Isolation of Cryptococcus Laurentii Associated with Bovine Mastitis Cases in India

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Short Report

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Abstract

Mycotic mastitis mainly caused by yeasts like *Cryptococcus* spp. and *Candida* spp. have been emerging since last decade due to several factors like indiscriminate use of antibiotics, immune suppression, corticosteroid therapy, teat injuries and faulty milking machineries. The fungus, *Cryptococcus laurentii* is a non-neoformans, encapsulated, basidiomycete, which was earlier considered to be saprophytic and non-pathogenic. Now it is increasingly being reported in humans, especially in the immune compromised patients. In this study, *Cryptococcus laurentii* was isolated and identified from bovine mastitic milk samples. This probably might be the first report of isolation and identification of *Cryptococcus laurentii* from mastitic milk sample of buffalo from India to best of our knowledge. On milk culture examination, typical creamy white color colonies were appeared on Sabouraud's Dextrose agar, which on Gram's staining gave budding yeast cells appearance. India ink staining revealed bright halo of capsules surrounding the yeast cells. All the isolates were positive for urease production and biofilm formation. Further confirmation was done using VITEK 2 compact system (BioMerieux) which was based on their biochemical tests profiles. Molecular confirmation was done by the PCR assay. Isolation and identification of this rare fungus from milk samples in present study raises a potential threat of zoonosis.

Introduction

Bovine mastitis is a multifactorial disease causing huge economic losses to the dairy sector. Mastitis can be caused by over 250 microorganisms (Shome et al. 2011) which include bacteria, fungi, viruses and protozoa. Relative importance of different infections is likely to vary in different geographical regions. In the past, very little importance was given to fungal/mycotic mastitis due to very less percent prevalence but since last decade increasing trend is seen in reports of bovine mycotic mastitis. This could be attributed to several factors like indiscriminate use of antibiotics, immune suppression, corticosteroid therapy, teat injuries and faulty milking machines (Gupta et al. 2018).

The most common fungal species isolated from cases of bovine mastitis are the *Candida* species. However, there have been reports of isolation of different other yeast like *Cryptococcus* species as well. The genus *Cryptococcus* is a heterogenous group of encapsulated fungi including important pathogenic species for human and animals, most commonly the *Cryptococcus neoformans* and non-neoformans *Cryptococcus* species is considered to be non pathogenic. Eight percent of non-neoformans *Cryptococcus* species include *Cryptococcus laurentii (C. laurentii)* and *Cryptococcus albidus* (Khawcharoenporn et al. 2006). Earlier *C. laurentii* was considered to be saprophytic and non-pathogenic and has also been diagnosed as the etiological pathogen able to cause human infections mainly in immunosuppressed patients (Cheng et l. 2001; Khawcharoenporn et al. 2006; Banerjee et al. 2013). Many researchers focused on its transmission and effect on immune compromised persons (Cheng et l. 2001; Khawcharoenporn et al. 2006; Gupta et al. 2018). Their findings explored its pathogenicity in multiple hosts and considered it as a pathogen of major concern. *C. laurentii* has been reported to be a rare and opportunistic yeast pathogen isolated from bovine mastitic milk samples. It has also been associated with biofilm formation and refractory to most of the antibiotic treatments (Ajesh and Sreejith, 2012). The

present study is probably the first report of *C. laurentii* isolated from bovine mastitic milk samples from India.

Material And Methods

Isolation and biochemical identification

The College Central Laboratory (CCL), LUVAS, Hisar, Haryana, India 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 isolation and identification of rare *C. laurentii*. Mastitic milk samples were plated onto blood agar, McConkey agar, and Sabouraud dextrose agar and incubated at 37^0C for 48-72 h. Fungi were identified phenotypically, on the basis of colony morphology and further stained by Gram's stain and Indian ink capsular stain. The presumptive isolates were also tested for urease activity and biofilm production on Congo red agar plate.

The isolates were identified and confirmed using VITEK 2 Compact system (bioMerieux) and different biochemical tests results of each isolates were analyzed.

C. laurentii var. laurentii MTCC 2898 (Microbial Type Culture Collection) and *Cryptococcus neoformans* ATCC 14116 strain (American Type Culture Collection) were used as positive and negative control strains for identification, respectively.

