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GENITAL MYCOTIC INFECTION OF THE REPEAT BREEDER MARES AND FIELD TRIALS FOR ITS TREATMENT

(With 4 Tables)

By

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الإصابة الفطرية للجهاز التناسلى في المهرات التي تعانى من الشياع المتكرر والمحاولة الحقلية لعلاجاتها

جابر احمد مجاهد ، ضرار رفعت ابراهيم ، حسن عبد الصبور على احمد لطفى السيد

أجرى هذا البحث بهدف دراسة وعزل الفطريات التي قد توجد في الجهاز التناسلي للأفراس التي تعانى من الشياع المتكرر والتي لقحت أكثر من ثلاث مرات من ذكور ذات خصوبة عالية. كان العدد الكلى لهذ الحيوانات ٧٥ فرسه دخلت المستشفى البيطري التعليمي بأسيوط بين يناير ٢٠٠٧ وديسمبر ٢٠٠٨ وتعاني من الشياع المتكرر تم عز ل فطريات من الجهاز التناسلي ل٤٠ منها. وقسمت الحيوانات إلى ٤ مجاميع (١٠ فرسات لكل مجموعة) الأولى عولجت بمحلول ملح والثانية بمطهر الاكريفلافين بتركيز ١:١٠٠٠ والثالثة بمضاد حيوي (استربتوبنيسيد) موضعي داخل الرحم والرابعة تركت بدون علاج كمجموعة ضابطة. أوضحت النتائج المتحصل عليها وتحليل المعزول من المسحات ان الإصابة كانت: اسبيرجيللس (٣٧,٣٣%) واكريمونيم (٨٢,٢٠%) بيسليومايسس (١١,١١%) وفيوساريم (٩,٣٣%) بنيسيليم (٧,٥٥%) ميوكور (٥,٣٣%) التيرناريا ودريكسلاريا (٢,٢٢%) وتريكوسيكيم وكلادسبوتيم (١,٧٨%) والكانديدا (٠,٠٠٤%). وكانت السموم الفطرية الملحوظة هى الافلاتوكسين ب ١وب ٢وج. ولوحظ ان نسبة الاخصاب الاولى هى على التوالى فى المجاميع الاربعة: ٤٠%، ٦٠%، ١٠%و ٠% ولذلك يمكن الأخذ فى الاعتبار بان الاكريفلافين والمحلول الملحي له تاثير جيد في علاج الشياع المتكرر في المهرات ذات الإصابة الفطرية فى أرحامها.

SUMMARY

The aim of this work was to identify the fungal isolates present in the genitalia of the repeat breeding mare and application of acriflavine and normal saline as an intrauterine lavage for its treatment. A total number

of 75 mixed bred mares were introduced to the Vet. Teaching Clinic, Assiut University, Department of Theriogenology between January 2007 and December 2008 included in this study. These animals had clinically normal genitalia but failed to conceive after at least 3 times of natural mating. Out of them, 40 mares showed positive mycotic infection. The mares were treated twice with one week interval. According to treatments the animals divided into four groups, 10 mares for each. First group (G1) was treated with saline, second group (G2) treated with acriflavine dissolved in distilled water in concentration 1:1000, third group (G3) treated with streptomycin 2 gm and the fourth group (G4) left without treatment. After the second treatment, all mares were left for one cycle then mated with highly fertile stallion then pregnancy diagnosis was done using ultrasonography at 30-45 days after mating. The obtained results revealed that, the common isolates genera from the swabs were Aspergillus (37.33%), Acremonium (20.82%), Paecilomyces (11.11%), Fusarium (9.33%), Penicillium (7.55%), Mucor (5.33%), Alternaria and Drechslera (2.22% each),), Trichothecium and Cladospotium (1.78% each), and Candida (0.004%). The most common Aspergillus subspecies were A. *fumigatus* and A. *niger*. The detectable mycotoxins were Alfatoxine B_1, B_2, G as well as Aflatoxine B_1 and B_2, Citrinin and Zearalenone which were extracted from A. *flavous*, A. *terrus*, A. *parasiticus* and Fusarium *oxysporum*, respectively. The conception rates were 40%, 60%, 10%, 0% in G1, G2, G3 and G4 group, respectively. Therefore, it could be advised that the use of sterile physiological saline and/or diluted acriflavine could be indicated to counteract such sort of genital fungal infections successfully.

