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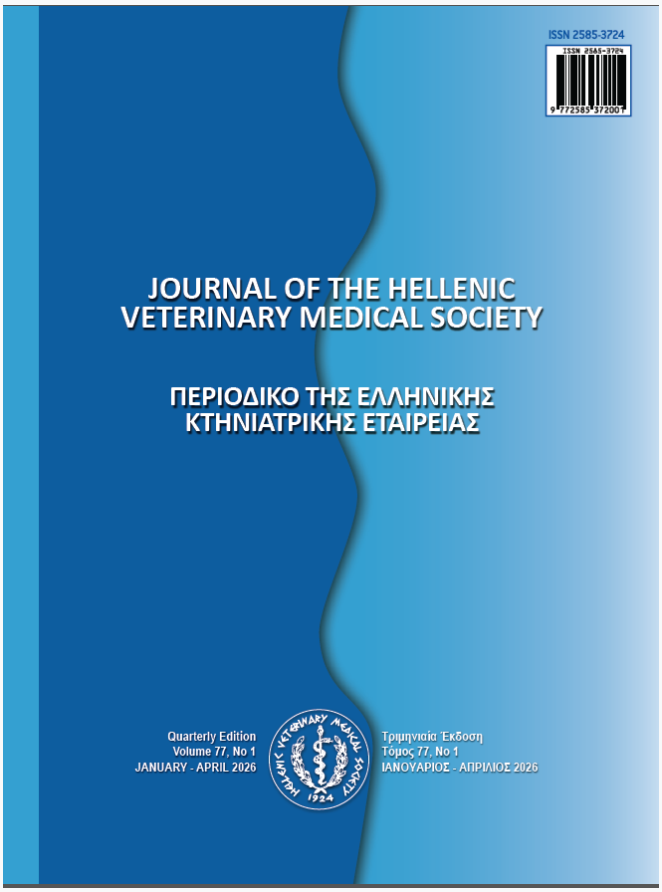

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Molecular detection and antifungal resistance profile of *Candida* spp. isolated from bovine mastitis cases

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ABSTRACT: Mycotic mastitis is an increasingly recognized problem in dairy herds, with *Candida* species emerging as significant etiological agents. Considering the growing concern about fungal mastitis in dairy production, this study was conducted to determine the prevalence and antifungal susceptibility profiles of *Candida* species isolated from bovine mastitis cases in Gujarat, India. A total of 608 bovine mastitic milk samples were collected and examined for fungal presence. Identification of *Candida* species was performed based on colony morphology, Gram staining and the germ tube test, with confirmation by PCR using genus-specific primers. Antifungal susceptibility patterns were evaluated using the standardized disc diffusion method. Results revealed that, fungal pathogens were present in 10.5% (64/608) of the samples, with *Candida* species specifically accounting for 1.64% (10/608) of the total bovine mastitis cases. Notably, all isolates were negative for germ tube production. Molecular analysis confirmed the phenotypic identification, with all suspected *Candida* isolates generating the expected amplicon size between 250-350 bp. Antifungal susceptibility testing indicated that fluconazole was the most effective agent (100% susceptibility), while ketoconazole showed limited effectiveness against the isolated strains.

Keyword: Antifungal susceptibility; *Candida* species; Dairy herds; Mycotic mastitis; PCR

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INTRODUCTION

Bovine mastitis is a persistent enigmatic issue in dairy animals worldwide, characterized by inflammation of udder tissues caused by physical injury or by a range of microorganisms, such as bacteria, mycoplasmas, viruses, fungi and algae. It is considered the most common disease leading to economic loss in dairy industries, primarily due to reduced yield and poor quality of milk (Gomes and Henriques, 2016).

