed 3X with PBS-T, and 25 mL of TMB membrane horseradish peroxidase substrate (SeraCare Life Sciences Inc, Milford, MA) was added to the membrane and incubated at room temperature until reaction fully developed (10–15 min). Protein band images on the membrane were taken using a Chem-iDoc™ Touch Imaging System (Bio-Rad) and analyzed using Image Lab Software version 5.2.1 (Bio-Rad). Images of the SDS-PAGE gels and the membranes were compared to identify immune-reactive protein bands.

2.11. Protein identification by sequencing from SDS-PAGE

After identification of bands containing proteins that react to hyper-immune serum, each dominant specific band (i.e., 10 immune-reactive protein bands) was excised using a clean scalpel.

We excised conserved immune-reactive bands across the majority of the strains shown by numbered arrows (1–10) (Fig. 1, bottom three panels) for sequencing from the SDS-PAGE gel of the cholic acid extraction method. The sequenced bands (1–10) were cut from the strain (Fig. 1, G) that we used to extract surface proteins for a vaccine in our previous experimental vaccine trial (Merrill et al., 2019). The excised bands were placed in a clean micro-centrifuge tube pre-rinsed with laboratory-grade water and sent to Bioproximity (Bioproximity, LLC, Chantilly, VA, USA), for protein identification following their protocol of GeLC-MS/MS (http://www.bioproximity.com).

2.12. Data analysis

Mass spectrometer raw data files were converted to MGF format using MS convert [https://doi.org/10.1038/nbt.2377]. Detailed search parameters were printed in the search output XML files. Briefly, all searches required 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, strict tryptic cleavage, 0 or 1 missed cleavages, fixed modification of cysteine alkylation, variable modification of methionine oxidation and expectation value scores of 0.01 or lower. MGF files were searched using the UniProt *Staphylococcus aureus* RF122 sequence library (tax ID 273036). MGF files were searched using XTandem [https://doi.org/10.1021/pr0701198] and by OMSSA [https://doi.org/10.1021/pr0499491]. All searches were performed on Amazon Web Services-based cluster compute instances using the Proteome Cluster interface. XML output files were parsed and non-redundant protein sets determined using Proteome Cluster [https://doi.org/10.1002/pmic.200900370]. MS1-based features were detected, and peptide peak areas were calculated using OpenMS [https://doi.org/10.1186/1471-2105-9-163]. Proteins were required to have 1 or more unique peptides across the analyzed samples with E-value scores of 0.01 or less.

