Subclinical endometritis in a mare by *Curvularia spicifera* and *Escherichia coli* co-infection

Maria Gil-Molino[1], Sofía G. Zurita[1], Iván Martín-Basconcillos[2], Francisco E. Martín-Cano[3], Cristina Gaona-Álvarez[4], Carmen González-Velasco[4] and Alfredo García-Sánchez[5]

[1] Service of sample processing and diagnosis, Clinical Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain
[2] Private veterinary practice in equine medicine and reproductive services, Madrid, Spain
[3] Reproduction Unit, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain
[4] Microbiology and Parasitology, Merida Hospital, Badajoz, Spain
[5] Department of Animal Production, CICYTEX-La Orden, Guadajira, Spain

Summary: Subclinical endometritis has a negative impact on the reproductive performance of the mares concerned. The present article describes the diagnosis of subclinical endometritis in a 14-year-old Oldenburg brood mare with a previous reproductive history of difficulty in becoming pregnant. Uterine cotton swabs were taken and sent to the laboratory for microbiological and cytological tests. *Curvularia spicifera* and *Escherichia coli* were isolated as causative agents. To the best of the authors' knowledge, this is the first time that *Curvularia spicifera* has been implicated as a causal agent of subclinical endometritis in a mare. A successful treatment was established by infusing 120 ml of 3% hydrogen peroxide in lactated Ringer's solution into the uterus to remove the fungi that could adhere to the luminal epithelium, followed the next day by further uterine lavage of lactated Ringer's solution. In addition, 7.5 mg/kg oral enrofloxacin was administrated for seven days.

Keywords: *Curvularia spicifera*, *Escherichia coli*, subclinical endometritis, mare, reproduction

Introduction

Equine endometritis is an important cause of infertility and subfertility in mares and is associated with major economic losses to the horse industry worldwide. Fungal endometritis is a relatively rare cause of infertility in the mare (only 1–7% of all cases of endometritis) (*Pasolini* et al. 2016) and usually occurs if their immune system is compromised or as a consequence of repeated intrauterine antibiotic treatments (*Coutinho da Silva* and *Alvarenga* 2011). However, it remains a challenge to the clinicians due to difficulties in diagnosing, a high rate of recurrence and a reserved prognosis for ongoing fertility. Fungal colonisation of the mare's uterus is almost certainly an opportunistic condition that is usually associated with previous antibiotic therapy or a history of pneumovagina (*Zafracas* 1975) and is based on the significant disruption and compromise of normal vaginal and/or uterine defence mechanisms (such as concomitant bacterial infection) or more generalised immunosuppression/compromise (*Scott* 2018). Either yeast or moulds are the etiological agents of fungal endometritis, with *Candida* spp. and *Aspergillus* spp. as the most common microorganisms isolated from mares with this condition (*Dascanio* et al. 2010), but more than 20 other fungal genera have been also identified in uterine cultures (*Coutinho da Silva* and *Alvarenga* 2011).

Members of the genus *Curvularia* have emerged over the past decade as human and animal pathogens of increasing clinical importance as causative agents of local and invasive infections. Among them, *C. australiensis, C. geniculata, C. hawaiiensis, C. lunata* and *C. spicifera* are the species most frequently isolated from clinical specimens (*Krizsán* et al. 2015). This pathogen has been described in keratomycosis, rhinitis/sinusitis and mycetomas in horses (*Boomker* et al. 1977, *Betbeze* et al. 2006, *More* et al. 2019).

Case report

The patient was a 14-year-old Oldenburg mare which had given birth in the last breeding season to a newborn foal with abnormal flexion of the corono-pedal joint or "contracted tendons". According to the owners, prior to the aforementioned birth, the mare was artificially inseminated and suffered embryo loss at least three times before to the 45th day following reproduction. The pregnancy was diagnosed by ultrasound on day 14 post ovulation and the semen was obtained from a public breeding stallion with proven fertility and tested for venereal diseases (equine infectious anaemia, equine viral arteritis and contagious equine metritis).

The mare's diet was based on grazing during the day and supplemented at night with roughage and concentrates inside her box. Her corporal condition was 5/9 according to the Henneke scale.

