Streptococcus equi subspecies *zooepidemicus* resides deep in the chronically infected endometrium of mares

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Abstract

Bacterial endometritis is considered a leading cause of infertility in the mare. Detection of the causative agent has traditionally relied on the use of the “guarded swab method”. Recently, however, attention has been directed towards alternative detection methods after Nielsen[6] found that a high proportion of infertile mares that were negative for *Streptococcus equi* subspecies *zooepidemicus (S. zooepidemicus)* based on culture from swabs (21/84=25%), were positive based on culture from endometrial biopsies (61/84=73 %) (p<0,0001).

Using fluorescence *in situ* hybridization (FISH), bacteria may be visualized within infected tissue, and their spatial distribution can be used to provide information about bacterial pathogenesis. The aim of the present study was therefore to develop a FISH procedure to visualize *S. zooepidemicus* in order to be able to detect the bacteria in the endometrium of experimentally infected mares and in broodmares diagnosed with clinical endometritis.

Using FISH, *S. zooepidemicus* could be visualized in the endometrium in a majority of the infected mares studied. In the young experimentally infected mares, *S. zooepidemicus* was

located superficially in the uterus, and could not be visualized 48 h after inoculation. However, in the chronic endometritis cases, *S. zooepidemicus* was found in distinct foci within the endometrium, often just below the luminal epithelia, but also further down, in the stratum compactum, 300 to 500 µm from the endometrial lumen. No *S. zooepidemicus* could be visualized directly on the luminal surface of the endometrium, but in some instances bacteria were present in the endometrial crypts.
Keywords: Endometritis; *Streptococcus equi* subspecies *zooepidemicus*, localization, chronic infections, equine, fluorescence *in situ* hybridization

Introduction

For more than 80 years endometritis has been considered a leading cause of infertility in the mare. [1, 2] *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) has historically been considered as the main pathogen in equine endometritis. [3-6] Forty years ago, Hughes and Loy[7] demonstrated that a population of mares could be described as either susceptible or resistant to endometritis based on their ability to clear an infection following experimental inoculation of *S. zooepidemicus.* Since then, substantial efforts have been devoted to identify factors that determine whether a mare is susceptible or resistant to endometritis. Compromised uterine clearance and myometrial contractility were demonstrated in susceptible mares[8, 9] and have been identified as important components of the pathophysiology of endometritis. [10-12]

The diagnosis of endometritis in the mare has routinely relied on using a guarded swab followed by bacterial culture and cytological evaluation of exfoliated endometrial material.[13,14] Recently, attention has been directed towards alternative detection methods, after it was shown by Nielsen[6] that the swabbing method was inadequate for detection of uterine pathogens, especially *S. zooepidemicus*. *S. zooepidemicus* were isolated from sixty-three of 84 culture positive mares (75%) based on endometrial biopsy whereas only 23 of the same 84 mares (27%) cultured *S. zooepidemicus* using the swabbing method ($p<0,0001$).

The higher proportion of positive mares using endometrial biopsy as compared to swabs, indicates that *S. zooepidemicus* may reside below the epithelial lining, in the deeper layers of the endometrium, and is therefore less likely to be detected by the non-invasive swabbing method. Information regarding the localization of *S. zooepidemicus* within the endometrium will affect our perception of *S. zooepidemicus* induced endometritis and potentially affect the choice of treatment regimen. Consequently, we wanted to investigate where in the endometrium *S. zooepidemicus* was located in mares suffering from endometritis.

Together with immunohistochemistry and polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH) offers an alternative to traditional detection methods based on bacterial culture.[15] The FISH technique relies on the use of hybridization between bacterial 16S ribosomal-RNA and fluorescent labeled complementary oligonucleotides. After hybridization, detection of individual bacterial cells can be made by fluorescence microscopy.[16] Using FISH, bacteria can be identified in their natural environment, and since spatial distribution within the infected tissue can be evaluated, information regarding bacterial pathogenesis can be generated.[17] Compared to standard epifluorescence microscopy, confocal laser scanning microscopy (CLSM) has the advantage of a pinhole to exclude fluorescence that originates outside the focal point of the objective lens, thus increasing the resolution of fine structures.[18] As 3D data can be acquired using CLSM, detailed visualization of small structures like *S. zooepidemicus* (0,5-2 µm) within the tissue of interest can be obtained.

