Revista Argentina de Microbiología
ISSN: 0325-7541
ram@aam.org.ar
Asociación Argentina de Microbiología
Argentina

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Revista Argentina de Microbiología, vol. 47, núm. 4, diciembre, 2015, pp. 282-294
Asociación Argentina de Microbiología
Buenos Aires, Argentina

Rev Argent Microbiol. 2015;47(4):282–294

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

Genotyping and study of the *pauA* and *sua* genes of *Streptococcus uberis* isolates from bovine mastitis

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Received 22 December 2014; accepted 22 June 2015
Available online 23 October 2015

KEYWORDS
Bovine mastitis;
Molecular typing;
Streptococcus uberis;
PauA;
SUAM

Abstract This study aimed to determine the clonal relationship among 137 *Streptococcus uberis* isolates from bovine milk with subclinical or clinical mastitis in Argentina and to assess the prevalence and conservation of *pauA* and *sua* genes. This information is critical for the rational design of a vaccine for the prevention of bovine mastitis caused by *S. uberis*. The isolates were typed by random amplified polymorphic DNA (RAPD) analysis and by pulsed-field gel electrophoresis (PFGE). The 137 isolates exhibited 61 different PFGE types and 25 distinct RAPD profiles. Simpson's diversity index was calculated both for PFGE (0.983) and for RAPD (0.941), showing a high discriminatory power in both techniques. The analysis of the relationship between pairs of isolates showed 92.6% concordance between both techniques indicating that any given pair of isolates distinguished by one method tended to be distinguished by the other. The prevalence of the *sua* and *pauA* genes was 97.8% (134/137) and 94.9% (130/137), respectively. Nucleotide and amino acid sequences of the *sua* and *pauA* genes from 20 *S. uberis* selected isolates, based on their PFGE and RAPD types and geographical origin, showed an identity between 95% and 100% with respect to all reference sequences registered in GenBank. These results demonstrate that, in spite of *S. uberis* clonal diversity, the *sua* and *pauA* genes are prevalent and highly conserved, showing their importance to be included in future vaccine studies to prevent *S. uberis* bovine mastitis.

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http://dx.doi.org/10.1016/j.ram.2015.06.007

PALABRAS CLAVE
Mastitis bovina;
Tipificación molecular;
Streptococcus uberis;
PauA;
SUAM

Genotipificación y estudio de los genes *pauA* y *sua* de aislamientos de *Streptococcus uberis* de mastitis bovina

Resumen Este estudio pretendió determinar la relación clonal entre 137 aislamientos de *S. uberis* obtenidos de leche de bovinos con mastitis clínica o subclínica en la Argentina, como así también la prevalencia y la conservación de los genes *sua* y *PauA* entre dichos aislamientos. Esta información es crítica para el diseño racional de una vacuna que prevenga la mastitis bovina por *S. uberis*. Los aislamientos se tipificaron molecularmente por amplificación al azar del ADN polimórfico (RAPD) y mediante electroforesis de campos pulsados (PFGE). Los 137 aislamientos mostraron 61 pulsotipos mediante PFGE y 25 tipos de RAPD diferentes. Los índices de Simpson calculados fueron 0,983 por PFGE y 0,941 por RAPD; esto evidencia el elevado poder discriminatorio de ambas técnicas. El análisis de la relación entre pares de aislamientos mostró un 92,6 % de concordancia entre ambas técnicas, lo que indica que cualquier par de aislamientos que fue distinguido por un método tendió a ser distinguido por el otro. La prevalencia de los genes *sua* y *puaA* fue del 97,8 % (134/137) y 94,9 % (130/137), respectivamente. Las secuencias de nucleótidos y de aminoácidos codificados por los genes *sua* y *pauA* de los 20 aislamientos de *S. uberis* seleccionados sobre la base de su tipo de PFGE y RAPD y origen geográfico tuvieron un porcentaje de identidad de entre 95 % y 100 % con respecto a todas las secuencias de referencia registradas en GenBank. Estos resultados demuestran que, a pesar de la diversidad clonal de *S. uberis*, los genes *sua* y *pauA* son prevalentes y están altamente conservados y deberían ser incluidos en futuros estudios de vacunas para prevenir mastitis bovina causada por *S. uberis*.

Introduction

Bovine mastitis is one of the most costly diseases of dairy cattle as a consequence of antibiotic treatment expenses, decreased milk production and quality, and increased animal replacement rate[6,30]. In Argentina, milk yield losses in cows suffering from mastitis were reported to be of about $4.3/cow/days[41]. Bovine intramammary infections (IMI) are caused by both contagious and environmental bacteria. *Streptococcus uberis* is one of the most prevalent environmental pathogens associated with subclinical and clinical IMI both in lactating and non-lactating cows[7,27]. In addition, this pathogen can persist in the mammary gland causing chronic IMI[47]. Traditional control procedures based on milking time hygiene and antibiotic therapy are considered adequate to reduce incidence of most contagious pathogens, but are often insufficient for the control of IMI caused by *S. uberis*[20]. Consequently, the interest has focused on the development of immunoprophylactic strategies. However, a significant obstacle in the design of an effective vaccine is the high level of genetic variability of different isolates of *S. uberis*, frequently involving virulence factor genes[18,31]. In this context, epidemiological studies of regional isolates are extremely useful to detect the frequency and distribution of bacterial types associated with IMI and to identify target molecules for the development of immunogens and therapeutic agents. Methods based on DNA analysis including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) have been successfully applied to fulfill this need. PFGE is the most discriminatory method for typing bacteria[22], whereas RAPD is a more straightforward method given its relatively lower costs, execution time demanded, less expensive equipment requirements and sensitivity. Both methods have been widely used to study the genetic variability of many bacterial species, including important human pathogens[22,37].

Several *S. uberis* virulence factors have been described[16,20,23,24]. Among them, plasminogen activator A (PauA)[35] and *S. uberis* adhesion molecule (SUAM)[2] have shown potential as protective immunogens. The former is a protease capable of activating plasmin, which in turn, degrades proteins producing small peptides and free amino acids used by bacteria as a nitrogen source. This factor has been related to early mammary gland colonization[21] while SUAM both to adherence and internalization, through its binding to lactoferrin[28], and to bacterial persistence in bovine mammary epithelial cells *in vitro*[2].

The wide genetic diversity observed in *S. uberis* indicates that virulence is not associated with any specific molecular type[43]. Therefore, immunologic prevention strategies against this organism should be directed to a multitude of different factors included in field isolates from individual herds. Nevertheless, the difficulty associated with *S. uberis* genetic variability can be currently overcome through bioinformatic tools that allow to analyze DNA sequence encoding genes from virulence factors which are found in a large number of isolates. Thus, epidemiological studies on gene distribution together with their sequence analysis will contribute to identify potential antigens as vaccine components.