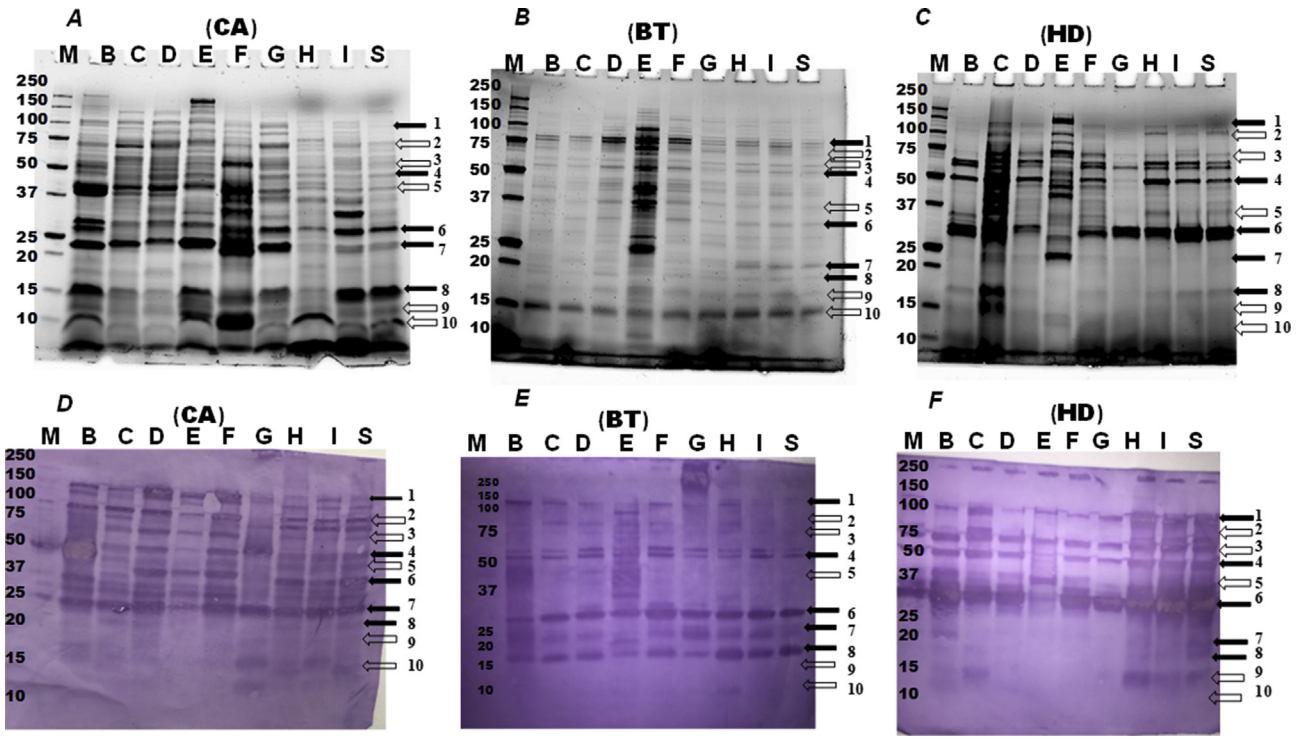

Fig. 1. SDS-PAGE and western blot results of surface proteins extracted from nine genetically distinct strains of *S. aureus* using cholic acid (CA), biotinylation (BT) or hexadecane (HD). The three panels in the top row (A, B and C) indicated protein bands by 1D SDS-PAGE and the three panels in the bottom row (D, E and F) were western blot results of same proteins transferred to nitrocellulose membrane from the SDS-PAGE. M = Protein molecular weight marker, Capital letters B–I showed dominant strains (PFGE types). The letter 'S' was the surface protein from the *S. aureus* challenge strain. The arrows (No. 1–10) indicated immunoreactive protein bands and the five black arrows (# 1, 4, 6, 7 and 8) showed strongly immuno-reactive proteins.

3. Results

3.1. *S. aureus* surface proteins extracted using cholic acid, hexadecane, and biotinylation

The total number of protein bands ranged from 14 – 23, 14–21, 9–18 for biotinylation, cholic acid, and hexadecane extraction methods respectively (Fig. 1 and Tables 1, 2, 3, and 4). The protein bands vary with the extraction method; however, there are 6, 11 and 6 conserved protein bands on SDS-PAGE across all nine strains with cholic acid, biotinylation and hexadecane extractions respectively (Fig. 1 and Tables 1, 2, 3, and 4). Overall, biotinylation had higher number of bands, followed by cholic acid and hexadecane but proteins extracted by cholic acid had thicker bands which indicated that this method provided a higher quantity of proteins (Fig. 1 and Tables 1, 2, 3, and 4). Most of the protein bands ranged from 10 - 100 kDa (Fig. 1, top three panels).

There were some conserved common protein bands on SDS-PAGE among three extractions methods. From each of the nine PFGE types (dominant strains) (Supplementary Fig-1), we observed multiple protein bands (Fig. 1, top three panels). Conserved protein bands of 15, 23, 30, 50, 40, 50, 70 and 100 kDa with cholic acid, 15, 23, 30, 37, 47, 50, 60, 75 and 80 kDa with biotinylation and 23, 30, 37, 50, 60, 70 with hexadecane were observed on SDS-PAGE (Fig. 1 and Tables 1, 2, 3, and 4).

Using CA extraction, there are unique bands for strains F at 20 kDa, B, F and G at 28 kDa, B and C at 32 kDa and H and I at 45 kDa. Similarly, for biotinylation extracted proteins, there are unique bands for strains E at 16 kDa, D, E and F at 25 kDa and C and E at 33 kDa. Hexadecane extracted proteins had unique bands for strains B, C, and E at 28 kDa, I at 75 kDa and E at 80 kDa. Overall, protein bands in CA extraction method were thick suggesting CA seems more efficient in extracting a large quantity of surface proteins.

3.2. Western blot

The total number of bands on Western blot ranged from 9 – 15, 8–13 and 7–11 for cholic acid, hexadecane, and biotinylation based extraction methods respectively (Fig. 1 and Tables 1, 2, 3, and 4). A detailed evaluation of the western blot bands revealed band variations among the protein extraction methods. However, there are 5, 6, and 8 conserved protein bands on Western blot across all nine strains with cholic acid, biotinylation and hexadecane extractions respectively (Fig. 1 and Tables 1, 2, 3, and 4).

