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Endometritis and Infertility in the Mare – The Challenge in Equine Breeding Industry–A Review

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Abstract

Most major infertility problems are complex and several factors can cause failure to produce offspring. In the last few years, much of the efforts of practitioners and researchers working in equine breeding industry have been directed to individuate the pathophysiological mechanisms underlying poor reproductive performances in mares. Endometritis is on the talk in much of the recent research as the most frequent cause of subfertility in mares that cycle normally but do not conceive and in mares that cycle normally and conceive but then suffer early embryonic death. Post-breeding persistent endometritis, bacterial and other infective endometritis and poor uterine clearance have all been discussed in an attempt to define risk factors and a diagnostic algorithm. The aim of this chapter is to perform a thorough review of recent literature about endometritis. The diagnostic algorithms are carefully examined, highlighting pros as well as pitfalls of each diagnostic aid. Suggested therapeutic protocols are examined in the effort to detect what is actually recommended and what would better benefit from further corroboration. The idea that a better etiopathogenetical understanding of the endometritis remains the key to access to a correct diagnostic protocol and to a successful therapeutic plan will inspire this chapter.

Keywords: Endometritis, Mare, Diagnosis, Ultrasound, Therapy

1. Introduction

Every year many mares fail to become pregnant. These failures represent a substantial economic and genetic loss to the horse industry. Most major infertility problems are complex and several factors, singly or in combination, can cause failure to produce offspring. In the last few years, much of the efforts of practitioners and researchers working in equine breeding industry have been directed to individuate the pathophysiological mechanisms underlying poor reproductive performances in mares. To clarify, infertility includes three “problems”

mares' types: mares that fail to cycle, mares that cycle normally but do not conceive and mares that cycle normally and conceive but then suffer early embryonic death. Endometritis is on the talk in much of the recent research as the most frequent cause of the last two conditions.

Post-breeding persistent endometritis, bacterial and other infective endometritis and poor uterine clearance have all been discussed in an attempt to define risk factors and a diagnostic algorithm, essential to reach a definitive diagnosis and to apply the appropriate therapeutic protocol. Breeding-induced endometritis is a normal physiological reaction in the horse, as it is believed that an inflammatory response is necessary for the effective removal of contaminating bacteria and excess spermatozoa introduced into the uterus. In a healthy uterus, the inflammation subsides within 48 hours, but the susceptible mares are not capable of resolving the inflammation triggered by sperm and develop persistent mating-induced endometritis (PMIE). A very strong relationship establishes between PMIE and infectious endometritis and a complex of mare (such as age, perineal conformation, uterine clearance and cervical competence) and microbial (such as induction of inflammation, epithelial adherence, resistance to phagocytosis and viscosity of secretion) factors contribute to the pathogenesis of endometritis. Traditionally, the mares that are prone to endometritis are called susceptible, in contrast to the "resistant" ones, not prone to uterine infection.

The aim of this chapter is to perform a thorough review of recent literature about endometritis. The cascades of inflammatory signals being complex and intertwined, the etiopathogenetical, diagnostic and prognostic roles of the recently studied inflammatory markers are discussed. In addition, the most common bacterial and fungal pathogens involved are reviewed, together with the recent advances in diagnostic procedures. In fact, the diagnostic algorithms are carefully examined, highlighting pros as well as pitfalls of each diagnostic aid. Suggested therapeutic protocols are examined in the effort to detect what is actually recommended and what would better benefit from further corroboration, with special attention to the correct use of antimicrobials and antibiotics, their common way of administration and contraindications. Consideration will be given to therapy alternatives such as proper breeding management, use of uterine lavage, oxytocin/prostaglandin administration and treatment of the biofilm formation. The idea that a better etiopathogenetical understanding of the endometritis remains the key to access to a correct diagnostic protocol and to a successful therapeutic plan will inspire this chapter.

2. Pathophysiology of endometritis in the mare

Endometritis is a major cause of infertility in the mare. It is an acute or chronic inflammation of the endometrium that can be classified based on both its etiology and pathophysiology. Susceptibility to persistent bacterial endometritis was characterized as early as 1969 [1]. Since then, the physiopathological mechanisms involved in persistent endometritis and its correlation with bacterial endometritis and infertility in the mare have been discussed in several studies [2–18]. Endometrium responds to the introduction of air, urine, semen, bacteria, fungi or yeasts through an inflammatory reaction that ultimately hesitates in restoring an environ-

ment suitable to receive the conceptus, as it migrates from the oviduct few days after the insemination. To induce endometritis in experimental conditions, either spermatozoa or bacteria such as *Streptococcus zooepidemicus* or *Escherichia coli* are commonly infused into the uterus. A subpopulation of mares, designated as susceptible, fails to resolve the endometritis and develops a persistent inflammatory condition, which affects fertility. Furthermore, if the mare is unable to clear bacteria that may have entered the uterus during breeding, a bacterial infection can develop [19, 20].

In conclusion, any difficulty in the physical clearance of inflammatory debris from the uterus after mating or foaling triggers the endometritis. Although the mechanisms for uterine clearance for different antigens are closely related, pathophysiology, clinical signs and therapy partially differ. For these reasons, breeding-induced endometritis and bacterial endometritis will be described separately.

2. 1. Transient and persistent mating-induced endometritis

The transient breeding-induced endometritis is a physiological reaction in the immediate hours after breeding. It is a local inflammatory response necessary to remove excess spermatozoa and bacteria introduced into the uterus [8]. This response is limited to local inflammation, because any hematological alteration in inflammatory parameters has not yet been detected during breeding-induced endometritis [21]. Furthermore, in healthy mares, the endometritis resolves within 24–48 hours, leaving the uterus clean and free from inflammation.

2. 1. 1. Mechanical clearance and inflammatory response

Semen and its extender play an important role in the induction of defense mechanisms, depending on seminal components and sperms numbers, concentration, viability and site of semen deposition. In mares mated, or artificially inseminated with fresh or cooled semen, seminal plasma activates the complement system that evokes massive migration of the polymorphonuclear leucocytes (PMNs), cytokines and mononuclear cells invasion that prepares the endometrium to receive the embryo [11, 22]. During the cryopreservation process, an important mechanism of modulation of the inflammatory response is lost due to the removal of seminal plasma, so that a severe inflammation follows the insemination with frozen/thawed semen [13]. Furthermore, seminal plasma contributes to the transport and survival of viable spermatozoa and the elimination of non-viable spermatozoa from the uterus, suppressing binding between neutrophils and viable spermatozoa [23].

The complement activation cascade led to the formation of leukotriene B₄, prostaglandin (PG) E and PGF₂α and other arachidonic acid metabolites, which act as chemo-attractants for PMNs in the uterus [2–5, 7]. The PMNs, on their part, drive the inflammatory response to cleaning the uterus: spermatozoa and bacteria destruction is performed by way of phagocytosis and release of neutrophil extracellular traps, composed of extensions of DNA and histones with antimicrobial action [15]. The intrauterine fluid is composed of neutrophils, inflammatory mediators and plasma proteins, including immunoglobulin and enzymes [3–4, 6–7]. The release of PGF₂α stimulates myometrial contractions combined with ciliary propulsion of the

mucus blanket, promoting the elimination of bacteria and dead inflammatory cells via the cervix or the lymphatics [9, 12].

In “resistant” mares, uterine fluid is eliminated and inflammation resolves within 5 days post-insemination and the fertilized oocyte descends in a uterus ready to implantation [24]. On the contrary, susceptible mares are unable to resolve the physiological breeding-induced inflammation and they surrender to persistent post-insemination endometritis. The accumulation of intrauterine fluid persists over five days post-ovulation in susceptible mares [17]. Therefore, the success of getting the mare pregnant is compromised by an unprepared endometrium and by the development of concomitant bacterial infections.

It is likely that at the origin of both breeding-induced and bacterial endometritis, there are common failures in the physical mechanisms of uterine clearance by uterine contractions. Indeed, mechanical clearance plays a key role in the uterine response to the contamination, and deficits in myometrial contractility could have a major responsibility in the pathogenesis of delayed uterine clearance. The increase in myoelectric activity, indicating an increase in uterine contractions, observed in healthy mares after the insemination, resulted delayed by 2 hours in susceptible mares. Additionally, susceptible mares showed a sharp decline in activity, dropping below baseline levels after 12 hours [10]. Furthermore, it was observed that more radiocolloid was retained in the uterus of susceptible mares 2 hours after infusion than in resistant mares [25]. Impaired myometrial function could be related to the increased intrauterine accumulation of nitric oxide (NO), produced in excess by inducible NO synthase (iNOS) during inflammation in susceptible mares, as confirmed by an increased endometrial expression of iNOS mRNA after insemination [26–28]. The NO is a smooth muscle relaxant and myometrial tissue was unable to respond to electrical stimulus in the presence of NO, during *in vitro* experiments [29].

Reaction to the insemination could involve humoral and cellular mechanism, with differences between resistant and susceptible mares. Several studies produced contradictory findings, but most authors agree that immunoglobulins are involved mainly in the endometrial response to bacteria than to semen. However, there are no data to demonstrate that susceptibility is a direct cause of altered immunoglobulins in the uterus [30–32]. Similarly, it was suggested that in resistant and susceptible mares, different inflammatory cells were involved and that susceptibility to persistent endometritis was primarily caused by a dysfunction in the adaptive immune response. Until now, there are no data to prove these hypotheses, and just a decreased opsonizing ability of the uterine secretions in sub-fertile mares compared with normal mares was demonstrated [9].

The inflammatory response in endometritis is a complex process involving multiple signaling pathways, initiated by the cytokines, produced by a variety of cell types, to allow the recognition of antigens and the recruitment of inflammatory cells [33]. Specifically, the inflammatory response is modulated by a delicate balance between the expressions of pro- and anti-inflammatory interleukin (IL). Another important pro-inflammatory cytokine involved with the inflammatory response is interferon- γ . It promotes the migration of inflammatory cells through vessel walls and leads to the activation of microbicidal functions, and to an upregulation of iNOS [34]. Few studies focused on these inflammatory pathways in the mare so far, and they gave contradictory results in relation to the experimental protocol and the timing of

evaluation [33, 35–37]. Specifically, timing is very important in the experimental design, since the difference between resistant and susceptible mares consist mainly in the persistence of the breeding-induced inflammatory response. Thus, results of the studies were sensibly different, measuring the expression of several IL at 24 hours or at 6 hours after breeding. However, an increased mRNA expression of IL8 and lower expression of IL10 were observed in susceptible mares compared to resistant mares 24 hours after insemination [38]. On measuring cytokines expression in mares at several time points within the first 24 hours after breeding, susceptible mares had lower expression of the inflammatory modulating cytokines IL10, IL1-receptor antagonist and IL6 when compared with resistant mares 6 hours after insemination. After 3–6 hours from breeding, there were no differences between resistant and susceptible mares in the degree of uterine inflammation, fluid retention, endometrial cytology and ultrasound imaging. However, on the basis of the differences in mRNA expression of cytokines observed, it was stated that a critical time in the development of persistent breeding-induced endometritis occurs around 6 hours after breeding [33].

