

Uterine Microbiology and Antimicrobial Susceptibility in Isolated Bacteria from Mares with Fertility Problems

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Albiñ A, Båverud V, Magnusson U: Uterine microbiology and antimicrobial susceptibility in isolated bacteria from mares with fertility problems. Acta vet. scand. 2003, 44, 121-129. – Uterine microbiology and antimicrobial susceptibility was investigated in 239 mares with fertility problems in a prospective study in Sweden. Uterine swab samples were collected with double guarded swabs and transported overnight before being cultured. The Minimum Inhibitory Concentrations (MIC) was determined for a panel of antimicrobials. From 152 of the 239 mares at least one bacterial species was isolated, most frequently *E. coli* (104 isolates), β -haemolytic streptococci (31) and fungi (16). β -haemolytic streptococci were more frequently ($p < 0.01$) associated with clinical endometritis than with repeat breeding. The opposite was true for *E. coli* ($p < 0.01$). Among β -haemolytic streptococcal isolates some resistance was noted for 4 of 11 tested antibiotics, however, all isolates were susceptible to the widely used penicillin G. Among *E. coli* isolates enrofloxacin was the only of the 10 tested antibiotics for which no resistance was noted. Resistance was most commonly noted to cephalothin (39% of the isolates), streptomycin (22%), trimethoprim/ sulphamethoxazole (15%) and ampicillin (11%). In conclusion, we show that both *E. coli* and β -haemolytic streptococci are frequently associated with fertility problems in mares and that antimicrobial resistance is a common feature of *E. coli* but also recognised for β -haemolytic streptococcal uterine isolates.

equine; subfertility; endometritis; uterine swab samples; bacteriology; resistance to antibiotics; E. coli, β -haemolytic streptococci; Streptococcus zooepidemicus.

Introduction

Uterine infections have long been recognised as one of the major causes of reduced fertility in the mare (Asbury 1986). These infections are most often caused by opportunistic micro-organisms and a variety of species have been isolated (Shin *et al.* 1979, Ricketts *et al.* 1993). The uterine infections often cause endometritis. Antibiotics are one component often used in the treatment of endometritis (Perkin 1999). For clinicians there is a need of rapid microbiological diagnosis so that adequate treatment of the infection can be performed while the mare is

still in oestrus (Ricketts & Mackintosh 1987, Ricketts 1989). Therefore some mares are treated with antibiotics without a preceding microbiological investigation, whereas sometimes bacteriological cultivation is performed by the clinicians themselves. If the treatment is performed without a microbiological diagnosis, the choice of antibiotic is often based on data from earlier studies, e.g. Shin *et al.* (1979) and Ricketts *et al.* (1993). However, the bacterial species isolated, as well as their susceptibility to antibiotics, may vary over time as well as from

one population of horses to another (Sternberg 1999). The variation may be attributable to differences in antibiotic treatment policies, stud-farm management, breed and clinical history of the sampled mares as well as microbiological culture routines. The present survey of uterine microbiology and antimicrobial susceptibility in mares selected for having fertility problems was conducted in Sweden where artificial insemination is commonly used. Also the policies for clinical use of antibiotics are regarded to be strict in Sweden (Franklin 1999, SVARM 2001). The aim of the present study was to determine the most common bacterial species in uterine samples from Swedish mares with fertility problems and the antimicrobial susceptibility of isolated bacteria. Such data should serve as a basis for updated recommendations on how to treat uterine infections in the mare.

Materials and methods

Sampling

Clinicians representing different types of stud farms, geographically located all over Sweden, were invited to send uterine samples from mares in oestrus to the National Veterinary Institute (SVA) for culture free of charge. During the spring and summer 1996 and 1997 swabs from 239 mares were submitted from 36 different clinicians.

