ISSN 0325-7541
ARTÍCULO ORIGINAL
Revista Argentina de Microbiología (2011) 43: 212-217

Phenotypic and genotypic characterization of *Streptococcus uberis* isolated from bovine subclinical mastitis in Argentinean dairy farms

MIRTA C. LASAGNO[1], ELINA B. REINOSO[1], SILVANA A. DIESER[1], LUIS F. CALVINHO[3], FERNANDA BUZZOLA[4], CLAUDINA VISSIO[2], CRISTINA I. BOGNI[1], LILIANA M. ODIERNO[11]*

[1]*Departamento de Microbiología e Inmunología y;* [2]*Departamento de Patología Animal, Facultad de Ciencias Exactas, Físico-Químicas y Naturales. Universidad Nacional de Río Cuarto, Ruta 36 Km 601, (5804) Río Cuarto, Córdoba;* [3]*Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria, (2300) Rafaela, Santa Fe;* [4]*Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, (1121) Buenos Aires, Argentina.*
**Correspondence: E-mail: lodierno@exa.unrc.edu.ar*

ABSTRACT

The aim of this study was to investigate the phenotypic and genotypic characteristics of *Streptococcus uberis* isolated from subclinical mastitis (SCM) cases, and to examine the possible association between both characteristics. A total of 32 *S. uberis* were isolated from 772 quarter milk samples (SCM > 250,000 cells/ml) collected from 195 cows selected randomly from 18 dairy farms located in Argentina. The *S. uberis* strains were characterized phenotypically by the presence of virulence factors as plasminogen activator factor (PAF), hyaluronidase (HYA), capsule (CAP) and CAMP factor, and were further characterized genotypically by pulsed-field gel electrophoresis (PFGE). *S. uberis* strains expressed plasminogen activator factor, hyaluronidase or capsule (65.5 %, 56.3 %, 59.4 %, respectively), but only 25 % of isolates were CAMP factor positive. Thirteen different virulence profiles were identified on the basis of the combination of virulence factors. Eighteen PFGE patterns with 90% of similarity were identified among 32 *S. uberis*. A great diversity of virulence profiles and PFGE patterns were present among dairy farms. *S. uberis* strains with the same PFGE pattern showed different virulence profiles. Bovine *S. uberis* strains causing SCM included in the present study showed heterogeneity in regard to their phenotypic and genotypic characteristics, and the PFGE patterns are not associated with the virulence profiles.

Key Words: *Streptococcus uberis*, bovine subclinical mastitis, pulsed-field gel electrophoresis, virulence factors

RESUMEN

Caracterización fenotípica y genotípica de *Streptococcus uberis* aislados de mastitis bovina subclínica en tambos de Argentina. El objetivo de este estudio fue investigar las características fenotípicas y genotípicas de *Streptococcus uberis* aislados de casos de mastitis subclínica (MSC) y examinar la posible asociación entre ambas características. Un total de 32 cepas de *S. uberis* fueron aisladas de 772 muestras de leche de cuartos mamarios (MSC > 25 0000 células/ml) colectadas de 195 vacas seleccionadas al azar pertenecientes a 18 tambos lecheros localizados en Argentina. Las cepas de *S. uberis* fueron caracterizadas fenotípicamente sobre la base de la presencia de factores de virulencia tales como el factor activador del plasminógeno (FAP), la hialuronidasa (HIA), la cápsula (CAP) y el factor CAMP. Además, fueron caracterizadas genotípicamente por electroforesis de campos pulsados (PFGE). Las cepas de *S. uberis* expresaron el factor activador del plasminógeno, la hialuronidasa o la cápsula (65,5 %, 56,3 % y 59,4 %, respectivamente), pero solo el 25 % fueron CAMP positivas. Sobre la base de la combinación de los factores de virulencia se identificaron 13 perfiles de virulencia diferentes. Asimismo, se identificaron 18 patrones de PFGE con un 90 % de similitud entre las 32 cepas de *S. uberis.* Se presentó una gran diversidad de perfiles de virulencia y patrones de PFGE entre los tambos. Cepas con el mismo patrón de PFGE presentaron perfiles de virulencia diferentes. Las cepas de *S. uberis* causantes de MSC en bovinos incluidas en el presente estudio mostraron heterogeneidad con respecto a sus características fenotípicas y genotípicas, y los patrones de PFGE no estuvieron asociados con los perfiles de virulencia.