Key words: *Mycotic infections, repeat breeder, uterine lavage, conception rate, mares*

INTRODUCTION

The biggest obstacles to produce good healthy newly born foals from the mares bred during a breeding season, are the infertility and subfertility (Samper, *et al.*, 2006). Many factors, which acting either alone or in combination with other, can cause infertility or subfertility. These factors can be categorized into infectious or non-infectious with the first being further divided into bacterial, viral and fungal agents. The most common source of fungi causing reproductive disease in the mares is probably from skin or fecal origin (Elvinger and Roberts, 1995). The uterine lumen of the normal fertile mare is sterile despite the fact that the reproductive tract is contaminated with bacteria from the act of breeding,

foaling and veterinary procedures. Mares with defective vulval conformation can suck air and bacteria into the vagina, which can develop into endometritis (Troedsson, 2006).

A variety of anti-mycotic agents have been used to treat fungal infection (Carter and Chengappa 1995). They added that, these agents exert their effect by interference with nutrient exchange across the fungal cell wall and cell membrane as well as by altering the permeability of the fungal cytoplasmic membrane and eventual cell death. Sometimes, the limiting factor in the treatment of fungal infections is the cost of medications resulting in shortened drug course or every other day therapy. The difficulty in treating equine fungal endometritis may be attributable to an insufficient treatment period, inadequate dose or inappropriate choice of anti-fungal drug. Most of the drug dosages used for treatments are extrapolated concentrations (Ley, 1994, Troedsson, 1997, Hess *et al.*, 2002, Scotty *et al.*, 2005). Unfortunately, the cost of anti-fungal medication often decreases the duration of therapy as well as the cost also may affect the route of administration preventing the use of systemic therapy.

The aim of the present study was to identify the fungal isolates commonly present in the genitalia of the repeat breeding mares. Moreover, the field application of some intrauterine treatments were also evaluated as well as estimates the subsequent fertility

MATERIALS and METHODS

1. Animals and gynecological examination:

A total number of 75 mixed breed mares were introduced to the Veterinary Teaching Clinic, Department of Theriogenology, Assiut University during the breeding season 2007/ 2008 with regular repeat breeder problem. These animals failed to conceive in spite of mating at least 3 times with good fertile stallions. Out of them 40 mares showed positive samples for mycology. The external genitalia, perineal region and the tail were inspected for signs of discharge and the cervix, uterus and ovaries examined manually through the rectal wall and ultrasonographically. The vagina and the vaginal portion of the cervix were examined visually through vaginoscopy.

2. Sample Collections:

The tail was wrapped with clean gauze, the perineal area was washed and rinsed 3 times and dried thoroughly before the genital tract was investigated. The protected sterilized cotton swab was carefully passed into vagina till portio-vaginalis under complete aseptic conditions. The sterile swab was then pushed from its protective sheath and moved

gently around the external os of the cervix. After retraction into its cover, the swab was gently removed. From each animal, other protected swabs were passed carefully through the cervix into the uterus then moved gently on the endometrium to obtain the samples. This process was repeated in positive cases in the next examinations to obtain the second samples after treatments. All swabs were brought on ice to the laboratory and kept at 4°C until processed.

3. Treatment regimens:

After scanning of the genital tract and samples collection, treatment was applied. Positive mares were divided into four groups randomly according to the type of treatments (each = 10 animals). First group (G1) was treated with saline (0.9% Sodium Chloride, ADWEC, Elnaser Pharmacial company, Egypt), acriflavine (Fulka Chemie, Switzerland) was dissolved in distilled water in concentration 1:1000 and used in second group (G2), streptomycin 2 gm (Streptopenicid®, CID, Egypt) was used for third group (G3) and the fourth group (G4) left without treatment. Intrauterine lavage using sterile rectal enema connected with long flexible rubber tube was used to infuse 500-1000 ml of the treatment solution inside the uterus and in the cervix, then allowed the fluid to pass out. The mares were allowed to run some distance to evacuate the residues of the fluid from the uterus. Examination, samples collection as well as treatments were repeated at least twice with one week interval. The untreated group was examined twice and samples collected twice without treatments. After treatment, all mares were left for one cycle then were mated in subsequent cycle at the second and fourth day of the estrus period by a good fertile stallion in the surrounding villages. Pregnancy diagnosis was done using ultrasonography for all mares at 30-45 days post service.

4. Isolation of Fungi:

The swabs were cultured onto Sabouraud's dextrose agar medium (SDA) supplemented with chloramphenicol (50 mg/L). Three plates were used for each swab and were incubated at 28°C for 7-10 days. The observed fungi were isolated and examined for identification through macro- and microscopic studies according to different guidelines (Raper and Thom, 1949, Raper and Fennel, 1965, Domasch *et al.*, 1980, Nierenberg, 1989). The identified fungi were transferred on SDA slants and kept until physiological studies.

