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What's new in bacteriology of the mare's genital tract

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Summary

Uterine bacterial infections are the major cause of reproductive failure in mares. The spectrum of bacteria involved has been carefully established over decades and is approved in recent studies. Streptococcus equi subspecies zooepidemicus (SEZ) is still the most important equine genitopathogenic bacterium. Considerable progress has been made to clarify the molecular basis of host-pathogen interaction as exemplified by SEZ. Genomic analyses of genes encoding putative virulence factors allow for the epidemiologic relevant typing of genitopathogenic bacteria. Moreover, these techniques are the basis of culture-independent sensitive and specific DNA-amplification assays as a measure to improve etiologic diagnosis. Recent advances in analyses of the innate immune system of the horse are expected to improve our understanding of the local activities of the genital immune system.

Keywords: mare, endometritis, bacteria, diagnosis, pathogenesis

Aktuelles zur Bakteriologie des Genitaltraktes der Stute

Bakterielle Infektionen des Uterus sind eine der Hauptursachen von Fruchtbarkeitsstörungen bei der Stute. Das beschriebene Spektrum der ursächlich beteiligten Bakterien ist aktuellen Studien zufolge immer noch ätiologisch relevant. Streptococcus equi subspecies zooepidemicus (SEZ) ist nach wie vor das wichtigste genitopathogene Bakterium bei der Stute. Am Beispiel von SEZ wird deutlich, dass unsere Kenntnisse über molekulare Grundlagen der Wirt-Pathogen-Interaktionen in den vergangenen Jahren erheblich erweitert werden konnten. Die molekulare Analyse von SEZ-Virulenzgenen zur epidemiologisch relevanten Feintypisierung der Erreger kann auf andere genitopathogene Bakterien übertragen werden. Molekulare Analysetechniken sind mittlerweile eine Alternative zum kulturellen Erregernachweis und können aufgrund der hohen Sensitivität und Spezifität zur Labordiagnostik der bakteriellen equinen Endometritis eingesetzt werden. Von besonderem Interesse sind die auch für das Pferd relevanten Erkenntnisse über die angeborene Immunität, die unser Verständnis über die bei der bakteriellen Endometritis ablaufenden immunologischen Vorgänge am Stutengenitale vertiefen könnten.

Introduction

Endometritis due to uterine bacterial infections has been established as a major cause of reproductive failure in mares. Bacteria involved in equine endometritis are for the most part considered to be opportunistic pathogens: they are capable of colonizing the lower genital tract as well as a variety of extragenital locations in the horse; yet they are normally barred from ascending the cervix and uterus by host defences, in particular by protective impacts of the endogenous vaginal flora and local activities of the innate and acquired immune system.

The pivotal factor in the pathogenesis of bacterial equine endometritis is the uterine clearance ability. This term is used to subsume all host factors which may exert significant influence on the clinical outcome of an uterine bacterial infection. They include the genetic constitution and the hormonal situation of the mare, the presence of anatomic deviations in the genital tract, age, and – most vital – local activities of the innate and acquired immune system. The latter are required to mount an endometrial inflammatory response assisting the physiological process of postmating clearance of the uterus. Disturbance of the clearance process may result in chronic active endometritis. Based on the major losses in the equine breeding industry the disease complex of bacterial uterine infections has been studied over the past decades (for review see *Hurtgen* 2006, *Causey* 2006). This report presents current aspects in bacteriology of the mare's genital tract.

Genitopathogenic bacteria in the mare

There is a limited number of aetiological bacteria concerned in equine endometritis. Even recent studies on the spectrum of genitopathogenic bacteria in the mare coincide with the broad experience gained over decades, i.e. the equine endometritis is in most cases caused by a rather small variety of bacterial species (*Albihn* et al. 2003, *Møller Nielsen* 2005). In particular uterine infections with Streptococcus (S.) equi subspecies zooepidemicus have long been established as the leading cause of bacteria-induced endometritis in the mare. Other relevant bacteria are members of the Enterobacteriaceae, namely Escherichia (E.) coli, Klebsiella species (sp.) of different capsule types, Proteus, and Enterobacter. By default of other more valid criteria, the etiologic relevance of E. coli is so far mainly deduced from in-vitro hemolytic growth on blood agar as an indicator for cell-damaging extracellular bioactive bacterial products. Recently, *Albihn* and coworkers (2003) described a significant correlation between the detection of E. coli in uterine swabs and a symptomless repeat breeding in mares. In conclusion, a characterisation of uterine E. coli isolates for virulence factors which may be involved in the development of endometrial inflammation in mares suf-

fering from reproductive failure seems adequate (*Chen* et al. 2003). In this context it is of particular interest to note that only a low percentage of these isolates exhibited hemolytic growth (*Albihn* et al. 2003).

