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Original Research

First Report of Molecular Characterization of Argentine Isolates of *Streptococcus equi* subsp. *equi* by Pulsed-Field Gel Electrophoresis

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ABSTRACT

Strangles is one of the most frequently diagnosed equine respiratory infectious diseases in the world. It is caused by *Streptococcus equi* subsp. *equi* (*S. equi*), and it is an acute infection characterized by pyrexia, nasal discharge, pharyngitis, and abscessation of lymph nodes. Frequently, healthy horses might continue to harbor *S. equi* after clinical recovery. Although the genetic distance between *S. equi* isolates is short, strains can be differentiated by pulsed-field gel electrophoresis (PFGE) and single locus sequence typing for epidemiological studies. The aim of this study was to characterize by PFGE Argentine isolates of *S. equi* obtained from horses with acute strangles and those that had recovered. Bacterial isolation and identification of 80 *S. equi* isolates by phenotypic and genotypic tests were performed using samples from 29 horses with acute strangles and 95 from healthy animals. Also, the isolates were characterized by PFGE using *Bsp*120I and *Sma*I. Visual comparison of macrorestriction patterns generated with both enzymes revealed three different DNA fragment profiles with variations of one or two bands. Interestingly, an identical profile was found in isolates from the same horse and from horses that were infected at the same time, and the horses recovered from strangles continue to carry the same strain. Some vaccinated horses have been mild infected for a different strain from that of carriers suggesting other source of infection. This is the first molecular characterization of Argentine isolates of *S. equi*, which shows the presence of three strains between 2010 and 2013 in Buenos Aires.

1. Introduction

Strangles is one of the most frequently diagnosed equine respiratory infectious diseases in the world caused by *Streptococcus equi* subsp. *equi* (*S. equi*) [1–4]. Strangles, which was first reported by Ruffus in 1251, is an acute infection characterized by pyrexia, nasal discharge, pharyngitis, and abscessation of submandibular and retropharyngeal lymph nodes [2,4,5]. An important complication of strangles is the extension of the infection to the guttural pouches, causing guttural pouch empyema and masses of purulent material called chondroids [2,4,6–8]. Also, clinically healthy horses might continue to harbor *S. equi* in guttural pouches and nasopharynx after clinical recovery [4,6,9–11], and may thus transmit the bacterium to susceptible naïve horses. Carrier horses are considered one of the main sources of infection, and these horses contribute to maintaining the disease in a same farm. Other complications of the disease include metastatic

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abscessation of different organs called "bastard strangles" [1,4,10] and immune-mediated complications such as purpura hemorrhagica [4,8,12]. Diagnosis of strangles is based on clinical signs, bacteriological isolation, and polymerase chain reaction (PCR) testing of nasal swabs, nasal or guttural pouch washes, or pus from abscesses [1,12]. A triplex PCR assay targeted the eqbE, SEQ2190, and SZIC genes has been more sensitive than culture tests, and this may indicate that the *S. equi* culture tests should not be considered the gold-standard test for this organism [13].

S. equi is β-hemolytic, Gram-positive coccus, of Lancefield group C and a host-restricted pathogen [3,10]. Virulence factors of *S. equi* include the hyaluronic acid capsule, antiphagocytic SeM protein, streptolysin, streptokinase, and pyrogenic superantigens [10]. *S. equi* is derived from an ancestral strain of *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) [14], which is a commensal of equine mucosa and an opportunistic pathogen of horses and other animals [10,15–17]. *S. equi* strain 4047 and *S. zooepidemicus* strain H70 genomes have been sequenced and compared by Holden et al [14]. *S. equi* strain 4047 has gained and lost genes in its evolution, which may have contributed to the increased pathogenesis of *S. equi* [14]. The gene gain events include the acquisition of 4 prophages (phiSeq1, phiSeq2, phiSeq3, and phiSeq4), which contain genes encoding phospholipase A2 (SlaA) and superantigens SeeH, SeeI, SeeL, and SeeM, and the integrative conjugative element ICESe2, which produces a potential siderophore (equibactin).

The presence of *S. zooepidemicus* could make the isolation of *S. equi* from nasopharyngeal swabs difficult, especially swabs of carrier horses, because of their similar appearance [18]. Differentiation of both subspecies is traditionally based on their different ability to ferment ribose, lactose, trehalose, and sorbitol [8,14]. Also, a molecular differentiation based on PCR testing of *S. equi* targeted the *seM* gene and genes for the superantigens *see*H, *see*I, *see*L, and *see*M has been used [19]. Webb et al. [13] have obtained PCR positive results in samples which were *S. equi* negative, but other β-hemolytic streptococci positive by culture, highlighting the difficulty of isolating *S. equi* from mixed cultures [13].

Subtyping of bacteria may be performed for molecular epidemiology [20] including to study bacterial population genetics, pathogenesis, natural history of infection, and epidemiological surveillance of infectious diseases [21,22]. Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) is a useful technique for genome fingerprinting of bacterial and yeast pathogens [20,22]. Nevertheless, an appropriate interpretation of the different patterns is the key for epidemiological studies [20–22].

