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MOLECULAR IDENTIFICATION AND SEQUENCING OF UTERINE BACTERIAL INFECTION ASSOCIATED WITH SUBFERTILITY PROBLEMS AND SUGGESTED TREATMENT PROTOCOLS IN ARABIAN MARES

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ABSTRACT

Reproduction failure in Arabian mares is mainly attributed to endometritis. To achieve successful solutions to endometritis, several diagnostic methods must be combined. 36 mares were diagnosed with endometritis, and 69.4% of mares scanned showed abnormal echogenicity and echotexture of endometrium by ultrasonography. This was confirmed by endometrial cytology via low-volume uterine flush, whereas 63.9% of smears contained more than 2–3 PMNs per HPF X100. Additionally, 86.1% of mares were positive for microbial culture. Sequencing of different isolates revealed that *Staphylococcus* was 44.4% more prevalent than *Brevundimonas* 27.8%, and *Alcaligenes* 27.8%. The mares were allocated to four groups; **G1** (n= 6) mares left without treatment as control. **G2** (KAN, n= 10) mares received three IM doses of kanamycin 10%. **G3** (DHS, n= 10) mares were given 3 intramuscular injections of dihydrostreptomycin. **G4** (PRF, n= 10) 30 mL autologous platelets rich fibrin (PRF), freshly prepared 6 to 10 minutes at 20-25°C, which was infused intrauterine within 6 hours after natural mating via one-way catheter to obtain its optimum therapeutic benefits. Twice mating was performed within 24-48 hours after ovulation induction of 30 mm follicles using 2500 IU of HCG, IV. A single fertile stallion per group was used. A noteworthy decrease in the endometrial thickness ($p<0.05$) between groups with an average of 8.35±0.32 mm, besides subsiding of the retained black anechoic intrauterine discharges. Moreover, a significant ($p<0.05$) enhancement in pregnancy percentage was noticed between the treated groups comparison versus the control. It could be concluded that bacterial infection can be involved in endometritis in Arabian mares, and also autologous PRF can be applied as an inexpensive substitute treatment.

***Keywords*:** Sequencing, ultrasonography, endometritis, echotextures.

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INTRODUCTION

The dilemma of equine endometritis is primarily classified as a major factor in infertility problems (Canisso *et al.*, 2020). There is a scientific consensus that endometritis in mares begins as a typical physiological process associated with an inflammatory response to breeding, involving immunological and mechanical reactions triggered by removing semen and pathogens from the uterus to provide a hospitable and fertile environment for successful conception (Christoffersen & Troedsson, 2017).

On the contrary, if the inflammatory response is not resolved within 48 hours after breeding, mares are at risk of developing persistent breeding-induced endometritis (PBIE) which can adversely affect their future productiveness (Canisso *et al.*, 2020). Indeed, several diagnostic methods will be accurate, rather than relying on one diagnostic method. So, Hurtgen (2006) reported that ultrasonography with endometrial cytology and uterine swabbing for microbial culture are collectively useful diagnostic tools of endometritis. Sure, ultrasonography can only provide a presumptive diagnosis of endometritis, based on the presence of ≥2 cm of retained anechoic intraluminal discharges throughout the oestrus phase (Brinkso *et al.*, 2003).

Additionally, these discharges can be detected via ultrasonography within 36 hours after breeding (LeBlanc, 2010), with an incidence rate range from 15% (Zent *et al.*, 1998) to 30% (Pycock & Newcombe, 1996). In addition to cytology by low-volume uterine flush, uterine swabbing of microbial examination, as well as cyto-brush for histological investigation (Katila, 2016) are recommended. The selection of appropriate antibiotics through sensitivity testing further enhances the accuracy and effectiveness of treatment (Maddox *et al.*, 2015).

A rapid and heightened production of pro-inflammatory cytokines including IL1β, IL-8, IFN-γ, IL1RA, and IL10 within the first 24h after mating. This is accompanied by the activation of polymorphonuclear cells (PMNs) which principally infiltrated into the uterus from 0.5 h post-breeding, and reach their peak within 4–8 h by enzymatic activities and bactericidal effects (Rebordao *et al.*, 2014). These cells engulf and remove pathogens, excess spermatozoa, and seminal plasma from the endometrium (Christoffersen & Troedsson, 2017).

On the other hand, low-volume uterine flush remains the most sensitive and accurate method to identify bacterial endometritis (Christoffersen *et al.*, 2015a). Whereas the retrieval of 5 PMNs per 100x field, and/or a bacterial monoculture, is confidently indicative of endometritis (Riddle *et al.*, 2007). Furthermore, uterine swabs prepared for uterine culture have revealed a wide variety of aerobic and anaerobic bacteria that gain access to the uterus through the relaxed gate of the cervix and uterus during foaling, postpartum period, natural mating and artificial insemination (AI) (Canisso *et al.*, 2016).

Recently, endometritis has been diagnosed using the genome sequencing method, which recognizes any shift in the predominant normal uterine microbiome and pathogenic bacteria (Heil *et al.*, 2018). Furthermore, New qPCR highly specified diagnostic tool which detects the innate immune genes such as equine β defensin1 (EBD1), lysozyme (LYZ) and secretory leukoprotease inhibitor (SLP1) in the endometrium of susceptible mares on the way to endometritis (Marth *et al.*, 2018a, b) It was evident that, the most frequently isolated microbes are Beta-haemolytic *Streptococci* (BHS), *Escherichia coli* (*E. coli*), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Tibary *et al.*, 2014).

Indeed, endometritis of mares was classified into acute, chronic, persistent post-mating,

and chronic degenerative forms based on the duration and severity of the infection (El-Shalofy *et al.,* 2021). It is noteworthy that treatment of endometritis based on enhancing endometrial clearance and myometrial contraction by short acting ecbolic as oxytocin administered after intrauterine lavage and local antibiotics (Scofield *et al.,* 2014) or PGF2α analogues with prolonged duration persisted up to 4h than oxytocin with adverse effects on progesterone secretion, which is necessary for maintaining pregnancy, and luteal function (LeBlanc & Causey, 2009).

