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Bacterial prevalence and antimicrobial susceptibility testing in uterine infections of mares from Rio Grande do Sul – Brazil

[*Prevalência bacteriana e susceptibilidade antimicrobiana em infecções uterinas de éguas no estado do Rio Grande do Sul*]

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ABSTRACT

One of the main obstacles of equine breeding is the presence of subfertile mares in the herd as they reduce pregnancy rates during the breeding season. Bacteriological exams made by collection of endometrial swabs is a reliable method of detecting the subfertility's origin and diagnosing uterine infections. In this retrospective study, 42 uterine swabs obtained from problem-breeding mares at the northwest and central region of Rio Grande do Sul – Brazil, were analyzed from 2016 to 2019. After microbiological culture, 30 swabs were positive for the presence of 37 microorganisms that cause endometritis. *Escherichia coli* (45.94%), followed by coagulase-negative (CNS) *Staphylococcus* sp. (18.92%), *Klebsiella aerogenes* (10.81%), *Corynebacterium* sp. (5.41%) and *Streptococcus* sp. (5.41%) were the most prevalent isolated microorganisms. The antimicrobials susceptibility tests revealed that ciprofloxacin, enrofloxacin and norfloxacin were the antibiotics with the greatest action *in vitro* against isolated microorganisms. In addition, neither Gram positive nor negative bacteria showed more resistance when compared to each other (p=0.239).

Keywords: antibiogram, equine, endometritis

RESUMO

Um dos maiores obstáculos da reprodução equina é a presença de éguas subférteis no plantel, as quais reduzem os índices de prenhez durante a estação. A realização de exame bacteriológico, por meio da coleta de swab *endometrial, é um método seguro no auxílio para detectar a origem da subfertilidade e diagnosticar infecções uterinas. Neste estudo retrospectivo, foram examinadas 42 amostras de* swabs *uterinos de éguas subférteis, das regiões noroeste e central do Rio Grande do Sul, durante o período de 2016 a 2019. Após cultura microbiológica, 30 amostras foram positivas para a presença de microrganismos causadores de endometrites.* Escherichia coli *(45,94%), seguido de* Staphylococcus *coagulase negativa - CNS (18,92%),* Klebsiella aerogenes *(10,81%),* Corynebacterium *sp. (5,41%) e* Streptococcus *sp. (5,41%) foram os microrganismos mais isolados. As provas de susceptibilidade aos antimicrobianos revelaram que ciprofloxacina, enrofloxacina e norfloxacina foram os antibióticos de maior ação* in vitro *contra os microrganismos isolados. Além disso, não houve correlação entre a classificação Gram e a taxa de resistência (P=0,239).*

Palavras-chave: antibiograma, equino, endometrite

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INTRODUCTION

Some considered endometritis as the main disease that causes subfertility and infertility, with a prevalence of 28 to 70% of mares with documented fertility problems (Leblanc and Causey, 2009; Rasmussen *et al*., 2015). Endometritis can be induced by copulation or artificial insemination (AI), being a normal event that occurs a few hours after semen deposition in the mare's reproductive tract. This process is important for the removal of bacteria and excess sperm introduced in the uterine lumen (Troedsson, 2006). Indeed, in all healthy animals, self-limiting endometritis is eliminated within 36 hours post-breeding, leaving the uterus clear without the semen-induced inflammation (Leblanc, 2003).

Most of the economic losses in equine reproduction are associated with failures in conception or abortions and when there is the development of postpartum placentitis or metritis (Pascottini *et al*., 2023). All mares that are incapable of cleaning the inflammatory products after breeding are called "susceptible mares" whereas "resistant mares" are those that perform rapid elimination of contaminants from the uterus and, consequently, are not prone to uterine infection (Leblanc, 2010). Uterine inflammation can be classified as acute, chronic, subclinical, postpartum, bacterial, fungal, viral, breed or AI induced and persistent. Each of these classifications is valid to describe the infection and inflammation that are affecting the uterus (Hurtgen, 2006).

There are some mechanisms capable of protecting the uterus against these diseases. Polymorphonuclear neutrophils (PMNs) are the first inflammatory cells to enter the uterine lumen followed by pro and anti-inflammatory cytokines because of uterine exposure to spermatozoa or bacteria (Troedsson and Woodwards, 2016). According to Leblanc (2010), culture and cytology are the most common techniques for diagnosing endometritis through the detection of uterine pathogens and inflammatory cells (neutrophils), respectively. Cytological evaluation involves the collection and interpretation of cells from the endometrium and uterine lumen, being a fast and inexpensive method capable of identifying the presence of endometritis in mares (Dascanio and Ferris, 2014).

