



# Isolation and morphological identification of fungi from endometrial samples of postpartum dairy cows<sup>#</sup>

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Citation: Neelima, P., Sumod, K., Promod, K., Chacko, L. and Paul, M.K. 2024. Isolation and morphological identification of fungi from endometrial samples of postpartum dairy cows.

*J. Vet. Anim. Sci.* **55**(2):354-359

DOI: <https://doi.org/10.51966/jvas.2024.55.2.354-359>

Received: 26.10.2023

Accepted: 25.01.2024

Published: 30.06.2024

## Abstract

Numerous studies on endometritis of postpartum dairy cows describe the bacterial aetiology of the condition. However, studies on fungal agents causing inflammatory conditions of the reproductive tract are limited. The objective of the present study was to identify the mycotic isolates from the reproductive tract of postpartum dairy cows and analyse their susceptibility to antifungal agents. In this study, 70 endometrial cytobrush samples were collected from dairy cows 40 - 120 days postpartum (dpp) and cultured in Sabouraud dextrose agar (SDA). The isolated fungal cultures were identified by Lactophenol Cotton Blue staining and were subjected to antifungal susceptibility testing. The frequency of isolation of fungi from endometrial samples was 15.71 per cent (11/70). *Aspergillus niger* was isolated from four (33.33%) samples, followed by *Penicillium* spp. (25%), *Aspergillus fumigatus* (16.67%), *Mucor* spp. (16.67%) and *Absidia* spp. (8.33%). Antifungal susceptibility testing revealed that 50 per cent of the isolates were susceptible to fluconazole, whereas only 25 per cent of the isolates were completely resistant to amphotericin-B. The prevalence of fungal infection in reproductive tract was higher in cows with an age of  $\geq 5$  years and a parity of  $\geq 2$ , though this was not significant. A significantly higher number of cattle with history of intrauterine antibiotic therapy were positive for fungal culture. However, the association between prevalence of fungi and reproductive complications was statistically non-significant.

**Keywords:** Postpartum dairy cows, fungal infections, *Aspergillus niger*, mycotic infections

<sup>#</sup>Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Endometritis is one of the most significant postpartum complications in cattle causing financial losses to the farmers. Hence, early diagnosis and treatment of endometritis is a crucial part of reproductive management in cattle. Even though there are extensive studies on bacterial pathogens that cause endometritis, limited information is available with respect to mycotic agents causing inflammatory changes in the reproductive tract of postpartum cows. Fungi are opportunistic pathogens that can establish in uterus or vagina if chronic infections are present (Stout, 2008). There are several predisposing factors which could facilitate the growth of fungus in the reproductive tract (Saini *et al.*, 2019). Repeated intrauterine antibiotic therapy in cattle is one of the major factors that disturb the normal microbial flora of the reproductive tract. The change in the vaginal flora reduces the competition for available nutrients and disturbs the vitamin synthesis by normal microflora which enables the fungal species to flourish. Anatomical defects of external genitalia such as horizontal displacement of vulva and deep ano-rectal fossa due to poor body condition can cause entry of air, dung and pooling of urine at the anterior part of vagina, leading to pneumo-vagina or uro-vagina in pluriparous cows. This could cause contamination of the caudal reproductive tract that facilitates ascending reproductive tract infections. Prolonged corticosteroid therapy, progesterone therapy, immunosuppressive medication, cytotoxic drugs and endocrine abnormalities in cattle can result in immunosuppression that facilitate establishment of infection by the opportunistic commensal fungi present in the reproductive tract. Stress, negative energy balance and vitamin and mineral deficiencies can also lead to immunosuppression and trigger the growth of fungal organisms in reproductive tract. Postpartum complications like dystocia and retention of foetal membranes provide favourable environment for the growth and establishment of fungal infection. Unhygienic artificial insemination (AI) and calving assistance in abnormal calving could facilitate the introduction of pathogenic organisms, including fungi into the uterus.

Fungi were isolated from reproductive disorders like vulvo-vaginitis, cervicitis and

mycotic abortions (Ali *et al.*, 2016). The treatment of fungal endometritis has been found to be challenging and positive response to the treatment was generally poor (Scott, 2018). Inadequate duration of therapy, incorrect choice or dosage of anti-fungal medication, failure to treat reservoirs of infection and inability to correct predisposing conditions such as pneumo-vagina could be the reasons for an unsuccessful course of treatment.

