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GENOTYPIC AND PHENOTYPIC DETECTION OF CAPSULAR POLYSACCHARIDES IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE INTRAMAMMARY INFECTIONS IN ARGENTINA

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ABSTRACT

Staphylococcus aureus (n=157) isolated from intramammary infections in Argentine dairy areas were evaluated for presence of *cap*5 and *cap*8 loci. Isolates carrying *cap*5 and *cap*8 were serotyped using specific antisera. Sixty four percent of the isolates were genotyped as *cap*5 or *cap*8 and 50% of them expressed CP5 or 8.

Key words: *Staphylococcus aureus*, capsular polysaccharides, bovine mastitis

Staphylococcus aureus capsular polysaccharides 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 (5). Conversely, antibodies against CPs have a protective effect since they can opsonize encapsulated *S. aureus* from bovine origin for phagocytic killing by PMN (5).

The existence of 11 CP serotypes has been proposed (13); however, only four types (CP1, CP2, CP5 and CP8) have been chemically characterized. Among them, CP5 and CP8 are the predominant serotypes in *S. aureus* isolated from human and bovine infections (13). Distribution of CP serotypes among *S. aureus* isolates from bovine milk from different countries shows variability (6, 15, 23). A study carried out in Argentina found that only 14% from 195 *S. aureus* isolates were typeable by serological methods (21). However, more than 70% of those isolates belonged to one province and only 9 isolates came from two provinces that concentrate about 60% of Argentina dairy production.

Capsular polysaccharides *in vitro* expression does not necessarily correlate with expression under *in vivo* conditions (11, 13). Therefore, surveys of CP prevalence taking into account only the *in vitro* phenotype, could underestimate the true distribution of virulent CP strains among a bacterial population. Up to now, the reports of CP prevalence have been mainly performed by *in vitro* phenotype analysis and only in few studies involving *S. aureus* from bovine origin, a subset of phenotyped isolates was typed by genetic methodology (21, 23).

Protection afforded by antibodies against CPs is related to their prevalence and type distribution in the population of isolates present in different regions (10). Therefore, the latter

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information is of paramount importance to estimate the usefulness of incorporating these components in a vaccine formulation. The aim of this study was to determine the prevalence and distribution of capsular genotype and phenotype of *S. aureus* isolated from bovine IMI in the four main dairy provinces of Argentina by genotypic and phenotypic methods.

One hundred and fifty seven *S. aureus* isolates were obtained between 2004 and 2007 from mammary secretion of cows with clinical or subclinical IMI, including a maximum number of 3 isolates from the same dairy herd. Isolates were confirmed to be *S. aureus* on the basis of conventional biochemical reactions. Isolates belonged to 83 dairy farms located in four Argentine provinces that concentrate more than 90% dairy production of the country: Santa Fe (n=91), Buenos Aires (n=31), Córdoba (n=22) and Entre Ríos (n=13). From these isolates, 43 were from clinical and 91 from subclinical IMI; while for the remaining 23 isolates, the clinical origin was not determined. Clinical IMI was defined as presence of clinical signs in the mammary quarter (swelling, heat, pain) and/or changes in the appearance of milk; while subclinical IMI was defined as absence of clinical signs but somatic cell counts > 200,000 cells/ml.

Genomic DNA was extracted from each isolate with a standard phenol-chloroform procedure (14). The presence of *cap*5k and *cap*8I loci was evaluated in all the isolates by Polymerase Chain Reaction (PCR). PCR was performed using genomic DNA as a template in a total volume of 25µl containing: 1x PCR buffer, 2mM MgCl2, 0.25 mM dNTPs (Genbiotech, Buenos Aires, Argentina), 1U/µl *Thermus aquaticus* DNA polymerase (PB-L, Argentina) and 0.2 µM of the primers Cap5k1 (5'-GTCAAAGATTATGTGATGCTAC TGAG-3´), Cap5k2 (5´-ACTTCGAATATAAACTTGAATCA ATGTTATACAG-3´), Cap8k1 (5´-GCCTTATGTTAGGTGA TAAACC-3´), Cap8k2 (5´-GGAAAAACACTATCATAGCA GG-3´) (Invitrogen Argentina, Buenos Aires) as described by Verdier *et al.* (24). Amplification was carried out on GeneAmp PCR System (Applied Biosystems, USA) using a program as follows: an initial 5-min denaturation step at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 1 min of extension at 72°C; with a final extension step at 72°C for 5 min. PCR products were analyzed by electrophoresis on ethidium bromide-stained 1.5% agarose gels (Biodynamics, B.A. Argentina). The sizes of the amplicons were 361 bp for capsular type 5 and 173 bp for capsular type 8.

