Short communication: Virulence profiles of *Staphylococcus aureus* isolated from bulk tank milk and adherences on milking equipment on Chilean dairy farms

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ABSTRACT

Staphylococcus aureus is an important intramammary pathogen for dairy cows that also is remarkably important for public health. Multiple virulence factors can be involved simultaneously during the pathogenesis of a staphylococcal disease, including adhesion proteins, extracellular enzymes, and toxins. The main objective of this study was to assess virulence factors that are associated with cow intramammary infection (IMI) and of human health concern among *Staph. aureus* isolates obtained from bulk tank milk (BTM) and adherences on milking equipment surfaces. A total of 166 *Staph. aureus* isolates from 23 dairy farms were characterized according to their virulence profiles. For virulence factors of importance in IMI, the presence of the virulence markers thermonuclease (*nuc*) and coagulase (*coa*) and virulence genes such as fibronectin (*fnbA*) and intercellular adhesion (*icaA*, *icaD*) were assessed. For virulence factors of public health concern, presence of antimicrobial resistance (*mecA* and *mecC*) and enterotoxin (*sea* and *seb*) genes were analyzed. Among all *Staph. aureus* isolates, 5 virulence profiles were found; the profile *nuc*⁽⁺⁾ *coa*⁽⁺⁾ *fnbA*⁽⁺⁾ *icaA*⁽⁺⁾ *icaD*⁽⁺⁾ *mecA*⁽⁻⁾ *mecC*⁽⁻⁾ *sea*⁽⁻⁾ *seb*⁽⁻⁾ was the most frequently observed (21 out of 23 dairy farms). No differences were found between the virulence profile frequencies of *Staph. aureus* from BTM and adherences on milking equipment surfaces. The virulence profiles most frequently observed included genes involved in the adherence and biofilm-forming ability of *Staph. aureus*, which could represent a potential advantage for the bacterium during the early stages of IMI colonization and for persistence on surfaces. Our results indicate a greater frequency of virulence factors of importance for IMI pathogenesis than virulence factors of public health concern, consistent with the dairy origin of isolates. The *mecA*, *mecC*, and *seb* genes

were not observed among *Staph. aureus* isolates analyzed in this study. However, the *sea* gene was detected in 3 *Staph. aureus* isolated from BTM, thus posing a potential public health threat. Our results emphasize the importance of understanding the epidemiology and dynamics of *Staph. aureus* on dairy farms as a tool for the improvement of udder health and milk safety.

Key words: *Staphylococcus aureus*, virulence factor, enterotoxin, biofilm, milking equipment

Short Communication

Staphylococcus aureus is an important pathogen for both human and animal health. In dairy herds, *Staph. aureus* is one of the most prevalent intramammary pathogens described around the world, including Chile (Munoz et al., 2018). Although milk from cows suffering from subclinical IMI caused by *Staph. aureus* may not show macroscopic changes, intramammary infecting bacteria can be shed in the milk and be a source of bulk tank milk (BTM) contamination. Extramammary sources of *Staph. aureus*, such as biofilms on milking equipment surfaces, can also contribute to BTM contamination (Latorre et al., 2020). Regardless of the initial source of *Staph. aureus* in milk, the presence of strains carrying genes that confer virulence traits, such as enterotoxin production or antimicrobial resistance, is a public health concern.

Virulence profiles (VP) of IMI caused by *Staph. aureus* have been previously reported (Åvall-Jääskeläinen et al., 2018). Nevertheless, few studies have assessed VP of *Staph. aureus* isolated from BTM (Kreusukon et al., 2012; Prenafeta et al., 2014) and, to our knowledge, no previous studies have analyzed the VP of *Staph. aureus* isolated from biofilms or adherences on milking equipment surfaces (AMES) in contact with milk during milking or milk cooling. The objective of this study was to assess and describe the presence of *Staph. aureus* virulence factors involved in the pathogenesis of cow IMI as well as virulence factors of concern for human health among *Staph. aureus* isolates obtained from

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BTM and AMES in Chilean dairy herds. This study represents the first report describing the VP of *Staph. aureus* isolated from in situ adherences or biofilms in milking equipment, and it is the first *mecC* assessment on *Staph. aureus* obtained from dairy operations reported in Chile.