Palabras clave: *Streptococcus uberis*, mastitis subclínica bovina, electroforesis de campos pulsados, factores de virulencia

INTRODUCTION

Streptococcus uberis is one of the most important environmental pathogens implicated in bovine mastitis, accounting for a significant proportion of subclinical intramammary infections (5, 6, 8, 10, 21, 22). In Argentina, *S. uberis* also was identified as one of the most frequent environmental agents of subclinical mastitis, being recovered from 2 % to 20 % of the regional dairy herds evaluated (4). Severe economic impact is caused

by subclinical infections, which may hamper the control of mastitis because they often go unnoticed and untreated, resulting in long duration of the infection (30). Moreover, economic losses are also caused as result of the failure to eradicate *S. uberis* mastitis through management that controls transmission of contagious mastitis (15). Molecular studies have yielded evidence for the predominance of particular strains in some herds suggesting that it could be the result of differences between strains in pathogen virulence (2, 21). Several potential *S. uberis* virulence factors have been proposed, including plasminogen activator factor (PAF) (23), hyaluronidase (HYA) (20, 24), hyaluronic acid capsule (CAP) (28), and CAMP factor (CAMP) (16), among others. Reports concerning the phenotypic expression of one or a few *S. uberis* virulence factors were limited to a very few strains and varies according to different studies (12, 17, 20). DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) is a reliable technique for resolution of clonal relationships (2, 6, 21). Recently, molecular epidemiological studies based on PFGE have been conducted on bovine *S. uberis* isolates from Europe (13), Australia (2, 21, 27), New Zealand (18), and North America (31). At present, nothing has been reported about the phenotypic and genotypic characterization among *S. uberis* isolates from bovine mastitis at various dairy farms in Argentina. Of particular clinical importance is the question concerning the existence of *S. uberis* strains responsible for subclinical mastitis, which differ with respect to their pathogenic and genotypic properties, requiring strategies towards prevention and treatment of the intramammary infections. In order to investigate this hypothesis, we first started a study to obtain *S. uberis* isolates from milk of dairy cows presenting subclinical mastitis in farms located in an area that comprises three major Argentinean provinces in the east-central region of the country. As pathogenicity of a certain strain might depend on its repertoire of virulence factors, we analyzed the isolates for their presence of various virulence factors by phenotypic methods. In addition, we investigated the genetic relationships among *S. uberis* strains by DNA macrorestriction analysis. The genotypes were then associated with phenotypic data from 18 dairy farms in order to determine the possible existence of *S. uberis* strains possessing specific virulence profiles or PFGE patterns associated with subclinical mastitis at various farms.

MATERIALS AND METHODS

Bacterial isolates

A total of 772 quarter milk samples collected from 195 cows selected randomly from 18 dairy farms (1-18) from the three major dairy provinces (Buenos Aires, Córdoba, Santa Fe) located in the east-central region of Argentina kept under grazing conditions were included in the present study. Farms were visited once between March and December 2008.

The quarter milk samples were collected from a single visit at milking time at the farms. Samples were subjected to total somatic cell count (SCC) in order to confirm the subclinical status (> 250,000 cells/ml) of the collected samples. The determination of SCC was performed with the Somatocount 300 System (Bentley Instrument, Chaska Minnesota, USA). Isolation of *S. uberis* was attempted from the milk samples with SCC values more than 250,000 cells/ml. A gland was defined as being subclinically infected with *S. uberis* when ≥ 3 CFU/10 µl were isolated from milk samples and only one colony type was present. The isolates were initially identified using standard conventional biochemical tests (19) and further confirmed using restriction fragment length polymorphism analysis of 16S rDNA (16S rDNA RFLP) as previously described (9).

Phenotypic characterization

(I). Detection of plasminogen activator factor. Plasminogen activation factor (PAF) was detected as described by Leigh (14). Bovine plasminogen was kindly supplied by Dr. James Leigh, School of Veterinary Medicine & Science, University of Nottingham, UK). Controls were performed using equal volumes of phosphate buffer saline and *S. uberis* culture filtrate. Activity was detected by diffusion from wells cut in PBS 1 % agarose (Promega, USA) containing 2 % (v/v) skimmed milk following 24 h incubation at 37 °C.

(II). Hyaluronidase activity assay. Determination of hyaluronidase (HYA) activity was performed as described previously by Azeredo *et al.* (1) using bovine testicular hyaluronidase (Sigma Aldrich, USA) as the positive control.