The main objectives of this work were to determine the clonal relationship between *S. uberis* isolates from milk

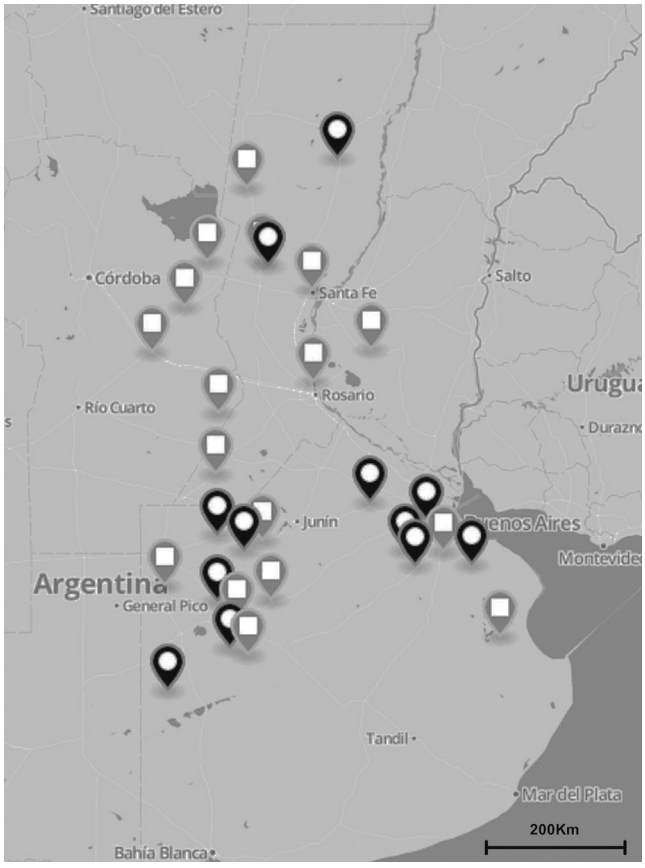

Figure 1 The total number ($n = 137$) of *S. uberis* isolates recovered from bovines suffering from subclinical or clinical mastitis were from 35 dairy farms located in the main dairy region of Argentina (black and gray globes). Gray globes indicate geographical origin of isolates selected for the *sua* and *pauA* sequencing.

of cows suffering from subclinical or clinical mastitis in Argentina and to assess the prevalence and conservation of the *pauA* and *sua* genes.

Materials and methods

S. uberis collection and DNA preparation

A total of 137 *S. uberis* strains were isolated from milk of 122 cows suffering from clinical and subclinical mastitis between November 2010 and July 2012. Milk samples were obtained from 35 dairy herds located in the main dairy regions of Argentina (Fig. 1). Clinical mastitis was characterized by evidence of inflammation (abnormal milk, heat, swelling or pain of affected quarters) while subclinical mastitis by lack of local or systemic signs of mammary inflammation and somatic cell counts ≥200,000 cells/ml.

S. uberis isolates were initially identified by standard conventional biochemical tests such as esculin hydrolysis, hippurate hydrolysis, growth on 6.5% NaCl, growth on bile-esculin-agar and the CAMP test[25]. Restriction fragment length polymorphism (RFLP) of 16S rDNA[14] was performed for molecular confirmation. *S. uberis* ATCC 27958 was used as reference strain.

Genomic DNA was extracted using a modification of the method described by Hill and Leigh[13]. The detailed protocol is available at pubmlst.org/suberis/info/protocol.shtml.

Molecular typing of *S. uberis* strains

Molecular typing was carried out by PFGE and RAPD-PCR. Pulsed-field electrophoresis was performed in accordance with a previously described method[5]. Briefly, electrophoresis was performed with the CHEF-DR III SYSTEM (Bio-Rad Laboratories) using 1% Pulsed Field Certified™ (BioRad Laboratories) in 0.5× Tris-borate-EDTA (TBE) buffer at 11.3 °C for 23 h. The electrophoresis conditions were as follows: initial switch time value of 5 s, final switch time of 35 s at a gradient of 6 V/cm. After electrophoresis, the gel was stained with ethidium bromide solution (0.5 mg/l) and then destained with deionized water. Then the DNA bands were visualized with a UV transilluminator and a digital image of PFGE patterns was obtained using Molecular Imager® Gel Doc™ XR+ System (BioRad, Laboratories Inc., Richmond, CA, USA).

RAPD-PCR was performed as previously described[45] using primer OPE-4. The amplified products were separated by gel electrophoresis in 1.5% agarose gel, stained with ethidium bromide. Banding pattern similarities were analyzed using the Dice correlation coefficient. Values for tolerance and optimization were set at 1.5% and 1%, respectively. The cluster analysis was based on UPGMA, Unweighted Pair Group Method with Arithmetic averages, and data were analyzed with the TREECON software for Windows[39]. Isolates indicating more than 3 DNA fragment differences and a similarity of < 80% following the dendrogram analysis were classified as different PFGE or RAPD types, whereas fragment variations and a similarity of > 80% following the dendrogram analysis were defined as PFGE or RAPD subtypes. Isolates showing identical patterns (100% pattern similarity index) or a similarity index of > 80% were interpreted as belonging to the same PFGE or RAPD type[1]. The typing experiments were repeated at least twice.

Calculation of concordance, Simpson's index of diversity and Wallace's coefficients

Simpson's index of diversity was calculated to measure the discriminatory power of the typing systems. This index indicates the probability that 2 strains randomly sampled from a population will belong to 2 different types[4]. Wallace's coefficients were used to explore the correlation between results produced by PFGE and RAPD. These were calculated using EpiCompare version 1.0 (http://www3.ridom.de/epicompare/). Group concordance was evaluated by cross-classification of all possible pairs of isolates as previously described[34]. All possible pairs of isolates were cross-classified according to matched or mismatched types. The resulting 2 × 2 table was evaluated by the chi-square test and the percentage of concordant cells was calculated using the GraphPad Prism 5 (Version 5, USA) software.

PCRs and DNA sequencing

DNA amplification for the *sua* gene was performed using oligonucleotide primers derived from published sequences[23]. Amplification of the *pauA* gene was performed using the following primers, which were designed using DNAstar Primer Select software (DNAstar, Madison, WI), 5′-TTTTTAATATTAATGCTTTTG-3′ and 5′-AGAAAAATTTAATGGATAC-3′. The reaction mixture was made with 150 ng/μl template DNA, 0.8 μM oligonucleotide primers, 0.2 μM of each of the four dNTPs, 1.25 U Taq polymerase and 3 mM $MgCl_2$, in a final volume of 50 μl. The expected amplicon size was 874 bp. PCR conditions were 5 min at 95 °C, 30 cycles of [1 min at 94 °C, 1 min at 49 °C and 1 min at 72 °C], 5 min at 72 °C.