2. 1. 2. Predisposing factors to persistent breeding-induced endometritis

Persistent endometritis has multifactorial pathogenesis, and properties of the bacteria and mare's characteristics are key components in the development and resolution of the inflammation [39]. Several predisposing factors are associated with the individual mare [17–18]. Young and fertile resistant mares and older and subfertile susceptible mares have been examined to clarify the differences in the ability to resolve uterine inflammation. Indeed, the aging and parity have been associated with altered systemic immune response [30–31]. Older mares present several predisposing factors to develop persistent uterine infections after breeding. Aging and parity influx on the ability of clearing debris, since mares develop over time anatomical or degenerative defects that interfere with uterine drainage. Other risk factors associated with persistent infections include the conformation or the acquired alteration of the internal and external reproductive organs. For example, not only poor vulvar conformation, incompetent vagino-vestibular sphincter, vaginal stretching or an incompetent cervix but also genital pathology, such as pneumovagina or vagino/cervical injuries can facilitate the entrance of pathogens, which normally lives over body surface [40]. Mares with cervical fibrosis secondary to a traumatic birth, or with the elongated, narrow cervix typical in aged maiden mares, accumulate uterine fluid easily because of the compromised cervical drainage [17]. Free fluid accumulation is also facilitated by a pendulous uterus or impaired lymphatic drainage and atrophy of endometrial folds [41–44].

Degenerative changes, such as an abnormal myometrium, periglandular fibrosis, vascular degeneration, lymphangiectasia, scarring and atrophy of endometrial folds or damage to the reproductive apparatus may also explain delayed uterine clearance of bacteria, fluid and debris [17]. The altered mucociliary activity does not impede the bacterial adhesion on the endometrium and expulsion of the inflammatory cells. Vascular degenerations inhibit hormones delivery to endometrium and disturb uterine drainage, reducing venous return in capillary beds. Prolonged endometrial edema and consequent persistent inflammation characterize the uterus of susceptible mares [43].

2. 2. Bacterial endometritis

The sexually transmissible disease in horses are caused by primary pathogens *Taylorella equigenitalis*, certain unspecified serotypes of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* capsule types 1, 2 and 5 [45]. The true venereal disease is caused by *T. equigenitalis* and is known as contagious equine metritis resulting in cervicitis, vaginitis and endometritis. Asymptomatic infected carrier stallions transmit the pathogens to the mares, which will present copious muco-purulent vaginal discharge within a week after breeding. However, this pathology is endemic in Europe and a more insidious form with minimal clinical signs has been recognized [46–47]. *P. aeruginosa* and *K. pneumoniae* inhabit the external genital of the stallion and infections are transmitted by coitus, insemination with infected semen and genital manipulations [48–49].

Contamination of the uterus by fecal and genital opportunistic flora may provoke an infection during mating or genital tract manipulations. A wide variety of opportunistic aerobic and anaerobic bacteria, fungi and yeasts, either alone or in synergy, have been occasionally implicated as causes of endometritis. In a report, *Streptococcus equi* subsp. *zooeconomicus* was responsible for approximately 65% of the cases while *Escherichia coli*, *K. pneumoniae* and *P. aeruginosa* accounted for approximately 10% [47]. The alteration of uterine defense mechanisms is attributed to individual microbial factors such as induction of inflammation, epithelial adherence, resistance to phagocytosis and viscosity of secretions [50]. Bacterial products can modify any properties of mucus, rendering cilia unable to expel uterine exudates. Changes in production, viscosity or elasticity of mucus and cilia function determinate the adverse effects on uterine clearance and hence, these can interfere with antibiotic penetration, resulting in treatment failure or antibiotic resistance [17].

2. 3. Chronic endometritis

Chronic infection and mixed population of microorganism cause severe, progressive and irreversible fibrotic condition that affects mare endometrium [51–54]. Long-standing influx of lymphocytes and plasmacells into the endometrium contribute to chronic degenerative changes, such as periglandular, perivascular or diffuse stromal fibrosis. This condition impairs endometrial function and future pregnancies, causing infertility [52–53].

3. Diagnosis of endometritis

The diagnosis of endometritis in the mare represents one of the crucial challenges for the equine clinical practice [55]. Equine practitioners have recently understood that the major cause of the progression from an acute to a chronic condition is the failure to identify the causative agent, given that affected mares do not always show evident clinical signs [55]. Hence, it is critical to make a correct etiological diagnosis, and to characterize the degree and the type of inflammation, in order to establish an effective treatment. Furthermore, it is not uncommon that mares with a long history of normal fertility can acquire post-breeding endometritis. In such cases, the clinician has no opportunity for prophylactic intervention [56].

The diagnostic algorithm for the identification of the causative agent comprises a detailed reproductive history and a complete clinical evaluation, including the transrectal palpation of the reproductive tract and the use of ancillary diagnostic aids [17, 57–58]. Among the latter, specific instrumental investigations, such as transrectal ultrasonography, vaginal and cervical exams, with both manual and endoscopic evaluation, and laboratory tools, such as uterine culture, endometrial cytology and biopsy, represent useful tools for the diagnosis of endometritis [17, 58]. Recently, myeloperoxidase has been investigated as a uterine inflammatory biomarker, but the relationship between its concentration and pathologic uterine conditions needs further studies [59]. To increase the pregnancy rates in “problem” mares, the practitioner should dispose a quick and reliable diagnostic technique to start the best treatment as soon as possible in the breeding season [56].

3. 1. Experimental identification of susceptible mares

Mares with endometritis begin to show intrauterine fluid accumulation following breeding, after the first three to four successful pregnancies. Uterine degenerative changes appear and worsen with age, the reproductive tract may become more pendulous and uterotonic drugs are no longer effective [57]. Furthermore, some reproductively normal, nulliparous as well as pluriparous mares exhibit an excessive uterine inflammatory response after being bred with frozen semen. The exuberant reaction may be a consequence of a low volume of seminal plasma and the lack of its beneficial action in decreasing the migration of neutrophils into the uterine lumen [57].

The delay in uterine clearance has an important role in the pathogenesis of uterine inflammation, since it influences the retention of endometrial inflammatory factors, whether they are bacterial or sperm-induced [60]. In fact, it was shown that mares susceptible to chronic uterine infections failed to clear adequately ⁵¹Cr-labeled microspheres when compared with mares with normal uteri [37, 60]. The uterus, studied with scintigraphy in mares with a delayed uterine clearance, showed to be oriented vertically, while in reproductively normal mares it was oriented horizontally. Hence the “baggy” uterus contributes to fluid accumulation and low clearance [37, 57]. However, although scintigraphy has been proven to be a useful method for studying post-breeding endometritis, it seems not to be a functional diagnostic tool in a clinical setting [61]. In addition to the uterine position, the myometrial function plays an important role in uterine clearance, as confirmed by electromyographic recordings [60]. Nevertheless, not all mares susceptible to endometritis exhibit a delayed uterine clearance if cervical dilatation is appropriate [25].

3. 2. Clinical identification of susceptible mares

3. 2. 1. History

Mares susceptible to developing a persistent post-breeding endometritis are more often pluriparous, barren, older than 12 years of age and with a poor perineal conformation [57, 62]. Nonetheless, some maiden mares, which show an insufficient degree of relaxation of their

cervix during estrus, may also develop persistent mating-induced endometritis, regardless of age. In such mares, the problem may be resolved with the first foal delivery [57].

3. 2. 2. *Clinical examination*

The mares' reproductive tract examination should include a body condition scoring and the notation of previous or existing foot problems [57]. Any changes in perineal, vulvar or cervical conformation should be evaluated before breeding [56–57, 63]. The two vulvar labia may not create a proper seal due to persistent relaxation of the vulva, and the rectum may be displaced cranially, stretching the vulva dorsally to the ischiatic arch and causing repeated fecal contamination of vulva and vestibule [56]. This kind of conformation is linked to the loss of fat in the perineal area due to a high level of fitness, and muscular fatigue and estrus exacerbate the perineal relaxation, but it can also be a heritable trait [63–64]. It is very important to check that the cervix opens properly in estrus and closes in diestrus [56].

3. 3. **Clinical signs**

Clinical signs of endometritis may be hidden, but vaginal discharge, short inter-estrus intervals and/or a shortened luteal phase and reduced fertility can be detected [17, 37, 65]. In bacterial endometritis, they can vary widely according to the pathogen, with symptoms markedly different between Gram-positive and Gram-negative bacteria [17]. A vaginal discharge may be seen more often during estrus [57]. However, the presence of evident clinical signs 48 hours post-breeding is of concern, since a non-inflammatory uterine environment is important for embryonic survival [16].

3. 4. **Imaging of the reproductive system: ultrasonography**

Ultrasonography (US) has always been considered an accurate and essential step in the diagnostic workup of endometritis [56, 59, 66]. The monitoring function of US is as important as the diagnostic one, since mares with subclinical endometritis require careful daily checking post-breeding [17]. Since its inception, the use of US has strongly impacted on the ability to detect the presence and to quantify the volume of uterine fluid accumulation, which is considered the main sign of endometritis, especially when observed after breeding; but this "hallmark" sign is not always present [17, 60, 67]. Intrauterine fluid presence has also been shown to be strongly related with low pregnancy rates, especially in mares older than 16 years, and to the biopsy score, revealing its diagnostic value in identifying the uterine inflammation [62, 67–68]. It may be detected as soon as 6 to 36 hours after breeding, but it can be noted at the pregnancy examination 14 to 16 days after breeding [57, 67]. Nonetheless, the more significant time to detect the uterine fluid collection is mid-to-late diestrus because the cervix is closed, and in a healthy uterus, fluid should be little or absent [68]. Intrauterine fluid has been also correlated to specific endometrial bacteria and cytological findings, with mares positive to non-pathogenic bacteria showing intra-uterine fluid in <40% of the US examinations [66–67]. The relevance of the uterine fluid accumulation can be evaluated by examining the depth, the echo-texture and persistence of the fluid [16]. Two or more centimeters of intra-uterine fluid during estrus, or between 6 and 36 hours post-breeding, are significant indices

of susceptibility to mating-induced endometritis [62, 67]. A high echogenicity in the uterine fluid is associated with the presence of neutrophils and debris, thus indicative of inflammatory components [16]. Short, thick and hyperechoic lines within the uterus may represent air or exudates [17]. In Figure 1, some characteristic appearances of the equine uterus are depicted.



Figure 1. Trans-rectal ultrasonographic evaluation of the equine uterus with a linear transducer (bandwidth 5–10 MHz). In A: normal appearance of the uterus during early diestrus, 48 hours post-ovulation. In B: mild intrauterine fluid accumulation, with moderate edema of uterine folds during early estrus. In C: moderate intrauterine fluid accumulation, with severe edema of uterine folds during estrus. In D: severe (over 2 cm in depth) intrauterine fluid accumulation in diestrus, 13 days after ovulation, “hallmark” of equine endometritis.

