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Phenotypic and molecular characterization of *Candida spp*. isolated from intramammary infections in dairy animals by PCR-RFLP

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ABSTRACT

Mastitis is caused by various microorganisms like bacteria, viruses, mycoplasma, algae and yeasts. Of them, fungal infections contribute to 2–13% of the cases, with *Candida* as the most common genus, although wide variations in prevalence and species are identified. In the present study, 06.66% (n = 40/600) prevalence was observed in mastitic milk samples of cattle and buffalo on the basis of culture examination on blood agar (BA), sabouraud dextrose agar (SDA) and candida differentiation agar (CDA). On SDA, the isolates produced white to creamy, smooth, pasty and convex colonies. However, different isolates could not be differentiated on the basis of colony characteristic on SDA medium. Chromogenic agar such as candida differential agar was able to differentiate different species of *Candida* on the basis of production of different coloured colonies. In the VITEK®2 compact system, different isolates were identified with diverse level of identification ranging from excellent to acceptable. Furthermore, all of the isolates were confirmed by amplification of internal transcribed spacer (ITS) region by polymerase chain reaction (PCR). For molecular characterization of *Candida* spp., the PCR products were digested by restriction enzymes (*HaeIII* and *TaqI*) for restriction fragment length polymorphism (RFLP) and various patterns were obtained. Furthermore, in-silico RFLP analysis was done with restriction enzymes *HaeIII* and *TaqI*, for different *Candida* species to obtain accurate fragment sizes. PCR-RFLP proved to be a simple, cost effective and accurate method as compared to the phenotypic based methods which are often difficult and time consuming for rapid and species level differentiation of *Candida* species involved in mycotic mastitis.

Keywords: Buffalo, *Candida* spp., Cattle, RFLP, Mastitis

Mastitis is inflammation of mammary gland associated with significant reduction in milk yield resulting in increased production cost and degraded milk quality (Kaur *et al*. 2025). It was estimated annual losses of around $35 billion (Ortiz-Duran *et al.* 2017) in dairy industry worldwide. India alone experiencing losses of 7165.51 crores (Bansal and Gupta 2009). Bovine mastitis is a multifactorial disease involving several microorganisms such as bacteria, viruses, mycoplasma, algae and yeasts (Kaur *et al.* 2024). Fungal infections, particularly yeast-related mastitis, account for a small portion (2 to 13%) of mastitis cases, but their incidence has been increasing with time (Costa *et al.* 2012, Cilvez and Turkyilmaz 2019).Yeast-related mastitis is often associated with environmental hygiene and repeated intramammary treatments. Yeasts thrive in damp, organic-rich environments and can be found on teats and milking equipment (Cavalheiro and Teixeira 2018). Several yeast species, including *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporon*, have been linked to mastitis in dairy cows, with *Candida* being the most commonly isolated genus (Tomanić *et al.* 2024). Specific virulence factors like Phospholipase B1 gene have been identified in *Candida* species (Gaffar *et al.* 2025). Some fungal species also exhibit haemolytic activity. In addition to this, *Candida*'s ability to form biofilms is a unique pathogenic trait that shields them from host immune responses and antifungal treatments (Cavalheiro and Teixeira 2018, Clivez and Turkyilmaz 2019).

Conventional identification of yeasts is based on morphological and physiological characteristics. These methods are time consuming and strongly influenced by culture conditions giving uncertain results often leading to misdiagnosis (Khalaf *et al.* 2021). To address these challenges, molecular DNA-based tests, such as PCR-RFLP analysis, have been developed for yeast identification. This method involves pattern comparison obtained by digesting specific target DNA with various restriction endonucleases. Studies have shown that this approach is highly effective for differentiating yeast species in mastitis milk samples and is faster and more accurate than traditional methods (Fadda *et al*. 2013). Therefore, the present study focused on isolation

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and identification of various *Candida* species responsible for intramammary infections in dairy animals using traditional phenotypic methods and molecularly internal transcribed spacer (ITS), PCR-Restriction fragment length polymorphism (PCR-RFLP) method.

MATERIAL AND METHODS

Isolation and identification of Candida spp. from mastitic milk samples by culture examination: The College Central Laboratory (CCL), LUVAS, Hisar receives milk samples from all over the state of Haryana for routine examination of mastitis. A total of 600 milk samples from buffaloes and cows with a history of chronic mastitis and prolonged antibiotic use were processed for isolation of *Candida* spp. A loopful (~10 µL) of the milk sample was streaked onto Blood agar (BA) plate to isolate individual colonies. Presumptive isolates were streaked on Sabouraud Dextrose agar (SDA). The colonies were also streaked on candida differential agar (CDA) and incubated at 37°C for 48 hr for appearance of characteristic-colored colonies and morphology as per manufacture's instruction. All the agar media used in present study were procured from Hi-Media, Laboratories Pvt. Ltd. Mumbai, India. The reference strain of *Candida albicans* ATCC 10231*, Candia parapsilosis* ATCC 22019*, Candida tropicalis* ATCC 750 were procured from Hi-Media, Laboratories Pvt. Ltd. Mumbai, India. *Candida rugosa* NCIM 3462 was procured from National Chemical Laboratory, Pune. The physiological data of animals found positive for different species of *Candida* is presented in Table 1.

