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RESEARCH ARTICLE

Prevalence and Drug Susceptibility of Mycotic Endometritis in Sahiwal Cattle in District Faisalabad, Pakistan

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A B S T R A C T

Mycotic infections of reproductive tract did not receive much attention in the past. Fungi cause reproductive problems by invading or colonizing the reproductive tract, which adversely affects the reproductive efficiency in cattle. Current study was designed to find out the prevalence of fungal pathogens from uterine samples of Sahiwal cattle and to perform antifungal susceptibility testing on the isolated fungal pathogens. For this purpose, 144 uterine samples were collected and cultured on Sabouraud's dextrose agar at 25ºC for two weeks. Fungi were identified based on the microscopic characteristics. The results showed that out of 144 samples, 4 were found positive, showing an overall prevalence of mycotic endometritis in Sahiwal cattle as 2.8% in Faisalabad. *Penicillium* and *Aspergillus* species were the isolated fungal pathogens. The prevalence of mycotic endometritis was significantly higher in cattle having the history of reproductive problems ($P<0.05$). However, the association between mycotic endometritis, and managemental conditions, parity, herd size, body condition score, and the breeding method was statistically non-significant. The mean endometrial thickness in Sahiwal cattle suffering from mycotic endometritis was significantly higher ($P<0.05$) than the value for non-endometritis cattle. The minimum inhibitory concentration (MIC) of Itraconazole (Rolac®) was 4.12 mg/ml against *Aspergillus spp.* and 8.6mg/ml against *Penicillium spp.* However, both *Aspergillus* and *Penicillium spp.* were resistant to Fluconazole (Fungone®). It is recommended that further studies should be undertaken to investigate the role and economic impact of fungal pathogens in the etiology of reproductive disorders that fail to respond to antibiotic treatment.

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INTRODUCTION

Pakistan is an agricultural country and its economy is based on the agriculture sector. In the socio-economic growth of Pakistan, livestock sector plays a crucial role. During 2018-19 livestock contributed 60.5% to the overall agriculture and 11.2% to the GDP, showing an increased growth rate of 4% compared to 2017-18. Pakistan has a cattle population of approximately 47.8 million heads, which produce 21691 thousand ton of milk (Anonymous 2018-19).

Reproductive efficiency is the primary determinant of the overall productivity and profitability of cattle production. The major causes for a decline in reproductive efficiency are various reproductive disorders (Abdisa, 2018). The occurrence of bovine reproductive disorders is on the rise over the years (Yoo, 2010). Major reproductive problems in cattle include endometritis, pyometra, retained placenta, abortion, anestrous, and repeat breeding. These problems have a significant impact on the reproductive efficiency of the animal (Abdisa, 2018).

Infectious agents are among major causes of reproductive disorders and have received high priority in the bovine industry (Yoo, 2010). Endometritis, metritis, and pyometra are the most important uterine infections (Mandhwani *et al.*, 2017). Endometritis is a standout among the most imperative reasons for infertility in bovines, bringing about high financial misfortunes in the dairy business (Emre *et al.*, 2017).

Many fungal species are generally acknowledged as animal pathogens. Fungi may cause reproductive failure either by invading and colonizing the reproductive tract or through ingestion and absorption of mycotoxins (Shokri and Yadollahi, 2017). The increase in the prevalence of mycotic endometritis has been attributed to several reasons, including regular and indiscriminate use of intra-uterine antibiotics, postpartum uterine contamination, and poor hygiene during artificial insemination procedures (Ahmadi*et al*., 2015).

The most significant effect of fungal infection of the genital tract is mycotic abortion. However, fungi have occasionally been discovered in other reproductive problems such as vulvovaginitis and endometritis (Shokri and Yadollahi, 2017). Intra-uterine infusion of 0.1% Lugol's iodine was found clinically efficacious in the treatment of fungal endometritis (Sharma and Singh, 2012; Ramsingh *et al*., 2013). To date, there are no reports of antifungal agent used in bovines (Saini*et al.,* 2019), especially on the scientific basis of susceptibility testing and evidence-based medicine.