Bacterial suspensions for preparation of typing sera were made from cultures of prototype *S. aureus* strains CP5 (Reynolds) and CP8 (Becker). These strains were isolated in 1979 from blood cultures at Kaiser permanent Hospital, North Hollywood, California (9) and were a kind gift from Dr. B. Poutrel (INRA, Nouzilly, France). Bacteria were grown on Columbia agar (Britania, Buenos Aires) supplemented with 2.5% NaCl, harvested and inactivated following previously described conditions (8). Two New Zealand white rabbits weighing 3 kg were immunized with each bacterial prototype according to the scheme described by Karakawa *et al.* (8, 12). Each rabbit serum was absorbed with *S. aureus* strain 57, to remove antibodies to noncapsular antigens as previously described (8), aliquoted and stored at -70°C. CP from prototype strains 5 and 8 and all isolates typed by genetic method (n=101) were isolated as described by Fattom *et al.* (3). Polysaccharides concentration was determined by phenol-sulphuric acid method (2), and presence of CP was visualized by SDS-Page and silver stain. Absence of proteins was verified by bicinchoninic acid assay (20) and SDS-Page followed by Coomassie Blue stain. ELISA assays were performed as follows: 5µg of purified CPs from isolates genotyped as carrying *cap*5 and *cap*8 were used as antigens to sensitize 96-well plates. Plates were blocked with PBS-powdered milk (5%) and incubated with CP5 or CP8 antisera (1/200), respectively. Finally, a goat anti-rabbit IgG conjugated to alkaline peroxidase was used as secondary antibody, and the reaction was developed with TMB (Zimed). All incubations were carried out at 37°C, for 60 minutes. Optical Densities (OD) were measured at 450 nm in an ELISA plate reader (Molecular Device). Chi square test was used to compare percent

distribution of capsular types between provinces and to assess association between percent distributions of capsular types with regard to clinical origin of the isolates.

Sixty four percent of the isolates were typeable by PCR with specific primers for loci *cap*5 or *cap*8; being the rest of the isolates nontypeable (NT). Eighty three (52.87%) isolates were genotyped as *cap*5 whereas eighteen (11.4%) as *cap*8. None of the isolates positive for either *cap*5 or *cap*8 genes was found to amplify both genes, confirming specificity of PCR used. Distribution of genotypes among isolates originated in different geographical areas is shown in Table 1. The prevalent capsular type among isolates from Córdoba, Santa Fe and Entre Ríos was *cap*5. Conversely, the majority of isolates from Buenos Aires were NT, while type 5 was predominant among typeable isolates from this province. CP genotype distribution and percent of NT isolates varied between provinces; however, differences were not significant (P=0.227). More than 50% of the isolates from each province could be genotyped by the PCR methodology. Among isolates from clinical IMI, 31 (72.1%) were genotyped either as *cap*5 or *cap*8, while only 12 (27.9%) were NT. Among 91 isolates from subclinical IMI, 53 (58.2%) were typed as *cap*5 or 8 and 38 (41.8%) were NT (Table 2). Differences between percentages of typeable isolates according to the clinical origin were not significant (P=0.12).

Table 1. Distribution of capsular polysaccharide genotypes and phenotypes 5 and 8 among *Staphylococcus aureus* isolated from bovine intramammary infections in four Argentinean provinces.

	Provinces									
	Santa Fe		**Entre Ríos**		**Córdoba**		**Buenos Aires**		**Total**	
	Genotype	**Phenotype**	**Genotype**	**Phenotype**	**Genotype**	**Phenotype**	**Genotype**	**Phenotype**	**Genotype**	**Phenotype**
*cap*5 (%)	46 (50.5)	19 (33.3)	10 (76.9)	6(54.5)	16 (72.7)	6(37.5)	11 (35.5)	8(47.1)	83 (52.9)	39(38.6)
*cap*8(%)	11 (12.1)	8(14.0)	1 (7.7)	0	0 (0)	0	6 (19.3)	4(23.5)	18 (11.4)	12(11.9)
NT(%)	34 (37.4)	30(52.7)	2 (15.4)	5(45.5)	6 (27.3)	10(62.5)	14 (45.2)	5(29.4)	56 (35.7)	50(49.5)
Total	91	57	13	11	22	16	31	17	157	101

References: NT: nontypeable.
No differences in CP genotype (P = 0.227) or phenotype (P = 0.179) distribution were found between provinces.

