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RESEARCH ARTICLE

Antimicrobial Susceptibility of Bacteria Isolated from Uteri of Thoroughbred Mares with Fertility Problems

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Summary

Endometritis is an important cause of subfertility with high economic impact in mares and is mostly associated with bacterial infections. The aim of this study was to investigate bacterial pathogens in uteri and the susceptibility of some clinical isolates against several antimicrobial agents frequently used to control bacterial endometritis in mares in Germany. A total of 247 uterine swabs taken from mares with fertility problems were cultured to isolate bacteria and *in vitro* antimicrobial susceptibility of ß-hemolytic *Streptococcus* spp. and *Escherichia coli (E. coli)* strains was determined. Totally, 151 samples (61.1%) were found culture positive. A total of 332 microorganisms including 331 bacteria and one fungus were isolated from the samples. From the bacteria, 21,9%, 15.9%, 15.4%, 12%, 10.5%, 5.7% and 3.0% of the isolates were identified as *Escherichia coli (E. coli)*, α- hemolytic streptococci, ß-hemolytic streptococci, *Bacillus* spp., γ-hemolytic streptococci, coliform bacteria and *Staphylococcus aureus (Staph. aureus)* respectively. The remaining isolates (15.6%) included 13 other bacterial species and one fungus. ß-hemolytic streptococci and *E. coli* strains were considered as frequently associated with fertility problems and antimicrobial susceptibility of these isolates against 14 antimicrobial agents including penicillin, tulathromycin, tetracycline, erythromycin, florfenicol, ceftiofur, amoxicillin, amoxicillin/clavulanic acid, enrofloxacin, gentamicin, cefquinome, colistin, marbofloxacin and sulfamethoxazole/trimethoprim was determined. All ß-hemolytic streptococci were found to be susceptible to penicillin, ceftiofur, amoxicillin/clavulanic acid, enrofloxacin, cefquinome, marbofloxacin and sulfamethoxazole/trimethoprim, whereas 29.3% of ß-hemolytic streptococci showed resistance against colistin. All *E. coli* strains and 50% of them were resistant to penicillin and erythromycin, respectively, wheres all *E. coli* isolates were sensitive to all other tested antimicrobial agents used in this study.

Keywords: *Mare, Endometritis, Bacterial Isolation, Antibiotic Susceptibility*

Fertilite Problemli Damızlık Kısrakların Uteruslarından Bakteri İzolasyonu ve Antimikrobiyal Duyarlılık

Özet

Kısraklarda endometritis yüksek ekonomik kayba sebep olan önemli kısırlık nedeni olup, çoğunlukla bakteriyel infeksiyonlar sonucunda ortaya çıkmaktadır. Bu çalışmanın amacı, Almanya'da kısraklarda bakteriyel endometritis patojenlerini belirlemek ve klinik izolatların çeşitli antimikrobiyal ajanlara duyarlılıklarını araştırmaktır. Bu çalışmada Almanya'da fertilite problemi olan kısraklardan toplanan 247 uterus numunesi mikrobiyal ve antibiyotik duyarlılığı yönünden değerlendirildi. Yüzellibir (%61.1) numune bakteriyolojik olarak pozitif bulundu. 331'i bakteri ve biri mantar olmak üzere toplam 332 mikroorganizma izole edildi. Bakteri izolatların % 21.9'u *Escherichia coli (E. coli)*, %15.9'u, α-hemolitik streptokok, %15.4'ü ß-hemolitik streptokok, %12'si *Bacillus* spp., %10.5'i γ-hemolitik streptokok, %5.7'i koliform bakteri ve %3'ü *Staphylococcus aureus* olarak identifiye edildi. Geriye kalan suşlar (%15.6), 15 farklı bakteri türü ve bir mantar suşundan oluştu. Kısrakların fertilitesini en sık etkileyen bakteriler olarak ß-hemolitik streptokok ve *E. coli* etkenlerine karşı antibiyogram testi uygulandı. Bu bakterilerin, penisilin, tulatromisin, tetrasiklin, eritromisin, florfenikol, seftiofur, amoksisilin, amoksisilin/klavulanik asit, enrofloksasin, gentamisin, sefkuinom, kolistin, marbofloksasin, sulfametoksazol/trimetoprim olmak üzere toplam 14 antimikrobiyal maddeye karşı duyarlılığı test edildi. Bu çalışmada, ß-hemolitik streptokok suşlarının tamamı florfenikol, seftiofur, amoksisilin/klavulanik asit, amoksisilin, enrofloksasin, sefkuinom, marbofloksasin ve sulfametoksazol/trimetoprim'e duyarlı olduğu, %29.3'ünün de kolistine karşı dirençli olduğu belirlendi. *E. coli* suşlarının tamamı penisillin'e, %50'si de eritromisin'e dirençli bulunurken, tamamının florfenikol, seftiofur, amoksisilin, amoksisilin/klavulanik asit, enrofloksasin, gentamisin, sefkuinom, kolistin, marbofloksasin, sulfametoksazol/trimetoprim'e duyarlı olduğu saptandı.

