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Molecular detection and genetic analysis of *Candida* species isolated from bovine clinical mastitis in India

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Abstract

Bovine clinical mastitis is an economically important disease in dairy industry worldwide resulting in reduction of milk yield and quality. Among mycotic mastitis, *Candida* spp. are commonly occurring opportunistic mycosis in immunocompromised animals. The micro-organism's causing mastitis has high zoonotic potential and has been linked with rapid growth and introduction of antimicrobial resistance between animals and humans. The present study was conducted to isolate and identify the common pathogenic *Candida* spp. from bovine mastitis cases in India. The isolates were phenotypically characterized by culturing on Sabouraud's dextrose agar, Hichrome *Candida* differential agar and germ tube production test. Antibiogram was also performed to determine their antifungal activities. The phenotypically positive isolates were confirmed by polymerase chain reaction (PCR) and genetically analyzed by targeting 18S-ITS1-5.8S-ITS2-28S region specific for *Candida* spp. and identified the yeast at the species level. The antibiogram showed the isolates were highly sensitive with ketoconazole, clotrimazole and miconazole. The PCR assay identified *C. lusitaniae* and *C. tropicalis* based on the two distinctive amplicon sizes (592bp and 737bp) respectively. Also, the sequence analysis and phylogeny confirmed *C. lusitaniae* in six sequences and *C. tropicalis* in one sequence. It is worth noting that in this study, the species identification was consistent among PCR and genetic analysis. Therefore, the PCR based identification system of the fungal species performed in this study could be an efficient and time saving tool for early diagnosis of clinical mastitis in milch animal, which allows prompt control and application of speedy effective treatment.

Introduction

Bovine mastitis is an economically important disease in dairy herds worldwide resulting in reduction of milk yield and change in milk quality. More than 140 different micro-organisms have been identified as etiological agents for mastitis.[1] Among mycotic mastitis in milch animals, *Candida* spp. are frequently occurring opportunistic pathogen in immunocompromised animals.[2] The major causative agent is *C. albicans,* however, the isolation of Non-albicans *Candida* (NAC) species has also been increasing in recent times. Some of the most frequent NAC species isolated are *C. glabrata* complex, *C. tropicalis, C. lusitaniae C. guilliermondii complex, C. dubliniensis, C. parapsilosis complex, C. tropicalis and C. krusei*.[3,4] The frequency and occurrence of the NAC species vary in geographical regions. The seriousness and effectiveness of mycotic infection depends on the species of the fungus involved as well as the infectivity percentage.

In general mastitis therapy, antibiotics are used more often and emphasis is not given on antifungal drugs. Many of these mycotic organisms do not respond to the antibiotics therapy, and treatment could be challengeable.[5] Different *Candida* species may exhibit varied patterns of antifungal susceptibility and it is essential to identify yeasts at the species level for accurate diagnosis and effective antifungal therapy. Identification of this diverse group of pathogens by conventional culturing methods is often difficult, time consuming and sometimes inconclusive. Therefore, for diagnostic purposes, polymerase chain reaction (PCR) assay plays a major role in detecting and identifying *Candida* spp.

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Therefore, the present study was conducted to isolate and identify the common pathogenic *Candida* spp. from bovine mastitis cases in Puducherry, India. The isolates were characterized phenotypically and further subjected to PCR and genetic analysis of the yeast at species level.

Materials and Methods

Clinical Samples. A total number of 30 milk samples were collected from the Teaching Veterinary Clinical Complex, Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry, India, with the history of long-standing clinical mastitis. After discarding the first few milk strips, 2.00 mL of the milk samples were collected into a sterile container and transported to the lab in ice.

Isolation and identification. A loopful of mastitis suspected milk samples were inoculated into Sabouraud dextrose broth (HiMedia, Mumbai, India) and incubated at 37.00˚C overnight. The incubated inoculums were streaked onto Sabouraud dextrose agar (SDA) for isolation of organism. On a daily basis, colony growths were observed for 72 hr and the samples with and without yeasts were noted. The loopful of inoculums were streaked onto the HiChrome Candida differential agar (selective and differential medium), which facilitates rapid isolation and differentiation of *Candida* species namely *C. albicans, C. krusei, C. tropicalis* and *C. glabrata* on the basis of colony coloration and morphology.[6] Then, the isolated yeast was characterized phenotypically based on their microscopic morphology using Gram staining, cultural and biochemical characteristics.[6]

Antifungal susceptibility test. The test was performed for the isolated *Candida* spp. as per the Bauer's standard disc diffusion method[7] using seven antifungal substances (HiMedia) such as ketoconazole (10.00 µg), miconazole (30.00 µg), amphoteracin-B (20.00 µg), clotrimazole (10.00 µg), fluconazole (10.00 µg), nystatin (10.00 µg) and itraconazole (10.00 µg).

