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PCR based detection of virulent genes in *Candida* spp. isolated from bovine mastitis

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Abstract
This study employed molecular identification techniques to characterize isolates of *Candida albicans, Candida tropicalis*, and *Candida krusei* obtained from cases of bovine mastitis. The results indicated that PCR targeting the 18Sr RNA gene successfully identified 95.45% of *Candida albicans* isolates, all (100%) *Candida tropicalis* isolates, and 80% of *Candida krusei* isolates. Furthermore, this study found a high detection rate (72.33%) of the LIP1 virulent gene in *Candida tropicalis* isolates, suggesting its potential significance in the virulence of this species. Additionally, the ALS1 gene was exclusive to *Candida albicans* isolates. The highest detection rate of the FKS1 gene was observed in *Candida krusei* isolates. Regarding the SAP1 gene, it was detected in only 30% of the *Candida* spp. isolates Sequencing of selective *Candida albicans* and *Candida krusei* isolates confirmed their identification, showing high similarity with previously published sequences. These findings contribute towards understanding the prevalence, distribution, and virulence factors of different *Candida* species in bovine mastitis.

Keywords: PCR-based detection, virulent genes, candida species, bovine mastitis, pathogenicity

Introduction
The significance of fungi causing mastitis in cows has lately come to light. Various yeasts and mould spp. have been isolated from cows suffering from mastitis in India and internationally. Although conventional methods are used to identify *Candida albicans*, these processes can take many days or weeks. (Tarini *et al.* 2010) [15]. Polymerase chain reaction (PCR) may be a viable alternative to traditional diagnostic methods for *Candida* infections; this technique addresses all issues of sensitivity, specificity, and delayed diagnosis (Kan 1993; Makimura *et al.* 1994; Miyakawa *et al.* 1993) [8, 9, 10]. The polymerase chain reaction (PCR), is fast, sensitive, and specific for detecting fungal DNA sequences (Pincus *et al.,* 2007) [14]. Furthermore, the fact that these molecular markers are environmental condition-insensitive and can be standardized across laboratories makes them safe (Atkins *et al.* 2004) [2]. *Candida albican's* ability to infiltrate numerous organs in the host has been connected to several virulence variables. (Mousa *et al,* 2016) [11]. Several investigations have shown that the process of adhesion to the host surface comprises *C. albicans* genes producing *agglutinin-like sequence* (*ALS*1), *secreted aspartyl proteinase* 1 (SAP1), and Set12-like transcription factor (*CPH*1) (Mousa *et al*., 2016) [11]. *Phospholipase B*1 (*PLB*1) is regarded as a key virulence factor, and phospholipases harm cell membranes by hydrolyzing membrane lipids. Only *PLB*1 has been found in animal candidiasis instances (Novarro-Garcia *et al*., 2001; Eldesouky *et al*., 2016; Mousa *et al*.,2016) [12, 6, 11]. Recent research has identified a diverse variety of virulence factors in *Candida parapsilosis* and *Candida krusei* strains derived from cow mastitis infections. Gene sequencing is an extremely important molecular method for detecting the genotypic changes that are occurring in the pathogenic gene associated with the infection

Materials and Methods
Ethical approval
All samples were collected as per standard sample collection procedure. This type of study does not require any ethical approval.

Sampling

A total of 235 milk samples were collected from cattle of various ages for this investigation. The adjacent villages in the districts of Pune and Satara of Maharashtra state constitute the sample collection area. A total of 66 milk samples were collected from cows suffering with subclinical mastitis &169 milk samples from cows suffering from clinical mastitis (Table no.1.) All the affected cows had a history of treatment with different antibiotics and most of these cases were of recurrent mastitis. About 10 ml of milk was aseptically collected from each affected animal after discarding the first few strips of milk. The samples were transported to the laboratory under cold conditions. Identification of yeast pathogens by traditional methods requires several days and specific mycological media. Chromogenic media contain chromogenic substrates that react with enzymes secreted by the target microorganisms to yield colonies of varying colours. A loopful of milk from each sample was streaked onto Hichrome Candida differential agar. Inoculated plates were kept at 37 ℃ for 48-72 hours. After observing the colony characters. The stock cultures were prepared on SDA and Hichrome Candida differential agar. Slant pure cultures were kept at 4°C until yeast-like growth was identified.

Molecular Detection *Candida* spp

The Extraction of Yeast Genomic DNA was performed with Hipur A Yeast Genomic DNA purification kit (Himedia) The procedure for DNA extraction was adopted as per the manufacturer's instructions Phenotypically positive isolates of *Candida albicans*. *Candida krusei* and *Candida tropicalis* were confirmed by polymerase chain reaction. The oligonucleotide sequence of the primers used in the study is given in (Table. No 2) Phenotypically positive isolates of *Candida spp*. were further used for the detection of virulence genes by polymerase chain reaction. The oligonucleotide sequence of the primers used for the detection of various virulent genes is given in (Table 3.) The PCR reaction mixture used for performing PCR is given in (Table No.4.) The Purified PCR products of *C. albicans* & *C. krusei* were selected.

