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Coagulase gene polymorphism of *Staphylococcus aureus* isolates from dairy cattle in different geographical areas

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SUMMARY

The objectives of this study were to investigate the coagulase gene polymorphism of *Staphylococcus aureus* isolates obtained from bovine mastitic milk and to determine the resistance of predominant and rare coagulase genotypes to bovine blood neutrophil bactericidal activities. A total of 453 isolates were collected from four countries: the Czech Republic, France, Korea and the United States. The isolates were subtyped into 40 types by restriction fragment length polymorphism (RFLP) of the coagulase gene. Twenty-three strains from predominant and rare genotypes were evaluated for their ability to resist neutrophil bactericidal activities. There were significant ($P < 0{\cdot}01$) differences in the average percent neutrophil killing of the predominant (16·7%) and rare (39·7%) genotypes when bacteria were opsonized with antiserum. The results indicate that the profiles of coagulase genotype differ among geographic locations, and only a few genotypes prevail in each location. In addition, the predominant genotypes were more resistant to neutrophil bactericidal activities than rare genotypes.

INTRODUCTION

Bovine mastitis is one of the most important diseases that affect the dairy industry. Mastitis can be caused by a variety of bacterial pathogens although *Staphylococcus aureus* is one of the major pathogens involved in subclinical and chronic mastitis [1, 2]. Current protocols for mastitis control include: teat dipping with disinfectants before and after milking, dry cow therapy with antibiotics to eliminate existing infections and reduce incidence of new infections, proper use and maintenance of milking equipment to prevent back flushing and teat damage, segregation of infected cows, avoiding introducing infected heifers to herd through replacement, and culling chronically infected cows and supplementation of micronutrients to increase host resistance to mastitis [3]. These measures have decreased the incidence of bovine mastitis, but *S. aureus* mastitis is still prevalent and is a major problem in the dairy industry today. A better understanding of the epidemiology of *S. aureus* will be beneficial for the improvement of current mastitis control protocols.

Subtyping is an important tool for epidemiological investigation of bacterial infection. In the past decade, numerous molecular techniques have been developed and used for identification and comparison of *S. aureus* isolates in epidemiological studies. These techniques include: phage typing, ribotyping, plasmid analysis, pulsed-field gel electrophoresis of genomic DNA fragments, multilocus enzyme electrophoresis (MLEE), random amplified polymorphic DNA (RAPD) and coagulase gene typing [4–10]. Phage typing and MLEE are labour intensive and a

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significant percentage of *S. aureus* isolates are untypable by phage typing. Ribotyping is time consuming, expensive and requires special equipment. Plasmid analysis has limited discrimative power because some isolates do not harbour plasmids and plasmids are inherently unstable. Pulsed-field gel electrophoresis needs a large amount of purified high-quality genomic DNA. RAPD is simple and easy to perform, but the DNA fragment produced by random PCR is hard to interpret. Among these methods, coagulase gene typing has proved to be a simple and effective means with a high specificity to identify coagulase-positive *S. aureus* isolates from mastitic milk of cows [11].

The defence of the mammary gland is mediated by non-specific and specific immune responses. Non-specific immune responses play an important role at the onset of a bacterial infection, while specific immune responses are critical during the later stages of disease by generating specific antibodies against the bacteria. Neutrophils are the major non-specific defence mechanism which comprise up to 90% of the total somatic cells of mastitic milk when bacteria invade the mammary gland and cause mastitis [12, 13]. Epidemiological investigations have suggested that only a few types of *S. aureus* predominate and most types are rare [8, 11, 14]. A limited report by Aarestrup and colleagues based on *S. aureus* isolates from Denmark suggested that predominant genotypes of *S. aureus* are more resistant to neutrophil phagocytic and bactericidal activities when compared to the rare genotypes [15]. However, these previous studies were restricted to a single geographical area. If the same observations can be made based on isolates obtained from around the world it may provide new insights concerning important host–pathogen interactions during disease pathogenesis. In this study we investigated the polymorphism of the coagulase gene of *S. aureus* isolated from bovine mastitic milk samples from a broad range of geographic locations. Furthermore, we evaluated the resistance of predominant *vs.* rare coagulase genotypes to bovine blood neutrophil bactericidal activities.