Moreover, most bacterial pathogens of the endometrium should be covered by the bactericidal effect of broad-spectrum penicillin (LeBlanc, 2009), kanamycin, ampicillin, and also some quinolones (Mi *et al.,* 2016), dimethyl sulfoxide (DMSO) Solutions of 2.5–30% are bacteriostatic for *Staphy. aureus, E. coli, Strept.* spp. and most Gram-negative bacteria (Yahya *et al.,* 2018), acetyl-cysteine 3.3% solution (Mokhtari *et al.,* 2017), fluoroquinolone (Launay *et al.,* 2009), aminoglycosides (Albihn *et al.,* 2003), or tetracycline (Mateu and Martin, 2001). Recently, platelet-rich plasma (PRP) has been proposed as an effective remedy for endometritis due to locally increased concentrations of bioactive autologous growth factors. For example, platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-b), and vascular endothelial growth factor, accelerate the healing and regeneration of compromised tissues when infused locally (Thomopoulos *et al.,* 2005).

Unfortunately, PRP has a relatively short biological half-life and rapid degradation of most growth factors, and is not effective alone (O'Connell *et al.,* 2008). From this point, platelets-rich fibrin can effectively overcome the disadvantages of PRP. It is noteworthy that platelets-rich fibrin (PRF) not only acts as a reservoir of growth factors and cytokines, but also provides sustained release of high concentrations of growth factors over an extended period to promote cell migration (Breen *et al.,* 2009). With promising future in the modulation of an inflammatory process, attributed to its rich contents of platelets, leukocytes, cytokines, stem cells, and numerous growth factors such as PDGF, TGF, IGF, EGF, VEGF, interleukins-1ß, 4 and 6 and adhesion factors (Pavlovic *et al.,* 2021). These factors are slowly released with effective healing properties for up to 15 days, with negligible minimal immunological reaction (Yu Ding *et al.,* 2020).

This investigation aims to clarify the isolation, identification and sequencing of bacteria associated with endometritis in Arabian mares, as well as the efficiency of different investigative approaches, as well as the roles of systemic antibiotics and autologous platelets-rich fibrin in managing equine endometritis and improving reproductive capabilities of Arabian mares.

MATERIALS AND METHODS

1. Animals

This study was conducted on 36 sub-fertile Arabian Mares aged 10 to 13 years, with a body condition score (BCS) ranging from 5 to 8. Mares were raised on three exclusive stud farms in Smouha and Abyss Zone, Alexandria governorate, Egypt, from March 2021 to December 2022. All mare care and experimental steps complied with animal use and care guidelines, which were ethically approved by Institutional Animal Care and Use Committees at the Pharmaceutical & Fermentation Industries Development Center of the General Authority of the City of Scientific Research and Technology Applications (SRTA-City) IACUCs # 87-2C-0823, Egypt. Every mare was housed and cared for using the same diet and management practices. The animals were kept in separate stables with beds on the floors and self-serve water sources.

2. Clinical and Reproductive Diagnosis

2.1. Ultrasonographic scanning and monitoring of Mare's genitalia

Mares secured by a head collar without sedation, faces completely evacuated. Scanning and monitoring of Mare's genital tracts within 48 h after breeding (LeBlanc 2010) by a real-time B-mode ultra-sonography (Sonoscape Co. Ltd., model M12, China). A fully lubricated trans-rectal linear array transducer (5-7.5 MHz) was used, to detect the echogenicity of retained anechoic discharges, as well as the echotexture or endometrial oedema pattern (thickness). Moreover, further scanning to assess treatment response (LeBlanc & Causey, 2009).

2.2 Uterine flushing techniques for cytological examination

Low-volume uterine flushing was performed to obtain endometrial samples for cytology. Approximately 60 mL of normal saline solution 0.9% was infused intrauterine, then recollected using a sterile disposable Rail's tube (ULTRA MED Rail's tube 14 FG, Egypt). Fixation and staining of the retrieved endometrial smear by Leishman's stain, and finally, examined under the oil immersion (1000 x) by light microscope (LeBlanc *et al.*, 2007).

2.3 Uterine swabs and sampling for microbial identification

The external genitalia and perineum of mares were washed with warm water and soap, and then disinfected by povidone iodine 1% solution. A double glove-guarded occluded swabs were introduced intrauterine via the cervical opening to avoid contamination by vaginal flora (Barbary *et al.*, 2016). The obtained uterine swabs were sent to the laboratory for bacterial isolation, identification and antibiotic sensitivity. The protected intrauterine samples were quickly inoculated into nutrient broth media at 37°C for 24 h. Then, a specific selective media was prepared for the isolation of salmonellosis. Afterward, the incubated broth samples were inoculated into Selenite-F-broth tubes at 37°C for 14–18 h. The culturing process was done by a special loop from the incubated nutrient broth tubes to be streaked onto plates of different media: nutrient agar, sheep blood agar, MacConkey agar, and mannitol salt agar. While for *Salmonella*, the incubated Selenite-F broth was streaked onto *Salmonella Shigella* agar and Brilliant green agar media. The inoculated plates were incubated at 37°C for 24– 48 h. The incubated plates were examined for colonial morphology, haemolysis on blood agar, and pigment production on nutrient agar, as well as characteristics on *Salmonella Shigella* agar, Brilliant green agar, and Mannitol salt agar. The developed colonies were picked up and delivered into nutrient agar slopes, then incubated at 37°C for 24 h for identification (Barbary *et al.*, 2016).

2.4 Bacterial isolates identification

The bacterial isolates were identified through stained by Gram stains and microscopic examination, followed by biochemical tests. The biochemical tests for Gram-negative bacteria were performed. Consequently, cytochrome oxidase, catalase, indole, methyl red test, Voges Proskauer test, citrate utilization test, detection of H_2S production, sugar fermentation test (for glucose, lactose, sucrose, dulcitol, sorbitol, arabinose, rhamnose, and xylose), motility test, lysine, and ornithine decarboxylation and Arginine Di hydrolase test, urease test, gelatin liquefaction test, and nitrate reduction test. Moreover, the Gram-positive cocci were microscopically examined for cell arrangement. Because some cells tend to form tetrads or grape-like clusters. In addition, the *staphylococci* were differentiated from certain species of *streptococci* by further testing, such as the Catalase test, Coagulase test, and haemolysis on blood agar (Estée Töröka, and Nick Day, 2005).

2.5 Molecular identification of bacterial isolates

Genomic DNA was extracted from overnight cultures using a DNA Extraction Kit (Shawki, 2024). PCR amplification of the 16S rDNA genes was performed with a Biometric PCR Thermocycler using 0.1µg

DNA as a template, and the 16S universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GATTACCTTGTTACGACTT) (Wang *et al.*, 2007).

