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STUDY OF THE ROLE OF *CHLAMYDIA*, *MYCOPLASMA*, *UREAPLASMA* AND OTHER MICROAEROPHILIC AND AEROBIC BACTERIA IN UTERINE INFECTIONS OF MARES WITH REPRODUCTIVE DISORDERS

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In six healthy mares and 24 mares showing reproductive disorders swab samples were taken from the fossa clitoridis to isolate *Taylorella equigenitalis*, and from the uterus to isolate mycoplasmas, ureaplasmas and other aerobic bacteria. Swab samples were also taken from the uterus for Chlamydia antigen ELISA and Chlamydia PCR studies. The uterus of 27 mares was examined cytologically, and biopsy samples were taken from the endometrium for histological examinations and for immunohistochemical examinations aimed at the detection of chlamydiae. *T. equigenitalis,* mycoplasmas, ureaplasmas and chlamydiae could not be detected from any of the mares examined. Aerobic facultative pathogenic bacteria were isolated from mares with endometritis in four cases. In 18 out of 22 mares with endometritis (82%) no infective agents could be demonstrated. Further studies are needed to elucidate the relative importance of non-infectious causes of endometritis and of anaerobic bacteria often detectable in the uterus in the aetiology of the reproductive disorders observed.

Key words: Mares, endometritis, infections, bacterium

Infertility in mares is most often due to inflammatory changes of the endometrium (Brook, 1984). Endometritis may develop as a consequence of sexually transmitted infections, as a result of mating in susceptible mares, and it may also be caused by chronic infections of the uterus (LeBlanc, 1999). In the latter two cases numerous facultative pathogenic bacterium species can be isolated from the uterus of affected mares (Shin et al., 1979; Ricketts et al., 1993). The commonest causes of bacterial endometritis include the aerobes *Streptococcus equi* subsp. *zooepidemicus, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and the anaerobes *Bacteroides fragilis* and *B. ureolyticus* (Ricketts and Mackintosh, 1987; Fodor et al., 1995; Langoni et al., 1997; LeBlanc, 1999).

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Equine abortion caused by chlamydiae has been described by several authors (Pienaar and Schutte, 1975; Dilbeck et al., 1985; Glávits et al., 1988; Bocklisch et al., 1991; Lehmann and Elze, 1997; Henning et al., 2000). However, the possible role of this pathogen in fertility problems has not been studied yet.

In humans, infections caused by *Ureaplasma* and *Mycoplasma* species are regarded as sexually transmitted diseases (Bihari, 1997). In the horse, mycoplasmas can often be detected on the mucous membrane of the external genital organs, while ureaplasmas have not been isolated so far (Bermudez et al., 1987). The aetiological role of these pathogens in the reproductive disorders of mares remains to be clarified.

The objective of this study was to demonstrate the possible role of uterine infections caused by aerobic and microaerophilic bacteria as well as chlamydiae, mycoplasmas and ureaplasmas in the fertility disorders of mares.

Materials and methods

At the time of oestrus, samples were collected in 14 horse herds from six healthy mares (four virgin mares and two mares having foaled once) and from 24 mares affected with reproductive disorders (conception failure, embryo resorption, abortion). The age of 25 mares was known; their average age was 10.5 years (ranges: 4–24 years).

Swab sampling (n = 30)

Before cleaning the vulva, swab samples were taken from the fossa clitoridis and placed into carbon-containing Amies transport medium. Subsequently, after having cleaned the vulva and the surrounding region, the following samples were collected using swabs protected from vaginal contamination (Equivet uterine swab, Kruuse, Marslev, Denmark). The first sample was taken into Stuart's transport medium for aerobic bacterial culture, the second into Howard's U4 liquid medium (Howard et al., 1978) for ureaplasma culture, then into Hayflick's liquid and solid medium (Hayflick, 1965) for mycoplasma culture, the third into a transport medium (IDEIA, Dako Co., Glostrup, Denmark) for Chlamydia antigen ELISA, while the fourth for Chlamydia PCR tests. All swab samples were transported to the laboratory in a refrigerator of 4 °C temperature within 4 h of sample collection.

Cytological examination of the uterus (n = 27)

In the case of five healthy mares and 22 mares affected with reproductive problems, samples were taken with a finger from the uterine secretion, smears were prepared, dried in air, and stained using a Dia-quick Panoptic staining kit

(Reagent Ltd., Budapest, Hungary). The smears were evaluated as described by Asbury et al. (1999).

Examination of uterine biopsy samples (n = 27)

Five healthy mares and 22 mares affected with reproductive disorders were examined. A previously disinfected biopsy apparatus (Kruuse, Marslev, Denmark) was introduced into the uterus, and an approx. 0.5 × 0.5 × 1 cm portion of the uterine mucosa was chipped off from the dorsal wall of the uterine body, at the junction of the uterine horns, under control by rectal palpation. The biopsy samples were fixed in 10% buffered formaldehyde solution for 24 h. Subsequently the samples were embedded in paraffin, cut into 4 μm thick sections, which were then stained with haematoxylin and eosin. The samples were evaluated on the basis of the criteria described by Kenney and Doig (1986).