Since 1980, bacterial culture has been used as a means to diagnose endometritis (Riddle *et al.*, 2007) and it can provide information on the diagnosis of infectious or non-infectious endometritis, such as bacterial growth associated with reduced pregnancy rate (Morel *et al.*, 2013). The antibiogram quickly allows, with 24 hours of incubation, to obtain information on sensitivity or resistance for the selected antibiotics, by measuring the zone of bacterial inhibition (Bishop and Mccue, 2014). The most common treatments for endometritis include the use of antimicrobials systemically or by uterine infusions (Zingher, 1996). In this case, the selection of antimicrobials to be used must be based both on determining the prevalence of the causative agents and on the knowledge of the susceptibility of these microorganisms to antimicrobials (Mccue *et al*., 1991)

It is crucial to address this pathology due to its significant economic impact. Therefore, an accurate diagnosis is necessary to enable the implementation of an effective treatment, thereby improving pregnancy rates and increasing the likelihood of mares carrying foals to term. This study aimed to identify the most prevalent bacterial species isolated from cases of equine endometritis in the state of Rio Grande do Sul and to evaluate the antimicrobial susceptibility of these pathogens to commonly used veterinary treatments.

ETHICAL ASPECTS

This research was not submitted to the *Ethics Committee on Animal Use* because it was a retrospective analysis of data collected during routine clinical procedures at veterinary hospitals. The animals involved were standard patients receiving necessary care and treatment, and no experimental interventions or deviations from the standard clinical protocol were performed for research purposes.

MATERIAL AND METHODS

The criteria of inclusion in this retrospective study were animals with history of subfertility and suggestive clinical and ultrasonographic findings of uterine infection, detected during

routine gynecological exams. The animals included in this study were from different breeds and placed in the northwest and center of state Rio Grande do Sul – Brazil. The 42 mares that matched the inclusion criteria were submitted to culture and antibiogram tests aiming for their diagnostic and treatment.

Gynecological examinations were performed with the mares restrained in individual stock and the animal's tail wrapped with a bandage. Rectal palpation, transrectal ultrasound and vaginoscopy were performed as part of the exam. For samples collection, the vulvar area was cleaned with neutral soap and water, repeated sufficiently to remove debris. The region was dried using disposable paper towels. In addition, a piece of cotton with NaCl 0,9% solution was used to clean the interior of the vestibule and remove fecal material and debris, thus preventing it from being improperly transferred to the uterus during the examination.

The material was collected using the double-guarded swab technique (Ferris, 2016). A palpation glove was sterilized by ultraviolet irradiation and with a sleeved arm, the swab was manually introduced through the vagina until the verification of the external ostium of the cervix, where the instrument was directed until it reached the uterine lumen. After the introduction, the metal rod was positioned in the body of the uterus, exposing the swab and rotating it consecutively three times to obtain the material (Overbeck *et al*., 2011).

The swabs were transported under refrigeration in test tubes with BHI (Brain Heart Infusion) medium (Difco, USA) to a referral microbiology laboratory of the region, where it was incubated at 37ºC for 24-48 hours. After that period, 10% defibrinated sheep blood agar plates (for aerobic culture) and chocolate blood agar plates (for microaerophilic culture) were smeared and incubated at 37ºC for 72 hours. The identification of microorganisms was conducted according to the recommendations of Quinn *et al*. (1994), and antimicrobial susceptibility tests using the disk diffusion method (Bauer *et al*., 1966). The criteria for the selection of the antimicrobial agents to be included and for interpreting the inhibition halos were those established by the Clinical and Laboratory Standards Institute (2024) guideline, and all the data were analyzed with the software RStudio 2024.06.14+372 based on R 4.4.1.

RESULTS AND DISCUSSION

In this study 30/42 mares (71.42%) presented positive uterine culture and 4/42 (9.52%) were with coinfection, presenting more than one microorganism in the analyzed samples. Moreover, 12/42 mares (28,57%) presented negative culture and did not require antibiogram. Gathering all the data, there was a higher prevalence of *Escherichia coli* (45.94%), followed by *Staphylococcus* sp. - CNS (18.92%), *Klebsiella aerogenes* (10.81%), *Corynebacterium* sp. (5.41%), *Streptococcus* sp. (5.41%), *Klebsiella pneumoniae* (2.70%), *Morganella* sp. (2.70%), *Proteus vulgaris* (2.70%), *Pseudomonas* sp. (2,70%) and *Serratia* sp. (2.70%) (Table 1).

