Activation of persistent *Streptococcus equi* subspecies *zooepidemicus* in mares with subclinical endometritis

M.R. Petersen\textsuperscript{a,1,*}, B. Skive\textsuperscript{b,1}, M. Christoffersen\textsuperscript{c}, K. Lu\textsuperscript{d}, J.M. Nielsen\textsuperscript{e}, M.H.T. Troedsson\textsuperscript{f}, A.M. Bojesen\textsuperscript{b}\textsuperscript{p}

\textsuperscript{a}The Fertility Clinic, Rigshospitalet, Section 4071, University Hospital of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark
\textsuperscript{b}Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøljen 4, DK-1870 Frederiksberg C, Denmark
\textsuperscript{c}Section of Veterinary Reproduction and Obstetrics, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Dyrlægevej 58, DK-1870 Frederiksberg C, Denmark
\textsuperscript{d}Hagyard Equine Medical Institute, Lexington, Kentucky, USA
\textsuperscript{e}Ansager Large Animal Hospital, Gartnerhaven 5, 6823 Ansager, Denmark
\textsuperscript{f}Maxwell H. Gluck Center for Equine Research, University of Kentucky, Lexington, USA

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**A B S T R A C T**

Endometritis in horses caused by *Streptococcus equi* subspecies *zooepidemicus* (S. zooepidemicus) may be underdiagnosed due to traditional diagnostic methods lacking sensitivity and specificity. We serendipitously identified a bacterial growth medium (bActivate) that appeared capable of inducing growth of dormant *S. zooepidemicus*, which subsequently allowed detection by standard diagnostics. To assess the effect of bActivate we compared its ability to activate dormant *S. zooepidemicus* in a group of potentially infected subfertile mares with phosphate-buffered saline (PBS). All mares had to test negative for *S. zooepidemicus* on a low-volume uterine lavage. Be negative on endometrial cytology and without clinical signs of endometritis to be included in the investigation. The mares were instilled with bActivate or PBS in the uterus. Growth of *S. zooepidemicus* was induced by bActivate in 64% (16/25) and PBS in 8% (1/12) of the mares, respectively (p < 0.002).

In vitro studies supported that some strains of *S. zooepidemicus* were able to form persistor cells tolerating 32-times of the minimal inhibitory concentration of penicillin compared to normal growing cells. Persistor cells had not acquired penicillin resistance, but seemed to tolerate the antimicrobial due to dormancy.

This is, to our knowledge, the first description of controlled growth induction of dormant bacteria from a subclinical infection. Moreover we demonstrated how endometritis can originate from a reservoir of dormant bacteria residing within the endometrium, and not only as an ascending infection. Further studies should aim at determining the prevalence of dormant *S. zooepidemicus*, impact of activation on diagnostic and treatment efficacy, uterine health and mare fertility.

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1. Introduction

Uterine infection, or more specific endometritis, remains a major cause of subfertility in the mare (Allen et al., 2007; Riddle et al., 2007) and *Streptococcus equi* subspecies *zooepidemicus* (S. zooepidemicus) is the most commonly isolated pathogen (Nielsen, 2005; Riddle et al., 2007; Overbeck et al., 2011). Efficient diagnosticians and treatment strategies are thus key to improved breeding management of mares to optimize fertility.

Diagnosis of infectious endometritis is based on clinical signs and/or recovery of a uterine diagnostic sample enabling identification of polymorphonuclear neutrophils (PMN) and/or presence of pathogens (Dimock and Edwards, 1928; Bain, 1966; Troedsson, 1999; Nielsen, 2005). Traditionally the double guarded uterine swab has been the preferred diagnostic method to recover material for uterine culture and cytology (LeBlanc, 2010). Although practical in a clinical setting, several studies have indicated alternative diagnostic methods e.g. endometrial biopsy (Nielsen, 2005), low volume lavage (LeBlanc et al., 2007; Christoffersen et al., 2015) and the double guarded cytobrush (Overbeck et al., 2011; Cocchia et al., 2011).