At the end of February, the mare had normal reproductive cycles, and a complete exploration, which included evaluation of the external genital tract, manual transrectal palpation and ultrasound of the internal reproductive organs, showed no alterations except for incomplete vulvar closure. This defect which resulted in a mild pneumovagina was not accompanied by any sign of irritation or vulvar discharge.

Endometrial samples for cytological and microbiological tests were taken at the beginning of oestrus. The perineal area was washed with iodine soap and rinsed with a hygienic wet paper towel a minimum of three times. Uterine samples were collected using double-guarded cotton swabs and transferred to AMIES® transport medium.

The samples were submitted to the Clinical Veterinary Hospital of the University of Extremadura for cytological and microbiological evaluation. The service of sample processing and diagnosis of the Clinical Veterinary Hospital did not show any sign of an ongoing inflammatory reaction (e.g. neutrophils) or the presence of yeasts or elongated fungal hyphae in the smears. Regarding microbiology, the sample was inoculated on blood agar, MacConkey agar and Saboureaud cloramphenicol agar. Mild to moderate bacterial growth was obtained on the MacConkey agar and blood agar after 24 hours of aerobic incubation at 37 °C. The colonies fermented lactose on MacConkey agar and were identified with the API 20E (bioMérieux) system as *Escherichia coli*. The antibiotic susceptibility was tested by the disk-diffusion method on Mueller-Hinton agar (Kirby-Bauer Method) with 15 antimicrobial agents. The following discs (Bio-Rad®) were used: ampicillin (AMP; 10 μg); ceftiofur (CTF; 30 μg); gentamicin (GEN; 10 μg); streptomycin (STR; 10 μg); doxycycline (DOX; 30μg); enrofloxacin (ENR; 5 μg); trimethoprim/sulphamethoxazole (TRS; 23.75/1.25 μg); ciprofloxacin (CIP; 5μg); Imipenem (IMP; 10 μg); rifampicin (RP; 5 μg), erythromycin (ERY; 15 μg), amikacin (AK; 30μg), cefquinome (CFQ; 10 μg), penicillin (PCN; 1U) and amoxicillin clavulanic acid (AMC; 10μg). *Escherichia coli* ATCC 25922 was used as a control strain. Data were interpreted using EUCAST epidemiological cut-off values (www.EUCAST.org). This bacterium showed a sensitivity to CTF, GEN, AK, ENR, STR, CIP, DOX and TRS, however, it was resistant to AMP, AMC, PCN, CFQ, ERY and RP. Intermedia resistant to IMP.

Abundant fungi developed on the Saboureaud Cloramphenicol agar plate after 72 hours of incubation at 37 °C. Colonies appeared glassy and their colour was initially grey and became blackish brown as they matured. Microscopical examination of the fungal culture revealed solitary conidiophores or in small groups, with cords, geniculated several times and hairy, cylindrical conidia with rounded ends. They were black from the reverse (Figure 1).

Initial identification of the filamentous fungus was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Considering the Brukers' cut-off value for reliability (LogScore > 1.70), the isolate was identified as *Curvularia verruculosa* with a score of 2.15.

This result was subsequently confirmed by direct sequencing of the polymerase chain reaction products obtained by amplification with the universal fungal primers ITS1 5'-TCCG-TAGGTGAACCTGCGG-3 and ITS4 5'-TCCTCCGCTTATT-GATATGC-3' (*White* et al. 1990) A BLAST search against the GenBank database revealed that the 570-bp (100% Identity) nucleotide sequence of the amplified product matched the internal transcribed spacer regions of fungal ribosomal DNA (rDNA) of *Curvularia spicifera* strain TN290D12 (Sequence ID: MH271090.1). Most studies have shown that sufficient variation exists within the ITS regions to allow for species identification (*Iwen* and *Krahmer* 2015).

According to these findings, the mare was treated with oral enrofloxacin 7.5 mg/kg SID for seven days in dioestrus and an uterine lavage was carried out to reduce the organism load and eliminate fungal organisms. The lavage consisted of 120 ml of 3% hydrogen peroxide solution infused into the uterus and kept overnight. The following day, the hydrogen peroxide was removed by lavage with a large amount of lactate Ringer's solution and a temporary Caslick's procedure was applied to the mare's vulva using metal staples to provide temporary closure of the vulva lips.

