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RESEARCH ARTICLE

Prevalence of Mycotic Endometritis in Buffaloes and Mares Maintained Under Different Managemental Conditions in District Faisalabad

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A B S T R A C T

In the present study, the prevalence of mycotic endometritis (ME) in buffaloes and mares in district Faisalabad, Pakistan was investigated. Uterine flush samples were aseptically collected from 144 buffaloes and 75 mares and incubated on Sabouraud Dextrose Agar for two weeks. The overall prevalence of ME was 3.5% in buffaloes and 2.7% in mares. Ultrasonographically, buffaloes with ME had thicker endometrium ($P<0.05$) than that of buffaloes without ME (9.66 ± 0.9460 mm vs 8.97 ± 0.0816 mm). Buffaloes bred through artificial insemination showed lower ($P<0.05$) incidence of ME than those bred through natural mating (2.3% vs 12.5%). In mares, stocking density (1.44% for 1-5 animals/paddock vs 33.3% for >10 animals/paddock), history of abortion and retained fetal membranes were significant risk factors ($P<0.05$) for the prevalence of ME. Management condition, body condition score and parity were not associated with the prevalence of ME in either species. In addition, herd size and reproductive problems had no effect on the prevalence of this problem in buffaloes. Antifungal susceptibility testing for buffalo isolates showed that all three isolated genera (*Penicillium*, *Aspergillus* and *Rhizopus*) were susceptible to itraconazole, *Penicillium* being the most susceptible. *Penicillium* and *Aspergillus* were resistant to fluconazole; however, *Rhizopus* was susceptible to fluconazole. In mares, out of the two isolates (*Penicillium* and *Candida*), *Penicillium* was susceptible to itraconazole and resistant to fluconazole, while Candida was resistant to both drugs. It is concluded that the prevalence of ME is not threatening in either species. However, buffaloes bred through natural mating; mares reared under high stocking density or those with history of abortion and retained fetal membranes have high risk of developing the disease.

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INTRODUCTION

Buffalo is the main dairy animal in Pakistan and contributes over 60% to the gross milk production in the country. The buffalo population in the country is second only to the cow population amongst livestock and is estimated to be 40.0 million heads. The horse population is about 0.04 million heads, while there are about 0.2 million heads of mules in the country (Anonymous, 2019). Horses are mainly used for sports and breeding purposes, while mules are used for pulling carts and for transportation purposes in hilly areas.

Despite the undeniable contribution of the livestock sector towards national GDP, buffalo is constantly lagging behind the cow in terms of reproductive performance. Reproductive problems are the principal cause of culling in buffaloes (Taraphder *et al.*, 2011), while repeat breeding is the most prevalent manifestation of reproductive failure in buffaloes in Pakistan (Rabbani *et al.*, 2010). The 8.9 percent incidence of repeat breeding in Pakistani buffaloes (Khan *et al.*, 2016) can be linked with overdependence on natural mating, as only 9.08 percent of buffaloes kept in households are artificially inseminated in Punjab, Pakistan (Anonymous, 2006). Endometritis and metritis are the most frequently encountered reproductive problems in buffaloes (Modi *et al.*, 2011). A high incidence of these problems has been reported in repeat breeder buffaloes (Dutt *et al.*, 2017).

Endometritis interferes with the normal fertility of the animal, resulting in low conception rates. Oxidative stress due to endometritis can reduce the ability of spermatozoa to produce competent embryos (Hendricks and Hansen, 2010). Uterine infection can negatively affect the follicular growth in the ovary and adversely affect blood estradiol levels (Dahiya *et al.*, 2018).

In most cases of bovine endometritis, bacterial species are involved. However, fungal involvement in reproductive problems is increasing due to the irrational use of antibiotics and reproductive hormones. Certain species of fungi have been associated with bovine abortion and endometritis (Clothier and Anderson, 2016). In India, Verma *et al.* (1999) reported the prevalence of fungal agents to be 18.6 and 21.8%, respectively in buffaloes and cows having reproductive problems like abortion, repeat breeding, endometritis, and cervicitis or vaginitis. Clinically healthy animals can also carry the fungal pathogens and potentially acquire fungal infections under fungi-favoring conditions (Shokri and Yadollahi, 2017).

