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RESEARCH ARTICLE

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

Hamza Hassan Khan[1]*, Zafar Iqbal Qureshi[1], Muhammad Salman Waqas[1], Muhammad Hussnain Rashid[1], Muhammad Saqib Saeed[1], Sultan Ali[2]* and Muzzammil Hassan[1]

[1]Department of Theriogenology, University of Agriculture Faisalabad, Pakistan
[2]Institute of Microbiology, University of Agriculture Faisalabad, Pakistan
*Corresponding authors: hamza.hassan2592@gmail.com; sultanali@uaf.edu.pk

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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, a total of 144 uterine samples were collected and cultured on Sabouraud's dextrose agar at 25°C for two weeks. Fungi were identified based on the microscopic characteristic. The results showed that out of 144 samples, 4 were found positive making an overall prevalence of mycotic endometritis in Sahiwal cattle 2.8% in Faisalabad. *Penicillium* and *Aspergillus* species were the isolated fungal pathogens. The prevalence of mycotic endometritis was significantly higher in cattle having a history of reproductive problem (P-value=0.03). 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 values of endometrial thickness in Sahiwal cattle suffering from mycotic endometritis was significantly higher (P-value=0.036) than 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, Fluconazole (Fungone®) was found resistant against both *Aspergillus spp.* and *Penicillium spp*. 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 do not 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 plays a crucial role. During 2018-19 livestock contributes 60.5% to the overall agriculture and 11.2% to the GDP, showing an increased growth rate of 4% during 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 cause for a decline in reproductive efficiency is reproductive disorders (Abdisa, 2018). The occurrence of bovine reproductive disorders is on the rise over the years (Yoo, 2010). Major reproductive problems in the cattle include endometritis, pyometra, retained placenta, abortion, anestrous, and repeat breading. These problems have a significant impact on the reproductive efficiency of the animal (Abdisa, 2018)

Infectious agents are a major cause 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 intrauterine (IU) 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 illness 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 check 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 antifungal susceptibility of 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).

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

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 intra-uterine samples were collected from repeat breeder cows (animals that had a regular estrous cycle but failed to conceive after three inseminations) and animals with the history of abortion, RFM, and 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).

Transrectal 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 inspected daily for fungal growth. Microscopic slides were prepared and stained with lactophenol cotton blue for the identification of fungi under a microscope (Derakhshandeh *et al*., 2015).

Antifungal susceptibility Test: Broth dilution method was performed to determine the antifungal susceptibility of the isolates. Itraconazole (Rolac®) and Fluconazole (Fungone®) were the antifungal agents used. 1ml of Sabouraud dextrose broth was added in six test tubes. 1 ml of antifungal drug solution was added into the first test tube making a total volume of 2 ml. 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

Overall prevalence: 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 raised under different managemental conditions (Fig. 1).

Prevalence of mycotic endometritis under different managemental conditions: Out of a total of 144 samples

51, 53, and 40 samples were collected from farms, conventional housing systems, and field conditions respectively. The prevalence of mycotic endometritis was 3.8%, 2.5% and 2% in conventional housing, field condition and farm respectively (Fig. 2). There was a statistically non-significant association (P>0.05) found between managemental conditions and the prevalence of mycotic endometritis.

Association of mycotic endometritis with Parity number: Considering the parity number as risk factors, three groups of the animals were made for data analysis. Out of a total of 144 samples, 31 belonged to parity number 0-2, 86 to parity number 3-4 and 27 to parity number >4. The prevalence of mycotic endometritis was 0%, 2.3% and 7.4% in animals with parity number0-2, 3-4 and >4 respectively (Fig. 3). Statistically non-significant association (P>0.05) was found between parity number and the prevalence of mycotic endometritis.

Association of mycotic endometritis with body condition score: Animals were divided into three groups with BCS <2.5, 2.5-3.5 and >3.5 for data analysis. Three samples were positive in animals with BCS <2.5 and one positive samples in animal with BCS group 2.5-3.3 while the third group of the animal having BCS >3.5 had no positive sample. The prevalence of mycotic endometritis was 6%, 1.3% and 0% in animals with body condition score (BCS) <2.5 and 2.5-3.5 and >3.5, respectively. (Fig. 4). The association between the prevalence of mycotic endometritis and body condition score (BCS) was non-significant (P>0.05).