Results and Discussion

The present study was conducted to ascertain the role of *Candida spp.* in bovine mastitis. A total of 235 milk samples were collected out of the 235 milk samples collected for mastitis in the current study, 50 (21.27%) tested positive for *Candida species* based on colony features on Chromogenic media. (Table no.5) In this investigation, the three species of *Candida* were isolated. Among these three species of *Candida*, the most frequently isolated form was *Candida albicans* (n = 22, 9.36%), followed by *Candida tropicalis* (n = 18, 7.65%) and *Candida krusei* (n = 10, 4.25%). The findings of this study correlate with those of Eldesouky *et al.* (2016) [6]. They reported a high prevalence of 41 (27.3%). of *Candida spp.* from 150 milk samples Six different *Candida* species have been discovered based on morphological, biochemical, and cultural characteristics. As per their findings, *Candida albicans* had the highest prevalence (29.3%), whereas the findings of this research work indicate a low prevalence of *Candida albicans* (9.36%). They reported a prevalence of *Candida tropicalis, Candida guilliermondii* (19.5% each), *Candida glabrata* (14.6%), *Candida krusei* (12.2%), and *Candida kefyr* (4.9%).A total of 22 isolates identified as *Candida albicans* were further identified by using 18Sr RNA.(Table no.6) The results from PCR showed that out of the 22 isolates, 21 (95.45%) tested positive, with an observed amplicon size of 415 bp.(.The plate no 4).current findings presented here are similar to those of AL-abidy *et al. (*2019) They documented the presence of an amplicon measuring 415 base pairs through the utilization of species-specific 18S rRNA PCR technique, and their results showed that 60 isolates out of 116 produced distinct bands corresponding to a molecular size of approximately 415 bp which exhibited *C. albicans* by PCR, while 15 isolates out of 116 were produced distinct bands corresponding to a molecular size of approximately 507 bp which exhibited *C. parapasilosis.* Out of the 235 collected samples of *Candida spp.,* a total of n=18/235 (7.66%) isolates were *Candida tropicalis*. These 18 pure isolates of *Candida tropicalis* were subsequently subjected to PCR using the species-specific primers for each of the *Candida* species. For the design of the species-specific primers, the nucleotide sequences of the DNA topoisomerase II genes of *Candida species* were used as described by Erbas *et al.* (2017) [7]. These primers were specific to the species and were used for molecular detection, following the standard conditions and procedures. The results obtained from polymerase chain reaction (PCR) demonstrated that all 18*C.tropicalis*isolates (100%) were tested positive, with an observed amplicon size of 777 base pairs. (Table. No.7 plate no.5) The findings of this study correlate with the findings of Dolgun *et al.* (2022) [4]. In their study they also reported a lower detection rate of (4.3%) of *C. tropicalis.* The polymerase chain reaction (PCR) outcomes revealed a positive result in (n=8/10,80%) of the *C. Krusei* isolates, with an observed amplicon size measuring 227 base pairs. (Table. no 8 plate no.6) All 50 *Candida spp*. isolates were validated by PCR, and their virulent genes were identified using species-specific virulent gene primers. The study focused on a total of four virulent genes. Here's a breakdown of the data summarized for all virulent genes detected in *Candida albicans, Candida tropicalis,* and *Candida krusei* isolates.

FKS1 Gene

The detection rate for the FKS1 gene in all three species was 10/50 (20%).

Candida krusei isolates had the highest FKS1 gene detection rate of 10/10 (100.0%).

FKS1 gene was not found in *Candida albicans* or *Candida tropicalis* isolates.

Similar results were reported by Du *et al*. (2018) [5] they reported the FKS1 gene in all 14 *C. krusei* isolates. (Table. no 9 Plate. No.9)

ALS1 Gene

ALS1 gene detection rate is 8.0% across all three species.

Only 4 out of 22 *Candida albicans* isolates had the ALS1 gene detected (18.18%). (Table. no. 9) *Candida tropicalis* and *Candida krusei* isolates showed the lowest detection rate of 0.0%. The low detection percentage (4/22,18.18%) of the ALS1 gene in *Candida albicans* isolates in this investigation contrasts with the findings of Orçun and Ozturk (2020) [13], who found a high 40% detection rate of the ALS1 gene in 5*C. albicans* isolates.