The western blot result (Fig. 1, bottom three panels) showed that hyperimmune serum from a cow previously vaccinated with *S. aureus* surface proteins (SASP) and protected from mastitis upon challenge (infection) with heterologous strain (S) cross-reacted with surface proteins from other 8 genetically distinct strains (PFGE types B through I) (Supplementary Fig. 1). Western blot results showed that most of the nine PFGE types have visible immune-reactive protein bands shown by numbered arrows (1–10) (Fig. 1, bottom three panels). Some of the bands were strongly (well-resolved, thick, dark and intense band) reacted to hyperimmune serum (*e.g.,* band numbers 1, 4, 6, 7 and 8) at an estimated molecular weight of 100, 50, 30, 23 and 15 kDa. Conserved protein bands of 15, 23, 30, 50 and 100 kDa with cholic acid, 15, 23, 30, 50, 60 and 100 kDa with biotinylation and 23, 30, 50 and 60 kDa with hexadecane were observed on Western blot (Fig. 1 and Tables 1, 2, 3, and 4). Protein bands at 23, 30 and 50 kDa on western blot (Fig. 1, bottom three panels, Tables 1, 2, 3, and 4) were conserved across all nine strains with all three extraction methods. These conserved common immune-reactive proteins across the different strains of *S. aureus* might serve as potential vaccine antigens to control *S. aureus* mastitis in dairy cows.

Table 1
Protein bands of nine *S. aureus* strains on SDS-PAGE and western blot with cholic acid (CA) extraction method. kDa = Kilodalton, WB = Western blot, Con = conserved proteins, Seq = sequenced bands.

CA Extraction method: SDS PAGE																										
Seq	10	9	8				7		6				5				4		3	2		1			
kDa	10	12	**15**	16	18	20	**23**	28	**30**	32	33	34	37	**40**	45	47	**50**	55	**60**	**70**	75	**100**	150		
B	+ +	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+		21
C	+ +	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+		19
D	+ +	+	+	+	-	-	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+		18
E	+	+	+	-	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+		16
F	+	-	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+ +	+	+	-		**16**
G	+ +	+	+	-	-	-	+	+	+	-	+	-	+	+	-	+	+	-	-	+	+	+	-		14
H	+ +	+	+	+	+	-	+	-	+	-	+	-	+	+	+	+	+	-	+	+ +	+	+	-		17
I	+ +	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	-	+ +	+	+	-		17
S	+ +	+	+	-	+	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+	-	+			15
WB			con				con		con					con			con			con		con	-		
B	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	+	-	-	+	+	+	-		9
C	-	-	+	-	-	+	+	+	+	-	+	+	+	-	-	-	+	-	+	+	+	+	-		13
D	-	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	-		13
E	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	-	+	-	+	-	+	+	-		9
F	-	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	-		13
G	+	-	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	-	-	+	-	+	-		11
H	-	-	+	-	+	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+		15
I	+	-	+	-	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	+		15
S	-	-	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	+		15

Bold shows protein conserved in most strains tested.

3.3. Evaluation of bacterial cell integrity

3.3.1. Gram stain

After surface protein extraction using cholic acid or hexadecane, *S. aureus* cells maintained the Gram-positive staining (Fig. 2B and C) with no difference from untreated control cells (Fig. 2A) indicating intact cell wall structure.

3.3.2. Electron microscopy

SEM micrograph results showed that *S. aureus* cells had smooth cell surfaces before surface protein extraction (Fig. 3A). The morphology of *S. aureus* cells exhibited very few dimples and blisters on their cell wall after cholic acid treatment (Fig. 3C). Hexadecane treated *S. aureus* cells had few blisters on their cell wall (Fig. 3B). TEM micrograph results showed that untreated control *S. aureus* cells had dense and homogenously distributed intracytoplasmic granules (Fig. 3D). The surface protein extraction by hexadecane did not change the intra-cytoplasmic architecture (Fig. 3E) compared to untreated control cells whereas extraction with cholic acid slightly reduced the homogenously distributed dense granules in the cytoplasm (Fig. 3F).

Table 2
Protein bands of nine *S. aureus* strains on SDS-PAGE and Western blot with biotinylation (BT) extraction method. kDa = Kilodalton, WB = Western blot, Con = conserved proteins, Seq = sequenced bands.

BT extraction method: SDS - PAGE																											
Seq	10	9	8					7			6			5				4	3	2			1			
kDa	10	12	**15**	16	17	19	20	**23**	25	28	**30**	33	34	**37**	43	45	47	**50**	**60**	**70**	75	80	**100**	150		
B	+	-	+	-	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-		17
C	+	-	+	-	+	+	-	+	-	-	+	+	-	+	-	+	+	+	+	-	+	+	-	-		14
D	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+		22
E	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+		23
F	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+		**20**
G	+	-	+	-	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+		19
H	+	-	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+		19
I	-	-	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+		18
S	-	-	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+		18
WB			con					con			con							con	con							
B	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	+	-		7
C	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	+	-		7
D	-	-	+	-	-	-	-	+	-	-	+	-		+	+	-	-	+	+	+	+	-	+	-		10
E	-	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-	-	+	+	+	+	-	+	-		11
F	-	-	+	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	+	-	+	-	+	-		9
G	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	+		7
H	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+	+	+	+	-	+	-		9
I	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	+	-		7
S	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	+	-		7

Bold shows protein conserved in most strains tested.