Association of mycotic endometritis with Reproductive problems: 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. Prevalence of mycotic endometritis was 13.3%, 7.7%, 1.0% and 0% in Sahiwal cattle with the history of abortions, RFM, repeat breeding and uterine prolapse, respectively (Fig. 5). History of a reproductive problems had a significant (P<0.05) effect on the prevalence of mycotic endometritis.

Association of mycotic endometritis with Herd size: 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=1-30, medium herd= 30-60 and large herd= 60-90 animals in the herd. Prevalence of mycotic endometritis was the highest (3.7%) in large herd whereas in medium and small herds prevalence was 2.4% and 2.1%, respectively (Fig. 6). The difference in the prevalence of mycotic endometritis among the different herd size groups was statistically non-significant (P>0.05) thus herd size was not a risk factor for fungal endometritis.

Association of mycotic endometritis with Breeding Methods: The prevalence of mycotic endometritis was 4.5% in the herd employing natural mating and 2.5% in the herds using artificial insemination (Fig. 7). There was a non-significant difference between breeding methods and the prevalence of fungal endometritis.

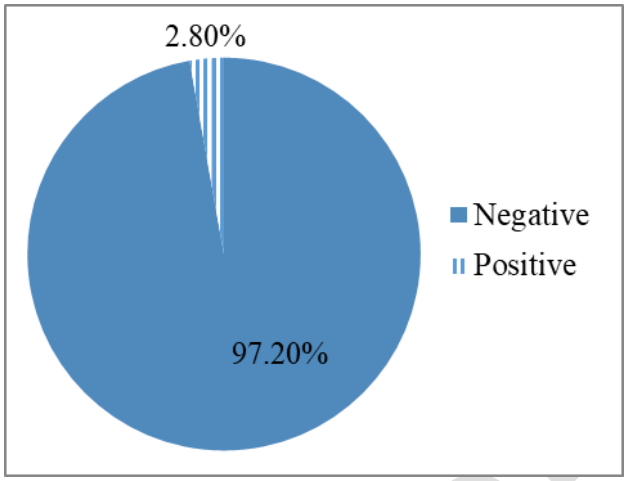

Fig. 1: Overall prevalence of mycotic endometritis in Sahiwal cattle.

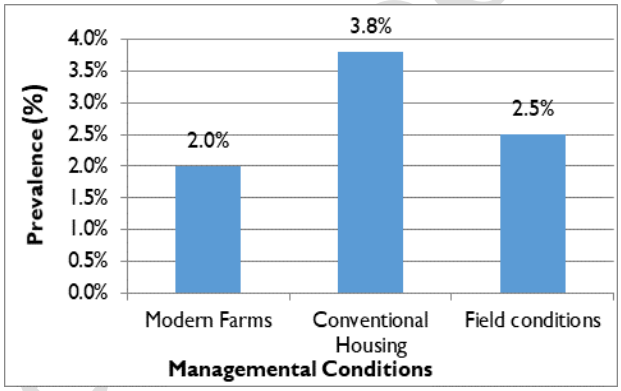

Fig. 2: Relationship of mycotic endometritis with different managemental conditions.