Another important US sign of endometritis is abnormal edema [17]. Edema may occur physiologically due to lymphangiectasia and under influence of estrogen, i. e. , during the early estrus. A modified version of the subjective system described by Samper has been developed to score uterine edema: grade 0 coincides with the absence of edema; grade I, in which it is difficult to identify uterine folds; grade II, characterized by some of the endometrial folds detectable and a cervix with a fish-bone aspect; grade III, with endometrial folds, easily identifiable and characterized by hyperechoic borders and hypoechoic centers (cartwheel); and grade IV for mares with “hyperedema”, in which thickened and bulging endometrial folds are abnormally thick, with hyperechoic border and marked central hypoechogenicity, and the normal architecture of the cartwheel is lost [59]. Cervical incompetence, abnormal vascular reaction to estrogens, a lymphatic pathology or an altered myoelectric activity are all possible non-inflammatory causes of hyperedema [17, 59]. Thus, some edema patterns, such as excessive edema pre- or post-mating, or edema pattern that does not extend throughout the uterine wall, represent a pathological feature [17]. In particular, hyperedema has been considered the marker of uterine pathology when found during the estrus phase, independ-

ently from the positivity of a uterine cytology, but not during the early estrus [59]. Sometimes uterine fluid and edema are associated, probably due to the severity of drainage deficiency [59]. Furthermore, the appearance and dynamics of uterine cysts can be studied by US. Their effect on pregnancy rate seems to be a quantitative one, with only severely affected mares showing a reduction of fertility [68].

The use of more advanced US software, like Color-, Power-flow- and Pulse-Wave- Doppler applications, allows the evaluation of uterine vascularization. In humans, abnormal uterine blood flow and higher uterine artery impedance have been observed in women with recurrent pregnancy loss and different causes of infertility. Poor blood flow of the gravid uterus has been correlated with advanced age and diffuse endometrial degenerative changes during early pregnancy in mares. Furthermore, disturbed uterine blood flow has been recently associated to other uterine pathologies such as uterine cysts and endometrial elastosis in subfertile mares [69].

3. 5. Endoscopic evaluation of the reproductive system

After repeated mating in a season, mares may accumulate intrauterine fluid and display classic signs of inflammation on vaginoscopy. Endoscopic examination is the only way to establish the degree and the clinical significance of superficial intrauterine lesions and it is useful in several pathological conditions. It can be used in the mare suspected of having subclinical endometritis due to focal lesions, intra-uterine adhesions and endometrial cups retention. It has proven to be useful in mares with a history of silent heats, which may display endometrial scarring or loss of endometrial folds [17]. The best time to perform endoscopy is during diestrus or early estrus, since it is easier to perform than in other phases. Before the examination, the uterine lumen is dilated with air or saline, but the best visibility is obtained with air. It is important to remember to discard air out of the uterus after the procedure, with a catheter or a pump, because of possible irritation [17].

3. 6. Sampling techniques for bacteriological, cytological and histological evaluation: uterine swab, cytobrush, low volume flush and endometrial biopsy

The diagnostic methods used to characterize endometritis are the uterine swab, the cytobrush, low volume flush and the endometrial biopsy [37, 70]. As always, the clinician must interpret the data resulting from such techniques together with the clinical signs and bearing in mind how the results of each technique vary, depending on the pathogen [17, 58, 61, 65, 70–73]. All the procedures described thereafter assume that the mares are restrained in an examination stock, the tail is wrapped in an examination glove and suspended to the stock, and the whole perineal area is cleaned and disinfected to avoid contamination from the environment [16, 58, 71]. Each method has its pro and cons and many comparisons of the different techniques have been indeed performed [58, 65, 70–73]. Histologic evaluation of endometrial biopsies is the “gold standard”, to which each technique has been compared to calculate the sensitivity and specificity [17, 65, 71]. Nevertheless, it should be remembered that all techniques can yield false-negative results if not conducted by expert clinician [37].

3.6.1. Uterine swab

The uterine swab collection methods for bacteriologic and cytological analysis are the mainstay for the diagnosis of acute endometritis in the mare [74]. Through this technique, bacteria as well as inflammatory cells can be collected and examined by culturing it, or smearing the swab for cytology [70, 74]. Uterine swabs are the most commonly used procedure because of low costs, ease of collection and safety of use; hence, routine pre-breeding uterine swabs should be always obtained from mares “at risk” [56, 65].

The double-guarded swab technique implicates the use of a double sheath, which allows minimizing vaginal contamination [16, 70–71]. The tip of the double-guarded swab is kept covered and free from lubricant as it is introduced into the reproductive tract. Then, it is advanced through the cervix into the uterus and the inner sheath is pushed through the outer sheath. The examiner starts moving the swab to sample the endometrial surface using a pushing and rolling motion for up to 1 min, redirecting the swab into different areas of the uterus. At the end of the sampling, the swab is pulled within the inner sheath, which is drawn back into the outer sheath, and the entire unit is then removed from the reproductive tract [16, 70–71].

Another kind of guarded uterine instrument is the Knudsen catheter that can be autoclaved and used repeatedly. It consists of a metal tube of 87 cm in length and an inner spiral metal rod, and it has a small hole at the tip, to allow advancing a cotton swab. The tube includes a thickened area, the olive, which marks the point of the catheter that should be placed at the outer cervical orifice [74].

For the classic uterine swab, cytological smears are prepared by gently rolling the side of the swab, and by pushing the end on a sterile slide. The swab is sent to laboratory for microbiological tests, including the tip in a transport container [16, 70–71]. On the other hand, the Knudsen catheter use provides the cotton swab for bacteriology, whereas the cytological sample is obtained by gently removing the material on the spiral rod [74]. Even if this technique, when properly adopted, is ideal for bacteriology, it leads to contrasting results for the cytological sampling. The use of this technique decreased strongly the number of mares improperly treated, but it may lead to cells deformation depending on the pressure applied during the smearing procedure [70, 74]. Nevertheless, too little pressure during the rolling procedure may lead to clumping of cells [74]. Moistening and gentle rolling have been proven to relief distortion and fragmentation of the collected cells [70]. Furthermore, this method can yield false negative results. In fact, about half of the cases of infectious endometritis resulted negative, since the microorganisms were located in the most pendulous part of uterus that is not easily reached. Conversely, false positive results can be associated with a contaminated sampling [55]. Furthermore, it collects a small superficial endometrial area, resulting in a small amount of total cells [70].

Otherwise, the degree of cellularity obtainable with the Knudsen catheter is influenced by the level of uterine secretion. Indeed, the contact of the smooth surface of the metallic spiral with a dry endometrial surface provides poor cellularity, whereas in case of abundant secretions, samples obtained show an excellent cellularity due to the larger surface area, without cells distortion and with infrequent red blood cells [74].

3. 6. 2. *Cytobrush*

Uterine brushes have been used for uterine cytology in humans, as well as in the equine species [60]. As described for the uterine swabs, a guarded technique is used, and the properly lubricated guarding tube is passed through the vagina to the base of a uterine horn or into the uterine body. Once in the area selected for the sampling, the outer tube is retracted far enough to expose the brush, and the cytobrush is rotated in a clockwise direction while in contact with the uterine wall [70]. The instrument is then retracted into the tube prior to removal from the uterus and then rolled on slides.

Cytobrush has been considered superior to the other methods for cytological sampling, since it is easier, more consistent and produces samples with higher cellularity than other techniques, but a gentle preparation of smear is mandatory to reduce the cells distortion [70, 74]. Cells fragmentation has been frequently observed in cytobrush smears probably due to the rigid fibers that damage the cells, as well as the common occurrence of red blood cells, which is an index of its invasiveness [70]. Proteinaceous material may contaminate the smears obtained with this technique, as well [70]. Nonetheless, it allows collecting cells both from the surface and from the depth of endometrium, up to glandular cells [70]. Such technique could preferentially be used to detect subclinical endometritis in field practice, for its inexpensiveness and safeness, particularly in comparison to endometrial biopsy [58, 65].

3. 6. 3. *Low volume flush*

The low volume flush is a method for uterine fluid collection [55]. It is almost as efficient as endometrial biopsy for microorganisms' isolation and can be performed during estrus or diestrus [55]. The procedure involves the use of a low volume (60 to 150 ml) of phosphate-buffered saline (PBS) or lactated Ringer's solution or physiological saline injected into the uterus. The practitioner, properly gloved, advances a sterile insemination pipette, or a Bivona catheter, *per vaginam* into the uterus, through which the solution is injected. Thus, the uterus is transrectally massaged to distribute the fluid evenly throughout the uterine lumen. Meanwhile, the pipette is moved back and forth and suction is applied to trap cells in the pipette. Finally, the effluent fluid is recovered in a sterile container. If the mare is in estrus, intravenous administration of 10 IU of oxytocin is suggested, to facilitate the release of fluid trapped in the edematous endometrial folds [16, 55, 70, 72].

The fluid is first evaluated macroscopically, holding the sample up to the light, for cloudiness and amount of mucus. The efflux is graded as clear, cloudy or clear with mucus strains. Both mucus and cloudiness are strongly correlated to the presence of *Streptococcus β haemolyticus* and *E. coli* [55, 72]. The recovered fluid is then centrifuged at 400 rpm for 10 min, the supernatant is discarded and the pellet is aliquoted in two parts, one for microbiological culture and the other one is resuspended in 1 ml of PBS and drops of the suspension are spilled onto slides for cytological evaluation [16, 55, 70, 72].

The low volume flush is a suitable method to collect cells over a larger surface area and provide more information about cells, mucus and/or exudates than other techniques [70]. It has been showed to be twice as sensitive as swab culture [71–72]. Indeed, it allows quick identification

of Gram-negative bacteria, e. g. , *E. coli* [55, 72]. Even if low volume flush requires a larger equipment to be performed than other procedures, it is considered rapid and accurate, and it may prove to be valuable for subclinical endometritis in the chronically infected mare. Nevertheless, this method may cause irritation of the endometrial mucosa and it may be more likely source of contamination from vaginal flora [60, 72]. Such contamination may yield false positive culture results; hence, other indices of endometritis should be added to improve the diagnostic power, such as a rise in pH or presence of debris [70, 72]. Moreover, the samples may show a high number of blood red cells, probably due to the transrectal manipulation of uterus and the scraping effect of the tip of the catheter [70].

Recently, a double-guarded low volume flush technique has been developed, with improved sensibility and specificity in identifying endometritis in the mare [18]. Overall, this new method represents a valid alternative to the classic low volume flush and seems to decrease the risk of contamination during sampling procedure. Furthermore, the availability of a disposable lavage tube and of a closed fluid-tubing system is favorable to be used on field and allows the execution by only one person [18]. On the other hand, the technique showed a poorer ability to find PMNs compared to biopsy [18].