Confirmation of Candida spp. using automated VITEK 2.0 compact system: The *Candida* isolates were confirmed using VITEK®2 compact system (BioMerieux, France), using YST cards as per manufacturer's instruction. The Vitek 2.0 compact system is a fully automated instrument that provides a rapid colorimetric based measurement for specific species identification (Melhem *et al.* 2014). The results were classified as excellent (96-99 % probability), very good (93-95 % probability), good (89-92 % probability), and acceptable (85-88 % probability) based on the numerical probability calculation by comparing the unknown bio pattern to the database of reactions for each taxon.

Molecular confirmation of Candida spp.: DNA extraction was performed using the Zymo Research Kit and DNA purification was done by Wizard® DNA Clean-Up System (Promega Biotech India Pvt. Ltd.) in accordance with the manufacturer's protocol. PCR amplification of universal primers targeting internal transcribed spacer regions (ITS1 and ITS2) for *Candida* spp. was performed using the forward primer V9G (5′-TTACGTCCCTGCCCTTTGTA-3′) and the reverse primer LS266 (5′-GCATTCCCAAACAACTCGACTC-3′) as previously published by Merseguel *et al.* (2015). Primers were synthesised from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India for this study. The PCR reactions for various *Candida* spp. were carried out in 25 µL volumes in a thermocycler (Bio-Rad) with reaction components as 12.5 µL 2x master mix (Promega Biotech India Pvt. Ltd.), 2.0 µl forward and reverse primer each of 10µM concentration each, 3.0 µL template DNA and 5.5µl Nuclease free water. The thermal cycler conditions used for PCR were initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 56°C for 30 sec, extension at 72°C for two min and final extension at 72°C for 10 min followed by final hold at 4°C. The PCR products obtained were subjected to 1.5% agarose gel electrophoresis and bands were visualized under gel documentation system (Azure biosystems).

PCR-RFLP of Candida spp.: The PCR products were subjected to restriction enzyme treatment. For this, a reaction mixture was prepared containing 5.70 µL nuclease free water (NFW), 0.70 µL 10X buffer and 0.60 µL restriction enzyme (*HaeIII* and *TaqI;* New England Biolabs with their catalogue numbers as R0108S and R0149S) in a PCR tube. Then 7 µl of the reaction mixture was transferred to a PCR tube along with 3 µL of PCR product. Initially the reaction mixture along with PCR product was incubated at 37°C for 1 hr. Results could not be obtained, therefore, the incubation period was increased to 3 hrs. After incubation 3 µl loading dye was added to the final digested product and then agar gel electrophoresis was carried out to get the restriction fragment pattern. The power for discrimination by different restriction enzymes was measured by the formula of Simpson's index of diversity (Hunter and Gaston 1988).

Nucleotide sequencing and analysis: DNA purification was done using Wizard DNA clean-up system (Promega Biotech India Pvt. Ltd.) in accordance with manufacturer's protocol. The sanger DNA sequencing of PCR products obtained from AgriGenome Labs Pvt. Ltd. The sequences were checked for their quality and were used to blast against sequences available in Genebank, NCBI database. The sequences were processed to get complete sequence. The poor-quality sequence of each forward and reverse sequence was trimmed off and good quality sequences were used to make a contig sequence. Evolutionary analyses were conducted in MEGA6 and phylogenetic tree was generated using the UPGMA method (Tamura *et al.* 2013).

RESULT AND DISCUSSION

Isolation and identification of Candida spp. from mastitic milk samples by culture examination: Milk samples received from respective quarters were inoculated on blood agar (BA) at 37°C in BOD. incubator overnight. After incubation, the small, creamy-white colonies that appeared more raised and larger than normal bacterial colonies were considered as presumptive isolates of *Candida*. The tentative isolates of *Candida* subjected to crystal violet staining and demonstrated presence of distinctive yeast cells along with attachment of bud to the yeast cells (Fig. 1A). The shape, size and pattern were almost similar for all the isolates. These presumptive colonies of *Candida* (n=40) on the blood agar produced white to creamy, smooth, pasty

Table 1. Physiological data of the animals found positive for presence of various *Candida* spp. in their milk

