Journal of Applied Genetics
https://doi.org/10.1007/s13353-018-0443-8

MICROBIAL GENETICS • ORIGINAL PAPER

Survey of potential factors involved in the low frequency of CP5 and CP8 expression in *Staphylococcus aureus* isolates from mastitis of dairy cattle from Argentina, Chile, and Uruguay

Maria Belen Ambroggio[1] · Melina Soledad Perrig[1] · Cecilia Camussone[2,3] · Nazarena Pujato[1] · Alicia Bertón[4] · Edgardo Gianneechini[5] · Silvia Alvarez[6] · Ivan Sergio Marcipar[1,3] · Luis Fernando Calvinho[2,7] · Maria Sol Barbagelata[1]

Received: 9 November 2017 / Revised: 20 February 2018 / Accepted: 10 April 2018

Abstract

Staphylococcus aureus produces capsular polysaccharides (CPs) both in vivo and under defined culture conditions being serotypes 5 and 8 the most prevalent. *S. aureus* isolates that fail to produce CP5 or CP8 are defined as non-typeable (NT). Loss of capsule expression, however, may lead to *S. aureus* persistence in a chronically infected host. The prevalence of NT strains of *S. aureus* isolated from bovine mastitis varies according to the geographic origin of the strain. The aims of this work were to detect phenotypically and genotypically the capsular profile of 144 *S. aureus* isolated from bovine mastitis in Argentina, Chile, and Uruguay and explore the factors that are considered to be associated with capsule expression as presence of IS*257*, IS*cap*, and *agr* typing of non-related collection. The detection of the IS*257*, IS*cap*, *cap* genes, and *agr* typing was performed using PCR. The detection and quantification of capsular polysaccharide production were performed by ELISA assays. We found that 96% of the *S. aureus* isolates investigated carried *cap5(8)* genes but over 75% of strains do not express capsule in the three countries studied. However, only 6 isolates from Argentina carried the IS*cap* element that totally suppressed the expression of the capsule, suggesting that other factors could influence on CP expression. Moreover, the *agr*I/NT association was statistically significant suggesting that this profile is a phenomenon observed not only in other parts of the world but also in our region.

Keywords *Staphylococcus aureus* · Capsule expression · IS*cap* · *agr* typing · Bovine mastitis

Communicated by: Agnieszka Szalewska-Palasz

✉ Maria Sol Barbagelata
msolbar@yahoo.com.ar

[1] Laboratorio de Tecnología Inmunológica, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, RN168, 3000 Santa Fe, Argentina

[2] Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria (INTA), Rafaela, Santa Fe, Argentina

[3] Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Buenos Aires, Argentina

[4] Colonia Cosmopolita, Departamento de Colonia, Lactodiagnóstico Sur Uruguay, Colonia del Sacramento, Uruguay

[5] Laboratory Veterinary Direction "Miguel C. Rubino", Paysandú, Uruguay

[6] Udder Health, Osorno, X Región, Chile

[7] Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina

Introduction

Staphylococcus aureus produces capsular polysaccharides (CPs) both in vivo and under defined culture conditions (Lee et al. 1993; Stringfellow et al. 1991); serotypes 5 and 8 are the most prevalent (Guidry et al. 1997; Sordelli et al. 2000; Sutra and Poutrel 1994; Tollersrud et al. 2000). *S. aureus* isolates that fail to produce CP5 or CP8 are defined as non-typeable (NT). CPs have been shown to confer resistance to phagocytosis by polymorphonuclear neutrophils (PMN), which are considered the main mammary gland line of defense against invading pathogens (Guidry et al. 1991; Xu et al. 1992). *Staphylococcus aureus* CPs have been classically considered as one of the main virulence factors of this bacterium (Karakawa and Vann 1982; Lee and Lee 2006; O'Riordan and Lee 2004). This paradigm is widely accepted as antibodies against CPs have a protective effect by opsonizing encapsulated *S. aureus* and promoting phagocytic killing by PMN (Cunnion et al. 2003; Guidry et al. 1991). In this line of