MATERIALS AND METHODS

Bacterial isolates

Staphylococcus aureus is a highly contagious pathogen and prevalence of this organism in a herd will depend upon herd health management and hygiene, immune status of the cows, and potential differences in bacterial virulence status. Isolates of *S. aureus* were collected from different herds located in a variety of geographical locations in order to accommodate for the variability associated with herd management regimes and other herd-specific factors that may affect host resistance to disease. Four hundred and fifty-three isolates of *S. aureus* were isolated from bovine mastitic milk samples from the Czech Republic, France, Korea and 19 states in the United States (Arizona, California, Colorado, Iowa, Illinois, Kentucky, Louisiana, Maine, Michigan, Minnesota, Missouri, New Hampshire, New York, Ohio, Pennsylvania, Tennessee, Washington, Wisconsin and Vermont). All isolates were cultured on trypticase soy agar (TSA) with 5% sheep blood (BiMed, St Paul, MN) and stored at −70 °C in trypticase soy broth (TSB) with 15% glycerol until needed. Isolates were identified based on colony morphology, haemolysis, Gram stain, production of catalase, coagulase and acetoin. The test for phenotypic expression of coagulase production was performed as a tube test using 0·5 ml of citrate stabilized rabbit plasma. One colony from an overnight culture was transferred to the plasma-containing tube, which was incubated at 37 °C and observed for clot formation after 1, 4, and 24 h. To differentiate *S. aureus* from coagulase-positive *S. hyicus* and *S. intermedius*, the acetoin test (Voges–Proskauer test) was used according to the recommendation by National Mastitis Council [16]. All isolates with a questionable acetoin test result were further identified by API Staph system (BioMerieux Vitek, Hazelwood, MO).

Coagulase gene typing

The coagulase gene typing was performed by a previously described method [11]. In brief, bacterial cell lysates were prepared from 1 ml of overnight TSB cultures. The bacteria were then pelleted and resuspended in 500 μl of 50 mM Tris-HCl buffer (pH 8·3) that contained 50 mM disodium EDTA. The cells were lysed with 15 U of lysostaphin (Sigma, St Louis, MO) at 37 °C for 1 h. Lysis was completed by adding 1 ml of lysis buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8·3, 1·5 mM $MgCl_2$, 1% of Triton X-100, 0·45% Nonidet P-40, 0·45% Tween-20, and 0·6 μg of proteinase K and incubated at 56 °C for 1 h. Proteinase K was inactivated by heating at 95 °C for 10 min.

The 3′ end region of the coagulase gene was amplified by using the nested primers as described

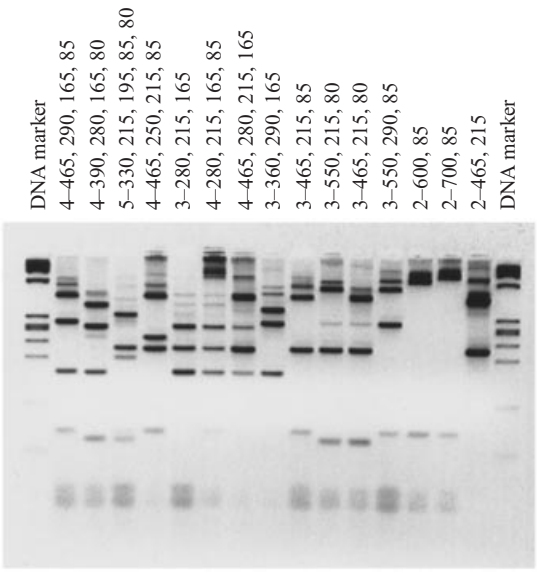

Fig. 1. An example of agarose gel electrophoresis of *S. aureus* coagulase PCR products. The PCR products were digested with restriction enzyme *Alu*I and separated in 4% NuSieve GTG agarose gel and detected in the presence of ethidium bromide under UV illumination. For convenience, the genotype profile of each isolate is denoted by the total number of the major bands followed by the estimated molecular weight of each band. For example, genotype 4-465,290,165,85 indicates that there are four major bands with the molecular weight of 465, 290, 165 and 85 bp, respectively. Phix 174/*Hae*III DNA is used as the marker (fragment size: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp).