Polymerase chain reaction assay. The isolated colonies were subjected to the genomic DNA extraction using Zybio's Nucleic Acid Extraction Kit by Magnetic Bead Method (Zybio Inc., Chongqing, China) according to the manufacturer's protocol. The extracted DNA from the isolated colonies were subjected to PCR assay with the genus specific screening primers (Table 1) which amplifies 250 - 350 bp product for the *Candida* spp.[8] Similarly the extracted DNA from the screened positive isolates were further subjected to another PCR assay to identify different *Candida* species with the species specific primers by targeting the 18S-ITS1-5.8S-ITS2-28S which amplifies the fragment sizes ranging from 592 bp to 1,100 bp depending upon the species (Table 1).[9] For the PCR assay the reaction mix consisted of 25.00 µL of 2.00 X Taq DNA polymerase Ampliqon Red Master mix, 0.50 µL 0.20 µM of each primer, 3.00 µL of template DNA and Nuclease Free water (NFW) for the final volume of 50.00 µL. The thermocycling conditions were as follows: 5 min at 95.00 ˚C (initial denaturation), 35 cycles of 1 min at 95.00 ˚C (denaturation), 45 sec at 56.00 ˚C (annealing), 45 sec at 72.00 ˚C (extension), followed by final extension (72.00 ˚C for 10 min) and hold at 4.00 ˚C.

Sequencing and sequence analysis. The amplified PCR products from the isolates using *Candida* spp. specific primers were gel extracted by QIAquick gel extraction (Qiagen, Germantown, USA) and sent for sequencing. The query nucleotide sequences were analyzed with corresponding 18S-ITS1-5.8S-ITS2-28S sequences of *Candida* spp. using multiple alignment program, Clustal Omega (http://www.ebi.ac.uk/clustalomega/).

Phylogenetic analysis. The phylogenetic relationship was analyzed based on the nucleotide sequences of 18S-ITS1-5.8S-ITS2-28S region of *Candida* spp. and other related genus obtained from different parts of the world (NCBI, GenBank®) with MEGA Software (version 11.0; Biodesign Institute, Tempe, USA) program using the maximum likelihood estimation (MLE), method based on Tamura *et al*. model.[10]

Results

In the present study, 7 out of 30 milk samples yielded fungal/yeast like colonies on SDA on an average of 24 to 72 hr after inoculation. The colonies were white with creamy consistency, 0.50 - 1.00 mm in diameter uniformly

Table 1. Oligonucleotide's primers used in this study for the identification of *Candida* species.

Primers	Sequence (5'-3')	*Candida* species	Amplicon Size (bp)	References
Screening primers	TCGCATCGATGAAGAACGCAGC TCTTTTCCTCCGCTTATTGATATGC	*Candida sp.*	250 - 350	8
***Candida* species specific primers**	AGCTTGCGTTGATTACGTCCCTGCCC TTCACTCGCCGCTACTAAGGCAATCCC	*C. albicans*	850	9
		C. glabrata	1,000	
		*C. tropicalis**	737	
		C. parapsilosis	731	
		C. krusei	800	
		C. guilliermondii	1,100	
		*C. lusitaniae**	592	
		C. dubliniensis	810	

* identified *Candida* species in the present study.

bounded and with fungal fringes on further incubation (Fig. 1A). In HiChrome *Candida* differential agar, one isolate showed blue to metallic blue colored raised colony (Fig. 1B) and six isolates showed creamy white to purple color colonies (Fig. 1C) which were later confirmed by PCR as *C. tropicalis* and *C. lusitaniae*, respectively. The isolates were negative for germ tube production test.

On Gram staining, microscopically the isolates revealed, they were Gram positive, oval shaped with budding stages and some with pseudohyphogenic yeast cells which were characteristic of *Candida* spp. (Fig. 1D).

