

Incidence of bacterial pathogens in equine uterine swabs, their antibiotic resistance patterns, and selected reproductive indices in English thoroughbred mares during the foal heat cycle

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ABSTRACT: Fertility problems of mares on a well-managed breeding farm with thoroughbred stallions have been ascribed mostly to contamination of the reproductive apparatus of females with pathogens, particularly those of bacterial origin. This study presents a summary of the frequency of bacterial pathogens isolated from 437 cervical swabs which were collected from English thoroughbred mares intended for mating between 2008–2014, as well as of resistance tests of these pathogens to seven commonly used antibiotics as follows: penicillin, gentamicin, tetracycline, sulfisoxazole, cefotaxime, marbofloxacin and enrofloxacin. In addition, the study reports the changes in the levels of plasma oestradiol and progesterone determined two to three days before and two to three days after the first post-partum ovulation in mares with positive and dubious bacteriological findings and percentage of barren mares and mares that conceived at first, second and third post-partum ovulations. It was observed that 21.5% of mares were barren even after the third post-partum cycles. The oestradiol levels determined two to three days before the first post-partum ovulation were significantly lower in mares positive for pathogenic microflora in their reproductive apparatus compared to mares with the dubious findings (25.1 ± 5.8 pg/ml vs. 69.7 ± 18.3 pg/ml; $P < 0.05$), while the mean progesterone levels did not differ significantly but displayed a rather wide range in positive mares (from 0.08 to 1.38 ng/ml) compared to dubious mares with only small variations (0.12 ± 0.03 ng/ml). Moreover, of the total number of cervical swabs taken shortly before the first post-partum oestrus from all the mares intended for mating as many as 69.7% were contaminated with pathogenic microflora (positive findings). Saprophytic microorganisms only (the dubious findings) were isolated from 29.7% of swabs. From the 307 positive swabs, we could identify 40.4% positive for β -haemolytic streptococci and 20.4% positive for *Escherichia coli*, the pathogens implicated in causing reproductive disorders. Tests of antibiotic resistance of the investigated pathogens revealed that both Gram-positive and Gram-negative bacteria showed high susceptibility to antibiotics such as cefotaxime, marbofloxacin and enrofloxacin. On the other hand, both these bacterial groups showed high resistance to routinely used broad-spectrum antibiotics, such as penicillin and tetracycline. Because further research is required for a full understanding of the mechanism of pathogenesis of post-breeding endometritis, we can only hypothesise that uterine contamination with pathogenic microflora, particularly with β -haemolytic streptococci and coliform bacteria, diagnosed before the first post-partum ovulation, could negatively affect the hormonal regulation of oestrus and result in mare fertility problems.

Keywords: equine; endometritis; antibiotics; progesterone; oestrogen

One of the most serious problems encountered in the reproduction of racehorses, associated with mare fertility problems, is contamination of the reproductive apparatus of these animals with pathogenic microorganisms, particularly those of

bacterial origin. These pathogens are introduced into the reproductive organs in several ways: (a) most frequently soon after the parturition when an opened cervix allows the microorganisms pass into the uterus from the vagina; moreover, retained

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lochia (amniotic fluid, portions of placenta) is a suitable substrate for multiplication of pathogens; (b) during oestrus, when the uterus may be contaminated with smegma from the preputial sac of stallions during natural mating of thoroughbreds. For example, LeBlanc (2003) reported that healthy mares can clear any excess fluid from the uterus within 8 h following coitus while sub-fertile mares may retain uterine fluid for more than two days after the covering. Unfortunately, the anatomy but also the physiology of the reproductive tract of mares delays uterine clearance and may lead to the development of endometritis. Numerous studies performed in horses have reported that the most common bacterial pathogens isolated from uterine swabs include β -haemolytic streptococci, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Pseudomonas* spp. and others (Dhingra and Sandhu 1987; Ricketts and Mackintosh 1987; Fodor et al. 1995; Langoni et al. 1997; LeBlanc 1999; Albihn et al. 2003; Szeredi et al. 2003; Frontoso et al. 2008). Infection of the reproductive tract with these agents may result in endometritis and infertility. For this reason it is very important for veterinarians to detect mares positive for uterine contamination and to initiate their antibiotic therapy. Because effective antibiotic therapy should be based on microbiological cultivation and the subsequent testing of antibiotic resistance of relevant pathogens – a time-consuming process even when performed in certified microbiological laboratories – veterinarians frequently choose a simple but flawed approach involving administration of broad-spectrum antibiotics and consider only the results of available relevant studies and their experiences. The careless preventive administration of “first-choice antibiotics”, practiced particularly between the seventies and nineties of the past century, resulted in the development of resistance to broad-spectrum antibiotics, such as penicillin and tetracycline, in many microorganisms.

