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FULL PAPER

Importance of broth-enrichment culture in equine endometritis diagnosis

Francesca Paola Nocera[1], Monica Ambrosio[1], Antonella Conte[2], Tommaso Di Palma[2], Sibilla Castaldo[2], Maria Pia Pasolini[1], Filomena Fiorito[1], Luisa De Martino[1]

[1]Department of Veterinary Medicine and Animal Production, Via Delpino 1, University of Naples "Federico II", Naples, Italy;
[2]Specialist in equine reproduction, Naples, Italy

SUMMARY

The objective of this study was to investigate the diagnostic accuracy of the standard microbiological protocol to assure the evaluation of bacterial endometritis in the equine clinical practice. Four hundred fifty-two equine uterine swabs were seeded on different types of agar plates and then in a broth-enrichment (Brain Heart Infusion Broth) before plating by using the same media the day after. The prevalence of positivity was 33.7% following direct plating and 66.3% following use of added enrichment-broth phase before seeding on solid media. Furthermore, the prevalence of isolated bacteria included *E. coli* (29.7%) and *Streptococcus equi* subsp. *zooepidemicus* (15.2%), both frequently associated with equine endometritis.
Our results indicate that the addition enrichment-broth culture significantly increases the rate of positivity for the detection of bacteria in equine uterine swabs compared to the direct plating of samples alone. Thus, this diagnostic technique may be recommended to increase the sensitivity of bacteriological analysis in mares with endometritis.

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INTRODUCTION

Endometritis is one of the main causes of infertility in mares and is often undiagnosed, since it frequently runs as a subclinical disease (LeBlanc and Causey, 2009). It increases with age and seems not to be associated with the number of foalings (Ricketts and Alonso, 1991; Hoffmann *et al.,* 2009; Aresu *et al.,* 2012). The best breeding period for mares is from 4 to 15 years, with peak fertility at around 6 to 7 years; fertility declines from 15 to 20 years and reproductive problems intensify in mares older than 20 years. Obviously, each mare is an individual with her own health history and genetic predispositions. The degree of endometritis, one of the causes of infertility in mares, increases with age; however, some studies report that it may not be associated with the number of foalings (Ricketts and Alonso, 1991; Hoffmann *et al.,* 2009; Aresu *et al.,* 2012).
It is generally acknowledged that a multidisciplinary approach is required to manage endometritis: clinical evaluation, imaging and laboratory sustenance are needed to perform a proper diagnosis (Tibary *et al.,* 2018). Specifically, bacteriology diagnostic procedures may be a valid tool to rapidly identify causative pathogenic bacteria (Nielsen *et al.,* 2010). However, endometrial cytology, culture and biopsy remain the basis for diagnosing any case of infertility. Uterine swabs, which are the most commonly used devices due to their low cost, are the chosen procedure in routine pre-breeding examination in subfertile mares (Overbeck *et al.,* 2011). It should nevertheless be highlighted that culture sensitivity from endometrial swabs is only 34% (i.e., risk of a false negative diagnosis of 66%) (Tibary *et al.,* 2018). Many mares with negative culture might produce a positive culture or cytology after initiation of breeding, suggesting the presence of a deep-seated persistent infection (Christoffersen *et al.,* 2012). Unfortunately, this equine uterine disease is not simple to diagnose quickly and properly. There are numerous studies that compare different sampling methods (brush, swab and flush) (Bohn *et al.,* 2014; Tibary *et al.,* 2018); however only a few evaluate and compare the microbiological procedures to establish which may may be a valid tool for proper endometritis diagnosis. According to standard microbiology techniques, the uterine swabs are inoculated onto different agar plates by rubbing and rolling the swab across a side of each plate. Then, streaking is extended from this area using a sterile wire

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Corresponding author:
Francesca Paola Nocera, DVM, PhD
E-mail: francescapaola.nocera@unina.it

loop, and the plates are incubated aerobically at 37°C for 24 h to quantify and identify the bacteria in the uterus.
In our experience, no growth is often detected with this standard technique. Probably, in some cases no reason for bacterial endometritis can be established, and the failure to detect bacterial infections in mares could be one of the main causes, due to poor sensitivity of current microbiological procedures. Indeed, the low presence of bacteria in a uterine swab could be correlated to the low presence of bacteria in the uterus that, in turn may increase to the stress of pregnancy.
Increasing the pregnancy rates in mares with bacterial endometritis is one of the biggest goals of equine veterinary clinicians, who need a correct diagnosis in order to perform the best therapeutic treatment during the breeding season.
The objective of this study was to compare the results obtained from direct-plating and the use of a broth-enrichment (Brain Heart Infusion Broth) before plating by using the same media. It is known that enrichment medium is used to increase the relative concentration of certain microorganisms in the culture and requires 24 h more incubation than does direct-plating.