The vast group of nonfermenters is dominated by Pseudomonas (P.) aeruginosa which is an opportunistic bacterium with a broad variety of pathogenic attributes causing infection (known mainly due to its relevance in human medicine). Another bacterial agent of global concern in equine infectious endometritis is Taylorella (T.) equigenitalis. This bacterium is closely related to its adaption to the horse and the relevant specific growth conditions. Recently, a phylogenetically close relative of T. equigentialis, i.e. T. asinigenitalis has been isolated from the genital tract of male donkeys (*Jang* et al. 2001). Under natural conditions T. asinigenitalis is apparently able to colonise also the equine male genital tract. Therefore, it is important to analyse whether this bacterial species is genitopathogenic in the mare (*Båverud* et al. 2006). Although mostly the bacteria-induced equine endometritis bases on monoinfection, we must consider that mixed infections do also occur. Mixed infections however – in particular those with gram negative fastidious anaerobes such as Bacteroides sp. – but also infections with fastidious bacteria such as mycoplasmas or even chlamydiae cannot be exposed to standard tests.

Streptococcus equi subspecies zooepidemicus: classification and pathogenicity

The genus Streptococcus (S.) consists of gram positive mainly anaerobic but aerotolerant cocci with an average diameter of 0.5 to 2.0 μm. The species S. equi comprises three subspecies (ssp.): ssp. zooepidemicus (further referred to as SEZ), ssp. equi (SEE), and ssp. ruminatorum (Fernandez et al., 2004). The species S. equi and S. dysgalactiae consisting of two subspecies ssp. dysgalactiae and ssp. equisimilis pertain to the Lancefield serogroup C. SEZ is regarded the archetypal species of the closely related SEE (*Chanter* et al. 1997, *Harrington* et al. 2002, *Timoney* 2004).

At a rate of up to 50% of the endometritis cases SEZ is globally the most considerable bacterial endometritis agent in mares (*Albihn* et al. 2003, *Causey* 2006). The wide prevalence of inapparent genital and extragenital infections with SEZ in the horse points to a close adaption of this pyogenic bacterium to this host. In diagnostics of bacterial endometritis – particularly in SEZ tests - cultures on blood agar are the acknowledged standard. SEZ are identifiable based on their colony morphology, β-hemolysis, cell morphology according to gram stain, and serologic identification of the cell wall polysaccharide antigen of the Lancefield serogroup C. To ensure classification the biochemical reaction pattern is tested, in particular the fermentation of carbohydrates. A positive lactose and sorbit reaction is characteristic for SEZ, whereas SEE results in consistent negative reactions. Both SEZ and SEE are able to ferment trehalose in contrast to other streptococci of the Lancefield serogroup C, S. dysgalactiae ssp. dysgalactiae and ssp. equisimilis. For the phenotypic differentiation of streptococci commercial biochemical test kits are applicable, too. On average 98% of our own tests to classify SEZ agreed with the commercial Rapid ID 32 STREP differentiation system.