previously [10]. The outer primers were: COAG1 5′-ATACTCAACCGACGACACCG-3′ and COAG4 5′-GATTTTGGATGAAGCGGATT-3′. Ten μl of cell lysate were added to a PCR mixture containing 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris-HCl, 1% Triton X-100, 1 μM of each primer, 200 μM of each dNTPs and 1 U of Taq polymerase to a final reaction volume of 40 μl. Each sample was subjected to 40 PCR cycles, consisting of 30 s at 95 °C, 2 min at 55 °C and 2 min at 72 °C. For the nested PCR amplification, 1 μl of the first PCR reaction was added to 39 μl of PCR mixture containing 1 μM of the second set of primers COAG2: 5′-ACCACAAGGTACTGAATC-AACG-3′ and COAG3: 5′-TGCTTTCGATTGTTC-GATGC-3′. The nested PCR amplification was performed with the same conditions as above. Ten μl of the second PCR reaction were digested at 37 °C for 1 h with 2 U of the restriction endonuclease *Alu*I (Promega, Madison, WI) according to the manufacturer's instruction. The digested DNA fragments were separated in 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) and detected in the presence of ethidium bromide under UV illumination.

Bovine blood neutrophil bactericidal assay

Bovine neutrophils were isolated from three lactating Holstein Dairy cows free of intramammary infection based on microbiological analyses of milk samples. Neutrophils were isolated by the technique described previously [15]. Bovine antiserum was collected from cows intramammarily infected with bovine *S. aureus* Newbould 305. The coagulase genotype of Newbould 305 is denoted as 4-465,290,165,85, which is the predominant type in the United States. The antiserum was pooled from five infected cows, heat inactivated at 56 °C for 30 min and stored at −20 °C in 5 ml aliquot before use.

Twenty-three *S. aureus* isolates were selected from both predominant ($n = 12$) and rare ($n = 11$) genotypes in isolates from France (predominant, $n = 3$; rare, $n = 3$), Korea (predominant, $n = 3$; rare, $n = 1$) and the US (predominant, $n = 6$; rare, $n = 7$). The selected predominant strains belong to the most frequently identified genotypes of each country, and the selected rare strains belong to the least frequently identified genotypes. Genotypes used for bactericidal assay are indicated by an asterisk in Table 2. Bacteria were prepared by initial culturing in 100 ml ultra-high temperature (UHT) skim milk at 37 °C overnight. After incubation, bacteria were harvested by centrifugation at 2000 g for 30 min at 4 °C and washed twice with 1 × PBS. The pellets then were resuspended in 5 ml of 1 × PBS. The concentration of bacteria was estimated by measuring absorbance at 600 nm and adjusted to $2{\cdot}5 \times 10^8$ c.f.u./ml in skim milk.

The resistance of bacteria to bovine neutrophil bactericidal activities was evaluated by the bactericidal assay. This assay was run in 5 ml sterile polypropylene tubes (Fisher Scientific Co. Pittsburgh, PA) by incubating $2{\cdot}5 \times 10^7$ c.f.u./ml *S. aureus* with $2{\cdot}5 \times 10^6$ cells/ml neutrophil in 1 ml of skim milk, in the presence or absence of 2·5% heat-inactivated bovine antiserum for opsonization. Controls without neutrophils were run in parallel. The mixtures were incubated at 37 °C for 30 min by nutating. After incubation, 0·05 ml of the mixture was transferred to 10 ml sterile ddH_2O (pH 11·0), incubated for 10 min at room temperature to lyse neutrophils and release bacteria. The number of viable bacteria was determined by plate count method where each sample was plated in duplicate. The mean colony forming

Table 1. *Coagulase genotype profiles among different herds*

Country	Herd ID	No.	Genotypes	Counts
France	A	5	3-280,250,215	5
	B	6	2-465,290	6
	C	7	1-800	1
			2-600,85	4
			4-290,280,250,85	1
			5-280,215,165,85,80	1
Korea	A	10	2-465,290	10
	B	13	4-465,290,165,85	12
			5-215,195,165,85,80	1
	C	10	2-465,215	1
			3-465,215,80	2
			3-360,250,215	3
			3-360,215,85	1
			4-330,290,195,85	3
US	A	20	2-700,85	2
			4-390,280,165,80	17
			4-465,290,165,85	1
	B	21	3-280,215,165	1
			4-465,290,165,85	20
	C	9	4-465,290,165,85	9

unit (c.f.u.) of bacteria following exposure to neutrophils and control cultures not exposed to neutrophils were used to calculate percent neutrophil killing as follows:

$$\text{Percent killing} = \frac{\left(\begin{matrix}\text{c.f.u. of}\\ \text{control}\end{matrix}\right) - \left(\begin{matrix}\text{c.f.u. following}\\ \text{neutrophil exposure}\end{matrix}\right)}{\text{c.f.u. of control}}.$$

