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Vaginal bacterial microbiota of an endangered donkey breed: a comparison between *Miranda* donkey breed (*Equus asinus*) jennies with and without reproductive problems

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ABSTRACT

This study provided an overview of the vaginal bacterial microbiota of *Miranda* donkey breed jennies with and without reproductive problems. This Portuguese autochthonous donkey bred is in danger of extinction. Bacteria isolated were predominantly Gram-positive and belonging to the genera of *Bacillus* spp, *Corynebacterium* spp., *Lactobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp.. The species found in the vaginal microbiota were diverse and didn't differ significantly between the jennies with and without reproductive problems. However the isolates of *Streptococcus zooepidemicus* of jennies with reproductive problems presented a higher number of the studied genes encoding virulence factors then the isolate without reproductive problems. This is the first study reporting vaginal bacterial microbiota of *Miranda* donkey breed jennies. Since there are few studies regarding the vaginal microbiota of equines, especially in this donkey breed, these results can be an asset for future studies.

Keywords: Equids; Donkey; *Streptococcus zooepidemicus*; Vaginal bacterial microbiota; Virulence factors.

1. Introduction

The *Miranda* donkey breed is the only Portuguese autochthonous donkey breed native of northeast of Portugal and it is currently endangered of extinction. Until 2012, it was reported that there were only 725 animals alive. The potentially reproductive population at the end of 2012 comprised 545 females and 72 males, alive and younger than 20 years old. Only around 15% of the females were foaling each year, a reported low fertility rate [1,2].

Commensal microbiota is a complex community of microorganisms that colonizes several biological systems in animals. So far, equine native microbiota has not been extensively characterized. These microbial communities are related to normal functions of different organisms and its characterization may provide important data about beneficial microbes useful for the promotion of equine's health. Many uterine pathogens inhabit the vagina. The knowledge of bacterial biota inhabiting the genital tract of the mare would be important to develop therapeutic strategies to control the setting of the disease [3].

Bacterial infections of genital tract are known to be an important cause of reproductive problems in equines. Bacteria involved in equine endometritis are, for the most part, considered to be opportunistic pathogens. They are capable of colonizing the lower genital tract as well as a variety of extragenital locations in the animal; yet they are usually barred from ascending to the cervix and uterus by

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host defences. Previous studies showed that the most common bacterial causes of uterine infection include *Streptococcus zooepidemicus*, *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Bacteria which are capable to establish endometritis are considered to differ from the physiological genital microbiota by several tracts that promote colonizing and causing damage to equine endometrium. The concept that some virulence mechanisms are sufficient to explain why a bacterium can cause endometritis may oversimplify the complex nature of the disease [4,5,6,7].

Taylorella equigenitalis is a Gram-negative bacterium responsible for contagious equine metritis (CEM), a sexually transmitted infection of horses that was first reported in 1977. In 2001 it was confirmed the existence of a second species within the genus *Taylorella* which was named *Taylorella asinigenitalis* and isolated from genital tract of donkeys. Until now *T. asinigenitalis* is not associated with CEM [8,9].

Uterine infections caused by *S. zooepidemicus* have been established as the leading cause of bacteria-induced endometritis in equines. The pathogenesis of endometritis induced by *S. zooepidemicus* is based on interactions between bacterial virulence factors and the infected host tissues. Understanding the role played by virulence factors can help elucidate the understanding of the pathogenesis of infection and can be used to identify points of treatment or vaccination. Known virulence factors of *S. zooepidemicus* include M-like proteins and superantigens [4,10,11,12,13] The designation M-like protein usually refers to proteins that bind fibrinogen and have antiphagocytic activity. The antiphagocytic of these proteins appears to be associated with their ability to inhibit deposition of the complement component C3b on the bacterial surface and their ability to bind fibrinogen which then inhibits the phagocytosis. Superantigens interfere with the development of a protective immune response, by disrupting antigen-specific T cell responses and inhibiting the production of antibodies [14,15].

The main aim of this study was to identify and characterize bacteria from vaginal samples of eight Miranda jennies with reproductive problems (included the females with an infertility story) and nineteen *Miranda* jennies without reproductive problems. The analysis of the presence of genes encoding virulence factors in *Streptococcus zooepidemicus* isolates (*sze*F, *sze*N, *sze*P, *szm* and *szp*) was also an aim of our study.

2. Material and Methods

2.1. Samples and bacterial isolates.

Fifty four vaginal exudates (two samples per animal) were collected between October of 2014 and February of 2015. Before collecting each sample, the genital zone of each jenny was washed with water to eliminate fecal contaminations. One of the samples collected from each jenny was transported refrigerated in Amies Transport Medium with charcoal (Remel) to preserve the viability of bacteria present in the sample. The other sample collected was transported at room temperature in Nutrient Broth (Oxoid), in order to preserve the viability of *Mycoplasma* spp..