Table 3
Protein bands of nine *S. aureus* strains on SDS-PAGE and western blot with Hexadecane (HD) extraction method. kDa = Kilodalton, WB = Western blot, Con = conserved proteins, Seq = sequenced bands.

HD extraction method: SDS - PAGE																						
Seq	10	9	8		7		6			5				4	3		2			1		T
kDa	10	**12**	15	19	**23**	28	**30**	33	34	**37**	43	45	48	**50**	**60**	64	**70**	75	80	**100**	150	
B	+	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	12
C	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	17
D	+	+	+	-	+	-	+	+	+	+	+	+	+	++	+	+	+	-	-	+	+	18
E	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	16
F	-	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	+	-	-	+	+	**14**
G	-	+	+	-	+	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	9
H	-	+	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	-	-	+	+	14
I	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	16
S	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	16
WB			con		con		con	con	con					con	con					con		
B	+	+	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	12
C	-	+	+	-	+	-	+	+	+	+	-	-	-	+	+	-	+	-	-	+	+	11
D	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	-	-	-	-	+	+	10
E	-	-	-	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	-	+	+	9
F	-	-	-	-	+	-	+	+	+	-	-	-	-	+	+	-	-	-	-	+	+	8
G	-	+	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	9
H	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	+	+	+	13
I	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	+	+	12
S	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	+	+	12

Bold shows protein conserved in most strains tested.

3.3.3. *Determination of the identity of 5 conserved immunoreactive S. aureus surface proteins by sequencing*

We sequenced conserved protein bands indicated by numbered arrowhead 1, 2, 3, 4, 5, 6, 8, 9 and 10 from H strain extracted by cholic acid. There were multiple visible immune-reactive surface protein bands, of which 5 were strongly immune-reactive proteins among most strains evaluated in this study (Fig. 1 black arrows). We found that out of several immune-reactive proteins, 5 proteins (band # 1, 4, 6, 7 and 8) were strongly (well-resolved, thick, dark and intense band) immune-reactive and conserved among most strains tested. Based on the sequence results, some of the identified proteins included staphylococcal cysteine protease (SCP) extracellular protein (Fig. 1, band # 5), autolysin protein (Fig. 1, band # 7), glyceraldehyde-3-phosphate dehydrogenase (GapC) (Fig. 1, band # 6), elongation factor Tu (Fig. 1, band # 4), small GTP-binding domain protein (Fig. 1, band # 10), IgG-binding protein SBI (Fig. 1, band # 3), lipoprotein (Fig. 1, band # 8), chaperone protein DnaK (DnaK) (Fig. 1, band # 1), alkyl hydroperoxide reductase (Fig. 1, band # 9) and other proteins involved in carbohydrate metabolism (Fig. 1, band # 1 and 2). We noticed from our sequence results that more than one proteins were matched to the peptides sequence from a single band (Fig. 1 bands # 1 and 10).

4. Discussion

Bacterial surface proteins play a pivotal role in host cells-*S. aureus* interaction, the pathogenesis of the infection, and the induction of the host immune response. Developing methods for *S. aureus* surface protein extraction, protein identification, and assessment of their role in the infectious process have great potential in the design of vaccines against *infection* caused by this pathogen.

We compared two surface protein extraction methods using cholic acid (1%) and n-hexadecane (99%) with the standard biotinylation method. We found multiple visible protein bands with all three extraction methods per isolate. Hexadecane adsorbs proteins when in a liquid state, but it desorbs the proteins when in a solid-state (Caldwell and Lattemann, 2004; Rosenberg et al., 1986). A recent study showed that incubation of bacteria with bile salts (*e.g.,* cholic acid) causes widespread protein unfolding and aggregation (Cremers et al., 2014). We exploited these biological properties of hexadecane and cholic acid to extract *S. aureus* surface protein.