The seven DNA sequences under this study were deposited in the NCBI GenBank® database and were assigned accession numbers of OP503374 to OP503380. The sizes of the contiguous obtained were 592 bp for the OP503374 to OP503379 and 737 bp for OP503380. Upon Basic Local Alignment Search Tool analysis, the six sequences (OP503374 to OP503379) were found to be maximally identical (98.00 - 99.00%) with the *C. lusitaniae* strains, however, one sequence (OP503380) had found to show maximal identity (98.00%) with *C. tropicalis* strains.

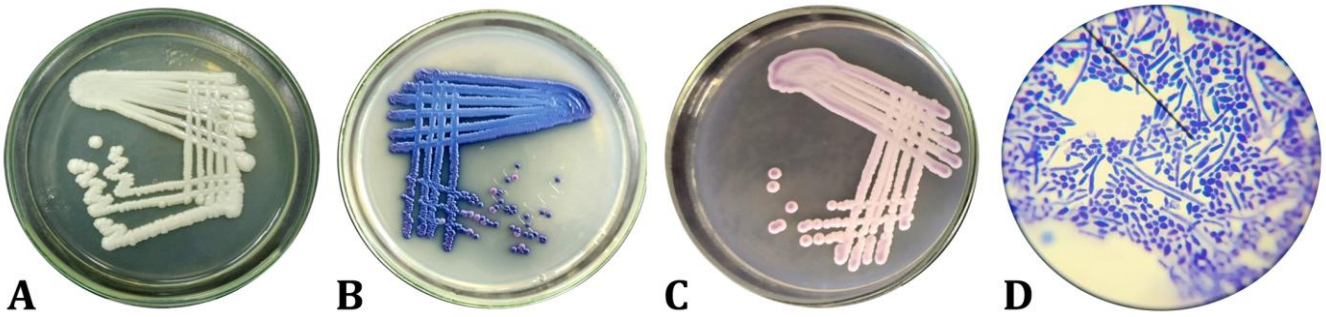

Fig. 1. Growth of *Candida* spp. observed on an average of 24 to 72 hr on 37.00 ˚C incubation **A)** In Sabouraud dextrose agar with the colony characteristics of white, creamy consistency, 0.50 - 1.00 mm in diameter uniformly bounded with fungal fringes. **B)** In HiChrome *Candida* differential agar; the *Candida tropicalis* isolate showing blue to metallic blue colored raised colonies, and **C)** In HiChrome *Candida* differential agar, the *Candida lusitaniae* isolate showing creamy white to purple color colonies. **D)** Isolate showing the microscopic morphology. Oval shaped, Gram-positive budding yeast with some pseudohyphogenic yeast cells of *Candida* spp. demonstrated by Gram's staining (1,000 ×).

The isolates showed complete resistance (100%) to amphoteracin-B, itraconazole, fluconazole and Nystatin nystatin, and complete susceptibility (100%) to ketoconazole, clotrimazole and miconazole in our study.

All the isolated colonies subjected to PCR assay using the screening primers yielded 250 - 350 bp amplicons specific for *Candida* spp. (Fig. 2). The positive isolates were further subjected to PCR with the *Candida* species specific primers (Table 1) targeting the 18S-ITS1-5.8S-ITS2-28S gene amplified. A product size of 592 bp was obtained for 6 isolates corresponding to *C. lusitaniae* and a product size of 737 bp was obtained for one isolate corresponding to *C. tropicalis* (Fig. 3).

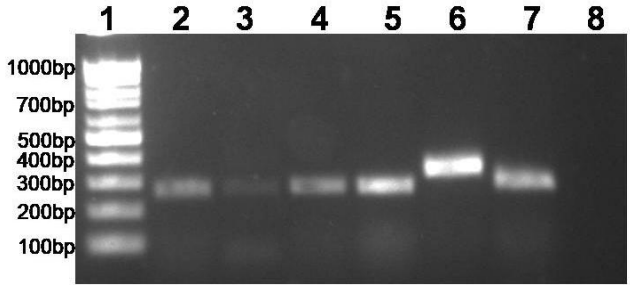

Fig. 2. The polymerase chain reaction assay showing approximately 250 - 350 bp amplicons targeting using screening primer pair. Lane 1: DNA Marker, Lanes 2 – 7: *Candida* spp. positive isolates, and Lane 8: Negative control showing no band.