(III). Capsule expression. Presence of capsule (CAP) was determined by Anthony staining (7). An encapsulated *S. uberis* (RC2) and unencapsulated *S. uberis* (RC3) isolated from bovine mammary secretions from individual cows were used as positive and negative controls, respectively.

(IV). CAMP factor reaction. Bacteria were screened for CAMP factor (CAMP) activity as previously described (11). Briefly, *S. uberis* strains were streaked perpendicular to a streak of beta-toxin-producing *S. aureus* on blood agar plates and after 6-20 h incubation at 37 °C, they were observed for haemolysis. All phenotypic assays were tested at least four times**.** Each virulence factor was considered positive or negative when 3 of the 4 assays were positive or negative, respectively. Virulence profiles were identified on the basis of the combination of virulence factors.

Genotypic characterization

Pulse field gel electrophoresis (PFGE). Genomic DNA for PFGE was carried out according to the rapid method described by Benson and Ferrieri (3). Banding patterns were digitalized using the MiniBisPRO gel documentation system (Micro Photonics Inc., Allentown, USA) and stored as Tagget Image Files Format (TIFF). These files were converted, normalized and analyzed with GelWorks 1D software (version 3.00, Ultra Violet products, England). The reproducibility of the PFGE patterns was examined by repeated testing of the same isolate on separate occasions on different gels. The relatedness of the fingerprints was assessed by using visual inspection of the band profiles according to criteria described by Tenover *et al.* (26). Dendrograms were generated by using the Dice coefficients and clustering was done by the unweighted-pair group method with arithmetic averages (UPGMA), and the cutoff was set at 90 % similarity level.

Statistical analysis. The statistical significance of probabilities of dependences between different virulence profiles and PFGE patterns was analyzed by Chi-square and Fisher tests. A *p*-value of 0.05 or less in the Fisher test was considered significant.

RESULTS

Phenotypic characterization

A total of 32 *S. uberis* were isolated from 772 quarter milk samples (subclinical mastitis > 250,000 cells/ml)

collected from 195 cows selected randomly from 18 dairy farms (1-18) located in the east-central region of Argentina. One to four isolates were obtained from each herd. A total of 32 *S. uberis* strains isolated from milk of cows with subclinical mastitis were characterized on the basis of the virulence factors expression. More than fifty percent of *S. uberis* strains expressed PAF, HYA and CAP (65.5 %, 56.3 %, 59.4 %, respectively), while only few (25 %) were CAMP factor positive. Confidence Intervals (95 % CI) of these values were (49.2 % to 82.1 %), (39.1 % to 73.4 %), (42.4 % to 76.4 %), (10 % to 40 %), respectively. Thirteen different virulence profiles (a-m) were identified on the basis of the combination of virulence factors. Virulence profiles e and g were the most frequent, with five strains each other. Virulence profiles a, b, e, f, g, i, and l showed between 2 and 5 strains each one. Different virulence profiles were found among dairy farms. In addition, we found two strains that expressed four virulence factors and three strains that did not express any factor (Table 1).

PFGE-genomic macrorestriction analysis

PFGE of chromosomal DNA digested with *Sma*I yielded 9 to 17 fragments in the 20 to 680 kb size range. Genome sizes calculated by addition of DNA fragments of various sizes varied; for most of the strains a genome size of 2.6 x 10^6 to 1.7 x 10^6 bp was calculated, which corresponded to the previously reported streptococci genome size (29). Figure 1 shows the dendrogram produced by the UPGMA algorithm. Eighteen different patterns (A-B-C-D-E-E_a-F-G-H-I-J-K-L-L_1-L_a-M-M_1-N) were identified among 32 *S. uberis* strains tested according to the Dice coefficient with similarity values of 90 %. A great diversity of PFGE patterns was present among dairy farms. Nineteen *S. uberis* strains were clustered in six groups (1 to 6) composed each one by strains with identical PFGE patterns. *S. uberis* strains with the same PFGE pattern showed different virulence profiles. However, some strains collected from different dairy farms shared PFGE patterns and had the same virulence profile. Two strains of group 1 had virulence profile e, two strains of group 3 had virulence profile e, two strains of group 4 had virulence profile g, and two strains of group 6 had virulence profile f. Any association between virulence profiles and PFGE patterns analyzed by Chi-square and Fisher tests was found among *S. uberis* strains.