Isolate no.	*Candida* species	Breed	Age (years)	Lactation	Pregnancy status
1	*C. tropicalis*	Non-descript	8	6th	Yes
2	*C. tropicalis*	Non-descript	6	3rd	Yes
3	*C. tropicalis*	Non-descript	6	3rd	Yes
4	*C. tropicalis*	Murrah	5	2nd	Yes
5	*C. tropicalis*	Non-descript	3	1st	Yes
6	*C. tropicalis*	Non-descript	5	2nd	Yes
7	*C. tropicalis*	Non-descript	6	3rd	Yes
8	*C. lusitaniae*	Non-descript	9	5th	Yes
9	*C. famata*	Non-descript	3	1st	Yes
10	*C. tropicalis*	Non-descript	5	2nd	Yes
11	*C. tropicalis*	Non-descript	6	3rd	Yes
12	*C. tropicalis*	Non-descript	4	2nd	Yes
13	*C. tropicalis*	Non-descript	4	1st	Yes
14	*C. albicans*	Non-descript	5	3rd	Yes
15	*C. tropicalis*	Non-descript	3	1st	Yes
16	*C. utilis*	Non-descript	5	2nd	Yes
17	*C. utilis*	Non-descript	5	2nd	Yes
18	*C. rugosa*	Non-descript	10	7th	Yes
19	*C. rugosa*	Non-descript	6	3rd	Yes
20	*C. tropicalis*	Non-descript	8	5th	Yes
21	*C. tropicalis*	Non-descript	4	1st	Yes
22	*C. tropicalis*	Murrah	5	2nd	Yes
23	*C. krusei*	Non-descript	5	2nd	Yes
24	*C. tropicalis*	Non-descript	3	2nd	Yes
25	*C. glabrata*	Non-descript	7	5th	Yes
26	*C. tropicalis*	Non-descript	4	1st	Undisclosed
27	*C. tropicalis*	Murrah	4	1st	Undisclosed
28	*C. tropicalis*	Non-descript	6	4th	Yes
29	*C. parapsilosis*	Non-descript	7	4th	Undisclosed
30	*C. tropicalis*	Murrah	4	1st	Yes
31	*C. tropicalis*	Murrah	6	3rd	Yes
32	*C. tropicalis*	Non-descript	5	2nd	Yes
33	*C. rugosa*	Non-descript	3	1st	Yes
34	*C. rugosa*	Non-descript	4	1st	Yes
35	*C. tropicalis*	Non-descript	3	1st	Yes
36	*C. tropicalis*	Murrah	6	4th	Yes
37	*C. tropicalis*	Non-descript	5	3rd	Yes
38	*K. ohmeri*	Non-descript	5	2nd	Yes
39	*C. tropicalis*	Murrah	4	1st	Yes
40	*C. rugosa*	Non-descript	3	1st	Yes

and convex colonies when grown on sabouraud dextrose agar (SDA) (Fig.1B). The different isolates could not be differentiated based on the colony characteristic on SDA medium. On Candida differential agar, out of the 40 isolates were incubated for 48 hour incubation, among them, 26 isolates of *C. tropicalis* produced blue colonies (Fig. 1C), a single isolate of *C. parapsilosis* produced creamy coloured colonies with slight purple tinge (Fig. 1D), a single isolate *C.* of *albicans* produced light green coloured colonies (Fig. 1E), 5 isolates of *C. rugosa* produced light green coloured colonies with some appearing creamy coloured appearance (Fig. 1F), a single isolate of *Candida krusei* produced pink with whitish border (Fig. 1G), 2 isolates of *Candida utilis* produced pale pink to pinkish purple (Fig. 1H), a single isolate of *Kodamaea ohmeri* produced green colored colonies (Fig.1I), a single isolate of *Candida glabrata* produced cream to white colour (Fig. 1J), a single isolate of *Candida lusitaniae* produced deep blue colour (Fig. 1K), while a single isolate of *Candida famata* produced blue with pinkish tinge (Fig. 1L).

Confirmation of Candida spp. using automated VITEK 2.0 compact system: Out of 600 milk samples, 40 samples (6.67%) were found positive for ten species of *Candida* by VIETK 2.0 compact system. Out of 40 isolates, 26 (65.0%),

