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Vaginal isolation of beta-haemolytic *Streptococcus* from bitches with and without neonatal deaths in the litters

AE Guerrero^{1,2} | MC Stornelli¹ | SB Jurado³ | G Giacoboni² | GH Sguazza⁴ |
RL de la Sota¹ | MA Stornelli¹ ¹Cátedra y Servicio de Reproducción Animal, La Plata, Argentina²Cátedra de Microbiología, La Plata, Argentina³Servicio Central de Microscopía Electrónica, La Plata, Argentina⁴Laboratorio de Virología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina**Correspondence**Maria A. Stornelli, Cátedra y Servicio de Reproducción Animal, La Plata, Argentina.
Email: astornel@fcv.unlp.edu.ar**Funding information**

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Contents

The aim of the study was to identify beta-haemolytic streptococci in the vagina of bitches who had delivered healthy litters and bitches who had delivered litters in which neonatal deaths occurred. Fifty-one bitches divided into two groups were used. Group 1 (G1) included 28 bitches that had delivered healthy litters and group 2 (G2) included 23 bitches that had delivered puppies who died in the neonatal period. Two vaginal samples were taken, one in proestrus and the other at the end of gestation (EG). Beta-haemolytic *Streptococcus* (BS) was isolated from 16 bitches (57%) in G1 and from 21 bitches (91%) in G2. The bacteriological cultures, serological tests (Streptex[®]) and PCR assay allowed identification of *Streptococcus canis* and *Streptococcus dysgalactiae* in G1 and G2. Ultramicroscopic studies allowed the observation of M Protein and capsules in strains of *S. dysgalactiae* and *S. canis* in G1 and G2. The *S. canis* strains isolated from G2 showed thicker capsules than *S. canis* strains isolated from G1 (234 ± 24.2 vs 151.23 ± 28.93 nm; $p < .001$). No differences were observed in capsule thickness between strains of *S. dysgalactiae* isolated from G1 and G2 (210 ± 13.54 vs 211.66 ± 19.67 nm; $p > .70$). All strains of beta-haemolytic *Streptococcus* isolated were penicillin sensitive. Penicillin was administered from EG to 5 days *post-partum* in 10 G2 females with isolation of BS (G2A). Saline solution was administered in eleven G2 females with isolation of BS (G2B). Ninety per cent of the puppies survived in G2A and 25% survived in G2B. Our results suggest BS is involved in canine neonatal deaths.

1 | INTRODUCTION

Pup mortality, both during parturition and in the neonatal period, is a common and big problem in kennels, although poorly documented in the veterinary literature (Gill, 2001; Münnich, 2008; Tønnessen, Borge, Nødtvedt, & Indrebø, 2012). Losses of puppies may occur *in utero*, during expulsion, after birth, in the first weeks of life and after weaning. The rate of perinatal death (stillborn puppies and neonates) is highly variable but highest during parturition, immediately after birth and in the first days of life. All together morbidity and mortality range from 5 to 35% (Mosier, 1981; Münnich, 2008; Poffenbarger, Chandler, Ralston, & Olson, 1990; Veronesi, Panzani, Faustini, & Rota, 2009). Pup mortality has been attributed to a wide

variety of causes including dystocia, congenital defects, low birth weight, infectious diseases, trauma and fading puppy syndrome (Gill, 2001; Mila et al., 2017). The principal cause of pup death was attributed to maternal factors and mothering failure (trauma, excessive licking, lactational failure and cannibalism; Andersen, 1957; Gill, 2001). Infectious diseases are the second most important cause of mortality and account for a large proportion of newborn losses (Münnich, 2008). In this way, infection with bacteria, rather than viruses, is the most common cause of death in the first week of age (Münnich, 2008; Nielsen, van der Gaag, Knol, & Shukken, 1998). However, information on sources of infections in newborn dogs is still limited. The environment and mothers are suspected as sources of infection (vaginal discharge, milk, faeces, oropharynx, skin) for

puppies (Davidson, 2003; Münnich & Lübke-Becker, 2004), similar to the situation in humans (Münnich, 2008). When puppies are exposed to bacteria and their defence mechanisms are overwhelmed, severe illness and newborn losses occur (Münnich, 2008). Reports show that the bacteria most frequently involved in neonatal mortality in dogs are *E. coli*, staphylococci, streptococci, *Klebsiella* spp., *Proteus mirabilis* and *Pseudomonas aeruginosa* (Bjurström, 1993; Davidson, 2003; Johnston, Kuztritz, & Olson, 2001; Mosier, 1981; Münnich, 2008; Sager & Remmers, 1990; Schäfer-Somi, Spargser, Breitenfellner, & Aurich, 2003). *Streptococcus canis* is the species most frequently identified from cases of septicemia in newborn puppies, but other species of beta-haemolytic streptococci, such as *Streptococcus agalactiae*, *Streptococcus equi subsp. zooepidemicus* or *Streptococcus dysgalactiae subsp. dysgalactiae* have also been implicated (Greene, 2012; Vela et al., 2012).

