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A comparison of microbiological and molecular detection of vaginal *Lactobacillus spp.* between mares and women

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Abstract

A possible explanation for endometritis in mares is ascendant contamination from the vagina. The presence of *Lactobacillus spp.* is considered to be important in women for a healthy vaginal environment; however, there are few studies in mares related to the presence of *Lactobacillus* in the vaginal flora of healthy mares. The present work aims to determine the occurrence of *Lactobacillus spp.* in the vaginal micro-environment of mares. A total of 35 crossbred multiparous mares, aged between 4 and 12 years, with no history of reproductive problems and with healthy reproductive tracts, were used. Two vaginal swabs were obtained from the mares during estrus for *Lactobacillus* isolation and PCR evaluation. Ten human female volunteers, aged between 24 and 35 years, sexually active, with no history of gynecological diseases and treatments in the past two years were used. *Lactobacillus spp.* were isolated from 5.7% of the mares' vaginal samples and from 90% of the women's vaginal samples. *Lactobacillus* DNA was detected by PCR in 22.9% of the mares' vaginal samples and in all of the vaginal samples from the healthy women. The primers used here were demonstrated to have *in silico* specificity for the detection of *L. equi* (AB425924.1), *L. pantheris* (DQ471798.1) and *L. mucosae* (DQ471799.1), but they did not anneal on *Enterococcus faecalis* (EU887827.1) *or E. faecium* (EU887814.1). In conclusion, this study showed a low occurrence of *Lactobacillus spp*. in mares, suggesting that this bacterium may not play a fundamental role in the equilibrium of the vaginal micro-environment of normal mares.

Keywords: *Lactobacillus*, mare, microbiota, PCR, vagina, woman.

Introduction

There are only a few studies concerning the mare vaginal flora. Newcombe (1978) and Hinrichs *et al.* (1988) described a vaginal commensal organisms consisting of a compound of non-pathogenic uterine microorganisms, although pathogenic bacteria (e.g., *Streptococcus zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) were isolated from the clitoral fossa and vestibular swabs of mares without clinical or histological evidence of endometritis and may represent a risk factor for bacterial ascendant infection (Hinrichs *et al*., 1988). However, Scott *et al*. (1971), studying material collected from the vagina of mares at a slaughterhouse, observed that approximately 90% of the mares showed pathogenic bacterial growth, and *S. zooepidemicus* was the predominant isolated bacteria. However, in this experiment the plating of the culture occurred up to 6 to 10 h after removal of the reproductive tract from the mares and they also incubated the swabs in broth for 6 h before plating, increasing the potential for overgrowth of bacteria.

Endometritis is a uterine disorder that involves endometrial inflammation and possibly endometrial infection and has been recognized as the main cause of low fertility in mares. Generally, the incidence of uterine problems is higher in embryo donors, probably because these animals are submitted to excessive manipulation of the genital tract (Losinno and Alvarenga, 2006). It is also postulated that the reflow of antibiotics and disinfectants used in repetitive uterine treatments can modify the vaginal flora, inducing microbiologic imbalance, creating a favorable environment to develop pathogenic agents such as fungi and causing ascendant contamination through the cervix (Silva and Alvarenga, 2011).

Lactobacilli are considered the primary microbiological barrier against women's genital pathogens. Between 50 and 90% of the aerobic bacteria present in women's vaginal flora are facultative *Lactobacilli* (Dembélé *et al*., 1998). In women, the production of lactic acid by *Lactobacillus spp.* seems to be essential for the maintenance of a healthy vaginal ecosystem. The bacteria are able to inhibit the growth of pathogens mainly by lowering the pH through the

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production of lactic and acetic acid and hydrogen peroxide (H_20_2; Hess *et al*., 2002; Linhares *et al*., 2010). Despite several studies describing *Lactobacillus* in women's vaginal flora (Aslim and Kilic, 2006; Witkin *et al*., 2007), the presence of *Lactobacillus* in the vaginal micro-environment of mares was only recently reported (Fraga *et al*., 2008). The present work aims to determine the occurrence of *Lactobacillus spp.* in vaginal samples from a group of mares (recipients in a commercial embryo transfer program) during estrus.