The PCR conditions consisted of 30 cycles, including an initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing at 55°C for 1 min, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. To categorize the bacterial isolates, the 16S rDNA-RFLP (Restriction fragment length polymorphism) technique was employed. Ten microliters of the amplified 16S rDNA (~1500 bp) were digested at 37°C for 5 minutes with 2 units of Hinc II fast digest restriction enzyme (Thermo-Scientific).

The resulting fragments were separated via electrophoresis on 2% agarose gels (FMC, Rockland, USA) containing 0.1 µg/ml Ethidium bromide. The gels were run at 100 V in 1X TBE buffer and then visualized and photographed in the multi-image light cabinet. A100 bp (base pair) ladder mix (Fermentase) was used as the molecular weight marker. Sequencing was performed using the ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer, Foster City, Calif.). Sequences were analyzed using the nBLAST program (National Centre for Biotechnology Information) to identify similarities and the database matches. Selected rDNA sequences were aligned using Clustal W software with the neighbour-joining method (Shingler 1996). and a phylogenetic tree was displayed using Tree View (Saitou and Nei 1987).

3. Antibiotic sensitivity test

According to Forbes *et al.* (2007), the antibiotic discs were tested, in order to select the most effective antibiotics and bacterial susceptibility (Table 3).

4. Processing of mare's autologous platelets rich fibrin (PRF)

PRF extracted according to (Choukroun *et al.*, 2006), about 100 ml of mare's venous blood was collected by 23-gauge needle and centrifuged in 10 ml sterile tubes without anticoagulant at 3000 rpm for 10 min. The centrifuged tubes were demarcated into three layers. Both upper and lower layers were discarded, meanwhile the middle layer contained a PRF clot only collected, and the harvested fibrin layer detached from the tubes. Finally, PRF prepared according to (Shashank & Bhushan, 2020), middle layer PRF obtained in a membrane form and then squeezed by a sterile gauze to extract its fluid loaded with cytokines and therapeutic growth factors from the fibrin clot, and mandatory uses within 6 to 10 minutes to obtain the optimum therapeutic benefits of PRF, avoid diffuse fibrin polymerization, or altered consistency.

5 Experimental design and treatment of mares:

Treatment commenced with uterine lavage during the oestrus period for three successive days, on the 1st, 2nd and 3rd days. Two liters of worm (40-45 °C) isotonic saline solution (El-Fath for drugs and cosmetics industry, New Borg El-Arab City, Alexandria, Egypt) were administered intrauterine via a large-bore catheter by gravity-driven installation, then removed (Leekha *et al.*, 2011). Then post lavage mares received 2.5 ml (25 IU) Oxytocin® (Oxytocin 10 I.U., ADWIA Co. S.A.E. 10th Ramadan, Egypt), as short-acting ecbolic to facilitate uterine evacuation (Stephen *et al.*, 2019). Then, all mares were allocated into four main groups based on diagnostic methods: endometrial swabbing, bacterio-logical examination, ultrasonography, and cytology.

Group I (n= 6):

Mares were left without treatment to serve as a control group.

Group II (KAN, n = 10):

Mares were treated during the oestrus phase with three successive intramuscular doses of Kanamycin 10% ® (12gm Kanamycin

Sulphate Equivalent to 10gm Kanamycin base) (ADWIA Co. S.A.E. 10th of Ramadan, Egypt) based on sensitivity testing**.**

Group III (STR, n = 10):
Mares were treated during the oestrus phase with three successive intramuscular doses of streptomycin® 10ml/100 kg.b.w (Dihydro-streptomycin sulphate 250 mg,) Alfas an international B.V. Kuipersweg 9,3449 JA, Woerden., The Netherlands. based on sensitivity testing.

Group IV (PRF, n =10):
Mares received 30 mL of freshly prepared autologous platelets rich fibrin (PRF) within (6 to 10) minutes of preparation to maximize its therapeutic benefits. Additionally, PRF was infused intrauterine via a one-way catheter at 20–25°C, within 6 hours after natural mating, to avoid disrupting spermatozoa colonization and its propagation in the female genitalia (Segabinazzi *et al.*, 2017).

6. Breeding of mares and pregnancy diagnosis:
The clinical signs and behaviour of mares during oestrus were carefully observed daily, by teasing them by known fertile stallions. Concurrently, ovarian follicular growth was monitored using ultrasono-graphy, on arriving the developing follicles up to 30 mm in diameter, ovulation induced by the administration of 2500 IU of HCG intravenously (Pregnyl, ® Misr Company, Cairo, Egypt). The natural mating was conducted twice within 24–48 hours after ovulation induction by known fertility stallions (one stallion per group). Additionally, all inseminated Mares underwent ultrasonography 24–48 hours after breeding to confirm ovulation. By the 20th day of natural breeding, pregnancy diagnosis via trans-rectal ultrasonography will detect the presence of amniotic vesicle.

8. Statistical analysis
The obtained numerical data of endometrium thickness were statistically analyzed using PROC GLM of SAS (2004) to test the effect of treatment and time (before and after) and the interaction between them. Differences among means were calculated. Also, conception rates from 1st and 2nd service and pregnancy rates were analyzed as binomial traits using a logistical regression model.

RESULTS

1. Bacterial sequencing and mare's reproductive performance
Based on RFLP results, five different groups were generated. The sequence analysis was carried out of an isolates from each group for 16S rDNA genes. (Fig. 1). *Alcaligenes* species, *Staphylococcus* species, and *Brevundimonas* species are the most common prevalent bacteria in percentages of 56.25, 37.5 and 6.25%, respectively (Fig. 1 & 2). And deposited in Gene Bank as Strain 7F mac OM 949994, 25 F mac OM 913617, 23 F mac OM 913900, and 29 F mac 912502. Moreover, PRF showed a positive impact on reproductive parameters. Endometritis must be confirmed by more than one diagnostic method. The phylogenetic tree (Fig. 2) revealed and classified the bacterial isolates according to prevalence. Moreover, Ultrasonography showed the changes that had already occurred in the uterine echogenicity and echotexture (Fig. 3 & 4), a significant variation ($P<0.05$) of endometrial thickness.