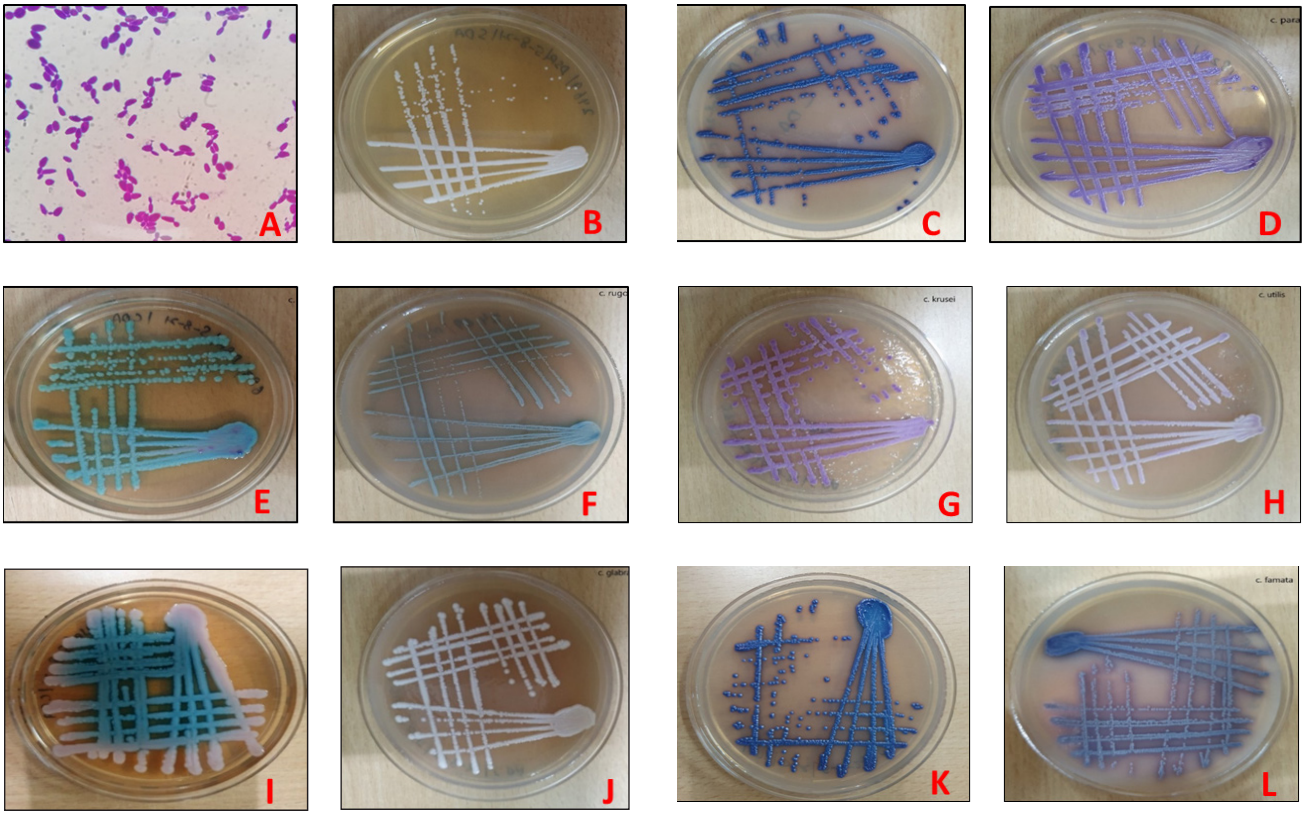

Fig.1. Identification and characterization of different *Candida* spp. isolated from mastitic milk samples. (A) Crystal violet staining of isolates showed presence of budding yeast cells, (B) Colony morphology of isolates on SDA- creamy to white, smooth, pasty convex colonies, (C) blue colored colonies of *Candida tropicalis*, (D) Creamy colored colonies with purple tinge of *Candida parapsilosis*, (E) green-colored colonies of *Candida albicans*, (F) Green colored colonies with few creamy colored colonies of *Candida rugosa*, (G) Pink with whitish border colored colonies of *Candida krusei*, (H) Pale pink to pinkish purple colored colonies of *Candida utilis*, (I) Green with pinkish border *Kodamaea ohmeri*, (J) Cream to white colored colonies of *Candida glabrata*, (K) Deep blue colored colonies of *Candida lusitaniae*, (L) Blue with pinkish tinge colored colonies of *Candida famata*

5 (12.5%), 2 (5.0%), 1 each (2.5%) were identified as *C. tropicalis, C. rugosa*, *C. utilis, C. parapsilosis*, *C. lusitaniae, C. albicans, C. krusei, C. glabrata, K. ohmeri, C. famata,* respectively.The probabilities of identification for each isolate were depicted in table 2. The average time taken for identification was approximate 18.25 (hrs) for all the samples.

In the present study, seven species *i.e. C. rugosa*, *C. utilis*, *C. parapsilosis*, *C. albicans*, *C. krusei*, *C. glabrata* (currently named *Nakaseomyces glabratus*), *K. ohmeri* were separately identified by the two identification methods *i.e*., CDA and Vitek 2.0. However, the three species i.e., *C. tropicalis*, *C. lusitaniae* and *C. famata* were separately identified by Vitek 2.0 compact system while on CDA they produced similar colours.

In recent years, the cases of fungi as a cause of mastitis are increasing (Mohammed and Yassein 2020) and the cases of mycotic mastitis are incurable or difficult to treat (Kalinska *et al.* 2017). Several species of the yeasts including various genera such as *Candida, Cryptococcus*, *Rhodotorula*, and *Trichosporon* have been associated with mastitis in dairy cows (Akdouche *et al.* 2018). In the present study, 06.67% samples were found to be positive for ten species of *Candida* by VITEK 2.0 compact system. Similar to the present study, Talukdar (2020) also reported 14.3% prevalence of mycotic mastitis caused by various *Candida* species. However, previous reports suggested that there was variation in both the prevalence and the *Candida* species reported in mycotic mastitis (Krukowski *et al.* 2006). Slightly higher yeasts and yeast-like fungi prevalence were reported by Khalaf *et al.* (2021), Asfour *et al.* (2009), Bekele *et al.* (2019) and Zhou *et al.* (2013) with percentages of 47.2%, 40.8%, 38.18% and 35.6% in Egypt, Ethiopia and China, respectively. This high prevalence may be due to insufficient milkers training, repetitive intramammary infusion, and poor teat hygiene prior to the intramammary infusion (Costa *et al.* 2012). Therefore, early detection and identification of yeast is essential for target antifungal therapy of mastitic cases and spread of this infection.