Published online: 03 May 2018

reasoning, CPs have been considered as vaccine components against *S. aureus* infections (Boyle-Vavra et al. 2015; Cunnion et al. 2003; Cheng et al. 2017; Tollersrud et al. 2001). Contrarily, it has been described that CP absence increases the capacity for persistence in the host (Pohlmann-Dietze et al. 2000). This phenomenon has been explained by an effective interaction between unmasked staphylococcal surface ligands and cell receptors leading to the internalization into epithelial cells. In accord, *S. aureus* bacteria that do not express capsule induce chronic mastitis in mice, suggesting that the absence of capsule synthesis may help the bacteria to persist within the mammary gland promoting *S. aureus* chronic infections (Buzzola et al. 2007; Tuchscherr et al. 2005).

The prevalence of NT strains of *S. aureus* isolated from bovine mastitis varies according to the geographic origin of the strain. It was previously shown that 86% (Sordelli et al. 2000) and 67.5% (Camussone et al. 2012) of *S. aureus* isolates from clinical and subclinical mastitis in Argentina did not produce serotype CP5 or CP8. The lack of *S. aureus* CP expression could be due to the lack of *cap5(8)* locus or to point mutations within the capsule-encoding essential genes, mutations in the *cap5(8)* promoter regions, or suppression of the *cap5(8)*locus (Cocchiaro et al. 2006). Also, there are strains that present mutations in genes that regulate capsule expression, such as *agr* or *arlRS* (Cocchiaro et al. 2006). Tuchscherr et al. (2007) demonstrated that 13 of 21 epidemiologically unrelated NT bovine isolates from Argentina failed to produce capsular polysaccharides because *cap5(8)* locus have a deletion mediated by IS*cap*. IS*cap* is an insertion sequence (IS) element with 93% identity to IS*257* (Cocchiaro et al. 2006). The widespread prevalence of IS*257* suggests that it has played a fundamental role in the emergence of staphylococcal resistance (Chandler and Mahillon 2002; Firth and Skurray 2006). The IS*257* like all ISs has been shown to contribute to genetic flexibility, thus increasing the range of genetic rearrangements. The recombination and the horizontal transfer of the IS elements have played an important role in the evolution of bacterial species (Chandler and Mahillon 2002; Lawrence et al. 1992) resulting in dynamic genomes, in which the DNA is introduced and removed from the chromosome (Ochman et al. 2000). The loss of *cap5(8)* genes from *S. aureus* isolated from bovine intramammary infections (IMI) provides an example of such genetic changes, where the IS*257* element is inserted in the middle of the *cap* genes by interrupting the coding sequence and inactivating gene expression (Tuchscherr et al. 2007).

The significance of *S. aureus* NT strains in bovine mastitis pathogenesis is not fully understood. A previous study in Argentina has shown that strains belonging to *agr* group I are more likely to be internalized in the epithelial cells and to persist in murine mammary glands than the strains belonging to other groups (Buzzola et al. 2007). The authors also found that *S. aureus* strains from the *agr*I group were mostly NT. Similar results were described by Bardiau et al. (2014) with Belgian *S. aureus* strains from bovine mastitis hypothesizing that this group of isolates (*agr*I/NT) may be better adapted to intracellular niche leading to chronic infection.

In a previous study performed with isolates from Argentina, we described that 33% of *S. aureus* from clinical or subclinical mastitis did not carry the capsule genes while the remaining 67% were genotyped as *cap5* or *cap8* but only 50% of them expressed CP5 or CP8 (Camussone et al. 2012). Interestingly, a study performed in 159 *S. aureus* bovine mastitis isolates from Brazil showed that all the isolates harbored the *cap* gene and that 69% of the isolates expressed CP5 or CP8 (Salimena et al. 2016). These results suggest that the deletion of the locus *cap* by the IS*cap* element is due to a local and non-regional phenomenon.