3. 6. 4. Endometrial biopsy

The endometrial biopsy is a very helpful tool for diagnosing the cause of subclinical endometritis, always relating the microscopic lesions to mares' age and reproductive history [17]. Its results about the changes within the endometrium are considered the most reliable. Indeed, it is used to look for degenerative and inflammatory changes, which are classified using both the histologic grading system proposed by Kenney & Doig and modified by Schoon and coll. and the classification of Ricketts [17, 65]. Endometrial biopsies are collected using a sterilized dedicated biopsy instrument, which is passed through the cervix within the uterine lumen. One arm is then inserted into the rectum to guide the biopsy forceps to the desired location, usually at the base of one uterine horn [58, 71]. Once the forceps is closed, it is withdrawn and the sample is macroscopically evaluated for consistency and size, and immediately put in the selected media, depending on its further use.

Bacteriological culture and cytology from endometrial biopsy were demonstrated to be superior to culture swabs, both for sensitivity and for positive predictive value [71]. Endometrial biopsy is safe, but it is not particularly practical [58]. A practical disadvantage is the time between sampling and histologic results, while bacteriological and cytological results are achievable in a short time [58]. Moreover, it is objectively more invasive than other techniques and needs specific equipment, requires further processing, such as shipping to skilled laboratories, time for examination and transmission of results. Hence, it takes longer to have final diagnosis [65, 71].

3. 7. Endometrial cytology

Endometrial cytology is an inestimable tool in assessing the endometrial inflammation, mainly through the detection of PMNs. Unfortunately, an agreement on the classification and

interpretation of cytological results has not yet been reached and different interpretative cut-offs were proposed [16, 70, 74–75]. Indeed, some authors record the number of PMNs as a percentage of all cells seen on a slide and the cut-off value for positive to endometritis ranges from 0.5 to 5%, according to the guidelines of Brook [16, 71]. The latter classification system categorizes cytological samples as: non-inflammatory (PMNs < 5%), mild inflammation (PMNs 5–15%), moderate inflammation (PMNs 15–30%) and severe inflammation (PMNs > 30%) [16, 70, 75]. Other authors record the amount of PMNs seen per microscopic high power field (HPF) examined. In details, cytological smears are graded as not inflammatory (0–2 neutrophils/field), moderate inflammation (2–5 neutrophils/field), severe inflammation (>5 neutrophils/field) or hypocellular (scant epithelial cells and no neutrophils) [70, 75]. The presence of uterine fluid during estrus has been found to be associated with an increased number of PMNs, and mares with intrauterine fluid on the second–third day of estrus were 1.4 times more likely to have more than 5 PMNs per HPF than those with no or mild inflammation [66–67]. Recently, it has been suggested to evaluate endometrial cytology using the percentage of PMNs in relation to epithelial cells, rather than counting the number of PMNs per HPF, when using the cytobrush for sampling [73]. Nonetheless, the number of PMNs per HPF was found to be inversely proportional to the pregnancy rate, i. e., mares without inflammation had pregnancy rates 1.3 and 3 times higher than those of mares exhibiting moderate or severe inflammation, respectively [75].

The amount of PMNs detectable is affected by various factors. Samples obtained in early estrus may not contain inflammatory cells, since PMNs' migration into the uterine lumen during the period of waning progesterone dominance is lower than during maximal estrogen dominance [70, 75]. Nonetheless, the time post-ovulation does not seem to display any effect on endometrial cytological parameters in non-bred mares. Moreover, a small resident amount of PMNs in the endometrium has been demonstrated from 24 to 96 hours after ovulation as well as during pro-estrus, in healthy mares [16, 65, 70–71]. The PMNs number increases in the uterine stratum compactum, but not in low volume flushes, after infusion of semen extenders, saline or seminal plasma, which are known to have an inflammatory effect [65].

Other important parameters useful to interpret the cytological sample are: the background content of the slides, i. e., if it is proteinaceous, contaminated with red blood cells, or clear; the quality of the cells harvested, i. e., if they are intact, distorted, or fragmented; the total cellularity, that is the number of cells per HPF; the ratio between PMNs and uterine epithelial cells; the presence of other inflammatory cells, e. g., eosinophils and monocytes, and their number per HPF; the presence and the number per HPF of vaginal epithelial cells; and the eventual presence of bacteria [55, 70].

Cytology is at the top among the diagnostic techniques, since it is a relatively inexpensive method to obtain results in a short time [73]. However, this method has a relatively high rate of false negative results and it does not provide information about the cause of the inflammation [58, 70]. That is why it should always be conducted together with bacteriology, as the detection of PMNs together with potential pathogens is a stronger indicator of endometritis, and the number of mares identified as positive to endometritis is significantly higher than with either technique alone [58, 65, 70–71]. Furthermore, cytological samples supported by positive cultures show on average twice neutrophils than those associated with negative cultures,

regardless of the virulence of the bacteria individuated [74]. Nonetheless, mares may have positive cytology with negative culture, and *vice versa* [55, 65]. Various interpretations have been made to explain the absence of a correlation between the two techniques, in particular to justify negative cultures, such as that uterine swabs may miss focal infections, the presence of antimicrobial preparations in the uterus, deep-seated infection or non-infectious irritation [65].

3. 8. Endometrial microbiology

A positive uterine culture before breeding is among the causes of infertility linked to endometritis [17, 75]. If a mare does not spontaneously eliminate an infection in 2 to 4 days, she is considered either persistently infected or on her way of becoming persistently infected, as supported by the study on uterine clearance of bacteria in healthy mares [60]. Mares are classified as resistant when able to clear intrauterine fluid, inflammatory cells, and bacteria within 48 hours from breeding, otherwise they are considered susceptible [61]. In addition, the mares may change their susceptibility over subsequent breeding seasons, in a gradual manner. Some of them exhibit a decreased endometrial quality, while others show a floating resistance [61]. Most susceptible mares exhibit minimal signs of inflammation prior to the first breeding of the year, likely because of prolonged sexual rest [57]. Although uterine culture may yield false positive or false negative results, aerobic endometrial culture is still the most common method for diagnosing infectious endometritis [65, 71]. Indeed, although bacteria may be recovered indicating an infectious endometritis, the underlying problem may be a persistent post-breeding endometritis, either not managed or treated suitably [57]. Bacteria and other microorganisms (yeast or fungi) may be found on cytological smears, free or phagocytized within neutrophils or macrophages, and should be scored based on their number per HPF [16]. Cultures may be positive for one or more bacterial species, but mixed cultures of more than three pathogens are usually considered as the result of contamination [58, 65, 71].

Various correlations have been demonstrated between endometrial bacteria and different US and endometrial cytological findings. Mares with intrauterine fluid were 1.4 times more likely to have 5 PMNs per HPF on cytological specimens than those with no or mild fluid [66]. Intrauterine fluid was more commonly detected when *hemolytic Streptococcus*, *Klebsiella* species, *Enterobacter cloacae* or yeast were isolated, compared with *E. coli*, *Staphylococcus aureus* and *Pseudomonas* species [66, 72]. Also intrauterine fluid and a cytology containing 2 PMNs per HPF were more commonly associated to *hemolytic Streptococcus* than *E. coli* [66]. Nevertheless, less than 40% of mares from which were isolated *E. coli*, *S. aureus*, *Pseudomonas* spp. , or non-pathogen bacteria, such as *Micrococcus* spp. , *Alpha Streptococcus* or *Bacillus* spp. , had intrauterine fluid at US, immediately before the uterine culture was done [66]. Intrauterine fluid was seen more frequently, in 45–55% of the US examinations, when β -*hemolytic Streptococcus*, *K. pneumoniae*, *E. cloacae* or yeast were isolated [66]. However, intrauterine fluid, especially during estrus, was not always associated with bacterial endometritis [66]. Furthermore, pathogens associated with uterine fluid were more likely to coincide to neutrophils findings on cytology and vice versa [67]. Hence, the uterine fluid indicates an acute inflammation, but not necessarily a bacterial infection. This is confirmed by the presence of other causes of acute and neutrophilic intrauterine collection, such as pneumovagina, irritating effect of semen, urine reflux into the uterus and excessive production of endometrial mucus [67]. Moreover, not all microbes cause a neutrophilic response and the amount of cytological specimens graded as

positive for inflammation varied among the microbial findings [75]. Indeed, *β-hemolytic Streptococcus* or *Klebsiella* yield more often positive uterine cytology, whereas *E. coli*, *S. aureus* and *Pseudomonas* spp. had fewer positive cytological results [66, 75]. Presence of PMNs was strongly associated with *S. equi* subsp. *zooepidemicus*, rather than *E. coli* [71, 74–75]. Among the main pathogens, *β-hemolytic Streptococcus*, in particular *S. equi* subsp. *zooepidemicus*, a Gram-positive bacteria, is more likely to cause an endometrial inflammatory response and it was more often associated with positive cytology, differently from other pathogens [74].

Various bacterial species have proven to be pathogenic in the mares' uterine environment, and each species have been isolated in different percentage. Most frequently isolated bacteria, the relative percentage, the sampling techniques adopted and the geographical area in which the study was conducted are summarized in Table 1. In general, *β-hemolytic Streptococcus* is strongly related to a rise in pH in the low volume flush efflux, probably due to super antigens, released by the bacteria itself, which beckon pro-inflammatory mediators into the uterine lumen [72]. They were reported as the most common microorganisms isolated from mares' uterus [71–72, 75]. The double-guarded low volume flush allowed to isolate more *β-hemolytic Streptococcus*, even if uterine lavage seems to be not proper to detect it, probably due to its deep localization in the endometrium [18]. Furthermore, in some *S. equi* subsp. *zooepidemicus* strains, a clear ability to form persistent cells, highly tolerant to penicillin, has been demonstrated. The prevalence of subclinical *S. zooepidemicus* endometritis in the mare is still to be determined, and in subfertile mares the prevalence has been determined to be as high as 64%. The activation of dormant bacteria, using bacterial growth mediums like *bActivate*, may improve significantly the sensitivity of traditional diagnostics [76].

On the other hand, *E. coli*, a Gram-negative bacteria, was associated neither with cytological evidence of inflammation nor with increased pH [65, 71–72, 74–75]. The absence of correlation between *E. coli* and positive cytology may be due to its inability to attract PMNs into the uterine lumen [18]. In fact, the pathogen–host relationship, and consequently the uterine inflammatory response, of *E. coli* appears to be different from that of *β-hemolytic Streptococcus* [72]. On the other hand, it appears to associate with moderate to heavy debris on cytological smears. The interpretation of cultures positive for *E. coli* is difficult, since only pure cultures and large numbers of colonies are considered significant, and an increased sensitivity seems obtainable by culturing low volume flush [71–72, 74]. *E. coli* is sometimes considered a contaminant, but it is believed to cause subfertility in the mare [65], and it has been associated with focal infections, in the form of granulomatous plaques identified with endoscopic evaluation in two mares [72]. Another pathogen is *P. aeruginosa*, a Gram-negative bacteria, which produces a biofilm, i. e. , an adhesive matrix that harbors bacterial microcolonies and resists as well to antibiotics as to the immune system [67]. *P. aeruginosa* is believed to be cause of chronic and persistent infections [67].