The primary aim of this work was therefore to develop a FISH procedure that allowed visualization of *S. zooepidemicus* in the endometrium of the mare, and to demonstrate the localization of the bacteria in the endometrium of experimentally infected mares as well as in broodmares diagnosed with clinical endometritis.

Materials and Methods

Endometrial biopsies were recovered from 4 mares experimentally infected with *S. zooepidemicus* and from 6 broodmares with symptoms of clinical endometritis. Except otherwise stated, all plastic wares were purchased from Nunc (VWR International, Albertslund, Denmark), and all reagents were from Sigma-Aldrich (Vallensbæk, Denmark). The media were prepared with freshly produced Milli-Q water (Millipore, Hedehusene, Denmark).

Endometrial biopsies from mares experimentally infected with *S. zooepidemicus*

Four reproductively normal Standardbred mares aged five to ten years and belonging to the teaching herd at the Faculty of Life Science, Department of Animal Science, were used for the experimental infections. The experimental mares were evaluated for cyclicity and clinical signs of endometritis (transrectal palpation/ultrasound and speculum examination of the vagina) every two to three days for a minimum of 14 days before bacterial inoculation. When mares were in estrus, they were inoculated with a *S. zooepidemicus* isolate cultured from a mare with clinical endometritis (10^9 colony forming units in 10 ml of brain-heart infusion broth). Endometrial biopsies were sampled from the left and right uterine horn before inoculation (0 h) and again 3h, 6h, 24h, 48h and 96h after inoculation.

Biopsies were collected as described by Nielsen.[6] In brief, the mare was placed in a set of stocks, the tail was wrapped, the perineal region washed, and a sterile biopsy speculum was introduced through the vagina into the cervical canal. Biopsies were recovered using an alligator forceps directed to the base of the left and right uterine horn. The biopsy instrument was not guided by rectal palpation. Following collection of a biopsy, a sterile pair of pincers was used to smear the biopsy onto blood agar (5% fetal calf blood, toxin free). Blood agar plates were incubated at 37 °C for 48 h and colonies of *S. zooepidemicus* were identified by the β-hemolysis, colony morphology (mucoid and non-pigmented) and Gram stain.

Experimental mares were re-evaluated by transrectal palpation and ultrasography, a speculum examination of the vagina, and a bacterial culture from endometrial biopsy 18 to 21 days after inoculation.

Endometrial biopsies from broodmares in clinical practice infected with *S. zooepidemicus*

Endometrial samples were collected from broodmares if clinical signs of endometritis were detected, e.g. >2 cm free fluid in the uterus during estrus before and/or after breeding, mucupurulent vaginal discharge, or repeated breeding without establishment of a pregnancy. Endometrial biopsies were collected and bacterial culture performed as described by Nielsen.[6] No information was available concerning the mares or the breeding management including pregnancy status. To increase the chance of visualizing *S. zooepidemicus* only endometrial biopsies from which β-haemolytic streptococci had been cultured were evaluated by FISH. Five biopsies were evaluated.

Fluorescence *in situ* hybridization (FISH)

Oligonucleotide probes. The *Streptococcus* specific oligonucleotide probe, Strept (5`Cy3-CACTCTCCCCTTCTGCAC-3`), used to target 16S rRNA from *S. zooepidemicus* in this experiment was developed by Trebesius et al.[19] As a positive control, the general bacteria probe Eub338 (5`Cy5-GCTGCCTCCCGTAGGAGT-3`) was used. [20] As a negative control, the reverse and complementary Strept probe called Non-strept (5`Cy3-GTGCAGAAGGGGAGAGTG-3`) was used. All probes were purchased from TAG Copenhagen, Denmark.

In situ hybridization of bacterial cells. Prior to hybridization, fixed bacterial cells were bound to poly-L-lysine (Sigma, St. Louis, USA). Teflon coated slides (Novakemi AB, Enskede, Sweden) and dehydrated by sequential washes in 70 and 96% ethanol (3 min. each). Ten μL of hybridization buffer (40% formamide, 20 mM Tris, pH 7.0, 0.9 M NaCl, 0.1% SDS) and 5 ng of probe were applied to each slide followed by hybridization in a humidified moist chamber at 46

^{o}C. The duration of the hybridization time was at least 1 h. Slides were washed in hybridization buffer (46 ^{o}C) for 10 m and subsequently transferred to a washing buffer (46 ^{o}C) for 15 m. The slides were finally rinsed in MilliQ-water (46 ^{o}C) and air-dried in the dark. The hybridization signal intensity was evaluated with the probes Strept, Non-strept, and EUB338.