Pulsed-field gel electrophoresis using the restriction enzymes *Sma*I, *Apa*I, and *Not*I were performed to characterize Streptococci in several epidemiological studies [15,16,23,24]. However, only a few of these studies were carried out to characterize *S. equi* isolates [25–27]. Other molecular characterizations of *S. equi* were performed using random polymorphic DNA amplification techniques (ERIC PCR, rep-PCR, and BOX PCR) and ribotyping with different restriction enzymes, but none of them could discriminate between the isolates [26]. Also, the single locus sequence typing (SLST), which is based on sequencing the 5'-region of the *seM* gene, is being used as an epidemiological tool to subtype the *S. equi* strains [27–31]. However, the N terminus of the SeM protein may be truncated in some isolates recovered from carrier horses [5,31]. Although the genetic distance between *S. equi* isolates is very short, clinical *S. equi* strains can be differentiated by PFGE, SLST [25–27], and also by genome sequencing with different discriminatory power.

Buenos Aires is the province with the highest stock of horses and equine stables in Argentina [32], and strangles is a major infectious disease affecting the equine population and consequently the equestrian industry. Commercial *S. equi* bacterin is available for prevention of strangles, although practitioners in Argentina have not had successful results with these one, so they prefer to vaccine horses with autologous immunogens. Knowledge of epidemiological characteristics of the disease in Buenos Aires province could contribute to improving the control of infection.

The aim of this study was to characterize by PFGE Argentine clinical isolates of *S. equi* obtained from horses with clinical signs and those that were clinically recovered in different stables in Buenos Aires province.

2. Materials and Methods

2.1. Samples

Samples were collected from horses with acute stage strangles and healthy horses recovered from the disease were collected from several stables in different parts of Buenos Aires province between 2010 and 2013 (Tables 1 and 2). The data of the animals and the stables were recorded, and clinical and epidemiological records were completed.

There needs to be a Ethical statement on animal care in the Method section.

Acute stage: samples from abscesses (submandibular, retropharyngeal, and inguinal lymph nodes), nasal swabs, nasopharyngeal swabs, and guttural pouches were collected from 29 horses in outbreaks ($n = 17$) occurring in 14 different stables (Table 1). Persistent stage: nasopharyngeal swabs from 95 healthy horses were collected by passing a guarded swab to the level of the common pharynx via the ventral nasal meats (Table 2).

2.2. Bacterial Isolation and Identification

The samples were plated on horse blood agar (blood agar base; Britania, Argentina), with 10% citrated adult horse blood and incubated at 37°C with a CO_2-enriched atmosphere for 48 hours. Differently sized β-hemolytic colonies (small [Ch]: 0.5–1 mm, medium [Md]: 2–3 mm, and large [Gd] ≥4 mm) were selected from each sample and subcultured for 24 hours. The Gram staining, catalase test, Streptococcal Grouping Kit (Oxoid, England), and API 20 Strep (bioMérieux, France) were used to identify *S. equi*. The isolates were preserved by freezing at −20°C in 20% glycerol.

2.3. Multiplex Polymerase Chain Reaction

Isolates were incubated in Todd Hewitt Broth (Britania, Argentina) at 37°C for 48 hours, and then washed twice in 1X TE buffer (Tris-HCl 1M, EDTA 0.5M, milli-Q water, pH 8).

Table 1
Horses with clinical signs.

Stable	City	Number of Outbreak	Outbreak	Horse	Sample	Isolates	*Bsp*120I PFGE Pulsotype	*Sma*I PFGE Pulsotype	PFGE Profile
1	Pilar	1	November/ 2010	E1	SM LN p	UBA1Ch, UBA1Gd	1	1	1/1
				E2	SM LN p	UBA2Ch, UBA2Gd	1	1	1/1
4	Baradero	2	March/2011	E7	SM LN p	UBA7	1	1	1/1
				E8	GP p	UBA8Ch, UBA8Md, UBA8Gd	1	1	1/1
5	Trenque Lauquen	3	May/2011	E9	SM LN p	UBA9Ch, UBA9Md	2	3	2/3
				E10	SM LN p	UBA10Ch, UBA10Md, UBA10Gd	2	3	2/3
6	Daireaux	4	May/2011	E11	SM LN p	UBA11Ch, UBA11Md, UBA11Gd	2	3	2/3
				E12	SM LN p	UBA12Ch, UBA12Md, UBA12Gd	2	3	2/3
9	Olavarría	5	June/2011	E15	SM LN p	UBA15Ch, UBA15Md, UBA15Gd	2	3	2/3
				E16	SM LN p	UBA16Md, UBA16Gd	2	3	2/3
		9	June/2012	E25	RF LN p	UBA25Ch, UBA25Md, UBA25Gd	2	3	2/3
				E26	ING LN p	UBA26Ch, UBA26Md, UBA26Gd	2	3	2/3
12	Mones Cazón	6	May/2012	E19	SM LN p	UBA19Ch, UBA19Md, UBA19Gd	2	3	2/3
				E20	SM LN p	UBA20Ch, UBA20Md, UBA20Gd	2	3	2/3
13	Daireaux	7	May/2012	E21	SM LN p	UBA21Ch, UBA21Md, UBA21Gd	2	3	2/3
				E22	SM LN p	UBA22Ch, UBA22Md, UBA22Gd	2	3	2/3
14	Luján	8	May/2012	E23	SM LN p	UBA23Ch, UBA23Md, UBA23Gd	2	3	2/3
				E24	SM LN p	UBA24Ch, UBA24Md, UBA24Gd	2	3	2/3
15	Trenque Lauquen	10	August/2012	E27	SM LN p	UBA27Md, UBA27Gd	1	1	1/1
				E28	SM LN p	UBA28Md, UBA28Gd	1	1	1/1
16	Capilla del Señor	13	April/2012	E31	SM LN p	UNICEN31	1	1	1/1
		11	August/2012	E29	SM LN p	UBA29Ch, UBA29Md, UBA29Gd	2	3	2/3
		15	May/2013	E35	SM LN p	UBA35	3	2	3/2
17	Roque Pérez	12	January/2013	E30	SM LN p	UBA30Md, UBA30Gd	2	3	2/3
18	Bahía Blanca	14	March/2013	E32	SM LN p	UBA32Ch, UBA32Md	2	3	2/3
				E33	SM LN p	UBA33Md	2	3	2/3
				E34	SM LN p	UBA34Ch, UBA34Md	2	3	2/3
19	Pilar	16	May/2013	E37	SM LN p	CRESAL37	2	3	2/3
21	Lobos	17	March/2013	E39	SM LN p	UBA39Ch, UBA39Gd	2	3	2/3