The role of non-pathogenic bacteria isolated from the mares' uterus is still unknown, but they may be the cause of decreased pregnancy rates [75]. Mares with bacteriological positivity but no cytological evidence of PMNs may be affected by contaminants [58, 72, 75]. However, their pregnancy rate resulted lower than those of the mares without any positivity to the tests. For example, *Bacillus* and *Micrococcus* are skin commensals that are not usually the cause of lowered pregnancy rates, but in barren and older mares with weakened physical barriers they may play a role in decreased fertility [75]. Even if *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. Trans-cervical medical procedures, particularly when involving instillation of substances that may possibly favor bacterial growth, represent a risk of iatrogenic contamination of the uterus with bacteria from the micro-flora of the caudal reproductive tract [76].

Bacterial Species	Prevalence (%)	SamplingMethod	Area	Reference
<i>S. equi</i> subsp. <i>zooepidemicus</i>	28.3	Cotton Swab /	Kentucky	[66]
<i>E. coli</i>	20.5	Low Volume Flush		
<i>S. equi</i> subsp. <i>zooepidemicus</i>	34	Cotton Swab	Kentucky	[75]
<i>E. coli</i>	17			
<i>Pseudomonas</i> spp.	11.7			
Non-pathogens (Micrococcus, α -Streptococcus, Bacillus)	11.3			
<i>E. coli</i>	27.6	Low Volume	Kentucky	[72]
Mixed	14.6	Flush		
<i>S. equi</i> subsp. <i>zooepidemicus</i>	20.9			
Mixed	16.7			
<i>S. equi</i> subsp. <i>zooepidemicus</i> + <i>E. coli</i>	7.8			
<i>S. equi</i> subsp. <i>zooepidemicus</i>	39	Cotton Swab		
<i>E. coli</i>	16			
<i>S. equi</i> subsp. <i>zooepidemicus</i>	19	Endometrial	Denmark	[71]
<i>S. aureus</i>	1	Biopsy		
<i>E. coli</i>	1	Cotton Swab		
<i>S. equi</i> subsp. <i>zooepidemicus</i>	31.7	Cotton Swab	Italy	[103]
<i>E. coli</i>	19.6			
Non-hemolytic	18.4			
Hemolytic	1.2			
<i>E. coli</i>		Cotton Swab	Sweden	[101]
Hemolytic	3			
Non-hemolytic	64			
<i>S. equi</i> subsp. <i>zooepidemicus</i>	20			
Gramnegative Coccus	8			
<i>E. coli</i>	30	Low Volume	Denmark/	[18]
<i>S. equi</i> subsp. <i>zooepidemicus</i>	11	Flush	Kentucky	
<i>E. coli</i> + β -Streptococcus	20			
<i>E. coli</i>	21	Swab		

Bacterial Species	Prevalence (%)	Sampling Method	Area	Reference
<i>S. equi</i> subsp. <i>zooepidemicus</i>	8			
<i>E. coli</i> + β - <i>Streptococcus</i>	4			
<i>E. coli</i>	12	Endometrial		
<i>S. equi</i> subsp. <i>zooepidemicus</i>	7	Biopsy		
<i>E. coli</i> + β - <i>Streptococcus</i>	3			
<i>S. equi</i> subsp. <i>zooepidemicus</i>	Not Specified	Cotton Swab / Low Volume Flush	Canada	[16]
<i>Bacillus</i> spp.	10	Cytobrush	Poland	[58]
<i>S. equi</i> subsp. <i>zooepidemicus</i>	6			
<i>E. coli</i>	5			
<i>Bacillus</i> spp.	11	Endometrial		
<i>S. equi</i> subsp. <i>zooepidemicus</i>	8	Biopsy		
<i>E. coli</i>	5			
<i>E. coli</i>	10.9	Cotton Swab /	Germany	[65]
<i>S. equi</i> subsp. <i>zooepidemicus</i>	9.1	Cytobrush /Endometrial Biopsy		
<i>E. coli</i>	Not Specified	Knudsen Catheter	Germany	[74]
Non hemolytic <i>S. equi</i> subsp. <i>zooepidemicus</i>				
<i>Klebsiella</i> spp.	Not Specified			
<i>Pseudomonas</i> spp.	Specified			
<i>S. equi</i> subsp. <i>zooepidemicus</i>	Not Specified	Cytobrush		
<i>E. coli</i>	Specified			
Non-hemolytic <i>Klebsiella</i> spp.				
<i>Pseudomonas</i> spp.	Not Specified			
<i>E. coli</i>	Not Specified	Cotton Swab		
Non-hemolytic <i>S. equi</i> subsp. <i>zooepidemicus</i>				
<i>Klebsiella</i> spp.	Not Specified			
<i>Pseudomonas</i> spp.	Specified			

Table 1. Prevalence of the most common bacterial species isolated from mares' uterus in various geographical areas

3. 9. Endometrial histology

In brief, the histological grading system for the endometrium ranges from I to III. Grade I is an essentially normal endometrium with minimal alterations. Grade II is a wide category, often divided into subcategories, IIA and IIB, from mild to moderate pathologic conditions. Grade

III includes severe and widespread inflammatory and/or degenerative changes of the endometrium [77]. This technique is considered the “gold standard” to diagnose endometritis, in particular the detection of PMNs infiltrating the luminal epithelium and the stratum compactum [71]. Grade IIB and III mares have also shown an increased incidence of intrauterine fluid retention, compared to grade I and IIA mares [61].

The endometrial lesions are further classified as inflammatory, i. e. , acute, sub-acute or chronic, and non-inflammatory lesions, e. g. , hypoplasia, hyperplasia or chronic degenerative conditions. The acute inflammatory lesions are characterized by at least one PMN per 5 HPF, whereas the chronic ones by lymphocytes are often accompanied by eosinophils [58, 71]. Anyway, the number of PMNs is affected by the phase of the estrus cycle, with more neutrophils especially during estrus, regardless the cause of endometritis, breeding as well as bacterial-induced. Degenerative processes are the sclerosis of uterine vessels, i. e. , “angiosis” also known as “pregnancy sclerosis” in humans, which increase with parity, the lymphangiectasia, secondary to vascular degeneration or persistent inflammation, the loss of epithelium and the epithelial hyperplasia, which are highly influenced by ageing, hormones and parity [57, 61, 70]. Even if the age combined with parity has been related with endometrial degeneration, and that increased parity is clearly positively correlated with increased age, old maiden mares may display much more marked changes than expected for their age [17, 61]. Sports mares differ in regard to their endometrial pathology from equine populations of nearly exclusively non-sports mares, i. e. , sports mares have a much higher prevalence of endometrial periglandular fibrosis [78]. An irregular glandular differentiation may occur physiologically during the transitional cycles, but when these findings are seen during the breeding season, they represent a pathological alteration correlated with a permanent or temporary reduced fertility [78].

In mares susceptible to post-breeding endometritis, findings may differ depending on when the examination is performed [57]. Prior to the first breeding of the year, endometrial biopsy score may be a category IIA, with pathological findings of mild, focal, subacute inflammation with or without lymphangiectasia [57]. Bacteria and PMNs are usually absent. After repeated mating in a season, these mares accumulate intrauterine fluid and may have interstitial edema within the uterine wall after ovulation. At this time, the endometrial biopsy score may worsen to a category IIB, with the primary lesions of diffuse, moderate, subacute inflammation, lymphangiectasia, and moderate to severe edema [57]. Then, positive uterine culture and neutrophilic uterine cytology can be observed [17].

3. 10. Novel and old biomarkers for endometritis: myeloperoxidase and nitric oxide

Myeloperoxidase is a pro-oxidant enzyme stored in and released by neutrophils during degranulation or after lysis [59]. In horses, myeloperoxidase has been demonstrated in different biological samples, such as plasma and broncho-alveolar lavage fluid, reaching variable concentrations. Its presence has been recently confirmed in the uterine lumen, both in physiological conditions, like during estrus, and it seems to be related to uterine inflammation [59]. Samples were collected by low volume flush and stored in tubes with EDTA, since it prevents coagulation and consequently degranulation of neutrophils, thus stabilizing

myeloperoxidase concentration. It has been noted that its concentration is high in mares affected by endometritis, showing a positive correlation both with positive cytological results and with the presence of intrauterine fluid [59]. Further studies to establish the threshold between normal and pathologic uterine concentrations of myeloperoxidase are needed.

Another intriguing biomarker is NO, a smooth muscle relaxant able to compromise the uterine contractility and, consequently, its clearance [60]. A higher amount of NO and a higher NOS expression in uterine biopsies were found in susceptible mares 13 hours after insemination, compared with resistant ones [26]. Although it is not clear whether the higher NO concentration in susceptible mares is the cause or the result of a delayed uterine clearance, the difference between the susceptible and the resistant mares suggests a possible role for it either directly or through a NO-associated pathway [26].

4. Treatment of endometritis

The purpose of the treatment protocol is always to prepare the endometrium for embryo descent, which happens about 6 days after-mating, but the choice of the exact management depends on the specific etiological diagnosis [56]. The main objective is to resolve endometrial inflammation by restricting the bacterial contamination and by improving uterine physical clearance. This goal is reached through the control of post-mating inflammation length and degree, and/or through the identification and the elimination of the microorganism involved in the uterine infection [67]. Resolution of underlying problems is necessary to succeed in endometritis treatment.

Any anatomical defects, which may contribute to development of infections and impairs the post-insemination fluid drainage, should be corrected with surgery, e. g. , Caslick's vulvoplasty or urethral extension [79]. Traditional therapy to improve physical clearance of uterine fluid is uterine irrigation, associated to the administration of ecbolic, either oxytocin (10–25 UI i. v. or i. m.) or cloprostenol (250 µg i. m.), 4 to 8 hours after breeding [44, 80–86]. In case of infectious endometritis, the key to resolve the pathology is the antimicrobial therapy, either with systemic or intrauterine administration. Mucolytic irrigations have been recommended before the antimicrobial infusion, in order to eliminate bacterial biofilm and to improve drugs absorption by the uterine mucosa [17].

New treatment strategies are being developed, thanks to the growth of knowledge about persistent uterine infection pathophysiology. For example, immunomodulatory therapy has been proven effective in modulating the impaired uterine inflammatory response in susceptible mares. Different biological products such as heterologous or autologous plasma, platelet-rich plasma infusions, autologous conditioned serum and mesenchymal stem cells have been tested [87–92]. Corticoids and *Mycobacterium* cell wall extract or *Propionibacterium acnes* have been confirmed to be useful to treat endometritis [93]. At the end of the treatment, its efficacy should be controlled. A clinic examination should always be performed and culture swab and/or a biopsy sample should be collected at 3–4 weeks from the beginning of the treatment.

4. 1. Uterine flushing

The uterine lavage is helpful to remove debris, microbes, neutrophils and other substances that interfere with the mucosal absorption of antimicrobial and with neutrophils action. This also improves the clearance by stimulating uterine contractility. The lavage contrasts infectious agents by mechanical irritation of endometrium, which helps recruitment of neutrophils and opsonins [17].