A total of 166 *Staph. aureus* isolates collected from 23 dairy farms of the Ñuble Region in Chile were analyzed. The 23 farms corresponded to farms that were *Staph. aureus* positive (i.e., *Staph. aureus* was isolated at least once from BTM or AMES samples) among 30 farms that voluntarily participated in the study. The 30 farms corresponded to 59% of active dairy farms in the region that complied with the selection criteria for participation in the study (i.e., farms that had stationary milking equipment and a refrigerated bulk tank for cooling and storage of milk and that did not hand-milk the cows were included). Dairy operations of the study had year-round and seasonal confinement of cows and had grazing systems. The number of lactating cows on the participating farms ranged from 5 to 1,020 cows, with an average of 112 and a median of 48 milking cows per farm. A total of 17 farms (64.8%) had milking units connected to a milk line, whereas the remaining 6 farms (35.2%) had milking units connected to milk cans that were used to collect the milk before it was transferred to the bulk tank for cooling and storage. Bulk tank milk samples were collected during 3 consecutive days on each farm following the National Mastitis Council guidelines (Hogan et al., 1999). Briefly, BTM samples from a single milking were aseptically collected in 100-mL sterile vials once the milk was cooled to refrigeration temperature in the tank and after agitation for at least 10 min. For *Staph. aureus* analysis, BTM samples were individually cultured in Baird-Parker agar (Oxoid, Basingstoke, UK) as described by Jayarao et al. (2004). In addition, samples of AMES (i.e., visible films or adherences attached to milking equipment surfaces, persisting after cleaning and sanitation procedures) were collected and cultured in ChroMagar *Staph. aureus* (ChroMagar, Paris, France) as described by Latorre et al. (2020). For both BTM and AMES putative *Staph. aureus* isolates, identification was confirmed by PCR using the methods described by Riffon et al. (2001). Virulence factors assessment was conducted for all 166 *Staph. aureus* isolates from both BTM (n = 113) and AMES (n = 53). For AMES, the analyzed *Staph. aureus* included isolates obtained from adherences on rubber liners (n = 8), milk collectors (n = 4), collector valve (n = 2), short milk tube (n = 2), long milk hoses (n = 4), bulk tank outlet (inner and outer area, n = 4), agitator blades (n = 2), milk cans (n = 13), receiving jar (n = 2), receiving jar cover (n = 2), milk hoses used to divert

milk from cows with mastitis or undergoing antibiotic treatment (n = 3), and milk line sections (n = 7).

For virulence genes analysis, *Staph. aureus* isolates were cultured for 6 h in BHI at 37°C, and bacterial DNA was extracted as described by Adwan (2014). Virulence genes analyzed on *Staph. aureus* isolates were fibronectin A (*fnbA*), intercellular adhesion A (*icaA*) and D (*icaD*), enterotoxin A (*sea*) and B (*seb*), and methicillin resistance (*mecA* and *mecC*); the virulence markers coagulase (*coa*) and thermonuclease (*nuc*) were also analyzed. Analysis was conducted using the primer sequences and PCR conditions described previously (Murakami et al., 1991; Vasudevan et al., 2003; Vancraeynest et al., 2004; Cremonesi et al., 2005; Srinivasan et al., 2006; Paterson et al., 2012). Negative controls (*Staphylococcus epidermidis* ATCC 12228 and ultrapure water) and positive controls were included on each PCR run. *Staphylococcus aureus* ATCC 29213 was used as a positive control for *icaA*, *icaD*, *fnbA*, *coa*, and *nuc* genes, whereas 2 reference strains from the Chilean Institute of Public Health, Nunoa, Chile (*Staph. aureus* SA190 and SA170) were used as positive controls for detection of *sea* and *seb* genes, respectively. For methicillin resistance genes, *Staph. aureus* ATCC 43300 and *Staph. aureus mecA*_{LGA251} were used as positive controls for detection of *mecA* and *mecC*, respectively. Virulence profiles of *Staph. aureus* isolates were analyzed using descriptive statistics, and difference between the proportion of VP in *Staph. aureus* from AMES and BTM was analyzed using chi-squared or Fisher's exact analysis.