## Material and methods

### Experimental animals

The present study was conducted at the Instructional Livestock Farm Complex (ILFC), College of Veterinary and Animal Sciences, Pookode and Livestock Research Station (LRS), Thiruvazhamkundu and organized farms in Wayanad District. Crossbred dairy cows between 45 and 120 days postpartum, aged between three to ten years and with a parity between one to seven were selected for the study. History of the cows was taken in detail regarding the age, breed, parity, previous history of any reproductive complications and history of unhygienic practices by laymen or unqualified practitioners.

### Primary isolation of fungi

A modified cytobrush (Gayathri *et al.*, 2020) was created by heat-fixing a human cytobrush to a sharpened stylet of an artificial insemination (AI) gun, which was then inserted into an AI sheath covered by a sanitary plastic sleeve. The cytobrush assembly was inserted into the anterior vagina. The sanitary sleeve was punctured at the external os of the cervix and the stylet was pushed forward to expose the cytobrush. Endometrial samples were collected by rotating the stylet in clockwise direction. After collection, the stylet was retracted back into the AI sheath within the uterus itself and the whole assembly was withdrawn from the genital tract.

Each cytobrush was rolled on the surface of Sabouraud Dextrose Agar (SDA) in a Petri plate using a sterile forceps while slightly pressing over the agar surface. The culture plates were sealed using parafilm and incubated at 25°C. The plates were examined

daily for the presence of fungal colony growth for up to two weeks. The characteristics of the mycelia were observed and analysed in those SDA plates which showed fungal growth.

### Identification of fungal cultures

Colony characteristics like colour, texture of the colony surface, extent of fungal growth and the presence or absence of pigmentation were observed from both obverse and reverse side of fungal culture plates and recorded. Staining of fungal isolates using Lactophenol Cotton Blue (LPCB) was carried out to see the microscopic morphology of the isolates under 400 × magnification.

### In vitro antifungal susceptibility test

Antifungal test was performed by disc diffusion method, according to CLSI guidelines (CLSI document M51) and manufacturer's instructions. The medium used was Mueller-Hinton agar supplemented with two per cent glucose and 0.5 mg/L methylene blue dye. The antifungal discs tested were amphotericin B (20 mg), fluconazole (10 mcg), miconazole (30 mcg), ketoconazole (10 mcg) and nystatin (50 mg). Within 15 min after the application of discs over the inoculated plate, it was incubated at 35 °C for 24 h. At the end of the incubation, the plates were examined and the diameters of the zones of complete inhibition were measured to the nearest whole millimetre, with a scale held at the bottom of the Petri plate, which was illuminated with reflected light. The mean

diameter of the zone of inhibition of each disc was compared with that of the standard zone of inhibition as per Beltaire *et al.* (2012) and the isolates were grouped as resistant (R), susceptible (S) or intermediately susceptible (I) for each selected antifungal disc.

### Statistical analysis

The data obtained were tabulated and analysed as per standard statistical procedures by SPSS version 24.0 and results were interpreted. Chi-square analysis and Yate's continuity correction were used for comparisons.

### Results and discussion

Out of the 70 samples, 11 (15.71%) were positive for fungal isolation which yielded 12 isolates. Among these 11 samples, single isolates were obtained from 10 cultures (90.9%) and one (9.09%) sample yielded two isolates. No fungal growth could be obtained from 59 samples. Sharma and Singh (2012) obtained mycotic isolates from 15.5 per cent of the uterine discharge samples from cows which were comparable with the results of the present study. Ahmed and Bhattacharyya (2015) also collected uterine samples from repeat-breeder animals and obtained fungal growth in 17.98 per cent of them (10 cows and 6 buffalo). The most common fungus isolated from the reproductive tract in cattle in the current study was *Aspergillus niger* (Fig.1) followed by *Penicillium* spp. (Fig. 2), *Aspergillus fumigatus*