The mare was inseminated with fresh semen during the next oestrus. The mare had a normal oestrus and ovulation was induced with 1500 UI human chorionic gonadotropin while she had a 37 mm preovulatory follicle. The next day, the mare showed no fluid in the uterus and had not yet ovulated. She was treated three times with 10 IU oxytocin intramuscularly every eight hours. The mare was scanned again 48 hours after insemination, showing a corpus luteum and no fluid was detected.
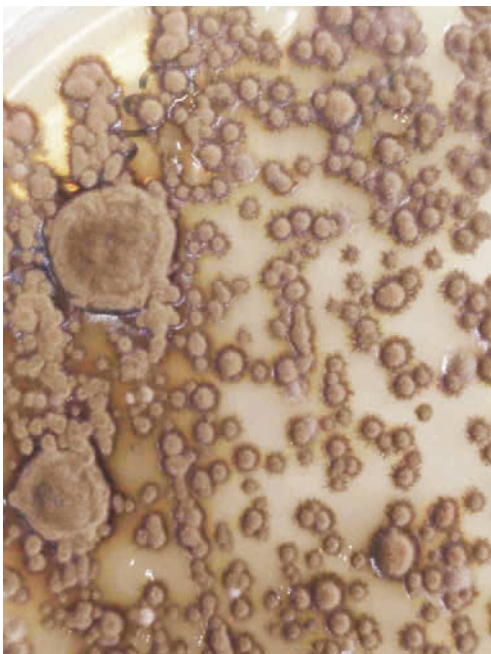
Fig. 1   Mature colonies of *C. spicífera* have a greyish brown surface | *Reife Kolonien von C. spicífera haben eine graubraune Oberfläche*

The diagnosis of pregnancy was made 14 days after ovulation and an embryonic vesicle of normal size was found. Pregnancy was monitored every two weeks up to 90 days of gestation without any alterations in the normal development of the foetus and placenta being detected.

Discussion

Based on the history, clinical findings and results of microbiological examinations, the mare suffered from subclinical endometritis, which occurs more often in aging mares than in younger counterparts. Subclinical endometritis has been described as a 'hidden' endometritis in mares which did not show the typical signs of endometritis (*LeBlanc* 2003). Its diagnosis is made through detecting uterine fluid, signs of vaginitis, vaginal discharge, short inter-oestrous intervals, inflammatory uterine cytology and positive uterine culture. However, these signs may be absent in subclinical cases (*LeBlanc* 2003, *Buczkowska* et al. 2014). In fact, the present case did not show intrauterine fluid or any sign of inflammatory uterine reaction. It is know that the degree of inflammatory response in infectious endometritis is highly dependent on the aetiological agent (*Sharp* and *Sharp* 1993). *E coli* infections are commonly associated with negative cytology (*Riddle* et al. 2007a, *Christoffersen* et al. 2015), and it has been suggested some *E coli* strains have a low chemotactic potential (*Christoffersen* et al. 2015). Furthermore, samples obtained in early oestrus may not contain inflammatory cells, because the migration of polymorphonuclear leukocytes into the uterine lumen during the period of declining progesterone dominance is lower than during peak oestrogen dominance (*Riddle* et al. 2007b, *Cocchia* et al. 2012).

Subclinical endometritis are commonly caused by Gram-negative organisms, fungi and *Streptococcus equi* subsp *zooepidermicus* (*Nielsen* 2005). In our case, *Curvularia spicifera* and *E. coli* were isolated as causative agents. *Curvularia* is a hyphomycete (mould) fungus which is a facultative pathogen or beneficial partner of many plant species and common in soil (*Priyadharsini* and *Muthukumar* 2017). Apart from its importance in plants, members of this genus also have been reported causing infections in animals, affecting both immunocompetent and immunocompromised individuals at different levels of severity (*Riddle* et al. 2007a, *Ben-Shlomo* et al. 2010). *Curvularia* spp. are among the causative agents of phaeohyphomycosis, rhinitis, sinusitis, wound infections and mycetomas in horses, and they are on the growing list of emerging fungal pathogens in humans. *C. spicifera* is the most isolated agent from this genus in human respiratory tract infections, infections complicating peritoneal dialysis, peritonitis, and deep and disseminated infections (*Krizsán* et al. 2015).