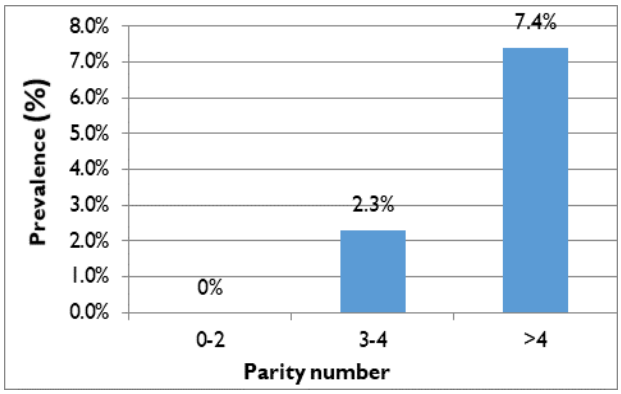

Fig. 3: Relationship of mycotic endometritis with parity number

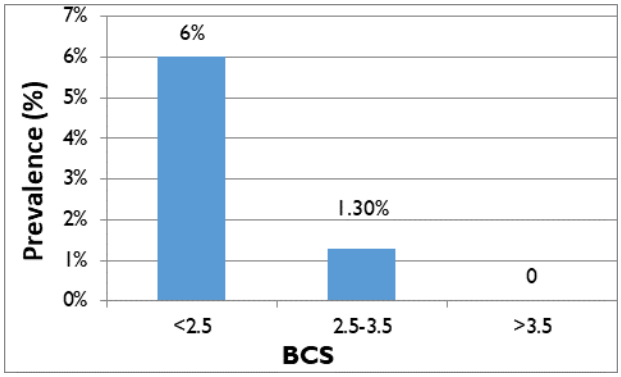

Fig. 4: Relationship of mycotic endometritis with BCS.

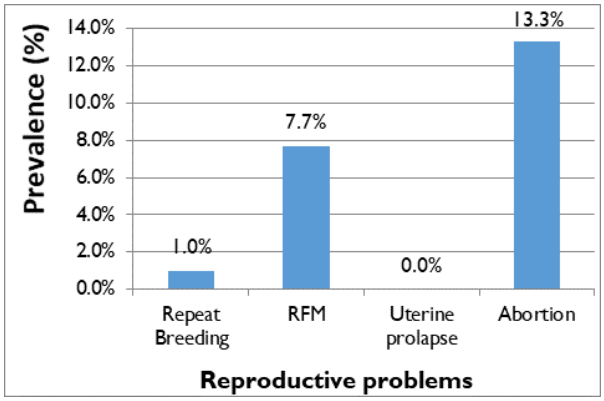

Fig. 5: Relationship of mycotic endometritis with reproductive problems.

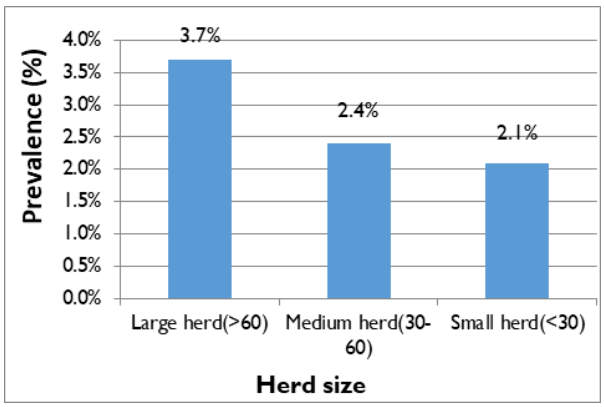

Fig. 6: Relationship of mycotic endometritis with Herd size.

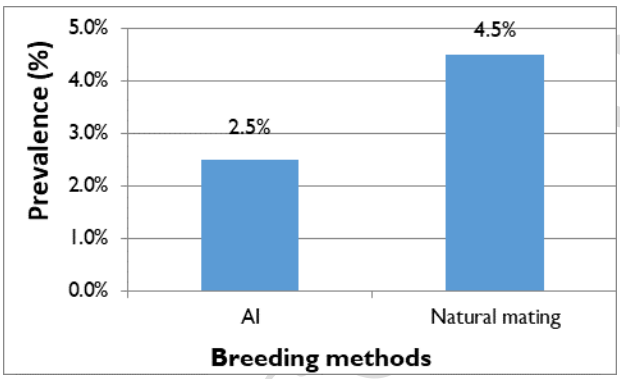

Fig. 7: Relationship of mycotic endometritis with breeding methods.