Surface protein of *S. aureus* has been extracted using several techniques such as cleaving surface proteins using soluble trypsin or proteinase K (Solis et al., 2010), immobilized trypsin on beads (Dreisbach et al., 2010), shaving by lysostaphin (Taverna et al., 2007; Vytvytska et al., 2002), biotinylation (Becher et al., 2009; Hempel et al., 2010) and immunoprecipitation (subtractive proteome analysis) (Glowalla et al., 2009; Holtfreter et al., 2016). Also, genome sequence with computer algorithm (bioinformatics assisted in silico sequence analysis) has been used for prediction of surface proteins from sequencing data (Holtfreter et al., 2016; Maione et al., 2005; McCarthy and Lindsay, 2010). Overall, biotinylation based extraction method is considered as a standard protocol for extraction of surface protein from Gram-negative and

Table 4
Total number of protein bands from nine *S. aureus* strains on SDS-PAGE and Western blot with cholic acid (CA), biotinylation (BT), and hexadecane (HD) extraction methods.

Method	# of total bands on SDS-PAGE									# of bands on WB									# of conserved	
	B	C	D	E	F	G	H	I	S	B	C	D	E	F	G	H	I	S	SDS-PAGE bands	Wb bands
CA	21	19	18	16	16	14	17	17	15	9	13	13	9	13	11	15	15	15	6	5
BT	17	14	22	23	20	19	19	18	18	7	7	10	11	9	7	9	7	7	11	6
HD	12	17	18	16	14	9	14	16	16	12	11	10	9	8	9	13	12	12	6	8

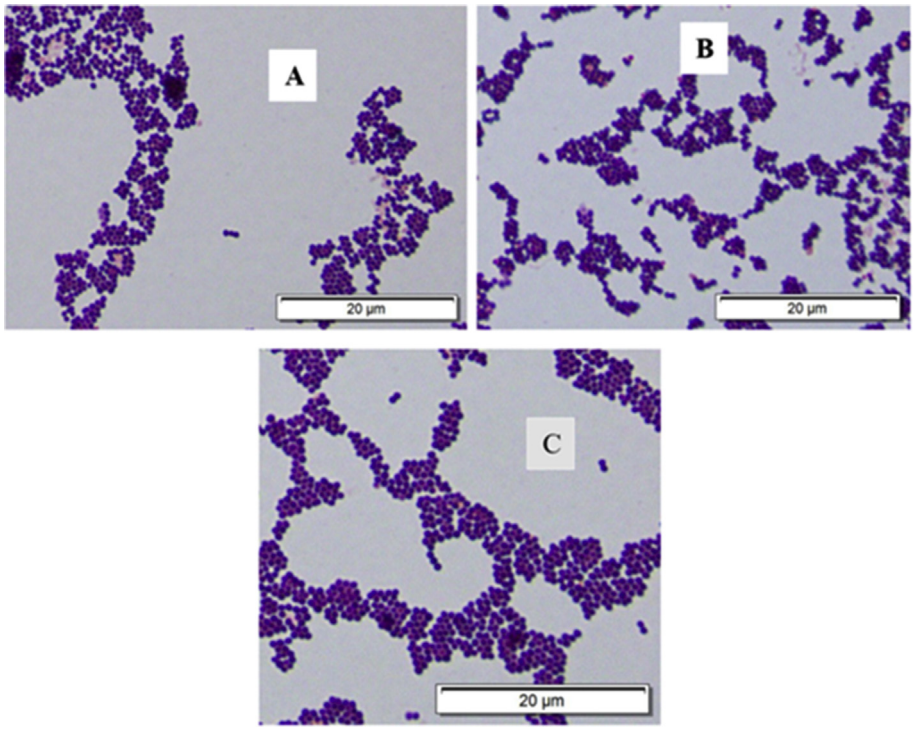

Fig. 2. Gram-stained *Staphylococcus aureus.* A) Gram-stained *S. aureus* before surface proteins extraction, B) Gram-stained *S. aureus* after surface protein extraction by hexadecane and C) Gram-stained *S. aureus* after surface proteins extraction by cholic acid.