The traditional methods used for yeasts identification are generally time consuming and often not very accurate (Imran *et al.* 2020). One of the methods of identification is the use of chromogenic agar which is easy in its application, rapid yielding and helpful in species-level identification, although Talukdar (2020) also reported errors in the identification of *C. rugosa* using this method

Table 2. Identification confidence level of Vitek 2.0 compact system

Organism	N	Identification confidence level					
		Excellent (96-99 %)	Very good (93-95 %)	Good (89-92%)	Acceptable (85-88%)	Low discrimination	Unidentified
Candida tropicalis	26	20	6	–	–	–	–
Candida rugosa	5	5	–	–	–	–	–
Candida utilis	2	2	–	–	–	–	–
Candida parapsilosis	1	1	–	–	–	–	–
Candida albicans	1	1	–	–	–	–	–
Candida lusitaniae	1	1	–	–	–	–	–
Candida krusei (P. kudriavzevii)	1	1	–	–	–	–	–
Candida glabrata (N. glabratus)	1	1	–	–	–	–	–
Candida famata	1	–	–	–	1	–	–
Kodamaea ohmeri	1	1	–	–	–	–	–

Note: N is the number of isolates

due to variability in colony colour. Many studies have evaluated the performance of chromogenic media in routine diagnosis of *C.* spp. and have found them to be rapid and efficient for better detection and identification of *Candida* spp. (Ozcan *et al.* 2010, Vijayakumar *et al.* 2012, Bhaskaran *et al.* 2020). But, variations in the colour intensity with time, similarity in colony colour of related species leading to misidentification, presence of chromogenic medium for some *Candida* species only, makes it difficult to identify using chromogenic medium. In addition, chromogenic medium couldn't be used as a sole identification tool as biochemical identification must be performed to avoid incorrect identification (Ozcan *et al.* 2010).

Molecular confirmation of Candida spp.: All the 40 *Candida* isolates already identified by conventional and automated methods were further subjected to molecular confirmation by PCR amplification of highly variable internal transcribed spacer regions ITS1 and ITS2. All the 40 isolates amplified internal transcribed spacer regions ITS1 and ITS2 with variable size of amplicons (Fig. 2).

The sanger DNA sequencing of PCR products obtained from AgriGenome Labs Pvt. Ltd. All the sequences were checked and analyzed for homogenicity with other *Candida* species worldwide, using '*nblast*' tool of Genbank, NCBI database. Phylogenetic tree was generated ITS partial gene sequence obtained from various *Candida* species of the present study and gene bank sequences (Fig. 3). *C. tropicalis, C. lusitaniae* and *C. famata* isolates from the present study showed close homology (more than 98%) and were clusterd in same group with other *Candida* species strains from public domain. However, *C. parapsilosis*, *C. albicans, C. utilis*, *K. ohmeri, C. rugosa*, *C. krusei* (currently named *P. kudriavzevii*), *C. glabrata* (currently named *N. glabratus*) were grouped in separate cluster than *Candida* species strains, reflecting their unique genetic makeup.

PCR-RFLP analysis of amplified product: Furthermore, the amplified PCR products of 40 isolates were processed for PCR-RFLP analysis by *HaeIII* and *TaqI* restriction endonuclease enzymes (Fig.4A-D). There were erroneous results especially with *TaqI* enzyme. However, with subsequent changes in the digestion conditions, desired patterns were obtained with the enzyme. *C. rugosa*, *C. utilis*, *C. krusei* (currently named *P. kudriavzevii*), *C. glabrata* (currently named *N. glabratus*), *C. albicans*, *C. parapsilosis*, *K. ohmeri* produced characteristic pattern and can be distinguished separately on basis of digestion pattern. However, *C. tropicalis*, *C. famata*, *C. lusitaniae*

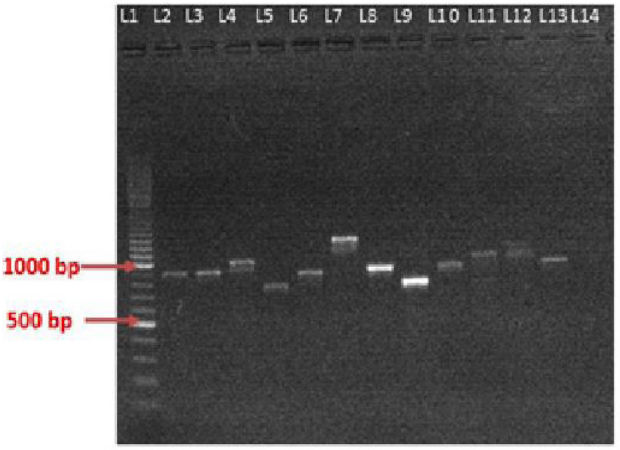

Fig.2. Agarose gel results of PCR amplification of *Candida* spp. (Lanes: L1-100 bp DNA ladder, L2: *C. lusitaniae*, L3: *C. famata*, L4- *C. utilis*, L5: *C. rugosa*, L6: *C. krusei* (currently named *Pichia kudriavzevii*), L7: *C. glabrata* (currently named *Nakaseomyces glabratus*), L8: *C. albicans*, L9: *K. ohmeri*, L10: *C. tropicalis*, L11: *C. parapsilosis*, L12: Positive control ATCC *C. arapsilosis*, L13: Positive control ATCC *C. albicans*, L14: Negative control)