In humans, *S. agalactiae* or Lancefield group B (GBS) has been identified as one of the most important causal agents of serious infections and neonatal sepsis (Schrag et al., 2000; Schuchat, 1998). Around 40% of all pregnant women present rectal and/or vaginal GBS colonization (Regan, Klebanoff, & Nugent, 1991). Vertical transmission from mother to the neonate has been documented (Edwards et al., 2008; Winn, 2007). In humans, the culture-based method is recommended for screening GBS. The standard method for the diagnosis of GBS colonization consists of culturing combined vaginal and anal swabs in a selective medium (Schrag et al., 2000). The antibiotic prophylaxis *intrapartum* for those women who have positive cultures for rectovaginal GBS colonization reduce neonatal infection (Bergeron et al., 2000; Boyer & Gotoff, 1986; Garland & Fliegner, 1991; Muller, Oostvogel, Steegers, & Dörr, 2006). Aqueous penicillin G is the drug of choice in women. Like in women, in bitches, beta-haemolytic streptococci vaginal colonization could produce vertical infection and neonatal disease. The aim of this work was to identify beta-haemolytic streptococci in the vagina of bitches who had delivered healthy litters and bitches who had delivered litters in which neonatal deaths occurred.

2 | MATERIALS AND METHOD

2.1 | Animals

Fifty-one clinically healthy, intact bitches were used in this study. Bitches were from 10 different breeds (English Bulldog, French Bulldog, German Shepherd, Dogo Argentino, Dogue de Bordeaux, Pug, Schnauzer, Bernese, Labrador Retriever, Great Dane), were aged between two and 6 years old and weighed between 7 and 40 kg. All bitches were referred to the University Veterinary Teaching Hospital of La Plata, Argentina to identify the proper timing of mating or insemination at the onset of the heat. Only bitches that became pregnant were included in the study. Animal care and experimentation complied with the International Guiding Principles for Biomedical Research Involving Animals (National Research Council, 2012). The Graduate School and the Laboratory Animal Care and Use Committees of the Faculty of Veterinary Sciences at National University of La Plata approved this study.

Animals were divided into two groups, group 1 (G1) included 28 bitches that have delivered healthy litters and group 2 (G2) included 23 bitches that have delivered puppies who died in the neonatal period in the previous two or three parturitions.

2.2 | Samples

2.2.1 | Vaginal cytology

Each bitch was observed for sexual behaviour and clinical signs of heat. Vaginal cytology samples were obtained at the onset of heat and every other day until the onset of diestrus. Vaginal cytology samples were obtained by introducing a sterile cotton-tipped swab into the vagina and gently rotating it against the floor and lateral walls of the vagina. The swab was gently rolled over a clean microscopic slide, and smears were air-dried and stained with a modified Wright's stain. Stained slides were examined at 100 and 400x to count parabasal, intermediate and superficial cells. According to the percentage and type of cells present in the slide, proestrus and diestrus were identified (Johnston et al., 2001).

2.2.2 | Bacteriological samples

Two vaginal samples were taken from each bitch, one in proestrus (PR; between three and 7 days after the beginning of heat; sample 1[S1]) and the other one at the end of gestation (EG; between 45 and 48 days after the first day of cytological diestrus; sample 2[S2]). Samples were taken from the cranial portion of the vagina using a sterile vaginal swab through a sterile vaginoscope. The swabs were immediately introduced into Stuart media and transported to the microbiology laboratory (Groppetti, Pecile, Barbero, & Martino, 2012).

2.3 | Sample processing

2.3.1 | Bacteriological study

Clinical samples were surface plated on Columbia agar (Oxoid, Britania S.A, CABA, Argentina) with 5% sheep blood, selective media for *Enterobacteriaceae* (MacConkey agar; Oxoid, Britania S.A, CABA, Argentina) and agar TKT (toxine, kristal-violet, tallium) for streptococci. Agar plates were incubated at 37°C aerobically for 24 or 48 hr. The result was negative if bacterial growth was not visible after 48 hr. In addition, cultural procedures on blood agar media were performed after a 2-day enrichment in fluid Thioglycollate medium (Becton Dickinson, Sparks, MD, USA) and Trypticase Soy broth (Becton Dickinson). Bacterial identification was carried out on all isolates using standard identification methods (Schäfer-Somi et al., 2003; Winn et al., 2006).