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\textsuperscript{*} Corresponding author. Fax: +45 35332757.
E-mail address: morten.petersen@gmail.com (M.R. Petersen).

\textsuperscript{1} Each author contributed equally.

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2012) to be equivalent or superior to the swab for culture and cytology. Treatment of endometritis has focused primarily on the uterine lumen. It was however recently found, that chronically infected mares often were infected with S. zooepidemicus localized deep within the endometrium, which may explain why more mares culture positive with growth of S. zooepidemicus from biopsy samples than from methods focusing on sampling the uterine lumen (Nielsen, 2005; Petersen et al., 2009 Petersen et al., 2009). Comparing localization of PMNs in either the luminal epithelium (cytology) or within the endometrium (histology), PMN’s were identified in twice as many mares on histology than cytology (Nielsen et al., 2012). Diagnostic and treatment strategies aimed at bacterial endometritis should thus consider the uterine lumen as well as deeper structures of the endometrium. Even though both a biopsy and a double-guarded swab were used for examination, 128 of 212 (61%) problem mares unable to become pregnant remained undiagnosed (Nielsen, 2005). Although infertility is multifactorial by nature, we hypothesized that an undetected bacterial infection should be considered in some of these mares. The suspicion gained support when we discovered a method that appeared able to initiate growth of resident S. zooepidemicus in culture-negative uteri of problem mares. The mechanism by which S. zooepidemicus is able to persist in the endometrial tissue without being eradicated by the immune system or antimicrobial treatment remains an open question.

Recently, increased focus has been directed towards bacteria able to enter a dormant state and form “persistor cells”. Bacterial persistor cells are characterized by being a slow-growing or dormant sub-population of cells tolerant to antibiotic treatment and other environmental stress factors and able to resume normal growth in a stochastic manner (Maisonneuve and Gerdes, 2014). The frequency of persistor formation is very low when the bacterial population is in the active/exponential growth phase, typically in the order of 10^{-6}–10^{-4}. However, environmental factors including starvation, quorum sensing, host (immune) induced effects or biofilm formation appear to trigger more cells to enter a dormant state (Keren et al., 2004; Maisonneuve and Gerdes, 2014). It is a challenge to study persister cells, which are genetically identical to the rest of the population and present in very small numbers. One approach is exposure of high doses of an antibiotic compound, which kills all normal growing cells, leaving behind the persister cells. To fulfill the definition of persister cells, these periodically tolerant cells should be as sensitive to the antibiotic as the population they derived from when they resume normal growth (Lewis, 2010). Persistor cells have been implicated in chronic/recalcitrant infections by Pseudomonas aeruginosa, Mycobacterium tuberculosis and Salmonella Typhimurium (Barry et al., 2009; Lewis, 2010; Helaine et al., 2014).

In the current investigation we hypothesized that S. zooepidemicus persister cells may cause subclinical endometritis in mares that go undetected by standard diagnostic methods. To test the hypothesis, we compared a putative bacterial growth medium (bActivate,) which we previously found able to re-activate dormant S. zooepidemicus in the endometrium of subclinically infected mares, with a similar volume of sterile phosphate-buffered saline (PBS) (in vivo study I). We also evaluated whether mares not selected by fertility would be subclinically infected (in vivo study II). To gain further insight into S. zooepidemicus’ ability to form dormant persister cells we aimed at establishing a persister assay in vitro. The in vivo investigations were thus supported by in vitro studies indicating that some strains of S. zooepidemicus indeed were able to form persister cells, tolerating at least 32-times the minimal inhibitory concentration (MIC) of penicillin compared to regular growing bacterial cells of the same strain.

2. Materials and methods

2.1. In vivo studies

A vehicle-control study was performed in a selected group of subfertile brood mares potentially subclinically infected with dormant S. zooepidemicus (study I) and in a group of mares not selected by fertility (study II). bActivate is a growth medium (US patent 863300782) potentially supporting growth of endometrial S. zooepidemicus in the mare. PBS was chosen as control because it is used as the dilution media in bActivate.

