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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).