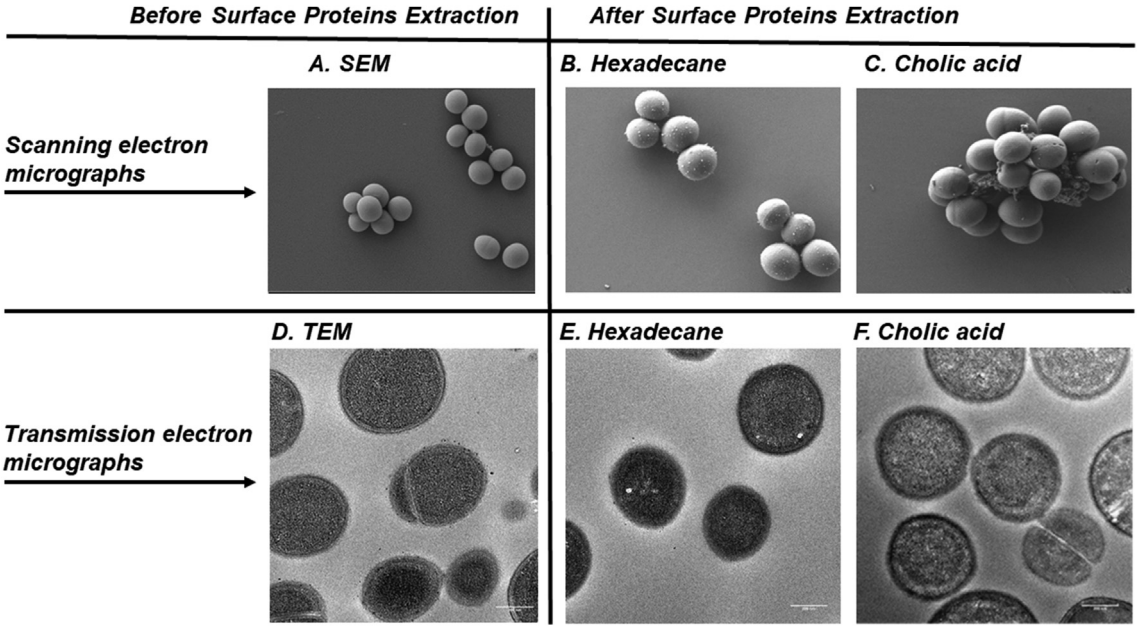

Fig. 3. Evaluation results of bacterial cell integrity by scanning and transmission electron microscopes before and after surface protein extractions. A) Scanning electron micromicrograph of untreated *S. aureus* before protein extraction, B) Scanning electron micrograph of *S. aureus* after surface protein extraction by hexadecane, C) Scanning electron micrograph of *S. aureus* after surface proteins extraction by cholic acid, D) Transmission electron micrograph of untreated *S. aureus* before protein extraction, E) Transmission electron micrograph of *S. aureus* after surface proteins extraction by hexadecane and F) Transmission electron micrograph of *S. aureus* after surface protein extraction by cholic acid. Bar = 200 nm.

Gram-positive bacteria (Becher et al., 2009; Hempel et al., 2010; Sabarth et al., 2002).

In the literature, the total number (inventory) of reported surface proteins of *S. aureus* is variable. Using immobilized trypsin extraction method followed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), about 96 surface proteins of *S. aureus* were identified from four strains (Dreisbach et al., 2010; Solis et al., 2010). Solis et al. (2010) identified 42 surface proteins (23 by trypsin and 26 by proteinase K) from laboratory-adapted *S. aureus* strains. Other authors reported a total of 48 (Gatlin et al., 2006), 26 (Taverna et al., 2007) and 15 (Vytvytska et al., 2002) *S. aureus* surface proteins using lysostaphin extraction followed by 2-D gel-based identification. Using a combination

of $^{14}N^{15}N$ metabolic labeling, biotinylation, and GeLC−MS/MS 98 surface proteins including 3 sortase substrates, 3 cell wall-associated proteins, 35 lipo-proteins, 23 membrane-associated proteins, and 34 signal peptide-containing proteins were identified. Using biotinylation-based extraction method Becher et al. (2009) reported 146 proteins, of which 48 were membrane proteins, 4 were covalently attached to peptidoglycan, 37 were lipoproteins, and 57 were cell wall-associated proteins containing a signal peptide. Others reported 95 surface proteins using similar biotinylation method (Hecker et al., 2018; Otto et al., 2014). Using immunoprecipitation (subtractive proteomic analysis) method 39 anchorless cell wall-associated proteins were identified (Glowalla et al., 2009). Using labeled immunoglobulin against individual surface proteins, 22 covalently anchored surface proteins were identified in a wild-type *S. aureus* strain expressing sortase protein (StrA), compared to an isogenic sortase mutant (Mazmanian et al., 2000). Using genome sequencing and *in silico* prediction, 22 covalently and 6 ionically cell wall attached surface proteins (McCarthy and Lindsay, 2010) and 70 lipoproteins (Graf et al., 2018) were identified. The objective of this study was to develop a novel *S. aureus* surface protein extraction method and test immune-reactivity of extracted proteins. Direct comparison of our findings with the findings of other studies (Dreisbach et al., 2010; Flower, 2003; Hecker et al., 2018; Maione et al., 2005; Otto et al., 2014; Solis et al., 2010; Taverna et al., 2007) was not possible since we did not determine the identity of all the extracted proteins. We focused only on the extraction method and immune-reactive protein bands based on western blot result.