Materials and Methods

Animals and sample collections

A total of 35 crossbred mares, aged between 4 and 12 years, were used. Ultrasound evaluations were performed to determine the estrous cycle period and to evaluate the uterus and ovaries before sample collection. No neutrophils were detected on cytological samples collected using a gynecological brush, and only mares with normal conditions of the uterine tract were used. All the mares were multiparous, with no history of reproductive problems and were randomly selected from a cohort of recipients in a commercial embryo transfer program. Ten healthy volunteer women were also used in this experiment, aged between 24 and 35 years, sexually active, with no history of gynecological diseases and treatments in the past two years.

The vaginal samples were collected from the mares during estrus when a pre-ovulatory follicle (>35 mm) was detected by ultrasound during October and December (the South American breeding season). First, the external genitalia and perineum were washed with neutral liquid soup and dried with paper towels. Briefly, an operator using powder-free plastic sterile gloves inserted a commercial swab protected with a sterile silicone tube into the vagina, and the cranial wall of the vagina was then swabbed for at least 15 sec.

Two vaginal swabs of the vaginal wall, proximal to the fornix, were obtained from the mares; one was transferred to Amies medium with charcoal (CB Products, Corumbataí, Brazil) and transported at 5°C for a period of 3 h to a reference laboratory for *Lactobacillus* isolation at the University of Campinas (Microbiology Laboratory of Women Health Center - UNICAMP), the other swab was transferred to a sterile plastic tube containing 500 µl PBS (saline-phosphate buffer 1X, pH 7.2) and the tube was sent to the Veterinary Clinical Science Laboratory of Molecular Biology at the College of Veterinary Medicine and Animal Science, UNESP-Univ Estadual Paulista, where it was stored at -20°C for posterior DNA extraction. Two swab samples from the deep vaginal wall were self-collected from volunteer women for *Lactobacillus spp.* isolation and PCR analysis. The women's vaginal samples were stored and processed using the same methodology previously described for the mares. The mares' vaginal pH levels were determined according to the methodology previously described (Fraga *et al*., 2008), using commercial strips (Merck, Germany) placed inside the vagina for 2 min. All the experiments were performed according to the Univ Estadual Paulista (UNESP) Institutional Animal Care and Use Committee. All human subjects were informed and signed a consent form for participation in the project.

Isolation of Lactobacillus spp.

Isolation of *Lactobacillus spp.* from the vaginal samples was performed according to the methodology previously described (Brolazo *et al*., 2009). The vaginal samples used for *Lactobacillus* isolation were inoculated on Man Rogosa and Sharpe (MRS; Oxoid, Basingstoke, England) agar plates. Briefly, the MRS agar plates were incubated at 37°C for 48 h in an anaerobic atmosphere (Forma Anaerobic System, Thermo Electron Corporation, Waltham, MA, USA) with 5% CO_2 (Forma Series II Water Jacketed CO_2 incubator - Thermo Electron Corporation). Morphologically distinct and well-isolated colonies were examined for cultural and morphological characteristics. The size, shape, color and texture of the colonies were recorded. The bacterial isolates were tested for catalase production by a catalase test and growth at 15 and 45°C. Cell morphology was examined after Gram staining (Harrigan and McCance, 1976).

Bacterial DNA extraction

Bacterial DNA was isolated from the vaginal swabs from the mares and the women and conserved in PBS medium using a QIAamp DNA Mini Kit (Qiagen®, Valencia, CA, USA) following the manufacturer's recommendations with some modifications described below. The samples were vortexed for 5 min. Next, the swab was removed, and the sample was centrifuged for pellet formation (10,000 x g for 8 min). The supernatant was removed and the bacterial cell pellet obtained was then resuspended in 80 µl of Lysozyme Buffer (10 mM Tris, 50 mM NaCl, 0.2% of sodium deoxycholate, 0.5% of N-Lauroylsarcosine, pH 7.2) and 20 µl (4,000 IU) of Lysozyme (USB®, Cleveland, OH, USA). After incubation at 37°C for 30 min, 20 µl of proteinase K (Qiagen®, Valencia, CA, USA) was added, and the mixture was incubated at 56°C for 10 min. Next, 200 µl Buffer AL (Qiagen®, Valencia, CA, USA) was added to the sample which was mixed by pulse-vortexing for 15 sec and then incubated at 70°C for 10 min to lyse the bacterial wall. Briefly, the lysate sample was transferred to a QIAamp Mini Spin (Qiagen®, Valencia, CA, USA) column and the bacterial DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation. To complete the removal of any residual contaminants, the DNA bound to the QIAamp membrane was washed with 2 centrifugation steps with Buffer AW1 and Buffer

AW2 (Qiagen®, Valencia, CA, USA), respectively. Immediately after, the purified DNA was eluted from the QIAamp Mini spin column in a concentrated form in 30 µl of Buffer AE (10 mM TrisCl, 0.5 mM EDTA, pH 9.0). The relative quality of the isolated bacterial DNA was determined by spectrometry, and the ratio of A260-A280 nm exceeded 1.8 for all preparations (Nanodrop® 2000 Spectrophotometer, Thermo Scientific™).