The PCR products from 20 isolates of *S. uberis* were selected for sequencing. The following criteria were taken into account for isolate selection: isolates had to belong to different geographical regions and/or had to be epidemiologically unrelated (Table 1; Figs. 1 and 2). DNA was purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) according to the manufacturers' protocol and eluted in 30 μl of sterile MilliQ water. Positive PCR products were sequenced in both directions using the same specific PCR primers for the *pauA* gene while for the *sua* gene the following oligonucleotides were added: sua2: 5′-GAA TTC ACA CAA TCT GAC GAG GT-3′; sua3: 5′-GAA TTC GAA GTT GGG GCA TAC-3′ and sua4: 5′-GAA TTC CCA AGT GCT CCG GTC T-3′ to the PCR primers. DNA samples (30–50 ng/μl) were sequenced by ABI3130XL sequencer analyzer (Applied Biosystems) from the sequencing service of the Biotechnology Institute of INTA Castelar, Argentina.

Molecular and data analysis

Forward and reverse sequences were aligned using Sequencher-DNA Sequencig Software Demo 5.1 (Gene Codes Corporation) and the consensus sequence files converted to FASTA format. Sequences were analyzed by searching the GenBank database of the National Center for Biotechnology Information via the Basic Local Alignment Search Tool (BLAST) network service. Then, the sequences were compared between them and against all existing allelic types from the database using the Codon Code Aligner software (Codon Code Corporation, Dedham, MA). The *pauA* sequences were also compared through the Food Microbe Tracker database at www.pathogentracker.net[48].

Results

Typability and discrimination of PFGE typing

The molecular epidemiological analysis of *S. uberis* isolates from milk identified by RFLP was performed on 137 isolates from 35 dairy farms. Digested chromosomal DNA generated 1–23 fragments ranging from 48.5 to 436.5 kb in size, which could be resolved by PFGE (Fig. 2a). Epidemiologically related groups were identified based on a minimum 80 % similarity. The analysis of the 137 isolates revealed 61 types of PFGE patterns, 29 of which were unique isolates, and 32 types, containing 2–8 isolates per type. Isolate distribution in groups was: 19 types with 2 isolates (PF 04, 9, 11, 12, 15, 16, 18, 21, 23, 25, 27, 37, 43, 45, 46, 47, 55, 56 and 57), 5 types with 3 isolates (PF 13, 19, 32, 33 and 40), 5 types with 4 isolates (PF 20, 31, 34, 35 and 39), 2 types with 6 isolates (PF14 and PF29) and 1 type with 8 isolates (PF1) (Table 1). Genotype grouping appeared to be neither related to the mastitis type (clinical or subclinical) nor to the farm origin. Epidemiologically related groups were observed among isolates from different farms. Similarities ranging between 80.1 % and 100 % were established in isolates retrieved from the same farm in only 11 types (PF 1, 4, 14, 19, 20, 29, 31, 32, 34, 35 and 47), suggesting a common source or horizontal transmission of the pathogen. Fifteen out of the 137 studied isolates gave a poor PFGE quality pattern; therefore, they could not be included in the analysis. These strains were classified as non-typeable, resulting in 90.5 % typability for this technique according to the Epicompare software analysis.

Typability and discrimination of RAPD-PCR typing

Products obtained after RAPD-PCR amplification were 0.5–8.0 kb in size (Fig. 2b). All samples exhibited at least one of these products: 1632 bp, 1800 bp, and 2430 bp. Differences among isolates were given by products ranging between 1800 and 1632 bp in size. The analysis was carried out in 137 samples using the same criteria for PFGE which allowed the identification of 25 clonal patterns. The patterns were assigned to 5 types with only one isolate and 20 types, containing 2–17 isolates. The composition of the groups was as follows: 4 types with 2 isolates (RD-7, 12, 20 and 22), 3 types with 3 isolates (RD-5, 8 and 15), 1 type with 4 isolates (RD-19), 5 types with 5 isolates (RD-4, 11, 16, 18 and 23), 1 type with 7 isolates (RD-14), 2 types with 8 isolates (RD-1 and 6), 1 with 9 isolates (RD-3), 1 with 11 isolates (RD-9), 1 with 13 isolates (RD-13), and 1 with 17 isolates (RD-10). As for PFGE, no association was found either between mastitis type or farm of origin. Thirteen isolates could not be assigned to any group given the poor quality observed in their electrophoretic pattern, and therefore they were classified as non-typeable resulting in 89 % typability for this technique according to the Epicompare software analysis.

Comparison of the typing methods

To compare the discriminatory power of PFGE and RAPD, we calculated the Simpson's diversity index for both methods (Table 2). PFGE and RAPD yielded different Simpson index values of 0.983 (95 % CI 0.978–0.989) and 0.941 (95 % CI 0.927–0.955), respectively. To compare the congruence between type assignments using PFGE and RAPD, we calculated the Wallace coefficients. Wallace coefficients for PFGE and RAPD indicated a weak bidirectional correspondence (0.042–0.172) between types generated by both methods. However, cross-classification of the isolates, based on matched or mismatched schemes by PFGE and RAPD, showed that the 2 typing systems were 92.6 % concordant (Table 3).