By phylogenetic analysis with the various *Candida* spp. retrieved from GenBank® further confirmed that the six *Candida* query sequences (OP503374 to OP503379) were clustering with the *C. lusitaniae* sequences whereas one *Candida* sequence (OP503380) was forming separate clade and clustering with *Candida tropicalis* sequences (Fig. 4).

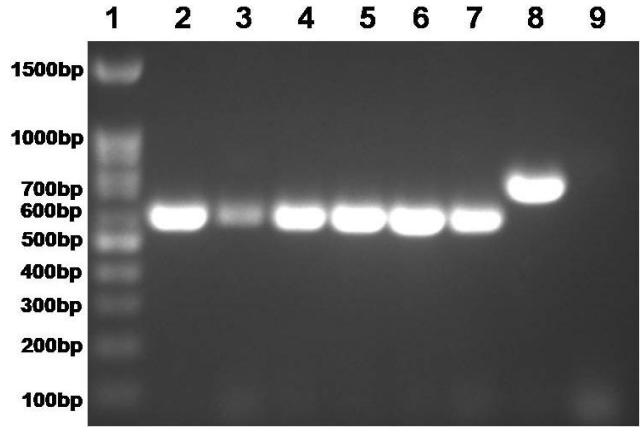

Fig. 3. The polymerase chain reaction assay showing two distinctive variable bands (592 and 737 bp) using *Candida* spp. specific primers. Lane 1: DNA Marker, Lanes 2 - 7: *C. lusitaniae* isolates amplifies 592 bp product (OP503374 to OP503379), Lane 8: *C. tropicalis* isolate amplifies 737 bp product (OP503380), and Lane 9: Negative control showing no band.