In vivo study I

2.2. Study population, inclusion and exclusion criteria

The mares selected for the investigation were subfertile/ problem client owned broodmares in Denmark (n = 20) and Kentucky, USA (n = 17). Only mares remaining non-pregnant despite breeding to a previously proven fertile stallion for at least three consecutive estrous cycles were included. Enrolled mares were all managed by veterinary practitioners specialized in equine reproduction. The majority of mares had been barren despite breeding in numerous cycles 8.6 ± 1.2 (mean ± SEM) and some had been barren for years. Before enrolling in the study mares were assigned to one of two treatment groups receiving a uterine instillation of PBS (10 mL) (n = 12) or bActivate (10 mL) (n = 25), respectively. Prior to instillation, a physical and a gynecological exam including transcervical palpation and ultrasonography of the reproductive organs, a vaginal speculum examination and uterine sampling was conducted. Only mares found to be healthy on the physical exam, without clinical symptoms of endometritis (e.g. uterine discharge or intraluminal fluid (>1 cm)) and without reproductive abnormalities precluding a normal gestation, such as a cervical tear, were included in the investigation. To be able to identify mares subclinically infected with S. zooepidemicus, mares found to be cytology positive or with growth of S. zooepidemicus before infusion with PBS or bActivate were excluded from the study. Growth of other uterine pathogens, e.g. Escherichia coli, was not an exclusion criterion. In other words strict selection criteria were applied and only mares initially identified as cytology and S. zooepidemicus-negative were included in the investigation.

2.3. Study design

As part of the initial gynecologic examination conducted in early estrus a diagnostic low volume uterine lavage (250 mL sterile 0.9% NaCl) was performed to obtain a bacterial culture prior to instillation of PBS or bActivate (pre-sample). Only mares negative for presence of PMN’s and streptococcal growth after 24 h of incubation were included in the study. The post-instillation sample (a double guarded swab) was obtained 24 or 48 h after uterine instillation of PBS or bActivate.

Mares were enrolled in the study during the breeding season of 2009 (Denmark) and 2011 (Kentucky, USA). During the initial studies in 2009 the post-instillation sample was recovered at 48 h, whereas the post-instillation sample in 2011 was recovered at 24 h. Another study conducted in 2010 including 37 mares compared culture results at 24 h and 48 h from the same mares. No difference was identified between sampling at 24 h and 48 h (data not shown). Based on these results post- sampling at 24 h was chosen in 2011.

2.4. Diagnostic procedures

Before obtaining uterine diagnostic samples or uterine infusions the tail of the mare was wrapped to avoid contact with the
perineal area. An area, 30 cm in diameter, around the perineum was washed with a disinfecting soap, rinsed with water and dried with paper towels.

The low volume uterine lavage pre-sample (Bivona catheter, product id: 340846, www.kruuse.com) was performed using a total of 250 ml sterile physiologic sodium chloride. The recovered lavage fluid was allowed to sediment for a minimum of one hour and the most ventral 50 ml including the sediment was recovered in a sterile conical 50 ml tube. Following centrifugation for 10 min. at 400 g, all but 2 ml of supernatant was removed. The remaining fluid was mixed and used to soak a sterile cotton swab subsequently streaked onto a blood agar (BA) plate (Brain Heart Infusion (BHI) agar, Oxoid, added 5% bovine blood) to allow bacterial growth.

The double guarded swab (Equivet, product id: 290955, www.kruuse.com) used for the post-sample was streaked on a BA plate for bacterial growth. The same swab was subsequently used for exfoliative cytology according to Nielsen (2005). All laboratory procedures were performed no later than 6 h after sample collection.

All BA plates were incubated aerobically for 24 h at 37 °C. Bacterial growth was identified according to colony morphology, Hancock stain-morphology, haemolysis, catalase and potassium hydroxide (3% KOH) tests. Colonies were counted on BA plates and scored: no growth/sterile: ≤ 10 colony forming units (CFU) or positive growth: >10CFU. If three or more different bacteria colonies were present on the BA plate, the result was recorded as contamination.