Mares

For all 239 mares included in the study, at least one fertility problem was noted. From the preceding season 89 barren mares and 33 abortions/resorptions were recorded. During the current season 121 repeat breeding mares were recorded. Repeat breeding was defined as starting a new oestrus cycle after artificial insemination (AI) or being bred by a stallion once or repeatedly during oestrus in at least one oestrus cycle (with a normal or changed length of the luteal phase). Clinical signs of endometritis

during the current season were noted in 89 mares. The designation clinical signs of endometritis in this study included at least one of the following criteria: vulvular discharge or fluid in the uterine lumen during the luteal phase, the latter diagnosed with ultrasonography, inflammatory cells on a cytological smear sample or significant bacterial growth. Reproductive problems during the current season may be combined with barrenness or resorption/abortion during the preceding season. Repeated breeding was often combined with some of the other reproductive disorders. The average age of the mares was 12.2 years. For 32 mares the age was not recorded, 32 mares were from 3-7 years, 85 from 8 to 13 years and 90 from 14 to 24 years old. The dominating breeds of the included mares were Swedish Warmblood (80), Standardbred Trotter (75), North-Swedish Trotter (39) and Thoroughbred (25). Twenty mares were either of other breeds or their breed was not recorded.

Since many clinicians were involved in the present study, it was important to establish a simple, straightforward sampling protocol in order to achieve good quality and reliable data. Hence, we limited the study to bacteriological sampling. For these samples, the external genitalia were carefully washed with soap and water and thereafter dried with paper. In order to minimise contamination of the sample by bacteria from the vagina and perineum the sampling was performed using a gloved hand in the vagina and double-guarded, occluded swabs enabling sampling from the uterus solely (Equi-Vet, Kruuse, Marslev, Denmark). The uterine culture swabs were transported in Amies' modified media with charcoal (SVA, Uppsala, Sweden) (Amies 1967, Engvall 1985) at ambient temperature and cultured within 24 h. This medium has been widely used in Sweden as an all-purpose transport medium for equine gynaecological swabs.

Bacteriology

All samples were cultured on 5% horse blood agar (SVA) and lactose bromocresol purple agar (SVA). The samples were inoculated on the agar plates and diluted by an inoculation loop obtaining 3 levels of dilution on the agar plate. Each bacteriological culture was inspected and bacterial growth was registered after 24 and 48 h incubation at 37°C.

Growth of *Pseudomonas* (*P.*) *aeruginosa*, *Klebsiella* (*K.*) *pneumoniae*, haemolytic *Escherichia* (*E.*) *coli* and β -haemolytic streptococci was always considered to be of significance (Shin *et al.* 1979, Ricketts *et al.* 1993, Langoni *et al.* 1997). Other bacterial isolates were typed and considered as significant if growth was in pure culture or dominating on the agar plate. From the same sample, 2 bacterial species might be isolated and typed. Bacterial growth was evaluated on horse blood agar plates according to the following guidelines: abundant, >100 CFU (colony forming units)/plate; moderate, 21-100 CFU/plate; sparse, 10-20 CFU/plate; insignificant, <10 CFU/plate. Conventional methods for isolation and identification of microorganisms were used (Quinn *et al.* 1994).

Mycology

Samples from 233 of the 239 mares were also cultured for fungi on sabouraud dextrose agar 2% (Difco laboratories, Detroit, M) with chloramphenicol (0.5 µg/ml, Fluka Chemi, Buchs, Switzerland) and incubated at 30°C for 5 days. In the identification of fungus yeast was not identified to species except for *Candida albicans* according to McGinnis (1980).

Antimicrobial susceptibility testing

A microtiter plate system (VETMIC™, SVA) was used for the antimicrobial susceptibility tests. The test was done according to the manufacturer's instruction. In brief, each well was inoculated with 50 µl of Mueller Hinton broth

Table 1. Species isolated in microbiological cultivation from uterine swabs from 152 mares. Two bacterial species might be isolated and typed from the same mare.