MATERIALS AND METHODS

Samples

In 2018-2019, veterinarians specialized in equine reproduction collected a total of four hundred fifty-two uterine swabs from mares before insemination. Uterine swabs were collected from mares diagnosed with suspected bacterial endometriosis by clinical equine veterinarians. The major fertility problems were associated with barren in preceding season (19.0%), resorption/abortion in preceding season (8.4%), repeated breeding after artificial insemination in preceding or in present season (39.8%), fluid in uterine lumen during luteal phase and/or vulval discharge (32.8%). The mares showing reproductive failure were from 4 to 24 years old; specifically, 40.6% were ≤10 years, 56.2% between 11 and 19 years, 3.2% ≥20 years.
Samples were collected for routine bacteriological examinations at the Microbiological Diagnostic Unit of the Department of Veterinary Medicine and Animal Production, University of Naples "Federico II" (Italy).

Microbiological analysis

For conventional bacteriological detection, equine uterine swabs were smeared on Columbia CNA agar, a selective medium for the isolation of Gram-positive microorganisms, on mannitol-salt agar (MSA), a selective medium to isolate staphylococci, and on MacConkey agar (MCA), a selective and differential medium allowing the growth of Gram-negative bacteria. All the plates (from Oxoid, Milan, Italy) were incubated aerobically at 37°C for 24-48 h. Samples were also cultured for fungi on Sabouraud dextrose agar (SDA) (Oxoid, Milan, Italy) incubated under normal atmospheric conditions at 30°C for up to 5 days.
In addition, the same swabs were also inoculated in brain heart infusion (BHI), which is a nutrient-rich non-selective enrichment medium for aerobic bacteria, then incubated aerobically at 37°C for 24 h (Oxoid, Milan, Italy). Turbid BHI tubes were sub-cultured on the agar plates as above described, specifically CNA, MSA, MCA and SDA for a further 24 h at the temperature mentioned above.
Once bacterial growth was detected, the microorganisms were further isolated and first screened by using standard, rapid techniques: colony morphology, cellular morphology by Gram's staining method, catalase and oxidase tests. Then the isolates were identified by matrix assisted laser desorption ionization-time of flight/ mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik, Germany).
E. coli ATCC 25922 and *S. equi* subsp. *zooepidemicus* ATCC 53698 were included as quality control microorganisms.

Statistical analysis

The statistical difference in positivity between direct-plating and enrichment before plating was investigated using Fisher's exact test. P values ≤0.05 were considered statistically significant at 95% confidence interval. Analyses were performed with the Statistical Program Easy Fisher Exact Test Calculator.

RESULTS

Four hundred fifty-two uterine swabs from owned mares were included in the study. All swabs were inoculated on agar plates and in broth. From the bacteriological examination, 267 (59.1%) yielded bacterial isolates, while 185 (40.9%) swabs did not yield any bacterial growth. Among the 267 positive specimens, only 90 (34.0%) resulted positive by direct plating, while the remaining 177 (66.0%) samples were found positive only after the additional broth-enrichment step. The comparison between the two different methods, calculated by the Fisher exact test, showed a statistical significance (P<0.0001).
The number of positive swabs was represented by samples inoculated in the broth and then plated. However, the swabs that resulted positive by direct-plating also allowed isolation of the same strains by using the broth-enrichment step, emphasizing that the broth-enrichment step does not represent an additional source of bacterial contamination.
Specifically, as showed in *Table 1*, we observed that the bacterial species most frequently isolated were *E. coli* (29.7%) followed by *S. equi* subsp. *zooepidemicus* (15.2%). Other bacterial isolates were found at lower frequencies as presented in *Table 1*. It is worth noting that direct-plating of uterine swabs allowed the isolation of 45 *E. coli* strains and 25 *S. equi* sub-

sp. *zooepidemicus* strains, while a further 43 *E. coli* strains and 20 *S. equi* subsp. *zooepidemicus* strains were isolated by using additional overnight broth enrichment. These results suggested that broth enrichment increased the recovery of *E. coli* and *S. equi* subsp. *zooepidemicus* by about 50%. Moreover, *Table 1* shows a reduction of isolation frequency by direct plating compared to the additional broth-enrichment step for most of the isolated microorganisms except for *E. coli*, *S. equi* subsp. *zooepidemicus* and *S. equinus*.