Keeping in view the importance of fungal endometritis and ultimately outcome of infertility in animals, it is imperative to monitor the prevalence and recommend effective therapy for the fungal infections associated with endometritis. To date, limited information is available regarding the prevalence of mycotic endometritis in the Sahiwal cattle or any other livestock species in Pakistan. Therefore, the present study was designed to investigate the prevalence of mycotic endometritis in the Sahiwal cattle kept under different managemental conditions and to explore susceptibility of isolated fungal species against antifungal drugs Itraconazole and Fluconazole to recommend an efficacious treatment for the field cases.

MATERIALS AND METHODS

Sample size: The sample size was determined by using the following formula (Arya*et al*., 2012).

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

Where

Z = Z statistic for a level of confidence of 95%= 1.96

P= expected prevalence= 10.5%

d = precision (when prevalence is expected to be between 10% to 90%) = 0.05

Prevalence of mycotic endometritis was unknown in Pakistan. Thus, the expected prevalence of 10.5% reported in India (Ramsingh*et al*., 2013) was used to determine the sample size.

$$Number\ of\ samples(n) = \frac{1.96^2 \times 0.105(1-0.105)}{0.05^2} = 144$$

Collection of uterine samples: A total of 144 uterine flush samples were collected during winter and early spring from repeat breeder cows (animals that had regular estrous cycle but failed to conceive after three inseminations) and animals with the history of abortion, RFM or uterine prolapse. A questionnaire was designed to collect information about the reproductive history, managemental conditions, herd size, parity, breeding methods and body condition score of the animals. This information was used to determine the association between fungal endometritis and these factors to explore potential risk factors.

For the samples collection, the animals were restrained and the perineal area was cleaned properly. Uterine samples were collected from the cows with infusion pipette (Santos and Bicalho, 2012). A total of 30 ml sterile normal saline was infused into the uterus, after gently massaging and elevating the uterus per rectum, a sample was aspirated back into the infusion pipette. The recovered fluid was aseptically transferred to an autoclaved plastic vial and placed in the icebox for transportation to the laboratory (Wang *et al.*, 2018).

Trans-rectal ultrasonography: Before the collection of uterine samples, transrectal ultrasonography was performed to detect the endometrial thickness. The examination was performed using ExaGo ultrasound machine with a 7.5 MHz frequency rectal probe. The thickness was measured at the level of the base of uterine horn. The thickness of the endometrium was measured from uterine lumen to the endo-myometrial junction using freeze, measure and distance function of the machine.

Mycological examination: In laboratory, the samples along with positive and negative control were inoculated on Sabouraud dextrose agar in Petri plate at 25ºC for 14 days. The culture was examined daily for fungal growth. Microscopic slides were prepared and stained with lactophenol cotton blue for the identification of fungi under microscope (Derakhshandeh *et al*., 2015).

Fungal susceptibility Test: Broth dilution method was performed to determine the antifungal susceptibility of the fungal isolates against antifungal agents Itraconazole (Rolac®) and Fluconazole (Fungone®). Briefly, 1ml of Sabouraud dextrose broth was added in six test tubes. Then 1 ml of antifungal drug solution was added into the first test tube making a total volume of 2 ml. Then 1ml from 1st test tube was taken and transferred into the 2nd test tube and repeated this up to test tube No. 6. One ml from the last test tube was taken and discarded. Pure culture (0.5ml) of the isolates was added to all test tubes. In positive control, only pure culture was added into the broth, while in negative control only antifungal drug was added into the broth. All test tubes were incubated at 37ºC for 2 days (Galgiani and Stevens, 1976).

Statistical analysis: Data were statistically analyzed by Chi-square test using SPSS software.

RESULTS

Prevalence and risk factors of mycotic endometritis: Out of 144 uterine samples collected from Faisalabad district, 4 were positive for fungal growth. Therefore, the prevalence rate of mycotic endometritis was 2.8% in the Sahiwal cattle in district Faisalabad (Fig. 1).