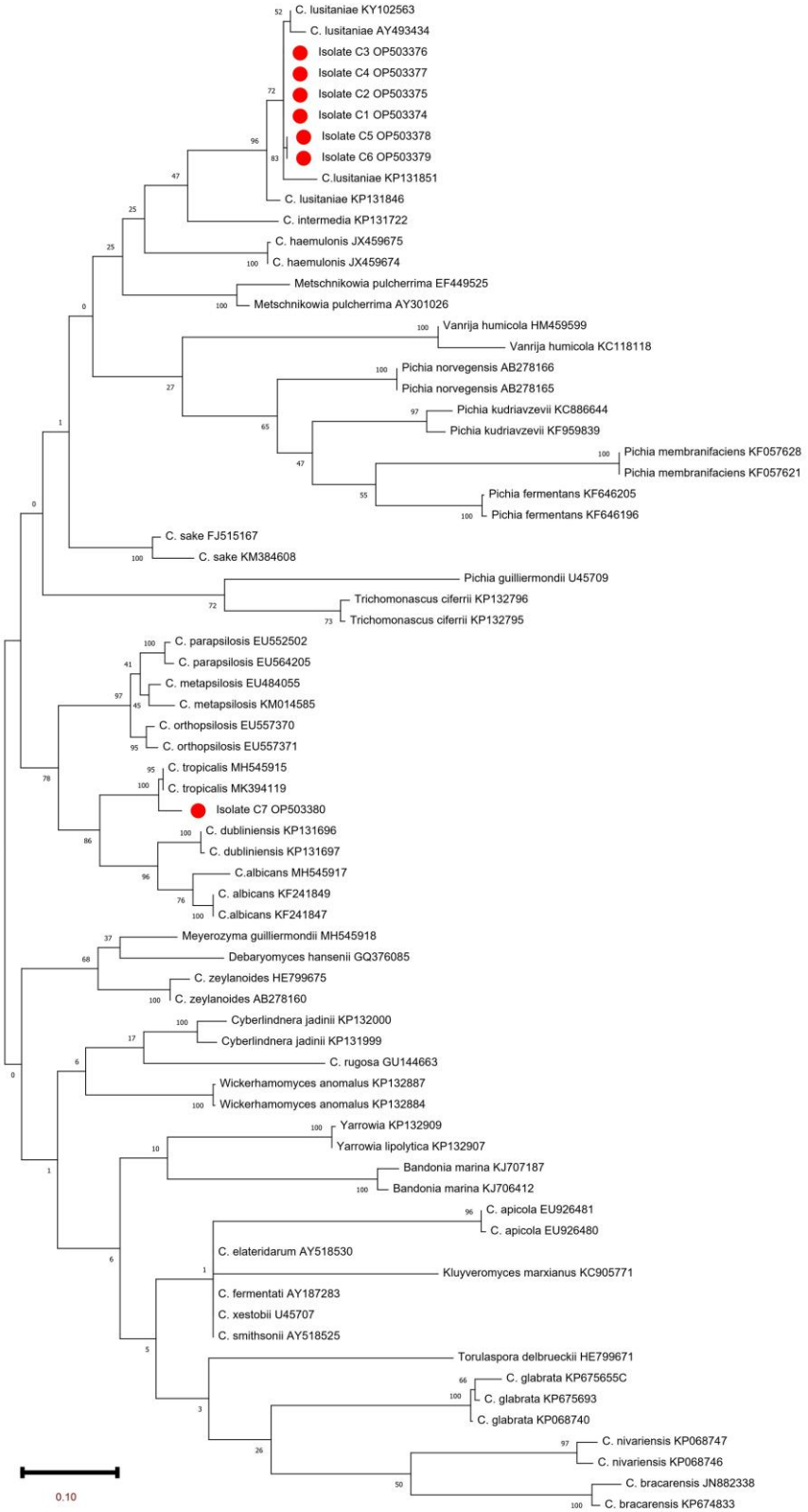

Fig. 4. The evolutionary history was inferred by using the maximum likelihood estimation (MLE) tree based on the Tamura *et al*. model.[10] The *Candida* query sequences (OP503374 to OP503380) shown with solid circle and other reference sequences available in the GenBank®. Bootstrap values are shown next to the branches in the phylogenetic tree.

Discussion

In this study, 23.00% of mastitis milk samples yielded fungal/ yeast like colonies which indicated the importance of fungal infections in clinical mastitis in bovine. The surveys carried out in several countries showed that the percentage of *Candida* infection had been increasing causing numerous mammary gland infections with as high as 26.00% reported in India,[11] 25.20% in Egypt[12] and 17.00% in Tanzania.[13] The colonies differentiation using HiChrome *Candida* differential agar helped us to identify two different species based on colony colors. Microscopic examination of colonies using Gram staining were characteristic of *Candida* spp. as reported by other author from bovine clinical mastitis milk samples.[14]

Increased candidiasis in clinical mastitis infection in recent years and varied susceptibility to the antifungal agents for the identified species *i.e.,* NAC showed the importance of susceptibility tests to avoid the use of non-specific antibiotics and antifungal for the treatment.

The diagnosis, particularly in systemic or invasive fungal infections, must be accurate and rapid in order to establish specific and timely treatment. Although cultures are the gold standard for detecting *Candidiasis*, they are time consuming as at least 3 days for isolation plus additional days for the species identification are required. Considering this, PCR assay targeting the 18S-ITS1-5.8S-ITS2-28S gene which amplifies distinctive variable bands for species level identification was done. The primers based on the 18S-ITS1-5.8S-ITS2-28S region, take advantage of the ITS regions intraspecific variability and the conserved sequences of the 18S, 5.8S, and 28S regions. By this strategy, eight of the most frequent pathogenic species-specific *Candida* spp. can be differentiated based on the amplicon size as described by García-Salazar *et al*.[9] In our study, two distinctive bands (592bp and 737bp) corresponding to *C. lusitaniae* and *C. tropicalis* could be identified. In addition, sequencing and phylogeny analysis also supported to further confirm the *Candida* species identification. Therefore, the PCR based species level identification system used in this study could be an efficient tool for early and specific diagnosis of *Candida* mastitis, so that early treatment can be initiated.

In the present study, the isolation of NAC such as *C. lusitaniae* and *C. tropicalis* in mastitis milk raised the risk of cow milk acting as a vehicle in transmission of these pathogenic yeasts and posed a zoonotic threat to the farmers, animal handlers and the consumers. The study also addressed the development of fungal resistance to the anti-mycotic agents and the importance of susceptibility tests to establish a specific and prompt treatment. Culturing techniques are gold standard for identification in *Candida* spp. The PCR based assay covering the ITS regions, the conserved sequences of the 18S, 5.8S, and 28S regions of *Candida* spp. could be an efficient method for rapid and confirmatory species level identification.

Conflict of interest

The authors declare no competing interests.

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