Fungal infections represent approximately 2-13% of all cases of mastitis in cows (Krukowski et al., 2006; Krukowski et al., 2001). Many fungi such as *Aspergillus* spp., *Candida* spp., *Cryptococcus neoformans*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Pichia farinosa*, *Rhodotorula glutinis*, *Saccharomyces fragilis* and *Trichosporon beigelii* are isolated from mastitic milk of animals throughout the world (Pal, 2018). Although the incidence of fungal mastitis is generally low, it has notably increased over the past decade, with *Candida* species being the most commonly identified mycotic agents causing bovine mastitis (Radostits et al., 2007; Krukowski et al., 2006).

The predisposing factors for fungal mastitis include poor milking hygiene, hot and humid environmental conditions, misuse of antibiotics, fungal- contaminated fodder, substandard barn sanitation and errors in diagnostic assessment (Pal, 2007; Rawat et al., 2020). Additionally, immunocompromised animals are particularly susceptible to fungal mastitis due to their weakened defence mechanisms, which allows opportunistic fungal pathogens to establish infection more readily (Seker, 2010).

Mycotic mastitis is challenging to treat because many fungi utilize antibiotics like tetracycline as an energy source rather than being inhibited by them (Tarfarosh and Purohit, 2008). Antibiotic treatments often overlook antifungal therapy, resulting in persistent or spreading infections within herds. Understanding fungal contributions to mastitis, enabling differential detection and targeted treatment is crucial. This study was conducted to investigate the prevalence and antifungal susceptibility profiles of *Candida* species in dairy cattle with mastitis.

MATERIALS AND METHODS

Study location and sample collection

The present study was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh, Gujarat, India, during October 2024–January 2025. A total of 608 bovine mastitic milk samples were analyzed. The samples were received at Veterinary Clinical Complex (VCC), Junagadh, from different parts of Saurashtra region, Gujarat. After discarding the first few milk strips, the mastitic milk samples were collected with all aseptic precaution into a sterile container and transported to the laboratory.

Isolation and identification

A loopful of mastitic milk samples was streaked onto Brain Heart Infusion agar (BHI) and Sabouraud Dextrose Agar (SDA) for identification of causative agent and incubated aerobically at 37°C for 24-48 hrs and 30°C for upto 1 week for bacterial (result not shown) and fungal isolation, respectively. The fungal colonies identified on SDA plate, comprising both filamentous molds and yeast-like colonies were isolated and identified as causative agents of fungal mastitis. Among the fungal isolates, only those belonging to the genus *Candida* were considered for this study. The yeast like colonies, that appeared white and creamy or pasty in consistency and exhibited Gram-positive with oval-shaped budding cells upon Gram staining were phenotypically identified as yeast cells (*Candida* spp.). Each yeast isolate was inoculated into microtubes containing 1ml of SDA broth and incubated at 37 ± 1°C for 24 h, then stored at −20°C with glycerol for subsequent analysis.

A germ-tube test was conducted for the phenotypic identification of *Candida albicans*. In this test, the suspected yeast isolates were inoculated into 0.5 ml of bovine serum and incubated at 37°C. The suspension was examined microscopically first after 2 hours and then at hourly intervals for upto 5 hours of post-inoculation. The presence of hyphae like projection from the yeast cells was considered a positive result, which is characteristic of *Candida albicans* (Quinn et al., 2011).

DNA extraction and molecular detection of *Candida* spp. by PCR assay

The stored isolates of *Candida* spp. were revived by streaking on SDA plates and the genomic DNA was extracted by heat and thaw method as per the standard protocol described by Sultana et al. (2018) with minor modifications. The purity and concentration of isolated DNA was assessed using µDrop™ Plate in µDrop plate reader (Thermo Scientific). The extracted DNA from the isolated colonies were subjected to PCR assay with the genus specific screening primer

(Table 1) with an expected product size between 250-350 bp for *Candida* spp.

The composition of reaction mixture and cycling conditions for PCR was set as per Ahmad et al. (2002). The amplification of target gene was carried out using a programmable thermal cycler (Verity, Applied Biosystems by life technology, Singapore). The amplified products were then electrophoresed on a 1.5% w/v agarose gel with ethidium bromide (0.5µg/ml) and a DNA ladder, performed in 1x Tris-Acetic acid-EDTA (TAE) buffer at 60 V for 60 min. The amplified product image was documented by the gel documentation system Bio-PrintST4® (VilberLourmat).