SAP1 Gene

The total detection rate in all three species was 36.0%. The Highest 40.0%detection rate of the SAP1 gene was observed in *Candida krusei* isolates followed by *Candida tropicalis at* 27.77%.The Lowest detection rate: 27.27% was recorded in *Candida albicans* isolates.(Table no 9. plate no,7)

LIP1 Gene

The total detection rate in all three species was 30.0%. The highest detection rate of the LIP1 gene72.33% was recorded in 18 *Candida tropicalis* isolates (Table.no.9.Plateno.8.)
This is congruent with Zhang *et al.* (2019) [16] findings, which found ten genes encoding LIPs (LIP1-10) in *C. albicans* and five comparable lipase-producing genes in *C. tropicalis*.

Table 1: Collection of milk samples from Satara and Pune District

District	Subclinicalmastitis	Clinical mastitis
Satara	34	93
Pune	32	76
Total	66	169
66 +169 = 235		

Table 2: Primer sequences used in PCR of different *Candida spp*

Candida species	Sequence (5'-3')	Product (bp)	Reference
Candida albicans (18srRNA)	F=GCCGCCAGAGGTCTAAACTT R=AGTTCAGCGGGTAGTCCTAC	415	AL-Abidy *et al.* 2019
Candida tropicalis	F=GGACAGTTTGGATGAAGATTTA R=GAGACCAGCCACGGACAAATT CAAC	777	(Erbas *et al.* 2017) [7]
Candida krusei	F=GAGCCACGGTAAAGAATACAC A R=TTTAAAGTGACCCGGATACC	227	(Erbas *et al.* 2017) [7]

Table 3: Primer sequences of Virulent genes of *Candida spp.*

Virulent gene	Sequence (5'-3')	Product (bp)	Reference
LIP1	F=CCAAGGAGTCTATGGCTCAGTTA R=TAAGTGTAAAGTTGTCGGTGTTC	1945	L.J. Zhangv *et al.* (2019) [16]
SAP1	F=GCTCTTGCTATTGCTTTATTA R=CATCAGGAACCCATAAATCAG	253	(Dikmen *et al.*, 2021) [3]
FKS1	F=ACTGCATCGTTTGCTCCTCT R=GAACATGATCAATTGCCAAC	500	(Du *et al.*, 2018) [5]
ALS1	F=ACATGTACTGTGAACGACGCT R=GACGACTGCCAGCACAAGTA	577	(AL-Abidy *et al.*, 2019) [1]

Table 4: PCR reaction mixture content

2xPCR master mix	12.5 µl
Nuclease– freewater	7.5 µl
Forwardprimer	1 µl
Reverseprimer	1 µl
DNAtemplate	3 µl

Table 5: Total no of *Candida albicans,Candida tropicalis & Candida Krusei* isolates

Species of *Candida*	The total no of positive isolates	Percentage of *Candida spp.*
Candida albicans	22/235	9.36%
Candida tropicalis	18/235	7.65%
Candida krusei	10/235	4.25%
Total no of *Candida spp.* isolated	50/235	21.27%

Table 6: Molecular detection of *Candida albicans* by18S rRNA

Total no of *Candida albicans* Identified by a conventional method	Total no of *Candida albicans* identified by 18S rRNA method	Percentage positivity
22	21	95.45%

Table 7: Molecular detection of *Candida tropicalis* by DNA topoisomerase II gene

Total no of *Candida tropicalis* identified by conventional method	Total no of *Candida tropicalis* identified by DNA topoisomerase II gene	Percentage positivity
18	18	100%

Table 8: Molecular detection of *Candida krusei* by DNA topoisomerase II

Total no of *Candida krusei* Identified by conventional method	Total no of *Candida krusei* identified by DNA topoisomerase II gene	Percentage positivity
10	8	80%

Table 9: Molecular detection of the virulent gene of *Candida* spp.

Sr.no.	*Candida spp.*	FKS1		ALS1		SAP1		LIP 1	
		No	%	No	%	No	%	No	%
1	*Candida albicans* (22)	0	00%	4	18.18%	6	27.27%	0	00%
2	*Candida tropicalis* (18)	0	00%	0	00%	5	27.77%	13	72.22%
3	*Candida krusei* (10)	10	100%	0	00%	4	40%	5	50%
4	Total=50	10	20%	4	8.%	15	30%	18	36%