Abbreviations: Ch, small colonies; E, *enfermo* (acute infection); Gd, large colonies; GP, guttural pouches; ING, inguinal; LN, lymph nodes; Md, medium colonies; p, puncture; PFGE, pulsed-field gel electrophoresis; RF, retropharyngeal; SM, submandibular; UBA, Universidad de Buenos Aires.
Comparison of PFGE pulsotypes of strains of *S. equi* isolated from horses with clinical signs of strangles in Buenos Aires province, between 2010 and 2013.

After that, the resultant cell pellet was resuspended in 250 μL of 1X TE buffer and DNA extraction was performed using 5 μL of proteinase K (15 mg/mL, Invitrogen, Germany) for 120 minutes at 56°C and for 15 minutes at 100°C.

Multiplex PCR with forward primers *sodA*-F (5′-CAG CAT TCC TGC TGA CAT TCG TCA GG-3′) and *seeH*-F (5′-AGC ATG ATT CTA ACT TAA TTG AAG CCG-3′), and reverse primers *sodA*-R (5′-CTG ACC AGC CTT ATT CAC AAC CAG CC-3′) and *seeH*-R (5′-TAG CAT GCT ATT AAA GTC TCC ATT GCC-3′) was performed as had been described by Alber et al. [19] to amplify the *sodA* and *seeH* genes encoding the superoxide dismutase A and the exotoxins SeeH, respectively. The PCR reaction was performed in a final volume of 25 μL containing 5 μL of 5X Green GoTaq Buffer (Promega, Madison, WI, USA), 1 μL of deoxynucleotide triphosphate (20 mM, Fermentas, Burlington), 1 μL of each primer (10 pmol/μL), 0.2 μL of GoTaq DNA Polymerase (Promega), 2 μL of DNA template, and 13.8 μL of water. Polymerase chain reaction was carried out using 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute by Mastercycler gradient thermocycler (Eppendorf, Spain). The amplification products were observed in 1.5% agarose gel (Biodynamics, Argentina) stained with ethidium bromide.

The reference strains ATCC 33398 and ATCC 700400 obtained from the ATCC Licensed Derivative Strains (Medica-Tec, Argentina) were included in the experiment as positive controls of the PCR. The UBA 393/07 *S. equi*s train obtained previously from a horse with acute strangles in Buenos Aires was used as a positive control for the multiplex PCR.

Table 2
Clinical healthy horses.

Stable	City	Date of Isolation	Horse	Sample	Number/ Date of Outbreak	Isolates	*Bsp*120I PFGE Pulsotype	*Sma*I PFGE Pulsotype	PFGE Profile
16	Capilla del	February/ 2013	P9	NF swab	11	UBAP9	2	3	2/3
	Señor		P22	NF swab	January/ 2013	UBAP22-1, UBAP-22-2	2	3	2/3
			P56	NF swab	11	UBAP56-1, UBAP56-2, uBAP56Gd	2	3	2/3
		June/2013	P88	NF swab	15	UBAP88Md, UBAP88Gd	3	2	3/2
			P90	NF swab	15	UBA90Md	3	2	3/2
			P91	NF swab	15	UBAP91-1Gd, UBAP91-2Gd	3	2	3/2
			P93	NF swab	15	UBAP93Gd	3	2	3/2
17	Roque Pérez	March/2013	P69	NF swab	July/2012	UBAP69Gd	2	3	2/3

Abbreviations: Gd, large colonies; Md, medium colonies; NF, nasopharyngeal; P, *portador* (carrier); PFGE, pulsed-field gel electrophoresis; UBA, Universidad de Buenos Aires.
Comparison of PFGE pulsotypes of strains of *S. equi* isolated from healthy horses recovered from four different outbreaks in Buenos Aires province, between 2012 and 2013 from two stables.

2.4. Pulsed-Field Gel Electrophoresis

Bacteria were grown on blood agar plates (bioMérieux, France) at 37°C for 48 hours, and then washed twice in 1X TE buffer. The inoculum was adjusted to 25% transmittance at the colorimeter (bioMérieux Vitek, Inc) and 240 μL of this suspension was mixed with 60 μL of lysozyme (Sigma-Aldrich) 10 mg/mL. After 3 hours at 37°C and 30 minutes at 60°C, the suspension was mixed with 300 μL of SSP (267 μL of 1.2% MP agarose [Roche, Germany], 30 μL of 10% sodium dodecyl sulfate, and 3 μL of proteinase K [Roche, Germany] 20 mg/mL) and was poured into plug molds (Bio-Rad, Hercules, CA, USA). After solidification at 4°C, the plugs were removed and incubated in 4 mL of lysis buffer (Tris-HCl 1M, EDTA 0.5M, sodium lauroyl sarcosinate, Milli-Q water and 0.3 mg/mL of proteinase K) overnight at 54°C in a shaking water bath. The plugs were washed twice in Milli-Q water and six times in 1X TE buffer at 50°C for 15–20 minutes each time.

