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* (Dembéle 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
production of lactic and acetic acid and hydrogen peroxide (H$_2$O$_2$; 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, Corumbatai, 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...
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. Rodriguez 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.

References


Newcombe JR. 1978. Comparison of the bacterial flora