2.3.2 | Identification of beta-haemolytic streptococci

After incubation, blood agar plates were inspected for beta-haemolytic colonies. The isolates were characterized by phenotype,

based on Gram stain and cell morphology. For beta-haemolytic streptococci, biochemical characterization was achieved (fermentation of lactose, maltose, cellobiose, sorbitol, arabinose, mannitol and trealosa, Bile Aesculin hydrolysis test [TP 2–Aesculin Hydrolysis Test], arginine hydrolysis test and Vogues Proskauer test) and colonies were submitted to the CAMP (Christie, Atkins, Munch, Petersen) test (Winn et al., 2006). In addition, Lancefield serological group reaction was determined (Daly & Seskin, 1988).

2.3.3 | Serological identification of streptococci

The streptococci serological identification was performed according to Lancefield classification (Lancefield, 1933) using the Streptex® Grouping Kit according to instructions defined by the manufacturer (Streptex, Clipper Boulevard West, Crossways Dartford, Kent, DA2 6PT UK; Daly & Seskin, 1988).

2.3.4 | Identification of streptococci by PCR amplification methods

The DNA was extracted from 10 colonies of *S. canis* or *S. dysgalactiae* grown in pure culture, suspended in 100 µl of PCR water incubated at 100°C for 10 min in a thermoblock, to produce a thermal lysis of the cell wall, and cooled in ice for 3 min. The supernatant, obtained by centrifugation at 4293 g for 5 min, was used as a template for PCR (Preziuso, Laus, Tejada, Valente, & Cuteri, 2010).

To identify *S. canis*, PCR was performed with primers: Camp-I: 5'-CAA TTA ACT AAT AAG GTA GAA CAG-3' and camp-II 5'-CTC TCT CAA AAC GGG TG-3' (Hassan, Khan, Abdulmajjood, & Lämmler, 2003). To identify *S. dysgalactiae*, PCR was performed with primers: dyCF 5'-AAG GAA ATG GAA CAC GTT AGG G-3' and adyR 5'-TCC TAC CAT GAC ACT AAT GTG TC-3 (Kawata et al., 2004). The oligonucleotide primers were custom synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA).

For DNA amplification, 12.5 µl of 2x PCR MasterMix (Promega; Fitchburg, Wisconsin, United State), 10 pmoles of each primer, 2.5 µl of DNA template and 8 µl of nuclease free water were mixed. PCR was performed in a thermal cycler (Cycler, Bio-Rad) using the following parameters: one cycle at 95°C for 5 min, 30 cycles at 94°C for 1 min, 58°C for 1.5 min and 72°C for 1.5 min, followed by one cycle at 72°C for 5 min.

The electrophoretic analysis was performed using 2% agarose gel (Biodynamics. CABA, Argentina) and stained with ethidium bromide, using a marker of 100 pb (Promega; Fitchburg, Wisconsin, United State). In each run, water instead of DNA was used as negative control. DNA of ATCC® 43497™ (*S. canis*) and ATCC® 43078™ (*S. dysgalactiae*) was used as positive controls. The positivity consisted of a band corresponding to 238 bp and 1508 bp, respectively.

2.3.5 | Antibiotic sensitivity

Antibiotic sensitivity for beta-haemolytic streptococci was carried out by the Kirby-Bauer method. Antimicrobials tested were penicillin

(10UI), vancomycin (30ug) and eritromycin (15ug; Britania S.A., CABA, Argentina) according to CLSI standards (CLSI, 2013; CLSI, 2016).

2.3.6 | Therapeutic trial

Twenty-one G2 bitches with isolation of beta-haemolytic *Streptococcus* were included in the therapeutic trial. G2 bitches were divided into two groups. In G2A ($n = 10$), benzathine Penicilin G (Penicilina G benzatínica®; Veterquímica) 24.000 IU/kg/48 hr (Bonagura & Tewdt, 2008) was administered from 50 days after the first day of cytological diestrus to five days *post-partum*. In G2B ($n = 11$), saline solution was administered from 50 days after the first day of cytological diestrus to 5 days *post-partum*.