Analysis of data

The frequency of a genotype in a country was calculated as: $\Sigma n_{\mathrm{i}}/T_{\mathrm{i}}$, where Σn_{i} was the counts of a genotype, T_{i} was the total number of isolates from the country. Genotypes were sorted based on the frequencies. Genotypes with the highest (predominant) or lowest (rare) frequencies were selected for bactericidal assay. The percentage of bacterial killings were transformed into arcsin $\sqrt{}$ killing (arcsine the square root of killing) with the killing expressed as a proportion. The transformed data was analysed by the GLM procedures in SAS 6.12 [17] by the model

$$Y_{ijkm} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \epsilon_{ijkm},$$

where Y_{ijkm} was the percentage of bacterial killing, μ was the general mean, α_i was the fixed effect of the ith frequency type, β_j was the fixed effect of the jth opsonization status, γ_k was the random effect of the kth cow, $\alpha\beta_{ij}$ was the interaction between frequency and opsonization, $\alpha\gamma_{ik}$ was the interaction between frequency and cows, $\beta\gamma_{\mathrm{jk}}$ was the interaction between opsonization and cows, $\alpha\beta\gamma_{ijk}$ was the interaction among frequency, opsonization and cows, and ϵ_{ijkm} was random variation. The results of data analysis were re-transformed into percentage of killing and presented in the paper.

RESULTS

Coagulase gene typing

In this study, 453 *S. aureus* isolates from 187 herds in 4 countries were genotyped by coagulase gene polymorphism. These include 20 isolates from 20 dairy herds in the Czech Republic, 40 isolates from 11 dairy herds in France, 115 isolates from 20 herds in Korea and 278 isolates from 136 herds from the United States.

Forty genotypes were identified and an example of agarose gel electrophoresis is shown in Figure 1. For convenience, genotype profile of each isolate were denoted by the total number of the major bands followed by the estimated molecular weight of each band. For example, genotype 4-465,290,165,85 indicates that there were four major bands with the molecular weight of 465, 290, 165 and 85 bp, respectively.

Table 2. *Frequency of* S. aureus *genotype in different geographical locations*

	Frequency (%)			
Genotypes	Czech Rep.	France	Korea	USA
1-800	0	2·5†	0	6·1
2-360,290	5	0	0	0
2-465,215	0	0	1·7	0·4†
2-465,290	25	30·0*	20·9*	0
2-600,85	5	12·5	0	6·1
2-700,85	0	5	0	9·3
3-280,215,165	0	0	0	6·5
3-280,250,215	0	12·5	0	0
3-290,195,85	0	0	0·9	0
3-360,215,85	0	0	1·7†	0
3-360,250,215	0	2·5	7·0	0
3-360,290,85	0	0	0·9	0·4
3-360,330,215	0	2·5	0	0
3-360,290,165	0	0	0	0·4†
3-465,215,80	10	0	15·7	2·5
3-465,215,85	0	0	0	0·7†
3-465,215,165	0	0	0	0·4
3-465,290,80	0	0	0	0·4
3-465,290,85	0	0	0	0·7
3-550,215,165	0	0	1·7	0
3-550,215,80	0	0	0	0·4†
3-550,290,85	0	5·0	0	0
4-280,215,165,85	0	0	0	0·7
4-280,215,195,85	5	0	0	0
4-280,250,215,80	0	7·5	0	0
4-290,250,195,85	0	0	3·5	0·4
4-290,280,250,85	0	2·5	0	0
4-290,280,165,85	0	0	0	0·7†
4-330,290,195,85	0	0	7·0	0·7
4-360,215,165,85	0	0	8·7	0·4
4-390,280,165,80	0	0	0	11·9
4-465,250,215,85	0	0	0	0·4†
4-465,280,215,165	0	0	0	0·7
4-465,290,165,85	45	10·0	10·4	43·5*
4-700,465,330,215	0	0	0	0·4
5-215,195,165,85,80	5	2·5	20·0*	0
5-280,215,165,85,80	0	2·5†	0	0
5-290,280,165,85,80	0	0	0	0·7
5-330,215,195,85,80	0	0	0	5·8
6-360,330,290,215,165,80	0	2·5	0	0
Total (40 types)	100	100	100	100

The frequency of a genotype is determined as the count of a genotype divided by the total number of isolates from the same country.

* Isolates were selected from these genotypes (predominant) for bactericidal assay.