We transferred proteins from SDS-PAGE to immunoblot membrane to test their immune reactivity. We found that out of several immune-reactive proteins, protein bands (100, 70, 50, 30, and 23 kDa) were strongly (well-resolved, thick, dark and intense band) immune-reactive and conserved among most strains tested. Proteins extracted by the cholic acid have thicker bands, which may indicate that cholic acid is more efficient in extracting large quantities of surface proteins compared with the other two methods. However, bands of proteins extracted by the biotinylation method are clear and sharp compared to other methods. The clear and sharp nature of the bands may indicate the presence of a single protein in the band. Proteins extracted by hexadecane has a lower number of bands compared to biotinylation and cholic acid.

Some of these proteins, including autolysin (Dreisbach et al., 2010; Solis et al., 2010), elongation factor Tu (Vytvytska et al., 2002; Taverna et al., 2007; Dreisbach et al., 2010), IgG-binding protein SBI (Protein A) (Taverna et al., 2007; Dreisbach et al., 2010; Solis et al., 2010), lipoprotein (Vytvytska et al., 2002; Gatlin et al., 2006), glyceraldehyde-3-phosphate dehydrogenase (GapC) (Taverna et al., 2007; Dreisbach et al., 2010), alkyl hydroperoxide reductase (Taverna et al., 2007) and chaperone protein DnaK (Taverna et al., 2007; Dreisbach et al., 2010) were previously identified using different extraction methods. The chaperone protein Dnak and elongation factor Tu were also reported as surface proteins from non-staphylococcal bacteria such as *Bacillus anthracis* (Delvecchio et al., 2006). We also identified superoxide dismutase, fructose-bisphosphate aldolase (Taverna et al., 2007), penicillin-binding protein 2 (Taverna et al., 2007) and ABC transporter proteins (Taverna et al., 2007; Dreisbach et al., 2010; Solis et al., 2010). We were unable to identify the following surface proteins within the 10 excised and sequenced immune-reactive protein bands in this study, although some studies have reported them as surface proteins. These proteins include catalase, capsular polysaccharide synthesis enzyme (Taverna et al., 2007), lipase A/esterase (Taverna et al., 2007), protein phosphatase/methicillin resistance surface proteins (Gatlin et al., 2006; Vytvytska et al., 2002; Dreisbach et al., 2010; Solis et al., 2010), dihydrolipoamide acetyltransferase, alkaline shock protein (Vytvytska et al., 2002), extracellular matrix-binding protein (EbP), and clumping factor B (fibrinogen-binding protein) (Taverna et al., 2007; Dreisbach et al., 2010; Solis et al., 2010). The lack of detection of these proteins could be explained by the scope of the current study since we only sequenced 10 immune-reactive bands despite the presence of multiple reactive surface proteins. At this point, we do not know for sure the immune-reactive protein in gel bands # 1 and 10. To identify the encoding genes of protein bands (#1–10) for further study by deleting or cloning the genes. In this study, we screened the immunogenic surface proteins using a combination of proteomics with immuno-proteomics. Some researchers use a computer (bioinformatics) based immunogenic protein prediction for screening vaccine antigens. A verification/validation using experimental animal trial is needed due to the inherent complexity of immune presentation and recognition process that complicates the bioinformatics based epitope prediction using *in silico* data. Thus, we believe that our immunoproteomic approach is not only simple and easy but also practical.

Some of the cell-surface proteins identified (listed above) in this study were known for their role as metabolic (housekeeping) enzymes. Other researchers also noted that these proteins are found on the surface of pathogens as multi-functional (virulence, glycolytic) proteins in addition to their metabolic (glycolytic) functions (Ebner et al., 2015; Pancholi and Chhatwal, 2003). It is possible that the glycolytic enzymes that we identified on the *S. aureus* cell-surface may be due to leakage from cytoplasm after cell lysis. However, we did not found any visible changes after we evaluated whether our extraction method disrupted the bacterial cell wall and resulted in the loss of Gram-positivity by Gram-staining. We also did not found changes after evaluation of bacterial cell by Scanning electron microsope (SEM) and Transmission electron microscope (TEM) to confirm the structural intactness of the bacterial cell wall after surface protein extraction. However, it is not possible to conclude with certainity that there was no minor leakage or increased bacterial cell wall permeability at all due to the lack of a reliable method to show such changes.