For the enzymatic restriction, 1/3 of the plugs were incubated with 10 U of *Bsp*120I (Thermo Scientific, Waltham, MA, USA) and 10 U of *Sma*I (Thermo Scientific) in 10% of an appropriate buffer at 37°C and 30°C, respectively, for 24 hours and after that, the plugs were stored in 0.5X TBE buffer (Tris 0.9M, boric acid 0.9M, EDTA 0.5M, Milli-Q water, pH 8) at 4°C.

DNA fragments were resolved by using a 1% gel (MP agarose Sigma-Aldrich in 0.5X TBE buffer) on a PFGE apparatus, CHEF-DR III (Bio-Rad, Spain), at 6 V/cm for 25 hours, with switching times ramped from 0.1 to 15 seconds at 14°C, with an angle of 120°. Gel were stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA) and photographed on Fluor-S transilluminator using Molecular Image Gel Doc (Bio-Rad, Spain). A lambda ladder marker (Mid-Range II PFG Marker, Biolabs, England) and *Salmonella* serotype Braenderup strain (H9812) digested with *Xba*I (Thermo Scientific) were used as molecular weight standards. In addition, the ATCC 33398 strain was used in the study.

2.5. Molecular Analysis

The genetic relationship among isolates was evaluated following the criteria of Tenover et al [33]. Also, degrees of similarity between strains based on *Bsp*120I digestion were calculated with 1.5% tolerance and 1.5% optimization by applying the band-based Dice similarity coefficient, and clustering analysis was performed using the UPGMA method by the BioNumerics software (Applied Maths, BVBA, Belgium).

3. Results

3.1. Bacterial Isolation and Identification

A total of 80 isolates of *S. equi* were obtained: 67 from 29 horses with clinical signs (Table 1) and 13 isolates from eight healthy horses (Table 2). Although we found some variation in the size of colonies, most were large and mucoid (Tables 1 and 2). The β-hemolytic bacteria such as *S. zooepidemicus* and *Streptococcus dysgalactiae* subsp. *equisimilis* were also obtained from nasopharynx of healthy horses and animals with acute strangles.

3.2. Multiplex Polymerase Chain Reaction

All isolates identified as *S. equi* by biochemical and Lancefield streptococcal grouping tests showed amplicons with sizes of approximately 230 bp and 500 bp corresponding to the *sodA* and *seeH* genes, respectively.

3.3. Pulsed-Field Gel Electrophoresis

All *S. equi* isolates displayed *Sma*I and *Bsp*120I restriction endonuclease digestion profiles. Visual comparison of macrorestriction patterns generated with the *Sma*I and *Bsp*120I enzymes revealed three different DNA fragment profiles with variations of one or two bands (Figs. 1–3). Pulsotypes were assigned with consecutive numbers. A dendrogram constructed using the UPGMA method with

BioNumerics software showed the strains shared over 95% similarity in their PFGE pattern (data not shown).

Fifty-four isolates from 20 animals with acute strangles and seven isolates from three carrier animals were classified in the same genetic profile (profile 2/3: pulsotype 2 with *Bsp*120I and 3 with *Sma*I), which therefore predominated. Interestingly, the other 19 isolates were classified into two patterns (profile 1/1: pulsotype 1 with *Bsp*120I and 1 with *Sma*I; and profile 3/2: pulsotype 3 with *Bsp*120I and 2 with *Sma*I) that were closely related to the outbreak strain because they have only one or two different bands. Twelve isolates from horses with acute strangles and six isolates from carriers showed the profile 1/1, and only one isolate from clinical strangles showed the profile 3/2.

It is worth pointing out that the same PFGE profile was found in isolates obtained from the same horse, and also the same PFGE profile was detected in isolates obtained from horses, which had acute infections at the same time.

Tables 1 and 2 show PFGE pulsotypes and epidemiological data of the isolates obtained from acute and persistent stage, respectively.

4. Discussion

Strangles in an endemic infectious disease affecting horses in Argentina. A high number of outbreaks has been produced in several farms in the last years that produces large economic losses. The present study describes molecular approaches using PFGE to characterize *S. equi* isolates to distinguish strains circulating in different stables in Buenos Aires, Argentina, and this information may contribute in the knowledge of the *S. equi* behavior. The samples from horses with acute strangles infection and healthy animals, between 2010 and 2013, represented a wide geographic distribution within Buenos Aires province.

As mentioned in section 1, several studies have demonstrated that the population of *S. equi* is genetically closely related [25–31,34–36]. In our study, isolates were classified in only three profiles using *Sma*I (Figs. 1 and 2) and *Bsp*120I (Figs. 1 and 3). The predominant profile, had pulsotype 2 with *Bsp*120I and 3 with *Sma*I (profile 2/3). The other isolates showed one or two fragments of difference with this strain (Figs. 1 and 2) and were classified as closely related. The reference strain ATCC 33398 was considered unrelated to the predominant strain because it had variations on more than seven bands (data not shown) and it may be related with the different geographic and temporal source of strains suggesting the epidemiological usefulness of the PFGE. Although Tenover et al. guidelines [33] are frequently used, other researchers [25–27] consider different strains of *S. equi* when the isolates show at least one band of difference in their PFGE profiles. The present study reveals the presence of three strains by PFGE and discriminatory power may be higher adding other molecular techniques.

