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Study on Mycoflora of female reproductive tract infections of large dairy animals in Punjab

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Abstract
Reproductive infections take a heavy toll on productivity of livestock and large dairy animals are often found infected by mycotic agents due to their general habits. In this study, 70 reproductive tract samples were collected from cows and buffaloes from different districts of Punjab suffering from infectious reproductive disorders and were processed for fungal examination using isolation and identification procedures. This examination yielded 41 isolates from 30 fungal cultures belonging to *Aspergillus* spp. (18) where *A. flavus* had the maximum isolates (6) followed by *Alternaria* spp. (9), *Penicillium* spp. (3) and other fungi like *Rhizopus* spp., *Mucor* spp., *Mortierella* spp., *Curvularia* spp. were isolated in less numbers.

Keywords: *Aspergillus*, *Mortierella*, mycotic infections

Introduction
Mycotic agents or fungal elements are important and ubiquitous part of any living being's environment. Some are beneficial and are put to purposed use while others are present as commensals on bodies of animals and human beings. These commensals, sometimes, become pathogenic when they encounter an immunocompromised host body and cause diseases in them. Apart from these, some fungal elements themselves are aggressively pathogenic to cause diseases, particularly in animals due to their housing and cleanliness conditions. Reproductive diseases in animals are usually of bacterial or protozoal origin, but fungal diseases are also not uncommon. Fungi are opportunistic pathogens which are capable of causing disturbed uterine or vaginal environment [1]. Several studies have reported prevalence of fungal endometritis in repeat breeder cows [2, 3]. Diseases could be due to direct invasion by mycotic agents like *Aspergillus* spp. or by predisposition of animals due to immunosuppression or as secondary mycotic infections by opportunistic fungi. Disorders like abortions, metritis are very commonly observed due to fungi; mycotic abortion in large dairy animals being a common one.

Materials and Methodology
A total of 70 reproductive tract disorder samples were collected from cases of abortion, retained placenta, repeat breeders, anoestrus, pyometra, metritis and endometritis consisting of vaginal mucous, cervical mucous, uterine discharges, uterine pus and aborted materials like placenta, caruncles, foetal stomach contents, amniotic fluid and placental fluid as tabulated in Table 1. Faecal material or dirt was removed from external genitalia followed by thorough cleaning with Cetrimide solution (2.25% w/v) and mopping of vulvar lips using sterilized cotton before sample collection. Cervical and uterine discharges were collected using sterilized plastic sheaths on artificial insemination guns and transferred to sterile vials. Mucous and mucous plugs were taken from vagina using hands covered with sterile gloves. All collected samples were transported on ice from dairy farms of Punjab and clinical cases from GADVASU clinical complex. If the processing had to be delayed, the samples were kept at 4 °C until processed. All the samples were collected as paired samples, over a fortnight i.e. samples were collected again from the same animal 15 days later after the first collection to ensure that the fungi isolated were not commensals but were actual pathogens or opportunistic invaders.

Table 1: Enumeration of types of samples collected from different animals with specific reproductive disorders

Type of infection/disorder	Cattle				Buffalo			
Type of specimen	**FSC**	**UD**	**VM/D**	**PL/F**	**FSC**	**UD**	**VM/D**	**PL/F**
Abortion	7	9	2	3	5	7	0	2
Pyometra/Metritis/Endometritis	0	13	0	0	0	2	0	0
ROP	0	2	1	1	0	2	0	0
Repeat breeding	0	4	2	0	0	2	5	0
Cervicitis	0	1	0	0	0	0	0	0

Samples were then processed according to their type. e.g. Mucous samples were vortexed at high speed for 15 seconds and sediments were collected. Tissue samples were triturated in sterile pestle and mortar using sterile sand. The triturate was collected in a 15ml centrifuge tube, centrifuged at 3000xg for 5 minutes. The supernatant was collected and used for inoculation. Discharges and fluid samples were used as such.