Preparation of tissue sections and in situ hybridization. After smearing onto blood agar, the biopsies were transferred into 10% phosphate-buffered formaldehyde for fixation, and the following day transferred to 70% ethanol and placed at 5 °C until slide processing. The biopsies were embedded in paraffin, cut into 5 µm thick sections, which were mounted on adhesive SuperFrost/plus slides (Menzel-Gläser, Braunschweig, Germany). From each biopsy 10 tissue sections were made, of which four would be hybridized with the Strept and four with the Eub probe respectively, while the remaining two slides would be labeled with the Non-strept probe.

Prior to hybridization, the slides were deparaffinized in xylene for 3 m and allowed to air dry. The tissues samples were then dehydrated by sequential washing in 70% and 90% ethanol (3 min each), and allowed to air dry. Lysozyme (5mg/ml in 100mM Tris (pH 8.0), 50 mM EDTA,) was then added and incubated for 20 min at 37 ^{o}C and finally washed in Mili-Q water. Hybridization was performed using 100 µl hybridization buffer (40% formamide; 20 mM Tris-HCL, pH=7.0; 0.9 M NaCl; 0.1% sodium dedocyl sulphate [SDS]) containing a total of 200 ng of probe per tissue section. Only one probe was used for each tissue section. Hybridization was conducted in a CMT-hybridization chamber (Corning Inc., Corning, NY, USA) at 46 ^{o}C overnight. The slides were then washed in prewarmed hybridization buffer at 46 ^{o}C for 15 m and transferred to a washing buffer containing 20% (vol/vol) formamide for 10 m at 46 ^{o}C. Finally the slides were washed in Mili-Q water, allowed to air dry before being mounted with a coverslip for microscopy.

Confocal Laser Scanning Microscopy

Imaging was performed on a Leica Confocal microscope (Leica TCS SP2; Leica Microsystems, Bensheim, Germany). The objective used were Leica Microsystems HC PL APO ×10/0.40 or HCX PL APO ×63/1.32 (oil).

To allow visualization of the endometrial tissue, autofluorescence was excited by the 458 nm laser and the emitted light was detected at the interval from 460 to 529 nm, whereas Cy3 and Cy5 were excited by the 543 nm and 633 nm lasers, respectively, and emitted light detected at the interval from 540 to 600 nm and 650 to 750 nm, respectively. Either single images or a stack of images were used to construct three dimensional visualization of the tissue. Image visualization and three-dimensional analysis were performed with the Leica TCS SP2 software.

Results

Verification of probe specificity

FISH on pure cultures of the *S. zooepidemicus* strain used for experimental infections demonstrated that the bacteria were labeled following probing with the Strept- and EUB338- probe, respectively. Bacterial morphology and number could be determined with both probes, whereas no signal was obtained when the Non- strept probe was applied.

Endometrial biopsies from mares experimentally infected with *S. zooepidemicus*

Results following experimental infections with *S. zooepidemicus* including clinical signs, bacterial culture, and FISH are outlined in Table 1.

Table 1: Clinical signs, results of bacterial culture and visualization of *S. zooepidemicus* by FISH at fixed time intervals following uterine inoculation with *S. zooepidemicus* (0 to 96 h).

Time from inoculation (h)	Clinical signs	Bacterial culture	FISH

0	No abnormalities noted	No growth from three mares. Two *S. zooepidemicus* colonies isolated from one mare.	No bacteria visualized
3 and 6	Increasing amount of fluid (echogenic on transrectal ultrasound evaluation) in the uterus. No discharge or small amount of discharge noted on the speculum examination.	Large (>40) number of *S. zooepidemicus* isolated from biopsies from each mare	Large number of bacteria visualized in the uterine lumen and/or aligned close to the luminal epithelium. Bacteria visualized in all biopsies recovered.
24	Echogenic fluid in the uterine lumen, mucupurulent exudates from the cervix	Large number of *S. zooepidemicus* isolated	*S. zooepidemicus* organized in clusters in distinct foci on the luminal epithelium. Very few if any bacteria visualized in the uterine lumen.