In general, some of these proteins identified by sequencing are multifunctional proteins involved in pathogenesis and metabolism. Some of the proteins identified by sequencing included *S. aureus* glyceraldehyde-3-phosphate dehydrogenase (GapC) (Goji et al., 2004) which is known to be involved in glycolysis and virulence. The other proteins were staphylococcal cysteine protease (SCP) family extracellular protein that are known to modulate biofilm formation (Mootz et al., 2013); elongation factor Tu (Monteiro et al., 2012); autolysin protein; small GTP-binding domain protein; IgG-binding protein SBI; lipoprotein; chaperone protein DnaK (dnaK); alkyl hydroperoxide reductase; and other proteins involved in metabolism (*Formate acetyltransferase*, inosine-5′-monophosphate dehydrogenase).

For example, autolysin proteins have both enzymatic (amidase and glucosaminidase) and adhesive functions playing a role in the colonization of host tissue via binding to extracellular matrix proteins (ECMP) (Heilmann et al., 2005). Elongation factor adheres to the surface of Hep-2 cells and has a multifunctional binding protein domains that include extracellular matrix proteins (ECMP), factor H, heparin, plasminogen and several complement factors, CD21 (Li et al., 2015; Widjaja et al., 2017; Yu et al., 2018) and elongation factor Tu of *F. tularensis* targets nucleolin on human cell surface for its internalization into the cell (Barel et al., 2008; Widjaja et al., 2017). Alkyl hydroperoxide reductase serves as broad-spectrum detoxification of host-mediated killing by H_2O_2, organic peroxides and peroxynitrite, thus increasing bacterial survival, persistence and colonization in the host (Cosgrove et al., 2007; Hébrard et al., .2009). Ornithine carbamoyltransferase induces interferon-gamma and proinflammatory cytokine genes expression in addition to its contribution for macrophage evasion (Hussain et al., 1999; Zhao et al., 2014) and phosphoglycerate kinase serves for biofilm formation and involves in production of energy (glycolysis) to ensure the survival of the bacterial (Roychowdhury et al., 2014). Both enzymatic proteins are highly immunoreactive (Zysk et al., 2000). Aldolase (FbaA) and glyceraldehyde-3-phosphate dehydrogenase belong to a family of dual-function proteins with roles in pathogenicity as an adherence factor (Ebner et al., 2016; Kerro-Dego et al., 2012) and host-cell cytotoxicity (Ebner et al., 2016). Several studies revealed that glyceraldehyde-3-phosphate dehydrogenase of *S. aureus* is localized on the cell surface of many pathogenic microorganisms (Delgado et al.,

2001; Gil-Navarro et al., 1997; Goji et al., 2004; Pancholi and Fischetti, 1992; Taylor and Heinrichs, 2002). It serves as an important pathogenesis, virulence, and adhesive factor to mammary epithelial cells in vitro and mastitis in vivo (Kerro-Dego et al., 2012). Staphylococcal GAPDH also induces strong immune responses against *S. aureus* in dairy cows (Kerro-Dego et al., 2006; Perez-Casal et al., 2006).

In general, the cholic acid and hexadecane-based methods are novel, easy and cheap for extraction of surface proteins of *S. aureus* as confirmed by SDS-PAGE and western blot. However, cholic acid-based extraction yielded a higher number of proteins in quantity as well as band diversity than hexadecane. The cholic acid extraction method is a suitable method for extraction of novel *S. aureus* vaccine candidates. Our protein sequence results indicated that a mixture of different proteins was present in a single band (# 1 and 10) indicating limitations of the 1D SDS-PAGE in resolving proteins with similar molecular weight. We recommend further evaluation by 2D SDS-PAGE, followed by Western blotting. In spite of all such future efforts for resolving on the 2D gel, the definitive confirmatory identification of the actual immune-reactive proteins can only be achieved by cloning and expression of each protein in the band (#1–10) and compare with the protein in wild-type strains as well as creating their isogenic mutants. Therefore, we aim to work in such a direction with the current potential candidate proteins to develop an effective vaccine to control *S. aureus* infections.

5. Conclusion

In conclusion, hexadecane- and cholic acid-based methods can be used for extraction of *S. aureus* surface proteins. Both hexadecane- and cholic acid-based extraction methods are cheaper and easier compared to biotinylation. Cholic acid and biotinylation methods provided multiple protein bands on SDS-PAGE and immunoblotting with hyperimmune serum from SASP vaccinated cow that was protected from mastitis upon experimental challenge recognized immune-reactive surface proteins. Some of these proteins react strongly (well-resolved, thick, dark and intense band) to hyperimmune serum across most of nine genetically different strains tested. These strongly immune-reactive surface proteins may serve as potential candidates for a vaccine against *S. aureus* mastitis in dairy cows.

Declarations

Author contribution statement

Oudessa Kerro Dego, Reta Abdi, John Dunlap, Barbara Gillespie, Desta Ensermu, Raul Almeida: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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