5. Mycotoxin production of the isolated fungi:

A total number of 225 isolated colonies during this investigation were screened for their ability to produce mycotoxins on yeast extract sucrose medium (YES). Erlenmeyer flasks (250 ml), each containing 50

ml of YES medium, were inoculated by the examined fungi and incubated as stationary culture at 28°C for 10 days. Three replicates of each flask were analyzed. At the end of the incubation period, the contents of each flask were homogenized with 50 ml chloroform for 5 min. in a high speed blender. Extraction was repeated three times. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulfate, filtered and dried to near dryness on a rotary evaporator. The residue was diluted with chloroform to one ml. The chloroform solution was analyzed for the presence of different mycotoxins using thin layer chromatography as described by (Gimeno, 1979).

RESULTS

1. Mycotic findings:

The isolated mycoflora from the mares with repeat breeder problem are presented in Table (1). 12 cases had in their swabs mixed colonies (100 colonies) and 28 cases contained single colonies (125 colonies). There were 11 isolated fungal species. The fungal isolates commonly present in cervical and uterine swabs are illustrated in Table 2. The common isolates genera from the swabs of repeat breeder mares were Aspergillus (37.33%), Penicillium (7.56%), Candida (0.44%), Mucor (5.33%), Alternaria and Drechslera (2.22% each), Fusarium (9.33%), Trichothecium and Cladosporium (1.78% each), Paecilomyces (11.11%) and Acremonium (20,89%). In the same table, the most common Aspergillus subspecies were A. *fumigatus* (38 colonies), A. *niger* (16 colonies), A. *carbonaris* (10 colonies), A. *terrus* and A. *versicolor* (4 colonies each) and A *parasiticus* and A. *utus* (one colony each). Moreover, Acremonium spp. Divided into A. *strictum* (27 colonies) and A. *roseum* (20 colonies) as well as, the common isolates colonies from Penicillium spp. were P. *chaysogenum* (8 colonies), P. *citrinum* (6 colonies), P. *corylephilum* (2 colonies) and P. *cyclopium* (one colony). The mycotoxin production from the obtained isolates is recorded in Table 3. Out of 84 Aspergillus isolates, 3 only had a toxigenis (A. *flavus*, A. *terrus* and A. *parasiticus*) and the toxin productions were Aflatoxin B_1,B_2,G_1, Aflatoxin B_1,B_2 and Citrinin respectively. However, only one isolate from 21 Fusarium oxysporum isolates produced Zearalenone toxin.

2. Conception rate:

The conception rates were 40%, 60%, 10%, 0% in saline (G1), acriflavine (G2) and antibiotic treated group (G3) and control group (G4), respectively (Table 4).

Table 1: General classification of the cultured colonies from the mares with repeat breeder problem.

Items	Number
-No. of examined repeat dreeding mares	75
-No. of positive samples for mycology	40
-No. of negative samples for mycology	35
-No. of mares used for treatment and follow up	40
-No. of isolstes species	11
-No. of isolates colonies	225
-No. of mixed colonies	100
-No. of single colonies	125
-No. of mixed cases	12*
-No. of single cases	28

* The isolated fungi from each case were 2 or 3 types.
* These types were: A. terreus, A. fumigatus and A. parasiticus (3 cases).
A. niguer and A. flavus (4 cases.). A. carbonaries and A. terreus (3caese).
Drechslera spicifera and Paecilomyles lilacinus (1 case).
Acremoniun strictum, Acremoniun roseum and A. flavus (1 case).

Table 2: Different types of fungal isolates from examined mares

Isoltes	Portio-vaginalis		Uterus		TOTAL	
	No.	%	No.	%	No.	%
1) Aspergillus	36	37.5	48	37.21	84	37.33
- A. *carbonaris*	6	6.25	4	3.1	10	
- A. *terreus africans*	3	3.13	1	0.78	4	
- A. *fumigatus*	14	14.58	24	18.61	38	
- A. *flavus*	5	5.21	5	3.88	10	
- A. *versicolor*	3	3.13	1	0.78	4	
- A. *niger*	5	5.21	11	8.53	16	
- A. *parasiticus*	-	-	1	0.78	1	
- A. *ustus*	-	-	1	0.78	1	
2) Penicillium	6	6.25	11	8.53	17	7.56
- P. *chaysogenum*	4	4.17	4	3.1	8	
- P. *cyclopium*	-	-	1	0.78	1	
- P. *citrinum*	-	-	6	4.65	6	
- P. *corylephilum*	2	2.08	-	-	2	
3) Candida	-	-	1	0.78	1	0.44
4) Mucor	4	4.17	8	6.20	12	5.33
5) Alternaria *alternata*	2	2.08	3	2.33	5	2.22
6) Fusarium *oxysporum*	13	13.54	8	6.20	21	9.33
7) Trichothecium *roseum*	3	3.13	1	0.78	4	1.78
8) Drechslera *spicifera*	3	3.13	2	1.55	5	2.22
9) Paecilomyces *lilacinus*	17	17.71	8	6.20	25	11.11
10) Acremonium	11	11.46	36	27.91	47	20.89
- A. *strictum*	3	3.13	24	18.61	27	
- A. *roseum*	8	8.33	12	9.30	20	
11)Cladosporium *cladosporioides*	1	1.04	3	2.33	4	1.78
TOTAL	96		129		225	