In mares, the prevalence of mycotic endometritis (ME) is rare (1-5%); however, other common problems of reproductive tract such as pneumovagina, bacterial endometritis and repeat breeding can lead to the development of this problem (Saini *et al.*, 2019). Moreover, impaired immune system and prolonged use of antibiotics can lead to the development of such infection in the uterus (Satué and Gardon, 2016).

Despite a significant threat posed by fungal agents to the reproductive performance in domestic animals, relatively little information is available on the prevalence of the fungi in association with reproductive problems in Pakistan. In the absence of such information, it is impossible to forecast the potential role of fungi in causing reproduction failure. In this scenario, the current study was conducted to survey the prevalence of ME in buffaloes and mares, to determine the risk factors for the development of the problem and to suggest suitable antifungal agents for the treatment of suspected field cases of fungal endometritis.

MATERIALS AND METHODS

Experimental animals: Female buffaloes and mares of different parity, having the history of repeat breeding and reared under different managemental conditions were selected. Samples were collected from buffaloes and mares from different parts of Tehsil Faisalabad (buffalo: SB Dairy Farm, Cattle Colony 217/R.B., Chak no. 218/R.B. Prokianwala, Cattle Colony Ghulam Muhammad Abad; mare: Chak no. 231/R.B. Risalewala, Canal Road, Chak no. 100/J.B. Kurriwala, Jhang Road). The sample size was calculated by using the following formula (Daniel and Cross, 2018):

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where,

Z = Z-statistic for a level of confidence (for 95% level of confidence, Z = 1.96), P = expected prevalence based on past studies (for buffaloes, P = 0.105; Ramsingh *et al.*, 2013; for mares, P = 0.05; Stout, 2008), d = precision (when prevalence is expected to be between 10 & 90%, d = 0.05). In total, 144 and 75 samples were collected from buffaloes and mares, respectively.

Housing and management: The housing of buffaloes was classified into three categories: field, conventional, and farm. The buffaloes reared in the subsistence system in small herds (of less than 10) were categorized in the field category. The conventional system referred to the traditional barns used for housing buffaloes. Farm conditions referred to commercial farming system facilities (Khan *et al.*, 2020).

For buffaloes, other information such herd size, parity, history of obstetrical problems, body condition score, and method of breeding was also recorded. Similarly, data regarding housing conditions, age, parity, population density, body condition score and history of reproductive problems were recorded for mares.

Collection of Samples: Uterine flush samples were collected by infusing normal saline solution into the uterine lumen and aspirating it back. before collecting the samples, the perineal area was washed and cleaned. in buffaloes, a sterile artificial insemination rod was inserted into the uterus, and 30 ml sterile normal saline solution was infused. in mares, a sterile foley's catheter was inserted into the uterine lumen, and 60 ml normal saline solution was infused. this fluid was aspirated back. the samples were recovered in sterile containers, transported to the laboratory in cold boxes and stored at 4ºc.

Ultrasonographic Examination of the Uterus: Transrectal ultrasonographic examination of the uterus was done in buffaloes before collecting flush samples. Honda hs-1500 ultrasound machine, with a 7.5 MHz rectilinear array, was used for measuring the endometrial thickness in the transverse plane of the uterine horns at the level of external uterine bifurcation. Ultrasound examination was not performed in mares to prevent unnecessary irritation and prevent damage to the rectal wall.

Processing of Samples for Fungal Growth: Collected samples were inoculated on Sabouraud Dextrose Agar (SDA) at 25 °C for two weeks, as described by Verma *et al.* (1999). Inoculated Petri plates were regularly observed for the appearance of any mycotic growth. Positive samples were used to perform antimicrobial susceptibility testing (AST) by the microdilution method. Itraconazole (Rolac®) and fluconazole (Fungone®) were used in the susceptibility testing. Negative and positive control plates were also incubated, along with the samples (Khan *et al.*, 2020).