Out of a total of 144 samples 51, 53 and 40 samples were collected from farms, conventional housing systems and field conditions, respectively. There was statistically

non-significant association found between managemental conditions and the prevalence of mycotic endometritis (Fig. 2). Moreover, out of a total of 144 animals, 31 belonged to parity number 0-2, 86 to parity number3-4, and 27 to parity number >4. Statistically, non-significant association was found between parity number and the prevalence of mycotic endometritis (Fig.3). Furthermore, out of a total of 144 samples, 50 belonged to BCS group <2, 75 to BCS group 2.5-3.3 and 19 to BCS group >3.5. Three samples were positive in animals with BCS <2.5 and one positive sample was in animal with BCS group 2.5-3.3, while the third group of the animals having BCS >3.5 had no positive sample. The association between the prevalence of mycotic endometritis and body condition score (BCS) was non-significant (Fig. 4).

Out of 144 cattle selected for study, 15 had history of abortion, 15 of retention of fetal membranes, 101 of repeat breeding and 13 of uterine prolapse. The prevalence of mycotic endometritis was significantly associated (P<0.05) with history of reproductive problems (Fig. 5). It was significantly higher (P<0.05) in the cows having history of abortion (13.3%) compared to RFM (7.7%), repeat breeding (1.0%) and uterine prolapse (0%). Furthermore, out of a total of 144 samples 54, 42 and 48 samples were collected from the large herd, medium herd, and small herd, respectively. The selection criterion for herd size was the number of animals in the herd: small herd= <30, medium herd= 30-60 and large herd= 61-90 animals in the herd. The difference in the prevalence of mycotic endometritis among different herd size groups was statistically non-significant (Fig. 6). Lastly, out of a total of 144 samples, 121 and 23 samples were collected from the animals that were bred through artificial insemination and natural breeding, respectively. There was a non-significant association between breeding methods and the prevalence of fungal endometritis (Fig. 7).

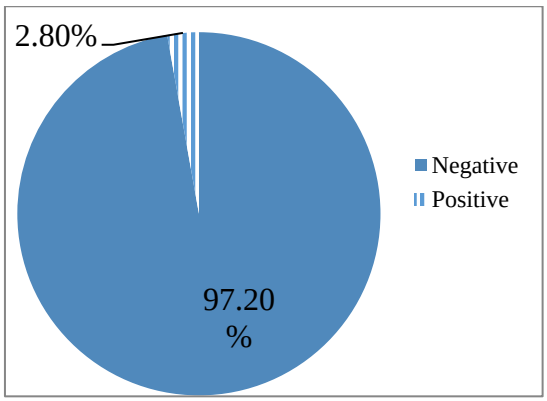

Fig. 1: Overall prevalence of mycotic endometritis in Sahiwal cattle.

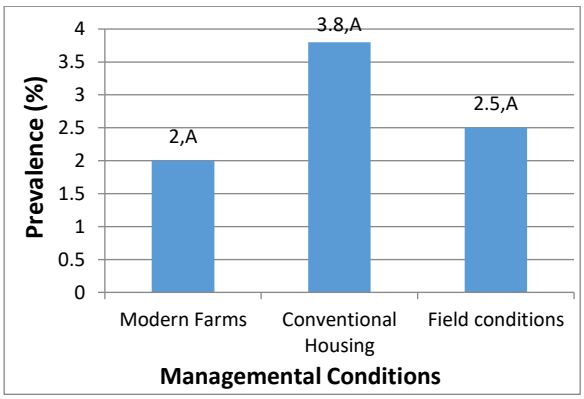

Fig. 2: Relationship of mycotic endometritis with different managemental conditions. Identical alphabets indicate non-significant difference.

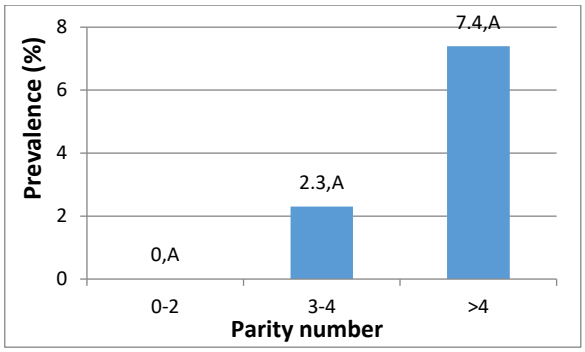

Fig. 3: Relationship of mycotic endometritis with parity number. Identical alphabets indicate non-significant difference.