Cells from the cell rich fraction of the centrifuged lavage samples were gently smeared on glass slides, dried at room temperature and stained using the diff-quick hemacolor stain (Diff–Quick, Fischer Scientific, Hampton, NH, USA). The slides were evaluated under light microscopy (×400 magnification). The cytological classification was based on numbers of PMNs present per 200 cells examined. The sample was considered positive for inflammation when ≥0.5% PMNs where present as described by Nielsen (2005).

2.5. Uterine instillation of PBS and bActivate

Immediately following recovery of the low volume lavage sample, a 10 ml uterine instillation of either bActivate (Bojesen and Petersen Biotech, Copenhagen, Denmark) or PBS (Sigma–Aldrich, Broendby, Denmark) was conducted. The hand and arm of the operator was covered in a sterile sleeve (Krutex, product id: 260685, www.kruuse.com) and a sterile insemination pipette (Insemination pipette, product id: 230732, www.kruuse.com) was digitally guided through the cervix and the 10 ml was deposited in the uterus.

In vivo study II

2.6. Prevalence of subclinically infected mares within a group of mares not selected on the basis of poor fertility

To determine the prevalence of subclinically infected mares within a group of mares not selected on fertility uterine samples were collected before and after uterine instillation of bActivate or PBS as described above.

2.7. Study design and population, diagnostic procedures, inclusion and exclusion criteria

As part of the general health evaluation of blood donor mares at Statens Serum Institute (Hvidsten, Denmark) in 2009, uterine samples were collected immediately before and 48 h after uterine instillation of bActivate or PBS. All mares within the heard were evaluated. These mares were part of a larger herd of horses, which donate blood for diagnostic purposes. Statens Serum Institute approved all conducted procedures.

A low volume uterine lavage sample was collected before activation by uterine instillation of 10 ml bActivate, while a guarded swab was collected 48 h after activation. A total of 14 mares were included in the study (bActivate: 8, PBS: 6).

In vitro studies

2.8. Strain selection

To investigate if S. zooepidemicus was able to form persister cells in vitro two strains previously obtained from mares suffering from endometritis were selected. Strain S31A1 originates from the microbial culture collection at Department of Veterinary Disease Biology, University of Copenhagen, Denmark, whereas strain 1-4a was obtained from a research mare at the Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY, USA.

2.9. Minimal inhibitory concentration determination

The minimal inhibitory concentration (MIC) of the strains was determined for penicillin G (penicillin G potassium salt, Sigma–Aldrich, Broendby, Denmark) in triplicate in a microdilution broth assay according to Clinical and Laboratory Standards Institute (CLSI, 2012; CLSI, 2014). Streptococcus pneumonia ATCC 49619 was included as a reference and quality control strain.

Persistor cell assay: To investigate if the streptococcal strains were able to form persister cells the strains were plated on BA and incubated overnight (ON) at 37 °C. A single colony was transferred from the BA plate into 15 ml of BHI broth (Brain Heart Infusion broth, CM 1135, Oxoid) in a 50 ml sterile plastic tube with a loose lid to allow for aeration. This was done in six replicates for each strain and the tubes were incubated ON at 37 °C with shaking (125 rpm). Optical density (OD) of the ON culture was measured and inoculation in 15 ml of fresh BHI broth was adjusted to a start OD 0.015. The cultures were incubated to the early-exponential phase (OD = 0.1) or late-exponential phase (OD = 0.5), respectively. At these time points samples were taken for CFU determination and 0.5 ug/ml penicillin G was added (Fig. 1). Cultures were incubated 20 h with penicillin G exposure. Subsequently broth samples of 1 ml were centrifuged (4000 g × 5 min), washed in PBS and the cells plated on BA and incubated at 37 °C ON and at room temperature for another 16–20 h where the CFUs were determined.

Following antibiotic challenge in the first assay, single colonies of the survivors/persister cells were passed on to an assay similar to the one described above for a total of three cycles. The MIC was determined at initiation and termination of the continuous cycle experiment.