It is interesting to point out the fact that our 80 *S. equi* isolates presented 16 constant fragment sizes with *Bsp*120I digestion and five constant fragment sizes with *Sma*I and only one or two different bands with both enzymes (Fig. 1). Our results may be related to the restriction sites of both enzymes in the *S. equi* reference strain 4047 genome [14]. *Sma*I cuts at 18 sites, most of which are in 16S rRNA, 23S rRNA, and tRNA sequences and also in phiSeq1. *Bsp*120I cuts at 45 sites, which are in 16S rRNA and in several other predicted coding sequences throughout the genome including three sites in phiSeq1, one site in phiSeq2, and one site in phiSeq4. A collection of 224 geographically and

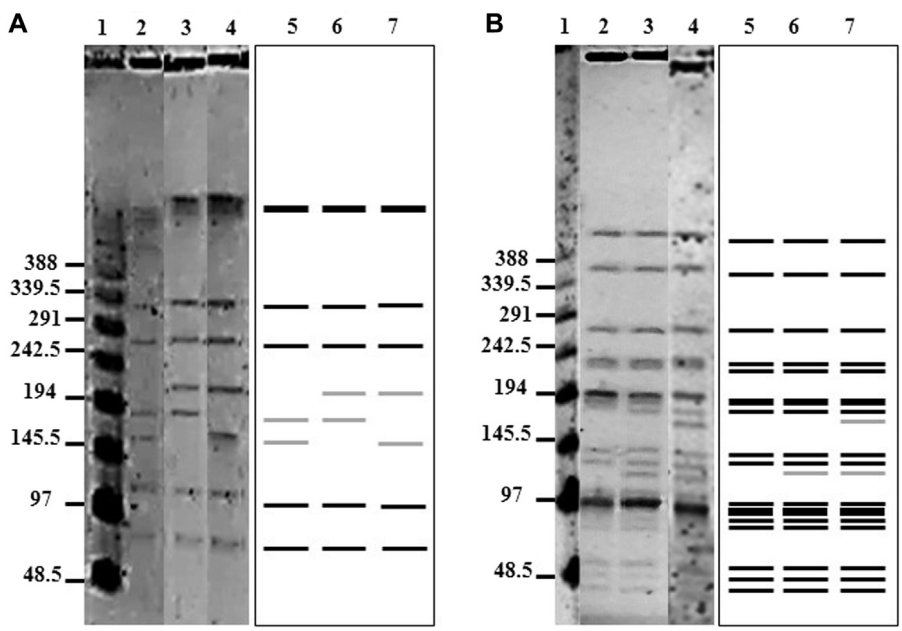

Fig. 1. Pulsotypes. PFGE pulsotypes of DNA of *S. equi* strains after *Sma*I and *Bsp*120I digestion and schematic PFGE pulsotypes where constant fragment sizes with each enzyme are displayed in black and variable bands in grey. (A) PFGE pulsotypes after *Sma*I digestion, lane 1: lambda ladder PFG marker; lanes 2, 5: pulsotype 1; lanes 3, 6: pulsotype 2; lanes 4, 7: pulsotype 3. (B) PFGE pulsotypes after *Bsp*120I digestion, lane 1: lambda ladder PFG marker; lanes 2, 5: pulsotype 1; lanes 3, 6: pulsotype 2; lanes 4, 7: pulsotype 3. PFGE, pulsed-field gel electrophoresis.

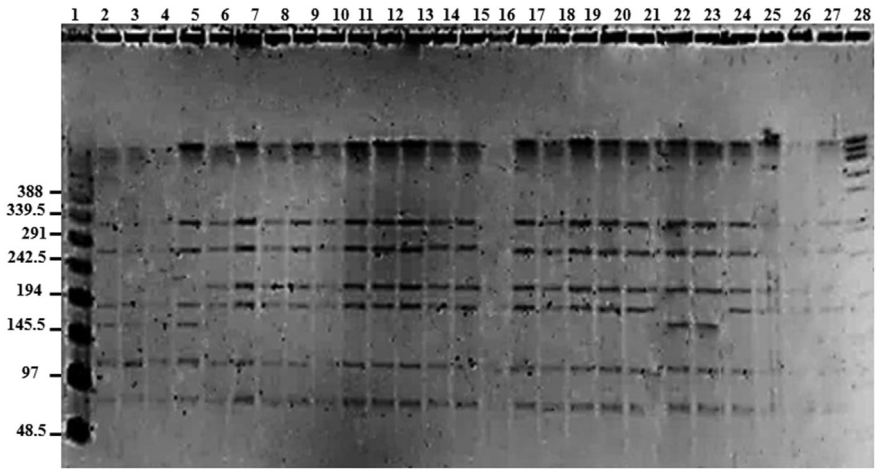

Fig. 2. *Sma*I PFGE pulsotypes. PFGE pulsotypes of DNA of Argentine *S. equi* strains after *Sma*I digestion, lane 1: lambda ladder PFG marker; lanes 2–5: *Sma*I pulsotype 1; lanes 6–15, 17–21, and 24–27: *Sma*I pulsotype 2; lanes 22–23: *Sma*I pulsotype 3; lane 28: *Salmonella* serotype Braenderup strain (H9812) digested with *Xba*I. PFGE, pulsed-field gel electrophoresis.

temporally diverse *S. equi* isolates have been sequenced by Harris et al. [34], and a low frequency of recombination within the *S. equi* population has been found. An interesting observation in the dynamics of the accessory genome is that phiSeq1 appeared more dynamic, with evidence of alternative but related prophages in the accessory genomes of isolates [34] and this could explain the different PFGE profiles obtained.