Detection of mycoplasmas and ureaplasmas (n = 30)

The broth media were incubated under aerobic conditions at 37 °C while the solid media in 5% CO_2 atmosphere, also at 37 °C, for 14 days. From the liquid media, samples were inoculated onto Hayflick's solid agar plates on days 3 and 7. The agar plates were examined under stereomicroscope daily.

Isolation of Taylorella equigenitalis *(n = 30)*

Swab samples taken from the fossa clitoridis were incubated on modified CEM selective agar and on Columbia-based chocolate agar containing 7% equine blood, at 37 °C, in 10% CO_2 atmosphere, for 48 h (Quinn et al., 1994).

Isolation of other bacteria (n = 30)

Uterine swab samples were incubated on common agar and on Columbia blood agar containing 7% equine blood under aerobic conditions at 37 °C for 24 h. Bacteria were identified on the basis of their colony morphology, staining according to Gram, and biochemical properties (API, bioMérieux, France).

Detection of Chlamydia antigen in uterine swabs by ELISA (n = 30)

The samples were stored at –20 °C until examined. For the examination, the IDEIA Chlamydia test kit (Dako Co., Glostrup, Denmark) was used according to the manufacturer's instructions. Colour intensity was measured at a wavelength of 492 nm (Labsystem Multiscan Plus photometer, Helsinki, Finland).

Chlamydia immunohistochemistry (n = 27)

Five healthy mares and 22 mares affected with reproductive disorders were examined. After deparaffination, antigen retrieval was performed with 0.1% prote-

ase XIV solution (Sigma Aldrich Co., St. Louis, MO, USA) at 37 °C for 10 min. The sections were incubated in 3% H_2O_2 solution for 10 min, with 2% skim milk powder for 20 min, then with 1:200 dilution of a monoclonal antibody recognising the amorphous locus of the Chlamydia lipopolysaccharide (LPS) capsule (Progen GmbH, Heilderberg, Germany) overnight, at room temperature. Antibody binding was detected with a horseradish-peroxidase-labelled streptavidin-biotin kit according to the manufacturer's instructions (Universal LSAB2 Kit-HRP, Dako Co., Glostrup, Denmark). The sections were treated with 3-amino-9-ethylcarbazole solution (Sigma Aldrich Co., St. Louis, MO, USA) containing also 0.01% H_2O_2 at room temperature for 10 min, counterstained with Mayer's haematoxylin for 20 sec, covered with glycerol-gelatin, and examined at × 100–400 magnification. A formalin-fixed, paraffin-embedded sheep cotyledon infected with *Chlamydophila abortus* was used as positive control, while the negative control was a series of sections incubated with an irrelevant monoclonal antibody.

Chlamydia PCR (n = 30)

The samples were stored at –70 °C until examined. DNA extraction was performed using a commercial kit (QIAamp Tissue Kit, QIAGEN). The DNA was amplified by nested PCR. A genus-specific primer hybridising with the *omp1* gene fragment (the gene fragment encoding the major outer membrane protein [MOMP] of Chlamydia) was used. The reaction products were detected by agarose gel electrophoresis, and the DNA was visualised with ethidium bromide (Schiller et al., 1997).

Results

Uterine cytology and examination of uterine biopsy samples

By the examination of uterine biopsy samples, endometritis was found in a total of 22 mares (81%). Three mares free of reproductive disorders were found to have mild endometritis, while among 19 mares with reproductive problems 11, 7 and 1 had mild, moderate and severe endometritis, respectively. By cytological examination this could be confirmed in only 13 mares showing reproductive problems. In the remaining 9 cases inflammation of the uterine mucosa could only be detected by histological examination of uterine biopsy samples. In 8 out of these 9 cases this inflammation was consistently mild. Fibrosis in the endometrium was found in 18 mares (67%), and it was accompanied by inflammation in all but two cases. Pneumovagina or abnormal position of the vulva was seen in only four out of the 30 mares. All of these mares also had endometritis (Table 1). In mares affected with reproductive problems, the endometrium was free from pathological changes in one case (category I), while it could be classified into category IIa, IIb and III in 6, 11 and 4 mares, respectively. The endometrium of

two of the healthy mares without any reproductive problems proved to be free from pathological changes (category I), and three mares could be assigned to category IIa.

Isolation of bacteria

Mycoplasmas, ureaplasmas and *T. equigenitalis* could not be detected from any of the mares examined. The role of bacteria in the development of endometritis could be demonstrated in four cases. In one mare with pneumovagina *P. aeruginosa*, in another animal showing abnormal position of the vulva *E. coli*, while in two other mares *Str. equi* subsp. *zooepidemicus* was isolated from the uterus in pure or almost pure culture. In further two cases pneumovagina and abnormal position of the vulva were found, but no pathogenic bacteria could be isolated from the uterus (Table 1).