Molecular confirmation of Cryptococcus laurentii

Phenotypically and biochemically confirmed isolates were subjected to DNA samples from all the isolates were extracted using Quick –DNA Miniprep PLUS kit (Zymo Research) and PCR amplification was performed targeting D1-D2 28S rDNA and 18S rRNA gene Internal transcribed sequence (ITS) region sequences (Barton, 2010). PCR using standard procedure was carried out using primer sets (Table 1) as reported earlier (Sugita et al. 2000).

Table 1
Primer sequence for PCR amplification

S. No	Target gene	OligoName	5'<—Sequence—>3'	Amplicon size (bp)	Reference
1	D1-D2 28S rDNA	CL/28S rDNA/F	GCATATCAATAAGCGGAGGAAAAG	600	Sugita et al. 2000
		CL/28S rDNA/R	GGTCCGTGTTTCAAGACGG		
2	18S rRNA gene Internal	CL/ITS/F	GTCGTAACAAGGTTAACCTGCGG	Variable	Sugita et al. 2000
	transcribed sequence (ITS)	CL/ITS/R	TCCTCCGCTTATTGATATGC		

Sequence analysis of Internal transcribed sequence (ITS)

The PCR amplified internal transcribed sequence (ITS) products of each isolate were sequenced by Sanger's Sequencer (Applied Biosystems). The sequences obtained were subjected to nucleotide BLAST (basic local alignment search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the similarity with the already prevalent gene sequences in GenBank (National Center for Biotechnology Information). Twenty nine reference sequences were obtained from public domain of genebank, NCBI with variable accession numbers. All sequences were aligned using Bio Edit (Hall et al. 2011) and MEGA6 (Tamura et al. 2013) software to study the variations in the nucleotide sequences and their phylogenetic cluster analysis.

Results And Discussion

Isolation and biochemical identification

A total of four isolates (Isolate number 421, 657, 1848, 2085) of *C. laurentii* were confirmed using phenotypic and biochemical test profile generated by automated VITEK 2 compact system. Automated VITEK 2 compact system revealed that isolate number 421, 657, 1848 and 2085 had 89%, 87%, 90%, and 88% confidence level (probability), respectively of being *C. laurentii* species. The isolates have typical creamy white colonies on Sabouraud's Dextrose agar (Fig. 1a) and blood agar plate. Gram's staining showed budding yeast cells (Fig. 1b) and India ink staining revealed bright halo of capsules surrounding the yeast cells (Fig. 1c). All the isolates were detected as biofilm producers (Fig. 1d) on Congo red agar plate by producing black coloured colonies and also tested positive for urease production (Fig. 1e). Biofilm formation is the important virulence factor of many microorganism including Candida and other fungi which are usually associated with chronic and recurrent infections. There are various methods to detect biofilm production, of them Congo red agar method is most convenient one (Saxena et al. 2014). Fourteen distinguished biotype patterns were observed on the basis of biochemical test result of each isolates by VITEK 2 compact system (Table 2). Biotype 1 comprised of 26 types of tests which were

tested positive by all the isolates. Biotypes 2 to 14 were tested positive by variable numbers of isolates (Table 2).

Molecular confirmation of Cryptococcus laurentii

All the four phenotypically and biochemically confirmed isolates amplified species specific 600 bp amplicon size of D1-D2 28S rDNA and 18S rRNA gene Internal transcribed sequence (ITS) region sequences producing variable size amplicon of *C. laurentii* by PCR using primer set reported earlier (Sugita et al. 2000).

Table 2

Biotyping of Cryptococcus laurentii **isolates on the basis of biochemical test results profile generated by VITEK 2 system**