Table 1 Characteristics of *S. uberis* isolates according to their PFGE type

PFGE type	RAPD type	Isolate	District	Mastitis type	Farm	*pauA*	*sua*
NT	RD-20	34[a]	Capitán Bermudez – Santa Fe	SCM	36	+	+
NT	RD-23	37[a]	Arrufó – Santa Fe	SCM	45	+	+
NT	RD-06	52	General Granada – Bs As	CM	31	+	+
NT	RD-11	64	Pehuajó – Bs As	SCM	3	+	+
NT	RD-07	68	Carlos Casares – Bs As	SCM	1	+	+
NT	RD-11	89	Navarro – Bs As	UAI	5	+	+
NT	RD-02	90	Mones Cazón – Bs As	CM	3	+	+
NT	RD-11	93	Mones Cazón – Bs As	SCM	9	+	+
NT	NT	109	Carlos Casares – Bs As	SCM	1	+	+
NT	RD-15	123	Rivadavia – Bs As	SCM	27	+	+
NT	RD-4	134	Navarro – Bs As	UAI	20	+	+
NT	RD-10	141	Navarro – Bs As	SCM	20	+	+
NT	RD-03	162	Navarro – Bs As	SCM	5	+	+
NT	RD-13	168	Vicente Casares – Bs As	CM	16	–	+
NT	RD-08	172	Navarro – Bs As	SCM	14	+	+
PF-01	RD-01	72	Carlos Casares – Bs As	CM	5	+	+
PF-01	RD-01	73	Navarro – Bs As	SCM	5	+	+
PF-01	RD-04	74	Navarro – Bs As	SCM	2	+	+
PF-01	RD-01	84	Navarro – Bs As	SCM	14	+	+
PF-01	RD-06	85	Navarro – Bs As	SCM	14	+	+
PF-01	NT	86	Navarro – Bs As	CM	14	+	+
PF-01	NT	87	Navarro – Bs As	CM	14	+	+
PF-01	RD-03	137	Navarro – Bs As	SCM	5	+	+
PF-02	RD-13	152	Handerson – Bs As	CM	22	+	+
PF-03	RD-22	36	Capitán Bermudez – Santa Fe	SCM	36	+	+
PF-04	NT	106	Mones Cazón – Bs As	CM	9	–	–
PF-04	NT	107	Mones Cazón – Bs As	CM	9	–	+
PF-05	NT	110	Carmen de Areco – Bs As	SCM	17	+	+
PF-06	RD-14	60	Navarro – Bs As	SCM	2	+	+
PF-07	RD-18	94	Lincoln – Bs As	SCM	11	+	+
PF-08	RD-10	155	Pehuajó – Bs As	SCM	23	+	+
PF-09	RD-01	20[a]	Rafaela – Santa Fe	CM	40	+	+
PF-09	RD-10	156[a]	Lincoln – Bs As	CM	11	+	+
PF-10	RD-07	92	Mones Cazón – Bs As	SCM	9	+	+
PF-11	RD-01	41	Handerson – Bs As	SCM	29	+	+
PF-11	RD-10	135	Carlos Casares – Bs As	CM	55	+	+
PF-12	RD-18	61	Navarro – Bs As	SCM	2	+	+
PF-12	RD-09	111	Carmen de Areco – Bs As	SCM	17	+	–
PF-13	RD-09	79	Carlos Casares – Bs As	SCM	1	+	+
PF-13	RD-04	112	Navarro – Bs As	SCM	18	+	+
PF-13	RD-09	128	Mones Cazón – Bs As	CM	3	+	+
PF-14	RD-10	49	Carmen de Areco – Bs As	SCM	12	+	+
PF-14	RD-08	59	Navarro – Bs As	SCM	2	+	+
PF-14	RD-14	117	Navarro – Bs As	SCM	5	+	+
PF-14	RD-3	140	Navarro – Bs As	CM	5	–	+
PF-14	RD-9	145	Villa María – Cba	CM	21	+	+
PF-14	RD-03	150	Handerson – Bs As	SCM	22	+	+
PF-15	RD-05	96	Carlos Casares – Bs As	SCM	1	+	+
PF-15	RD-25	108	Mones Cazón – Bs As	CM	9	+	+
PF-16	RD-09	91	Curarú – Bs As	SCM	6	+	+
PF-16	RD-10	98	Vicente Casares – Bs As	SCM	15	+	+
PF-17	RD-23	76[a]	Colonia María – Cba	CM	8	+	+
PF-18	RD-23	39[a]	Pehuajó – Bs As	CM	19	+	+
PF-18	RD-10	118	Navarro – Bs As	CM	10	+	+
PF-19	RD-10	119	Navarro – Bs As	CM	10	+	+
PF-19	NT	121	Navarro – Bs As	SCM	5	+	+
PF-19	RD-13	160	Navarro – Bs As	SCM	5	+	+

Table 1 (*Continued*)

PFGE type	RAPD type	Isolate	District	Mastitis type	Farm	*pauA*	*sua*
PF-20	NT	57	Navarro – Bs As	SCM	18	+	+
PF-20	RD-09	80	Mones Cazón – Bs As	SCM	3	+	+
PF-20	RD-06	81	Mones Cazón – Bs As	SCM	3	+	+
PF-20	RD-08	142	Navarro – Bs As	SCM	5	+	+
PF-21	RD-24	11[a]	Nogoyá – Entre Ríos	CM	24	+	+
PF-21	RD-19	70	Navarro – Bs As	CM	2	+	+
PF-22	RD-14	122	Carmen de Areco – Bs As	SCM	12	+	+
PF-23	RD-03	78	Navarro – Bs As	CM	10	+	+
PF-23	RD-03	116	Navarro – Bs As	CM	5	+	+
PF-24	RD-06	104	Vicente Casares – Bs As	CM	16	–	–
PF-25	RD-22	42[a]	Handerson – Bs As	SCM	29	+	+
PF-25	RD-06	101	Vicente Casares – Bs As	CM	16	+	+
PF-26	RD-13	171	Navarro – Bs As	SCM	14	+	+
PF-27	RD-12	50	Carmen de Areco – Bs As	SCM	12	+	+
PF-27	RD-06	58	Navarro – Bs As	SCM	2	+	+
PF-28	RD-13	154	Pehuajó – Bs As	SCM	23	+	+
PF-29	RD-09	67	Carlos Casares – Bs As	SCM	1	+	+
PF-29	RD-16	100	Vicente Casares – Bs As	CM	15	+	+
PF-29	RD-10	102	Vicente Casares – Bs As	CM	16	+	+
PF-29	RD-10	131[a]	Carlos Casares – Bs As	CM	56	+	+
PF-29	RD-16	167	Vicente Casares – Bs As	CM	16	+	+
PF-29	RD-16	170	Vicente Casares – Bs As	SCM	25	+	+
PF-30	RD-06	151	Handerson – Bs As	SCM	22	+	+
PF-31	RD-13	124	Navarro – Bs As	SCM	5	+	+
PF-31	RD-15	125[a]	Rivadavia – Bs As	CM	27	+	+
PF-31	RD-13	126	Mones Cazón – Bs As	CM	3	–	+
PF-31	RD-15	130	Mones Cazón – Bs As	CM	3	+	+
PF-32	RD-16	147[a]	Villa María – Cba	CM	21	+	+
PF-32	RD-16	148	Villa María – Cba	CM	21	+	+
PF-32	RD-09	149	Handerson – Bs As	SCM	22	+	+
PF-33	RD-14	146	Villa María – Cba	CM	21	+	+
PF-33	RD-01	165	Vicente Casares – Bs As	CM	16	+	+
PF-33	RD-13	175	Vicente Casares – Bs As	CM	15	+	+
PF-34	NT	139	Navarro – Bs As	CM	5	–	+
PF-34	RD-10	143[a]	Navarro – Bs As	SCM	5	+	+
PF-34	RD-04	158	Navarro – Bs As	SCM	5	+	+
PF-34	RD-03	161	Navarro – Bs As	SCM	5	+	+
PF-35	RD-10	65	Brandsen – Bs As	SCM	32	+	+
PF-35	NT	127	Navarro – Bs As	CM	5	+	+
PF-35	RD-10	133	Carlos Casares – Bs As	SCM	26	+	+
PF-35	RD-10	144	Navarro – Bs As	SCM	5	+	+
PF-36	RD-03	132	Carlos Casares – Bs As	CM	26	+	+
PF-37	NT	38	Pehuajó – Bs As	CM	19	+	+
PF-37	RD-20	40[a]	Saliquelló – Bs As	CM	28	+	+
PF-38	RD-19	69	Navarro – Bs As	CM	28	+	+
PF-39	RD-01	44[a]	Handerson – Bs As	SCM	2	+	+
PF-39	RD-23	45	Navarro – Bs As	SCM	2	+	+
PF-39	RD-06	51	Pehuajó – Bs As	CM	23	+	+
PF-39	NT	95	Carmen de Areco – Bs As	SCM	12	+	+
PF-40	RD-12	48[a]	Corral de Busto – Cba	SCM	30	+	+
PF-40	RD-18	54	Navarro – Bs As	SCM	18	+	+
PF-40	RD-13	159	Navarro – Bs As	SCM	5	+	+
PF-41	RD-05	83	Mones Cazón – Bs As	SCM	3	+	+
PF-42	RD-10	153	Pehuajó – Bs As	SCM	23	+	+
PF-43	RD-17	166[a]	Vicente Casares – Bs As	CM	16	+	+
PF-43	RD-11	169	Vicente Casares – Bs As	SCM	25	+	+
PF-44	NT	75[a]	Sancti Spiritu – Sta Fe	CM	7	+	+
PF-45	RD-18	88	Curarú – Bs As	SCM	6	+	+