2.10. Growth kinetics

To assess the normal growth pattern of the included strains, growth curves were established using similar conditions as in the persister assays, but conducted prior to and without addition of antimicrobial (Fig. 1).

2.11. Persister frequencies

The persister frequency was calculated by dividing the CFU/ml after the penicillin challenge (CFU persist) by the CFU/ml before penicillin was added (CFU zero).
2.12. Statistics

In in vivo study I and II Fisher’s exact test with a two-tailed p-value was applied to test frequency of mares with or without growth of S. zooepidemicus following uterine instillation of either bActivate or PBS.

In the in vitro study Column statistics, Log transformation and Two-Way ANOVA followed by Tukey’s multiple comparison test was performed to describe and normalize data and to test whether persister formation, was affected by the strain as one factor and the growth phase as the other factor, and if the strain and growth phase did interact with each other regarding the persister formation. We used GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA.

The level of significance was set to p < 0.05.

3. Results

In vivo studies

3.1. Study I—uterine installation of PBS or bActivate in problem mares

A total of 51 broodmares were evaluated using the selection criteria described above. The mares were between 6 and 22 years of age (mean 11.9 years, SEM 4.3), had produced two to eight foals (mean 4.6 foals, SEM 2.1) and were Thoroughbred (28), warmblood (10) or standardbreds (13).

A total of 37 mares were found to have no clinical signs of endometritis, be cytology-negative, had no growth of S. zooepidemicus on the pre-sample and were thus eligible to be enrolled in the study. The majority of the 14 excluded mares were culture positive for S. zooepidemicus (12), found to be contaminated (1) or was positive at the cytological examination (1). Four of the enrolled mares cultured positive with other uterine pathogens on the pre-sample; E. coli in three mares and Pseudomonas aeruginosa in one mare. By chance these four mares were instilled with bActivate. Two of the mares remained culture positive for E. coli and P. aeruginosa, but negative for S. zooepidemicus, while the other two mares cultured positive for S. zooepidemicus and E. coli or S. zooepidemicus only, respectively.

3.2. bActivate versus PBS activation

After inoculation with bActivate S. zooepidemicus was isolated from a significantly higher number of mares than those inoculated with PBS, 64% (16/25) versus 8% (1/12) respectively (p < 0.002), (Table 1).

The majority of mares were found to be cytology positive (34/37) post inoculation with PBS or bActivate. The three cytology negative mares were infused with either PBS (1 mare) or bActivate (2 mares).

3.3. Study II—uterine instillation of bActivate in mares not selected by fertility

A total of 21 blood donor mares aged 5–21 years (11.4 ± 1.0 years (mean ± SEM)) were examined to be included in the study. Of these, seven mares had to be excluded as they were found to be cytology positive at the pre-instillation sample (n = 2) or culture positive for S. zooepidemicus (n = 5). Four mares were culture positive for E. coli (n = 1) or P. aeruginosa/Klebsiella spp. (n = 3) on the pre-instillation culture. A total of 14 mares were thus eligible to be admitted into the investigation.

3.4. bActivate versus PBS activation

After instillation of either bActivate or PBS none of the blood donor mares were found to be culture positive for S. zooepidemicus (Table 1). Following uterine instillation of PBS two mares were culture positive for E. coli, but the four mares initially found to be culture positive (E. coli or other pathogens) were culture negative following instillation with bActivate.

Mares instilled with bActivate or PBS were found to be either cytology positive (3 and 5) or negative (3 and 3).

In vitro studies

3.5. MIC determinations

Prior to testing strain S31A1 and 1-4a’s ability to form persister cells the Minimal Inhibitory Concentration (MIC) was determined to decide, which concentration of penicillin should be added to induce bactericidal effect on all the normal cells in the population. The MIC was 0.0156 µg/ml penicillin G potassium for both strains prior to the performance of the continuous assay. After the four cycles the MIC of both strains was re-evaluated. The MIC did not change for strain 1-4a, whereas the MIC was 0.0312 µg/ml for strain S31A1 at the end of the fourth cycle. This magnitude of

| Table 1 |
| Number of mares with induced growth or not of S. zooepidemicus following uterine instillation of PBS or bActivate in mares selected for reduced fertility (problem mares) or not (blood donor mares). |
|-----------------|-----------------|-----------------|
| Mares selected due to reduced fertility (Problem mares) | Mares not selected due to reduced fertility (Blood donor mares) |
| Growth of S. zooepidemicus | Growth of S. zooepidemicus | |
| **Total** | **Total** | **Total** |
| PBS | 1 | 11 | 12 |
| bActivate | 16 | 9 | 25 |
| Total | 17 | 20 | 37 |