Biotype	BIOCHEMICAL TEST (VITEK 2)	421	657	1848	2085
1	L-MALATE assimilation, Leucine-ARYLAMIDASE, ARGININE, GLYCEROL assimilation, ARBUTIN assimilation, GENTOBIOSE assimilation, D-GLUCOSE assimilation, LACTOSE assimilation, METHYL-A-D-GLUCOPYRANOSIDE assimilation, D-CELLOBIOSE assimilation, D-MALTOSE assimilation, D-RAFFINOSE assimilation, D-MANNOSE assimilation, L-RHAMNOSE assimilation, D-SORBITOL assimilation, SACCHAROSE/SUCROSE assimilation, D-TURANOSE assimilation, D-TREHALOSE assimilation, L-ARABINOSE assimilation, D-GALACTURONATE assimilation, D-XYLOSE assimilation, ACETATE assimilation, CITRATE (SODIUM) assimilation, L-PROLINE assimilation, 2-KETO-D-GLUCONATE assimilation, D-GLUCONATE assimilation	+	+	+	+
2	ALPHA-GLUCOSIDASE	+	+	+	-
3	D-GALACTOSE assimilation, D-MELEZITOSE assimilation, ESCULIN hydrolysis, L-GLUTAMATE assimilation	+	+	-	+
4	D-MELIBIOSE assimilation	+	+	-	-
5	ERYTHRITOL assimilation, XYLITOL assimilation	+	+	+	-
6	BETA-N-ACETYL-GLUCOSAMINIDASE	+	+	-	-
7	N-ACETYL-GLUCOSAMINE assimilation	+	-	+	+
8	Tyrosine ARYLAMIDASE	-	+	+	-
9	DL-LACTATE assimilation,	-	+	-	+
10	NITRATE assimilation	-	+	-	-
11	GLUCURONATE ASSIMILATION	-	-	+	+
12	L-SORBOSE assimilation, UREASE	-	-	+	-
13	GAMMA-GLUTAMYL-TRANSFERASE	-	-	-	+
14	L-Lysine-ARYLAMIDASE, AMYGDALIN assimilation, PNP-N-acetyl-BD-galactosaminidase 1	-	-	-	-

Sequence analysis of Internal transcribed sequence (ITS)

The PCR amplified products of 18S rRNA ITS of each isolates were sequenced by Sanger's Sequencer (Applied Biosystems). All of them showed 100% similarity with ITS sequences of *C. laurentii* species on nucleotide BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences with different geographical regions were retrieved from public domain and aligned using Bio Edit (Hall et al. 2011).

Phylogenetic tree was constructed using MEGA6 (Tamura et al. 2013) by maximum likelihood method based on whole ITS region of *C. laurentii* and related species with 1000 bootstrap replications. While tracing the sequences for fungi on NCBI, none of them was found to be of Indian origin.

Of the four isolates reported in the present study, two isolates (isolate number 421 and 1848) were in close homology with each other while two isolates (isolate number 647 and 2085) were clustered distinctly (Figure 3). None of them were clustered or grouped with *C. laurentii* isolates originating from the rest of the world. Phylogenetic analysis revealed their unique identities. All other isolates from USA and European origin were mixed and grouped in separate cluster. To best of our knowledge, none of the isolates were reported earlier from India. Probably this is the first report of *C. laurentii* isolated from mastitic milk of bovine in India.

Fungal infections, by both yeast and filamentous, are now considered as opportunistic agents causing severe illness in immune compromised hosts (both humans and animals) demonstrating its public health importance. They were previously considered to be non-pathogenic in most of the cases or their pathogenesis was not clearly understood. Various researchers have been working to characterize both most common as well as rare fungal agents in both animals and humans (Spanamberg et al. 2009; Dalanezi et al. 2018). *C. laurentii* has been associated with fungemia and pulmonary infection in humans ((Banerjee et al. 2013). Low birth weight neonate in humans from India has also been reported earlier (Gupta et al. 2018).