Table 1 (*Continued*)

PFGE type	RAPD type	Isolate	District	Mastitis type	Farm	*pauA*	*sua*
PF-45	RD-09	129	Mones Cazón – Bs As	CM	3	+	+
PF-46	RD-11	55	Navarro – Bs As	SCM	18	+	+
PF-46	RD-13	103	Mones Cazón – Bs As	CM	9	+	+
PF-47	RD-04	46	Corral de Busto – Cba	CM	30	+	+
PF-47	RD-10	47[a]	Corral de Busto – Cba	CM	30	+	+
PF-48	RD-19	66	Brandsen – Bs As	SCM	32	+	+
PF-49	RD-09	63	Pehuajó – Bs As	SCM	3	+	+
PF-50	RD-19	56	Navarro – Bs As	SCM	18	+	+
PF-51	RD-10	138	Navarro – Bs As	CM	5	+	+
PF-52	RD-13	157	Navarro – Bs As	SCM	5	+	+
PF-53	RD-09	62	Pehuajó – Bs As	SCM	34	+	+
PF-54	RD-14	97[a]	Las Varillas – Cba	SCM	13	+	+
PF-55	RD-05	82	Mones Cazón – Bs As	CM	3	+	+
PF-55	RD-14	163	Navarro – Bs As	SCM	5	+	+
PF-56	RD-01	43	Handerson – Bs As	SCM	29	+	+
PF-56	RD-18	71	Vicente Casares – Bs As	CM	4	+	+
PF-57	RD-14	115	Navarro – Bs As	CM	18	+	+
PF-57	RD-13	164	Vicente Casares – Bs As	CM	16	+	+
PF-58	RD-21	15[a]	Santa Fe – Santa Fe	SCM	35	+	+
PF-59	RD-13	105	Vicente Casares – Bs As	CM	16	+	+
PF-60	RD-03	114	Navarro – Bs As	CM	18	+	+
PF-61	RD-23	77	Navarro – Bs As	SCM	2	+	+

CM, clinical mastitis; SCM, subclinical mastitis; UAI, unavailable information.
[a] Selected for sequencing.

Table 2 Simpson's diversity indices of the genotyping methods for all typed isolates

	No. of types	No. of isolates per type (mean ± SD)	Simpson index (95% CI)
PFGE	61	2 ± 1.4	0.983 (0.978–0.989)
RAPD	25	5 ± 4.1	0.941 (0.927–0.955)

Table 3 Cross-classification of all possible pairs of isolates based on matched or mismatched PFGE and RAPD types

	RAPD-PCR	
	Match	Mismatch
PFGE		
Match	16	77
Mismatch	369	5533

$p < 0.001$ chi-square test. Concordance: 92.6%.

Detection and evaluation of the *sua* and *pauA* genes

PauA, encoded by the *pauA* gene, and SUAM, encoded by the *sua* gene, have been described as two of *S. uberis* major virulence factors and as potential vaccine immunogens against this pathogen. We studied the distribution of these genes among a population of *S. uberis* from our country and analyzed their sequences in 20 selected isolates (Table 1; Fig. 1). Table 1 and Fig. 1 show *S. uberis* isolates selected according to geographical differences and/or epidemiological dissimilarity. The encoding genes for SUAM and PauA were present in the majority of *S. uberis* isolates from different Argentinean dairy areas. The *pauA* gene was detected in 94.9% (130/137) whereas the *sua* gene in 97.8% (134/137).

Nucleotide sequences of the *sua* gene from the 20 *S. uberis* selected isolates showed 99% identity with respect to the unique reference sequence from the GenBank (DQ232760.1)[2]. In concordance with the identity of nucleotide sequences, the amino acid sequences from mastitis isolates showed between 97% and 99% identity with respect to the reference sequence (GenBank ABB52003.1)[2]. Amino acids encoded by codons with single mutations in the *sua* gene are shown in Table 4. About 5–13 amino acid changes were detected in the 20 isolates. It is worth mentioning that the changes in encoded amino acids were repeated in the different isolates and suggesting conserved mutations (Table 4).