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variation is however, considered within acceptable variation of reproducibility according to CLSI standards (CLSI, 2012, p. 5).

### 3.6. Persister frequencies

The persister assays were performed in biological triplicates for both strains and the persister frequencies were assessed in the early exponential and late exponential growth phases during four consecutive cycles to evaluate the level of persister cell formation and whether the tolerance to antibiotics changed during the experiment (Fig. 2). Before further analysis of the data the mean of the triplicates were calculated. To statistically test the differences in ability to form persister cells between the groups, the mean frequency from each cycle were pooled according to strain and growth phase.

A significant difference of persister frequency between strains (p = 0.0004) and between growth phases (p < 0.0001) was observed. No significant interactions between the growth phases and the strains were found. The multiple comparisons test found significant difference within the same strain regarding the early- and late-exponential growth phase and between the two strains regarding the early-exponential phase, whereas the difference between the two strains in the late exponential phase was not statistically significant (Fig. 2).

The surviving cells after penicillin challenged showed heterogenic colony morphology and expression of hemolytic activity.

### 4. Discussion

Formation of persister cells seems to be a general and conserved trait often involving toxin-antitoxin systems, which are potent regulators of a broad range of metabolic pathways present in nearly all bacterial species (Nguyen et al., 2011; Gerdes and Maisonneuve 2012; Amato et al., 2013; Fasani and Savageau, 2013). In the present study we showed that *S. zooepidemicus* is able to form persister cells in vitro. Little has been reported on persister frequencies in *Streptococcus* sp. but a study of *Streptococcus suis* persister cells using penicillin G, found the exponential phase persister frequency considerably higher than what we found in the current study (Willenborg et al., 2014). This may be explained by a difference in the definition of exponential phase, where Willenborg et al. (2014) used an OD of 0.2, which corresponds to mid-exponential phase in our setup, where the persister frequency has been reported to increase dramatically (Lewis, 2007). The stationary phase frequencies reported by Willenborg et al. (2014) are more comparable to the late exponential phase frequencies we found.

We also found a significant difference in persister frequencies between the two growth phases in the present study (p < 0.0001), which is in accordance with previous reports (Keren et al., 2004; Luidalpö et al., 2011; Willenborg et al., 2014).

Variation in persister formation between independent cultures is to be expected, and have been attributed to variation in ability to form colonies after antibiotic exposure (plating efficacy), and to the effects of the antibiotic on the frequency of persister formation (Johnson and Levin, 2013). In the present study variation was mostly observed in the early exponential phase and the inter-assay variation was larger than the intra-assay variation. One explanation could be the exact timing of the antibiotic challenge, which was technically highly demanding between assays and to a lesser extent within assays, as the cultures had a doubling time of 15–18 min. The early exponential phase seemed more variable in persister frequencies as very small numbers of persisters were observed. Minute variation in persister numbers will therefore impact the relative abundance and give rise to less accuracy and more variation (Sutton, 2011).

Here we demonstrated using two independent *S. zooepidemicus* strains, a clear ability to form persister cells highly tolerant to penicillin in vitro and we found the frequency of formation significantly dependent on the growth phase. What this may do to the potential ability to form persister cell in vivo remains to be investigated. However these findings in addition to other potential virulence factors e.g. capsule expression (Robhe and Chhatwal, 2012), may, at least partly, explain why some strains of *S. zooepidemicus* can form persistent subclinical infections in the reproductive tract of mares as observed in the present study.