Anahtar sözcükler: *Kısrak, Endometritis, Bakteriyel izolasyon, Antibiyotik duyarlılığı*

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INTRODUCTION

Bacterial infections of the uterus are known to be an important cause of reduced fertility in mares [1,2]. In the horse industry, endometritis due to uterine infections of bacterial origin brings about 25-60% economical losses, leading to infertility [3-5]. Frequent pathogenic bacteria causing infertility in mares are introduced to the uterus at occasions such as natural breeding, artificial insemination, postnatal infections (pneumovagina infections), and mostly during a delayed postnatal cleaning of the uterus [6-9]. Swabs taken from the lumen of the uterus have been commonly used for diagnosis of endometritis and infertility for long years. Careful evaluation of clinical signs, uterine culture, ultrasonography, cytology and histopathologic examinations are prerequisites for a correct diagnosis and successful cure of uterine infections in mares [9-11].

Bacterial infections are accepted to be the major reason of reproduction failure in mares. Bacterial isolation from the uterus can be shown as an evidence of endometritis [12,13]. Bacterial uterine infections cause conception and embryo survival failures despite many repeated breedings during the season. Moreover, bacterial pathogens of the uterus affect the elasticity and stickiness of the uterine mucus. It is stated that *Klebsiella pneumoniae* and other gram negative bacteria lead to the viscosity of the mucus causing a decrease of mucocilliar activity, while ß-hemolytic streptococci decrease the viscosity of the mucus. If uterus cleaning delays, subclinic endometritis occur [14]. In the same study, it has been mentioned that an increase of prostaglandin (PGE2), leucotriene B4 and arachidonic acide metabolits and a subsequent increase of vascular permeability cause the entrance of *Streptococcus equi* ssp. *zooepidemicus* into the uterus [3].

Aerobic bacteriologic culture from uterine samples of mares is the commonly used method for diagnosis of endometritis. Bacterial agents which are responsible for endometritis are *Streptococcus equi* ssp. *zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus* spp., *Pasteurella* spp., *Taylorella equigenitalis*, *Corynebacterium* spp. and fungi. A previous study has shown, that the degree of endometrial inflammation caused by *Escherichia coli* is more severe than caused by *Streptococcus* spp.[10]. Another study has emphasized that *Escherichia coli* showes an affinity to the urogenital system by uropathogenic virulence factors such as pap, sfa, hlyA, cnf1 and fim [15].

The purpose of this study was to determine bacteriological agents isolated from swabs obtained from the uteri of thoroughbred mares with infertility problems between 2006 and 2008 and to evaluate their antibitiotic susceptibility in Germany.

MATERIAL and METHODS

Between 2006 and 2008, a total of 247 uterine swabs taken from infertile and non-pregnant thoroughbred mares were delivered within the same day to the Bacteriological Diagnostic Laboratory at the Institute for Hygiene and Infectious Diseases of Animals, Justus-Liebig University Gießen. All endometrial samples were collected under sterile conditions either in sterile Phosphate Buffer Saline or Stuart Transport Medium (Oxoid; CM0111). Prior to sampling the external genital region and rima vulva of the mares was cleaned and disinfected several times with paper towels soaked in alcohol based antiseptic. A Polanski speculum was placed into the vagina and the cervix was made visible. Then a disposable swab (Equivet uterine culture swab; Kruse, Marslev, Denmark) or Knudsen catheter was used to take endometrial samples.

Uterine swabs were examined by conventional cultural methods. Bacteriological isolation was performed onto 5% defibrinated Blood Agar (Oxoid; CM0271), Gassner Agar (Merck; 1282) and serum bouillon. All plates and serum bouillon were incubated at 37°C for 24 to 48 h. The number of colonies on primary plates was assessed semi-quantitatively and scored as absent (no colonies), small numbers (1-50 colonies, +), moderate numbers (51-200 colonies, ++) and large numbers (>200 colonies, +++) [16]. Subculturing was performed from suspicious colonies. Biochemical tests and API kits (Biomerieux) were used for identification of subcultures.