Nucleotide sequences of the *pauA* gene from the 20 selected *S. uberis* mastitis isolates showed between 95% and 100% identity with respect to all the 55 sequences registered in GenBank and to 30 sequences registered in the Food Microbe Tracker database. Similarly, the amino acid sequences showed 96–100% identity with respect to the reference sequences. Amino acids encoded by codons with single mutations in *pauA* are shown in Table 5. About

Table 4 Amino acids encoded by codons with single mutations in the *S. uberis* adhesion molecule coding gene *sua* of *S. uberis*

Isolate	Position in consensus sequence																										
	115	142	145	148	151	157	181	199	208	271	319	367	376	379	391	400	409	457	460	469	481	829	886	1117	1195	1318	1375
SU44; SU47	**Leu**	**Lys**	**Cys**	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU48	**Leu**	**Lys**	**Cys**	Asn	Val	Arg	Phe	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	**Arg**	**Lys**	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU20	Ser	**Lys**	**Cys**	Asn	Val	**Gly**	Phe	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU01	Ser	**Lys**	**Cys**	**Ser**	Val	**Gly**	Phe	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU34	Ser	**Lys**	**Cys**	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU15	Ser	Ile	**Cys**	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	**Thr**	**Lys**	Val	**Arg**	**Gly**
SU75	Ser	Ile	Tyr	Asn	Gly	Arg	Phe	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	**Phe**	Gln	Gly
SU76	Ser	Ile	Tyr	Asn	Gly	Arg	**Leu**	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	Thr	Glu	Asp	Leu	**Val**	Glu	Ala	Ala	Asn	Glu	Val	**Arg**	**Gly**
SU37	Ser	Ile	Tyr	Asn	Val	Arg	**Leu**	Asn	Lys	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	**Asp**
SU39	Ser	Ile	Tyr	Asn	Val	Arg	**Leu**	Asn	Lys	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	Gly
SU156	Ser	Ile	Tyr	Asn	Val	Arg	Phe	**Ser**	Lys	Thr	**Arg**	Ala	Asp	**Ile**	Thr	Glu	Asp	**Val**	Ala	**Lys**	Ala	Ala	Asn	Glu	Val	**Arg**	Gly
SU131	Ser	Ile	Tyr	Asn	Val	Arg	Phe	**Ser**	**Arg**	**Ser**	Gln	Ala	Asp	**Ile**	Thr	Glu	Asp	**Val**	Ala	Glu	Ala	Ala	Asn	Glu	Val	**Arg**	Gly
SU11	Ser	Ile	Tyr	Asn	Val	Arg	Phe	**Ser**	**Arg**	Thr	Gln	Ala	Asp	Thr	Thr	Glu	Asp	**Val**	Ala	**Lys**	**Gly**	Ala	Asn	Glu	Val	Gln	Gly
SU166	Ser	Ile	Tyr	Asn	Val	Arg	Phe	**Ser**	**Arg**	Thr	Gln	Ala	Asp	**Ile**	Thr	Glu	**Asn**	**Val**	Ala	Glu	Ala	**Glu**	Asn	Glu	Val	**Arg**	Gly
SU97	Ser	Ile	Tyr	Asn	Val	Arg	Phe	Asn	Lys	Thr	**Arg**	Ala	Asp	Thr	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	Gly
SU40; SU147	Ser	Ile	Tyr	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	**Val**	**Glu**	Thr	Thr	Glu	**Asn**	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	**Arg**	Gly
SU42	Ser	Ile	Tyr	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	Ala	Asp	**Ile**	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	**Arg**	Gly
SU125	Ser	Ile	Tyr	Asn	Val	Arg	Phe	Asn	Lys	Thr	Gln	Ala	Asp	**Ile**	Thr	Glu	Asp	Leu	Ala	Glu	Ala	Ala	Asn	Glu	Val	Gln	Gly

Isolate	Position in consensus sequence																									
	1390	1633	1717	1786	1795	1804	1873	2005	2008	2023	2080	2104	2195	2395	2422	2455	2458	2464	2477	2506	2512	2524	2545	2749	Total Mutations
SU44; SU47	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	9
SU48	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	12
SU20	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	10
SU01	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	11
SU34	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	8
SU15	Ile	Pro	Pro	Thr	Ala	Ala	Ile	Ala	Leu	Asp	Leu	Ser	**Arg**	Asn	Arg	Glu	**Ala**	Asp	Glu	Val	**Lys**	Thr	**Ile**	**Val**	10
SU75	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	**Lys**	Thr	**Ile**	Ile	8
SU76	Ile	Pro	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	**Phe**	Ser	Lys	**Ser**	Arg	**Lys**	**Ala**	Asp	Glu	**Met**	Glu	**Ala**	Val	Ile	11
SU37	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	Glu	Thr	Val	Ile	7
SU39	Ile	Pro	Ala	Thr	Ala	**Thr**	**Val**	**Ser**	Leu	Asp	Leu	Ser	Lys	**Ser**	Arg	**Lys**	**Ala**	Asp	Glu	**Met**	Glu	Thr	Val	Ile	8
SU156	Ile	**Ser**	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	**Phe**	Ser	Lys	**Ser**	**Lys**	**Lys**	**Ala**	Asp	**Ala**	Val	Glu	Thr	Val	Ile	13
SU131	Ile	Pro	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	**Phe**	Ser	Lys	**Ser**	**Lys**	**Lys**	**Ala**	Asp	**Ala**	Val	Glu	Thr	Val	Ile	12
SU11	Ile	Pro	Ala	Thr	**Val**	Ala	Ile	Ala	Leu	Asp	Leu	Ser	Lys	Asn	Arg	**Lys**	**Ala**	**Ala**	Glu	Val	Glu	Thr	Val	Ile	9
SU166	Ile	Pro	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	Leu	Ser	Lys	**Ser**	**Lys**	**Lys**	**Ala**	Asp	Glu	Val	Glu	Thr	Val	Ile	11
SU97	Ile	Pro	Ala	Thr	**Val**	Ala	Ile	Ala	Leu	Asp	Leu	Ser	Lys	Asn	Arg	**Lys**	**Ala**	**Ala**	Glu	Val	Glu	Thr	Val	Ile	5
SU40; SU147	Ile	Pro	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	Leu	Ser	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	**Lys**	Thr	**Ile**	Ile	6
SU42	Ile	Pro	Ala	Thr	Ala	Ala	Ile	Ala	Leu	Asp	**Phe**	Ser	Lys	**Ser**	**Lys**	**Lys**	**Ala**	Asp	**Ala**	Val	Glu	Thr	Val	Ile	8
SU125	**Thr**	Pro	Ala	**Ile**	Ala	Ala	Ile	Ala	**Arg**	**Asn**	Leu	**Asn**	Lys	Asn	Arg	Glu	Val	Asp	Glu	Val	**Lys**	Thr	**Ile**	Ile	8

The difference in amino acid sequence from the majority are bolded.