Characterization of Fungi: The growth of fungal colonies on petri plates was further characterized based on colony features and microscopic examination. Genus identification was made by examining fungal hyphae under a microscope, as described previously (Noman *et al.*, 2017).

Calculation of prevalence: Prevalence (p) of mycotic endometritis was calculated by using the following formula (Thrusfield and Christley, 2018):

$$P = \frac{\text{number of samples positive for fungal agents}}{\text{total number of samples}} \times 100$$

Statistical analysis: The collected data for prevalence were analyzed on IBM SPSS® (version 25.0), and Pearson's Chi-square test was used to compare the prevalence of mycotic endometritis under different potential risk factors shown in tables 1 and 2 for buffaloes and mares, respectively. For buffaloes, t-test was used to compare mean endometrial thickness between animals with mycotic endometritis and those negative for mycotic endometritis.

RESULTS

Prevalence of mycotic endometritis: The overall prevalence of mycotic endometritis (me) in buffaloes was found to be 3.5% (5/144). When the prevalence of me in buffaloes under different risk factors was considered, it was found that different management conditions, parity of the animal, its body condition score, history of reproductive problems and herd size had non-significant effect on the prevalence of me in females of this species (Table 1).

However, the method of breeding was found to be significantly associated with the prevalence of ME in buffaloes. It was significantly higher (P<0.05) in the buffaloes that were bred through natural breeding (12.5%) compared to 2.3% in animals bred through artificial insemination (Table 1).

Similarly, the thickness of the endometrium was also significantly associated (P<0.05) with ME in buffaloes. The mean endometrial thickness was significantly higher (9.66 ± 0.9460 mm) in buffaloes positive for ME than animals free from ME (8.97±0.0816 mm; Fig. 1). Ultrasonographic cross section of the uterus of an experimental buffalo negative for mycotic endometritis is shown in Fig. 2.

In mares, the overall prevalence of ME was 2.7% (2/75). The prevalence was higher (33.3%) in stables that housed more than 10 mares, while it was only 1.44% where 1-5 mares were kept together, the difference was significant (P<0.05). No positive samples were recovered from the stables housing 5-10 mares (Table 2). The prevalence of ME in mares was also significantly associated with history of reproductive problems (P<0.01). Animals that had the history of either retained fetal membranes or abortion had 100% prevalence each, while no prevalence could be recorded in mares with history of repeat breeding or genital prolapse (Table 2). However, the age, parity and body condition score of the mare had no effect on the prevalence of ME in females of this species (Table 2).

Antifungal susceptibility testing: The microscopic visualization showed that out of five isolates recovered in buffaloes, two were *Aspergillus* and *Penicillium* each, and one isolate was *Rhizopus.* The MIC of itraconazole (Rolac®) against *Penicillium spp.* was observed to be 4.12 mg/ml, while against *Aspergillus spp.*, the MIC value was 8.25 mg/ml (Table 3). MIC value for *Rhizopus spp.* was highest at 16.50 mg/ml. *Penicillium spp*. and *Aspergillus spp.* were observed to be resistant to fluconazole (Fungone®). Fluconazole (Fungone®) had a MIC of 12.5 mg/ml against *Rhizopus spp*.

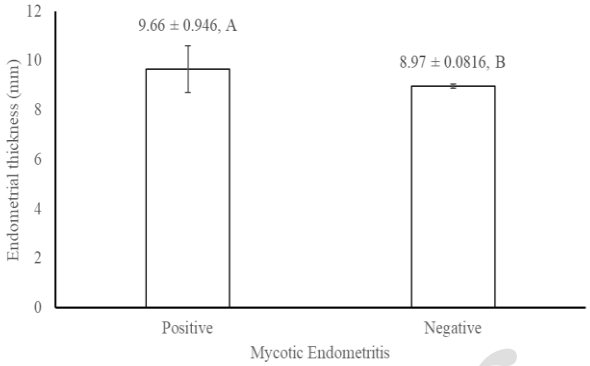

Fig. 1: Mean values (± SEM) of endometrial thickness with respect to mycotic endometritis; Values with different letters show significant difference (P<0.05).