The choice to treat or not a mare with uterine lavage relies on the presence of post-mating intrauterine fluid and on the degree of edema. An US examination is performed 4 to 8 hours post-mating: when no or little edema or slight accumulation of fluid is detected, the lavage is not performed. Otherwise, when the intrauterine fluid is more than 2 cm in depth, the mare is treated with immediate uterine irrigation [17]. To avoid the interference with conception, the best timing for uterine flushing is 4 hours post-breeding [94]. Thus, a further US examination is advisable 24 hours post-breeding to evaluate if additional uterine irrigations are needed. Whenever US results are impracticable, a preventive uterine lavage between 4 and 12 hours after breeding should be performed [47].

The procedure for a therapeutic uterine lavage is identical to that described for the low volume uterine flush, except for that a large-bore (e. g., 8 mm inner diameter × 11 mm outside diameter) Bivona catheter with a balloon cuff is used, and that a volume of 1 to 2 liters of warm solution is typically infused and then removed by gravity [95]. The warm uterine flushing should be combined with uterine massage per rectum to uniformly diffuse the fluid and to stimulate the myometrium contraction. Lavage is generally repeated until the effluent results are clear or not too turbid.

Uterine infusions are performed with isotonic saline solution or other balanced electrolytes solutions, such as lactated Ringer's solution. A low volume, 5–10 ml, of povidone-iodine solution can be added to 1 L of saline as preventive antimicrobial and antifungal treatment. This management is cost-effective and easy to be prepared, stored and delivered. However, it has been reported that intrauterine administration of 1% povidone-iodine solution during days 0 and 2 post-ovulation in healthy mares did not induce the histological changes of endometritis, but altered progesterone concentrations and reduced the expression of endometrial progesterone receptors, without affecting estrogen receptors. It was suggested that these changes could reduce embryo survival [96]. Ecboolics are administered as described to eliminate completely the infused fluids [17].

4. 2. Antibiotic treatment

Identifying and destroying bacterial infections might be the key to resolve some chronic reproductive problems. On the other hand, the decision to use antibiotics should rely on clear diagnostic evidences, which is not always the case. Hence, the key factor in treating endometritis with antibiotics is to confirm the presence of a true bacterial infection [97]. In endometritis, the infection is frequently limited to the endometrium and intrauterine infusion of antimicrobials is the most common approach treatment. However, the analysis of uterine biopsies showed that *Streptococcus zooepidemicus* was present deep within the endometrial tissue in

infertile mares [98]. In these cases, treatments limited to intra-uterine infusions may be unsuccessful, especially if the antibiotic did not achieve deep-tissue concentrations, adequate to kill the microorganism [97]. Infectious endometritis must be treated by intrauterine infusion, during estrus, with the appropriate antimicrobial administered daily for 3 to 7 days and after uterine irrigations, which eliminate organic material that may interfere with the antibiotic function. Intrauterine infusion volumes of antibiotics from 30 to 200 ml have been suggested to achieve distribution throughout the uterine lumen [99]. The infusion of small volumes is advisable, since larger volume infusions result in reflux through the cervix and inadequate distribution over the endometrium. Anyhow, the solution should be water-soluble and non-irritant.

Due to the increased spread of antimicrobial resistance, the choice of the antibiotic should be made considering the susceptibility pattern obtained from uterine culture [67]. Sometimes it is not possible to obtain a culture, either because the owners refuse the procedure or because there is no time to wait for results. In these cases, broad-spectrum antibiotics are the first choice to treat endometritis. Criteria for the choice are based on the consideration of the most common bacteria isolated from mares' reproductive tract: *S. equi* subsp. *zooepidemicus* (Gram positive), *E. coli* (Gram negative), *K. pneumoniae* (Gram negative), *P. aeruginosa* (Gram negative), *S. aureus* (Gram positive), and *Bacteroides* spp. (Gram negative, anaerobe) [19, 71, 100–103].

The effect of various antibiotics has been evaluated with controversial results. Gentamicin (1-2 g) or Amikacin (1-2 g) Gram-negative coverage have both been demonstrated to significantly reduce the activity of neutrophils [17]. On the basis of the results of an online survey of veterinarians concerning antibiotic use in equine reproduction, the most common antimicrobials used for intrauterine infusions in the mare before receiving culture/ antibiotic sensitivity results were ceftiofur (21%), followed by gentamicin (19%), ticarcillin with clavulanic acid (13%), ampicillin (12%), other (12%), procaine penicillin (5%), amikacin (5%), potassium penicillin (3%) and ticarcillin (3%). The category "other" included combination of penicillin and gentamicin (2%), penicillin and neomycin (2%), ampicillin and gentamicin (1%), oxytetracycline, framomycin, framycetin, cefquinome, cefazolin or chloramphenicol [97]. Dosages for antibiotics and antimicrotics commonly administered either systemically or through intrauterine irrigation to treat endometritis are summarized in Table 2.

Antibiotic intrauterine infusion in the estrus cycle may be realized either before or after breeding, preferably after ovulation is confirmed. The use of antimicrobial that may potentially have spermicidal properties on the day of insemination/mating generates controversial opinions. The uterine lavage or infusion is executed at least 4 hours after the insemination, so that the oviduct colonization with the sperm is not compromised. Then, 2 days after ovulation the progesterone levels increase, and the uterine defense mechanisms efficiency consequently decrease. For this reason, performing the infusion between 4 hours after insemination and 2 days after ovulation is considered to be harmless for the conception [104]. On the other hand, the antimicrobial uterine infusion in progesterone phase has been associated with increased incidence of resistant bacterial and fungal infection [105–106].

During acute endometritis, the time length of the antimicrobial treatment should depend on the degree of endometritis, according to endometrial biopsy. In slight endometritis 3 days,

in moderate one 5 days and in severe endometritis 7 days are necessary [99]. Concerns about inducing secondary fungal infections and/or antibiotic resistance are some of the reasons why the intrauterine antibiotic infusions have been reduced. Indeed, uterine lavages with saline associated to the use of ecbolics are now commonly accepted, and antibiotic therapies are more targeted at specific isolated organisms, in conjunction with methods to disrupt biofilms [97].

The combination of local and systemic antimicrobial treatment must be performed when clinical examination results in evident general illness, in case of infection or inflammation involving the deeper layers of the uterus, when repeated antimicrobial intrauterine infusions fail to cure, and when the recontamination is alarming. Systemic therapy during diestrus is preferable, since it is associated to lower fluctuation in antibiotic tissue levels and further contamination of the uterus should not happen. Trimethoprim-sulfa, ampicillin, penicillin and gentamicin are commonly used as systemic therapy. Oral administration of enrofloxacin at 5 mg/kg is recommended twice daily, to reach endometrial levels above the minimum inhibitory concentration of many endometritis-associated bacteria. In adequate doses repetition or drug interaction may create resistant microorganism and superinfections, such as yeasts or fungal overgrowth, which makes it particularly difficult to treat.

Molecule	Dosage (mg/kg)	Route	Intrauterine Dosage	Bacterial Susceptibility	Ref.
Amikacin	10 q24h	IV/IM	1–2 g	Gram negative	[128]
Ampicillin	29 q12–24h	IV/IM	1–3 g	Gram positive & negative, <i>E.coli</i>	[128]
Carbenicillin			2–6 g	Gram negative	[97]
Ceftiofur	2–4 q12–24h	IV/IM	1 g	Gram positive, some Gram negative, <i>S. zooepidemicus</i>	[128]
Doxycycline	10 q12h	OS		Broad-spectrum	[97]
Enrofloxacin	5.5 q24h 7.5 q24h 4.0 q12h	IV slow OS OS		Gram negative, Resistant bacteria	[128]
Gentamicin	6.6 q24h	IV/IM	1–3 g	Gram negative	[128]
Kanamycin			1–3 g	<i>E. coli</i>	[97]
Metronidazole	15–25 q24h	OS		Anaerobic Gram negative	[97]
Neomycin			2–4 g	Gram negative, <i>E. coli</i>	[97]
Oxytetracycline	6.6 q12h	IV slow			[97]
K-penicillin	22,000 IU/kg q6h	IV	5 × 10 ⁶ UI	Gram positive, <i>S. zooepidemicus</i>	[128]

Molecule	Dosage (mg/kg)	Route	Intrauterine Dosage	Bacterial Susceptibility	Ref.
Procaine penicillin	22,000 IU/kg q12h	IM	4.5–6 × 10 ⁶ UI	Gram positive	[128]
Ticarcillin			3–6 g	Gram positive, <i>Pseudomonas</i>	[97]
Ticarcillin + clavulanic acid			3–6 g /200 mg	Broad spectrum [<i>Staphylococcus</i> , <i>Enterobacter</i> , <i>Bacillus</i>]	[97]
Trimethoprim sulfa	30 q12h		OS	<i>S. aureus</i> , <i>E. coli</i> , <i>Klebsiella</i> spp., <i>Proteus</i> , some <i>Nocardia</i> spp.	[128]
Amphotericin B	0.3–0.9 q24–48h	IV	100–200 mg q24h	Broad spectrum	[128]
Clotrimazole			400–700 mg q24h	Yeast	[128]
Fluconazole	14 loading then 5 q24h	IV/OS	100 mg q24h	Yeast	[128]
Itraconazole	5 q12–24h	IV/OS		Broad spectrum	[128]
Ketokonazole	20 q12h	Via Nasogastric intubation in 1–2 h		Broad spectrum	[128]
Miconazole			500–700 mg q24h	Broad spectrum	[128]
Nystatin			0.5–2.5 × 10 ⁶ UI q24h	Yeast	[128]

Table 2. Most commonly used antibiotics and antimicrobics to treat equine endometritis, dosages for either systemic or intrauterine use, and bacterial susceptibility

4. 3. Mucolytics and chelating agents

Persistent endometritis could be associated with hypersecretion of mucus by endometrial epithelium and the presence of exudate renders aminoglycosides chemically inert and generally interferes with antibiotic penetration. Some Gram-negative and positive bacteria or fungi produce biofilm that confers antibiotic resistance [107–108]. The pathogens confirmed to produce biofilm include *S. epidermis*, *E. coli*, *E. cloacae* and the most potent biofilm producer *P. aeruginosa*.

Administration of mucolytic drugs is used to eliminate excessive mucus and exudates for the negative effect of the latter both on intrauterine antibiotics and on sperm transport to the oviduct. Despite controversial viewpoints, intrauterine effects of mucolytic oral administration have not been confirmed. However, it was stated that oral N-acetylcysteine (NAC) treatment, even if it does not reduce the viscosity of uterine mucus, has an anti-inflammatory effect on the equine endometrium [109]. Different kinds of intrauterine mucolytics are used and include NAC, dimethyl sulfoxide (DMSO) and kerosene.