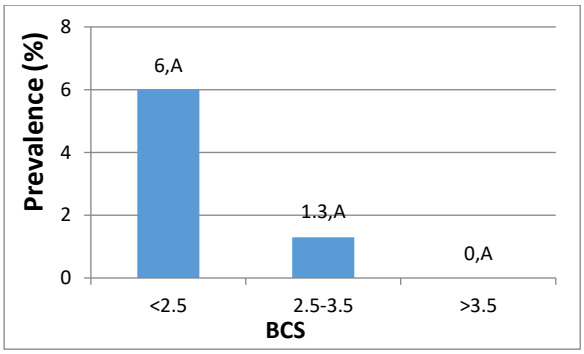

Fig. 4: Relationship of mycotic endometritis with BCS. Identical alphabets indicate non-significant difference.

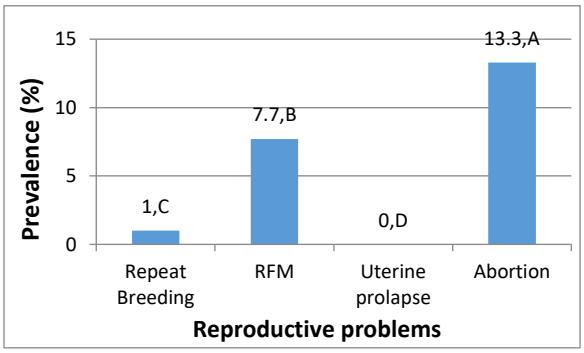

Fig. 5: Relationship of mycotic endometritis with reproductive problems. Different alphabets indicate significant difference (P<0.05).

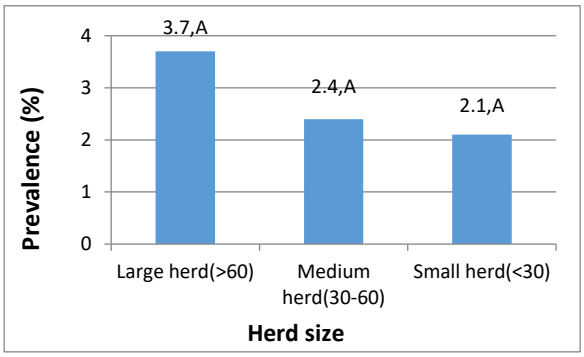

Fig. 6: Relationship of mycotic endometritis with Herd size. Identical alphabets indicate non-significant difference.

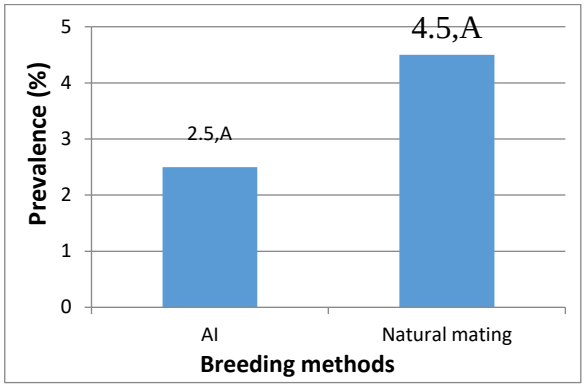

Fig. 7: Relationship of mycotic endometritis with breeding methods. Identical alphabets indicate non-significant difference.

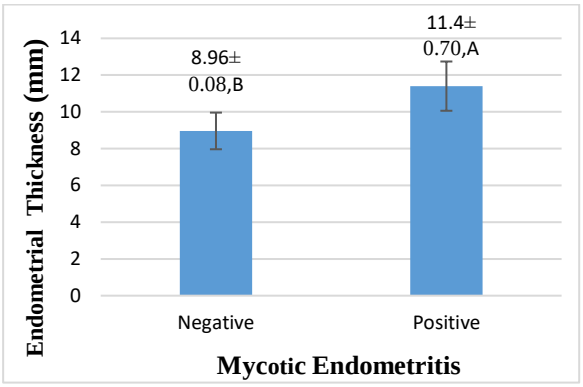

Fig. 8: Mean endometrial thickness in relation to mycotic endometritis. Different alphabets indicate significant difference (P<0.05).