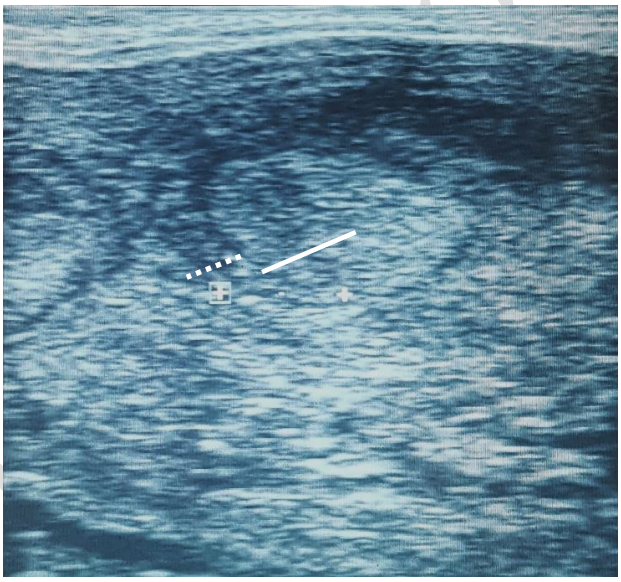

Fig. 2: Ultrasonographic cross section of the uterus of an experimental buffalo negative for mycotic endometritis; the solid bar spans the diameter of endometrium, while dotted line shows the diameter of myometrium.

In mares, out of the two isolates recovered, one belonged to Candida and the other to Penicillium species. Candida spp. were resistant to both itraconazole and fluconazole. However, Penicillium spp. was susceptible to itraconazole with MIC value of 4.12 mg/ml, but resistant to fluconazole (Table 3).

DISCUSSION

The overall mean prevalence of ME in buffaloes included in the present study was 3.5%. Similarly, the prevalence of ME in cows in Pakistan is not alarming, since the prevalence of ME in district Faisalabad has recently been reported as 2.8% (Khan *et al.*, 2020).

In the present study, a significant difference was observed between the mean values of the endometrial thickness in the buffaloes with ME and those without ME. Since ME is often associated with chronic infection, subsequent persistent use of antibiotics, and lowered immunity, a thicker uterine wall can be anticipated in the cases of ME due to the chronicity of the condition (Saini *et al.*, 2019).

Table 1: Effect of different risk factors on the prevalence of mycotic endometritis in buffaloes

Factor		Prevalence (%)	Positive/ total samples	Significance
Managemental condition	Farm	1.9	1/52	NS
	Conventional rearing system	5.4	3/56	
	Field conditions	2.8	1/36	
Parity	0-2	-	0/32	NS
	3-4	3.5	3/85	
	>4	7.4	2/27	
BCS	<2.5	5.2	1/19	NS
	2.5-3.5	3.2	2/61	
	>4	3.1	2/64	
Reproductive problems	Repeat breeding	2.0	2/96	NS
	RFM	6.7	1/15	
	Abortion	11.7	1/9	
	Genital prolapse	7.7	1/13	
	Dystocia	-	0/11	
Herd size	Small	1.8	1/57	NS
	Medium	5.0	1/20	
	Large	4.5	3/67	
Breeding method	Artificial insemination	2.3	3/128	*
	Natural breeding	12.5	2/16	

NS Non-significant; *Significant at P<0.05.