The NAC is a mucolytic agent that disrupts disulfide bonds between mucin polymers, thereby reducing mucus viscosity, and in addition possesses antioxidant and possibly antimicrobial properties [110–112]. Furthermore, its properties help the sperm transport to the oviduct. Intrauterine post-breeding infusion of 3.3% solution of NAC did not result to be harmful to mares' endometrium, and it decreased the extracellular mucus thickness and staining intensity in healthy mares, but not in mares with active bacterial endometritis. The same NAC protocol applied in mares repeatedly bred and with a history of mucus hypersecretion, combined with post-mating uterine lavage, oxytocin, and infusion of an antibiotic, was correlated to good pregnancy rates [113]. The safety of NAC on mucosal surfaces is clear, based on its use as a mucolytic for respiratory disease in humans and treatment for meconium impaction in foals [113]. Interestingly, some recent findings demonstrate that intrauterine application of NAC in healthy mares in estrus may reduce the endometrial response to irritants [114]. The intrauterine administration of 0.6% NAC solution, prepared by adding 30 ml of 20% NAC to 150 ml of sterile saline, performed 48 hours before breeding, improves the pregnancy rates [110]. The effect of post-breeding intrauterine infusion of 30% DMSO solution showed to be effective in improving the endometrial biopsy score, and it led to higher pregnancy rates compared with mares infused with saline [115]. The improvement of the pregnancy rate, following the infusion of 50 ml of commercial kerosene, operates differently from the other mucolytics. The mechanisms hypothesized were that kerosene activates endometrial glands and reduces the mucus through the destruction of the uterine epithelium. A moderate to severe endometritis results from kerosene uterine administration, with severe edema and production of serum-like exudates. Half of the treated mares manifest necrosis of the endometrium [116].

Advanced strategies include uterine infusion of buffered chelating agents. Their mechanism of action is not completely clear, but they alter the integrity and permeability of the bacterial cell wall by chelation of the calcium and/or magnesium on the outer membrane. To explicate this action, the chelating agents have to be in indirect contact with the bacterial cell wall [67]. Recommended treatment is the infusion of 200 to 500 ml of the first-generation *Tris-EDTA* (ethylenediaminetetraacetic acid 3.5 M plus tromethamine 50 mM) or third-generation *Tricide* (8 mM disodium EDTA dehydrate plus 20 mM 2-amino-2-hydroxymethyl-1, 2-propanediol) [117–120]. After 12–24 hours, the uterus should be flushed to remove debris and accumulated dead cells. The efflux should be examined; and if it is cloudy or mucus is present, the chelating treatment should be repeated one more time [58].

4.4. Ecbolics

Uterine irrigation should be associated with systemic administration of ecboolic drugs, which increase clearance of the fluid through cervix, stimulate myometrial contraction and impair lymphatic drainage from the uterus. Oxytocin is an equine uterine ecboolic commonly utilized in doses ranging from 10 to 25 IU i. v. or i. m. Higher doses should be avoided, since it may impair pregnancy rates [86]. The administration of oxytocin, in estrus and 48 hours post-breeding, induces high-amplitude uterine contractions lasting from 30 minutes to 1 hour. However, oxytocin half-life is very short and repeated administrations enhance treatment effectiveness [81, 121–122].

These drugs may be ineffective in aged maiden mares in which the cervix often fails to dilate. In such cases, oxytocin therapy should be associated with topical application to the cervical epithelium of misoprostol, a synthetic PGE1 analogue, before breeding, or substituted by cloprostenol administration to sustain low-amplitude uterine contraction for 2 to 4 hours. Intramuscular administration of cloprostenol or PGF2 α at a dosage of 250 μ g is recommended, before the ovulation or within 12 hours. However, administration post-ovulation is not advisable, since it decreases serum progesterone concentration and consequently the pregnancy rates. The cloprostenol-induced contractions last longer; therefore, cloprostenol is more suitable for mares with lymphatic stasis. Side effects, such as sweating and abdominal cramping, are minimal, transient, inconstant and dose-dependent [123].

The best timing for ecbolic administration after uterine lavage associated to the antimicrobial infusion has not been established, yet. When treating anxious or excitable mares, sedatives with α 2-adrenergic agonist action, such as xylazine or detomidine, should be used. These sedatives optimize oxytocin effect and increase the intrauterine pressure, whereas acepromazine, α 1-adrenergic antagonist, can cause a reduction of uterine contractions after oxytocin treatment [47].

4. 5. Immunomodulators

Higher levels of anti-inflammatory cytokines were observed in resistant mares compared with susceptible ones. The rationale for using immunomodulators is to equilibrate the expression of pro- and anti-inflammatory cytokines in susceptible mares and to rehabilitate the homeostasis of the local inflammatory response.

Glucocorticoids have been demonstrated to suppress the immune response, decreasing gene expressions of the pro-inflammatory cytokines, IL-1 α , IL-6, IL-8, while enhancing defense mechanisms and stimulating a higher anti-inflammatory response. Thus, short-term steroid therapy may be beneficial for treating post-mating endometritis in the mare [124]. An increase of pregnancy rates after single injection of dexamethasone within 1 hour post-mating, approximately 0.1 mg/kg i.v., associated with routine therapy, has been demonstrated in mares with history of fluid accumulation after breeding. Oral administration of 9-alpha-prednisolone acetate at 0.1 mg/kg twice daily for 4 days, beginning 48 hours before breeding, and the association of dexamethasone, administered within 1 hour after mating, and prednisolone, administered once daily before and after mating, were confirmed to be efficacious only in susceptible mares, decreasing uterine edema, and reducing and clarifying intra-uterine fluid [125–126].

Other immunomodulatory treatments have been tested to elicit a general increase in immune system activity by means of non-specific cell-mediated response and modulating the release of cytokines [127]. *Mycobacterium phlei* wall extract and *Propionibacterium acnes* were reported to be efficient in uterine infection caused by *S. equi* and *S. zooepidemicus* and in chronic endometritis, respectively [17, 93]. In 2014, mesenchymal stem cells and autologous conditioned serum were used to modulate successfully the uterine inflammatory response to spermatozoa in healthy mares [90]. However, the former seems to be more reliable than the latter, and additional studies are ongoing to determine the effects of mesenchymal stem cells on

“problem” mares. Preliminary data on small volume intrauterine infusion of platelet-rich plasma, either before or after insemination, suggest an improvement in conception rate, probably due to a more appropriate uterine environment for embryo survival. Both the efficacy and the exact mechanisms of action of these treatments need further elucidation, but they seem to be triggered through activating platelets, cytokines and growth factors, and they seem to regulate cell migration, attachment, proliferation and differentiation, and to promote extracellular matrix accumulation [89, 91–92, 128].

5. Fungal endometritis

Whereas a rich literature describes the etiopathogenesis of equine microbial endometritis, few papers are dedicated to the role of fungi as responsible for uterine inflammation in the mare. The incidence of fungal infection in mares affected by endometritis is probably significantly lower than the incidence of bacterial infection, but fungal endometritis is more challenging to treat and is associated to a guarded-to-poor prognosis for future fertility [129]. Fungal growth was demonstrated in different rates, ranging from 1 to 7%, considering either the total number of reproductive cultures submitted to laboratories or the percent of cultures positive for fungal compared to the total number of mares affected by endometritis [130–134]. The term “fungi” is referred to heterotrophic, aerobic or facultative anaerobic, non-motile organisms widely distributed in the environment [132]. They could have characteristics of both yeasts, round to oblong single-cell organisms, and molds, that are long, branched, filamentous structures [135]. The filamentous cells are also named hyphae and are often organized in small masses, called mycelia, sometimes isolated in fluid from uterine lavage. Cytological and histological specimens may demonstrate both yeasts, most adapted to grow in liquids, and hyphae, better suited to penetrate deeper inside the endometrial tissue [136].

5. 1. Aetiology

The most common organisms isolated during equine fungal endometritis are *Candida* spp. , *Aspergillus* spp. and *Mucor* spp. [137]. However, many other organisms have been reported (Table 3). Recently, *Cladophialophora bantiana* has been isolated in a 15-year-old Standard bred mare that displayed infertility and uterine fluid accumulation with numerous black, hairy granules. *C. bantiana* is an emerging pathogen, causing cerebral phaeohyphomycosis in humans, while animal cases are rare [138]. The inflammatory mechanisms associated with these types of endometritis have not been clarified until now, and probably endometritis involving fungus or yeast will be feared as long as the inflammatory mechanisms associated will not be studied [134].

The most common source of fungi causing reproductive disease in the mare is probably from skin or fecal origin and the primary reservoir for infectious agents is the caudal reproductive tract, vagina and external genitalia [132]. Fungal elements that cause reproductive disease are generally opportunistic and their growth is considered to be secondary to underlying factors impairing the local humoral and, particularly, cell-mediated immunity. Mares with uterine

fungal infections may have predisposing conditions such as: anatomical defects, i. e. , an incompetent vulvar seal from multiple Caslick's procedure or trauma, or a tilted vulvar conformation; cervical trauma and inadequate closure of the cervix due to dystocia or fetotomy; immunosuppression; malnutrition; endocrine diseases; a history of antibiotic treatment; veterinary manipulations due to frequent artificial inseminations and intrauterine treatments [132, 137–139]. Even if fungal endometritis has been reported also in mares without the history of previous uterine therapies, this pathology has been more often documented in older multiparous mares, treated for bacterial endometritis in the past [136].

One of the most discussed predisposing factors is the prolonged use of antibiotics, which is believed to modify the normal flora of the caudal reproductive tract, disrupting the biological barriers and allowing yeast overgrowth. Alteration of the vaginal flora may also destroy organisms producing anti-fungal substances and/or competing for available nutrients, interfere with the local microbial synthesis of vitamins or change vaginal pH [132]. Intrauterine antibiotics reach the vagina when cleared by the increased myometrial tone or through passive leakage, affecting the vaginal flora and the fungal colonization. Repeated reproductive manipulations, such as mating, or inseminations, or infusions and irrigations during the estrus may transfer microorganism from the caudal reproductive tract into the uterus. In mares in which deficit of uterine clearance mechanisms exist, opportunistic fungi find an endometrial environment favorable to their proliferation [132]. Administration of progesterone may be another predisposing factor due to reduction of neutrophilic phagocytic ability, decreased myometrial activity and increased cervical tone. Furthermore, a moist environment, the exposure to large numbers of fungi and the presence of necrotic, ischemic or infected foci may contribute to the fungal infection [132]. Fungal endometritis due to *Cladophialophora bantiana* was diagnosed in an infertile mare with a history of dystocia, fetotomy and cervical laceration [138]. Nevertheless, subclinical fungal endometritis was also demonstrated in an 8-year-old Hanoverian mare at her first breeding, with no further reproductive available history [140].

Fungi have been isolated from urethral cultures (*Mucor* spp.), fresh semen (*Absidia* spp.) and extended semen (*Candida* spp.) from stallions [141]. The transmission of fungal infection through the natural coitus or artificial insemination has not been demonstrated until now, in the horse. However, in humans, there is some evidence that the male may harbor fungi as asymptomatic carriers. This can cause the re-infection of the sexual partner. Thus, when multiple mares are diagnosed with fungal endometritis, cultures of the stallion's penis, prepuce and semen should be performed [136].