All *Staph. aureus* isolates were positive for *coa*, *nuc*, and *fnbA* genes, whereas *mecA*, *mecC*, and *seb* genes were not detected. A total of 5 VP were observed among the *Staph. aureus* isolates analyzed (Table 1). The VP *nuc*⁽⁺⁾ *coa*⁽⁺⁾ *fnbA*⁽⁺⁾ *icaA*⁽⁺⁾ *icaD*⁽⁺⁾ *mecA*⁽⁻⁾ *mecC*⁽⁻⁾ *sea*⁽⁻⁾ *seb*⁽⁻⁾ (VP1) was observed on 90 (79.6%) and 41 (77.4%) of the BTM and AMES isolates, respectively. This VP1 was observed on 21 out of the 23 dairy operations. On dairies where *Staph. aureus* was isolated from both BTM and AMES (n = 8), this VP1 was observed in all samples (Table 2). The VP *nuc*⁽⁺⁾ *coa*⁽⁺⁾ *fnbA*⁽⁺⁾ *icaA*⁽⁻⁾ *icaD*⁽⁺⁾ *mecA*⁽⁻⁾ *mecC*⁽⁻⁾ *sea*⁽⁻⁾ *seb*⁽⁻⁾ was the second VP (VP2) most frequently observed (15.9 and 20.8% of BTM and AMES *Staph. aureus* isolates, respectively). Overall, no differences were observed between the source of the isolate (BTM or AMES) and the frequency of VP of *Staph. aureus* ($P \leq 0.05$; Table 1).

Coagulase and thermonuclease virulence marker gene results are consistent with phenotypic characteristics of *Staph. aureus*, for which biochemical tests such as nuclease and coagulase test or the presence of these

Table 1. Virulence profiles (VP) of *Staphylococcus aureus* isolated from bulk tank milk (BTM) and adherences on milking equipment surfaces (AMES)

Item	VP ¹			BTM (n = 113)		AMES (n = 53)		Total (n = 166)		P-value ²
	<i>icaA</i>	<i>icaD</i>	<i>sea</i>	No.	%	No.	%	No.	%	
VP1	+	+	–	90	79.6	41	77.4	131	78.9	0.74
VP2	–	+	–	18	15.9	11	20.8	29	17.5	0.58
VP3	+	+	+	3	2.7	0	0.0	3	1.8	0.45
VP4	–	–	–	1	0.9	1	1.9	2	1.2	1
VP5	+	–	–	1	0.9	0	0.0	1	0.6	0.55

¹All isolates were *coa*⁽⁺⁾ *nuc*⁽⁺⁾ *fnbA*⁽⁺⁾ *seb*^(–) *mecA*^(–) *mecC*^(–).

²Differences between BTM and AMES were calculated using chi-squared or Fisher's exact test ($P < 0.05$).

genes on putative isolates are commonly used for species identification (Cremonesi et al., 2005; Grispoli et al., 2019). The *fnbA* gene was highly conserved among *Staph. aureus* isolated from BTM and AMES samples in our study (100%). This prevalence of *fnbA* is rather high and contrasts with previous reports. For example, frequencies of *fnbA* on *Staph. aureus* among different samples include 43% on bovine quarter milk with IMI, 28% on teat skin, 21.4% on BTM, and 0.8% on humans (Haveri et al., 2008; da Costa et al., 2014; Khoramian et al., 2015). High fibronectin frequency in our study may

be explained by a high incidence of participating farms isolating *Staph. aureus* in 2 or more consecutive BTM samples, an indication of underlying IMI in the herds (Jayarao et al., 2004). Fibronectin A has been described as an important protein involved in *Staph. aureus* adhesion to host fibronectin, elastin, and fibrinogen (Kerro Dego et al., 2002). Moreover, internalization of *Staph. aureus* in bovine and human epithelial cells has been described to be assisted by fibronectin A adhesion (Jett and Gilmore, 2002; Pereyra et al., 2016). Furthermore, the presence of *fnbA*-positive *Staph. aureus* on AMES in

Table 2. Virulence profiles (VP) of *Staphylococcus aureus* isolated from bulk tank milk (BTM) and adherences on milking equipment surfaces (AMES) on Chilean dairy farms