Fig. 9: Ultrasonographic measurement of the thickness of endometrium (13.5mm) in cows infected with mycotic endometritis.

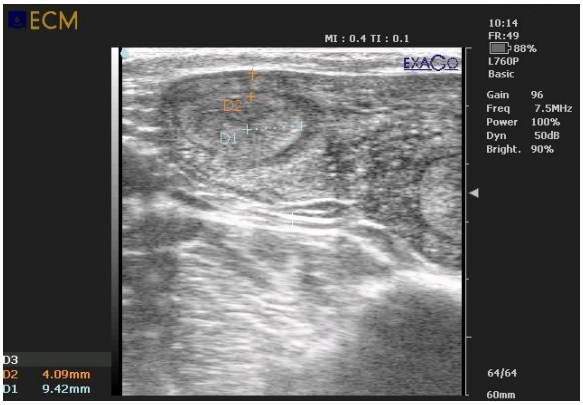

Fig. 10: Ultrasonographic image of endometrial thickness of a non-infected cow. D1 is the measurement of endometrial thickness while D2 is thickness of myometrium.

Table 1: Susceptibility of the isolated *Aspergillus* and *Penicillium* to Fluconazole and Itraconazole

Antifungal drugs	Fungus	MIC (mg/ml)
Fluconazole (Fungone®)	*Aspergillus*	Resistant
	Penicillium	Resistant
Itraconazole (Rolac®)	*Aspergillus*	4.12
	Penicillium	8.6

Mycotic endometritis and the endometrial thickness: The mean value of the thickness of endometrium was significantly higher (P<0.05) for the cows suffering from fungal endometritis than the value for non-infected cows (11.4±1.34mm vs. 8.96±0.10mm; Fig. 8). The respective measurements with the identical setting of the machine are shown in Fig. 9 and 10.

Identification of fungi and their susceptibility: Two genera, *Penicillium* and *Aspergillus*, were identified based on the fungi morphology on lactophenol cotton blue stained slides. The minimum inhibitory concentration of itraconazole (Rolac®) was 4.12 mg/ml against *Aspergillus spp.* and 8.6mg/ml against *Penicillium spp.* (Table 1). Aspergillus was more sensitive to Itraconazole (Rolac®) than Fluconazole (Fungone®). Both *Aspergillus* and *Penicillium spp.* were resistant to Fluconazole (Fungone®).

DISCUSSION

Many fungal species are generally acknowledged as animal pathogens. Protracted antibiotic therapy, intrauterine infusions, postpartum uterine contamination and unhygienic measures during artificial insemination predispose the animal to fungal endometritis (Sharma and Singh, 2012).

In the current study, the overall prevalence of mycotic endometritis in Sahiwal cattle was 2.8% in district Faisalabad. Two different fungal genera i.e. Aspergillus and Penicillium were isolated from the uterine samples of the cows. Ramsingh *et al.* (2013) recorded 10.5% fungal endometritis from repeat breeder endometritic cows, while 15.5% of fungal agents from uteri of repeat breeder cows were isolated by Sharma and Singh (2012). In this study, a lower prevalence of mycotic endometritis was observed, which might be due to lower animal density (Verma *et al.*, 1999). Moreover, the samples were collected during winter and early spring when humidity is low, which is an important growth factor for fungi (Kaur and Kumar, 2019). Non-significantly higher fugal endometritis in the cattle reared under conventional housing systems than those reared under field condition and modern farms may be due to poor barn hygiene, leading to contamination during calving, inappropriate feeding, and injury. Cheong *et al.* (2011) reported that the housing system was a significant risk factor, and the incidence of subclinical endometritis was lesser in the cows kept on free-stall than loose housing systems (bedded pack).Parity number showed a statistically non-significant association with the prevalence of mycotic endometritis. Non-significant increase in the prevalence of mycotic endometritis with

increased parity might be due to the stronger defense mechanism and rapid uterine involution in younger animals (Adnane *et al.,* 2017). High prevalence in the animals with parity number >4 is probably due to repeated contact of the reproductive tract to environmental factors, which results in increased uterine infections. Adnane *et al.* (2017) also reported that the endometritis was more prevalent in multiparous cows (third or more lactation) than primiparous animals.