Since there are few regional studies on the prevalence of genes and capsular expression, except for those carried out in Argentina and Brazil (Buzzola et al. 2007; Camussone et al. 2012; Salimena et al. 2016; Sordelli et al. 2000; Verdier et al. 2007), one of the aims of the present work was to detect phenotypically and genotypically the capsular profile of *S. aureus* isolated from bovine IMI in Argentina, Chile, and Uruguay. The *cap*5 and *cap8* genes were not detected among the isolates from Argentina previously by Camussone et al. (2012) and were defined as capsular genotype (NT). In addition, the relevance of factors that are considered to be associated with capsule expression as presence of IS*257*, IS*cap*, and *agr* typing of non-related collection was investigated.

Materials and methods

Bacterial isolates

A total of 51 bovine *S. aureus* isolates obtained from mammary secretions of cows with clinical or subclinical IMI from Argentina, 55 from Chile, and 38 from Uruguay, including a maximum number of three isolates from the same dairy herd, were included in the study. Isolates from Argentina were obtained between 2004 and 2007, while those from Uruguay and Chile, between 2011 and 2012. Isolates from Chile were from Región de Los Lagos, de Los Ríos, and de la Araucanía, while isolates from Uruguay were from the West Littoral and Traditional dairy areas. The isolates from Argentina were defined as NT in a previous study (Camussone et al. 2012).

Clinical mastitis was defined as presence of clinical signs in the mammary quarter (swelling, heat, and pain) and/or changes in the appearance of milk, while subclinical mastitis was defined as absence of clinical signs but somatic cell counts > 200,000 cells/ml.

Isolation and identification of *S. aureus* were performed according to standard microbiological procedures (Lee et al. 1993). All bacteria were stored in trypticase soy broth

(Britania, Argentina) medium with 20% glycerol at −20 °C until use. Species identification was confirmed by polymerase chain reaction (PCR) amplification of *S. aureus*-specific sequences according to Martineau et al.(1998).

Prototype *S. aureus* strains included *S. aureus* Reynolds and Becker (O'Riordan and Lee 2004) which produce CP5 and CP8, respectively, and do not carry IS*257* or IS*cap*. Strains MBC204 and 214 carry IS*cap* in place of the *cap5(8)* locus (Cocchiaro et al. 2006).

DNA manipulations

Genomic DNA was extracted and purified from *S. aureus* strains using a standard procedure (Pitcher et al. 1989). The presence of the IS*257* element was detected by amplification of specific sequences by PCR using primers IS*257*-Fw/IS*257*-Rv and IS*cap* with AdhE-Fw/IS*257*-Rv (Table 1). The *cap5* gene was detected with primers Cap5-k1/Cap5-k2 (Verdier et al. 2007), and the *cap8*-specific gene was amplified with Cap8h-k1/Cap8h-k2 (Table 1). Isolates that neither yielded amplified *cap5* nor *cap8* fragments were defined as NT. The detection of *cap5* and *cap8* genes and IS*257* element was evaluated in all the isolates but the location of IS*257* element was assessed in negative *cap* and positive IS*257* strains. To detect IS*cap* element, PCR was performed using AdhE-Fw and IS*257*-Rv primers (Table 1). These oligonucleotides anneal to *cap5(8)* locus flanking genes. The IS*cap* fragments obtained by PCR were sequenced. The BLAST software package was used to determine sequence homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/). The *agr* groups were determined by a multiplex PCR described previously by Gilot et al. (2002). This PCR allows the amplification of a 441-bp DNA fragment of the *agr* group 1 strains, of a 575-bp DNA fragment of the *agr* group 2 strains, of a 323-bp DNA fragment of the *agr* group 3 strains, and of a 659-bp DNA fragment of the *agr* group 4 strains.

Capsule purification and serotyping

For capsule purification, prototype *S. aureus* strains Reynolds (CP5) and Becker (CP8), MBC204 and MBC214 (IS*cap*$^{+}$), and all bovine *S. aureus* isolates were grown on Columbia agar (Britania, Argentina) supplemented with 2.5% NaCl, harvested, and inactivated as previously described by Karakawa et al. (1985). CP typing was performed to all *S. aureus* isolates by an enzyme-linked immunosorbent assay (ELISA) with CP5- or CP8-specific antibodies as described previously (Camussone et al. 2012). The cut-off point between CP-positive and CP-negative isolates was calculated with the average of the OD of prototype strains MBC204 and MBC214. The reactivity of the bovine isolates was evaluated by comparison with control *S. aureus* strains. Each bovine isolate was tested in duplicate. Isolates yielding no reactions to CP5 and CP8 antibodies were defined as NT.