Microorganisms	Number	%
<i>Actinobacillus</i> spp/ <i>Pasteurella</i> spp	1	<1
<i>Corynebacterium</i> spp	2	1
<i>Enterobacter aerogenes</i>	4	3
<i>Enterobacter agglomerans</i>	3	2
<i>Enterobacter</i> spp	1	<1
<i>Enterococcus</i> spp	2	1
<i>Escherichia coli</i> , non-haemolytic	99	64
<i>Escherichia coli</i> , haemolytic	5	3
<i>Klebsiella pneumoniae</i>	1	<1
Gramnegative coccus	12	8
Gramnegative rods, inactive	11	7
<i>Pseudomonas aeruginosa</i>	1	<1
<i>Pseudomonas</i> spp	5	3
<i>Sphingomonas paucimobilis</i> ¹	1	<1
<i>Staphylococcus</i> spp, coagulase neg	3	2
<i>Streptococcus</i> spp, α -haemolytic	1	<1
<i>Streptococcus</i> , β -haemolytic	31	20
<i>Streptococcus equi</i> subsp. <i>equi</i>	1	
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>	4	
<i>Streptococcus equi</i> subsp. <i>zooepidemicus</i>	21	
<i>Streptococcus</i> , β -haemolytic ³	5	
Fungi ²	16	
Yeast, not <i>Candida albicans</i>	13	
Yeast ³	2	
Mould	1	

¹previous name *Pseudomonas paucimobilis*

²233 out of 239 mares were cultured for fungi

³not further typed

(Merck, KgaA, Darmstadt, Germany) to which 10³ to 10⁴ CFU of the bacteria to be tested were added. The wells were sealed with transparent adhesive tape and incubated at 35-37°C for 16-18 h. The lowest concentration of an antibiotic completely inhibiting bacterial growth was registered as Minimum Inhibitory Concentrations (MIC). Results were categorised by using the breakpoints for resistant, intermediate and sensitive recommended by the NCCLS for bacteria

Table 2. Distribution (no. of isolates) of MIC values for 104 *Escherichia coli* isolates of the 10 antibiotics tested. Vertical lines show the break points between sensitive (S), intermediate (I) and resistant (R). The S-isolates are to the left and the R-isolates to the right of the lines.

Antimicrobial tested	MIC (mg/L)										Range tested ¹	S-I-R % isolates
	≤0,12	0,25	0,5	1	2	4	8	16	32	>32		
Ampicillin ²					20 ^{H1}	50 ^{H2}	19	3	11 ^{H2}		2-16	86 3 11
Cephalothin						5	14	44 ^{H1}	31 ^{H4}	10	4-32	18 43 39
Chlor- amphenicol					4	22	71 ^{H5}	6	1		2-16	94 6 1
Enrofloxacin		101 ^{H5}		1	2						0,25-2	97 3 0
Gentamicin				48 ^{H2}	NT	52 ^{H3}		3	1		1-16	96 0 4
Neomycin ²					58 ^{H2}	NT	38 ^{H3}	1	2	4	2-32	93 3 4
Nitro- furantoin ³						3	17	76 ^{H5}	7	1	4-32	99 - 1
Oxy- tetracycline				6	NT	79 ^{H4}	12 ^{H1}	2	5		1-16	81 12 7
Streptomycin					1	NT	51 ^{H1}	23 ^{H3}	6	23 ^{H1}	2-32	51 27 22
Trimethoprim- sulpha- methoxazole ^{3,4}	79	NT	6 ^{H5}	3	NT	NT	1	15			0,12-8	85 - 15

¹ When the MIC value was above the range tested, the value for the next titration step (the value just above the range) was used.

² One strain not tested.

³ The vertical line shows the break point between S and R, no I sensitivity is given.

⁴ The MIC value for trimethoprim tested in combination with sulfamethoxazol (1:20) is given.

^{H1-5} The number of haemolytic *E. coli* isolates.