2. Incidence and prevalence of endometritis in mares by different diagnostic methods
Ultrasonographic scanning of mares (Fig. 3) monitored that 25/36 (69.4%) of mares had intraluminal discharges indicative of Endometritis. Moreover, 23/36 (63.9%) and 31/36 (86.1%) of Mares were positive for endometritis by endometrium cytology and bacterial isolation, respectively, (Table 1).

The percentage of isolated bacterial strains from mares with endometritis is shown in Table (2).

The presence of 1-2 neutrophils per magnification in 100 high-power fields

HPF (Fig. 5) confirms endometritis.

The bacterial isolates showed a different intensity response to antibiotics. Moreover, the majority of bacterial isolates (*Staphylococcus* species, *Brevundimonas* species, and *Alcaligenes* species) were sensitive to kanamycin and dihydrostreptomycin (Table 3).

It was clear that both PRF and Kanamycin achieved lowered values of endometrial thickness (6.91±0.32mm) (7.56±0.49 mm) on arrangement than Dihydrostreptomycin (8.24±0.57mm) and control (10.70±0.39 mm) (Table 3). Moreover, PRF exerted an impressive effect on endometrial thickness, conception, and pregnancy rates identical to the results of antimicrobials.

Table 1: Incidence and prevalence of endometritis diagnosed by different methods

Method of diagnosis	sensitive mares	percentage of endometritis
ultrasonography	25/36	%69.4
cytology	23/36	%63.9
microbial culture	31/36	%86.1

Table 2: Percentage of isolated bacterial strains from mares with endometritis.

Closest bacteria	Percentage
Brevundimonas diminuta	44.4%
Staphylococcus equorum	27.8%
Alcaligenes sp.	27.8%

Table 3: Sensitivity of different bacterial isolates (strains) to antibiotics.

antibiotics	Bio disc code	strains				
		5FA	7Fmc	23Fmc	25Fmc	29Fmc
Kanamycin	K30	0.6	2.4	1.8	0.9	2.3
Dihydrostreptomycin	S10	1.3	1.7	1.1	4.4	1.7
Amoxi-clavulanic acid	AMC30	-ve.	-ve.	1.4	-ve.	1.8
Penicillin	P10	-ve	-ve	1.1	1.3	1.3
Cefotaxime	CTX30	-ve.	1.9	-ve.	-ve.	- ve.
Chloramphenicol	C30	-ve	-ve	-ve	2.4	1.3
Ceftriaxone sodium	Cro3	-ve	1.7	1.7	-ve	2.1
Enrofloxacin	ENR	-ve	2.2	-ve	2.3	-ve
Aztreonam	ATm10	-ve	2.1	-ve	-ve	1.3

*Each value expresses the average of three experiments.
* -ve means negative results.

Table 4: Efficacy of treatment protocols on the endometrium oedema or thickness (mm) of treated Mares.

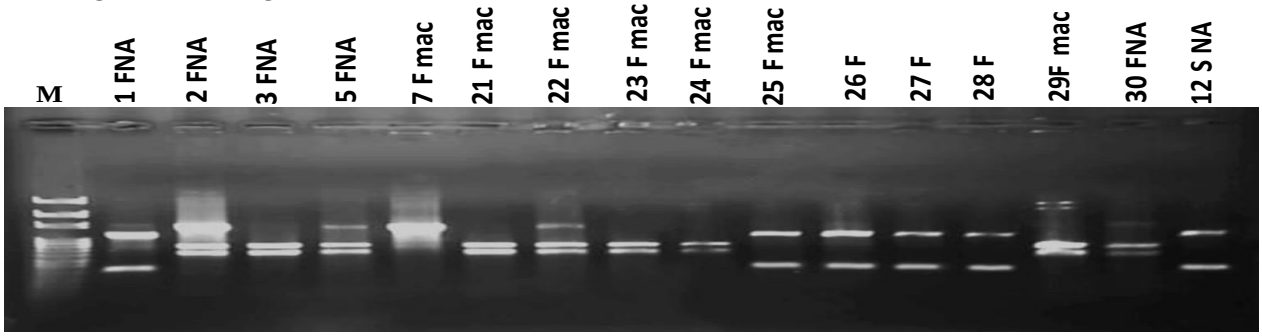
Treatment	Before	After
Control	12.00 ± 0.54^{Aa}	10.70 ± 0.39^{Ab}
Kanamycin	11.17 ± 0.47^{Aa}	7.56 ± 0.49^{BCb}
Dihydrostreptomycin.	12.08 ± 0.66^{Aa}	8.24 ± 0.57^{Bb}
PRF	12.00 ± 0.65^{Aa}	6.91 ± 0.32^{Cb}
Average	11.81 ± 0.29^{a}	8.35 ± 0.32^{b}

$^{A-C}$ Means within the same column with different superscript capital letters are different ($P<0.05$)
$^{a-b}$ Means within the same row with different superscript small litters are different ($P<0.05$).

Table 5: Efficiency of sensitive antibiotics and platelets Rich Fibrin (PRF) on reproductive performance parameters of treated Mares.

Treatment	Conception rate		Pregnancy rate
	1st Service	2nd Service	
Control	10	0	10^{c}
Kanamycin	20	13	30^{bc}
Dihydrostreptomycin	40	33	60^{ab}
PRF	60	50	80^{a}
Chi-Square	5.797^{ns}	1.806^{ns}	9.276^{**}

^{ns} not significant, ** Significant at $P = 0.025$



Groups							Closest bacteria
G 1	7 F mac						*B. diminuta*
G 2	1 FNA	25 F mac	26F	27 F	28F	12 SNA	*S. equorum*
G 3	3 FNA	21 F mac	22 F mac	30FNA			*Alcaligenes sp.*
G 4	29F mac						
G 5	3 FNA	21 F mac	23 F mac	24 F mac			

Fig. 1: RFLP profiles of 16S rDNA genes cut with restriction enzyme *HincII* of 16 isolates, respectively. LaneM is a 100 bp DNA ladder