Antimicrobial sensitivity test was performed by using the disc diffusion method described firstly by Bauer et al.[17] and evaluated according to the Standards of National Committee for Clinical Laboratory (NCCLS) [18]. Test was performed for *E. coli* and all ß-hemolytic streptococci. Following antibiotic discs were used in the test: Penicillin (10 U) (P), tulathromycin (30 µg) (TUL), tetracycline (30 µg) (TE), erythromycin (15 µg) (E), florfenicol (30 µg) (FF), ceftiofur (30 µg) (EFT), amoxicillin/clavulanic acid (20/10 µg) (AMC), amoxicillin (10 µg) (AML), enrofloxacin (5 µg) (EN), gentamicin (10 µg) (GM), cefquinome (30 µg) (CEQ), colistin (25 µg) (CO), marbofloxacin (5 µg) (MAR), sulfamethoxazole/trimethoprim (23.75/1.25 µg) (SXT).

RESULTS

Out of 247 collected samples, 151 (61.1%) were found to harbor bacteriae by conventional isolation and identification methods. No bacteriologic isolation was achieved from 96 samples (38.9%). More than one species of bacteria was isolated from 90 samples (59.6%) (Data not shown).

A total of 332 isolates were detected from culture positive samples. Most frequently isolated bacteria were *E. coli* (21.9%), α-hemolytic streptococci (15.9%), ß-hemolytic streptococci (15.4%), *Bacillus* spp. (12%), γ-hemolytic *streptococci* (10.5%) coliform bacteria (5.7) and *Staphylococcus aureus* (3%). The other isolates such as *Staph. epidermidis*, *Acinetobacter* spp. and *Corynebacterium* spp. and other species are detailed in *Table 1*.

Table 1. *Microorganisms and their ratios from uterus swabs of Thoroughbred mares between 2006 and 2008*
Tablo 1. *2006-2008 yılları arasında safkan kısrakların uterus svaplarından elde edilen mikroorganizmalar ve oranları (%)*

Microorganism	Number of Isolation (n)	Isolation Rate (%)
ß-hemolytic *Streptococci*	51	15.4
Escherichia coli	73	21.9
Escherichia coli hemolytic	1	0.3
Coliform bacteria	19	5.7
γ- hemolytic *Streptococci*	35	10.5
α- haemolytic *Streptococci*	53	15.9
Bacillus spp.	40	12.0
Actinobacillus equuli	3	0.3
Corynebacterium spp.	7	2.1
Proteus spp.	1	0.3
Staphylococcus epidermidis	14	4.2
Staphylococcus aureus	10	3.0
Pseudomonas spp.	2	0.6
Alcaligenes dentrificans	1	0.3
Enterobacter cloacae	1	0.3
Enterococcus faecalis	1	0.1
Enterococcus spp.	7	2.1
Acinetobacter spp.	9	2.7
Erwinia spp.	1	0.3
Nocardia spp.	1	0.3
Flavobacterium spp.	1	0.3
Fungus	1	0.3
TOTAL	332	100

E. coli and *ß*-hemolytic streptococci strains yielded moderate growth (++) in 40% and 45% of the cultures or abundant (+++) in 50% and 45% of the cultures, respectively (Data not shown).

In antimicrobial susceptibility tests, all *ß*- hemolytic streptococci were found to be susceptible to penicillin, ceftiofur, amoxicillin/clavulanic acid, enrof1oxacin, cefquinome, marbofloxacin and sulfamethoxazole trimethoprim, whereas 29.3% of *ß*-hemolytic streptococci showed resistance against colistin. All *E. coli* strains and 50% of them were resistant to penicillin and erythromycin, respectively, wheres all *E. coli* isolates were sensitive to all other tested antimicrobial agents used in this study *(Table 2)*.

DISCUSSION

Common bacterial agents isolated from uterine disorders and infertility problems are *Escherichia coli*, *ß*-hemolytic *streptococci*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae, Taylorella equigenitalis, Streptococcus equi* ssp. *zooepidemicus* [1,5,6,14]. In this study, 21 different bacteria and one fungus were isolated from uterine swabs of mares. Microbial isolates related to uterine disorders and their resistance and susceptibility to selected antimicrobial agents were discussed in this section.

In previous studies, the rate of mares with fertility problems due to bacterial infection were reported to be 30%, 32%, and 39%, respectively [19-21]. However, a more recent study reported that bacteria could be isolated from 66.2% of mares with fertility problems [22]. In our study, mares were selected from animals with reproductive disorders and 61.1% of them had significant growth of microorganisms.