Aerococcus viridans was the third most frequently isolated bacterium, and positivity obtained only by the broth-enrichment technique was three times higher than direct-plating. *Pseudomonas aeruginosa* presented a frequency percentage of 5.4 with 10/16 strains isolated by using broth enrichment. Interestingly, among *Klebsiella* species, *Klebsiella oxytoca* was the most frequently detected (2.4%), with 6/7 strains isolated by using broth-enrichment.

Staphylococcus spp was the most representative genera among the isolated opportunistic bacteria; however, only 7/57 strains were isolated by direct-plating. Precisely, the species identified most frequently were *S. aureus* (4.7%), *S. lentus* (2.7%), *S. xylosus* (2.4%) and *S. hycus* (2.4%).

Candida spp was detected in five clinical samples only after broth enrichment, and was found both alone (two samples) and as a coinfection agent together with bacterial pathogens (three samples). Furthermore, among the 267 positive uterine swabs, only 29 (10.9%) showed the presence of two different bacterial species, with the most frequent combination represented by *S. equi subsp. zooepidemicus* and *E. Coli* (24.1%).

DISCUSSION

The method for carrying out a uterine culture depends on a variety of factors, such as cost, time to perform

Table 1 - *Frequency of microorganisms isolated from 267 equine uterine swabs.*

Microorganisms	**Direct plating*	***Broth + plating*	*Total number of isolates*	*Frequency of isolation (%)*
Escherichia coli	45	43	88	29.7
Streptococcus equi subsp. zooepidemicus	25	20	45	15.2
Steptococcus equinus	4	2	6	2.0
Streptococcus dysgalactiae subsp. Dysgalactiae	2	3	5	1.7
Streptococcus dysgalactiae subsp. equisimili	2	3	5	1.7
Streptococcus uberis	2	2	4	1.4
Streptococcus agalactiae	2	1	3	1.0
Streptococcus gallolyticus	1	-	1	0.3
Streptococcus gallolyticus spp pasteurianus	-	1	1	0.3
Streptococcus mitis	-	1	1	0.3
Aerococcus viridan	6	18	24	8.1
Pseudomonas aeruginosa	6	10	16	5.4
Pseudomonas luteola	2	3	5	1.7
Pseudomonas fluorescens	1	-	1	0.3
Pseudomonas putida	-	1	1	0.3
Klebsiella pneumoniae	1	3	4	1.4
Klebsiella oxytoca	1	6	7	2.4
Staphylococcus aureus	2	12	14	4.7
Staphylococcus lentus	1	7	8	2.7
Staphylococcus xylosus	2	5	7	2.4
Staphylococcus hycus	1	6	7	2.4
Staphylococcus capitis	-	6	6	2.0
Staphylococcus caprae	-	5	5	1.7
Staphylococcus simulans	-	4	4	1.4
Staphylococcus saprophyticus	1	3	4	1.4
Staphylococcus haemolyticus	-	2	2	0.7
Other bacteria	4	13	17	5.7
Candida spp	-	5	5	1.7
Total	111	185	296	100.0

*The results refer to microorganism growth from direct plating of 90 swabs (the same microorganisms were isolated from the respective turbid broth-enrichment cultures).
**The results refer to microorganism growth from additional broth enrichment cultures of 177 swabs that had given a negative result to direct plating.

the procedure, type of laboratory services and its available equipment. The best time to perform a uterine culture is the early estrus. Generally, culture swabs are immediately plated on different agar media. In this study, we observed the failure to detect bacterial presence in a large number of equine uterine swabs, probably due to the poor sensitivity of direct plating. We speculate that mares for which the test yields negative results might nevertheless harbor bacteria at the uterine level, albeit at very low levels, and those bacteria could proliferate under stress conditions resulting in abortion or failure to conceive.

Infectious endometritis is one of the main causes of reduced pregnancy rates in mares, and many broodmares are affected by persistent or chronic infection of the uterine endometrium that is often undiagnosed (Pasolini *et al.,* 2016). Moreover, in the US, infectious endometritis is generally described as the third most common medical problem in American horses (Riddle *et al.,* 2007). Aerobic pathogenic bacteria, such as *E. coli*, *Streptococcus equi* spp. *zooepidemicus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* are mainly involved in uterine infections (Frontoso *et al.,* 2008; Benko *et al.,* 2015; Tibary *et al.,* 2018). In addition, more than one bacterial species can often be simultaneously responsible for endometritis in a mare (Tibary *et al.,* 2014; Rua *et al.,* 2018); unusual bacteria can also be responsible for endometritis (Nocera *et al.,* 2017).