Antifungal susceptibility test

The test was performed using disk diffusion method for yeast isolates as per the Bauer's standard disc diffusion method described by Hudzicki, (2024). For this study, commercially available antifungal discs (Himedia, Mumbai, India) were used, namely clotrimazole (10.00 µg), itraconazole (10.00 µg), miconazole (50.00 µg), ketoconazole (10.00 µg) and fluconazole (25.00 µg). The zones of inhibition were measured and determined based on CLSI guideline (CLSI, 2020).

RESULTS

Isolation and identification

In the present study, a total of 10.5% (64/608) isolates from mastitic milk samples yielded fungal growth on SDA medium (mycelial, filamentous colonies and yeast like colonies). The overall prevalence of *Candida* spp. among bovine mastitic milk samples was 1.64% (10/608) based on colony characteristics (Figure 1), Gram-positive staining and the presence of characteristic oval-shaped budding cells (Figure 2). Among the fungal isolates, *Candida* spp. accounted for 15.6% (10/64) of the total fungal mastitis cases. The germ tube test performed for confirmatory identification of *Candida albicans* was negative for all isolates, indicating that all belonged to non-albicans *Candida* (NAC) species.

Figure 1. White and creamy or pasty consistency yeast cell colonies in Sabouraud dextrose agar (SDA).

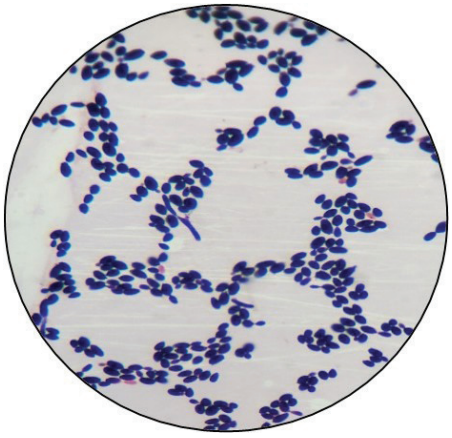

Figure 2. Microscopic image of oval shaped, Gram-positive budding yeast cell.

Molecular detection of *Candida* spp. by PCR assay

The genus specific screening primer previously designed by Ahmad et al. (2002) was used for the confirmation of *Candida* spp. genotypically. All the 10 isolates phenotypically confirmed as *Candida* spp. was subjected to PCR assay using the screening primer. All the isolates yielded amplicon size

Table 1. Genus specific primer used in this study for the identification of *Candida* species.

Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
Screening primer	F: TCGCATCGATGAAGAACGCAGC R: TCTTTTCCTCCGCTTATTGATATGC	250 - 350	Ahmad et al. (2002)

F: Forward and R: Reverse

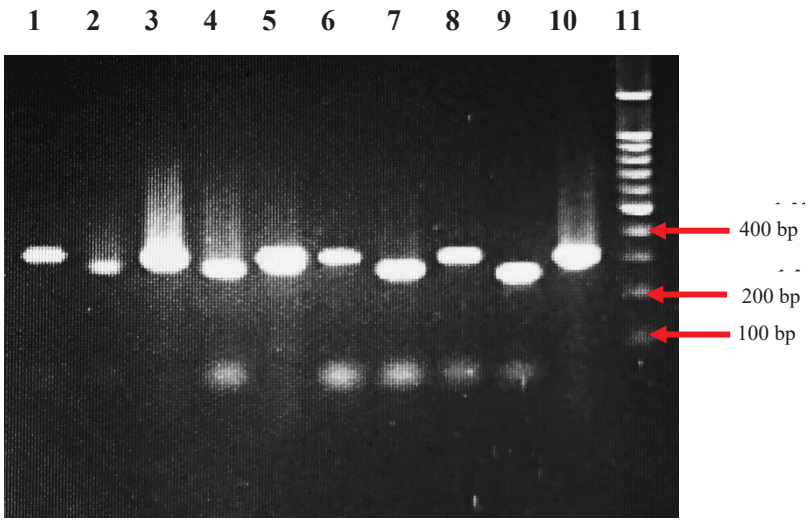

Figure 3. Agarose gel electrophoresis of PCR assay showing approximately 250 - 350 bp. Lane 1 to 10 = Samples, Lane 11= 100bp plus DNA Ladder.