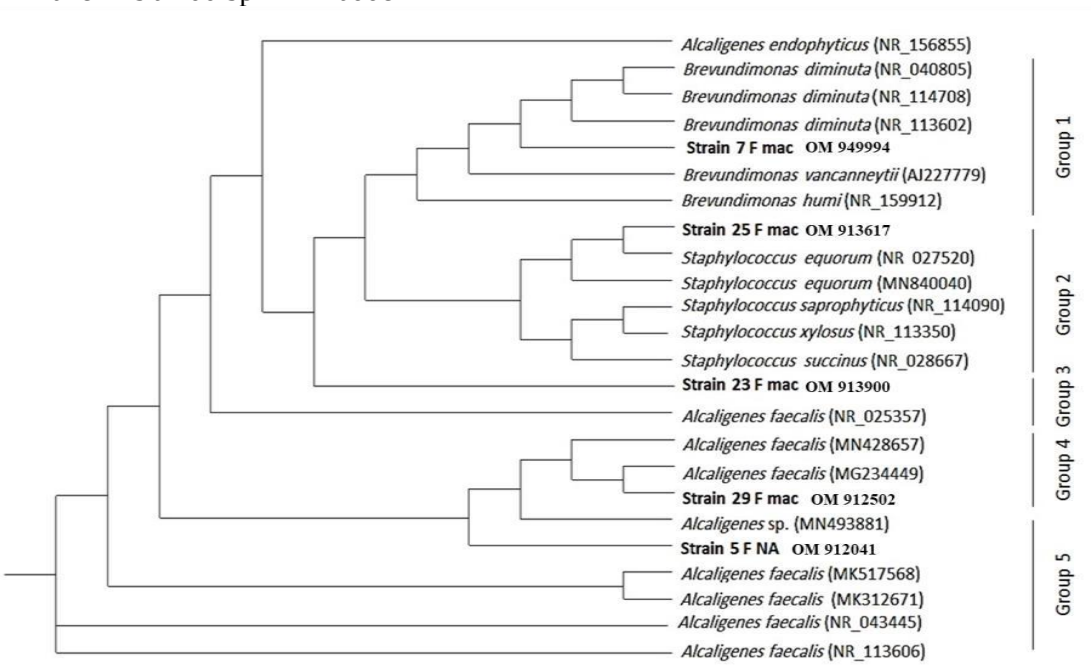


Figure 2: The phylogenetic tree; shows the relationships between five selected isolates out of 16SrDNA-RFLP analysis profiles (in boldface), and their accession numbers in GenBank.

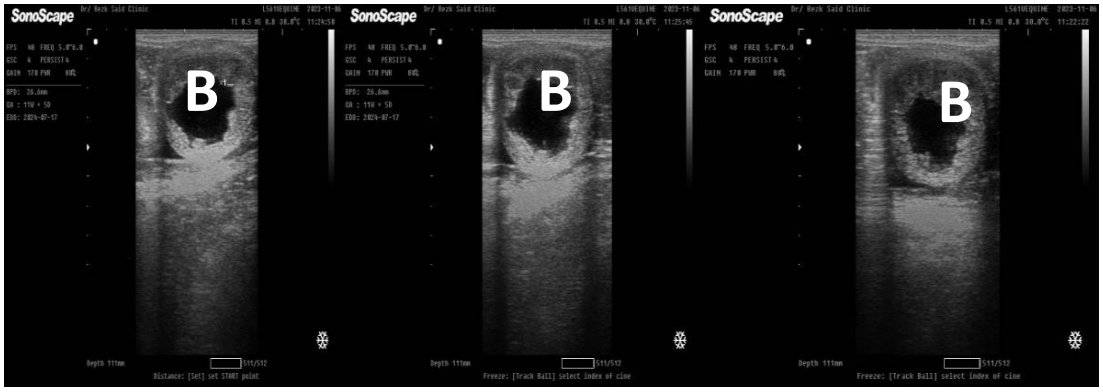


Figure 3: Presumptive diagnosis and ultrasonographic scanning of endometritis in mares.
A- refers to anechoic retained intrauterine discharges with different degrees of echogenicity according to the nature of discharges.
B- refers to increased endometrial echotexture (thickness) or abnormal oedema pattern due to endometritis of the mare.

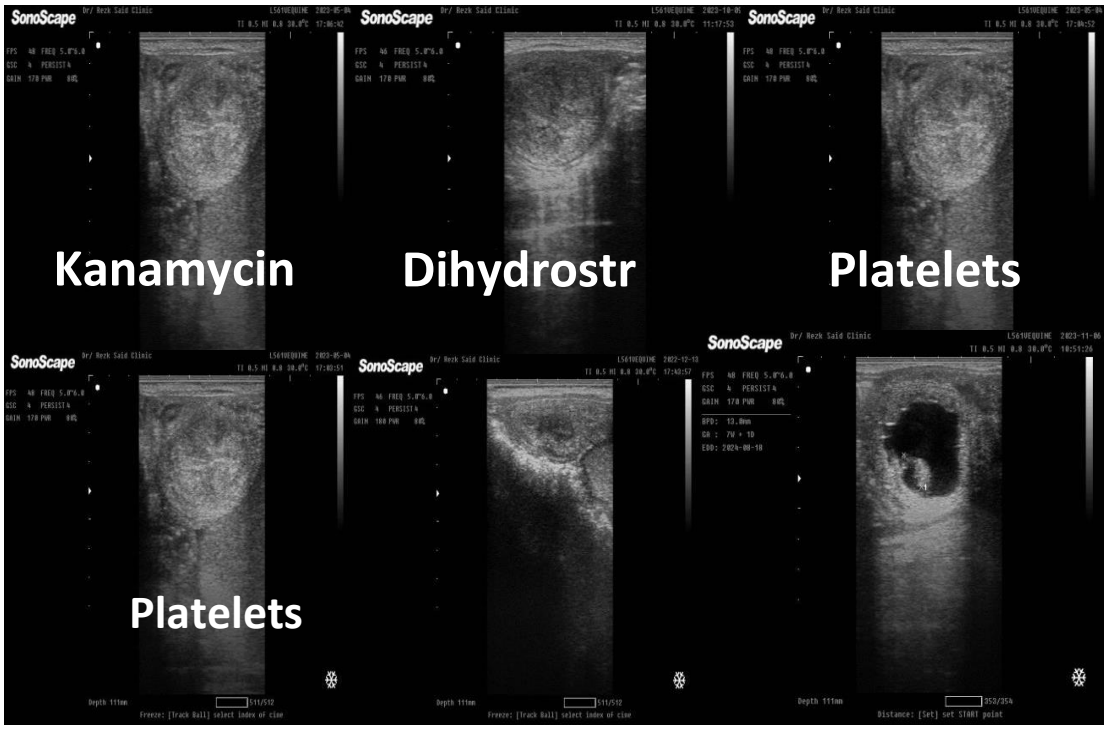


Figure 4: Ultrasonography scanning of uterine architectures and echogenicity revealed subsiding of retained intrauterine discharges after treatment