NT = not tested, the titration step is not included in the VetMICTM system.

isolated from animals (1999). Currently, no recommendations are available from the NCCLS for spiramycin, streptomycin, fusidic acid, nitrofurantoin and enrofloxacin. Therefore, for these antimicrobials the values recommended by the manufacturer were used. Quality control strains included *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. The MICs of the quality control strains were always within recommended ranges (NCCLS, 1997).

Statistical analysis

The analysis of frequencies of the various fertility problems connected with microbiological diagnosis was made by chi-square analysis within the frequency procedure in SAS (SAS Inc., 1990).

Results

Bacteriology

From 152 positive samples out of 239 sampled mares one or 2 significant bacterial species were isolated and identified. Ninety-two (38%) of the positive samples yielded growth of one single species in pure or almost pure culture. Thirty-one (13%) mares yielded 2 species and

Table 3. Distribution (no. of isolates) of MIC values for 31 β -haemolytic streptococcal isolates of the 11 antibiotics tested. Vertical lines show the break points between sensitive (S), intermediate (I) and resistant (R) isolates. The S are to the left and the R to the right of the lines.

Antimicrobial tested	MIC (mg/L)											Range tested ¹	S-I-R % isolates
	≤0,06	0,12	0,25	0,5	1	2	4	8	16	32	>32		
Ampicillin	30	1	NT	NT								0,12-16	100 0 0
Cephalothin							31					4-16	100 0 0
Chlor- amphenicol						15	15	1				2-16	100 - 0
Clindamycin ²					28	NT	3					1-4	90 0 10
Erythromycin ³			31									0,5-4	100 0 0
Gentamicin						NT	6	9	10	6		1-16	19 29 52
Neomycin						1	NT	3	5	10	12	2-32	13 48 39
Oxytetra- cycline					9	NT	15	6		1		1-16	29 48 23
Penicillin G	31		NT	NT		NT	NT					0,06-8	100 0 0
Spiramycin ⁴							31					4-32	100 - 0
Trimethoprim - sulphameth- oxazole ^{4,5}	17	5	6			NT	NT	1	2			0,12-8	90 3 7

¹ When the MIC value was above the range tested, the value for the next titration step (the value just above the range) was used.

² All isolates equal to or less than 1 were regarded as S since this was the lowest concentration tested.

³ All isolates equal to or less than 0.5 were regarded as S since this was the lowest concentration tested.

⁴ The vertical line shows the break point between S and R_t, no I-sensitivity is given.

⁵ The MIC value for trimethoprim tested in combination with sulfamethoxazol (1:20) is given.

NT = not tested, the titration step is not included in the VetMICTM system.

29 (12%) mares yielded 1 species dominating on the agar plate together with sparse non-specific mixed culture. In samples from the mares without significant growth 57 (24%) yielded no growth at all and 30 (13%) growth of non-specific mixed culture.

The bacterial species mostly isolated was *E. coli*, yielding 104 isolates, thereof 64 isolates in pure or almost pure culture (Table 1). When the bacterial growth was quantified, 72 *E. coli* isolates yielded abundant, 21 moderate and 11 sparse growth. From two mares, two different

isolates of *E. coli* were isolated. Only 5 of the 104 *E. coli* isolates were haemolytic *E. coli*, 3 of these in pure culture. When the bacterial growth was quantified, 3 of these haemolytic *E. coli* yielded abundant, 1 moderate and 1 sparse growth.

The second most frequently isolated species was β -haemolytic streptococci yielding 31 isolates, thereof 12 grew in pure culture (Table 1). When the bacterial growth was quantified, 12 *Streptococcus* isolates yielded abundant, 12 moderate and 7 sparse growth.

Relation between fertility problems and microbiological diagnosis

From repeat breeding mares, as well as from mares with clinical symptoms of endometritis, *E. coli* was the most frequently isolated species. β -haemolytic streptococci were more frequently ($p < 0.01$) associated with clinical endometritis than with repeat breeding (68 versus 23% of β -haemolytic streptococcal isolates). The opposite was true for *E. coli* ($p < 0.01$) (38 versus 53% of *E. coli* isolates).