† Isolates were selected from these genotypes (rare) for bactericidal assay.

More than one isolate may be selected from one type.

To compare coagulase genotype profiles among different herds, nine dairy herds with multiple *S. aureus* isolates from different cows were listed in Table 1. The geographic distribution and frequency of all 40 genotypes are shown in Table 2. Of the 20 isolates from the Czech Republic, the 2 most common types accounted for 70% with the most common type 4-465,290,165,85 accounted for 45%. The remaining 5

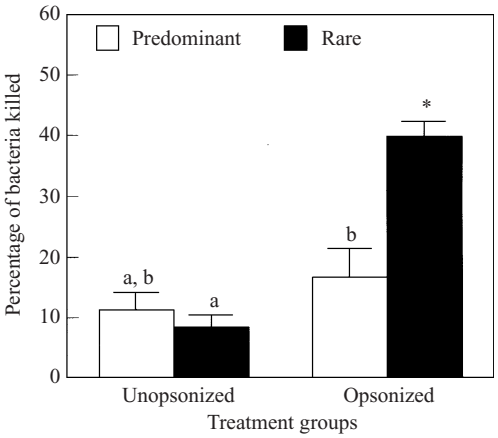

Fig. 2. Bovine blood neutrophil bactericidal activity against either opsonized or unopsonized *S. aureus*. a, b, c Bars between treatment groups with different letters differ significantly ($P \leqslant 0{\cdot}01$). * Bars within treatment groups with an asterisk differ significantly ($P \leqslant 0{\cdot}01$).

types accounted for 30% with each individual type accounting from 5 to 10%. Among the 40 French isolates, 14 different genotypes were observed. Of the 14, the 3 most common types accounted for 55% of the total genotypes. The most common genotype 2-465,290 accounted for 30%. The remaining 11 composed 45% of the total with each individual type accounting from 2·5 to 10%. Of the 115 Korean isolates, the 3 most common types 2-465,290; 5-215,195,165,85,80 and 3-465,215,80 accounted for 20·9 and 20 and 15·7%, respectively. The remaining 10 types accounted for 43·4% with each individual type accounting from 0·9 to 10·4%. From 278 USA isolates, one single genotype (4-465,290,165,85) accounted for 43·5%. The remaining 24 types accounted for 56·5% with each individual type accounting from 0·4 to 11·9%.

Bovine neutrophil bactericidal assay

The results of bactericidal assay are shown in Figure 2. When bacteria were not opsonized by antiserum, the average percentages of killing were 11·3% (standard error, S.E. = 1·7) for the 12 strains belonging to the predominant *S. aureus* types, and 8·4% (S.E. = 1·5) for the 11 strains belonging to the rare types. There was no significant difference in bactericidal effects ($P = 0{\cdot}54$). When bacteria were opsonized with 2·5% bovine heat-inactivated antiserum, the average percentages of killing were 16·7% (S.E. = 2·4) for the predominant *S. aureus* types and 39·7% (S.E. = 1·7) for the rare types. The difference was statistically significant ($P < 0{\cdot}01$) for the opsonized bacteria. Opsonization significantly increased the percentage of *S. aureus* killing by neutrophils in rare types ($P < 0{\cdot}001$). For predominant genotypes, opsonization did not enhance the percentage of killing significantly ($P = 0{\cdot}32$). There was no significant effect by cows ($P = 0{\cdot}81$). A significant effect of interaction between genotype status and opsonization of neutrophil killing was observed ($P < 0{\cdot}01$). Since the antiserum was collected from cows infected with *S. aureus* Newbould 305, which belongs to the predominant genotype in USA (genotype 4-465,290,165,85), the results of bacterial killing with opsonization may be biased. To determine this effect, we compared the data of bacterial killings between Newbould and non-Newbould predominant types. The mean percentage of bacterial killing of 6 strains of Newbould type was 9·7% (S.E. = 3·3), while the percentage of killing was 24·7% (S.E. = 3·2) for 6 strains of non-Newbould type. There was no statistically significant difference ($P = 0{\cdot}089$) between the types.

DISCUSSION

Coagulase production is an important phenotypic determinant used for the identification of *S. aureus* isolates from human and animal infections. There are conflicting data concerning the importance of coagulase as a major virulence factor. It is currently believed that coagulase may not be an important virulence factor of *S. aureus* based on site-directed allele replacement studies [18]. But in the case of gene variability, coagulase genotyping has proven to be a simple and powerful epidemiological tool to discriminate between coagulase-positive *S. aureus* strains [10, 11, 19, 20]. This study showed that there could be different genotypes among *S. aureus* isolates from different cows in a single herd, which supports previous studies revealing some genetic heterogeneity among bacterial isolates from a single herd [14, 21, 22]. However, this heterogeneity is minor and in general, only one genotype of *S. aureus* will prevail in the herd (Table 1).