DISCUSSION

Endometritis of Mares is one of the most important causes of conception failure and great economic losses. In the present study, the incidence of endometritis is 30 to 35%, similar to 25 to 60% (Samper, 2006), and a higher incidence of up to 70% (Troedsson, 1999). This may be attributed to virulence and type of pathogen. It is advisable to apply several diagnostic methods, rather than relying on one diagnostic tool to confirm endometritis of mares, such as ultrasonography, endometrial cytology by flushing endometrium, intraluminal swapping for bacterial isolation, identification, and bacterial sequencing (Traub-Dargatz *et al.*, 1991). So, the principles of diagnosis applied in this study including ultrasonography, endometrium

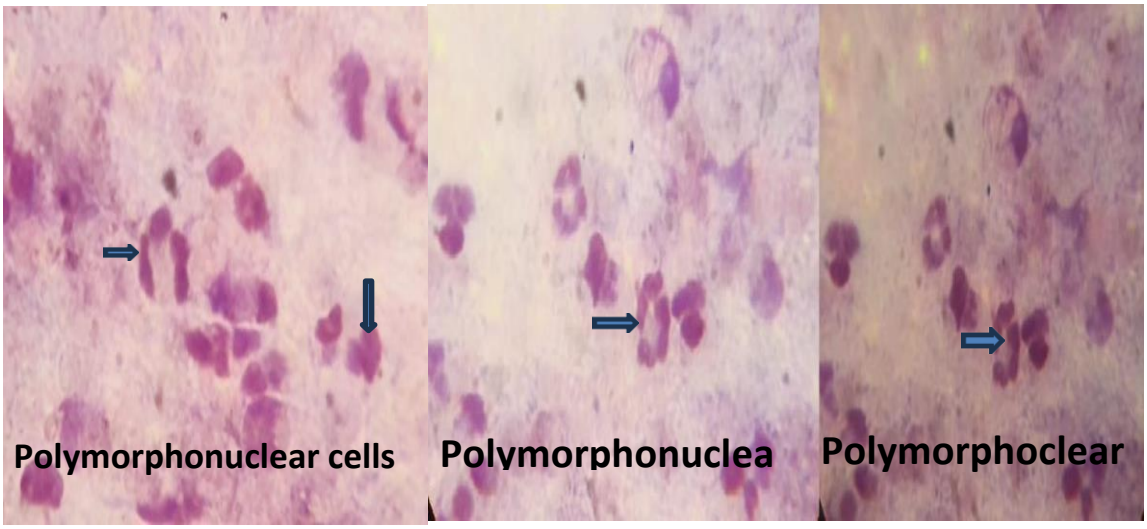


Figure 5: Photomicrograph of uterine flush, for endometrial cytology, the smear showing the presence of polymorphonuclear cells (PMNs) neutrophils Leishman's stain (×100) HPF field.

cytology and swapping techniques. These confirm endometritis existence in 69.4%, 63.9% and 86.1% of arrangements (Table 1), which is consistent with Ghallab *et al.*, (2023). Whereas the same diagnostic tools for endometritis showed 61.5%, 47.7%, and 92.3%, respectively. Actually, 69.4%, of mares in the present study positively monitored retained intrauterine discharges of endometritis and abnormal patterns of the endometrium. Unfortunately, ultra-sonography provides only a presumptive diagnosis of endometritis. Furthermore, 44-55% of scanned mares contain intrauterine exudates (Burleson *et al.*, 2010), 39%, and 65.4% (Pycock and Newcombe, 1996), which were similar to 69.4% in the present study. Furthermore, these retained intrauterine discharges of endometritis can be observed during the oestrus phase, and only 3.8% of mares retained intraluminal discharges during the dioestrus phase and associated with prolonged infertility troubles and diminished luteal phase Brinsko *et al.* (1991). These results are consistent with the detection of anechoic intrauterine exudate post-mating (Fig.3), which clarifies the accumulation of intrauterine discharges with different degrees of echogenicity, and echotextures according to the nature of these fluids, which appear as a black anechoic image with intra-uterine hyperechoic dots. Also, abnormal echotexture or oedema patterns of endometrium were increased according to duration, the severity of inflammation, and the type of micro-organisms agreed with the hypothesis of (Kahn, 1994, and Newcombe1998).

Contrary to previous concepts, ultrasonographic scanning is a helpful guide to detect uterine wall echotexture and echogenicity of uterine exudates during oestrus phase, post-mating and during the luteal phase. But it is not a prerequisite for the accumulation of uterine fluids to be conclusive evidence of the occurrence of endometritis, because subclinical cases were diagnosed without intraluminal retention of discharges (Troedsson, 2000). On this basis, ultrasonography should be supported by endometrial cytology, to detect cases of subclinical endometritis that aren't associated with accumulated uterine discharges.

In fact, the endometrial cytology displays the neutrophilic activity index or polymorphonuclear cells count (PMNs) (Fig.5), which is a reliable and confirmatory diagnostic method of

endometritis (Burleson *et al.,* 2010). It was prominent in the present study that 63.9% of mares were positive for endometrial cytology (Table1) and agreed with 60% (Nielsen, 2005, and Rebordao *et al.,* 2014). Furthermore, PMN facilitates uterine clearance by complement activation, chemotaxis and phagocytosis of uterine pathogens (Troedsson & Woodward, 2016). Furthermore, the endometrial cytology of a uterine specimen is a highly efficient diagnostic test, and more sensitive than microbiological investigation, to detect the number of polymorphonuclear leukocytes (PMNs) proportional to other endometrial cells. However, bacterial isolates don't fundamentally assure the existence of endometritis. (Riddle *et al.,* 2007, and LeBlanc, 2010).

In the present study, 86.1% of uterine swabs were positive for bacterial existence, which was consistent with 67% of positive swabs (Ibrahim *et al.,* 2015), 39.1% (Szeredi *et al.,* 2003) and 92.3% (Riddle 2007, LeBlanc & Causey 2009). Whereas, there is a significant relationship between bacterial infection and endometritis of mares. Contrary to the present findings, Nielsen (2005) found that uterine swabbing gives negative results and is less accurate than uterine biopsies, and supported by the results of Brinsko *et al.,* (2011), who stated that the presence of microbial isolates is not necessarily associated with endometritis of mares.

