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Chronic endometritis-causing bacteria in Arabic mares

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ABSTRACT

Bacterial infections of the uterus are known to be an important cause of infertility in the mare. The objective of this study was to determine the species of bacteria isolated from the uterus of infertile Arabic mares, and investigate how identified bacteria are related to parity. A total of 18 Arabic mares with a history of long-term infertility were evaluated. The age range of mares was 4-23 years. For statistical analysis, logistic regression and Chi-square test were used by Proc Logistic of SAS. In this study, low-volume uterine flush and culture techniques were used. *P. aeruginosa* was the most prevalent bacterium isolated from 26.32% of mares as pure or in conjunction with *E. coli, K. pneumoniae*, or *Citrobacter spp*. Furthermore, 23.68% of bacterial infertility cases were related to *E. coli.* Pure growth of *E. coli* was observed only in one case. However, mixed growth with *P. aeruginosa, S.zooepidemicus,* and *S. aureus* was very prevalent. The present study revealed that the most prevalent bacteria isolated from chronic endometritis in Arabic mares were gram-negative bacteria ($p < 0.05$), while in some cases may be accompanied by gram-positive bacteria. *C. albicans* was isolated in only 8% of mares with chronic endometritis. Moreover, older age and higher parity number of the mares were not related to the presence of intrauterine fluid or the species of bacteria ($p > 0.05$). It can be concluded that the most prevalent bacteria isolated from infertile Arabic mares with chronic endometritis are gram-negative bacteria.

Keywords

mare, uterus, endometritis, bacteria, parity

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Abbreviations

P. aeruginosa: Pseudomonas aeruginosa
E. coli: Escherichia coli
K. pneumoniae: Klebsiella pneumoniae
S. aureus: Staphylococcus aureus
S. zooepidemicus: Streptococcus zooepidemicus
T. equigenitalis: Taylorella equigenitalis
C. albicans: Candida albicans

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Introduction

Despite using sophisticated anti-microbial agents, endometritis is one of the major causes of mare infertility and has been reported to be the third most common medical condition in horses [1, 2]. Bacterial infections of the uterus are known to be an important cause of endometritis and reduced fertility in mares [3]. There is no normal flora in the uterus of cycling mares. If the culture technique is good, any organism isolated from the uterus is a potential cause of infection and infertility [4]. The principal bacterial pathogens involved in endometritis are S. zooepidemicus, *E. coli, P. aeruginosa, K. pneumoniae, S. aureus, and T. equigenitalis*. In addition, the most common fungi responsible for endometritis include *C. albicans* and *Aspergillus* [5].

The uterus is repeatedly exposed to these contaminants at breeding, parturition, and gynecological examinations [5]. However, the uterus has defense mechanisms to clear contamination. These mechanisms consist of anatomic (physical) barriers, cellular phagocytosis, and physical evacuation of uterine contents [6]. There is a rapid migration of neutrophils in the normal uterus which destroys bacteria rapidly within 24 h. Afterwards, inflammatory materials are eliminated mechanically. The absence of defense mechanisms leads to the formation of uterine infections. Moreover, susceptible mares often have fluid remaining in their uterus, and this fluid is evacuated from the uterus with delay. These mares have low fertility because they fail to provide a suitable environment for the growth of embryos [7]. The main point in the successful management of mares with such problems is recognition shortly before or after mating [8, 9]. In order to evaluate the mares with infertility problems, knowing the history of the mares, bacteria isolated from genitalia, age, breed, quality of husbandry, as well as knowledge about the last parturition, and abnormal cycles are important [10, 11].

It should be noted that the frequent occurrence of acute endometritis may cause chronic endometritis with mucociliary dysfunction (12). In chronic endometritis, biofilm forms in the endometrium that provides an adhesive environment for bacteria. Some bacteria, such as *E. coli*, produce a biofilm that protects itself and other microorganisms from the inflammatory response (13). Chronic endometritis is related to some factors, such as mare age, cervical problems, and perinea dysfunction (14). The role of bacteria in chronic endometritis has been proven. The capability of bacteria to cause endometritis results from sticking to the endometrium, inducing inflammation, biofilm production, and resistance to phagocytosis (15). In broodmares, persistent endometritis is a frequent cause of sub-fertility (12). Classically, antibiotics act against fast-proliferating bacteria. Therefore, the bacteria outside the biofilm might be damaged, while the bacteria in the center of the biofilm stay alive and lead to chronic endometritis (16).