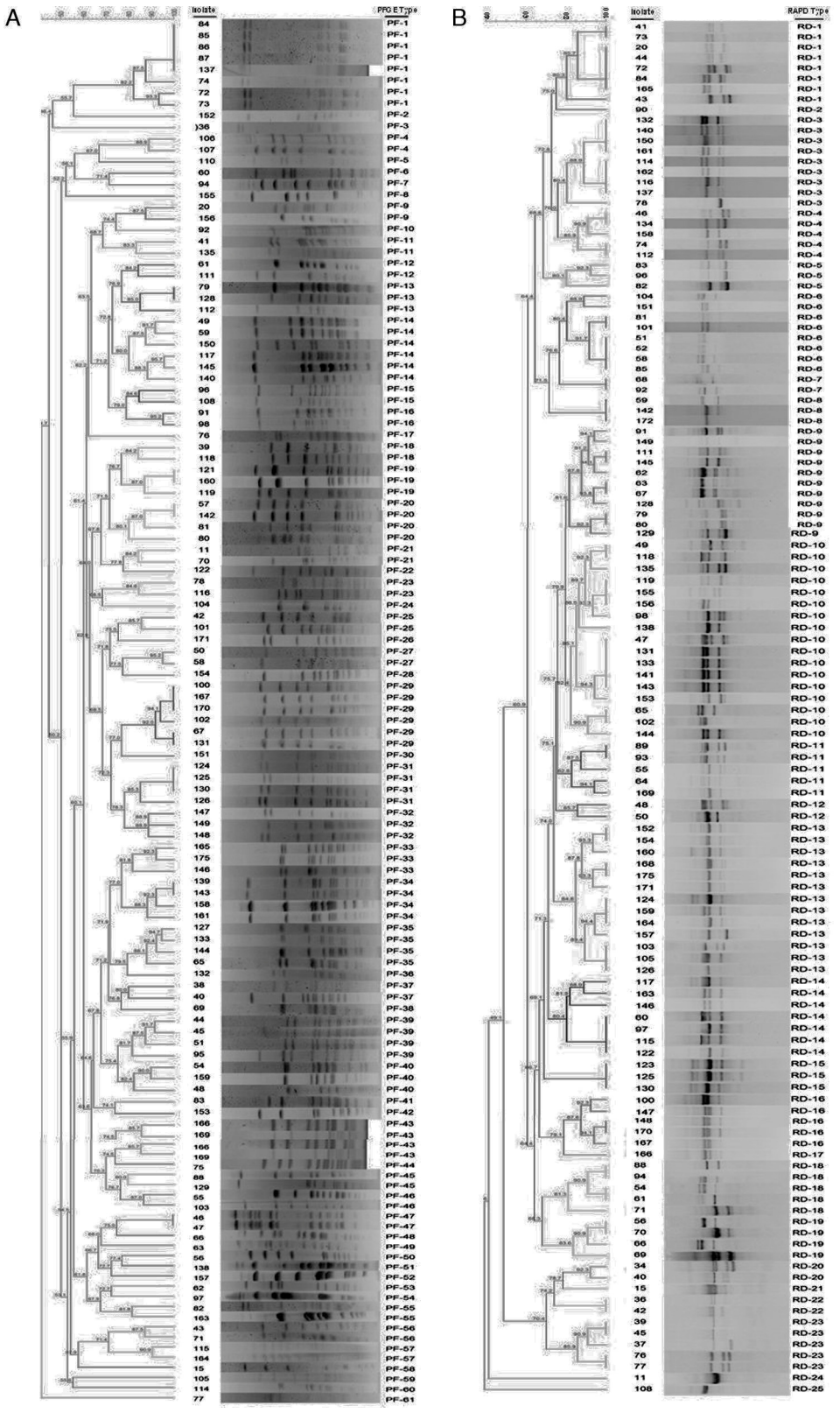

Figure 2 Dendrogram of pulsed-field gel electrophoresis (PFGE) (A) and random amplified polymorphic DNA (RAPD) (B) profiles of 137 *Streptococcus uberis* subclinical mastitis isolates collected from 35 dairy herds. Isolate code and PFGE type (A) and RAPD type (B) of each strain are also represented in the dendrogram. The dendrogram was produced by using Dice coefficients and an unweighted pair group method using arithmetic averages (UPGMA).

Table 5 Amino acids encoded by codons with single mutations in the plasminogen activator coding gene *pauA* of *S. uberis*

Isolate	Position in consensus sequence																	
	43	67	70	217	298	343	371	502	532	553	619	631	718	724	733	751	853	Total mutations
SU11	**Ala**	Val	Ala	**Asn**	Ser	Arg	Arg	Val	His	Asp	Lys	**Leu**	Pro	Asp	Tyr	Gln	Lys	3
SU75; SU37	Gly	**Ile**	Ala	Asp	Ser	Arg	Arg	Val	His	Asp	Lys	Arg	Pro	Asp	Tyr	Gln	Lys	1
SU34	Gly	Val	**Val**	Asp	Ser	**Gln**	**Gln**	Val	His	Asp	Lys	**Leu**	**Gln**	**His**	Tyr	Gln	Lys	6
SU97	Gly	Val	Ala	**Asn**	Ser	Arg	Arg	Val	His	Asp	Lys	Arg	Pro	Asp	Tyr	Gln	Lys	1
SU15	Gly	Val	Ala	Asp	**Thr**	Arg	Arg	**Ile**	His	**Asn**	Lys	Arg	**Gln**	Asp	Tyr	Gln	Lys	4
SU131	Gly	Val	Ala	Asp	Ser	Arg	Arg	Val	**Asn**	Asp	**Glu**	Arg	Pro	Asp	Tyr	Gln	Lys	2
SU42	Gly	Val	Ala	Asp	Ser	Arg	Arg	Val	His	Asp	Lys	Arg	Pro	Asp	**Phe**	Gln	Lys	1
SU39	Gly	Val	Ala	Asp	Ser	Arg	Arg	Val	His	Asp	Lys	Arg	Pro	Asp	Tyr	**Leu**	Lys	1
SU44; SU76	Gly	Val	Ala	Asp	Ser	Arg	Arg	Val	His	Asp	Lys	Arg	Pro	Asp	Tyr	Gln	**Gln**	1

The difference in amino acid sequence from the majority are bolded.

1–6 mutations that resulted in amino acid change were detected in 11 isolates. Sequence data of the *sua* and *pauA* genes were registered in GenBank under accession numbers KT006548–KT006587.

Discussion

S. uberis is a well-known pathogen causing bovine IMI worldwide. However, there is scant epidemiological information on *S. uberis* isolated from bovine milk in Argentina[19]. In the present study, PFGE and RAPD were used for the molecular characterization of *S. uberis* isolated from cows belonging to herds located in the main dairy areas of Argentina. Based of this information a reliable representative set of isolates was selected for the study of the *pauA* and *sua* genes.