The above-mentioned explains why a study of this character is very important for the strategy of use of antibiotics in equine veterinary practice. This is particularly so because only a limited body of information is available in this area and the national control programs focus only on food-producing animals.

MATERIAL AND METHODS

The examined stud farm raises only English thoroughbred stallions which are used for natural cov-

ering of English thoroughbred mares. The health of stallions is checked regularly and they are subjected to serological testing for infectious equine anaemia and infectious metritis annually, before the onset of the mating season. On the other hand, it is difficult to obtain information on the health status of mares brought to the stud farm as they come from various private farms. Therefore, after arrival at the stud farm, the urogenital apparatus of each mare should be regularly examined, as mentioned later.

A cervical swab is taken and examined microbiologically from each mare intended for mating at six to seven days post-partum and the entire reproductive apparatus of the animal is subjected to rectal and ultrasonographic examination in order to detect the first approaching post-partum oestrus by observation of the following signs: relaxed cervix with mild uterine tonus, oedematous endometrium, dominant ovarian follicle (diameter ≥ 30 mm), absence of uterine content (no cavity). If all these criteria are met the “healthy” mare is bred by one mount of the desired stallion. When, on the next day, the presence of the ovulation stigma is not confirmed by ultrasonographic examination, the mare is bred again every other day until the ovulation is confirmed (but maximally up to three mounts). In the case of retained uterine fluid the following procedure is used: (a) if the cavity with fluid is less than 2 cm in diameter, the mare is administered oxytocin *i.m.* at a dose of 10–20 IU; in principle, if the fluid is still present after 6 h, oxytocin is administered again and the uterus is examined after an additional 6 h; repeated positive finding indicates the 3rd (last) administration of oxytocin; (b) if the cavity with more than 2 cm diameter persists, uterine lavage with saline solution is performed and oxytocin is administered immediately after using the procedure described previously. In general, the last administration of oxytocin should take place within 48 h after ovulation. Natural covering of a mare which had excess fluid in the uterus is arranged according to a schedule described for a healthy mare but the natural cover must take place minimally 6 h before the therapeutic intervention.

All bacterial pathogens isolated from cervical swabs were subjected to antibiotic resistance testing and only on the basis of results (obtained no later than on Day 3 following the sampling) effective antibiotic therapy of mares with persisting positive uterine pathological findings was initiated.

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Levels of oestradiol and progesterone were determined in plasma obtained by centrifugation of venous blood withdrawn from *v. jugularis* into vacuum tubes (Vacutest 5 ml sterile tube). Both oestradiol E2 and progesterone were determined using available commercial kits (Oestradiol E2 Enzyme Immunoassay Kit and Progesterone Enzyme Immunoassay Test Kit) using the Enzyme Immunoassay method.

Before collecting the cervical swabs, the external genitals of mares were rinsed twice with a 0.1% solution of iodopovidone and dried. A sterile swab with double protection intended for sampling of biological material from the uterine cervix of mares (EquiVet uterine culture swab, Kruuse, Denmark) was introduced into the uterine cervix controlled by a hand protected by a rectal glove to minimise its contamination by vaginal microflora.