2.3.7 | Ultramicroscopic study

When beta-haemolytic streptococci were isolated, to perform after species identification and to study the presence or absence of M protein and capsules, isolated colonies were observed using a transmission electron microscope (TEM). Colonies were suspended in sterile saline solution for direct observation using negative staining. In addition, after initial isolation, bacteria were transferred into a blood agar media and incubated for 24 hr or 48 hr at 37°C under aerobic conditions to obtain a sufficient number of bacterial colonies to process the sample for TEM. Then, colonies were processed to obtain ultrathin sections and were finally observed with TEM. To do negative staining, a bacterial suspension drop was put on a copper mesh (400 mesh) coated with collodion for 5 min. After that, the excess was removed on a filter paper. Bacteria adhered to the mesh were negatively stained with phosphotungstic acid 2% for 40 s and then observed with TEM (JEM 1200 EX II; JEOL Ltd., Tokyo, Japan). To obtain ultrathin sections, colonies were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer with 0.5% of alcian blue and post-fixed in 1% osmium tetroxide (OsO₄). The samples were centrifuged; the pellets were dehydrated and then embedded in epoxy resin. Ultrathin serial sections (90 nm) were picked up on copper grids, counterstained with uranyl acetate and lead citrate and examined in a JEM 1200 EX II (JEOL, Japan) TEM microscope at 80 Kv.

The bacterial morphology and M protein and capsule presence were studied. Capsule thickness was measured on micrographs taken during the electron microscopic observations. For each beta-haemolytic *Streptococcus* strain, mean values were calculated from 10 to 15 *Streptococcus* cells per micrograph.

2.4 | Statistics analyses

Data were analysed by ANOVA. The model used included the effect of group (bitches with history of delivering health puppies [G1]; bitches with history of delivering puppies that died in the neonatal period [G2]), stage of cycle that bacteriological sample was taken (proestrus [PR], end of gestation [EG]); and type of bacteria isolated (*Staphylococcus* spp. [STA], beta-haemolytic *Streptococcus* [BHS], *E. coli*, *Proteus* spp., non-fermenting Gram-negative bacilli [NFGNB], *Enterococcus* spp.

TABLE 1 Percentage of bacteria isolated from vaginal samples obtained from Group 1 bitches

Bacteria	S1 ^A	S2 ^B
β -haemolytic <i>Streptococcus</i>	57.1%	35.7%
<i>Staphylococcus</i> spp.	42.8%	46.28%
<i>Escherichia coli</i>	21.42%	28.57%
NFGNB ^C	14.28%	14.28%
<i>Enterococcus</i> spp.	3.6%	3.6%
<i>Proteus</i> spp.	8.57%	5.71%

S1^A, proestrus [PR] sample; S2^B, end of gestation [EG] sample; NFGNB^C, non-fermenting Gram-negative bacilli.

TABLE 2 Percentage of bacteria isolated from vaginal samples obtained from Group 2 bitches

Bacteria	S1 ^A	S2 ^B
β -haemolytic <i>Streptococcus</i>	78.3%	69.6%
<i>Staphylococcus</i> spp.	43.47%	34.8%
<i>Escherichia. Coli</i>	21.7%	21.7%
NFGNB ^C	13.1%	4.3%
<i>Enterococcus</i> spp.	13.1%	13.1%
<i>Proteus</i> spp.	8.7%	8.7%

S1^A, proestrus [PR] sample; S2^B, end of gestation [EG] sample; NFGNB^C, non-fermenting Gram-negative bacilli.

[ENTB]). The type of bacteria isolated was analysed by categorical data analysis using the GENMODE procedure of SAS (2003) with binomial distribution and logit link function. The *Streptococcus* capsule thickness was analysed by general linear models using the MIXED procedure of SAS® (2003). Data are shown as least squares means \pm standard error (LSM \pm SE), and statistical significance was set at $p < .05$.

3 | RESULTS

In vaginal samples, *Staphylococcus* spp., β -haemolytic *Streptococcus*, *E. coli*, *Proteus* spp., NFGNB and *Enterococcus* spp. were found (Table 1 and Table 2). Vaginal samples showed bacterial growth of two, three or four genera. No difference was observed between groups in bacterial genera isolation. The biochemical, physiological and serological tests used for typifying β -haemolytic streptococci allowed identification of *Streptococcus dysgalactiae* and *Streptococcus canis* in vaginal samples. The PCR reaction confirmed the species of *streptococcus* typified by biochemical and serological methods.