DISCUSSION

S. uberis is a well-recognized worldwide environmental agent of subclinical mastitis in bovines. In Argentina, data on virulence factor profiles and PFGE patterns of *S. uberis* are not available. In the present study, we investigated the phenotypic and genotypic characteristics of *S. uberis* isolates from cows with subclinical mastitis in dairy farms of the east-central region of Argentina, a highly populated area where many of the most important dairy farms and dairy industries in the country are located.

A number of potential virulence factors have been identified in different studies (20). Among these, Leigh (14) investigated the activation of plasminogen from a variety of mammalian species among five clinical isolates of *S. uberis* in the UK. The presence of plasminogen activator factor was analyzed in the present study. The detection of PAF could be observed for twenty-one (65.6 %) *S. uberis* strains of the present investigation. The findings of plasminogen activator factor in a moderate proportion of

Table 1. Phenotypic characteristics of 32 *S. uberis* strains from subclinical mastitis from 18 dairy farms in the east-central region of Argentina

Virulence profile	PAF[(1)]	HYA[(2)]	CAP[(3)]	CAMP[(4)]	Dairy Farms [Number of isolates]
a	-	-	-	-	II [1], VII [1], XII [1]
b	+	-	-	-	II [1], XI [1]
c	-	-	-	+	III [1]
d	-	-	+	-	VIII [1]
e	+	-	+	-	I [1], VI [1], VIII [1], IX [1], XI [1]
f	-	+	+	-	VIII [1], XI [1], XIII [1], XV [1]
g	+	+	-	-	IX [2], XVI [2], XVIII [1]
h	+	-	-	+	XII [1]
i	+	+	+	-	IV[1], X [1], XVI[1], XVII [1]
j	+	-	+	+	V [1]
k	+	+	-	+	XVI [1]
l	-	+	+	+	VI [1], XII [1]
m	+	+	+	+	V [1], XIV [1]

[(1)]plasminogen activator factor, [(2)]hyaluronidase, [(3)]capsule, [(4)]CAMP factor

S. uberis isolates tested suggest a role for this molecule in the pathogenesis of the bacterium.

Hyaluronidase has long been considered to be a streptococcal virulence determinant (20, 24). In this study we found that HYA production could be observed for 18 of 32 (56.3 %) *S. uberis* strains. A single published study about the frequency of HYA with a different technique in relation to used in present study showed low proportion (35.6 %, 47/132) of *S. uberis* strains HYA positive (12).

An additional potential virulence factor investigated in the present study was the hyaluronic acid capsule. The capsule of *S. uberis* has been implicated in conferring resistance to phagocytosis by bovine neutrophils (20). Presence of CAP was detected in 59.4 % of the *S. uberis* strains examined. This result is consistent with previous reports where 44 % of *S. uberis* strains from bovine milk in US were found to produce capsule (17).

The lack of expression of plasminogen activator factor, hyaluronidase or capsule in 34.4 %, 40.6 % and 43.8 % of the strains, respectively, could indicate that these factors may not play a crucial role in the pathogenesis of *S. uberis*. This fact may indicate that others virulence factors could be involved.

Another potential virulence factor analyzed in the present study was the CAMP factor (11, 12). A positive CAMP reaction could be observed for only 25 % *S. uberis* strains in our study. Similarly, Lämmler (13) found 28 % CAMP positive *S. uberis* strains. On the other hand, Skalka and Smola (25) reported that 71.6 % of *S. uberis* strains were CAMP factor positive. The role of CAMP factor in pathogenicity is unclear, although it cannot be ruled out as a putative virulence factor.

In this work, we found a high number of virulence profiles associated with intramammary infections demonstrating that *S. uberis* strains with different virulence profiles were able to cause subclinical mastitis.

Despite the large number of different virulence profiles, the occurrence of *S. uberis* strains with identical profiles was found. In this study the distribution of virulence factors showed that not all of them were present in each strain.

In addition to the phenotypic characterization of *S. uberis*, we investigated the genetic relationship of the isolates by using a molecular typing system to evaluate overall DNA polymorphism (PFGE). A high degree of genetic variation of PFGE patterns was found in *S. uberis* among farms under study. These results demonstrate that multiple *S. uberis* PFGE patterns were able to cause subclinical mastitis. These data shows the genetic diversity in *S. uberis* strains as was demonstrated by the PFGE results in previous studies (6, 18, 21). Douglas *et al.* (6) found 330 different PFGE patterns among 343 *S. uberis* from mastitis in dairy cows, from 15 different farming regions in New Zealand. McDougall *et al.* (1). using PFGE found 173 different PFGE types among 234 *S. uberis* isolated