48	Echogenic fluid in the uterine lumen, mucupurulent exudates from the cervix – reduced amounts in three of the four mares compared to 24 h.	10 to 20 colonies of *S. zooepidemicus* isolated from all mares.	Few and small clusters of *S. zooepidemicus* visualized on the luminal epithelium. Bacteria visualized in biopsies from two of the four mares.
96	Echogenic fluid in the uterine lumen identified in one of the four mares. Vaginal discharge noted from one of the four mares.	None or max 3 *S. zooepidemicus* colonies could be identified from three of the mares. A total of 14 *S. zooepidemicus* colonies were isolated from one mare (mare with clinical symptoms, left + right horn).	No bacteria visualized in biopsies from the four mares.

Mares experimentally infected with *S. zooepidemicus* presented clinical signs as expected (Table 1). At 24 h after inoculation mucupurulent discharge originating from the uterus was noted in all four mares. The amount of fluid in the uterus and vaginal discharge had decreased by 48 h in three of the four mares. By transrectal ultrasound, a medium amount (2 cm) of slightly echogenic intrauterine fluid could be identified in one of the four mares at 96 h after inoculation, and *S. zooepidemicus* was cultured from the biopsies from this mare. Three of the mares cleared the

infection within 96 h, whereas one mare did not. *S. zooepidemicus* was cultured from this mare when she returned to estrus 19 d after inoculation. The mare was then treated on three consecutive days with penicillin (5 million IU), uterine lavage (1-3 L of Ringer's lactate) and oxytocin (10 IU, IM q. 8 h, Intervet®, Intervet Denmark AS). Bacterial culture from a uterine biopsy performed 2 d after the last treatment was negative.

Autofluorescence from the endometrial tissue, following excitation from the 458 nm laser, was adequate to outline the endometrial structures including luminal and glandular epithelium as well as uterine glands and vessels within the stratum compactum. Differentiation of cells in general, e.g. identification of inflammatory cells, was not possible with the described techniques. In most situations, a standard epifluorescence microscope equipped with the correct filters is adequate to evaluate tissue processed for FISH, and preferred over CLSM because of price and ease of use. Endometrial tissue autofluorescence was helpful in determining endometrial architecture, but autofluorescence prevented discrimination between artifacts and probe signals when a standard epifluorescence microscope was used, even though specific filters were applied. This problem was overcome when CLSM was used.

Using the described FISH protocol, *S. zooepidemicus* could be visualized in tissue sections originating from endometrial biopsies. When biopsies were collected 3 to 6 h after inoculation, *S. zooepidemicus* was localized in large numbers in the uterine lumen and /or in close association with the luminal epithelium. The number of bacteria decreased substantially, especially in the lumen of the uterus when biopsies were collected 24 h after inoculation. At this time, *S. zooepidemicus* appeared in small clusters on the luminal epithelium. Due to the high resolution of the confocal microscope, the characteristic round shape of the *S. zooepidemicus* could be discerned. In biopsies collected 48 h after inoculation, few and very small clusters of *S. zooepidemicus* could be visualized on the luminal epithelia, but only in biopsies from two of the four mares, even though

bacteria were demonstrated upon culture from all recovered biopsies. No *S. zooepidemicus* could be visualized in biopsies recovered 96 h after inoculation, although *S. zooepidemicus* was cultured from one of the four mares.

Endometrial biopsies from broodmares in clinical practice infected with *S. zooepidemicus*

Biopsies collected from five broodmares from a clinical practice from which *S. zooepidemicus* had been isolated were processed as described above. Using FISH, *S. zooepidemicus* could be visualized in the endometrium from three of the five mares. In these likely chronic endometritis cases, *S. zooepidemicus* were localized primarily in distinct foci just below the luminal epithelium, but also deeper in the stratum compactum 300 to 500 µm from the endometrial lumen. No *S. zooepidemicus* could be visualized directly on the luminal surface of the luminal endometrium, but in some instances bacteria were present in the endometrial crypts. Tissue remodeling with increased amounts of fibrotic tissue was, in some instances, observed in areas where bacteria were visualized; but in most cases presence of bacteria did not seem to entail changes in tissue morphology.