Table 2. Distribution of *S. aureus* isolate types CP5 or CP8, according to mastitis clinical origin.

	Clinical		**Subclinical**	
	Genotype	**Phenotype**	**Genotype**	**Phenotype**
Typeable(%)	31(72.1)	14(45.2)	53(58.2)	27(50.9)
NT(%)	12(27.9)	17(54.2)	38(41.8)	26(49.1)
Total	43	31	91	53

References: NT: nontypeable.
No statistical association between genotype (P =0.12) or phenotype (P=0.262) and clinical origin of the isolate was not found.

The isolates genotyped as *cap*5 and *cap*8 were then tested with anti-CP5 and anti-CP8, respectively. The results of serotyping are shown in Table 1. Fifty percent of isolates genotyped as *cap*5 and *cap*8 were shown to produce CPs by ELISA for detecting either capsular type. Thirty nine isolates (38.6%) reacted to anti-CP5 serum and 12 (11.9%) to anti-CP8. From 83 isolates genotyped as *cap*5, 39 (46.9%) were capable of expressing CP; while from 18 isolates genotyped as *cap*8, 12 (66.6%) were capable of expressing CP.

A statistical association between the expression of CP5 or CP8 capsules and clinical origin of the isolate was not found (Table 2). From 84 isolates genotyped as *cap*5 and *cap*8 for which clinical status was known, 31 (37%) where from clinical IMI, and among these isolates 14 (45.2%) and 17 (54.8%) were serotypeable and non serotypeable, respectively. Among isolates from subclinical origin, 53 from the 84 isolates were

genotyped as *cap*5 and *cap*8 (63%), and from these isolates 27 (50.9%) and 26 (49.1) were serotypeable and non serotypeable, respectively.

Prevalence of isolates expressing CP5 and CP8 in this study (32.2%) was lower than those observed in most previous reports of other countries (6, 15, 23). A previous study carried out in Argentina, including 195 isolates, demonstrated that only 14% could be typed by specific antisera against CP5 or CP8 (21). In the present study, a higher prevalence of isolates expressing CP5 than previously reported (21) was observed; however, the percent of isolates expressing CP8 was similar in both studies. Differences in proportion of isolates expressing CP5 between studies can be explained mainly by the isolates geographical origin and the time frame of both studies. While most isolates from the previous study belonged to Buenos Aires province, *S. aureus* isolates included in the present investigation were obtained from the four main dairy provinces of Argentina. In addition, in the present study, to avoid bias produced by clonal dissemination, we included a maximum of 3 isolates per dairy farm to assure bacterial isolate diversity within each geographical area considered.

The low proportion of phenotype expression with respect to genotype presence was also reported in bovine mastitis isolates from Europe and USA (23). Genotype-phenotype disparity could indicate a restriction in phenotype expression due to differences between *in vivo* vs *in vitro* culture conditions (16, 17). This implies that conventional phenotypic evaluation can underestimate isolates ability to express CP *in vivo*. In addition, genotype-phenotype disparity could be due to the fact that some isolates carry *cap* genes but lack capsule expression due to mutations within capsule genes (1).

We found no association between genotype or phenotype and clinical origin of the isolate. In a previous study a significant association between CP8 expression and mastitis clinical manifestations was observed only for isolates from Ireland and Iceland (23). Recent studies have shown an association between *S. aureus* genotypes and IMI clinical and epidemiological features (4, 7); however associations in these latter cases were established with patterns including several rather than individual genes (4).

Presence of CP alone is considered to be insufficient to generate a protective immune response; however, their inclusion in a multicomponent vaccine would be useful to improve control of *S. aureus* IMI (18, 19, 22). In addition, relevance of CP as candidates for generating protective responses is underscored by the fact that a commercial vaccine currently available for *S. aureus* mastitis control contains capsulated strains expressing 3 serotypes of CP present among the population of bovine isolates in USA (10). Sixty four percent of the isolates evaluated in this study carried *cap*5 and *cap*8 genes, which emphasizes the importance of including these components for rational design of mastitis vaccines.

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