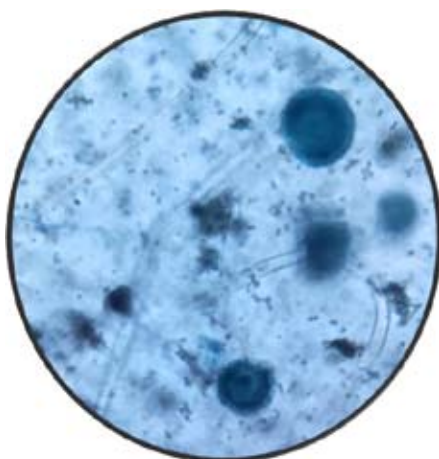
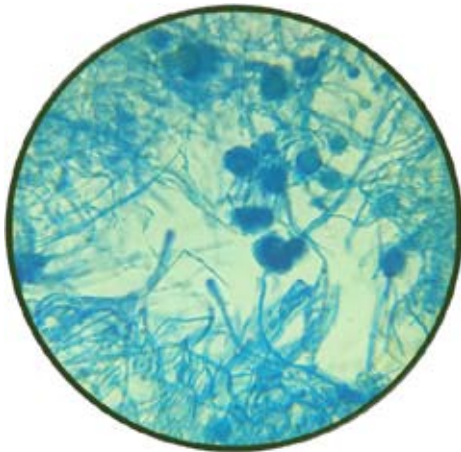


Fig. 1. Microscopic view of *Aspergillus niger*



Fig. 2. Macroscopic view of *Penicillium* spp.



**Fig. 3.** Microscopic view of *Aspergillus fumigatus*

(Fig. 3), *Mucor* spp. and *Absidia* spp. (Table 1). Khan *et al.* (2020) collected uterine fluids from Sahiwal cows with reproductive disorders and recorded a total prevalence of 2.8 per cent for mycotic endometritis in cattle in Faisalabad. The primarily isolated fungal pathogens in their study were *Penicillium* and *Aspergillus* species. These findings coincided with those of the current study. The lower prevalence of mycotic agents might be due to lower humidity and hot and dry climate of the area. Kour *et al.* (2023) collected discharges from various parts of reproductive tract of cattle and buffaloes ( $n = 70$ ) with reproductive disorders, which yielded 41 fungal isolates from 30 samples. The higher prevalence might be due to selection of animals with complicated parturition or puerperium in the study.

#### ***In vitro* antifungal susceptibility testing**

Fluconazole was found to be effective against 50 per cent of the isolates and 33.33 per cent of isolates were sensitive to ketoconazole, nystatin and amphotericin-B. Miconazole was found ineffective against all 12 fungal isolates. Only 25 per cent of the isolates were resistant to amphotericin-B. Beltaire *et al.* (2012) recorded 75 per cent sensitivity for amphotericin-B and nystatin, 38 per cent sensitivity for ketoconazole and 100 per cent resistance against fluconazole in mould isolated from reproductive tract of mares with fungal endometritis.

#### **Relationship of age and parity to fungal culture**

**Table 1.** Types of fungal isolates from uterine samples

Fungal isolates obtained	Number	Percentage (%)
<i>Aspergillus niger</i>	4	33.33
<i>Penicillium</i> spp.	3	25.0
<i>Aspergillus fumigatus</i>	2	16.67
<i>Mucor</i> spp.	2	16.67
<i>Absidia</i> spp.	1	8.33
<b>Total</b>	<b>12</b>	<b>100</b>

Out of 11 postpartum dairy cows positive for fungal culture, 45.46 per cent belonged to the <5 years age group, while 54.54 per cent belonged to the  $\geq 5$  years age group. In the 59 cows negative for fungal culture, 87.80 per cent belonged to the <5 years age group, though this was not significant ( $p > 0.05$ ). The study found that 20 per cent of positive cows had a parity of < 2, while 80 per cent had a parity of  $\geq 2$ . Among negative cows, 14.55 per cent had a parity of < 2 and 85.45 per cent had a parity of  $\geq 2$ , a statistically non-significant difference (Table 2).

Endometritis was more prevalent among pluriparous cows which were in their third or later lactation than in primiparous due to recurring contact of the reproductive tract to the external environmental factors during each parturition and inseminations, which resulted in increased uterine infections (Khan *et al.*, 2020). Our study shows that the incidence of endometritis attributable to factors associated with higher age and parity is not significant in dairy cows in North Kerala.