PCR analysis and sequencing of Lactobacillus spp. 16S ribosomal RNA gene

PCR reactions were performed in duplicate in a total of 20 µl each, which contained 4 µl of template DNA, 0.3 µm of each of the forward and reverse primers, 10 µl of GoTaq® Green Master Mix (Promega™, Madison, WI, USA) and 4.8 µl nuclease-free water. In addition, a ''no template'' control was performed in duplicate to show that contamination was absent. The PCR conditions on the EP-Gradient thermocycler (Eppendorf®, Hamburg, Germany) were set as follows: initial denaturation at 94°C for 5 min and 40 cycles at 94°C for 30 sec (denaturation), 62°C for 30 sec (annealing) and 72°C for 60 sec (extension), followed by a final extension at 72°C for 5 min. The specific PCR primers were Lacto-Forward, 5'-TGGAAACAGRTGCTAATACCG-3'; and Lacto-Reverse, 5'-GTCCATTGTGGAAGATTCCC-3' for amplification (≈ 230 bp) of the V2.l-V3 16S ribosomal RNA gene *Lactobacillus spp*. region as previously described (Byun *et al*., 2004). Before PCR, this set was tested *in silico* (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to confirm its specificity to detect *Lactobacillus spp*. as previously described in horses (Fraga *et al*., 2008).

The PCR products were analyzed by 1.5% agarose gel electrophoresis (Invitrogen®, Carlsbad, CA, USA), stained with GelRed™ (Biotium™, Hayward, CA, USA) and visualized under ultraviolet light (ImageQuant™, GE© Healthcare, USA). The molecular weights were estimated by comparison with a known molecular weight marker of 100 bp (Norgen®, Ontario, Canada).

To confirm that the amplicon was directly related to *Lactobacillus* species, PCR products (samples from 2 mares and 2 women) with a predicted size of 230 bp were purified using the QIAquick® PCR Purification Kit (Qiagen®, Valencia, CA, USA). After purification, the DNA purity and quality were evaluated at 260/280 nm using a Biophotometer® (Eppendorf®, Hamburg, Germany). Automated direct sequence analysis with 3500 Genetic Analyzers (Applied Biosystems, Foster City, CA, USA) and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was performed in duplicate using 5 µl each of the forward and reverse primers and 10 µl PCR product. The sequences and electropherograms obtained were analyzed using Sequencing Analysis 5.3.1 software (Applied Biosystems, Foster City, CA, USA) and aligned using the CLUSTAL X software. The sequence obtained was blasted (http://blast.ncbi.nlm.nih.gov/) to verify its sequence homology against previously available sequences deposited in GenBank™ (http://www.ncbi.nlm.nih.gov/).

Results

In this study, *Lactobacillus spp.* were isolated from 5.7% (2/35) of the mares' vaginal samples and 90% (9/10) of the women's vaginal samples. These positive samples were catalase-negative, Gram-positive rods, producing no gas and no growth was observed at 15°C. Bacterial DNA was detected by PCR in 22.9% (8/35) of the mares' vaginal samples; moreover, the two samples with a positive culture were also positive in the molecular study. All the vaginal samples (10/10) from the women were positive in PCR analysis.

The primers used here were demonstrated to have *in silico* specificity for the detection of *L. equi* (AB425924.1), *L. pantheris* (DQ471798.1) and *L. mucosae* (DQ471799.1), but they did not anneal on *Enterococcus faecalis* (EU887827.1) *or E. faecium* (EU887814.1).

The DNA sequence obtained from the positive PCR products from the mares and the women were blasted (http://blast.ncbi.nlm.nih.gov) with the genome sequence deposited in GenBank™, and it was determined that all of the DNA sequences had 100% identity to the *Lactobacillus spp.* 16S ribosomal RNA gene. Furthermore, the mare DNA sequence had 94, 93 and 92% identity with *L. mucosae*, *L. equi* and *L. pantheris,* respectively. The mean pH value of the vaginal samples collected from the all mares was 7.0 ± 0.5. There was no difference ($P > 0.05$) between mares that presented (pH = 7.0 ± 0.5) and did not present *Lactobacillus spp.* in the vaginal sample.