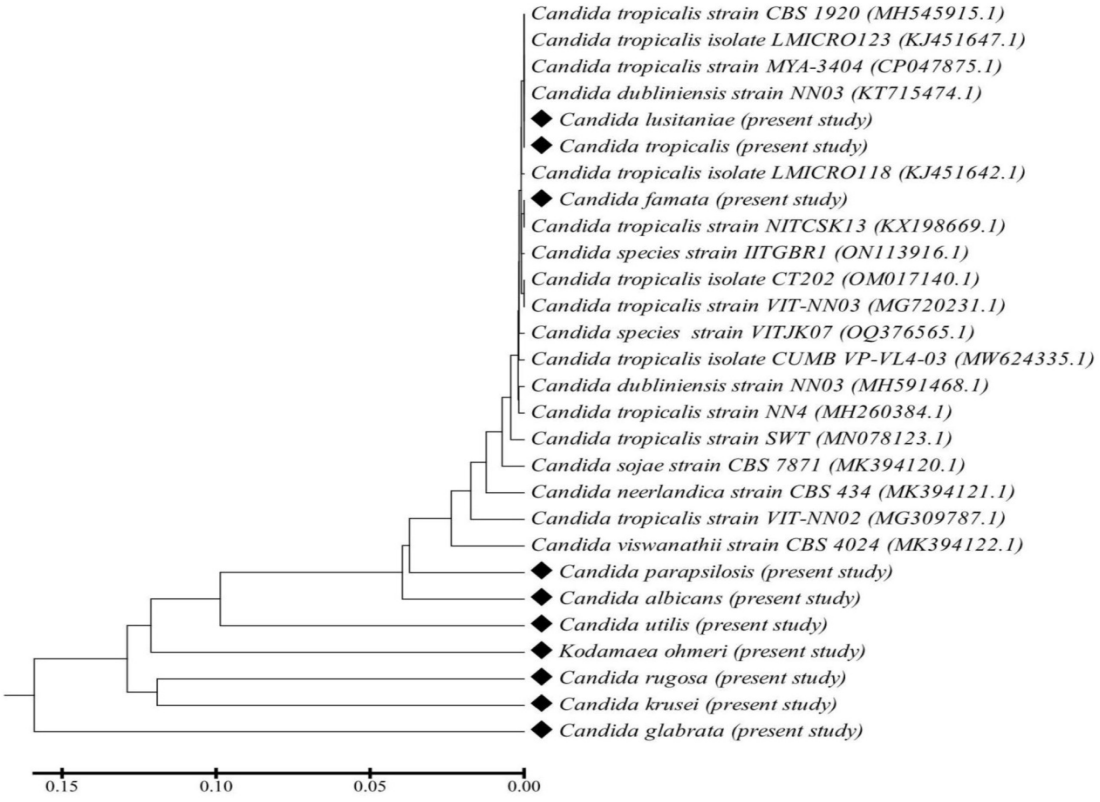

Fig. 3. Phylogenetic tree analysis of highly variable internal transcribed spacer regions (ITS) partial gene sequence obtained from various *Candida* species of the present study and public domain by UPGMA joining method using MEGA 6 software. *Note*: [*C. krusei* (currently named *Pichia kudriavzevii*), *C. glabrata* (currently named *Nakaseomyces glabratus*)]

produced similar digestion pattern and could not be distinguished from each other by the used restriction enzymes. PCR-RFLP analysis of *C. lusitaniae* and *C. famata* yielded characteristic pattern for both *HaeIII* and *TaqI* enzyme. While for *C. tropicalis*, characteristic pattern was obtained with *HaeIII* only but spurious bands were obtained with *TaqI* (Fig. 4A). For *C. parapsilosis* and *C. utilis*, characteristic patterns were obtained with *HaeIII* only but spurious bands were obtained with *TaqI* (Fig.4B). For *C. rugosa*, characteristic patterns were obtained with both enzymes. For *C. krusei* (currently named *P. kudriavzevii*) and *C. glabrata* (currently named *N. glabratus*) characteristic patterns were obtained with *HaeIII* only but spurious bands were obtained with *TaqI* (Fig. 4C). For *K. ohmeri* and *C. albicans* characteristic bands were obtained with *HaeIII* only but spurious bands were obtained with *TaqI* (Fig. 4D).