In G1 and G2, β -haemolytic *Streptococcus* isolated was biochemically characterized as *S. canis* and *S. dysgalactiae*. In G1,

Lancefield serological group and PCR reaction identified nine strains as G Lancefield group (*S. canis*) and seven as C Lancefield group (*S. dysgalactiae*). In G2, Lancefield serological group and PCR reaction identified 16 strains as G Lancefield group (*S. canis*) and five as C Lancefield group (*S. dysgalactiae*; Table 3).

The percentage of samples (S1 and S2) with β -haemolytic *Streptococcus* in the vagina was higher in G2 compared with G1 (73.91 vs 46.43 \pm 0.43%; $p < .05$). No differences were observed between S1 and S2 in any group. No differences were observed between G1 and G2 or S1 and S2 in other isolated bacterial genera.

The β -haemolytic streptococci isolated showed no bacterial resistance for any of the antibiotics used in the antibiotic sensitivity test.

In the ultramicroscopic study, capsules (Figure 1) and M protein (Figure 2) were observed in *S. canis* and *S. dysgalactiae*. Capsules were observed by direct observation using negative staining in 21 of the 22 strains of *S. canis* and *S. dysgalactiae* studied (Table 4). Seventeen strains of *S. canis* and *S. dysgalactiae* were observed in ultrathin sections. M protein was observed in the 17 samples.

No differences were observed in capsule thickness between strains of *S. canis* and *S. dysgalactiae* (218.95 \pm 21.56 vs 210.71 \pm 10.39 nm; $p > .62$). The *S. canis* strains isolated from G2 (S1 and S2) showed capsules thicker than *S. canis* strains isolated from G1 (234 \pm 24.2 vs 151.23 \pm 28.93 nm; $p < .001$). No differences were observed in capsule thickness between strains of *S. dysgalactiae* isolated from G1 and G2 (210 \pm 13.54 vs 211.66 \pm 19.67 nm; $p > .70$).

No stillbirths were recorded in either G1 or G2. The therapeutic trial showed differences in neonatal survival in G2A compared with G2B. Fifty-seven puppies were born in G2A. The 10 bitches included in G2A delivered healthy litters and 90% (51/57) of puppies survived. Six puppies (10%) died in the neonatal period. Sixty-eight puppies were born in G2B. The 11 bitches included in G2B delivered healthy litters but only 25% (17/68) of puppies survived. Fifty-one puppies (75%) died in the neonatal period.

4 | DISCUSSION

Vaginal microbiota findings in our work were similar to the findings reported by other authors (Carneiro, Toniollo, & Schocken-Iturrino, 2005; Gropetti et al., 2012; Laurusevičius, Jūratė, & Henrikas, 2008; Watts, Wright, & Whithear, 1996). *Staphylococcus* spp. were isolated in healthy bitches during proestrus and at the end of gestation in a lower percentage compared to other studies that reported percentages up to 76.19% in healthy bitches and up 71.42% in bitches with reproductive pathologies (Carneiro et al., 2005). In the same way, *E. coli* was found in this study in a lower percentage

Bitches	β -hemolytic <i>Streptococcus</i>	<i>S. canis</i>	<i>S. dysgalactiae</i>
G1 ^A (n = 28)	57.14% (n = 16)	32.14% (n = 9)	25.00% (n = 7)
G2 ^B (n = 23)	91.3% (n = 21)	69.56% (n = 16)	21.73% 7 (n = 5)

A Group 1, bitches with healthy litters; ^BGroup 2, bitches with newborn deaths in the litters.

TABLE 3 Percentage of bitches with β -haemolytic streptococci (*Streptococcus canis* or *Streptococcus dysgalactiae*) isolated from vaginal samples from bitches