Concerning this study, a wide diversity of bacteria isolated from the uterine lumen with a variable ratio, including *Staphylococcus species* 44.4%, representing the main source of intrauterine discharges and more prevalent than *Brevundimonas species* 27.8%, *Alcaligenes* 27.8% species, respectively (Fig. 1 and 2, Table 2). These results agreed with (Frontoso *et al.,* 2008; Barbary*et al.,* 2016). Also, Derbal *et al.* (2018) found that the majority of endometritis cases of mares are due to infection by *Staphylococcus spp*. 35.1%. On the contrary, (Allen & Pycock 1989, and Asbury & Lyle, 1993) concluded that *S. aureus* was the least common cause of the usual isolation of *Brevundimonas* species in the present study, following previous findings (Ghasemzadeh-Nava *et al.,* 2004; Frontoso *et al.,* 2008; Barbary *et al.,* 2016). Also, *Alcaligenes* pathogens isolated and sequenced in the present study represent 27.8% and were matched with results of (Troedsson, 2004; Le Blanc *et al.,* 2007, and Frontoso *et al.,* 2008). However, some cases of endometritis show negative microbial culture (Brinsko *et al.,* 2011).

It is noteworthy that there is a strong relationship between bacterial isolates and the existence of intrauterine discharges. Moreover, not all types of uterine pathogens produce uterine discharges. Whereas Staphylococcus is mainly associated with clinical endometritis and intrauterine fluid accumulation (Burleson *et al.,* 2010).

Clinically, the priorities must be directed to know the actual and predisposing factors of endometritis, as well as preventing the progression of post-breeding physiological inflammation into pathological type. This can be avoided by optimizing time of treatment as early as possible to overcome the pathological endometritis. This include a combination of ecbolic and uterine lavage within 48 hrs of breeding, to enhance physical elimination of inflammatory debris and/or micro-organisms (LeBlanc & Causey, 2009; Rose *et al.,* 2018). Also, antibacterial administration (Pycock & Newcombe, 1996, and Rose *et al.,* 2018), and immunomodulatory therapies (Woodward *et al.,* 2015). In the same context, the principles of (Knutti *et al.,* 2000; Pycock,2009), were taken as a guide in the present study, 25 IU Oxytocin administered directly after uterine lavage during oestrus phase due to higher active

oxytocin receptors gene expression in myometrium muscles compared to luteal phase (Annandale *et al.,* 2018) to provide high frequency and amplitude of myometrium contractions for 30 min. However, this effect still presents for 48 hrs after ovulation (LeBlanc & Causey, 2009).

To evacuate uterine discharges during oestrus, with 2 to 3 liters of warmed (42° to 45° C) saline solution until the fluid recovered is clear for 2 to 3 days before application of recommended systemic or local antibiotics, according to a sensitivity test (Table 3). It was evident that uterine lavage is recommended to get rid of retained intrauterine discharges using sterile saline or lactated Ringer's solution (LeBlanc & Causey, 2009). Moreover, the appropriate timing of uterine lavage, neither performed immediately before breeding (Vanderwall & Woods, 2003) nor at 0.5 or two hours after mating, decreases pregnancy rates (Brinsko *et al.,* 1990). So, the uterine lavage in this study was performed during the oestrus phase to overcome the previously mentioned detrimental effects of uterine lavage on pregnancy rate. Moreover, LeBlanc (2009) stated that local intrauterine infusion of antibiotics is more effective than the systemic approach. On the contrary, (Davolli *et al.,* 2018) stated that local intrauterine antibiotics can be inhibited by several factors such as inflammatory debris, non-physiological pH of used drugs, cervical invasion during treatment, increased drug volumes, drug resistance development, the possibility of iatrogenic infection, in the adequate covering of the endometrium by specific antibiotics, and the concentration of local antibiotics must exceed the minimum inhibitory concentration (MIC) of target bacteria (Witte *et al.,* 2010). Indeed, these disadvantages of local intrauterine treatment can be avoided by systemic antibiotic administration, whereas high concentrations of antibiotics in plasma and endometrium tissues can be achieved by using ceftiofur (Scofield *et al.,* 2014), gentamicin, neomycin,amikacin (Witte *et al.,* 2018), sulfadiazine and trimethoprim (Davolli *et al.,* 2018).

In the present study, *Staphylococcus aureus* and *Alcaligenes* isolates were sensitive to dihydrostreptomycin and kanamycin, which were consistent with previous results (Albihn *et al.,* 2003, and Frontoso *et al.,* 2008; Davis*et al.,* 2013, and Mitchell *et al.,* 2018). Unfortunately, the antibiotics used did not provide radical solutions to treat endometritis due to challenges, such as the occurrence of microbial resistance and the high cost of antibiotics. From this standpoint, it was logic to use cheap, easy-to-prepare and applicable therapeutic alternatives, such as platelets-rich fibrin PRF, which has proven and successful therapeutic effects due to its contents of cytokines and growth factors with prolonged release (Pavlovic *et al.,* 2021), which were noticed after application of 30 ml sterilized autologous PRF freshly prepared 6-10 minutes intrauterine, within 6 hours after natural mating to avoid disturbing spermatozoa.

This efficacy was reflected as, an improvement in the pattern of endometrial oedema, conception and pregnancy rate (Thanoon *et al.,* 2019, and Pavlovic *et al.,* 2021) as well as disappearance of post-mating intraluminal anechoic discharges which are secreted by *Staphylococcus*, *Brevundimonas* species, *Alcaligenes* species (Fig. 1 and 2).

The enhancement in the reproductive parameters in this study may be attributed to medical values of Platelets-rich fibrin, which enhance epithelium regeneration and healing by encouraging angiogenesis, cell recruitment, proliferation, differentiation of healthy tissues. This agreest with hypotheses of (Barbon *et al.,* 2019) who stated that, Platelets-rich fibrin contains high level of growth factors and immune cytokines. In addition to circulating stem cells and leukocytes

(Allawi *et al.,* 2020) or inhibiting the inflammatory process due to high PRF contents of PDGF and TGF-β1 (Wu W *et al.,* 2017, and Mudalal *et al.,* 2019).