Discussion

In this study, the presence of *Lactobacillus spp.* in the vaginal swabs of mares was observed in only 5.7% (2/35) of the culture samples. These data are in contrast to a recent publication (Fraga *et al*., 2008), in which the authors isolated *Lactobacillus spp.* from the vaginal flora of about 70% of 26 evaluated mares. *Lactobacillus* DNA detection was also low in our studied mares, with only 22% of the samples shown to be positive using PCR. The reasons for the discrepancy between our data and the Fraga *et al*. (2008) study are difficult to explain because the same techniques were used to isolate the *Lactobacillus* from the collected vaginal samples. It is important to note that in our human female group, 9 out of 10 (90%) of the women's samples cultured were positive for *Lactobacillus spp*., confirming the efficacy of the employed methodology.

The data presented here, obtained using molecular methods, support a recent study in which a

traditional PCR technique was shown to be more sensitive than microbiological culture (Ferris *et al*., 2010). These authors identified the presence of bacterial DNA in the uterus of mares in 33% of samples, versus 22% of the same samples sent to microbiological culture. We also observed that PCR was more efficient to detect the presence of *Lactobacillus* in both the mares' and the women's vaginal samples. This method is able to detect DNA from bacteria that died before the culture procedure, also contributing to the observed results.

The set of primers used here was previously designed to detect a consensus region of the 16S ribosomal RNA gene from *Lactobacillus* spp. (Byun *et al*., 2004) and was able to detect a wide diversity of this species, e.g., *L. brevis* (M58810), *L. casei* (AY196975), *L. crispatus* (AF257097), *L. delbrueckii* (AJ414691), *L. fermentum* (AF302116), *L. gallinarum* (AJ417737), *L. gasseri* (AF519171), *L. oris* (X94229), *L. plantarum* (AL935253), *L. rhamnosus* (AF243146), *L. reuteri* (L23507) and *L. salivarius* (AF089108). They were also demonstrated to have *in silico* specificity for the detection of the same *Lactobacillus* species previously described in the vaginal flora of mares. Furthermore, they did not show specific annealing in the sequence of the *Enterococcus* species described in the vaginal flora of mares (Fraga *et al*., 2008).

It is important to note that although the mare amplicon sequencing showed high identity to the *Lactobacillus* sequences deposited at GenBank™ (e.g., *L. mucosae*, *L. equi* and *L. pantheris*), the sequence obtained here was not deposited in GenBank™ because we used primers designed to amplify a consensus region (Byun *et al*., 2004), and it is not possible to characterize which specific species were present in these positive samples because multiple *Lactobacillus* DNA amplification could be presented at the same time (Fraga *et al*., 2008). The same situation occurred with the women's amplicon sequencing that confirmed that the analyzed product was *Lactobacillus*, but we were not able to characterize the specific species due to the possible multiple DNA *Lactobacillus* organisms present in these samples.

In this study, in most of the women's vaginal sample cultures (9/10), it was possible to isolate *Lactobacillus,* which agrees with other publications where *Lactobacillus* is routinely isolated in women and is the dominant bacterial species found in the vaginal micro-flora (Brolazo *et al*., 2009). According to Fraga *et al*. (2008), the vaginal pH of normal mares is 7.0, which agrees with the findings of the present work. However, the pH in women is approximately 4.5 (Reid *et al*., 2001), which can partly explain the difference in the abundance of *Lactobacillus* in the vaginal flora between humans and equines. It is well known that an important function of *Lactobacillus* is to acidify the vaginal environment. Rodríguez *et al*. (2011) isolated a very low number of *Lactobacillus* colonies from vaginal samples from cows, concluding that *Lactobacillus* is not directly related to the healthy state of the cow reproductive tract as it is in women. The normal vaginal pH of cows is also approximately 7.0 (Schilling and Zust, 1968), which is another indication of a low prevalence of *Lactobacillus* in the vaginal environment of this species compared to women.

In conclusion, the occurrence of *Lactobacillus spp.* in mares in our study was low, suggesting that this bacterium may not play a fundamental role in the equilibrium of the vaginal flora of reproductive normal mares.

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