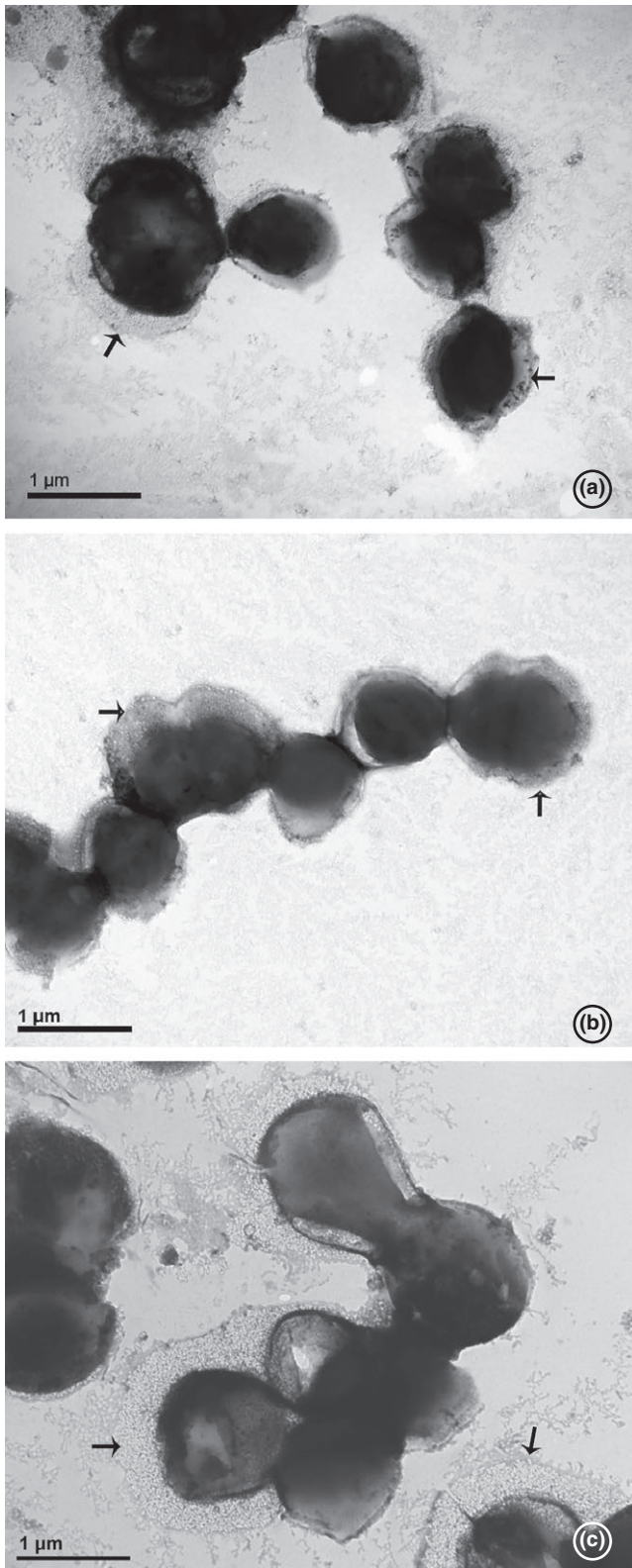


FIGURE 1 Electron micrograph of *Streptococcus canis* and *Streptococcus dysgalactiae* (negative staining). Arrows show capsule. Bar, 0,2 µm. A: *S. dysgalactiae*. B: *S. canis* (G1). C: *S. canis* (G2)

compared with another study in healthy bitches (66.66%) and in bitches with reproductive pathologies (71.42%; Carneiro et al., 2005). On the other hand, *E. coli* was found by Groppetti et al.