After inoculation of plates containing blood agar and Endo agar with the content of swabs, the plates were incubated under aerobic conditions at 37 °C for 24 h and observed for haemolytic reaction. *Proteus* bacteria were identified on the basis of their characteristic “swarming pattern” of growth. If staphylococcus colonies appeared on the plates the incubation lasted 48 h. All plates that did not show haemolysis and contained no colonies characteristic of the potential pathogenic bacteria were considered as dubious, containing only common saprophytic microflora. In contrast, each colony of haemolytic or potentially pathogenic bacteria was inoculated onto a specific diagnostic agar. Antibiotic resistance was determined employing Mueller-Hinton agar and in the case of staphylococci, blood agar. The swabs were sent to an accredited microbiological laboratory (Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Kosice, Slovak Republic) and the results regarding antibiotic resistance of bacterial pathogens were obtained on Day 3 following the sampling, or on Day 4 at the latest.

The examined stud farm evaluated the success of natural mating using ultrasonographic examination on Days 15 (diagnosis of twins in the uterus), 27, 42 and 48 post-ovulation. Findings were confirmed using ultrasonography. If the ultrasonographic examination performed on Day 15 post-ovulation failed to confirm pregnancy, the mare was administered a synthetic analogue of prostaglandin $F_2\alpha$ at a dose of 1 ml *pro toto*, and after three to five days examined for the signs of oestrus.

The number of mares that conceived at the first, second and third post-partum ovulation was obtained from the evidence of the veterinarian responsible for reproduction on the stud farm.

Statistical procedures. The statistical software GraphPad Prism 2004 was used for the statistical analyses. The hormonal indexes were analysed using the unpaired Student's *t*-test. These results are expressed as mean \pm SD.

RESULTS

During the investigated period more than one fifth of all mares (21.5%) showed fertility problems after natural mating and remained barren even after the third post-partum oestrus cycle. Of all mares that were pregnant at ultrasonographic examination on Day 48 after the last ovulation, 68.5% conceived at the first, 24.0% at the second and 7.5% at the third oestrous cycle after the parturition.

The level of oestradiol two to three days before the first post-partum ovulation was significantly lower in mares positive for potential pathogens in their reproductive apparatus compared to mares with dubious findings (25.1 ± 5.8 pg/ml vs. 69.7 ± 18.3 pg/ml, $P < 0.05$), while the level of progesterone showed no significant differences. However, in mares with positive findings the range of progesterone levels was much wider, from 0.08 up to 1.38 ng/ml, while in the dubious mares the range was quite narrow ($0.12 \pm$

Table 1. Levels of oestradiol and progesterone in plasma during first post-partum cycle in mares with dubious and positive results in microbial cultivation

	2–3 days before ovulation		2–3 days after ovulation	
	dubious finding	positive finding	dubious finding	positive finding
Oestradiol (pg/ml)	$69.7 \pm 18.3^{a,b}$	25.1 ± 5.8^b	20.9 ± 6.4^a	18.2 ± 11.8
Progesterone (ng/ml)	0.12 ± 0.03^a	0.41 ± 0.55	9.51 ± 5.98^a	5.52 ± 6.07

All results are presented as means \pm SD

^{a,b}means with the same superscript in a row are statistically significant at the level $P < 0.05$

Table 2. Results of bacteriological examination of cervical swabs from mares and frequency of detection of individual bacterial pathogens

Micro-organisms	Total number of isolates	Total frequency of isolates (%)	Frequency of pathological isolates (%)
Positive findings			
<i>Staphylococcus aureus</i>	27	6.3	8.8
<i>Staphylococcus intermedius</i>	6	1.4	2.0
<i>Staphylococcus</i> spp.	21	4.9	6.8
Non-haemolytic <i>Escherichia coli</i>	60	14.1	19.5
Haemolytic <i>Escherichia coli</i>	18	4.2	5.9
<i>Pseudomonas aeruginosa</i>	2	0.4	0.7
<i>Pseudomonas</i> spp.	11	2.5	3.6
β -haemolytic <i>Streptococcus</i>	124	29.0	40.4
<i>Streptococcus</i> spp.	33	7.7	10.7
<i>Corynebacterium</i> spp.	1	0.2	0.3
<i>Serratia</i> spp.	1	0.2	0.3
<i>Proteus</i> spp.	15	3.5	4.9
<i>Bacillus</i> spp.	1	0.2	0.3
<i>Klebsiella</i> spp.	5	1.2	1.6
<i>Enterobacter</i> spp.	1	0.2	0.3
Dubious findings			
Saprophytic micro-organisms	130	30.3	
Total number of swabs	427		

0.03 ng/ml). Moreover, comparison of oestradiol levels before and after ovulation showed that oestradiol levels in the dubious mares were significantly lower after ovulation while in the positive mares no significant change was observed (Table 1).