Table 1. Micro-organisms isolated from uterine swabs and their frequency of isolation

Microorganism	Number of times isolated	Frequency of isolation (%)
Escherichia coli	17	45.94
Staphylococcus sp. (CNS)	7	18.92
Klebsiella aerogenes	4	10.81
Corynebacterium sp.	2	5.41
Streptococcus sp.	2	5.41
Klebsiella pneumoniae	1	2.70
Morganella sp.	1	2.70
Proteus vulgaris	1	2.70
Pseudomonas sp.	1	2.70
Serratia sp.	1	2.70
Total	37	100

These results corroborate other studies, which mention that, infectious endometritis is usually associated with opportunistic pathogens such as *Streptococcus zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* spp. and *Klebsiella pneumoniae* (Nielsen *et al*., 2010; Troedsson, 2011), often resulting from an infection of the uterus by genital and fecal flora, which when found in the culture medium of the endometrium, is diagnosed as positive (Causey *et al*., 2008). Furthermore, *Corynebacterium* sp. that is usually found in the soil, can access the mare's perineum due to soiling and relaxation of

the area resulting in infection (Assad and Pandey, 2012). In chronic uterine inflammation, there is disruption of the epithelium and increase in protective mucus production (Causey *et al*., 2008), allowing these bacteria to adhere to the mucosa.

Bandeira *et al*. (2017) when evaluating 74 uterine swabs collected from mares suspected of endometritis, obtained similar results to this study, with a higher frequency of infections by *Escherichia coli*. Like Ruy (2014) who obtained 28% samples of *Escherichia coli* out of 50 bacterial isolations. In another study, *Escherichia coli* was also the most isolated bacterium (25%) in cases of endometritis according to the presence of the inflammatory process on the cytological examination (Oliveira *et al*., 2010).

The method of collecting material through a swab for culture is the most used in the veterinarian routine (Nielsen *et al*., 2010). However not all mares with bacterial endometritis are identified with the "swab" because the swab encounters only an area of 1-2 cm of endometrium directly cranial to the cervix (Leblanc *et al.*, 2007). The correct interpretation of the mare's endometrium cultures requires the consideration of possible false positives and false negatives. Blanchard *et al*. (1981) associated false positive cultures with the contamination of the instrument in the environment, the external genitalia and the vagina. Although, the use of a sterile double-guarded swab, like the ones used in this study, is the most reliable method of avoiding external contamination (Cooke and Crabtree, 2020). In this study, the vulvar conformation of the selected mares was not evaluated, but it may contribute to the occurrence of endometritis. According to Leblanc (1997), *Escherichia coli* is commonly found in mares with vulvar and perineal anatomical defects, which predispose to pneumovagina and fecal contamination. *Escherichia coli* is a gram-negative aerobic species (Hartman and Bliss, 2011), considered the second most found agent in endometrial cultures in the equine species (Brinsko *et al*., 2011). Anzai *et al*. (1996) suggest that staphylococcal endometritis is originated from other sources of infection such as cutaneous infectious processes of the stallion, being *Staphylococcus* sp. transmitted during breeding.

The use of broad-spectrum antibiotics is common in the veterinary routine at the farms. The described antibiotic choices for endometritis, according to the practitioners' experience, are trimethoprim-sulfonamides, penicillin's, fluoroquinolones, aminoglycosides and cephalosporins (Köhne *et al*., 2020, 2024; Morris *et al*., 2020). However, rates of bacterial resistance to commonly preferred antibiotics, including gentamicin, penicillin and trimethoprim sulfonamides are well described (Davis *et al*., 2013; Mitchell *et al*., 2018).

The *in vitro* microbial sensitivity testing of the agents indicated ciprofloxacin (92.86%), enrofloxacin (92.59%) and norfloxacin (81.48%) as the most effective antimicrobials (Table 2 and 3). In addition, the antibiotics that showed high resistance potential were penicillin G (92%), ampicillin (88.46%) and erythromycin (62.50%). These results corroborate those obtained previously by Langoni *et al*. (1994), who pointed out the high sensitivity of Gram-negative and Gram-positive bacteria isolated from equine endometritis to ciprofloxacin. Although ciprofloxacin has a good susceptibility result, this antimicrobial is not safe for systemic use in horses. According to Yamarik *et al.* (2010) the administration of ciprofloxacin to horses is contraindicated due to the high incidence of life-threatening complications. The probable development of colitis associated with antimicrobials after oral administration of ciprofloxacin was reported by Weese *et al*. (2002). Nonetheless, intrauterine ciprofloxacin had no local or systemic adverse effects after the treatment of infectious endometritis in mares (Trundell *et al*., 2017).