A chronological occurrence and spatial distribution of the strains was observed (Table 1). The strain with profile 1/1 was first identified in two near cities, Pilar and Baradero, in November 2010 and March 2011, respectively, and then, in Capilla del Señor in April 2012 and in Trenque Lauquen in August 2012. The strain with the most frequent profile (profile 2/3) was also the most widely distributed and it was found in Trenque Lauquen, Daireaux, Olavarría, Mones Cazón, Luján, Capilla del Señor, Roque Pérez, Bahía Blanca, Pilar, and Lobos between May 2011 and May 2013. Finally, the strain with profile 3/2 was isolated only from Capilla del Señor in a horse with acute infection and carriers in May 2013 and June 2013, respectively. Although the profile 2/3 was found in different cities of Buenos Aires province, the

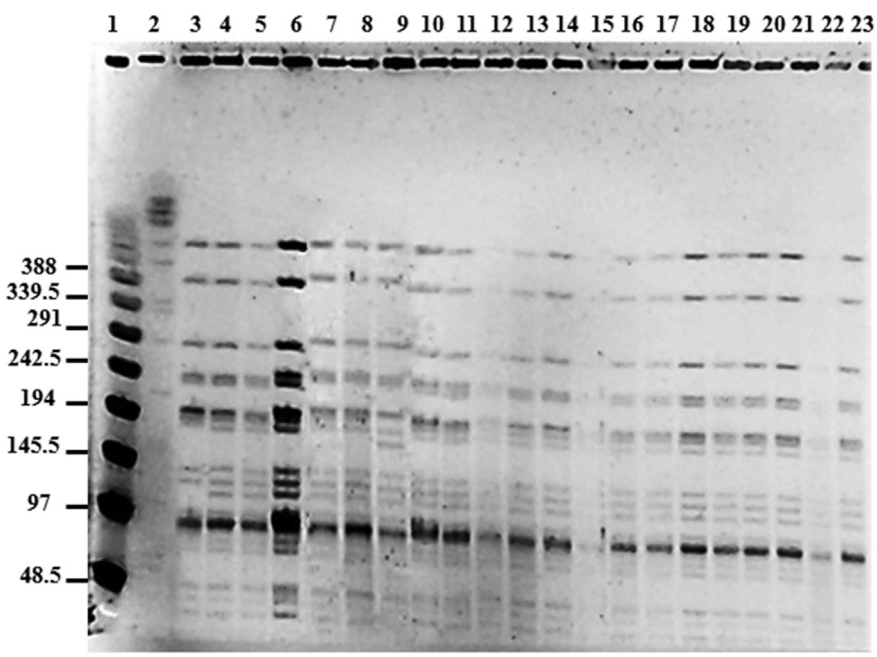

Fig. 3. *Bsp*120I PFGE pulsotypes. PFGE pulsotypes of DNA of Argentine *S. equi* strains after *Bsp*120I digestion, lane 1: lambda ladder PFG marker; lane 2: *Salmonella* serotype Braenderup strain (H9812) digested with *Xba*I; lane 3: *Bsp*120I pulsotype 1; lanes 4–8, 10–14, 16–21, and 23: *Bsp*120I pulsotype 2; lane 9: *Bsp*120I pulsotype 3. PFGE, pulsed-field gel electrophoresis.

three PFGE profiles were identified in the largest equine productive area of Buenos Aires province (near of Ciudad Autónoma de Buenos Aires). These may be possible because the constant movement of the horses in this geographical area permitting the mixing of different strains.

Our data highlighted the presence of different strains in the same stable. Also, our results showed that the PFGE profiles of isolates from carriers and the PFGE profile of isolates obtained from horses that were infected in a previous outbreak were always the same. These occurred in the horses that belonged to the stable 16 in Capilla del Señor (Table 1): the strain with profile 1/1 was the first one in April 2012, the strain with profile 2/3 was the second one in August 2012, and the strain with profile 3/2 was the last one in May 2013. Interestingly, in Capilla del Señor, the profile 2/3 was identified in isolates that had been obtained in the outbreak 13, in August 2012, and the same profile had been found in isolates from horses recovered from this outbreak (horses P9 and P56) 7 months later. Also, the profile 2/3 was identified in isolates from the foal P22 that had been the only one infected 1 month before, and the profile 3/2 was found in isolates that had obtained in the outbreak 15, and the same profile 1 month later was identified from recovered animals (horses P88, P90, P91, and P93). Unfortunately, the samples from the horses recovered from the outbreak 13 that occurred in April of 2012 in Capilla del Señor could not be taken. Also, the PFGE profile 2/3 was identified from horses with acute infection (E30) and carriers (P69) from the same stable in Roque Pérez. All these findings suggest that the horses recovered from the same outbreak of strangles continue to carry the same strain in agreeance with descriptions by other researchers [6,9,37].

The same strain was isolated from horses with acute infection in 2011 and 2012 in stable 9 in Olavarría (E15, E16, E25, and E26). In this case, persistently infected horses were able to maintain the strain in the stable and spread the infection to naïve horses from one year to another because these carriers were not treated to eliminate the infection. On the other hand, the susceptible animals from the stable in Capilla del Señor were vaccinated three times per year with autologous immunogens manufactured with strains isolated in the farm, as is the usual practice in Argentina and Brazil. Nevertheless, vaccinated animals have been mild infected with a different strain from that of carriers in Capilla del Señor, which suggest that the carriers were not the source of these infections. Another explanation may be that the isolates may mutate in the nasopharynx or guttural pouches, and then these carriers have been the source of infection for susceptible horses. Although the epidemiological relevance of strangles carriers has been widely studied [2,4,7,9,37] and it is possible that different healthy horses play a role as reservoirs of *S. equi* [34,35], and these three strains may coexist in the same stable in Capilla del Señor.