Pulsed-field gel electrophoresis typing

For determining the clonality of *S. aureus cap5(8)*$^{-}$IS*cap*$^{+}$ isolates, pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested (Promega) chromosomal DNA fragments was assessed (Tenover et al. 1995) using a CHEF-DR II apparatus (BioRad Laboratories, CA, USA) as previously described (Quelle et al. 2003). Selected strains which carried *cap5(8)* genes from different geographic regions were included. The similarity between PFGE types was evaluated by the Dice coefficient. The resultant similarity matrix was analyzed by the unweighted pair group method using arithmetic averages (UPGMA), and data were analyzed with the TREECON software for Windows (Van de Peer and De Wachter 1994).

Statistical analysis

Non-parametric data was analyzed with the chi-squared and Fisher tests using the GraphPad Prism version 4.00 Software for Windows. p values lower than 0.05 were considered statistically significant.

Results and discussion

Distribution of *cap5(8)* genes among *S. aureus* bovine isolates

We assessed the presence of locus *cap5(8)* in the genomic DNA of each bovine *S. aureus* isolate using specific primers (Table 1). From 55 *S. aureus* Chilean isolates, 37 (67.3%) carried *cap5* gene and 18 (32.7%) *cap8* gene. From 38 Uruguayan isolates, 17 (44.7%) and 21 (55.3%) isolates harbored *cap5* and *cap8*, respectively. In Argentina, a high prevalence of NT isolates that did not carry *cap5(8)* genes was reported (Cocchiaro et al. 2006). Unexpectedly, in our study from 51 isolates, only 6 (11.8%) were NT, whereas the remaining 45 (88.2%) were genotyped as *cap8*. It should be taken into account that these 51 isolates were defined as NT within a group of 150 isolates previously studied in Argentina (Camussone et al. 2012; Sordelli et al. 2000), where primers designed by Verdier et al. (2007) were used for CP genotyping. In this work, new primers were designed to re-assess *cap8* gene (Table 1) since primers designed in previous studies (Bardiau et al. 2014; Tuchscherr et al. 2007) were found to produce very low amount of amplicons, making *cap8* gene detection difficult even in the positive control. High percentage of *cap8*-positive strains found in the present study confirmed the low sensitivity of previous primers to detect *cap8* gene. It is noteworthy that all the isolates and controls of *cap5*

Table 1 Primers used in this study

Primer	Sequence (5′-3′)	Ref.
Cap5-k1	5′-GTCAAAGATTATGTGATGCTACTGAG-3′	Verdier et al. 2007
Cap5-k2	5′-ACTTCGAATATAAACTTGAATCAATGTTATACAG-3′	
Cap8h-k1	5′-TGTGGGATTTTTGTAGCTTT-3′	This work
Cap8h-k2	5′-CGGGTGACTAAAAATACTCG-3′	
IS*257*-Fw	5′-TGTCATTACTGTAGCCGTTG-3′	This work
IS*257*-Rv	5′-TTTTGCCGTATTGAGACTTT-3′	
AdhE-Fw	5′-CTTTGGCTGGTATGGCATTT-3′	This work

and *8* were analyzed with both pairs of primers. Those isolates and controls that were positive for *cap5* were negative for *cap8* and vice versa.