Table 1

Correlation of abnormal anatomical features of the vagina and the perivaginal region, facultative pathogenic bacteria isolated from the uterus, and the presence of endometritis

	Abnormal position of the vulva	Pneumo-vagina	Bacteriological examination	Endometritis		Category
				Uterine cytology	Uterine biopsy	
Case 1	–	+	*Pseudomonas aeruginosa*	+	++	IIb
Case 2	–	+	–	+	+	IIb
Case 3	+	–	–	+	++	III
Case 4	–	–	*Streptococcus equi* subsp. *zooepidemicus*	+	+	IIb
Case 5	–	–	*Streptococcus equi* subsp. *zooepidemicus*	+	+++	III
Case 6	+	–	*Escherichia coli*	+	+	IIb

– = absent/ no bacteria/ no inflammation; + = present/ mild inflammation; ++ = moderately severe inflammation; +++ = severe inflammation

Chlamydia ELISA, immunohistochemistry and PCR

By immunohistochemical, ELISA and PCR methods chlamydiae could not be detected in any of the cases.

Discussion

In earlier studies, cytological examination of the uterus detected endometritis in 8% and 15% of mares, respectively, from the uterus of which no pathogenic bacteria could be isolated (Wingfield Digby and Ricketts, 1982; Waelchli et al., 1988). According to some assumptions, such cases of endometritis may be caused by microaerophilic and anaerobic bacteria, mycoplasmas, viruses or other – non-infectious – aetiological factors. A further possible explanation is that sometimes bacteria may be present in the uterine cavity in very small numbers or restricted to the endometrium, which may hamper their detection (Waelchli et al., 1988). Of these possible causes, the role of microaerophilic bacteria, chlamydiae, mycoplasmas and ureaplasmas was investigated in this study.

Like in a previous Hungarian study (Fodor et al., 1995), the causative agent of contagious equine metritis (*T. equigenitalis*) could not be isolated from any of the mares in this study. The role played by the facultative pathogenic bacteria isolated in this work in the aetiology of equine endometritis has been known for a long time. Such bacterium species can permanently colonise the uterus of so-called 'susceptible' mares and produce inflammation (LeBlanc, 1999). Abnormal position of the vulva and the development of pneumovagina may equally predispose mares to uterine infections. Such infection was actually found in two of four cases of pneumovagina or vulvar abnormality seen in this study. At the same time, in two other mares no bacteria could be detected in the uterus despite the abnormal anatomical features.

Chlamydia trachomatis is one of the commonest sexually transmitted infections in humans (Széll and Szalka, 1998). The finding that chlamydiae can be detected in the ejaculate of 3.4% of stallions (Vezník et al., 1996) raises the question whether these pathogens can cause uterine infection in mares. Despite the fact that according to the findings of serological surveys 13.2% of the Hungarian horse population is infected with Chlamydia (Csukás et al., 1984), this bacterium could not be detected from the uterus of mares by two different antigen detection methods and by PCR, the technique currently considered to be the most sensitive method of detection (Széll and Szalka, 1998).

Mycoplasmas and ureaplasmas are causative agents of important sexually transmitted infections in humans (Bihari, 1997). Mycoplasmas have already been detected from aborted equine fetuses, and these bacteria occur also in the external genital tract (Kirchhoff et al., 1973; Langford, 1974). At the same time, in this study mycoplasmas and ureaplasmas could not be isolated from the uterus of any of the mares examined.

Langoni et al. (1997) observed a close correlation between the occurrence of anaerobic bacteria in the uterus and the development of endometritis. They reported that the most often isolated anaerobe was *B. fragilis*. This bacterium could be detected in the uterus of mares in 28% of the cases (Ricketts and Mackintosh,

1987). Therefore, it is possible that anaerobic bacteria played a role in some of the cases of endometritis in which no pathogens were detected in the present study.

According to the current state of knowledge, the individual susceptibility of mares is the most important factor in the development of endometritis. In these animals, the development of endometritis can often be attributed to non-infectious agents, such as spermatozoa, air, urine or glandular secretions accumulated in the uterine cavity (LeBlanc, 1999). In the present studies, no infectious agents could be detected from the majority (82%) of endometritis cases. Further research is needed to elucidate the relative importance of non-infectious agents and anaerobic bacteria (which were not studied in this work) as aetiological factors in the background of such cases.

To the best of our knowledge, this is the first reported work in which uterine infection of mares caused by *Chlamydia*, *Mycoplasma*, *Ureaplasma* species and other microaerophilic and aerobic bacteria was studied in parallel. Despite the relatively low number of samples examined, it appears that infection by *Chlamydia*, *Mycoplasma*, *Ureaplasma* species and *T. equigenitalis* does not play a role in the reproductive disorders of mares in Hungary.

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