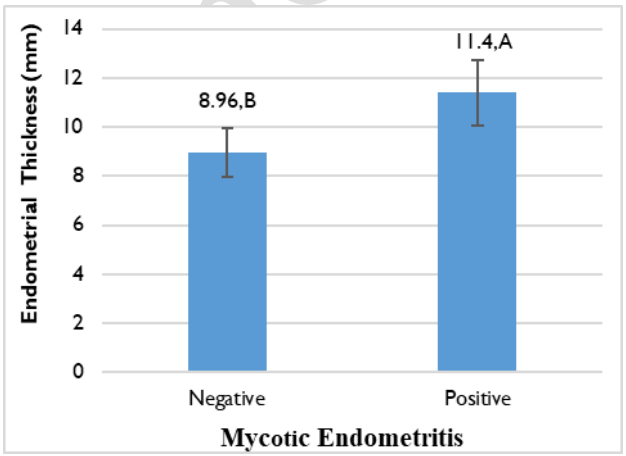

Fig. 8: Mean values of endometrial thickness in relation to mycotic endometritis.

Fig. 9: Ultrasonographic measurement of the thickness of endometrium wall in infected cattle.

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

Table 1: Antifungal susceptibility Test Results. Different superscripted letters indicate significant difference (P-value<0.05)

Antifungal Drugs	Fungus	MIC (mg/ml)
Fluconazole (Fungone®)	*Aspergillus*	Resistant
	Penicillium	Resistant
Itraconazole (Rolac®)	*Aspergillus*	4.12[a]
	Penicillium	8.6[b]

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

Identification of Fungi and Minimum inhibitory concentration: 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.* 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 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)

The results of the current study showed that the prevalence of mycotic endometritis was non-significantly higher (3.8%) in the cattle reared under conventional housing systems than those reared under field condition (2.5%) and modern farms (2%). Though, it was non-significant the difference 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. However, the prevalence rate was higher 7.4% in the cattle with parity number >4 compared to 2.3% in the cattle with parity number 3-4, and 0% in the cattle with parity number 0-2. This might be due to the stronger defense mechanism of younger animals and more rapid uterine involution (Adnane *et al*. 2017). High prevalence in the animals with parity number >4 is probably due to recurring contact of the reproductive tract to environmental factors, which result 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.

A statistically significant relationship between mycotic endometritis and reproductive problems was found. The prevalence rate was higher (13.3%) in the cattle having a history of abortion, followed by 7.7% and 1.0% in the cows with a history of retained fetal membranes and repeat breeding, respectively. The risk of infection and thus chances of metritis may rise as a result of the reproductive infection. Necrotic foci present due to retention of fetal membrane is considered 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 a large herd, small herd, and medium herd respectively. The higher prevalence of fungal endometritis at larger herds is probably due to lower management practices, rapid and large-scale disease transmission due to more frequent animal contact (Kaoud, 2015)Different body condition score groups (<2.5, 2.5-3.5, >3.5) were studied and analyzed statistically.

Statistically non-significant relationship was found between the prevalence of mycotic endometritis and body condition score. 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 reason that animals with a body condition score of less than 2.5 have compromised defense mechanisms, 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 on the incidence of endometritis was also found by Gautam *et al*. (2010).

The prevalence of mycotic endometritis was 4.5% in the natural mating system, and 2.5% for artificial insemination. Though the difference was non-significant, it 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 best strategy to prevent venereal diseases (Mulu *et al*., 2018).

Itraconazole (Rolac®) and fluconazole (Fungone®) were used for antifungal susceptibility testing of the isolated fungal pathogens. Itraconazole was found significantly more sensitive against *Aspergillus spp.* than *Penicillium spp*. while Fluconazole (Fungone®) was resistant against *Aspergillus* and *Penicillium spp.* For the treatment of mycotic endometritis, antifungals should be selected on the basis of 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. The tablet can be dissolved in a suitable liquid for intrauterine infusions. Many other antifungals are available in the country however the products (e.g., cream) are not suitable for intrauterine therapy in the cattle.

The mean values of the endometrial thickness in Sahiwal cattle with mycotic endometritis were significantly more than those without mycotic endometritis. The normal thickness of endometrium in cattle is 7-9.5 mm before ovulation and 8-7.4 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 11.4mm.

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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