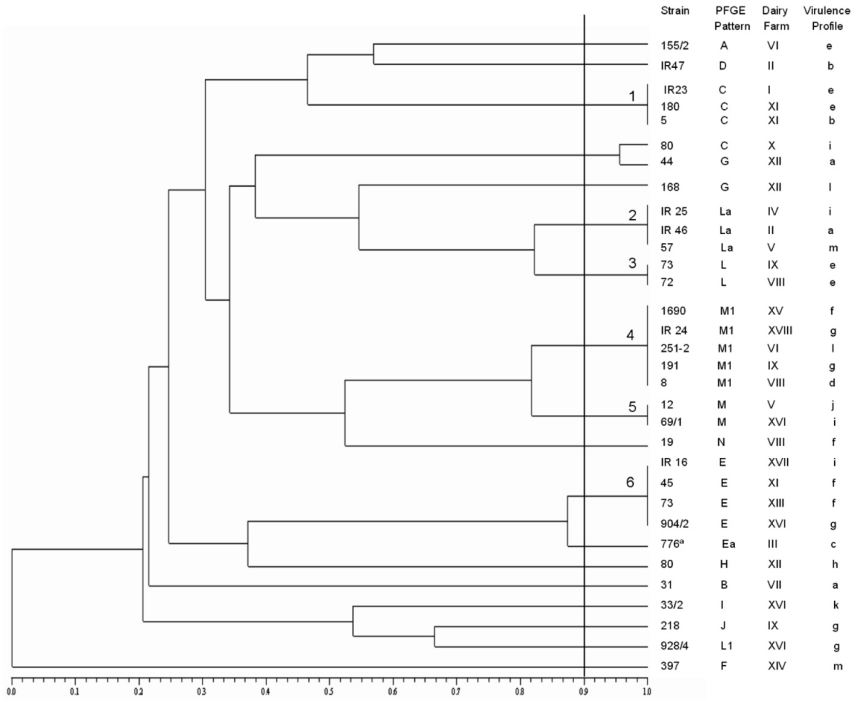

Figure 1. Dendrogram of similarity among the observed PFGE macrorestriction patterns of *Sma*I-digested DNAs from 32 *S. uberis* strains. The Dice coefficient was used for calculating the similarities and clustering among the patterns. Eighteen patterns (A-B-C-D-E-E_a-F-G-H-I-J-K-L-L_1-L_a-M-M_1-N) were identified at 90% similarity. Groups 1 to 6 composed each one by strains with identical PFGE pattern. 1-18: dairy farms, a-m: virulence profiles.

from New Zealand. Phuetkes *et al.* (21) reported that 62 distinct PFGE patterns could be observed among 138 *S. uberis* collected from dairy farms in Australia. However, the existence of predominant *S. uberis* strains has been reported (21) a larger study would be required in dairy farms of our country to confirm the genetic diversity found by other researchers in *S. uberis.*

The current study describes that different virulence profiles were detected even among *S. uberis* strains belonging to a given PFGE pattern. Finally, the association between virulence profiles and PFGE patterns was investigated and any association was found among *S. uberis* strains recovered from subclinical mastitis in Argentina.

We concluded that a high degree of phenotypic and genotypic variability is characteristic of *S. uberis* strains causing subclinical mastitis, and that the PFGE patterns of bovine *S. uberis* strains are not associated with the virulence profiles.

To our knowledge, this is the first report to describe phenotypic and genotypic characteristics of *S. uberis* isolates from milk of dairy cows presenting subclinical mastitis in Argentina.

A better knowledge of genotypic traits of *S. uberis* strains might contribute to determine the possible existence of PFGE patterns or virulence profiles of this pathogen as causative agent of subclinical mastitis at various farms, and might help in the design of strategies towards prevention and treatment of the *S. uberis* infections.

Acknowledgements. This work was supported by grants from SECyT (Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto) and FONCyT (Agencia Nacional de Promoción Científica y Tecnológica). The authors thank J. A. Giraudo (Dpto. Patología Animal, Fac. Agronomía y Veterinaria, Universidad Nacional de Río Cuarto), M.V. J. Chávez and M.V. Liliana Tirante (LactoDiagnóstico Sur, Olivos, Buenos Aires) for providing the isolates. C. Vissio and S.A. Dieser are fellows from CONICET and E. B. Reinoso is member assistant of the research career of CONICET.

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Recibido: 04/01/11 - Aceptado: 15/07/11