For the samples transported in Amies Transport Medium with charcoal, a dilution in saline solution was prepared before performing streak plate method in the following culture mediums: BHI agar (Liofilchem) with 5% Sheep Blood, Cetrimid agar (Merck), Chromocult Coliform agar (Merck), Columbia CNA agar (BBL) with 5% Sheep Blood, Columbia (Oxoid) Chocolate agar with 5% Horse Blood and streptomycin, Columbia (Oxoid) Chocolate agar with 5% Horse Blood, Amphotericin B and Violet Crystal and MacConkey (Merck) agar. These plates were incubated for 24-48h at 37ºC, with exception of Columbia Chocolate agar plates that were incubated for 5-7 days at 37ºC in a jar with microaerophilic environment. It was also used 0.5 ml from the dilution made to inoculate in a tube of Rappaport-Vassiliadis Enrichment Broth (Oxoid), to test if the samples presented *Salmonella* sp.. The tubes with Rappaport-Vassiliadis Broth were incubated in a water bath at 37ºC for 24-48h. On the other hand, the samples transported in Nutrient Broth were centrifuged at 3000 rpm for 5 min; the supernatant was aspired with a syringe and then filtered to the surface of Mycoplasma agar base with selective Mycoplasma supplement. Spread plate technique was performed and the plates were incubated for 4-14 days at 37ºC in a humid atmosphere. These plates were viewed under the low power objective (10x) of the optical microscope, in order to investigate the presence of typical "fried egg" colonies.

The different bacteria isolated were identified by colony morphology, Gram-staining, catalase test and oxidase test. Identification to the species level was made using API 20 Strep, API 20 E and API 20 NE systems. PCR amplification of the 16S ribosomal DNA was also performed for the isolates that indicated belonging to *Streptococcus* spp. to identify and confirm which isolates were *Streptococcus zooepidemicus*. The sequences of the oligonucleotide primer and the thermal cycler programs are given in table 1.

2.2. Antimicrobial susceptibility test.

Antibiotic susceptibility of *Streptococcus zooepidemicus* isolates was tested by the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013) for 9 antimicrobials: ampicillin (10 μg), cefepime (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), linezolid (30 μg), quinupristin -dalfopristin (15 μg), tetracycline (30 μg) and vancomycin (30 μg).

Table 1 - Oligonucleotide primers and PCR programs used in present study. The oligonucleotide primers were synthesized by Invitrogen™.

Target	Oligonucleotide primer	Sequence (5'–3')	PCR program	Reference
16s rDNA	16SP0	GAAGAGTTTGATCCTGGCTCAG	1x (95°C 5min), 25x (95°C 30s, 56.5°C 30s, 72°C 2min), 1x(72°C 10min)	[21]
	16SP6	CTACGGCTACCTTGTTACGA		
szeF	*szeFF*	CATAAAGTTAGTCGTGCAGAG	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szeFR	CGATGACGATGATTCACATCA		
szeN	*szeNF*	GACACCGGTAACATTTCAAGAG	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szeNR	GGGTTGACCACTCTTGTAG		
szeP	*szePF*	TCCAGTTGAGAAATCCTGGC	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szePR	CCTAAAAATTTCGACATCAAGTG		
szm	*szmF*	ATAAAGAAGTTCCTGTCAT	1x (95°C 2min), 30x (94°C 30s, 55°C 30s, 72°C 1min), 1x(72°C 10min)	[12]
	szmR	CAACAGACAGGAGACTGTTGC		
szp	*szpF*	ACAAAAGGGGAATAAAATGGC	1x (95°C 2min), 30x (94°C 30s, 60°C 30s, 72°C 150s), 1x(72°C 5min)	[13]
	szpR	TTTACCACTGGGGTATAAGGCTT		

Table 2 - Vaginal bacterial microbiota of *Miranda* jennies.

Identified bacteria	Number of isolates (Jennies with reproductive problems) N=46	Number of isolates (Jennies without reproductive problems) N=103	P-value	Genotype of virulence factors detected (if applicable)
Aerococcus viridans	-	3	0.242	-
Aeromonas hydrophila	-	1	0.516	-
Bacillus spp.	13	19	0.143	-
Corynebacterium spp.	12	9	0.076	-
Enterococcus faecium	-	3	0.242	-
Escherichia coli	-	1	0.516	-
Gemella haemolysans	-	1	0.516	-
Lactobacillus spp.	6	26	0.404	-
Micrococcus spp.	1	1	0.520	-
Mycoplasma spp.	-	5	0.115	-
Neisseria spp.	1	2	0.884	-
Oligella urethralis	-	1	0.516	-
Pantoea Agglomerans	-	2	0.349	-
Pseudomonas chlororaphis	-	6	0.242	-
Rhodococcus spp.	5	1	0.028	-
Staphylococcus spp.	4	17	0.461	-
Streptococcus acidominimus	-	1	0.516	-
Streptococcus oralis	2	-	0.026	-
Streptococcus spp.	-	3	0.242	-
Streptococcus zooepidemicus	2	1	0.144	szeF, szeN, szeP (67%) szeF, szp (33%)

2.3. Virulence factor genes.

The presence of genes encoding virulence factors (*sze*F, *sze*N, *sze*P, *szm* and *szp*) for *Streptococcus zooepidemicus* isolates was also analyzed by PCR. The sequences of the oligonucleotide primers and the thermal cycler programs are given in table 1.