Molecular methods such as the DNA fingerprinting of the bacterial DNA using different restriction enzymes are useful for an epidemiologically relevant typing of SEZ. Recent interesting sequences are the sodA gene encoding a superoxide dismutase and the chaperonin 60 encoding gene cpn60 of both, SEE and SEZ (*Alber* et al. 2004). Even the well known gene encoding the M-like protein of SEZ is suitable to establish genetic differentiation of SEZ (*Timoney* et al. 1991, *Timoney* 2004). Genetic analyses have shown that horses could be infected simultaneously with a number of different RFLP types of SEZ (*Timoney* et al. 1991). In our analysis of equine SEZ and SEE we used PCR-coupled RFLP-analysis. A total of 109 S. equi strains analysed in said test showed an amplification product of approx. 1100 bp in size. In contrast, 19 representatives of other streptococcal species gave no amplification signal. The HaeIII restriction enzyme analysis of 98 SEZ isolates from the genital tract of mares showed 16 different RFLP-patterns which were further confirmed by the DNA sequence analysis of the PCR products. In PCR-coupled HaeIII-RFLP-analysis of paired SEZ isolates obtained from mares prior to and after antibiotic treatment of the streptococcal endometritis, eleven out of twelve RFLP-patterns of the SEZ pairs were identical. Our conclusion is that SEZ is able to persist in the genital tract of the mare despite antibacterial therapy probably due to tolerance against beta-lactam antibiotics i.e. penicilline.

The pathogenesis of SEZ-induced endometritis bases on interactions between bacterial virulence factors and the infected host tissues. SEZ composes a non-antigenic anti-phagocytic hyaluronic acid capsule tested relevant mainly in SEE. Nevertheless, based on the close relationship amongst both subspecies it is an indicative virulence factor for SEZ, too. Yet another virulence factor of SEZ are M-protein-like surface proteins capable of depriving the bacterium of unspecific and specific immune reactions caused by attaching to the Fc-region of IgG and IgA. The M-like protein also binds fibrinogen (*Timoney* et al. 1997). Streptococci binding fibrinogen to the M-like protein attach to phagocytes, yet they are not internalized. The possible cause of the M-like protein's anti-phagocytic properties was studied with the A-streptococci. The C-repeat region showed a binding site for factor H (alternative pathway of complement activation). The B-repeat region is supposed to have a binding site for fibrinogen (*Fischetti* 1989). Even SEZ strains are able to resist complement-mediated cell lysis which might be mediated by the M-like protein (*Causey* et al. 1995). The antigenicity of the M-like protein of SEE is mainly responsible for the mounting of a protective immune response. The entire SEE genome is highly preserved and even isolates from different continents do not vary. By contrast, SEZ shows a high genetic and antigenic variability particularly within the homologue of the SEE M-like protein gene (*Timoney* et al. 1991). *Timoney* and *Guan* (1996) characterized opsophagocytic and protective epitopes of a SEE M-like protein using monoclonal antigen. They found yet another SEE M-like protein (SzPSe) very similar to the M-like protein (SzP) of SEZ, however without the ability to build cross protective antibodies against SEE (*Timoney* et al. 1995). The heterogeneity of the SEZ M-like proteins is obviously due to homologous recombination between intragenic repeats. SEE has probably lost the ability of analogous intragenic recombination – a phenomenon also observed in the human pathogen S. pyogenes (*Timoney* et al. 1991). The vast variability of

the M-like protein of SEZ is mainly responsible for the fact that a protective cross immunity cannot be mounted (*Causey* et al. 2006). One major function of the M-like protein surface structure of SEZ could be the binding of so-called $\alpha 2$ macroglobulines which are physiologically released by host tissues as a protective measure against proteases released by phagocytic cells. This protective ability of $\alpha 2$ macroglobulines may pass on to bacteria such as SEZ after binding to the bacterial surface.

After membrane-associated components of the innate immune system like Toll-like Receptors (TLR) were established in the mammalian endometrium and first reports on the detection of TLR2 und TLR4 in the equine lungs have been published. One may speculate whether the innate immune system of the equine endometrium is capable of recognising - via TLRs - so-called pathogen-associated molecular patterns (PAMPs) of bacterial invaders (*Suri* et al. 2006). Eleven TLRs have been identified so far, each with a distinct specificity (*Underhill* and *Ozinsky* 2002). Complexes of TLR1 and TLR2 recognize PAMPs present on the surface of gram-positive bacteria i.e. lipoprotein and peptidoglycan (*Witkin* et al. 2007). Recognition of those PAMPs triggers a sequence of events leading to the release of pro-inflammatory cytokines and activation of the adaptive immune system. Against this background, it makes sense to study the presence of TLRs in the equine genital tract and their impact on host defenses.

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