Dairy farm	VP ¹	VP frequency (%)	Source of <i>Staph. aureus</i> ²		
			AME CTE	AMES CM	BTM
A	VP1, VP2, VP4	37.5, 50, 12.5	ND	+, +, +	+, +, –
B	VP1	100	ND	+	+
C	VP1	100	ND	ND	+
D	VP1	100	ND	ND	+
E	VP1, VP2	83.3, 16.7	ND	ND	+
F	VP1	100	ND	ND	+
G	VP1, VP2, VP4	33.3, 50, 16.6	ND	ND	+, +, +
H	VP1, VP2	60, 40	ND	+, +	+, +
I	VP2, VP3	25, 75	ND	ND	+, +
J	VP1, VP2	91.7, 8.3	+, –	+, +	+, +
K	VP1	100	ND	ND	+
L	VP2	100	ND	ND	+
M	VP1, VP2	38.4, 61.5	ND	+, +	+, +
N	VP1	100	ND	ND	+
O	VP1	100	ND	ND	+
P	VP1	100	ND	+	+
Q	VP1, VP2	20.0, 80.0	ND	ND	+, +
R	VP1	100	ND	ND	+
S	VP1	100	ND	ND	+
T	VP1	100	ND	ND	+
U	VP1, VP5	85.7, 14.3	ND	+, –	+, +
V	VP1	100	ND	ND	+
W	VP1	100	ND	+	+

¹All isolates were *coa*⁽⁺⁾ *nuc*⁽⁺⁾ *fnbA*⁽⁺⁾ *seb*^(–) *mecA*^(–) *mecC*^(–). VP1 = *icaA*⁽⁺⁾ *icaD*⁽⁺⁾ *sea*^(–); VP2 = *icaA*^(–) *icaD*⁽⁺⁾ *sea*^(–); VP3 = *icaA*⁽⁺⁾ *icaD*⁽⁺⁾ *sea*⁽⁺⁾; VP4 = *icaA*^(–) *icaD*^(–) *sea*^(–); VP5 = *icaA*⁽⁺⁾ *icaD*^(–) *sea*^(–).

²Source of isolation of *Staph. aureus*. AMES CTE = milking equipment adherences or biofilms on surfaces in contact with teat ends (rubber liners). AMES CM = milking equipment adherences or biofilms on surfaces in contact with milk (milk collectors, collector valve, short milk tube, long milk hoses, bulk tank outlet, agitator blades, milk cans, receiving jar, receiving jar cover, milk hoses used to divert milk from cows with mastitis or undergoing antibiotic treatment, and milk line sections). ND = not detected.

pieces of milking equipment in close contact with teat ends could pose an increased risk for IMI due to a putative improved advantage of *fnbA*-positive *Staph. aureus* during the attachment phase of IMI pathogenesis.

Most studies assessing *icaA* and *icaD* are focused on *Staph. aureus* isolated from milk samples from clinical or subclinical mastitis, with frequencies ranging from 87 to 100% (Vasudevan et al., 2003; Castelani et al., 2015; Khoramian et al., 2015; Pereyra et al., 2016). However, to our knowledge, no previous reports on *icaA* and *icaD* are available for *Staph. aureus* from AMES, and only a few have been described for BTM isolates (Kreausukon et al., 2012; Prenafeta et al., 2014). Even though the frequency of both genes (78.9%) among our analyzed isolates is lower than that reported in other studies (Vasudevan et al., 2003; Castelani et al., 2015; Khoramian et al., 2015; Pereyra et al., 2016), the presence of these genes could represent an important mechanism for adhesion to biotic or abiotic surfaces, such as milking equipment surfaces. It is noteworthy that about one-third of our isolates were obtained from adherences or biofilms in milking equipment, which could also be responsible for BTM contamination due to *Staph. aureus* sloughed from these biofilms (Latorre et al., 2020). Consequently, the findings in our study may be skewed toward the presence of *Staph. aureus* with increased capabilities for adherence in both milking equipment and BTM. After surface adhesion, the polysaccharide intercellular adhesin, which is codified by the intercellular adhesion operon *icaABCD*, can be secreted around *Staph. aureus* (de Souza da Cunha, 2014). Accumulated polysaccharide intercellular adhesion plays a key role in the development of biofilms, which not only contributes to the persistence of pathogens on dairy farms (Latorre et al., 2011) but also confers the bacteria physical protection against the immune system, antimicrobials, and disinfectants that are commonly used in dairy operations. In our study, confirmation of AMES as biofilms (i.e., visualization of both bacteria attached to a surface and the presence of an exopolymeric matrix) was done only in the pieces of milking equipment that could be removed to conduct microscopy analysis (Latorre and Munoz, 2015; Latorre et al., 2020). However, the macroscopic physical properties of AMES in the pieces that were not microscopically assessed were consistent with those in the pieces in which the presence of biofilms was confirmed. Therefore, our results highlight the importance of investigating new sources of *Staph. aureus* such as adherences or biofilms in milking equipment, which may play a role in animal and human infections due to the close contact of parts and pieces of the equipment with teat ends, milker hands, and milk.