2.4. Statistical analysis

Data were first analysed using Shapiro-Wilk normality test to test the normal distribution of the values. To test if there were significant differences between the bacteria isolated from jennies with reproductive problems and bacteria isolated from jennies without reproductive problems an Independent-Samples Kruskal-Wallis test was performed. These tests were done using IBM SPSS Statistics 19. *P*-values less than 0.05 were considered significant.

3. Results

From a total of 54 vaginal exudates from 27 jennies, 149 bacterial isolates were obtained. Among these isolates, 130 were identified as Gram-positive bacteria, 14 as Gram-negative bacteria and 5 bacteria without Gram determined (identified as *Mycoplasma* spp.). The most isolated genera were *Bacillus* spp. (22.15%), *Corynebacterium* spp. (13.42%), *Lactobacillus* spp. (21.48%) and *Staphylococcus* spp. (14.09%). All genera and bacterial species isolated can be found in the table 2. The species found in the vaginal microbiota were diversified and didn't differ much between the jennies with and without reproductive problems. *P*-values only were less than 0.05 in *Rhodococcus* spp. and *Streptococcus oralis* showing significant differences between jennies with and without reproductive problems. All P-values can be found in the table 2. It was not isolated any bacteria that could be compatible to *Taylorella equigenitalis* or *Taylorella asinigenitalis*.

Only one isolate of *Streptococcus zooepidemicus* showed resistance to tetracycline, even though the other two *S. zooepidemicus* isolates were found to have an intermediated resistance phenotype to this antibiotic.

On both two isolates of *S. zooepidemicus* from jennies with reproductive problems, the genotype of virulence factors detected was *sze*F, *sze*N, *sze*P, *szp*. In the isolate of *S. zooepidemicus* from a jenny without reproductive problems we only detected the gene *szp* and the gene *sze*F.

4. Discussion

Changes in genital bacterial microbiota are a common sign of reproductive disorders. The knowledge of the bacterial microbiota inhabiting a particular physiological niche, in this case the genital tract, would be necessary to develop therapeutic strategies that do not affect the healthy vaginal microenvironment [16, 17].

In this study, different genera of non-pathogenic bacteria and also some considered pathogenic were isolated from the vagina of both healthy and jennies with reproductive problems. Previous studies investigated the vaginal microbiota of mares and concluded that this community can be composed by diverse genera such as *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp. or *Bacillus* spp.. On some cases, bacteria like *Escherichia coli*, *Pseudomonas aeruginosa* or *Klebsiella pneumonia*e can also be found to inhabit in the vagina. Our findings are similar to these previous studies suggesting that the vaginal microbiota of *Miranda* jennies does not differ much from the vaginal microbiota of mares [16,18,19].

Streptococcus zooepidemicus is the most frequently isolated pathogen from the uterus of the mare. The prevailing hypothesis is that isolates of *Streptococcus zooepidemicus*, residing in the lower reproductive tract, cause infectious endometritis, by an ascending infection in a random manner, primarily governed by the uterine defence mechanisms of the mare. The clitoral *fossa*, clitoral sinuses and the vagina have been suggested as possible bacterial reservoirs [20]. In this study, we isolated *Streptococcus zooepidemicus* from jennies with and without reproductive problems. The genotype of virulence factors detected from the isolates of the jennies with reproductive problems, which presented more encoding genes of virulence factors, was somehow different from the genotype of jennies without reproductive problems. Despite this, we cannot come to any conclusion since the number of isolates studied is too small to draw definitive conclusions.

5. Concluding Remarks

To our knowledge this is the first study providing an overview of the vaginal bacterial microbiota of the Miranda donkey breed. Bacteria isolated were predominantly Gram-positive and belonging to the genera of *Bacillus* spp, *Corynebacterium* spp., *Lactobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp.. Since there are few studies on this matter focusing this issue, especially in this autochthonous donkey breed that is near extinction, the results of this study may be an asset for future investigations.

In order to find out the reason of the fertility problems of *Miranda* jennies, it would be important to evaluate in the future, all the points through which the diagnosis of uterine infections must pass, to exclude the possibility of these problems being caused by a bacterial infection. The study of genital microbiota of *Miranda* donkey breed males will also be important to knowledge if it includes pathogenic bacteria which can infect jennies during mating. Other problems not related with microorganisms should also be taken into consideration in the future in order to find out the reason for the reproductive problems and low fertility rates in this breed.

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