LeBlanc demonstrated that the most common organisms isolated from chronic endometritis in old mares included *S. zooepidemicus, E. coli, K. pneumoniae, P. aeruginosa, C. albicans,* and *Aspergillus* [13]. This researcher described in 2010 that chronic endometritis is more common in old mares [17]. Some authors have introduced the main cause of acute endometritis as *S. zooepidemicus, E. coli* (haemolytica), *P. aeruginosa,* and *K. pneumoniae* [18, 19].

Clinicians face various problems in impregnating mares with endometritis. Consequently, this study aimed to determine the causes of endometritis resistant to routine treatments. We tried to identify common bacteria involved in chronic endometritis of Arabic mares in Iran using differential and specific culture media. Clinicians can use the results of this study for choosing their treatment strategy. Furthermore, it has been tried to determine the relationship between the type of bacteria isolated from the infectious uterus and the number of parity.

Results

Eighteen Arabic mares with a history of infertility (normal anatomy and physiology but non-pregnant after four matings) were included in the study. The age range of mares was 4-22 years, of which three mares were maiden, seven had 1-5 parities, and eight had 6-13 parities. In estrus, when the dominant follicle was 35-40 mm, the uterine examination by ultrasound showed that the mare had endometrial edema with the accumulation of intrauterine fluid (≥ 2 cm). There was no significant association between the number of parity and intrauterine fluid accumulation. In this study, all the mares had bacterial or yeast growth on the uterus sample in the estrus phase. Figure 1 shows the presence of each endometritis-associated organism in pure or mixed forms. As can be seen, *P. aeruginosa* and *E. coli* were isolated more than others.

P. aeruginosa was the most prevalent isolated bacteria (25% of mares, Figure 1) that could be pure or in combination with *E. coli, K. pneumoniae, or Citrobacter spp.* In one case, *P. aeruginosa* infection was observed along with *S. zooepidemicus* and in another with *S. aureus*. Furthermore, 24% of bacterial infertility was related to *E. coli* (Figure 1). Pure growth of *E. coli* was found only in one case but mixed growth with *P. aeruginosa, S. zooepidemicus*, and *S. aureus* was very prevalent. In one mare, *E. coli* grew with *C. albicans*. The pure growth of *S. zooepidemicus* and *S. aureus* was not seen and all gram-positive bacteria were observed

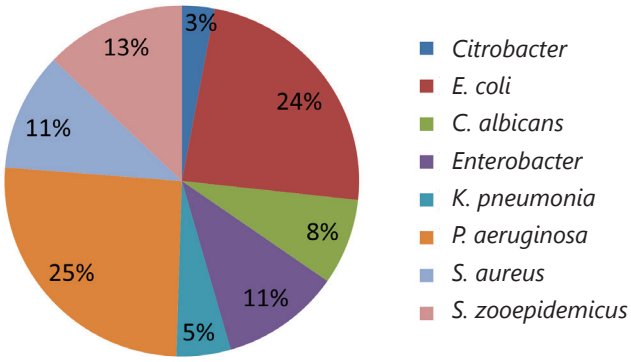

Figure 1.
Percentage of each organism isolated from infertile mares uterus.

Table 1.
Percentage and proportion of bacteria and yeast isolated from the uterus of infertile mares

Type	Gram-negative bacteria	Gram-positive bacteria	*C. albicans*
Pure Growth	7 (39%)	0	0
Mix Growth	11 (61%)	9 (100%)	*3 (100%)*

Table 2.
Result of Genmod for *C. albicans* and gram-negative and gram-positive bacteria

Organism	LSM ± SE[1]
C. albicans	$0.91^{a} \pm 0.58$
Gram-negative	$7.88^{b} \pm 0.21$
Gram-positive	$2.73^{a} \pm 0.34$

[1]Values indicate least square means (LSM) ± standard error (SE)

mixed with gram-negatives.

From another perspective, gram-negative bacteria were the most prevalent isolated organisms (39% pure growth and 61% with gram-positive bacteria or *Candida*). In this study, no pure growth of *Candida* or gram-positive bacteria was observed. All the growths of *C. albicans* or gram-positive bacteria were mixed with gram-negative bacteria (Table 1). In order to estimate the least squares means, we analyzed the data with PROC GENMOD. The least squares means are presented in Table 2. There was no significant association between the number of parity and the presence of intrauterine fluid or the class of microorganisms in endometritis ($p < 0.05$) (Table 3). The results for analysis of logistic regression on organism classification and parity are presented in Table 4.