#### **Association of reproductive complications with positive fungal culture of uterine samples**

Out of 70 postpartum dairy cows, 32.85 per cent had reproductive complications, with 26.08 per cent positive and 73.91 per cent negative for fungal culture. Out of 47 cows without complications, five were positive and 42 negatives for fungal culture. However, no significant association ( $p > 0.05$ ) between reproductive complications and incidence of fungal culture from uterus was observed (Table 2). Our findings are in contrast to the findings

**Table 2.** Factors associated with positive fungal growth in the reproductive tract of postpartum cows

Criteria	Growth of fungal culture			Test statistic value (P value)
	Positive (%)	Negative (%)	Total (n=70)	
Age groups (< 5 years)	5 (9.76)	36 (87.80)	41	0.925 <sup>1</sup> (0.607 <sup>ns</sup> )
Age groups (≥ 5 years)	6 (20.69)	23 (79.31)	29	
Parity < 2	3 (20.00)	12 (80.00)	15	0.013 <sup>2</sup> (0.607 <sup>ns</sup> )
Parity ≥ 2	8 (14.55)	47 (85.45)	55	
With history of reproductive complications	6 (26.08)	17 (73.91)	23 (32.85)	2.783 <sup>1</sup> (0.095 <sup>ns</sup> )
Without history of reproductive complications	5 (10.63)	42 (89.36)	47 (67.14)	
With history of intrauterine antibiotic therapy	7 (58.33)	5 (41.67)	12	16.114 <sup>2</sup> (0.00 <sup>***</sup> )
Without history of intrauterine antibiotic therapy	4 (6.90)	54 (93.10)	58	
October- February	9 (39.13)	14 (60.87)	23	11.70 <sup>1</sup> (0.000 <sup>***</sup> )
March- June	2 (4.26)	45 (95.74)	47	

(NS - non-significant ( $p > 0.05$ ), \* - significant ( $p < 0.05$ ),

\*\* significant ( $p < 0.01$ ); 1- Chi square value, 2 - Yates continuity correction value)

of Khan *et al.* (2020), as they reported that retention of foetal membrane after parturition could leave necrotic foci and a residual tissue for the growth of pathogens including fungi in the uterus.

#### **Association of history of intrauterine antibiotic therapy and positive fungal culture of uterine samples**

Among the 70 postpartum dairy cows studied, 12 (17.14%) had a history of treatment with repeated intrauterine antibiotics and 58 (82.85%) had no history of intrauterine antibiotic therapy. Out of the 12 cows with the history of repeated intrauterine antibiotic therapy, seven (58.33%) were positive and five (41.67%) were negative for fungal culture. Among the 11 cows positive for fungal culture, a significantly ( $p < 0.01$ ) greater number of animals (63.64%) had the history of intrauterine antibiotic therapy (Table 2). Retrograde flow

of the antibiotics from uterus to the vagina after an intrauterine therapy could disrupt the normal microbiota of the vagina which reduced the competition for nutrients and enabled the opportunistic commensal fungal organisms present in the caudal reproductive tract to shift into pathogenic fungal organisms and establish infections (Saini *et al.*, 2019).

#### **Association between incidence of uterine positive fungal culture and the effect of season**

The study was conducted from a time period of October 2022 to June 2023. A total of 23 samples were collected during the time period from October to February, of which nine (39.13%) samples were positive for fungal culture while 14 (60.87%) samples were negative. The remaining 47 samples were collected from March to June, out of which only two (4.26%) samples were positive for

fungal culture and 45 (95.74%) samples were negative (Table 2). The relative humidity in Wayanad district of Kerala from the months of Oct-Feb is an average of 90 per cent and from the months of Mar-Jun is an average of 58 per cent, which is significantly lower. Shokri and Yadollahi (2017) reported that high density of animals and topographic and climate variations, particularly high relative humidity, could lead to higher prevalence of fungal endometritis.

### Conclusion

The overall incidence of mycotic agents in the reproductive tract of postpartum dairy cows was 15.71 per cent. Antifungal susceptibility test revealed that fluconazole was effective against 50 per cent of isolates, whereas only 25 per cent of the isolates were completely resistant to amphotericin-B. The prevalence of incidence of fungus in reproductive tract was not significantly associated with age and parity. Significantly higher number of cattle with a history of intrauterine antibiotic therapy was positive for fungal culture. However, the association between positive fungal cultures and reproductive complications were statistically non-significant.

### Acknowledgments

The financial support provided by Kerala Veterinary and Animal Sciences University is acknowledged.

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