Table 3: Mycotoxin production of the isolated fungi

Fungal species	No. of isolates	No. of toxigenis	Mycotoxin detected
*Aspergillus	84	3	
- A. flavus	10	1	Aflatoxin B1,B2,G1
- A. terrus	4	1	Aflatoxin B1,B2
- A. parasiticus	1	1	Citrinin
* Fusarium oxysporum	21	1	Zearalenone

Table 4: Conception rate (C.R) after treatment

Items	Saline	Acriflavine	Streptomycin	untreated
No. of conceived mares	4/10	6/10*	1/10	0/10
% of C.R	%40	60%	10%	0%

* Significant difference ($p<0.05$)

DISCUSSION

Mycotic infection of the female genital organs in the mare is not as common as that of bacteriological origin, but recognition of a fungus as the causal agent is very important (Dascanio *et al.*, 2001). In the current study, there were a variety of fungi that have been identified from the swabs. The most common isolated fungi from all samples were Aspergillus spp. (37.33%). This report was in agreement with that reported by (Elvinger and Roberts, 1995, Elvinger and Roberts, 1996, Petrites-Murphy *et al.*, 1996, Dascanio *et al.*, 2000, Dascanio *et al.*, 2001). Concerning candida ioslates (0.004%) in this study, the obtained result coincide with that mentioned by (Doyle, 1969), but varied from that reported by (Zafracas, 1975, Pugh *et al.*, 1986, Dascanio *et al.*, 2001) who demonstrated that candida spp. isolated as the most common yeast causing fungal pathogen infecting the mare`s reproductive tract. This variation in isolated candida spp. may be attributed to that reported by (Garcia-Tamayo *et al.*, 1982, Dascanio *et al.*, 2000) who mentioned that some yeast organisms such as candida spp. have the ability to become invasive to tissues as well as have been showen to penetrate and grow intracellularly within epithelial cells. The mechanism of colonisation is thought to be initial adherence to epithelial cells through a manno-protein called adhesin. This also makes the organism more

difficult to remove. However, the high incidence of this yeast as candida may be attributed to the type of sampling where the authors mentioned that a biopsy from the mare's reproductive tract may be diagnostic of fungal endometritis (Hurtgen and Cummings, 1982, Freeman *et al.*, 1986).

Moreover, the obtained results revealed that Penicillium spp., Paecilomyces spp. and Fusarium spp. were identified as the fungal pathogen infecting the mare`s reproductive tract. These results agreed with that reported by (Blue, 1983, Chengappa *et al.*, 1984, Elvinger and Roberts, 1995). Regarding the perecent of positive fungal isolates, the obtained results indicated that, this perecnt was higher than that calculated by (Bain, 1966, Zafracas, 1975, Pugh *et al.*, 1986).

The obtained results which regarded the use of normal sterile saline were in agreement with that reported by (Pascoe *et al.*, 1989, Dascanio *et al.*, 2001). They concluded that, lavage of the uterus with sterile saline without additives may decrease the total numbers of organisms and stimulate an inflammatory response that may assist resolution of the infection. Lowering the pH of intrauterine saline solutions (to 3.7) has been demonstrated to cause a significant increase in prostaglandin secretion. An increase in prostaglandin release may affect corpus luteal function and lead to an increase in myometrial activity to aid in the evacuation of uterine contents (LeBlanc *et al.*, 1994). The comparison between the microflora which recovered in the swabs before and after treatment as well as the histopathological pictures of the endometrium were not the points in this work.

As a conclusion of the present study, the use of sterile physiological saline and/or diluted acriflavine could be indicated to counteract such sort of genital fungal infections successfully. Moreover, these medications are cheaper than other anti-fungal medication and easier application. Mares should be examined through palaption per rectum and by transrectal ultrasonography to detect the best time for breeding. Pre- or post-mating antibiotic therapy should be limited to prevent altering normal reproductive flora.

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