In order to examine the independent effect of each variable on endometritis, logistic regression analysis was performed. The analyzed variables included organism classification and parity. Only the variable "organism classification" was found to be related to endometritis ($p < 0.05$). No relationship was found between the number of parity and endometritis ($p > 0.05$). Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were computed for all variables included in the final model (Table 5).

Table 3.
Percentage of isolated bacteria and yeast from the uterus of infertile mares in different parity

Organism/parity No.	0	1-5	6-13	Total	Y, N, or P
No growth	0	0	0	0	-
C. albicans	0	2.63	5.26	7.89	Y
Citrobacter spp	0	0	2.63	2.63	N
E. coli	5.26	10.53	7.89	23.68	N
Enterobacter spp	2.63	7.89	0	10.52	N
K. pneumonia	0	0	5.26	5.26	N
P. aeruginosa	0	10.53	15.79	26.32	N
S. aureus	2.63	7.89	0	10.52	p
S. zooepidemicus	5.26	5.26	2.63	13.15	p
Total	15.78	44.73	39.46	100	Yeast + P + N

p : Gram-positive, N: Gram-negative, and Y: Yeast

Discussion

The objective of the present study was to identify the type of bacteria in the uterus of mares with a history of long-term infertility and to assist practitioners' management in endometritis cases. The current study showed that the furthermost rampant bacteria in chronic endometritis of Arabic mares are gram-negative bacteria ($p < 0.05$) sometimes accompanied by gram-positive bacteria. In addition, intrauterine fluid accumulation and bacterial species were not related to the age and parity of mares ($p > 0.05$).

According to the technique described by Katila, a double-guarded method was used for collecting uterine lavage fluid [20]. Blood agar and chocolate agar were used for isolating aerobic and anaerobic bacteria from the collected uterine fluid. According to Brooks et al., blood agar and chocolate agar are complex, non-selective media, which support the growth of different bacteria (21). Furthermore, MacConkey agar, eosin methylene blue agar, mannitol salt agar, triple sugar iron agar, and C.E.M.O. agar base were used as differential culture media. These differential culture media were selected based on Jawetz medical microbiology textbook (21). Catalase and oxidase assays were also used to identify gram-positive bacteria. These techniques have been previously described by Murray and others [22].

According to Figure 1, in our study, *P. aeruginosa* was the cause of 25% of chronic endometritis cases in Arabic mares, with *E. coli* in the second place accounting for 24% of chronic endometritis patients. In chronic endometritis cases, the particles of biofilm were observed in the uterine lavage. *P. aeruginosa* is regarded as a venereally transmitted pathogen by some clinicians [23-26]. In one study in Saudi Arabia, *P. aeruginosa* was one of the most common bacteria associated with endometritis in mares, camels, and cows (27). According to Frontoso et al., 4%-10% of mares and 36% of stallions can harbor P. aeruginosa in their genitalia [28]. It is thought that by completely replacing natural mating with artificial insemination, endometritis caused by *P. aeruginosa* will be reduced.

In 2017, Ryan A. Ferris and colleagues declared that the clinical isolates of *P. aeruginosa* from the equine uterus can produce a biofilm [29]. In other words, *P. aeruginosa* can lead to chronic endometritis that resists treatment. Due to the high prevalence of *P. aeruginosa* in infertile uterine fluid (Figure 1), practitioners should consider these issues when dealing with long-term infertility and uterine fluid in the ultrasonic examination. Because of the venereal transmission of *P. aeruginosa*, we believe that we should not allow mating until complete cure and we strongly recommend that natural mating be replaced by artificial insemination. It is also recommended that all hygiene principles be strictly followed during artificial insemination.

In a study by Frontoso et al., bacteria were isolated from 49% of infertile mares and 18.4% of cases related to *E. coli* [28]. In our study, bacteria were isolated from all mares that remained infertile after four matings. Moreover, in this study, after *P. aeruginosa*, *E. coli* was the second most important bacterium isolated from the uterus of infertile mares. According to the results of the present study and clinical observations, it is thought that most cases of chronic endometritis caused by *E. coli* are related to problems in the perinea.

Gram-negative bacteria, such as *P. aeruginosa* and *E. coli* are highly capable of forming biofilms [21]. A biofilm is the organized life of bacteria within an extracellular matrix [30]. It seems that in our study, biofilm formation by gram-negative bacteria was the main cause of long-term infertility.

Biofilm formation consists of four stages, including initial surface attachment, microcolony formation, formation of biofilm architecture, and biofilm propagation [31]. *S. aureus* biofilm has been observed in cases of contaminated catheters [21], which is likely to cause *S. aureus* to enter the uterus and cause biofilm formation on the endometrium.