Capsule expression among *S. aureus* bovine isolates

The assessment of CP5 and CP8 polysaccharides expression by ELISA analysis was performed for all the isolates. Thirteen (24%) Chilean *S. aureus* isolates expressed CP (12 CP5 and 1 CP8) and 10 (26%) isolates from Uruguay expressed CP (8 CP5 and 2 CP8). Forty-two (76%) Chilean and 28 (74%) Uruguayan isolates were defined as NT. Also, among Argentine isolates, a predominant proportion of NT (84%) was detected. Several studies have shown great variability on the prevalence of CP according to the geographical region (Guidry et al. 1997; Han et al. 2000; Hata et al. 2006; Khichar and Kataria 2014.; Poutrel et al. 1988; Salimena et al. 2016; Verdier et al. 2007). In South America, information about prevalence of CP serotypes from *S. aureus* isolated from bovine IMI is limited (Camussone et al. 2012; Salimena et al. 2016; Sordelli et al. 2000). Previous studies in Argentina indicated a low prevalence of isolates capable of expressing capsule (Camussone et al. 2012; Sordelli et al. 2000). Regarding Chile and Uruguay, this is the first report about distribution and prevalence of CP serotypes of *S. aureus* from bovine IMI. Similar to findings in Argentina, a low prevalence of capsulated isolates was detected both in Chile and Uruguay with the predominance of CP5 *S. aureus* isolates.

All together, these results show that both *cap8*-positive strains and *cap5*-positive strains are mostly NT as well as Argentina, Chile, and Uruguay suggesting that *cap5*- and *cap8*-positive isolates tend to be acapsulated (Table 2; $p = 0.001$ chi-squared test). Many factors have been described to be involved in the low proportion between capsule phenotypic expression and genotypic detection. These factors may be due to (i) differences in the growth conditions in vivo versus in vitro culture or (ii) the presence of mutations in essential genes encoding capsule, in promoter regions or capsule regulatory genes, causing a reduction in the capsular expression (Boyle-Vavra et al. 2015; Cocchiaro et al. 2006).

Presence of IS element in bovine *S. aureus* isolates

Considering the high prevalence of *cap5(8)* genotypes, we explored the potential mechanisms that might mediate the low capsule expression observed in the isolates from the three studied countries.

A previous study indicated that the insertion element IS*257* into the *cap* locus (IS*cap*) would be responsible for the phenomenon of decreased capsular expression observed in Argentina (Tuchscherr et al. 2007). IS*257* has been shown to contribute to staphylococcal genetic flexibility, enhancing the rate of genetics rearrangements (Simpson et al. 2000). Loss of cluster *cap* in bovine *S. aureus* provides an example of such genomic changes (Tuchscherr et al. 2007). To determine the prevalence of IS element (IS*257* or IS*cap*), PCR was performed on genomic DNA from each *S. aureus* isolate. Inverted repeats of IS*257* were not included in primer design (Table 1). IS*257* element was detected in 33 (60%) of *S. aureus* isolates from Chile and in the 92% of isolates from Argentina and Uruguay. Among IS257^{+} isolates from Argentina, Chile, and Uruguay, only 6 (13%), 10 (30%), and 9 (25.7%) expressed CP, respectively.

Only 6 isolates from Argentina that carried the IS*257* element did not amplify *cap*5 or *cap8* genes (Table 2) while all the IS257^{+} isolates from Chile and Uruguay amplified *cap5* or *cap8* genes. To confirm whether the absence of *cap5(8)* (*cap5(8)*$^{-}$) locus was a result of an IS*cap*-mediated deletion, specific PCR was performed. PCR reactions with genomic DNA from these 6 NT IS257^{+} strains yielded a 1.2-kb fragment similar to that obtained from prototypic strains MBC204 and MBC214, which have IS*cap* replacing the *cap5(8)* locus (Cocchiaro et al. 2006). The fragments were sequenced and nucleotide analysis revealed 99% identity with IS*cap* (Gen Bank: EF177828.1). These findings suggest that IS element in these 6 NT, *cap5(8)*$^{-}$, IS257^{+} strains was placed at a site consistent with deletion of the *cap5(8)* genes.