Table 2. Antibiotic susceptibility of *ß*-hemolytic *Streptococci* and *Escherichia coli* isolates (%)
Tablo 2. *ß*-hemolitik *Streptococci* ve *Escherichia coli* izolatlarının antibiyotik duyarlılığı (%)

Antibiotic Discs	*ß*-hemolytic *Streptococci*			*Escherichia coli*		
	S (%)	MS (%)	R (%)	S (%)	MS (%)	R (%)
Penicillin	100					100
Tulathromycin	93.2	6.8		50.0	50.0	
Tetracycline	81.8	15.9	2.3	100		
Erythromycin	95.4	2.3	2.3		50.0	50.0
Florfenicol	97.7		2.3	100		
Ceftiofur	100			100		
Amoxycillin/ Clavulanic acid	100			100		
Amoxycillin	97.7		2.3	100		
Enrofloxacin	100			100		
Gentamicin	84.6	13.6		100		
Cefquinome	100			100		
Colistin	68.2	2.5	29.3	100		
Marbofloxacin	100			100		
Sulfamethoxazole/Trimethoprim	100			100		

S: *Sensitive* ***MS:*** *Moderate-Sensitive* ***R:*** *Resistant*

The variations between the reports above-mentioned may be due to the prophylactically use of antibiotics, which may also result in increasing resistance of bacterial strains. Antibiotic resistance more and more becomes a health problem of major importance [23,24]. Also sampling technique influences culture results [25,26].

In the present study, most of the isolated nonhaemolytic *E. coli* and *ß*-hemolytic streptococci yielded moderate or abundant growth. This may indicate an infection in the uterus rather than a vulvovestibular contamination. Non-hemolytic *E. coli* and *ß*-hemolytic streptococci were the most frequently isolated microorganisms associated with fertility problems in the mare. In contrast to our study, it has earlier been suggested that non-hemolytic *E. coli* isolates from equine uterine are non-pathogen [27]. However, a similar study has declared that non-hemolytic *E. coli* may cause fertility problems in mares which seems to be more associated with repeat breeding without clinical symptoms than with clinical symptoms of endometritis [6]. *Staph. aureus* is reported to be a rather frequently isolates species from the equine uterus of mares [19,20]. Ten (3%) *Staph. aureus* isolated from the samples may also be responsible for disorder and/or infection in mares' uterus investigated in the present study.

The global rise in antibiotic resistance has been linked to an increased use of antibiotics [28]. In farm applications, antibiotics are used for prophylactic purposes rather than treatment. They are used prophylactically before breeding or treatment of endometritis [19] as well as in semen extenders [29].

In vitro antimicrobial susceptibility test was performed for selected isolates representing pathogenic bacteria such as ß-hemolytic streptococci and *E. coli* which are considered to be responsible for infertility. *ß*-hemolytic streptococci were found to be moderately resistant (29.3%) to colistin, whereas high susceptibility of *ß*-hemolytic streptococci was observed to penicillin, ceftiofour, amoxicillin/clavulanic acid, enrofloxacin, gentamicin, cefquinome, marbofloxacin, and sulfamethoxazole/trimethoprim. All *E. coli* isolates showed resistance to penicillin but were found to be susceptible to florfenicol, tetracycline, ceftiofur, amoxicillin/clavulanic acid, enrofloxacin, gentamicin, colistin, cefquinome, marbofloxacin and sulfamethoxazole/trimethoprim. These results of the study showed that antimicrobial susceptibility of both *ß*-hemolytic streptococci and *E. coli* isolates from uteri of thoroughbred mares in Germany seems to be higher than those of reported in other studies. A similar study reported that *ß*-hemolytic streptococci are susceptible against sulfamethoxazole/trimethoprim, enrofloxacin, gentamicin with a ratio of 100%, 97% and 96%, respectively [6]. In the same study, *E. coli* isolates were found to be susceptible against amoxycilline/clavulanic acid, enrofloxacin, and sulfamethoxazole/trimethoprim with a ratio of 71.9%, 78.1%, and 73.5%, respectively. Another study declared that ß-hemolytic streptococci isolates were susceptible to amoxycilline/clavulanic acid, enrofloxacin and sulfamethoxazole/trimethoprim in rates of 82.7%, 17% and 17%, respectively [7]. Gentamicin susceptibility of *E. coli* strains was found to be 86% and 96% in the studies performed in USA and Sweeden, respectively [6,30].

In conclusion, types and rates of bacteria isolated from the uterus of mares can vary by region during years. Although our results indicated high susceptibility of bacteria, which were responsible for infertility in thoroughbred mares, to antimicrobial agents commonly used for treatment in Germany, systematic bacteriological examination should be performed and positive cultures should be evaluated for resistance of bacteria to determine antibiotics for effective treatment. Infertility factors in mares should also be monitored systematically and regularly to achieve healthy mares.

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