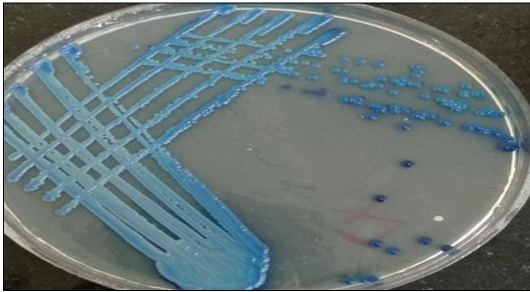

Plate 1: *Candida albicans* on Hichrome Candida differential agar

Plate 2: *Candida tropicalis* on Hichrome Candida differential agar

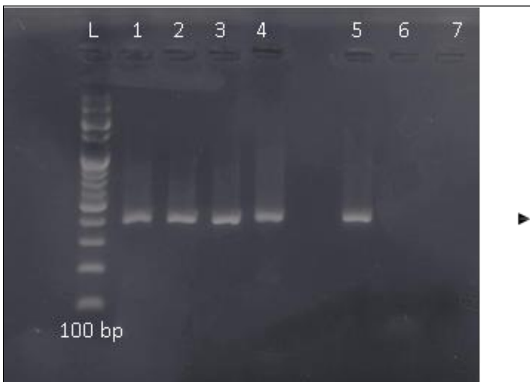

Plate 3: *Candida krusei* on Hichrome Candida differential agar

Plate 4: Agarose gel electrophoresis showing amplified PCR product *Candida albicans415bp* Lane 1, 2, 3, 4, 5, positive samples Lane 6 negative control L ladder

Plate 5: Agarose gel electrophoresis showing amplified PCR product *Candida tropicalis*Lanes2,3,4,5, positive sample for *Candida tropicalis* Lane 6 negative control Lladder

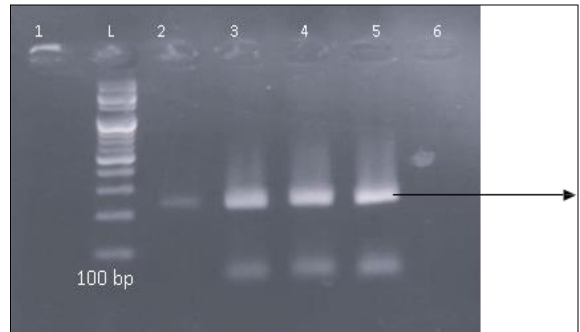

Plate 6: Agarose gel electrophoresis showing amplified PCR product of *Candidakrusei* Lane2,3,4,5, positive sample for *Candida krusei* Lane6negativecontrol Lladder

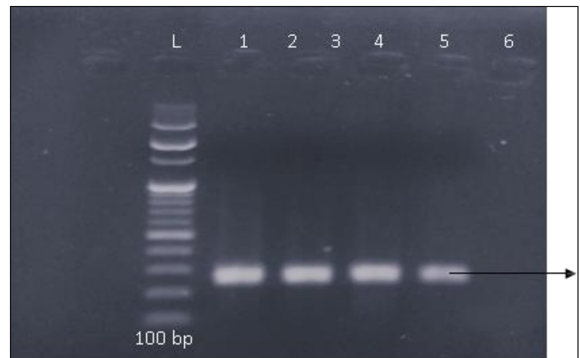

Plate 7: Agarose gel electrophoresis showing amplified PCR product *Candida albicans* SAP gene Lane, 1, 2, 3, 4, 5, positive sample for virulent gene SAP Lane 6 negative control Lladder

Plate 8: Agarose gel electrophoresis showing amplified *Candida tropicalis* LIP gene Lane1,2,3,4,5,positivesampleforLIPgene Lane 6 negative control L ladder

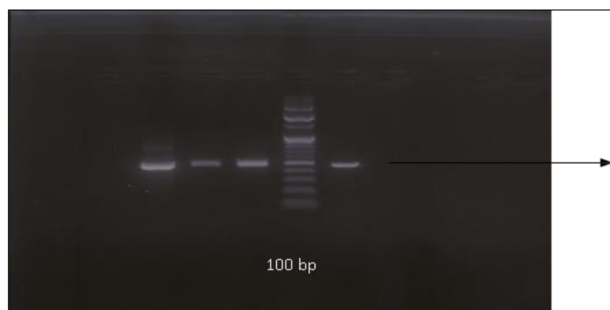

Plate 9: Agarose gel electrophoresis showing amplified *Candida krusei* FKS gene Lane1,2,3,4, positive sample for virulent gene FKS Lane 5 negative control L ladder

Conclusion

The findings of the current investigation lead to the following conclusions:

Throughout the study, three *Candida* species were found. *Candida albicans* was the most frequently isolated species (9.36%), followed by *Candida tropicalis* (7.65%) and *Candida krusei* (4.25%). The antifungal susceptibility test for *Candida albicans* revealed that ketoconazole was the most effective drug (95.45%), closely followed by fluconazole (90.90%). & itraconazole (77.27%). The prevalence of virulent genes in various species of *Candida* isolated revealed the highest detection rate for the FKS1 gene (100.0%) in *C. krusei isolates* followed by the SAP1 gene (40.0%). The *Candida tropicalis* isolates exhibited the greatest detection rate of the LIP1 gene (72.33%). The ALS1 gene was found solely in *Candida albicans* isolates and was absent from *Candida tropicalis* and *Candida krusei.*

Conflict of Interest

Not available

Financial Support

Not available

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