Regarding reproductive pathologies, members from the genus *Curvularia* have not been described frequently as causative agents. There are no reports of this organism in endometritis or abortion in human or horses. The only reference that specifically attributes a case of abortion to a *Curvularia* spp. organism was published in 1992 and affected a cow (*Knudtson* and *Kirkbride* 1992). Based on that, this is, to the best of our knowledge, the first description of subclinical endometritis induced by *E. coli* and *Curvularia spicifera* in a mare.

The method used to confirm the identity of the fungus at the species level was ITS sequencing (*Alex* et al. 2013, *da Cunha* et al. 2013). Although some doubts have been raised regarding the use of ITS to discern certain closely related species (*Begerow* et al. 2010, *Revankar* and *Sutton* 2010, *da Cunha* et al. 2013), the morphological characteristics of the conidiophores and the conidia, in addition to the 100% matching with *Curvularia spicifera* obtained by polymerase chain reaction, supported the outcome of this technique. (Fig. 2)

The treatment applied to the mare consisted of oral enrofloxacin and an uterine lavage with 3% hydrogen peroxide. The antibiotic treatment against *E. coli* was decided based on the susceptibility test, but the non-specific uterine lavage was chosen as a treatment due to the limited availability of an antifungal test and its prolonged testing times. This election was in accordance with several reports describing that these type of therapeutic measures should be considered as a first line treatment option if sensitivity results are not available (*Scott* 2018). The most effective antifungal treatment against this genus is Amphotericin B (*Krizsán* et al. 2015). This treatment was not applied because the lavage was successful but should be considered in other cases with similar aetiology (*Scott* 2018). It should be mentioned that the susceptibility to antifungal therapies in the genus *Curvularia* varies greatly between species and also between isolates, therefore, it is important to determine it isolate by isolate (*Krizsán* et al. 2015).

This report reinforces the necessity of performing a complete reproductive evaluation of every mare prior to the breeding season. Mares suffering from fungal endometritis typically have a prolonged history of difficulty in becoming pregnant. Older multiparous mares may develop poor perineal conformation, which increases the susceptibility to uterine infections. It is also believed that fungi or yeasts that colonise the uterus usually originate from a reservoir in the vagina and external genitalia (*Dascanio* et al. 2010) and are transported iatrogenically into the uterus during insemination or intrauterine treatment.
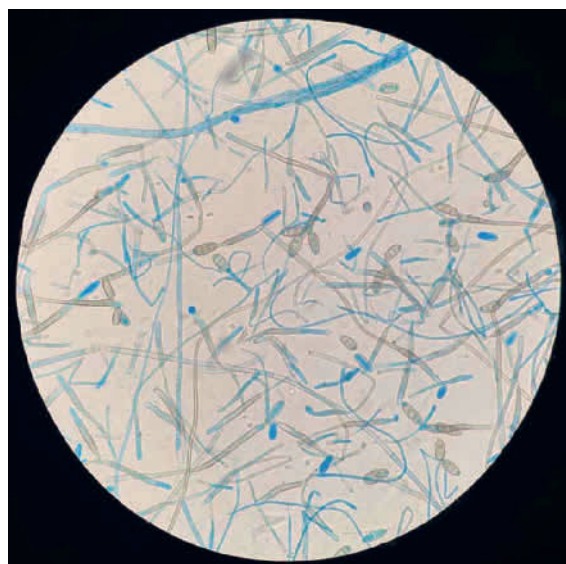
Fig. 2 Microscopic image (40×): *Conidiophores* are elongate. *Conidia* are cylindrical with 3 to 5 septa. | *Mikroskopisches Bild (40 ×) Die Konidiophoren sind länglich. Konidien sind zylindrisch mit 3 bis 5 Septen.*

Conclusions

It is essential for the equine veterinarian to diagnose subclinical endometritis and identify the causative pathogenic agents (ME, 2014). The veterinarian should be aware that, similar to this case, *Curvularia spicifera* may be among the fungal species concerned. The practitioner must have a rapid and reliable diagnosis in order to start the best treatment as early as possible during the breeding season.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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