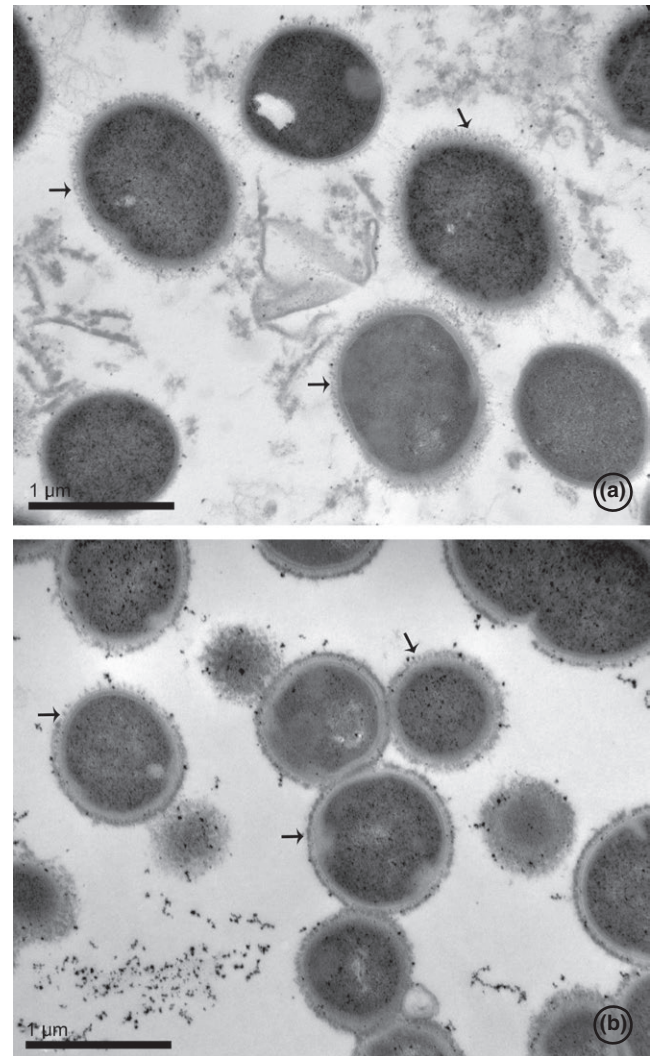


FIGURE 2 Electron micrographs of *Streptococcus dysgalactiae* and *Streptococcus canis* (ultrathin sections). Arrows show M protein. Bar, 0,2 µm. A: *S. dysgalactiae*. B: *S.*

(2012) during proestrus in lower percentages than in the current study (2.9%). In the present study, beta-haemolytic *Streptococcus* was the bacteria more frequently isolated. Our finding agrees with data reported by Allen and Dagnall, who found beta-haemolytic *Streptococcus* as one of the more frequently isolated bacteria from healthy pregnant or nonpregnant bitches, and infertile bitches with vaginal discharge (Allen & Dagnall, 1982). In our study, beta-haemolytic *Streptococcus* was the bacteria most frequently isolated in G2 in proestrus and at the end of gestation. The percentage of beta-haemolytic *Streptococcus* observed in the present study in S1 in G1 and G2 were higher from those reported by Groppetti et al. (2012), who founded a smaller percentage (20.6%) in healthy bitches during proestrus.

Streptococcus canis was the most frequently beta-haemolytic *Streptococcus* isolated in our study. These bacteria were previously isolated by other researches from the vagina of bitches in heat (Allen & Dagnall, 1982; Watts et al., 1996); and *Streptococcus dysgalactiae* was previously isolated from the canine pharynx (Acke et al., 2015;

Group	IS ^A / <i>S. canis</i>	BC ^B	WC ^C	IS ^D / <i>S. dysgalactiae</i>	BC ^B	WC ^C
G1 ^E	4	3	1	3	3	0
G2 ^F	11	11	0	4	4	0

IS^A/*S. canis*, number of strains isolated of *S. canis*; BC^B, number of strains with bacterial capsule; WC^C, number of strains without bacterial capsule; IS^D/*S. dysgalactiae*, number of strains isolated of *S. dysgalactiae*; G1^E, bitches with healthy litters; G2^F, bitches with newborn deaths in the litters.

Schrieber, Towers, Muscatello, & Speare, 2014). However, according to our knowledge, it has not been isolated from the canine reproductive tract.

The *S. canis* and *S. dysgalactiae* isolation in the bitch vagina in G1 and G2 showed that the microorganisms were present in both groups of bitches. The G2 bitches at the end of gestation had a higher percentage (69.6%) of beta-haemolytic *Streptococcus* isolated compared with the percentage reported in women (40%) in the last third of gestation (de-Paris et al., 2011; Winn, 2007). On the other hand, the percentage of beta-haemolytic *Streptococcus* found in G2 bitches was twice that found in G1. These findings could support the notion of bacteria transmission to the neonate during the parturition similar to findings in human (de-Paris et al., 2011; Schuchat, 1998; Winn, 2007). In humans, vertical transmission and neonatal illness was reported in 75 per cent of the newborns from mothers with vaginal colonization of beta-haemolytic *Streptococcus* (de-Paris et al., 2011; Winn, 2007). This process could also happen in the dog since beta-haemolytic *Streptococcus* was isolated in the vagina in G1 bitches at the end of gestation.

No difference was observed between groups in the bacterial genera isolation except in beta-haemolytic *Streptococcus*. These findings suggest *Staphylococcus* spp., *E. coli*, *Proteus* spp., non-fermenting Gram-negative bacilli [NFGNB] and *Enterococcus* spp. are not involved in neonatal deaths in the bitches included in this work.