Mycology

From 15 mares yeast was isolated (13 of these further typed as not being *Candida albicans*) and from 1 mare mould was isolated. These were all in mixed culture with bacteria.

Antimicrobial susceptibility

Among the 104 *E. coli* isolates, resistance was most common to cephalothin, streptomycin, trimethoprim/sulphamethoxazole (TMP) and ampicillin (Table 2). Several isolates were resistant to more than one antimicrobial. Enrofloxacin was the only one of the 10 tested antimicrobial agents for which no resistance was noted. The 5 haemolytic *E. coli* isolates were all classified as susceptible to TMP and gentamicin (Table 2).

Among the 31 β -haemolytic streptococcal isolates resistance was most common to gentamicin, neomycin, oxytetracycline, and to TMP (Table 3). All isolates were classified as susceptible to the β -lactam antibiotics, penicillin G and ampicillin, and also to cephalothin, erythromycin, spiramycin and chloramphenicol.

Discussion

Mares included in this study were selected for reproductive disorders and only 32% of them yielded no significant growth of microorganisms. This figure should be compared with

studies where mares have not been selected for reproductive problems. In these studies 70% (Redaelli & Codazza 1977), 68% (Shin et al. 1979) and 61% (Ricketts et al. 1993) of the mares yielded no significant growth. Other possible reasons for the difference in outcome between studies could be that different breeds have been studied and/or there was different breeding management between the studied populations. Also the sampling technique and microbiological culture routines may influence the results.

Our data suggest that *E. coli* is the microorganism most frequently associated with fertility problems in the mare and that β -haemolytic streptococci are the second most frequent. Further, *E. coli* seems to be more associated with repeat breeding without clinical symptoms than with clinical symptoms of endometritis. The opposite relation seems to apply to β -haemolytic streptococci. This finding may be of interest for clinical considerations.

The overall dominance of *E. coli* relative to β -haemolytic streptococci in uterine swab samples was consistent in both years of sampling. The dominance of *E. coli* is in contrast to studies from other countries, where β -haemolytic streptococci have been the bacteria most commonly isolated. We cannot give a causal explanation to this dominance of *E. coli* in the present study. Other studies have mostly been performed in normal populations of mares (Shin et al. 1979, Ricketts et al. 1993), which may be one explanation of this difference. But also a study in barren mares (Langoni et al. 1997) shows this dominance of β -haemolytic streptococci.

Further, it has earlier been suggested that non-haemolytic *E. coli* is a non-pathogen in the equine uterus (Barrelet 1995). In our study, 99 of the 104 *E. coli* isolates were non-haemolytic *E. coli*. Given the clinical history of these 99 mares, and the fact that most of the non-

haemolytic isolates yielded abundant growth in pure or almost pure culture, we suggest that also non-haemolytic *E. coli* in the equine uterus may cause fertility problems.

In the present study, fungi were the third most frequent microbiological finding. Yeasts or mould were always isolated together with bacteria. Unfortunately, it was not possible to tell whether fungi or bacteria caused the primary infection. Fungal infections of the non-pregnant equine uterus were earlier said to be uncommon (Redaelli & Codazza 1977), but more recently fungal infections have been believed to be more frequent, possibly due to the widespread use of antibiotics and the increasingly intensive management and manipulation of reproduction in mares (Blue 1987, Le Blanc 1997).

Staphylococcus aureus is reported to be a rather frequently isolated species from the equine uterus in a normal population of mares (Ricketts *et al.* 1993, Shin *et al.* 1979). In our study this species was not isolated at all, only coagulase-negative *Staphylococcus* spp. were isolated from 3 mares. Notable in the present study is also that well-known uterine pathogens such as *P. aeruginosa* and *K. pneumoniae* were only isolated from one mare each.