A statistically significant relationship between the prevalence of mycotic endometritis and reproductive problems was found. The risk of infection and thus chances of metritis may rise because of the reproductive infection. Necrotic foci present due to retention of fetal membranes is considered as a significant predisposing factor for fungal endometritis(Saini *et al.,* 2019). In the current study, the prevalence rate was 3.7, 2.4 and 2.1% in the large, small and medium sized herds, respectively. The higher prevalence of fungal endometritis in larger herds is probably due to lower management practices, rapid and large-scale disease transmission due to more frequent animal contact (Kaoud, 2015). Moreover, statistically non-significant relationship was found between the prevalence of mycotic endometritis and body condition score of cows. However, within body condition score groups, an increasing trend in the prevalence was observed from fattened to poor body condition score. This might be due to the fact that animals with a body condition score of less than 2.5 have compromised defense mechanism, more complications during parturition, increased incidence of dystocia and are more prone to retention of fetal membranes (Benti and Zewdie, 2014). Non-significant association of body condition score with the incidence of endometritis was also found by Gautam *et al.* (2010).Though the difference in the prevalence with respect to breeding method was non-significant, it was lower in cows bred through artificial insemination than those bred through natural mating, which may be due to better hygiene conditions during artificial insemination than the natural mating. Reproductive infections are more common in natural mating than artificial insemination and artificial insemination is also the best strategy to prevent venereal diseases (Mulu *et al.*, 2018).

The mean value of the endometrial thickness in Sahiwal cattle with mycotic endometritis was significantly higher than that for cows without mycotic endometritis. The normal thickness of endometrium in cattle is 7.0-9.5 mm before ovulation and 7.4-8.0 mm after ovulation (Souza *et al.,* 2011). However, the mean value of the endometrial thickness in Sahiwal cattle with mycotic endometritis observed in the present study was increased to 11.4±0.70 mm. Itraconazole (Rolac®) and fluconazole (Fungone®) were used for susceptibility testing of the isolated fungal pathogens. Itraconazole was found significantly more effective against *Aspergillus spp.* than *Penicillium spp*. However, both *Aspergillus* and *Penicillium spp.* were resistant to Fluconazole (Fungone®). For the treatment of mycotic endometritis, antifungal should be selected based on susceptibility testing (Beltaire *et al.*, 2012). The use of antifungal agents in bovine has not been reported as extensively as the use of antibiotics (Saini *et al.,* 2019). Itraconazole (Rolac®) and fluconazole (Fungone®) were studied in the current project due to their availability in the tablet form, which can be easily dissolved in a suitable liquid for intra-uterine infusions. Many other antifungal are available in the country (e.g., antifungal cream), however these are not suitable for intra-uterine therapy in the cattle.

Conclusions: The overall prevalence of mycotic endometritis in Sahiwal cattle in district Faisalabad was 2.8%. The prevalence of mycotic endometritis was non-significantly higher in animals maintained under conventional housing system, animals with BCS <2.5, parity number >4 and bred through natural mating. The prevalence was significantly higher in the animals having a history of reproductive problems. It is recommended that further studies should be undertaken to investigate the role of fungal pathogens in the etiology of reproductive disorders that do not respond to antibiotic treatment.

Authors contribution: HHK, ZIQ, MSS and SA designed the study. HHK, MH and MHR performed the field work/experiment. HHK, SA performed the lab work/experiment. HHK, MSW analyzed the data, wrote and reviewed the manuscript. ZIQ guided and supervised the study.

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