In the ultramicroscopic study, we observed M protein and capsule in *S. canis* y *S. dysgalactiae*. The M protein and capsule observed in the beta-haemolytic *Streptococcus* studied in this work could be involved in the pathogenicity of the bacteria. Ultramicroscopic studies showed the presence of M protein in *S. canis* and *S. dysgalactiae* in ultrathin sections. Our findings agree with previous reports by other researchers (Brandt & Spellerberg, 2009; De Winter, Low, & Prescott, 1999; Vasi, Frykberg, Carlsson, Lindberg, & Guss, 2000). The capsule was described in *S. dysgalactiae* (Brandt & Spellerberg, 2009). However, according to our knowledge, there are no previous reports about capsule description in *S. canis*. In the same way, the capsule was observed in *S. equi* and *S. pyogenes* using negative staining, the same staining used in the current study (Bustos, Muñoz, & Guida, 2015; Cleary & Cheng, 2006; Fazio & Fischetti, 2004). Vanrobaeys et al. reported that the thickness of the *S. galloyticus* capsule is related to the virulence of the strain studied. The most virulent strains have thicker capsules (Vanrobaeys, De Herdt, Charlier, Ducatelle, & Haesebrouck, 1999). The *S. canis* strains isolated in G2 showed thicker capsules than strains isolated in G1. This finding could be related to the pathogenicity of the strain isolated in G2 and the neonatal death observed in puppies delivered by G2

TABLE 4 Capsule observation using negative stain in strains of *Streptococcus canis* and *Streptococcus dysgalactiae* isolated from vaginal samples from bitches

bitches. In contrast, no differences were observed in thickness of the capsules in *S. dysgalactiae* strains isolated from G1 and G2. Several pathogenicity factors of *S. dysgalactiae* are known (M protein, streptolysin O, streptolysin S, streptokinase, streptodornase [DNase], pyrogenic exotoxin G and hyaluronidase; Brandt & Spellerberg, 2009; Timoney, 2010). It is likely that in the current study, the strains of the groups G1 and G2 of *S. dysgalactiae* differ not in the thickness of the capsule but in other factors of virulence not studied in this work.

Streptococcus canis and *S. dysgalactiae* strains isolated from vaginas in G1 and G2 showed penicillin and vancomycin sensitivity agreeing with *S. canis* strains isolated from bitch vaginas and cow milk by Hassan, Akineden, and Usleber (2005), and Pinho et al. (2013) and *S. dysgalactiae* strains isolated from the human pharynx (Rantala, 2014; Woo, Fung, Lau, Wong, & Yuen, 2001). In contrast, Lysková, Vydřalová, Královcová, and Mazurová (2007) found some *S. canis* strains with vancomycin resistance isolated to external ears canal, vaginal mucosa, pharynx and oral cavity, conjunctivae, skin and the rectum in dogs. Giannechini et al. (2014), found some *S. dysgalactiae* strains with erythromycin resistance isolated from dairy cows with subclinical mastitis. Woo et al. (2001) isolated erythromycin resistance *S. dysgalactiae* strains from human.

The current study showed that penicillin administration before and after parturition reduced neonatal death in G2. These findings agree with the reduction of neonatal infection and death in humans by penicillin administration during the parturition in women with vaginal colonization of beta-haemolytic *Streptococcus* (Verani, McGee, Stephanie, & Schrag, 2010).

Our results suggest beta-haemolytic *Streptococcus* could cause neonatal deaths when bitches have vaginal colonization. The antibiotic administration in pregnant bitches that a history of neonatal deaths and beta-haemolytic streptococci vaginal colonization could avoid neonatal deaths. The use of antibiotics in all bitches to reduce the incidence of neonatal death is not recommended because of antibiotic resistance. But, in bitches that have delivered puppies who died in the neonatal period, in previous parturitions, in which beta-haemolytic *Streptococcus* have been isolated, the administration of penicillin could improve neonatal survival and reduce neonatal deaths. Therefore, the bacteriological study of vaginal samples obtained from bitches with a history of delivering puppies that died in the neonatal period would be an important tool to selectively treat these bitches before parturition to reduce/minimize neonatal death. More studies are needed on this topic to clarify the proper use of antibiotics and the role of beta-haemolytic *Streptococcus* in the neonatal deaths when the bitch presents vaginal colonization.

CONFLICT OF INTEREST

The authors have declared no conflict of interests.

AUTHOR CONTRIBUTIONS

Guerrero AE helped to design, conduct the experiment, and process the samples, analysed data and drafted manuscript. Stornelli MC, Jurado SB, Giacoboni G and Sguazza GH, helped to conduct the experiment and process the samples. Stornelli MA and de la Sota RL helped to design and conduct the experiment and critically revised the manuscript.

ORCID

MA Stornelli  <http://orcid.org/0000-0001-7285-1814>

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