Broth enrichment is frequently used to enhance the recovery of potential bacterial pathogens, especially for samples which are transferred to the laboratory with prolonged times (Kaur *et al.,* 2017). In this study we observed that the use of broth enrichment prior to plating on a solid selective medium significantly increased the number of positive swabs. Indeed, the isolation of some pathogenic bacteria such as *E. coli* and *S. equi* subsp. *zooepidemicus* was doubled by using the broth-enrichment technique. In particular, isolating *S. equi* subsp. *zooepidemicus* is critical as it is the principal cause of chronic deep infection of the endometrium in mares (Petersen *et al.,* 2009). Moreover, early identification of *S. equi* subsp. *zooepidemicus* is needed to facilitate appropriate medical intervention, since this bacterium is considered an emerging zoonotic pathogen that may also cause severe illness in humans (Pelkonen *et al.,* 2013; Finazzi *et al.,* 2015; Kittang *et al.,* 2017).

Considering that the severity of the infection is linked to the growth of colonies in culture plates (Wolfsdorf, 2016), the lack of bacterial growth in culture plates for some samples is understandable in case of low or moderate bacterial presence in the mare's uterus. Generally, culture swabs are immediately plated onto blood agar or other appropriate media. In our study, 267 of the 452 uterine samples inoculated yielded bacterial growth, but about 50% of them were recovered only when the broth-enrichment step was performed before direct plating on solid media. In addition, the type of isolated bacterial species and mares with characteristic clinical signs or the age of the mares did not seem to be correlated.

Among the bacteria isolated, *E. coli* (29.7%) and *Streptococcus equi* subsp. *zooepidemicus* (15.2%) were the most common strains identified by both procedures. Compared to our previous study, where we identified *Streptococcus* group C and *E. coli* as the most frequent species associated with bacterial endometritis (Frontoso et al., 2008), here we found a surprising and significant increase in *E. coli* isolation rate, reaching 29.7% of the total number of isolates. More recently, Pisello *et al.* (2019) reported *E. coli* (27.9%) as the most frequently isolated microorganism, followed by *Streptococcus equi* subsp. *zooepidemicus* (24.9%). Other articles report that the most common bacterial isolates are *Streptococcus equi* subsp. *zooepidemicus* and *E. coli*, with higher percentages for the former (Samper and Tibary, 2006; Tibary *et al.,* 2014; Petersen *et al.,* 2015).

Furthermore, in this study, *E. coli* was the most frequently isolated microorganisms, by both direct plating as well as broth enrichment. These results suggest that broth enrichment is a valid diagnostic procedure to improve the isolation and identification of potential uterine bacterial pathogens in subclinical equine endometritis. It is worth noting that a positive uterine culture before breeding is among the causes of infertility linked to endometritis (LeBlanc and Causey, 2009). According to the literature, mixed cultures of more than three pathogens are usually considered indicative of contamination (Nielsen, 2005; Buczkowska *et al.,* 2014). In our hands, both the direct-plating and the broth-enrichment methods showed a high rate (40.9%) of negative results and, when positive, showed the growth of only one and, rarely, two bacterial species, supporting the accuracy of the procedure.

The diagnosis of endometritis in mares is one of the crucial challenges for the equine clinical practice (Cadario, 2014) and accurate criteria for determining what constitutes mild endometritis in mares have yet to be established. Identifying and eliminating bacterial infections might be the key to solving some chronic reproductive problems.

In conclusion, we recommend the use of broth enrichment before spreading swabs on solid media plates in order to increase the sensitivity of equine uterine swabs. Enrichment methodology increases the positivity of cultures and provides a more accurate method to identify etiological agents, thus representing a reliable alternative to standard diagnostic routine procedures. Furthermore, correct diagnosis allows an adequate and efficient treatment of bacterial endometritis in mares, enhancing the reproductive performance and limiting the spread of microorganisms with zoonotic potential.

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Declaration of Competing Interest
The authors declare that they have no conflicts of interest.

List of abbreviations
CNA, Columbia CNA agar; MSA, Mannitol Salt agar; MCA, MacConkey agar; SDA, Sabouraud dextrose agar; BHI, Brain Heart infusion; MALDI-TOF/MS, matrix assisted laser desorption ionization-time of flight/ mass spectrometry; ATCC, American type culture collection.

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