As previously mentioned, autologous vaccines have usually used in Argentine. These vaccines are prepared from a broth culture of freshly isolated *S. equi* inactivated with formaldehyde and then inoculated in naïve horses to prevent strangles. Clinical efficacy of this type of vaccine has been reported by practitioners and by Brazilian researches. Moboni et al. [38] have been obtained an increased serum bactericidal activity and have reported a lower incidence and severity of strangles in vaccinated horses concluding that autologous immunogens may confer a satisfactory protection against strangles in endemic farms. Clinical efficacy may be related with some immunogenic difference between isolates or with the fact that recently isolated strains may have a better immunogenic power than laboratory strains used as bacterins.

Finally, our results are in agreement with other studies that used several molecular methods to differentiate isolates of *S. equi* and concluded that PFGE as a useful discriminating method [25–27]. Nevertheless, PFGE is not very good at discriminating closely related strains as it relies on large-scale variation events or variation within the limited number of restriction site sequences. Other molecular studies including SeM subtyping of *S. equi* should be performed to increase the discriminatory power of the technique and to compare local strains with other strains isolated in other countries. Moreover, genome sequencing would greatly improve the knowledge of these strains.

5. Conclusion

This is the first molecular characterization of Argentine isolates obtained between 2010 and 2013 in Buenos Aires province, Argentina. The relevance of this work resides in the fact that identical profiles were found in isolates from the same clinical sample and the same outbreak and it may indicate that PFGE is a useful tool for tracing sources and possible spread of the infection. Our findings also suggest that the horses recovered from strangles continue to carry the same strain in their nasopharynx. Some vaccinated horses have been mild infected for a different strain from that of carriers suggesting other source of infection so the impact of the vaccination with autologous immunogens should be investigated. Additional studies based on molecular characterization are needed to further investigate the epidemiological relationship between these isolates.

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References

[1] Sweeney C, Timoney J, Newton J, Hines M. *Streptococcus equi* infections in horses: guidelines for treatment, control, and prevention of strangles. J Vet Intern Med 2005;19:123–34.
[2] Boyle A. *Streptococcus equi* subspecies *equi* infection (strangles) in horses. Compend Contin Educ Vet 2011;33:E1–7.