We found a high prevalence of IS*257* in isolates from the three analyzed countries. However, only 6 (12%) isolates from Argentina showed the IS*cap* element (*cap5(8)*$^{-}$ IS257^{+}). Although the prevalence of IS*cap* obtained in this study was significantly lower than that of the previously reported (62%) (Tuchscherr et al. 2007), our results support the hypothesis

Table 2 Capsular profile (genotype/phenotype) and *agr* group frequency distribution

		Argentina	Chile	Uruguay	*agr* groups				
					I	II	III	IV	Others*
Capsular genotype/phenotype	*cap5*/NT	0	*25*	*9*	*28*	0	1	0	5
	cap5/CP5	0	12	8	14	0	1	0	5
	cap8/NT	*37*	*17*	*19*	*57*	6	8	1	1
	cap8/CP8	8	1	2	4	2	3	1	1
	NT/NT	*6*	*0*	*0*	*6*	0	0	0	0
Total		*51*	*55*	*38*	*109*	8	13	2	12

Capsular profile (genotype/phenotype) and agr group frequency distribution. The differential numbers are highlighted in italic

*Others: include null *agr* or double bands

that the deletion of the *cap5(8)* locus is mediated by IS*cap* in *S. aureus* bovine mastitis isolates. However, other factors are also involved and could be more relevant than IS*cap* insertion.

Clonal relationships among IS*cap*$^+$ *S. aureus* isolates

*Sma*I digestion of the 6 NT (*cap5(8)*$^-$IS*cap*$^+$) *S. aureus* isolates, 9 selected isolates (based on its porting *cap5(8)* genes and its geographic region), and prototypic strains MBC204 and MBC214 (Fig. 1) was performed. A dendrogram was constructed on the basis of the levels of similarity, and a cut-off point of 80% was considered to define the groups. PFGE band analysis revealed the presence of seven pulsotypes (named A, B, C, D, E, F, and G). The pulsotype G comprised the highest number of isolates ($n = 10$). All NT *S. aureus* and MBC204 control strain were included in this group. Nine isolates of this pulsotype were from Argentina and one from Chile. The pulsotype C included 2 isolates (one from Argentina and one from Chile), and the rest were distributed into individual groups.

As mentioned previously, all the NT *S. aureus* detected in this work were grouped in the G pulsotype. In addition, 3 *cap5*-positive isolates were included within this group. Therefore, the 6 NT strains could be considered genetically related to the *cap5*$^+$ strains and could have a common ancestor as described by Tuchscherr et al. (2007).

Relation between the *agr* group and CP type

To determine the *agr* group of isolates from the three countries, a multiplex PCR was performed. Most strains isolated belonged to *agr* group I (76%). Nine percent of the strains were identified as being in *agr* group III, whereas the remainder were in *agr* groups II and IV (6 and 1%, respectively) and 12 (8%) were untypeable. *S. aureus* strains of *agr* group I were mostly NT (83%) including the six NT IS*cap*$^+$ and the rest were either CP5 (13%) or CP8 (4%) (Table 2). The *agr* strains of groups II and IV were either CP8 or NT and the strains from *agr* group III were CP5, CP8, or NT. When the distribution of bovine mastitis isolates from Argentina, Chile, and Uruguay