Intrauterine infusion of enrofloxacin is not recommended and may cause significant inflammation, mucosal necrosis and endometrial fibrosis (Rodriguez *et al*., 2012). However, systemic enrofloxacin is recommended in the treatment of bacterial endometritis caused specifically by the subspecies *Staphylococcus zooepidemicus* and *Staphylococcus equi (*Luque *et al*. 2006). Albihn *et al*. (2003) conducted *in vitro* studies comparing susceptibility to various antibiotics and presented that enrofloxacin was the only antimicrobial that obtained higher rates of susceptibility toward the most common

endometritis pathogens. A Fisher's exact test was performed to evaluate the relation of resistance rate and Gram classification (p=0.239). No association was found, therefore neither Gram positive nor negative bacteria showed more resistance when compared to each other.

Table 2. Percentage of Gram-negative bacterial species sensitivity (S, S%), resistant (R, R%) and intermediate sensitivity (I, I%) to the most tested antibiotics

Antibiotic	S	R	I	S%	R%	I%	(n)
Ampicillin	1	20	0	4.76	95.24	0.00	21
Cephalothin	4	11	4	21.05	57.89	21.05	19
Cephalexin	4	10	8	18.18	45.45	36.36	22
Ceftiofur	8	2	3	61.54	15.38	23.08	13
Ciprofloxacin	22	0	1	95.65	0.00	4.35	23
Enrofloxacin	21	3	1	84.00	12.00	4.00	25
Erythromycin	1	12	6	5.26	63.16	31.58	19
Gentamicin	19	6	0	76.00	24.00	0.00	25
Norfloxacin	18	2	2	81.82	9.09	9.09	22
Penicillin G	0	19	0	0.00	100.00	0.00	19
Sulfazotrim	11	12	1	45.83	50.00	4.17	24
Tetracycline	12	7	5	50.00	29.17	20.83	24

Table 3. Percentage of Gram-positive bacterial species sensitivity (S, S%), resistant (R, R%) and intermediate sensitivity (I, I%) to the most tested antibiotics

Antibiotic	S	R	I	S%	R%	I%	(n)
Ampicillin	2	3	0	40.00	60.00	0.00	5
Azithromycin	3	0	0	100.00	0.00	0.00	3
Cephalothin	0	2	1	0.00	66.67	33.33	3
Cephalexin	2	1	0	66.67	33.33	0.00	3
Ceftiofur	1	0	1	50.00	0.00	50.00	2
Ciprofloxacin	4	1	0	80.00	20.00	0.00	5
Doxyciclin	2	1	0	66.67	33.33	0.00	3
Enrofloxacin	4	1	1	66.67	16.67	16.67	6
Erythromycin	2	3	0	40.00	60.00	0.00	5
Gentamycin	3	3	0	50.00	50.00	0.00	6
Marbofloxacin	4	0	0	100.00	0.00	0.00	4
Norfloxacin	4	1	0	80.00	20.00	0.00	5
Penicillin G	2	4	0	33.33	66.67	0.00	6
Sulfazotrim	5	2	0	71.43	28.57	0.00	7
Tetracycline	3	1	2	50.00	16.67	33.33	6

Treatments for uterine infections must be based both on the determination of the etiologic agents causing the infection and on the results of susceptibility tests to antimicrobials (Moreno *et al*., 1995). According to Claude *et al*. (1983), the indiscriminate use of antibiotics, based upon the practitioners' experience, can lead to the development of resistance of microorganisms. In clinically predisposed patients the veterinarian must do a continuous monitoring of vaginal or cervical discharge, uterine ultrasonography, and the occurrence of pneumovagina or age-related factors that can reduce the natural barriers to contamination.

CONCLUSIONS

The bacteria identified in this study can be mostly related to breeding or AI contaminations that evolved to uterine contamination and the development of endometritis. Furthermore, some predisposing factors such as advanced age, vulvar conformation or deficient uterine immune response must be associated. Monitoring the predisposed mares may help reduce the rates of reproductive issues. Regarding the resistance, there must be taken into account the relevance of identifying the causer agent to effectively

increase pregnancy rates in problem breeding mares.

CONTRIBUTORS

RCF Pereira: study conception, investigation, methodology, resources, supervision, writing (review and editing). LB Paz: data curation, validation, visualization, writing original draft. NA Martins: data curation, formal analysis, validation, writing original draft. JVG Mucha: data curation, visualization, writing original draft. RC Santos: data curation, visualization, writing original draft. LCPM Melo: visualization, writing (review and editing). R Pozzobon: visualization, writing (review and editing). FD De La Côrte: visualization, funding acquisition, writing (review and editing).

DATA AVAILABILITY STATEMENT

The research data are available within the article itself.

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