Bovine mastitis caused by fungi is considerably less frequent than that caused by bacteria and sometimes the infection can become chronic and more difficult to treat. With the wide use of antibiotics across the world, more and more yeast species are being reported from clinical samples. Researcher detected 41.33% of all the tested samples as positive for mycotic mastitis (Yassein et al. 2016). Of them, 55.64% were yeast isolates and 7.24% were *Cryptococcus neoformans*. Seven isolates of *C. laurentii* were reported in blood and urine samples of clinically suspected cases by VITEK 2 Compact system (Nath et al. 2018). Rare opportunistic fungal species has also been found in milk samples nowadays causing risk to public health (Krukowski and Saba 2003). Accurate diagnosis and differentiating bacterial infections from infections in cases of bovine mastitis has become very crucial to minimize potential risks and epidemiological importance. Automated microbial identification such as VITEK 2 compact system has been frequently in use for diagnosis of uncommon fungal pathogens. However their sensitivity and specificity towards variable pathogens may be altered due to numerous reasons. Some organisms are misidentified by commercial automated systems (Nath et al. 2018). Modern molecular techniques involving Internal Transcribed Sequence (ITS) region in clinical mycology can yield better accuracy in the etiological analysis of bovine mastitis. The D1/D2 region of 28S rDNA region serves for identification of many genera of yeasts as it is sufficiently variable to recognize species with little nucleotide divergence (Kurtzman and Robnett 1997; Sugita et al. 2000; Dalanezi et al. 2018). To the best of our knowledge, this might be the first report of rare *C. laurentii* species from bovine mastitic milk sample in India. The pathogenic potential of the organism is unknown. However, more studies are needed to understand its pathogenicity as primary pathogen causing mastitis in bovines. Additionally, understanding the common

patterns of resistance against non-*neoformans* cryptococcal infections especially *C. laurentii* will prevent further treatment failure and economic losses associated with bovine mycotic mastitis.

Declarations

Acknowledgements

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Conflict of Interest

The authors declare that they have no competing interests.

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Author contributions

RC developed the hypotheses, designed and performed the experiments, analyzed and interpreted data, and drafted the manuscript; RC, NKK and PG administrated the overall research project, oversaw the experiments, and reviewed the manuscript; RY, GS, SJT. assisted with the experiments, analyzed & interpreted the data, and critically revised the manuscript.

Data Availability

“The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

Consent to participate

Research involved no human subjects.

Ethics Approval

The milk samples used in the study were directly received in the laboratory from the animal owners for bacterial isolation and antibiotic sensitivity testing. History of the animal with the symptoms of mastitis was recorded at time of sample submission. The milking/milk sample collection procedure does not involve invasive procedure therefore ethical permissions are not indicated. The verbal consent was obtained from the animals owners for the samples under study.

Consent to publish

The authors affirm that the verbal consent was obtained from the animals owners for publication of data obtained for the samples under study.

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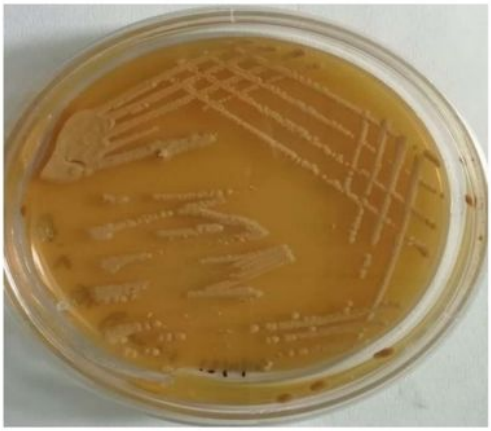
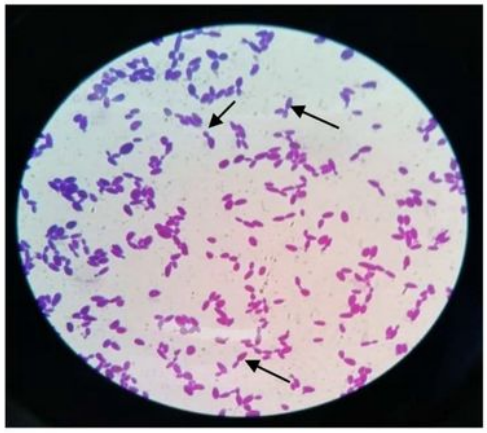
Figures



1(a)



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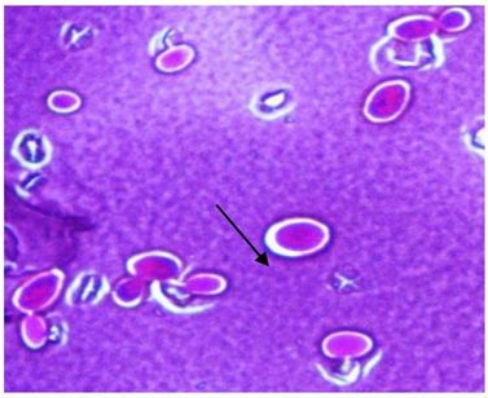
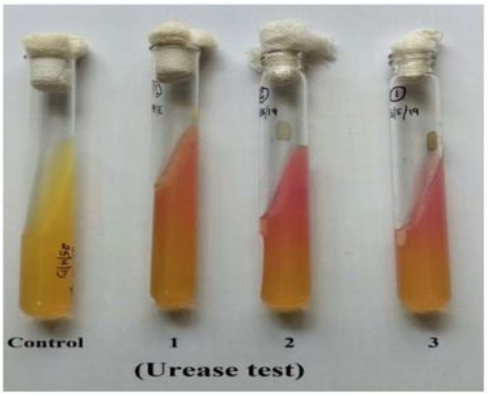