Interestingly, we recently reported that *S. zooepidemicus* strains implicated in equine endometritis seem to belong to a genetically distinct subpopulation among the reproductive tract-associated *S. zooepidemicus* (Rasmussen et al., 2013). Whether ability to form persister cells is a common feature for this group of strains should be investigated in the future.

Due to the strict inclusion criteria applied in this investigation only severely subfertile mares were enrolled in *in vivo* study I. The use of research mares would have allowed a more optimal study design, e.g. a cross-over design, however identification and selection of this subgroup of mares is very difficult and expensive to conduct in a research setting. All mares were previously treated with antibiotics as infectious endometritis is a leading cause of subfertility in the mare. Only antibiotics with demonstrated high *in vitro* efficacy to *S. zooepidemicus* were used, both in Kentucky and in Denmark. Future studies should investigate the impact and correlation of antibiotic use *in vivo* on persister formation *in vitro*.

While *S. zooepidemicus* is a commensal and common bacterium of the caudal reproductive tract, the cervix has been considered an efficient barrier maintaining a sterile uterine environment. Transcervical instillation of either bActivate or PBS, as in the current investigation, thus represents a risk of iatrogenic contamination of the uterus with bacteria from the microflora of the caudal reproductive tract. Although the latter cannot be entirely ruled out as a cause of the positive post-instillation cultures it is important to emphasize that in 11 out of 12 mares (92%) infused with PBS and nine out of 25 mares (36%) infused with bActivate, no growth of streptococci was registered. If the growth of streptococci in the remaining culture-positive mares was a result of contamination it would also be more likely that a mixed bacterial flora and not pure streptococcal growth was detected. Furthermore, the mares that became culture-positive with *S. zooepidemicus* were typically highly infected with florid growth on the BA plates,
something that would not be expected within 24–48 h if growth was due to random contamination. Based on the above we consider the risk of contaminating the uterus with microorganisms from the caudal reproductive tract as part of the activation procedure limited.

The prevalence of subclinical *S. zooepidemicus* endometritis in the mare is still to be determined. These initial studies indicate a substantial difference dependent on whether mares are selected for fertility or not, as a prevalence of 64% (16/25) was found within the evaluated problem mares, while none of the mares not selected on the basis of poor fertility was found to be subclinically infected. Future studies evaluating a large group of mares, used in active breeding or not, should be conducted.

In this work, we suggest activation of dormant bacteria as a novel method to diagnose subclinical *S. zooepidemicus* endometritis. This is, to our knowledge, the first report where this is demonstrated in vivo. Our findings potentially have large implications as the use of an anti-dormancy factor such as β-Activate appears to improve the sensitivity of traditional diagnostics significantly. As *S. zooepidemicus* is the most commonly isolated bacteria from the uterus of the mare (Dimock and Edwards, 1928; Nielsen 2005; Riddle et al., 2007; Overbeck et al., 2011), the impact of these findings are potentially substantial. The capacity of *S. zooepidemicus*, to form persister cells that tolerate antibiotic treatment and other environmental stress factors along with a method potentially able of reversing this state in subclinically infected mares, is likely to change the future reproductive management in problem mares.

5. Conclusion

*S. zooepidemicus* is the most commonly isolated pathogen from the uterus of the mare. Here we demonstrate how *S. zooepidemicus* can establish a subclinical dormant persister infection in the endometrium of the mare, which can be activated by uterine infusion of β-Activate. Following activation standard diagnostic cultures can be used to identify presence of an infection or not. In vitro studies demonstrate formation of persister cells tolerant to 32-times of the minimal inhibitory concentration of penicillin compared to normal growing cells. Future studies must evaluate the prevalence of subclinical *S. zooepidemicus* endometritis and the impact of activation on diagnosis and treatment of uterine infections caused by *S. zooepidemicus*, as well as the effect on uterine health and fertility.

Conflict of interest

This is to confirm that Morten R. Petersen and Anders M. Bojesen are cofounders of BP Biotech Aps, producing and selling β-Activate. The other authors have are not involved in β-Activate nor BP Biotech Aps in any way.

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