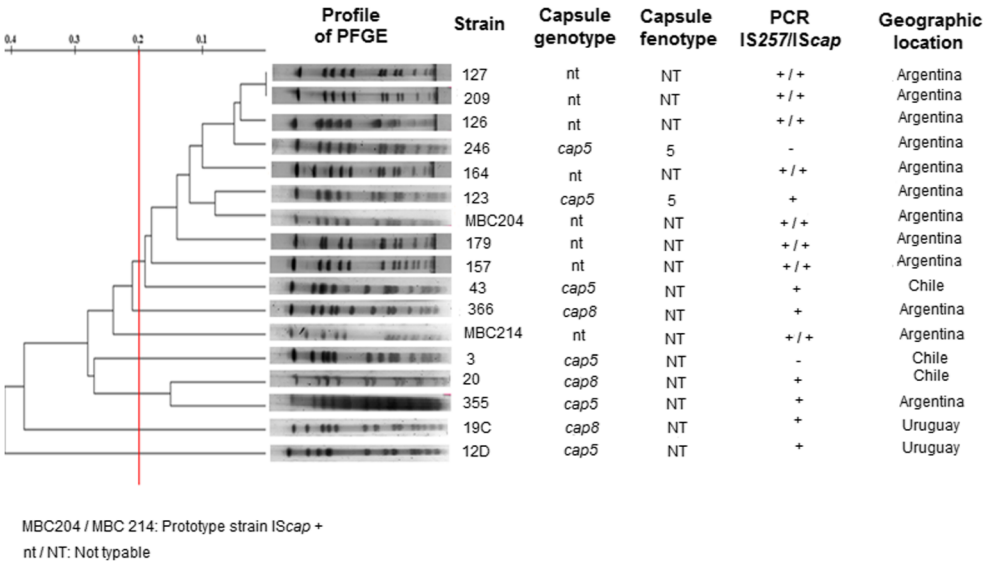

Fig. 1 PFGE patterns, genotype and phenotype of capsules, and the presence of IS*257*/IS*cap* in 15 *S. aureus* isolates from mammary secretions of cows with clinical or subclinical mastitis from Argentina, Chile, and Uruguay. PFGE discriminated seven pulsotypes and the NT isolates were found within the same clonal subtype, G_1

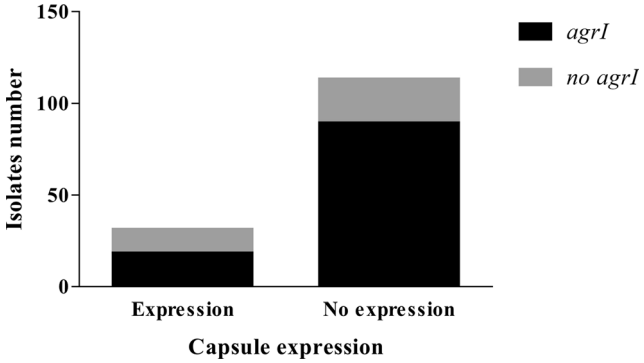

Fig. 2 The expression or no expression of the capsule in *S. aureus* isolates belonging to the *agr*I group and other *agr* groups. Isolates *agr*I tend to not express capsule statistically significantly ($p = 0.0098$; chi-squared test)

that did not express capsule was analyzed, it was found that these isolates carried *agr*I more frequently than the other groups of *agr* ($p = 0.0098$; chi-squared test) (Table 2; Fig. 2). The acapsulate phenotype contributes to the chronicity and survival of the bacteria since these organisms tend to internalize in mammary epithelial cells avoiding thereby host immune response (Buzzola et al. 2007). Buzzola et al. (2007) observed that isolates of *agr* groups II, III, and IV were internalized less efficiently, suggesting that these isolates may be more susceptible to attack by the host immune response because they tend to remain in larger amounts in the extracellular environment. These authors speculated that the high capacity of *S. aureus agr* group I to invade epithelial mammalian cells might be due to specifically suppressed action of the *agr* locus. Taking in mind that *S. aureus* lineage in bovines from Argentina, Chile, and Uruguay is NT and belongs to *agr* group I, the accessory gene regulator (*agr*) of *S. aureus* type I could influence the non-expression of the capsule. Bardiau et al. (2016) showed that more *agr* group I strains are acapsulated and had a higher invasion rate than *agr* group II strains in mammalian epithelial cells. However, as we did not assess the functionality of the *agr* system of all strains, we cannot determine whether the absence of CP expression is directly linked to regulation by the *agr* locus, or whether other factors, such as those related to the culture medium used, could play a role.

Finally, this is the first work that described the prevalence of capsular genes and their expression in Chile and Uruguay. Over 75% of strains from the three countries studied do not express capsule. However, only 6 isolates from Argentina carried the IS*cap* element that totally suppressed the expression of the capsule, suggesting that other factors could be influencing on CP expression. Moreover, the *agr*I/NT association was statistically significant suggesting that this profile is a phenomenon observed not only in other parts of the world but also in our region.

Funding information M.S.P. was a fellow from CONICET, and I.S.M. and C. C. are independent and assistant members of the research career of CONICET. This work was supported by grants from FONCyT (M.S.B., PICT Bicentenario 2010-1309), (L.F.C. and I.S.M., PICT 1175) and from Fundación Banco de la Provincia de Santa Fe (M.S.B.)

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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