Table 2: Effect of different risk factors on the prevalence of mycotic endometritis in mares

Factor		Prevalence (%)	Positive/ total samples	Significance
Stocking density	1-5	1.44	1/69	*
	5-10	-	0/3	
	> 10	33.3	1/3	
Age (years)	< 5	-	0/5	NS
	5-10	-	0/23	
	10-15	4.3	2/47	
Parity	0-2	-	0/40	NS
	3-4	3.2	1/31	
	> 4	25.0	1/4	
Reproductive problems	Repeat breeding	-	0/72	**
	RFM	100	1/1	
	Abortion	100	1/1	
	Genital prolapse	-	0/1	
Body condition score	1-3	4.5	1/22	NS
	4-6	2.4	1/42	
	7-9	-	0/11	

NS Non-significant, *Significant at P<0.05; ** Significant at P<0.01.

Table 3: Minimum inhibitory concentration (MIC) of the two antifungal drugs against different fungal species isolated from experimental buffaloes and mares

Animal species	Antifungal drug	Isolated fungal species	MIC (mg/ml)
Buffalo	Itraconazole	Aspergillus	8.25
		Penicillium	4.12
		Rhizopus	16.50
	Fluconazole	Aspergillus	Resistant
		Penicillium	Resistant
		Rhizopus	12.5
Mare	Itraconazole	Candida	Resistant
		Penicillium	4.12
	Fluconazole	Candida	Resistant
		Penicillium	Resistant

It was observed that management did not have a significant effect on the prevalence of ME in buffaloes. These findings are in accordance with the observations made by Bruun *et al.* (2002), who concluded that housing did not have a significant association with metritis in dairy cows. Parity was also found not to be associated with the occurrence of ME; similar observations have been made in a study conducted by Gilbert *et al.*, (2006). However, other studies in dairy cows ((Bruun *et al.*, 2002; Ghanem *et al.*, 2002; Bonneville-Hébert *et al.*, 2011) have documented a significant association between the parity and occurrence of endometritis and repeat breeding. Increased age with increased parity number may be a risk factor for infertility and reproductive failure due to aging (Urzua *et al.*, 2018). In the current study, this non-significant association between occurrence of ME and parity in buffaloes can be attributed to the unequal distribution of samples across the parity groups.

Results of this study also revealed that the prevalence of ME was not associated with body condition score, history of different reproductive diseases, and herd size in buffaloes. Although, the literature suggests that poor body condition score (Carneiro *et al.*, 2014; Kadivar *et al.*, 2014), history of other reproductive problems (Kim and Kang, 2003; Salasel *et al.*, 2010) and herd size (Kaneene and Miller, 1995) significantly affect the prevalence of endometritis. The different findings of the current study in this regard compared to literature may be due to overall low prevalence of ME in both species. Only five mycotic endometritic buffaloes and two mycotic endometritic mares were used to calculate the association between the prevalence of ME and these risk factors. The sample size of animals with mycotic endometritis is too small to be used for accurate determination of risk factors. Thus, studies with larger initial sample size are needed for selection of higher number of mares, cows, and buffaloes with ME. Such studies with higher sample size seem to be logistically difficult due to the lower prevalence of ME, as the present study demonstrated.

The prevalence of ME in mare was 2.7% and this is comparable with past studies where the prevalence was reported to be under 5.0% (Dascanio *et al.*, 2001; Stout, 2008). However, there have been no studies conducted to evaluate the effect of different risk factors on the prevalence of ME, and the present study seems to be the first such instance of reporting ME with different risk factors.

Conclusions: Based on the results of this study it was concluded that the overall prevalence of ME was 3.5% in buffaloes and 2.7% in mares. Breeding method and stocking density were significantly associated with the prevalence of ME in buffaloes and mares, respectively. Drug susceptibility testing showed that *Aspergillus* and *Penicillium* isolates were more susceptible to itraconazole, while *Rhizopus* isolate was susceptible to fluconazole only. *Candida spp.* were resistant to both drugs. In buffaloes, increased endometrial thickness was suggestive of mycotic endometritis.

Authors contribution: MHR, ZIQ, AM, HN and SA designed the study. MHR, SH, HHK, MMH and SA performed practical and laboratory work, while MHR, SW and SA analyzed the data and prepared the manuscript. All authors reviewed the manuscript.

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