The length of contig sequences and the results of *in silico* analysis are shown in table 3. Characteristic fragment sizes were obtained with the seven *Candida* species i.e. *C. rugosa*, *C. utilis*, *C. krusei* (currently named *P. kudriavzevii*), *C. glabrata* (currently named *N. glabratus*), *C. albicans*, *C. parapsilosis*, *K. ohmeri* while the three *Candida* species *i.e. C. tropicalis*, *C. famata*, *C. lusitaniae* produced similar fragment sizes and could be distinguished from each other by any of the restriction enzymes. Phylogenetic tree generated by PCR RFLP using *HaeIII* enzyme generated four clusters with discriminatory power of 0.53 (Fig. 5A). Whereas, *TaqI* enzyme generated three clusters with discriminatory power of 0.60 (Fig. 5B). Thus, a discriminatory power (D value) of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, an index of 0.0 would indicate that all members of a strain population were of an identical type. An index of 0.50 would mean that if one strain was chosen at random from a strain population, then there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first (Hunter and Gaston 1988). Thus, the *in silico* RFLP analysis results confirmed with the *in vitro* RFLP digestion results.

In the present study, PCR-RFLP assay was developed for identification of all the ten isolates. It was based on the use of fungal-specific universal primer pair to amplify the internal transcribed spacer region (ITS) region of all the ten *Candida* species followed by restriction fragment length polymorphism for species identification using *HaeIII* and *TaqI* restriction endonucleases. In our study, the seven species i.e., *C. rugosa, C. utilis, C. parapsilosis, C. albicans, C. glabrata* (currently named *N. glabratus*), *K. ohmeri* were separately identified by the three identification methods i.e., CDA, Vitek 2.0 and PCR-RFLP. But the three

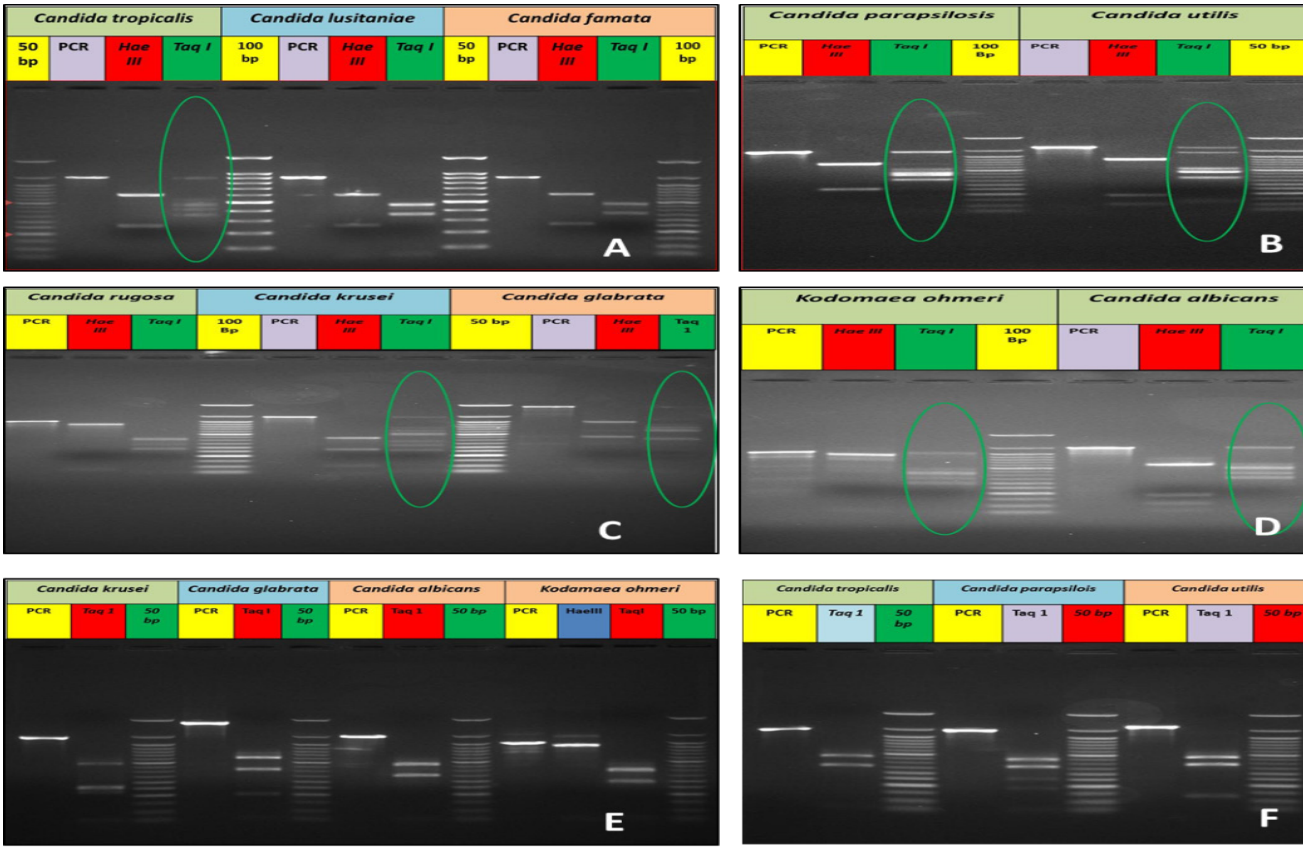

Fig. 4. PCR-RFLP of different *Candida* spp. isolated from mastitic milk samples *Note*: [*C. krusei* (currently named *Pichia kudriavzevii*), *C. glabrata* (currently named *Nakaseomyces glabratus*)]