Our investigations showed that 69.7% of cervical swabs were positive for pathogenic micro-organisms; of those (307 positive findings) 40.4% contained β -haemolytic streptococci and 20.4% *Escherichia coli* (19.5% non-haemolytic, 5.9% haemolytic). From 30 swabs we recovered more than one pathogen by cultivation; for example from eight swabs a combination of β -haemolytic streptococci and haemolytic *Escherichia coli* and from four swabs β -haemolytic streptococci and *Staphylococcus aureus*. On the other hand, common saprophytic microflora alone was diagnosed in 29.7% of all cervical swabs collected from mares (Table 2).

Table 3 presents the results of antibiotic resistance testing of the isolated pathogens. It was revealed that both Gram-positive and Gram-negative bacteria were highly susceptible to cefotaxime, marbofloxacin and enrofloxacin. On the other hand, a

high proportion of the tested bacteria of both types showed resistance to broad-spectrum antibiotics such as penicillin and tetracycline. For example, all six isolates of haemolytic *Escherichia coli* and 83.4% of isolated non-haemolytic *Escherichia coli* were resistant to penicillin. Tetracycline inhibited the growth of 66.6% of haemolytic and 45.8% of non-haemolytic *Escherichia coli*, while other *Escherichia coli* isolates showed high or intermediate resistance to this antibiotic.

DISCUSSION

Racehorse breeders strive to cover mares already during the first cycle, the so-called “foal heat”, or during the second one at the latest. In this ideal case, the mare will come into heat in February and the foal is born in January of the following year. Such early covering is advantageous for the mare owner and the basic idea behind it is as follows: (a) the first and second post-partum oestrus will occur again in February, so the entire cycle will be repeated regularly; (b) the foal born in January

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Table 3. Susceptibility of isolated Gram-positive and Gram-negative bacterial pathogens from cervical swabs of mares to common antibiotics

Gram-positive bacteria	β-haemolytic streptococci (90 isolates)			<i>Streptococcus</i> spp. (27 isolates)			<i>Staphylococcus aureus</i> (10 isolates)			<i>Staphylococcus intermedius</i> (2 isolates)			<i>Staphylococcus</i> spp. (15 isolates)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Antibiotics tested															
Penicillin	93.3	–	6.7	85.2	3.7	11.1	90.0	–	10.0	100	–	–	66.7	–	33.3
Gentamicin	8.9	8.9	82.2	44.4	7.4	48.2	100	–	–	50	50	–	60.0	6.7	33.3
Tetracycline	44.4	16.7	38.9	40.7	–	59.3	100	–	–	50	–	50	73.3	13.3	13.3
Sulfisoxazole	73.5	10.8	15.7	51.9	11.1	37.0	70.0	30.0	–	50	–	50	46.7	13.3	40.0
Cefotaxime	94.1	–	5.9	100	–	–	100	–	–	100	–	–	88.9	11.1	–
Marbofloxacin	84.4	13.1	2.3	74.1	18.5	7.4	100	–	–	100	–	–	93.3	6.7	–
Enrofloxacin	83.1	11.3	5.6	58.3	33.3	8.4	100	–	–	–	–	–	100	–	–
Gram-negative bacteria	Haemolytic <i>Escherichia coli</i> (6 isolates)			Non-haemolytic <i>Escherichia coli</i> (25 isolates)			<i>Pseudomonas</i> spp. (1 isolate)			<i>Proteus</i> spp. (4 isolates)			<i>Klebsiella</i> spp. (5 isolates)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Antibiotics tested															
Penicillin	–	–	100	8.3	8.3	83.4	–	–	100	25	–	75	–	–	100
Gentamicin	83.3	–	16.7	54.2	4.2	41.6	100	–	–	50	–	50	100	–	–
Tetracycline	66.6	16.7	16.7	45.8	4.2	50.0	100	–	–	25	–	75	60.0	–	40.0
Sulfisoxazole	16.7	–	83.3	25.0	8.3	66.7	–	–	100	25	–	75	80.0	–	20.0
Cefotaxime	100	–	–	81.8	8.3	8.3	–	–	–	100	–	–	–	–	–
Marbofloxacin	100	–	–	91.8	4.1	4.1	100	–	–	75	–	25	100	–	–
Enrofloxacin	100	–	–	81.8	18.2	–	100	–	–	–	–	–	100	–	–