As shown in previous studies[1,32,36,43], PFGE has proven to be a highly discriminatory method. In the present study, PFGE was able to resolve many isolates that were indistinguishable by RAPD-PCR. Some isolates from different herds that were defined as belonging to the same type (100% similarities) were associated with different types of mastitis (both clinical and subclinical), in agreement with previous studies[15,29]. The PFGE patterns for *S. uberis* showed great variation; among the 137 isolates (collected from 122 cows on 35 farms), 61 distinct PFGE profiles were observed. This high level of heterogeneity is in accordance with classical epidemiological studies from Argentina and other countries[1,19,32,42]. However, we found eleven cases of one type isolated from different animals in the same dairy herd. This finding suggests that cows were infected by the same organism, either from a common source or by spread from one quarter or cow to another. Poor milking hygiene and faulty milking machine functioning could contribute to *S. uberis* transmission among cows[47]. The total epidemiological data reported in this work allows to consider *S. uberis* as an environmental opportunistic pathogen, with a limited number of dominant types and a great variety of strains.

RAPD typing relies on non-stringent reaction conditions for the amplification of arbitrary target sites[26]. Low stringent reaction conditions are associated with difficulty to achieve high pattern repeatability[10]. Target DNA concentration conditions and the thermal cycling program were standardized in our fingerprinting method to yield constant and reproducible results. The analysis of the 137 isolates distinguished 25 distinct RAPD profiles. Conserved band pattern obtained with RAPD-PCR disagreed with those previously reported by groups from New Zealand and United States[11,45]. These discrepancies indicate that the method does not allow to compare results from different geographical origins. However, RAPD allows to perform epidemiological studies in less time at lower costs[45].

The comparative analysis of RAPD and PFGE results indicated that isolates belonging to the same type according to RAPD exhibited different PFGE patterns. The discriminatory power and congruence between both methods were compared using the Simpson's index of diversity and Wallace's coefficients. Our results indicated that both typing methods had a high discriminatory power between epidemiologically non-related isolates as indicated by the Simpson's index of diversity. Although the Simpson's diversity index of the FPGE method was higher than that of the RAPD method, the difference was modest. The congruence between types defined by PFGE and RAPD yielded low values, as reflected by the Wallace coefficients (0.042 and 0.172), indicating a weak bidirectional agreement among types generated by both methods. PFGE is a technique of choice for short-term or local epidemiological studies, since even minor genetic changes can lead to a three-fragment difference in the PFGE banding pattern[1]. Moreover, several studies have shown that PFGE has greater discriminatory power than MLST[32,38,40]. Gillespie and Oliver[12] compared RAPD and PFGE for differentiation of *S. uberis* isolates, concluding that PFGE had higher discriminatory power than RAPD on the basis of the number of groups that could be differentiated by each method. Similar results were obtained in the present study. However, considering the high concordance between both methods (96.2%), we can conclude that any given pair of isolates distinguished by one method tended to be distinguished by the other. To our knowledge, this is the first study reporting a statistical analysis for comparison of these two techniques applied for *S. uberis* molecular epidemiology.

A successful vaccine should confer broad protection against a multitude of strains. This approach requires a detailed knowledge of the epidemiology and pathogenesis of the organism. Despite the severe economic impact caused by this infection, the virulence factors associated with *S. uberis* pathogenesis are not well understood[2].

Plasminogen activators such as PauA have been proposed as important intermediaries to obtain nutrients for optimal growth of the organism[44]. In addition, the successful establishment of IMI depends on adherence, internalization, and intracellular persistence[28]. The SUAM was found to play a central role in *S. uberis* adherence to bovine mammary epithelial cells[8], contributing to infection persistence. These two virulence factors have been previously studied as potential immunogens[3,21]. However, it is unknown whether genetic variability could have modified the effectiveness they demonstrated in preclinical trials. In the present study, 94.9% of the strains studied carried the *pauA* gene. The prevalence of the *pauA* gene was previously reported as ranging from 61.5% in Argentina to 100% in India[17,33,36]. To get further insight, we studied the nucleotide and amino acid sequences from 20 selected *S. uberis* mastitis isolates. We observed an identity greater than 95% with respect to all the 55 sequences registered in GenBank and to the 30 sequences registered in the Food Microbe Tracker database. Furthermore, amino acid sequences showed a low number of mutations. Mutations at positions 43, 67, 217, 343, 371, 631, 718 and 724 of the consensus sequences coincided with those described by Zadoks *et al*[47]. In fact, the mutations observed in other positions found in this work were not described until now. Therefore, *pauA* is present in most isolates and interestingly, it is a highly conserved gene. Correspondingly, the presence of the *sua* gene was detected in 97.8% of the 137 isolates. Previous works reported a prevalence of the *sua* gene of 83.3%[33] in Argentina, 84.6% in India[36] and 100% in New Zealand, United States and England[23,46]. In addition, the nucleotide and amino acid sequences from the 20 selected *S. uberis* mastitis isolates had an identity greater than 97% with respect to the reference sequence (ABB52003.1). The study of amino acid sequences showed several repeated mutations in different isolates. Yuan *et al*. described similar results in *sua* gene conservation among isolates from different countries[46]. The relevance of the present findings, compared with previous studies, relies on the higher number ($n = 137$), the strict criteria used for the selection and broad distribution of the isolates analyzed. All together these results demonstrate the high prevalence and conservation of the *sua* and *pauA* genes in Argentinean *S. uberis* isolates, which was also reflected in their amino acid sequence analysis. To our knowledge, this is the first study that compared these two virulence factors in sequences from field isolates versus reported GenBank sequences from different countries.

It has been suggested that the genetic clonal diversity of *S. uberis* is an obstacle for the development of an effective vaccine since a broadly-reactive immunogen with field strains has not been obtained so far[9,21]. However, previous works and the present study demonstrated that *S. uberis* isolates belonging to different PFGE profiles showed conserved gene sequences[32]. In addition, it has to be taken in account that the minimal variation observed in gene carriage highlights a potential problem with respect to the development of subunit vaccines against *S. uberis*, since vaccines based on a single antigen may not provide protection against all strains of the pathogen even using conserved genes. Thus, we found that those isolates that did not carry the *sua* gene harbor the *pauA* gene and vice versa (Table 1), and only in two isolates (1.45%) none of these genes were detected. All together, our results showed that the *pauA* and *sua* genes are conserved across different geographical areas and are present in most *S. uberis* isolates despite the high genetic variability observed, which provides further support for the use of these virulence factors as potential vaccine components against *S. uberis*.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Funding

This work was supported by grants from FONCyT (M.S.B., PICT Bicentenario 2010-1309), Q5 (F.R.B., PICT Bicentenario 2010-0733), (L.F.C. and I.S.M., PICT 1175) and from Fundación Banco de la Provincia de Santa Fe (M.S.B.).

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank M.V. Liliana Tirante (Lactodiagnóstico Sur, Olivos, Buenos Aires) for providing the isolates and Dr. Andrea Puebla (Instituto de Biotecnología, CICVyA Castelar, INTA) for sequencing service. M.S.P. is a fellow from CONICET, I.S.M. and F.R.B. are independent and adjunct members of the research career of CONICET.

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