Most bacteria isolated from the clinical cases of mare endometritis include *S. zooepidemicus, E. coli, K. pneumoniae,* and *P. aeruginosa* [24]. LeBlanc (2008) showed that K. pneumoniae is one of the main causes of infertility in mares, and has been resistant to most antibiotics [24], but in our clinical study, *K. pneumonia* accounted for 5% of chronic endometritis cases. In the literature, *Enterobacter spp*. was not discussed as the main causative agent of infertility [4, 23, 24], which is inconsistent with the results of our study (10% of all chronic endometritis cases).

In our study, OR estimates showed that the incidence of gram-negative-related endometritis was approximately 20 times higher than the incidence of gram-positive-related endometritis (Table 5). Pure growth of gram-negative bacteria and mix growth were seen in 39% and 61%of infertile mares, respectively. No pure growth of gram-positive bacteria was

Table 4.
Result for the analysis of logistic regression on organism classification and parity

Effect	df	Wald *Chi*-Square	Pr > ChiSq
Organism classification	2	6.4463	0.0398*
Parity	2	3.7371	0.1543ns

*: significant difference ($p < 0.05$); ns: non-significant effect

Table 5.
Odds ratio estimates for statistical comparison between different investigated factors

Effect	Point estimate	95% Wald confidence limits	
Candida vs gram-positive	0.017	< 0.001	1.134
Gram-negative vs gram-positive	20.453	0.541	773.304
Parity 0 vs Parity 6-13	0.032	< 0.001	1.295
Parity 1-5 vs Parity 6-13	0.712	0.035	14.386

observed in this study (Table 1).

Ferrer et al. [32] reported that the most commonly isolated bacterial species was E. coli (30.7%), and mares with mixed growth most commonly grew a combination of gram-negative and gram-positive bacteria (65.5%). Furthermore, endometritis due to *T. equigenitalis* was reported by many clinicians and researchers [33, 34]. In the current study which was performed on a limited number of mares with chronic endometritis, *T. equigenitalis* was not isolated.

Results of the present study revealed that the most prevalent bacteria isolated from infertile Arabic mares were gram-negative bacteria (68% of the isolated bacteria), and in some cases were accompanied by gram-positive bacteria. Therefore, in mares with a history of long-term infertility, if the clinician does not have access to the microbiology laboratory for an antibiogram, it would be better to use broad-spectrum antibiotics that are more effective against gram-negative bacteria. Furthermore, the findings of this study indicated that the increasing age and parity of mares were not related to the presence of intrauterine fluid or the species of bacteria. In mares with chronic endometritis, mucolytic agents, such as DMSO and N-acetylcysteine, are recommended for biofilm disruption and better effects of antibiotics.

Materials and Methods

Animals

In this study, 18 Arabic mares with a history of infertility were used. The mares were examined by ultrasonography (SIUI CTS-900, equipped with a 5 MHz linear-array transducer Guangdong, China) for ovarian follicle diameter, uterine status, and the existence of uterine fluid. Mares with a history of infertility, uterine fluid accumulation, and ovarian follicles of > 35 mm in diameter (n=18, aged 4-22 years) were enrolled in the study.

Sampling

Primarily, the tails of mares were wrapped and pulled to the side. The vulva was thoroughly washed with detergents. Next, uterine lavage for bacteriological examination was taken. Briefly, an infusion of 60 ml of normal saline into the uterus was taken with a double-guarded catheter. After centrifuging uterine reversal fluid, the pellets were used for microbiological examinations.

Microbiological culture

Samples were cultured directly onto blood agar and chocolate agar and were incubated at 37 C and 5% CO_2. Bacterial growth was investigated after 24 and 48 h. Afterwards, gram staining was performed on isolated bacterial colonies. In addition to gram staining, catalase and oxidase assays were also used to identify gram-positive bacterial species. The grown bacteria were re-cultured on differential media, namely MacConkey agar, eosin methylene blue agar, mannitol salt agar, triple sugar iron agar, and C.E.M.O. agar base.

Statistical analysis

For statistical analysis, PROC LOGISTIC for logistic analysis was used. The significance level for the Chi-square test was $p < 0.05$. Differences in the presence of different infectious organisms were analyzed using a generalized model with PROC GENMOD. All analyses were carried out using SAS 9.2.

Authors' Contributions

BQP and GM conceived and planned the experiments. BQP and MK carried out the experiments. AR contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors have no conflict of interest to declare.

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