[3] Waller AS. Strangles: taking steps towards eradication. Vet Microbiol 2013;167:50–60.
[4] Waller S, Sellon D, Sweeney C, Timoney P, Newton R, Hines M. Streptococcal infection. In: Sellon D, Long M, editors. Equine infectious diseases. 2nd ed. Ed Saunders Elsevier; 2013. p. 265–77.
[5] Waller AS, Paillot R, Timoney JF. *Streptococcus equi*: a pathogen restricted to one host. J Med Microbiol 2011;60:1231–40.
[6] Newton J, Wood J, Dunn K, DeBrauwere M, Chanter N. Naturally occurring persistent and asymptomatic infection of the guttural pouches of horses with Streptococcus equi. Vet Rec 1997;140:84–90.
[7] Erol E, Locke SJ, Donahoe JK, Mackin MA, Carter CN. Beta-hemolytic *Streptococcus* spp. from horses: a retrospective study (2000-2010). J Vet Diagn Invest 2012;24:142–7.
[8] Sjöblom A. Complications to strangles in horses presented at referral hospitals. Final Project. Uppsala, Suecia: Faculty of Veterinary Medicine and Animal Science; 2014.
[9] Newton JR, Verheyen K, Talbot NC, Timoney JF, Wood JL, Lakhani KH, Chanter N. Control of strangles outbreaks by isolation of guttural pouch carriers identified using PCR and culture of *Streptococcus equi*. Equine Vet J 2000;32:515–26.
[10] Timoney JF. The pathogenic equine streptococci. Vet Res 2004;35: 397–409.
[11] Bustos CP, Munoz AJ, Di Gennaro E, Rossano M, Guida N. Adenitis equina: Detección de portadores de *Streptococcus equi* subsp. *equi*. Revista de Medicina Veterinaria 2012;93:28–35.
[12] Taylor SD, Wilson WD. *Streptococcus equi* subsp. *equi* (Strangles) infection. Clin Tech Equine Pract 2006;5:211–7.
[13] Webb K, Barker C, Harrison T, Heather Z, Steward KF, Robinson C, Newton JR, Waller AS. Detection of *Streptococcus equi* subspecies *equi* using a triplex qPCR assay. Vet J 2013;195:300–4.
[14] Holden MT, Heather Z, Paillot R, Steward KF, Webb K, Ainslie F, Jourdan T, Bason NC, Holroyd NE, Mungall K, Quail MA, Sanders M, Simmonds M, Willey D, Brooks K, Aanensen DM, Spratt BG, Jolley KA, Maiden MC, Kehoe M, Chanter N, Bentley SD, Robinson C, Maskell DJ, Parkhill J, Waller AS. Genomic evidence for the evolution of *Streptococcus equi*: host restriction, increased virulence, and genetic exchange with human pathogens. PLoS Pathog 2009;5:e100346.
[15] Las Heras A, Vela AI, Fernández E, Legaz E, Domínguez L, Fernández-Garayzábal JF. Unusual outbreak of clinical mastitis in dairy sheep caused by *Streptococcus equi* subsp. *zooepidemicus*. J Clin Microbiol 2002;40:1106–8.
[16] Kuusi M, Lahti E, Virolainen A, Hatakka M, Vuento R, Rantala L, Vuopio-Varkila J, Seuna E, Karppelin M, Hakkinen M, Takkinen J, Gindonis V, Suponen K, Huotari K. An outbreak of *Streptococcus equi* subspecies *zooepidemicus* associated with consumption of fresh goat cheese. BMC Infect Dis 2006;6:36.
[17] Lindahl SB, Aspán A, Båverud V, Paillot R, Pringle J, Rash NL, Söderlund R, Waller AS. Outbreak of upper respiratory disease in horses caused by *Streptococcus equi* subsp. *zooepidemicus* ST-24. Vet Microbiol 2013;166:281–5.
[18] Preziuso S, Vincenzo C. A multiplex polymerase chain reaction assay for direct detection and differentiation of β-hemolytic streptococci in clinical samples from horses. J Equine Vet Sci 2012;32:292–6.
[19] Alber J, El-Sayed A, Lammler C, Hassan A, Weiss R, Zschock M. Multiplex polymerase chain reaction for identification and differentiation of *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equi*. J Vet Med B 2004;51:455–8.
[20] Barrett TJ, Gerner-Smidt P, Swaminathan B. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. Foodborne Pathog Dis 2006;3:20–1.
[21] Struelens MJ. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 1996;2:2–11.
[22] Parizad EG, Parizad EG, Valizadeh A. The application of pulsed field gel electrophoresis in clinical studies. J Clin Diagn Res 2016;10:DE01–4.
[23] Pillai P, Srinivasan U, Zhang L, Borchardt SM, Debusscher J, Marrs CF, Foxman B. *Streptococcus agalactiae* pulsed-field gel electrophoresis patterns cross capsular types. Epidemiol Infect 2009;137:1420–5.
[24] Sánchez del Rey V. Estructura y diversidad de la población de "Streptococcus suis" presente en el ganado porcino, jabalí y conejo silvestre. PhD Thesis. Universidad Complutense de Madrid; 2015.
[25] Lindmark H, Jonsson P, Engvall E, Guss B. Pulsed-field gel electrophoresis and distribution of the genes *zag* and *fnz* in isolates of *Streptococcus equi*. Res Vet Sci 1999;66:93–9.
[26] Lanka S, Borst LB, Patterson SK, Maddox CW. A multiphasic typing approach to subtype *Streptococcus equi* subspecies *equi*. J Vet Diagn Invest 2010;22:928–36.
[27] Lindahl S, Söderlund R, Frosth S, Pringle J, Båverud V, Aspán A. Tracing outbreaks of *Streptococcus equi* infection (strangles) in horses using sequence variation in the *seM* gene and pulsed-field gel electrophoresis. Vet Microbiol 2011;153:144–9.
[28] Ivens P, Matthews D, Webb K, Newton J, Steward K, Waller A, Robinson C, Slater J. Molecular characterisation of 'strangles' outbreaks in the UK: the use of M-protein typing of *Streptococcus equi* ssp. *equi*. Equine Vet J 2011;43:359–64.
[29] Libardoni F, Vielmo A, Farias L, Matter LB, Pötter L, Spilki FR, Vargas AC. Diversity of *seM* in *Streptococcus equi* subsp. *equi* isolated from strangles outbreaks. Vet Microbiol 2013;162:663–9.
[30] Moloney E, Kavanagh KS, Buckley TC, Cooney JC. Lineages of *Streptococcus equi* ssp. *equi* in the Irish equine industry. Ir Vet J 2013;66:10.
[31] Chanter N, Talbot NC, Newton JR, Hewson D, Verheyen K. *Streptococcus equi* with truncated M-proteins isolated from outwardly healthy horses. Microbiology 2000;146:1361–9.
[32] Ministerio de Agricultura, Ganadería y Pesca, MAGyP, Argentina. 2013, Available at: http://www.minagri.gob.ar/. Accessed August 2016.
[33] Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233.
[34] Harris SR, Robinson C, Steward KF, Webb KS, Paillot R, Parkhill J, Holden MT, Waller AS. Genome specialization and decay of the strangles pathogen, *Streptococcus equi*, is driven by persistent infection. Genome Res 2015;25:1360–71.
[35] Kelly C, Bugg M, Robinson C, Mitchell Z, Davis-Poynter N, Newton JR, Jolley KA, Maiden MC, Waller AS. Sequence variation of the *seM* gene of *Streptococcus equi* allows discrimination of the source of strangles outbreaks. J Clin Microbiol 2006;44:480–6.
[36] Webb K, Jolley KA, Mitchell Z, Robinson C, Newton JR, Maiden MC, Waller A. Development of an unambiguous and discriminatory multilocus sequence typing scheme for the *Streptococcus zooepidemicus* group. Microbiology 2008;154:3016–24.
[37] Newton J, Wood J, Chanter N. Strangles: long term carriage of *Streptococcus equi* in horses. Equine Vet Education 1997;9:98–102.
[38] Moboni G, Leivas Leite F, Moboni F, Ziech R, Libardoni F, Marchioro S, Castagna de Vargas A. Uso experimental de vacuna autógena contra adenite equina. In: Proceedings of XVIII Jornadas de Jóvenes Investigadores de la AUGM. Santa Fé, Argentina, October 19, 20 and 21, 2010, Argentina: Asociación de Universidades del Grupo Montevideo; 2010. p. 320.