5. 2. Diagnosis of fungal endometritis

History of mares affected may reveal the presence of predisposing factors to fungal infections. Frequently, mares are presented with a history of infertility and of repeated use of intrauterine diagnostic and therapeutic procedures [132]. Clinical signs range from subclinical infection to profuse vaginal discharge [138]. Sometimes, at the physical examination, a gray vulvar discharge is evident [132]. The presence of mycelia, as granules, in the fluid from uterine lavage is strongly indicative. The diagnosis of fungal endometritis relies on endometrial cytology, uterine culture or uterine biopsy [136]. Microscopic examination of uterine fluid or uterine

Fungi	Reference
<i>Actinomyces</i> spp.	[132]
<i>Aspergillus fumigatus</i>	
<i>Aspergillus</i> spp.	
<i>Aureobasidium pullulans</i>	
<i>Candida albicans</i>	
<i>Candida</i> spp.	
<i>Rhodotorula</i> spp.	
<i>Coccidioides immitis</i>	
<i>Trichosporon beigeli</i>	
<i>Fusarium</i> spp.	
<i>Hansenula</i> spp.	
<i>Monosporium</i> spp.	
<i>Mucor</i> spp.	
<i>Nocardia</i> spp.	
<i>Paecilomyces</i> spp.	
<i>Penicillium</i> spp.	
<i>Rhodotorula</i> spp.	
<i>Scedosporium apiospermum</i>	
<i>Saccharomyces cerevisiae</i>	
<i>Torulopsis candida</i>	
<i>Cryptococcus neoformans</i>	
<i>Trichisporon</i> spp.	
<i>Cladophialophora bantiana</i>	[138]

Table 3. Most common fungi isolated from the mares' uterus

swab cytology is an important diagnostic tool for fungal endometritis [138]. Cytological smears may show yeast or hyphae. Yeasts appear as 3 to 5 µm round-to oval single-cell organism and often have a capsule surrounding them that exclude dyes. This capsule is visible by altering the focal point when viewing the microscope slide and is considered a protective barrier against phagocytosis. Hyphae are less common and appear as subtle filamentous multicellular organisms [132, 136]. Yeasts are usually associated to superficial infections; on the contrary, hyphae are generally associated to deeper-seated fungal infections, requiring a longer treatment. Fungi should be always isolated and identified by cultures, to allow for antifungal drug sensitivities to be performed. They may be cultured initially on blood agar, and then passed on Sabaroud's agar [132]. Time for their growth is sensibly higher than bacteria and

the laboratory should be aware that a search for fungal organism is requested. Otherwise, after the routine time of observation for microbial growth, the culture could be deemed negative, because of the early reporting of the results [142]. A microculture system, Dermatobac[®], originally developed for the isolation of fungi in human medicine, was recently tested to evaluate its sensitivity in the diagnosis of equine fungal endometritis. Dermatobac[®] proved to be efficient, showing conclusive information after only 24 hours of culture [139]. A rapid, sensitive and repeatable quantitative polymerase chain reaction assay (qPCR) was also developed for the detection of fungal DNA from equine endometrial samples. The qPCR assay identified fungal DNA in samples from 12 of 17 mares suspected of having fungal endometritis and proved to be potentially useful as an adjunct to microbial culture and cytological examination in clinical situations to provide identification of fungal organisms in a timely manner [143]. The clitoral fossa and vaginal mucosa should be cultured in addition to the uterine lumen because they could be the reservoir for fungal organism, allowing recontamination after successful uterine treatments [132].

Equine fungal endometritis is usually accompanied by bacteria, among which *β-hemolytic Streptococci* group C and *E. coli* are the most common species. The underlying factors impairing the local immunity, which promote the fungal growth, may favor the microbial growth as well [138]. Especially if bacteria are in low number, they can be missed at the culture, confounding the diagnosis. In these cases, after the treatment with antifungal drugs, in the absence of other concurrent microorganisms, a bacterial endometritis develops, and infertility persists. It is also possible that the microbial infection has its origin in the alteration of the vaginal microflora due to the antifungal treatments. For these reasons, it is important for early identification of mixed fungal and bacterial infection at either the cultural or the cytological examinations [136]. Endometrial biopsy may also be used in the diagnosis of fungal endometritis, when cytology is inconclusive or to confirm the successful treatment of uterine infection. Fungal elements may be observed either adherent to the luminal surface, within endometrial gland lumens or within the endometrium [132].

5. 3. Therapy of fungal endometritis

Many difficulties can prevent a successful treatment of mares affected by fungal endometritis, due to invasive forms that are not exposed to intrauterine therapies, poor uterine clearance mechanism, inadequate immune response or to recontamination from posterior reproductive tract sources, mixed or chronic infections. Therapy for fungal endometritis is made of a combination of predisposing factors correction, uterine lavage and intrauterine infusion of antifungal agents [137]. Any defect in the physical barriers to uterine infection, at the level of perineum, vulva, vulvo-vaginal folds and cervix, must be surgically corrected whenever possible [136]. All the tissue of the reproductive tract that may harbor the fungal elements should be treated. These include the clitoris, clitoral fossa, vagina, cervix and uterus.

Large volume uterine lavage may be used with the aim to decrease the overall fungal number, increase the infiltration of inflammatory cells and the myometrial tone and expose organism to antifungal solutions. Some substances and drugs are usually added to saline, which may interfere with the fungal growth by means of different mechanisms. The use of DMSO is

reported at concentrations of 10–20%, because of its *in vitro* activity against the growth of *Candida albicans* [132]. A potential antifungal effect has been attributed to diluted iodine (0.05–1%) and to acetic acid (vinegar, 2% – 20 ml of white vinegar in 1 L of 0.9% saline) [136]. Intrauterine iodine should be used with caution, because of the danger of tissue damage and adhesions in particularly sensitive mare, even if it was demonstrated that a 1% povidone-iodine infusion during days 0 and 2 post ovulation in healthy mares did not induce histological changes compatible with endometritis [96]. Ecobolic drugs, such as oxytocin (10–20 IU intramuscularly or intravenously) or prostaglandins may be administered after the lavage to facilitate the expulsion of residual uterine fluid [136]. After the uterine lavage, the treatment should be completed with the intrauterine and/or systemic administration of an antifungal drug, ideally chosen on the basis of the results of cultural examination and susceptibility [136].

The two main categories of antifungal agents are the polyene and the imidazole drugs, which interfere with the formation of fungal ergosterol, resulting in an increased permeability of the cell wall and eventual cell death. The polyene antibiotics include amphotericin B, nystatin and natamycin, whereas clotrimazole, econazole, ketoconazole, fluconazole and itraconazole are the most used azole derivatives [132]. Clotrimazole, miconazole and ketoconazole have two nitrogen molecules; fluconazole and itraconazole are called triazoles because of the presence of three nitrogen atoms in an azole ring [137]. Common antifungal agents used in the treatment of fungal endometritis are reported in Table 2. Amphotericin B (100–200 mg), clotrimazole (300–600 mg) and miconazole (500–700 mg) are active against *Candida* spp., *Aspergillus* spp. and other dimorphic fungi, and are the antifungal drugs generally in use by intrauterine infusion. Nystatin (0.5–2.5 million UI) and fluconazole (100 mg) are active only against *Candida* spp. [97]. Although many antifungal agents are available for intrauterine therapy, few data are available about the disposition of antifungal drugs in endometrial tissue after oral administration. A recent study showed that the oral administration of fluconazole (14 mg/kg once, followed by 5 mg/kg every 24 hours) resulted in mean plasma and endometrial tissue levels above the minimal inhibiting concentration for *Candida Albicans* for the entire treatment period, without potential sequel. Thus, oral fluconazole can be considered in a multimodal and multidrug treatment protocol to treat fungal endometritis in the mare [137]. Knowledge of commonly encountered fungi infecting the mare's reproductive tract and their respective drug susceptibilities should improve treatment efficacy in mares with fungal endometritis, when culture results are not available or incomplete. A study reports that the spectrum of fungal isolates from uterine samples from mares with reproductive problems includes 99 to 100% of organisms susceptible to the polyenes, while response to the azoles varied from 47 to 81%. Yeast isolates were 100% susceptible to the polyenes and least susceptible to miconazole (48%) while isolates of mold with septated hyphae were most susceptible to natamycin (100%) and least susceptible to fluconazole (0%). Results from this study suggest that polyenes are effective against uterine fungal isolates *in vitro* and may be the empiric treatment of choice for fungal endometritis, when cultural and susceptibilities examination are lacking. Furthermore, resistance to specific azoles was also observed increasing over time [129].

The study about the potential use of chitin inhibitors for the treatment of fungal infections in the horse gave contradictory results. These substances have insecticidal properties due to their

ability to inhibit chitin synthesis, polymerization and deposition. It was supposed that they might interfere with the formation of new chitin in the cell wall, but have no effect on the fungal cell walls that have already formed. Thus, their potential effect may consist in the prevention of a new fungal growth and, thus, reinfection. Lufenuron is an insecticide chitin inhibitor that was evaluated as a treatment for endometrial fungal infections. Preliminary results were promising, and intrauterine lavages, performed with lufenuron suspended in sterile saline solution, were effective in eliminating fungal endometritis in four mares [144]. However, further studies did not confirm the efficacy against *Aspergillus* spp. or *Fusarium* spp. in vitro and indicated that very low concentrations in whole blood of horses were achieved after oral administration [145]. Further investigations about the efficacy of chitin inhibitors are necessary before they can be indicated for the treatment of fungal endometritis in the horse [136].

Another suggested protocol consists in morning lavage with 2% acetic acid solution followed by intrauterine irrigation with the specific antifungal antibiotic in the afternoon. The length of this therapy should be of 7–10 days. If uterine culture and cytology obtained in the first day of the next estrus are negative, the mare is bred with the minimum number of insemination and using sterile techniques and double-guarded pipette, to prevent accidental seeding with organisms from reservoir sources [136].

The prognosis for future fertility of mares affected by fungal endometritis is guarded to poor. The success of the therapy relies on the timely identification and treatment of delayed uterine clearance, poor perineal conformation, or fungal colonization of the more distal reproductive tract, the correct length of the treatment period, the appropriate choice and dosage of the antifungal [132]. The uterine biopsy for many of these mares indicates significant fibrosis, probably due to fungal infection, as well as to a previous bacterial infection and age-related changes. Once the fungal infection is resolved, a uterine biopsy may be advised to quantify the resulting uterine damage [136].

6. Conclusions

Endometritis is the butler of the equine reproduction, and it is the main suspect in all cases of impaired fertility of the mare. Every year the literature is enriched by new interesting reports about this subject, which throw even more light on the knowledge of its pathogenesis. The events occurring during the uterine inflammatory response are complex and intertwined. A thorough understanding of the physiological response to uterine contamination, which is necessarily correlated with the act of the insemination, may allow timely identification of susceptible mares, in which these mechanisms get jammed within the first hours after breeding. Type and dose of treatments, as well as timing of administration, should be correctly targeted towards susceptible and/or affected mares to optimize the results and reduce the cost and the resources used. Continued research is welcome in this area of equine medicine, so that treatment strategies can be further improved.

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