Samples were then inoculated onto Sabouraud Dextrose Agar (SDA) and BHI (Brain Heart SInfusion) agar and were incubated at 25 °C from 5-15 days till visible colonies were obtained. Duplicate sets of plates for each sample were incubated at 37 °C for growth of yeast-like fungal elements. Obtained colonies were subjected to macroscopic (Observe and reverse view morphology) and microscopic (LPCB staining and observation under 40X power) examination and biochemical tests, wherever applicable. Slide culture technique was also employed to observe intact morphology in the fungal cultures.

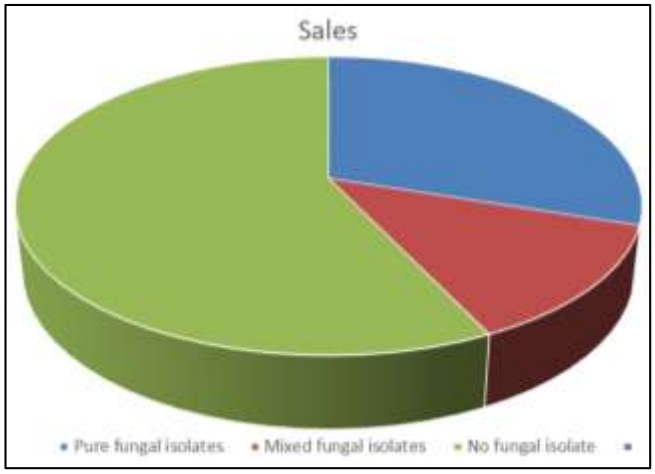
Results and Discussion

Disease-causing mycotic agents enter host body by two mechanisms. One is direct invasion which is caused by highly pathogenic fungal elements while other infections are opportunistic since fungi are ubiquitously present and can attack the host once its immunity is lowered.

In this study, 30 samples yielded fungal elements upon incubation at 25 °C and 37 °C among which 41 isolates were obtained as shown in Table 2. Among these 30 samples, 21 (70%) samples yielded single isolates while 9 samples (30%) yielded mixed isolates. However, 40 samples yielded no fungal growth at all as shown in the table below.

Table 2: Type of fungal cultures obtained

S. No.	Type of fungal culture obtained	Number of cultures
1.	Pure fungal isolates	21
2.	Mixed fungal isolates	09
3	No fungal isolate	40
Total samples inoculated		70



Upon identification, a total of 14 fungal species from 10 genera were isolated from 30 different reproductive disorder samples. Here, genera isolated were *Alternaria* spp. (21.95%), *Aspergillus* spp. (43.9%), *Candida glabrata* (2.4%), *Curvularia* spp. (2.4%), *Fusarium* spp. (4.8%), *Mortierella* spp. (2.4%), *Mucor* spp. (4.8%), *Penicillium* spp. (7.3%), *Pithomyces* spp. (4.8%) and *Rhizopus* spp. (4.8%). Details of isolates obtained were as given in Table 3. All the fungal isolates conformed to the typical characteristics of their genera and species [4] as per Fig 1-3.

Table 3: Different fungal isolates obtained from clinical samples and their distribution

S. No.	Fungal isolates obtained		Number		
1.	*Alternaria* spp.		9	Pure	5
				Mixed	4
2.	*Aspergillus* spp. (Total=18)	*Flavus*	6	Pure	4
				Mixed	2
		Fumigatus	4 (all pure growths obtained)		
		Niger	5	Pure	2
				Mixed	3
		Ochraceous	1 (from mixed growth)		
		Other spp.	2 (both pure growths obtained)		
3.	*Candida glabrata*		1 (from mixed growth)		
4.	*Curvularia* spp.		1 (from mixed growth)		
5.	*Fusarium* spp.		2	Pure	1
				Mixed	1
6.	*Mortierella* spp.		1 (from mixed growth)		
7.	*Mucor* spp.		2	Pure	1
				Mixed	1
8.	*Penicillium* spp.		3	Pure	1
				Mixed	2
9.	*Pithomyces* spp.		2	Pure	1
				Mixed	1
10.	*Rhizopus* spp.		2 (from mixed growths)		
Total number of isolates			41		

Among these isolates, one fungal species was *Candida glabrata*. To identify and confirm it, biochemical tests were conducted which yielded results as shown in the Tables 4 and 5.