between 250-350 bp confirming their identity as *Candida* species (Figure 3).

Antifungal susceptibility test

The *in vitro* antifungal sensitivity test (AFST) performed using 5 antifungal agents to check the phenotypic resistance among the 10 genotypically confirmed *Candida* spp. isolates revealed varying degrees of sensitivity (Figure 4 and 5), with fluconazole showing the highest sensitivity (100%), followed by miconazole (80%), clotrimazole and itraconazole (50% each), while ketoconazole revealed the lowest sensitivity (40%).

DISCUSSION

Fungal mastitis in dairy cattle exhibits significant geographic variability worldwide. In the present study, 10.5% of mastitic milk samples yielded fungal growth, with *Candida* spp. accounting for 1.64% of bovine mastitis cases in the Saurashtra region of Gujarat, India, indicating the presence of fungal pathogens in the etiology of mastitis. Our findings

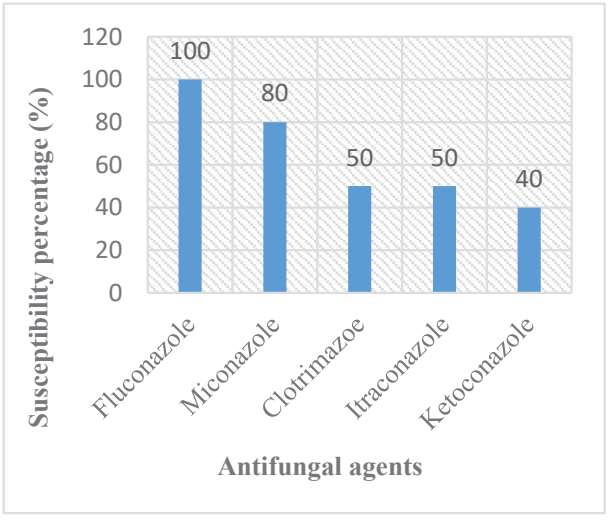

Figure 4. Graphical representation of isolates of *Candida* spp. showing varying degree of susceptibility against commercially available antifungal agents.

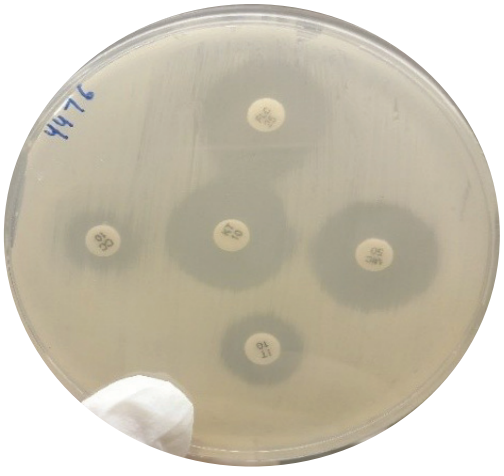

Figure 5. *In vitro* Antifungal sensitivity testing of *Candida* spp. isolates against commercially available antifungal agents using the disk diffusion method.

align with studies that reported fungal prevalence in mastitic milk of 9.6% in Poland (Krukowski et al., 2001), 12.07% in Brazil (Costa et al., 1993) and 10.17% in Algeria (Ksouri et al., 2015). In contrast, a higher percentage of 21.1% of mycotic mastitis was reported from bovine and ovine mycotic mastitic samples (Raheel et al., 2023).