Table 3. Length and sequence polymorphism of amplified regions in tested *Candida* spp. generated *In-silico*

Organism	Gen Bank Access. Num.	PCR fragment length (bp)	*HaeIII* fragments (bp)	*TaqI* fragments (bp)
Candida tropicalis	CP047875.1	913	43/267/603	8/18/59/361/467
Candida lusitaniae	CP047875.1	913	43/267/603	8/18/59/361/467
Candida famata	CP047875.1	913	43/267/603	8/18/59/361/467
Candida parapsilosis	MH545914.1	908	14/264/630	8/18/59/372/451
Candida utilis	MK394133.1	1008	111/195/702	18/128/390/472
Candida rugosa	GU144663.1	770	28/74/668	18/56/286/410
Candida krusei (P. kudriavzevii)	MH545928.1	889	32/38/104/279/436	18/58/325/488
Candida glabrata (N. glabratus)	CP048242.1	1271	62/451/758	8/18/59/210/418/558
Kodamaea ohmeri	MN268772.1	784	51/733	8/18/57/299/402
Candida albicans	CP025165.1	924	62/117/188/557	8/18/59/361/478

species i.e., *C. tropicalis, C. lusitaniae* and *C. famata* were separately identified only by Vitek 2.0 system while on CDA these species produced similar colours and showed similar pattern in PCR-RFLP. The results of this study are in concurrence with prior studies (Juyal *et al.* 2013, Vijayakumar *et al.* 2012, Daef *et al.* 2014). However, Sankari *et al.* (2019) reported that *Candida* differential agar method of speciation is unreliable compared to PCR–RFLP. The results on differential agar were not in agreement with PCR–RFLP. Percentage of disagreement was 40.2, 50.0, 100.0 and 25.0 for *C. albicans*, *C. krusei* (currently named *P. kudriavzevii*), *C. glabrata* (currently named *N. glabratus*) and *C. tropicalis,* respectively. In the present study, restriction digestion of the ITS amplification product with *HaeIII* and *TaqI* produced the predicted specific patterns for each species. Using this method, all ATCC stains as well as clinical isolates were identified successfully. Moreover, the results of PCR-RFLP analysis of the clinical isolates examined were comparable with those obtained on CDA. However, the results were not

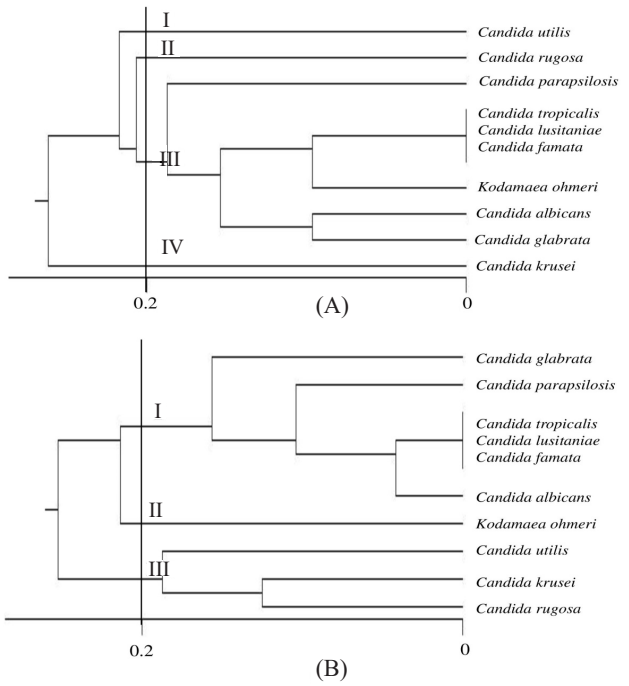

Fig. 5. Phylogenetic tree generated by PCR RFLP of ITS gene sequence obtained from various *Candida* species of the present study using *HaeIII* enzyme (A) and *TaqI* (B) enzyme. Note: [*C. krusei* (currently named *Pichia kudriavzevii*), *C. glabrata* (currently named *Nakaseomyces glabratus*)]

comparable with VITEK 2.0 compact system as digestion of *C. tropicalis, C. lusitaniae and C. famata* with *HaeIII* and *TaqI* yielded similar patterns and therefore additional enzymes for differentiation of these species are still required.

The findings of present study conclude that PCR-RFLP analysis may be used as diagnostic and differentiating tool for detection and differentiation of *Candida* species. Further, PCR-RFLP method is more reliable for identifying *Candida* species than *Candida* differential agar even though it may be a preferred method in a resource - limited lab setting. However, further studies are required to design PCR-RFLP as a rapid, sensitive and specific method for detection and identification of *Candida* species directly from milk samples from mastitic animals.

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