Table 4: Biochemical employed for identification of *Candida glabrata*

Growth with CYH at 25 °C	Growth on SDA at 37 °C	Germ tube test	Urease production (25 °C)
-	+	-	-

CYH-Cycloheximide, SDA-Sabouraud Dextrose Agar

Table 5: Carbohydrate utilisation tests employed for *Candida glabrata*

Carbohydrate utilisation tests								
Gal	**In**	**La**	**Ma**	**Rf**	**Suc**	**Tre**	**Xy**	**Glu**
_	_	_	_	_	_	+	_	+ (fermentative)

Gal-Galactose, In-Inositol, La-Lactose, Ma-Maltose, Rf- Raffinose, Suc-Sucrose,
Tre-Trehalose, Xy-Xylose, Glu-Glucose

Fig 1: Isolate of *Aspergillus fumigatus*

Fig 2: Iasolate of *Aspergillus ochraceous*

Fig 3: Microscopic view of *Aspergillus fumigatus*

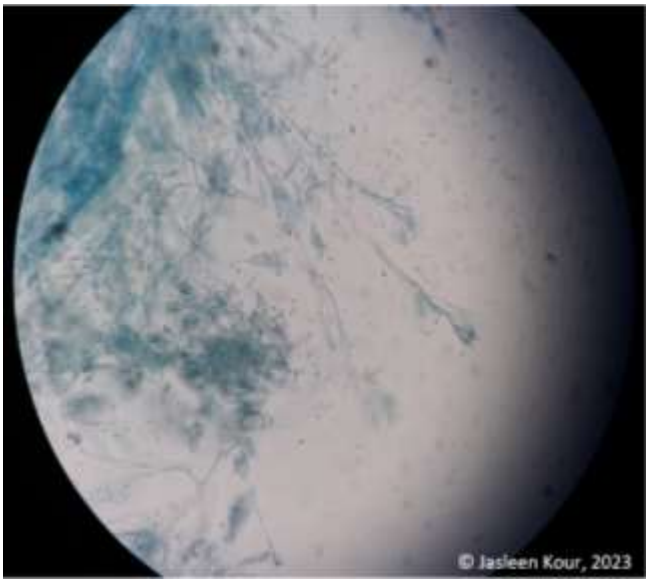


Fig 4: Microscopic view of *Penicillium* spp.

Fungi, being majorly abundant microbiota of our ecosystem, have easy access to act as secondary invaders in an infection or as opportunistic pathogens. In a report studying cervico-vaginal fluids for fungal elements, *Penicillium* spp. and yeast have been found in cervix and vagina of infertile/repeat breeder cows as 27.14% and 28.57% respectively [5]. In another study of pathogenic fungi prevalence [6], it was reported that 17.98% of causative agents were fungal element in cases of repeat breeders with Asperg*illus fumigatus* and *Penicillium* spp. to be the most common fungi. In another study [7], it was reported that isolation of a total of 20 fungal species related to 8 genera from 25 samples of vaginal swabs was done which were collected from cases of cows which suffered from abortion. The main recovered genera of fungi were *Aspergillus* spp. (80%), *Fusarium* spp. (16%), *Penicillium* spp. (32%), *Alternaria* spp. (8%) and *Candida albicans* (40%) which is similar to the present study. In another experiment [8], nine different genera were isolated, i.e. *Aspergillus*, *Candida*, *Fusarium*, *Mucor*, *Penicillium*, *Absidia*, *Geotrichum*, *Rhodotorula and Rhizopus* from repeater cows and buffaloes. In a study conducted [9], *Candida albicans* was found to be the cause of endometritis in buffaloes as an opportunistic yeast. In addition, the characteristics of all the fungal isolates obtained were similar to those described by Larone [10]. Biochemical tests and carbohydrate utilization tests were also conducted for various fungi all of which were in corroboration with the writings of Larone [10] and Quinn [4].

Conclusions

Mycotic infections are prevalent in substantial amounts in Punjab region among large dairy animals according to this study wherein *Aspergillus flavus* has been found to be the most common and most pathogenic fungal element affecting reproductive tract and its functions, mostly isolated from abortion cases. Other fungal elements have been found to be opportunistic pathogens.

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Declaration

None of the authors have any conflicts of interest.

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