The lower prevalence of *Candida* spp. observed in our study (1.64%) could be attributed to differences in environmental conditions, farm management practices or antibiotic usage. The observed prevalence rate is comparable to that reported in South Korea and Denmark, where a 1.3% prevalence of *Candida* spp. in mastitic milk samples was observed (Yeo and Choi, 1982; Aalbek et al., 1994). However, our results were substantially lower than those reported in Poland (Dworecka-Kaszak et al., 2012) and Brazil (Sartori et al., 2014), where *Candida* spp. prevalence rates of 11% and 12.8% were observed. Similarly, higher prevalence rates of 26% and 23.3% have been reported in India by Pachauri et al. (2013) and Devanathan et al. (2024), respectively from bovine clinical mastitic cases. These considerable variations highlight the geographic diversity, environmental conditions, stress and immunological status of animals in the epidemiology of fungal mastitis worldwide.

All 10 isolates tested negative in the germ tube test, indicating they likely belong to NAC species, which aligns with previous reports identifying *C. krusei*, *C. tropicalis* and *C. parapsilosis* as predominant over *C. albicans* in bovine mastitis (Tarfarosh and Purohit, 2008; Dworecka-Kaszak et al., 2012). However, contrary to our findings, *C. albicans* has been reported as the most frequently isolated *Candida* species from mastitic milk samples, with a prevalence of 8% (Mousa et al., 2016).

The presence of *Candida* isolates in all 10 samples was conclusively confirmed through PCR assay using genus-specific primer, which yielded the expected 250-350 bp amplicons for *Candida* species. Similar primer sets have been successfully utilized by Devanathan et al. (2024) to detect and differentiate fungal species, emphasizing their reliability in fungal diagnostics. Molecular approaches like PCR offer greater specificity and sensitivity than conventional culture-based and biochemical methods, enabling reliable identification of *Candida* species, including non-cultivable or slow-growing yeasts and reducing the risk of misidentification due to phenotypic variation.

Antifungal resistance among *Candida* species is a growing concern, especially with increasing NAC prevalence and their varying susceptibility profiles (Sanguinetti et al., 2015). Increased usage of antimycotics has led to the emergence of resistant isolates, with some *Candida* species exhibiting natural resistance to agents like fluconazole, amphotericin B and ketoconazole (Sonmez and Erbas, 2017; Whaley et al., 2017).

During the present study, fluconazole showed 100% efficacy, while miconazole, clotrimazole, itraconazole and ketoconazole exhibited varying degrees of sensitivity. This concurs with reports showing fluconazole as an effective treatment for Candida mastitis in goats (Sudhakara et al., 2018) and increased fluconazole susceptibility over time (Lyon et al., 2010). However, fluconazole resistance exceeding 50% has been noted in *Candida albicans* isolates (Dos Santos and Marin, 2014), while other studies found 100% susceptibility to ketoconazole but resistance to fluconazole and other agents (Sonmez and Erbas, 2017). These discrepancies underline the variable antifungal susceptibility profiles of *Candida* species and emphasize the necessity of AFST. Given limited antifungal options and emerging multidrug resistance, routine AFST prior to therapy is critical for effective treatment, resistance monitoring and optimal clinical outcomes (Berkow et al., 2020; Durand et al., 2021).

CONCLUSIONS

Fungal mastitis, though less common than bacterial forms, presents unique diagnostic and therapeutic challenges that can significantly impact dairy herd productivity and animal welfare. Our findings suggest that routine diagnostic protocols for bovine mastitis should incorporate both fungal culture and molecular detection methods to accurately identify fungal pathogens. Further studies are warranted to determine the specific *Candida* species involved, particularly through sequencing of the internal transcribed spacer (ITS) regions, which provides precise species-level identification. An integrated approach-combining advanced molecular diagnostics, resistance profiling and improved management protocols is essential for the effective control of mycotic mastitis and for safeguarding udder health in dairy herds.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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