In our study, *Staph. aureus* virulence factors of public health concern (i.e., antimicrobial resistance and en-

terotoxin production genes) were not detected or were observed with low frequency. Only 3 BTM *Staph. aureus* isolates (1.8%), which were obtained from a single dairy operation, were positive for the enterotoxin A gene. Staphylococcal enterotoxins are proteins that can cause enteric diseases and emetic symptoms in people who consume contaminated food with sufficient quantities of staphylococcal enterotoxins, including dairy products (Johler et al., 2018; Necidová et al., 2019). Even though raw milk consumption is not recommended due to the high risk of the presence of foodborne pathogens (Oliver et al., 2005), it is a rather common practice in the rural population of Chile, particularly on dairy farms (Latorre and Munoz, 2013). It is noteworthy that the 3 *sea*-positive *Staph. aureus* isolates were recovered from 2 out of 3 BTM samples from a single farm. This finding may represent a serious risk for human health as raw milk consumption has been reported by farm workers on this farm (A. A. Latorre and M. A. Munoz, unpublished data). Even if milk is pasteurized, staphylococcal enterotoxin A is a heat-stable protein that remains detectable after treatment of milk over 92°C (Necidová et al., 2019). Therefore, staphylococcal enterotoxin surveillance is crucial to improve dairy foods safety and protect consumer health (Miao et al., 2017).

Antimicrobial resistance is of great concern for both human and animal health. To our knowledge, this study is the first report of *mecA* and *mecC* assessment on *Staph. aureus* isolated from milk and milking equipment on Chilean dairies. Our results are consistent with other studies conducted in foods in Chile (specifically, in the pork meat supply), in which the absence of both *mecC* and *mecA* genes among *Staph. aureus* isolates has also been described (Velasco et al., 2018). However, methicillin resistance genes *mecA* and *mecC* have been previously detected in human and veterinary *Staph. aureus* isolates in other studies conducted in European countries and Australia (Worthing et al., 2016; Lakshundi and Zhang, 2018).

Although the present study shows the findings obtained in a limited number of farms from a single region of Chile, the diversity of farms of this study (i.e., in terms of the number of milking cows, farm facilities and management, and milk quality parameters) could be found in many dairy operations in Chile as well as on farms from different parts of the world. Moreover, although only a selected proportion of the virulence factors described for *Staph. aureus* were analyzed in our study, the inclusion of genes involved in adherence and biofilm formation from *Staph. aureus* isolated from in situ adherences or biofilm contributes to the current knowledge about *Staph. aureus* on-farm epidemiology. Several questions remain to be addressed, such as the role of the infected mammary gland as one of the po-

tential sources of *Staph. aureus* for both AMES and BTM. Nevertheless, genetic relation among *Staph. aureus* from BTM, AMES, and IMI has recently been well documented in one of the 23 dairy farms of our study (Latorre et al., 2020).

Addressing the emerging importance to assess methicillin-resistant *Staph. aureus* sources in animal and food production, the present study is the first to evaluate *mecA* and *mecC* in *Staph. aureus* isolates from Chilean dairy operations. Virulence profiles of *Staph. aureus* isolated from adherences or biofilms on surfaces in contact with milk and BTM were not different despite being obtained from different farm sources. Reservoirs in dairy operations such as biofilms or adherences on surfaces in contact with milk can be a source of *Staph. aureus* that are capable of invading and infecting the mammary gland.

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