Examination of endometrial tissue labeled with the EUB338 probe (positive control) revealed a similar bacteria localization pattern as observed using the Strept probe. Control hybridizations without any probe added (negative control) did not result in any specific hybridization signal.

Discussion

Using FISH, we were able to show a rapid decline in the number of *S. zooepidemicus* during the first four days following experimental infection of young mares in estrus. However, in endometrial samples from clinical cases, presumably representing a more chronic infection, *S. zooepidemicus* was demonstrated to reside deeply within the endometrium. To our knowledge this has not been demonstrated before.

In our initial visualization experiments it was possible to visualize *S. zooepidemicus* in biopsies recovered 3 and 6 hours after inoculation, but not in biopsies recovered 24 hours after inoculation, despite the presence of a large number of bacteria in cultures of smears from the same biopsies. It is known that the success of FISH is dependent on optimization of tissue fixation and permeabilization to allow the probe to access the bacteria.[17, 21] In an attempt to increase the permeation of the 16S rRNA probes, the tissue sections were treated with either lysozyme or protein kinase K .[21] A pilot study detected no difference between samples treated with either of the two permeabilizing agents. Increased permeabilization made it possible to visualize *S. zooepidemicus* in the endometrium, independent of time from inoculation. Why permeabilization was necessary to allow visualization in samples recovered 24 h after inoculation, but not after 3 or 6 h, is difficult to determine. The explanation could be the presence of a bacterial capsule 24 h after inoculation, since *S. zooepidemicus* capsule synthesis is usually lost following primary *in vitro* culture. [22]

A high degree of autofluorescence originated from the endometrial tissue, especially using epifluorescence microscopy when excitation was induced with a mercury lamp. This was particularly true when dilated uterine glands were present in the endometrium, likely due to mineralized deposits within these dilated glands [23] and perhaps autofluorescence originating from leucocytes, as shown in humans. [24] Autofluorescence was reduced when CLSM was employed, probably because excitation was induced with only one specific wavelength at a time.[18] The reduced autofluorescence, combined with the capacity to freely select emission wavelength, whereby probe signal could be differentiated from background, made specific visualization of *S. zooepidemicus* in the endometrium possible.

Localization of *S. zooepidemicus* deep in the endometrium of chronically infected mares explains why Nielsen[6] found a higher number of mares were diagnosed with endometritis by culture from a biopsy than from a swab. A recent study using low volume lavage to diagnose

endometritis indicated that *E. coli* in some situations is more frequently isolated (42.2%) than β-hemolytic *Streptococcus* (37.6%).[25] Other studies using a swab to diagnose endometritis, also conducted in Kentucky, found β-hemolytic streptococci to be the pathogen most commonly isolated.[5] The difference with respect to pathogens isolated clearly relates to the diagnostic method used and warrants further investigation. The FISH technique might be useful in the future to illuminate differences in the pathogenesis of *E. coli* and *S. zooepidemicus* induced endometritis in the mare.

Recently, restriction fragment length polymorphism (RFLP) was used to identify the genetic profile of *S. zooepidemicus* clones present in mares with endometritis.[26] Genetic profile or RFLP pattern were evaluated in 12 mares before and after treatment with antibiotics. Following treatment RFLP patterns were identical to those before treatment in 11 of the 12 mares, indicating treatment failure. The authors concluded that *S. zooepidemicus* persisted in the endometrium despite treatment. Different systemic or intrauterine treatments are likely to affect streptococci residing in the deeper layers of the endometrium differently. As described by Schlafer,[27] the use of molecular based techniques such as RFLP, amplified fragment length polymorphism,[28] PCR, FISH, etc. are likely to impact our knowledge of the pathogenesis of endometritis and facilitate optimal treatment protocols in the years to come.

The impact of *S. zooepidemicus* residing deep in the endometrium of chronically infected mares on endometrial function is difficult to predict. Recently, it has been suggested that persistent active inflammation in the endometrium can lead to increased mucus production.[29] Riddle, et al.[5] demonstrated the lowest pregnancy rate in mares with the highest degree of endometrial inflammation and correlated a high level of inflammation to isolation of β-hemolytic streptococci. Chronic infections with *S. zooepidemicus* deep in the endometrium might induce inflammation and increase mucus production potentially reducing pregnancy rates. We foresee that

by using the approach described here we will eventually arrive at a better understanding of *Streptococcus*- induced endometritis.

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