S = sensitive; I = intermediate; R = resistant

is “earlier” and will hold an advantage in performance tests (the tests are carried out only after reaching the age of two years) compared to foals which were born later. It is highly probable that the older foal will appear more mature in every aspect compared to the younger one. Such a strategy, however, faces some risks, because the anatomy and physiology of mares makes it more difficult to clear the uterus of all debris and fluid. Further, and as mentioned previously, most fertility problems in mares are associated with the inability or delay of these animals to get rid of the contaminating microflora after natural mating (LeBlanc 2003). Recent evidence showed that increased susceptibility of the urogenital apparatus of mares to infection is closely related to the level of oestrogen (Luthje et al. 2013), which corresponds to our observations of lower levels of oestrogen in mares positive for pathogenic microflora in the uterus before the first post-partum ovulation in comparison with mares with only common saprophytic microflora. In addition, during the post-partum period, oestrogen supports regeneration and growth of endometrium,

the thickness of which is one of the decisive factors in embryo implantation (Navot and Bergh 1991; Michalas et al. 1996; Groothuis et al. 2007). This fact can explain, on the one hand, our observations of a higher than 20% infertility rate in live cover mares, and on the other hand, the fact that almost 70% of cervical swabs from mares collected before the first oestrus cycle were positive for pathogenic microorganisms.

As reviewed in Edwards (2005), progesterone and oestradiol have a number of important physiological roles related to immunomodulation and inflammation. Although most of the relevant studies were performed on human or laboratory female animals, all data suggest that these sex steroid hormones play a key role in the modulation of interactions between bacterial microorganisms and the host’s environment (Garcia-Gomez et al. 2013). This bacterial-host communication can mediate the activation of virulence factors of microbial pathogens thus affecting the pathogenesis and prognosis of infection in their host organism (Hughes and Sperandio 2008). From a patho-physiological

point of view, the aforementioned mechanisms are only partially understood and require further research. Based on our findings, we can state that the presence of pathological microflora in the genital apparatus of mares after the parturition seems to have a negative influence on the hormonal (oestradiol/progesterone) balance during oestrus. Furthermore, infection with some Gram-negative endotoxin-producing bacteria could also result in disruptions to hormonal responses (Salkowski and Vogel 1992; Barish et al. 2005).

Similar studies conducted between the seventies and nineties of the past century indicated that up to 40% of mares that failed to conceive were positive for pathogenic microflora in their reproductive apparatus (Redaelli and Codazza 1977; Shin et al. 1979; Ricketts et al. 1993). As mentioned previously, these were the years characterised by “heedless” use of antibiotics mainly for prevention of various infectious diseases which, however, resulted in development of resistance of bacterial strains particularly to routinely used broad-spectrum antibiotics. Moreover, studies published some years later by Albihn et al. (2003), Baranski et al. (2003) and Frontoso et al. (2008) already reported that the incidence of bacterial infections in the reproductive apparatus of mares ranged between 50% to almost 70%. These results point to an increasing trend in the incidence of bacterial infection of the reproductive organs of mares and are in agreement with our results which showed that at least one bacterial pathogen was isolated from cervical swabs of 70% of the investigated mares. It is obvious that the principal cause of such a situation is a troublesome inheritance, namely, the high antibiotic resistance of bacteria, which has literally become a worldwide health problem. Moreover, it was observed that animals serve as a reservoir of bacterial resistance genes which can subsequently pass to bacteria circulating in the human population. The first scientific study that indicated the potential existence of a phenomenon of transfer of genes resistant to antibiotics which colonised the intestines of healthy dogs to bacteria causing enterococci infections in humans was published by De Graef et al. in 2004. Thus, studies involved in the monitoring of individual bacterial pathogens and their resistance to antibiotics are very important also for human medicine.