Our study showed that only a few genotypes predominated in each country. Comparison of predominant types among countries indicated heterogeneity of predominant types in different geographic locations and also homogeneity of a few types. For example, genotype 2-465,290 was predominant in the Czech Republic, France and Korea, but it was not found in the USA, while genotype 4-465,290,165,85 was a common type in all four countries. Out of 187

Danish isolates [15], the most common type (35% of the isolates) designated as 5-330,215,195,85,80 in this study was found in 5·8% of US isolates, but was not found in the isolates from the Czech Republic, France and Korea. This phenomenon may be explained by the coevolution of pathogens and their host, in addition to differences in reservoirs, management, trading and the environment in each geographical area.

The coevolution of pathogens and their hosts is important to the genetic diversity of both pathogens and hosts [23, 24]. When pathogens of multiple genotypes infect a host, they compete for resources and transmission, and the genotype with increased virulence is favoured [25]. Increased virulence could be due to the nature of bacteria more resistant to killing by host phagocytic cells. The pathogens that can escape host defence mechanism will be more successful at establishing infection. This theory is supported by our study of bovine neutrophil killing of different *S. aureus* genotypes. We have shown that the predominant genotypes of *S. aureus* were the types that were more resistant to neutrophil killings, whereas the rare types were more susceptible. The differences in predominant types in different geographical locations may be explained by the coevolution of *S. aureus* with genetically diverse hosts and their geographical environment which have differences in reservoirs, management and other factors. Considering the fact that only a few *S. aureus* genotypes predominated in a defined geographical location, it may be more efficient to control *S. aureus* mastitis by targeting the important virulence factors relevant to the pathogenesis of predominant genotypes.

Comparison of the mean bacterial killings between Newbould 305 and non-Newbould predominant types implied that antiserum generated from cows infected with Newbould 305 strain has similar effects in opsonization of all types, and opsonization was not biased to Newbould 305 type. In addition, opsonization of rare types significantly increased the mean percentage of bacterial killings by neutrophils, which suggested antibodies to common antigens were produced. However, opsonization of predominant types did not significantly increase the mean percentage of killings, which indicated that the predominant types might have unique characteristics that allow them to overcome neutrophil phagocytic and bactericidal activities [15]. These unique characteristics may include a number of different factors. First, predominant types may express rigid and thicker capsules that inhibits neutrophil phagocytosis. The capsule is a polysaccharide layer coating the cell wall. It allows antibodies to cell wall and complement to penetrate, but masks recognition of antibody by neutrophil [26, 27]. The ability of inhibiting phagocytosis is related to the structure of the capsule, with the rigid capsule being more effective than the large, flaccid capsule [28]. Second, predominant types may express higher levels of virulence factors that interfere with bovine neutrophil function. Already known factors include protein A and α-toxin. Protein A is a bacterial cell wall associated protein which binds strongly to the Fc portion of bovine IgG_2 [29]. Since IgG_2 is the major isotype involved in opsonization for neutrophil phagocytosis in bovine [30, 31], sequestration of IgG_2 by protein A would impair neutrophil function. α-Toxin is a pore-forming toxin that can cause neutrophil membrane perturbation and cell death, which results in a reduction of bovine neutrophil phagocytic capacity [32]. If these virulence factors are produced in higher levels in predominant types, they would allow these bacteria to be more resistant to phagocytosis. Third, predominant types may express unknown unique virulence factors that could help them escape killing by neutrophils. In a word, predominant types must have special properties that help them overcome host defence mechanisms and establish intrammary infection successfully.

The mechanisms which allow predominant types to be more resistant to neutrophil bactericidal activities are yet to be elucidated. For future studies, we will look for the unique or enhanced expressed virulence factors produced by predominant types. It is anticipated that these studies will provide valuable insight regarding the pathogenesis of the major mastitis-causing *S. aureus* types. The identification of virulence factors that contribute to the pathogenesis of *S. aureus* mastitis could be used to generate an efficacious subunit mastitis vaccine and lead to alternative strategies to control *S. aureus* mastitis.

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