The therapeutic trials showed an improvement in the endometrial thickness or endometrial oedema pattern with a significant value ($P<0.05$) after treatment in platelets rich fibrin (6.91±0.32 mm), kanamycin (7.56±0.49 mm), dihydro-streptomycin (8.23±0.57 mm) and control (10.70±0.39 mm) respectively, (Table 4). Similar results were mentioned by (Zine El-Abidine, 2008, and Ghallab *et al.,* 2023). Also, Abd-El-Razek *et al.* (2019) found that systemic enrofloxacin together with uterine ecbolic can improve the pregnancy rate between mares with endometritis and these results agreed with the results in this study (Table 5). Moreover, similar results were reported, where both ciprofloxacin and kanamycin can inhibit microbial growth in mares with endometritis (Barbary *et al.,* 2016). Moreover, El-Shalofy *et al.* (2021) obtained a significant difference between microbial isolates and selected antibiotics, as follows; Cefepime 57.1%, kanamycin 19.1%, levofloxacin 14.3% and dihydro-streptomycin 9.5%.

The most observed notes in the present study were the disappearance of intrauterine anechoic fluid (Fig. 4) and decreasing the endometrial thickness after the application of different treatment protocols. Furthermore, the post-mating infusion of PRF intrauterine and systemic dihydro-streptomycin achieved an enhancement in the conception rate of 1st and 2nd service, when compared to kanamycin and control, which were matched with 87.5% conception rate using cefepime (Abou El-Amaiem, 2016). Moreover,an improved pregnancy rate of 80% was achieved in PRF followed by Dihydrostreptomycin 60% and Kanamycin 30%, respectively, compared to 10% in the control group (Table 5).

CONCLUSION

Endometritis of mares is an imminent threat to reproductive efficiency that deserves scrutiny. Therefore, to obtain an accurate judgment of endometritis, more than one diagnostic method must be used, such as ultrasonography, endometrial cytology, microbial culturing and sequencing rather than relying on one diagnostic method. Moreover, microbial culture and sequencing revealed that the most common isolatesare *staphylococcus* species, *Alcaligenes* species and *Brevundimonas* species. Also, Platelets-rich fibrin can be used as a therapeutic and alternative to antibiotics, whereas its results were nearly like the results of dihydrostreptomycin and kanamycin, in terms of improved reproductive efficiency. The results reflected in uterine echogenicity and echotextures, endometrial oedema pattern, improved conception and pregnancy rate. Autologous PRF can be used as a low-cost alternative source to antibiotics and effective therapy for modulating the inflammatory process of post-mating induced endometritis in Arabian mares.

Author contribution

EK, SAZ, RSG: conceived and designed the study and analyzed data. EK, SAZ, RSG: analyzed data, and wrote the first draft of this manuscript. EK, SAZ, RSG: performed the software and wrote the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in the submitted manuscript.

Conflict of interest: All authors declare that they all have no conflict of interest.

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Ethical statement

The present work was ethically approved by Institutional Animal Care and Use Committees at the Pharmaceutical & Fermentation Industries Development

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التعرف الجزيئي وتسلسل العدوى البكتيرية الرحمية المرتبطة بمشاكل العقم وبروتوكول العلاج المقترح في الأفراس العربية

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يُعزى فشل التكاثر في الأفراس العربية بشكل رئيسي إلى التهاب بطانة الرحم. ولتأكيد التشخيص وإيجاد حلول فعّالة لهذه المشكلة، يجب الجمع بين عدة طرق تشخيصية. في الدراسة الحالية، تم تشخيص التهاب بطانة الرحم في ٣٦ فرسًا باستخدام تقنيات متعددة. حيث أظهرت الموجات فوق الصوتية أن ٦٩,٤٪ من الأفراس تعاني من أنسجة غير طبيعية وإفرازات داخل الرحم. تم تأكيد هذه النتائج باستخدام تمييز الخلايا البطانية، الذي كشف أن ٦٣,٩٪ من المسحات تحتوي على أكثر من ٢–٣ خلايا متعددة الأشكال (PMNs) لكل حقل رؤية عالي التكبير .(HPF X100) بالإضافة إلى ذلك، جاءت ٨٦,١٪ من عينات المسحات إيجابية للإصابة البكتيرية. أظهر تسلسل العزلات البكتيرية تنوعًا كبيرًا، حيث تمثلت المكورات العنقودية في ٤٤,٤٪ من الحالات، تلتها *Brevundimonas* بنسبة ٢٧,٨٪، و*Alcaligenes* بنسبة ٢٧,٨%

تم تقسيم الأفراس إلى أربع مجموعات كالتالي:

١. **المجموعة الأولى (الضابطة) :**بدون علاج.
٢. **المجموعة الثانية :**تلقت جرعة داخل الرحم من كاناميسين بتركيز ١٠.%
٣. **المجموعة الثالثة :**تلقت ثلاث جرعات عضلية من دايهيدروستريبتوميسين.
٤. **المجموعة الرابعة :**تلقت ٣٠ مل من الفيبرين الغني بالصفائح الدموية (*Platelet-Rich Fibrin - PRF*)، المُحضّر حديثًا خلال ٦–١٠ دقائق عند درجة حرارة ٢٠–٢٥ درجة مئوية، وتم حقنه داخل الرحم في غضون ٦ ساعات بعد التزاوج الطبيعي.

تم إجراء التزاوج مرتين خلال فترة ٢٤ إلى ٤٨ ساعة بعد تحفيز الإباضة، باستخدام ٢٥٠٠ وحدة دولية من الهرمون المحفز (HCG) لتحقيق توسع واضح للبصيلات بقطر ٣٠ ملم. تم استخدام فحل واحد لكل مجموعة لضمان التناسق.

لوحظ انخفاض كبير في سمك بطانة الرحم بين المجموعات المعالجة ($P<0.05$)، بمتوسط عام ٨,٣٥±٠,٣٢ مم.

تم تسجيل تراجع واضح في الإفرازات السوداء داخل الرحم، مما يشير إلى تحسن حالة الرحم. علاوة على ذلك، أظهرت نسبة الحمل تحسنًا ملحوظًا ($P<0.05$) في المجموعات التي تلقت العلاج مقارنة بالمجموعة الضابطة.

تشير النتائج إلى أن العدوى البكتيرية تلعب دورًا كبيرًا في التسبب بالتهاب بطانة الرحم لدى الأفراس العربية. كما يُعد استخدام الفيبرين الغني بالصفائح الدموية (*PRF*) بديلاً علاجيًا واعدًا، نظرًا لقدرته على تحسين الحالة الالتهابية ودعم نسبة الحمل.