The bacterial species isolated may be influenced by the stud farm management and the breeding regime used. In Sweden as well as in this study, the two dominating breeds are the Swedish Warmblood and the Standardbred Trotter. These breeds are mainly bred by AI, 71% and 88% for the Swedish Warmblood (Hästavel, 1998) and the Standardbred Trotters (STC, 1999), respectively.

Also the sampling technique influences the culture results. In the present study, most of the isolated *E. coli* and β -haemolytic streptococci yielded moderate or abundant growth of the isolated bacteria, indicating that these isolates represented an infection in the uterus rather

than a vulvovestibular contaminant (Hinrichs *et al.* 1988, Waelchli *et al.* 1992). In this study, samples were transported overnight and when a time-span of this kind exists between collections and cultures the choice of transport medium is likely to influence the culture results (Shin *et al.* 1979, Ricketts *et al.* 1993).

A general global rise in antibiotic resistance has been linked to an increased use of antibiotics (Fox 1997, Swartz 1997). In stud farm practice antibiotics have long been used both prophylactically before breeding, as a treatment of endometritis (Kenney *et al.* 1975, Shin *et al.* 1979) as well as in semen extenders (Burns *et al.* 1975, Kenney *et al.* 1975). In the present study, resistance to several commonly used antimicrobials was recorded. Notably as much as 15% of the *E. coli* isolates were resistant to TMP and 4% of the isolates to gentamicin. With respect to gentamicin, McCue *et al.* (1991) report a sensitivity in only 86% of *E. coli* isolates from equine endometrial swabs collected in the US. Our corresponding figure is 96%. This difference might be due to differences in how often the drug is used. The distribution of MIC-values for resistant *E. coli* isolates is rather consistent when comparing isolates from different species and organs (SVARM 2001). All β -haemolytic streptococcal isolates were uniformly sensitive to 6 of 11 tested antibiotics, which is in accordance with Shin *et al.* (1979). As expected, all streptococcal isolates were sensitive to β -lactam antibiotics.

Conclusion

The key findings from this study of mares with a history of fertility problems were firstly: *E. coli* was the overall most frequently isolated bacterial species, while uterine pathogens such as *P. aeruginosa* and *K. pneumonia* were rare. Secondly: β -haemolytic streptococci were more frequently associated with clinical endometritis than with repeat breeding, whereas the opposite

applied to *E. coli*. Thirdly: the noticed resistance to antibiotic suggests that a proper microbiological diagnosis and antimicrobial susceptibility testing are required for successful antimicrobial therapy.

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Sammanfattning

Mikrobiologi och antimikrobiell känslighet hos bakterier isolerade från uterus hos ston med fruktsamhetsproblem.

Mikrobiologisk status i uterus och antimikrobiell känslighet undersöktes hos 239 ston med fruktsamhetsproblem. Undersökningen utfördes i Sverige. Prov från uterus togs med dubbelskyddad svabb och transporterades innan odling till laboratoriet under natten. Minsta inhiberande koncentration (MIC) bestämdes för ett urval av antibiotika.

Från 152 av de 239 stona isolerades minst ett bakterie species, vanligast *E. coli* (104 isolat), β -hemolyserande streptokocker (31) och svamp (16). β -hemolyserande streptokocker associerades mer frekvent ($p < 0.01$) med klinisk endometrit, än med omlöpnings. Motsatsen gällde för *E. coli* ($p < 0.01$).

Bland β -hemolyserande streptokockisolat noterades viss resistens mot 4 av 11 testade antibiotika, dock var alla isolat känsliga för den allmänt använda penicillin G. Bland *E. coli* isolat var enrofloxacin det enda av de 10 testade antibiotika för vilket ingen resistens noterades. Resistens noterades mest frekvent för cefalotin (39% av isolaten), streptomycin (22%), trimetoprim/ sulfametoxazol (15%) och ampicillin (11%). Sammanfattningsvis så visades att *E. coli* ofta associeras med fruktsamhetsproblem hos sto och att antimikrobiell resistens är vanligt förekommande bland *E. coli* isolat.

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