Our investigations showed that β -haemolytic streptococci and *Escherichia coli* were the pathogens most frequently isolated from cervical swabs of

mares and it is well known that both these pathogens are involved in reproduction disorders of females. Also other authors, such as Koskinen and Katila (1987), Purswell et al. (1989), Waelchli et al. (1993), Langoni et al. (1997) and Frontoso et al. (2008) observed that the dominant, most frequently detected microorganisms cultivated from cervical swabs of mares were β -haemolytic streptococci followed by coliform bacteria. On the other hand, Albihn et al. (2003) reported in their study that *Escherichia coli* was isolated from 67% of swabs (of those 64% were non-haemolytic and 3% haemolytic *Escherichia coli*), while β -haemolytic streptococci were detected only in 20% of cervical swabs of mares with fertility disorders. This indicates that the microbiological findings in the reproductive apparatus of mares vary considerably and may be affected by multiple factors. One can assume that the presence of *Escherichia coli* in 20.4% of swabs may indicate a lower level of management (zoohygiene or inspection of health status) in some private breeding units as many mares with such findings were brought only for the purpose of natural cover by an English thoroughbred stallion kept on the investigated farm. Also the variability in incidence of haemolytic *Escherichia coli* strains (unambiguously associated with reproductive disorders in females) in comparison with non-haemolytic strains, reported in studies by Barrelet (1995), Albihn et al. (2003), Frontoso et al. (2008) as well as in our study indicate that both types of *E. coli* strains are important pathogens or potential pathogens populating the reproductive apparatus of mares after parturition. In agreement with other studies (Brown et al. 1979; Atherton and Pitt 1982; Frontoso et al. 2008) our results showed that *Klebsiella* spp. and *Pseudomonas* spp. constituted only a small proportion of pathogens isolated from the cervical swabs of mares. For example, *Staphylococcus aureus* bacteria were recovered both from the uterus of healthy mares (Ricketts et al. 1993) as well as mares with reproductive disorders (Frontoso et al. 2008). Many potentially pathogenic bacteria survive in the reproductive apparatus and their multiplication is suppressed by common saprophytic bacteria but when the immune responses are affected (e.g. after parturition) they can induce reproductive disorders.

As mentioned previously, the antibiotic resistance of some bacterial strains has become a serious health problem as resistance to multiple antibiotics has been commonly described (Cohen 1992; Gibbons 1992; Siu 2002; Frontoso et al. 2008)

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and was observed also in our study. Few studies are available on the development of antibiotic resistance of bacteria in horses. Sauer et al. (2003) described an increased resistance to antibiotics in bacteria causing ulcerous keratitis in horses from 1991 to 2000. However, more frequent are studies which compare their results on antibiotic resistance with those published previously. In such cases one must consider the fact that resistance to antibiotics is a complex problem involving various bacterial species, various mechanisms of pathogen resistance and transfer as well individual characteristics of the susceptible host (Guardabassi et al. 2004). Nevertheless, compared to our results, very similar findings on antibiotic resistance of bacteria to commonly used antibiotics such as penicillin, gentamicin, tetracycline and entrogloxacin were presented by Frontoso et al. (2008). One can thus speak about a certain stabilisation of the problem related to antibiotic resistance which can be ascribed to the strict regulation of the use of antibiotics implemented after the appearance of the WHO document entitled “The medical impact of the use of antimicrobials in food animals” in the year 1997.

Because a complete understanding of the mechanism of post-breeding endometritis pathogenesis requires further research we can only hypothesise that uterine contamination with pathogenic microflora (particularly β -haemolytic streptococci and coliform bacteria) diagnosed shortly before the first post-partum ovulation may have a detrimental influence on the hormonal regulation of oestrus which can subsequently result in fertility problems in these mares. In addition, it is obvious that resistance to multiple antibiotics has developed in bacterial pathogens cultivated from the uterine swabs of English thoroughbred mares.

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