1(c)

1(d)



1(e)

Figure 1

(a) Colonies appeared creamy white with yeast like odour on SDA **(b)** Budding yeast cell seen on Gram's Staining **(c)** Bright halo of capsules surrounding the yeast cells by India ink **(d)** Biofilm forming *Cryptococcus laurentii* (Black colony) on Congo red agar **(e)** Urease test positive after 7 to 8 hrs unlike *Cryptococcus neoformans* positive in 15 min-2 hrs

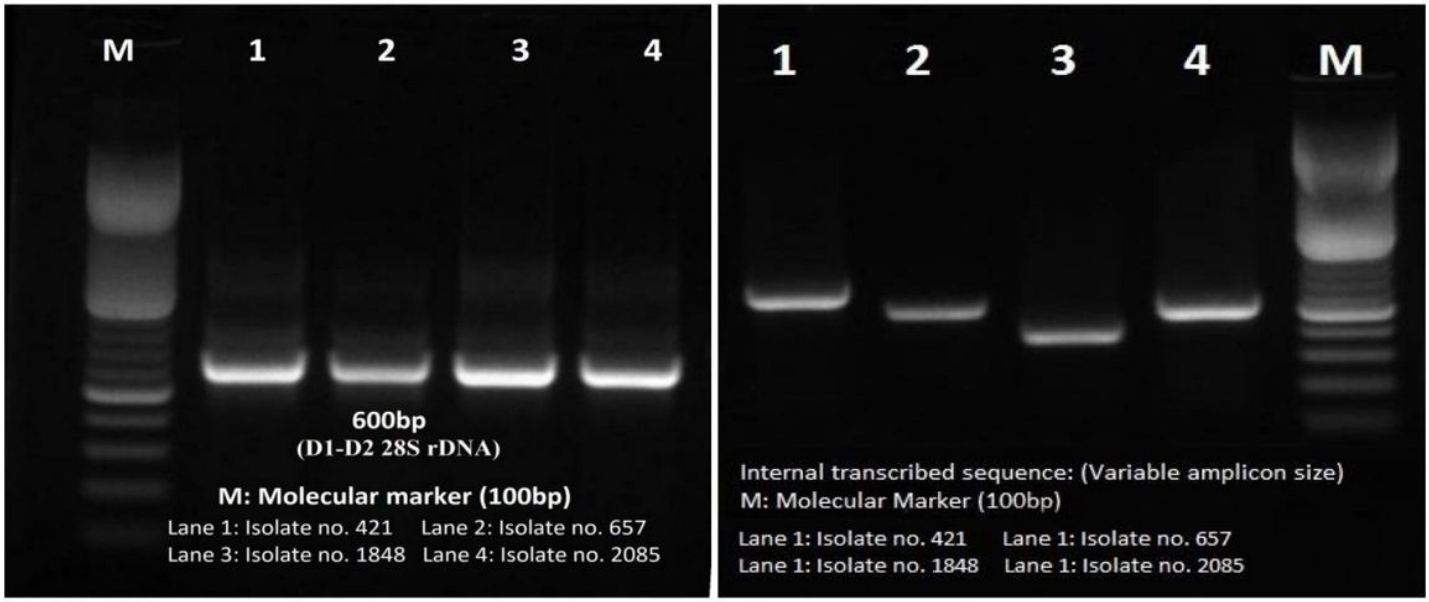


Figure 2

(a) Molecular confirmation of *Cryptococcus laurentii* isolates by PCR amplification of D1-D2 28S rDNA Sequences **(b)** PCR amplification of internal transcribed sequence with variable amplicon size

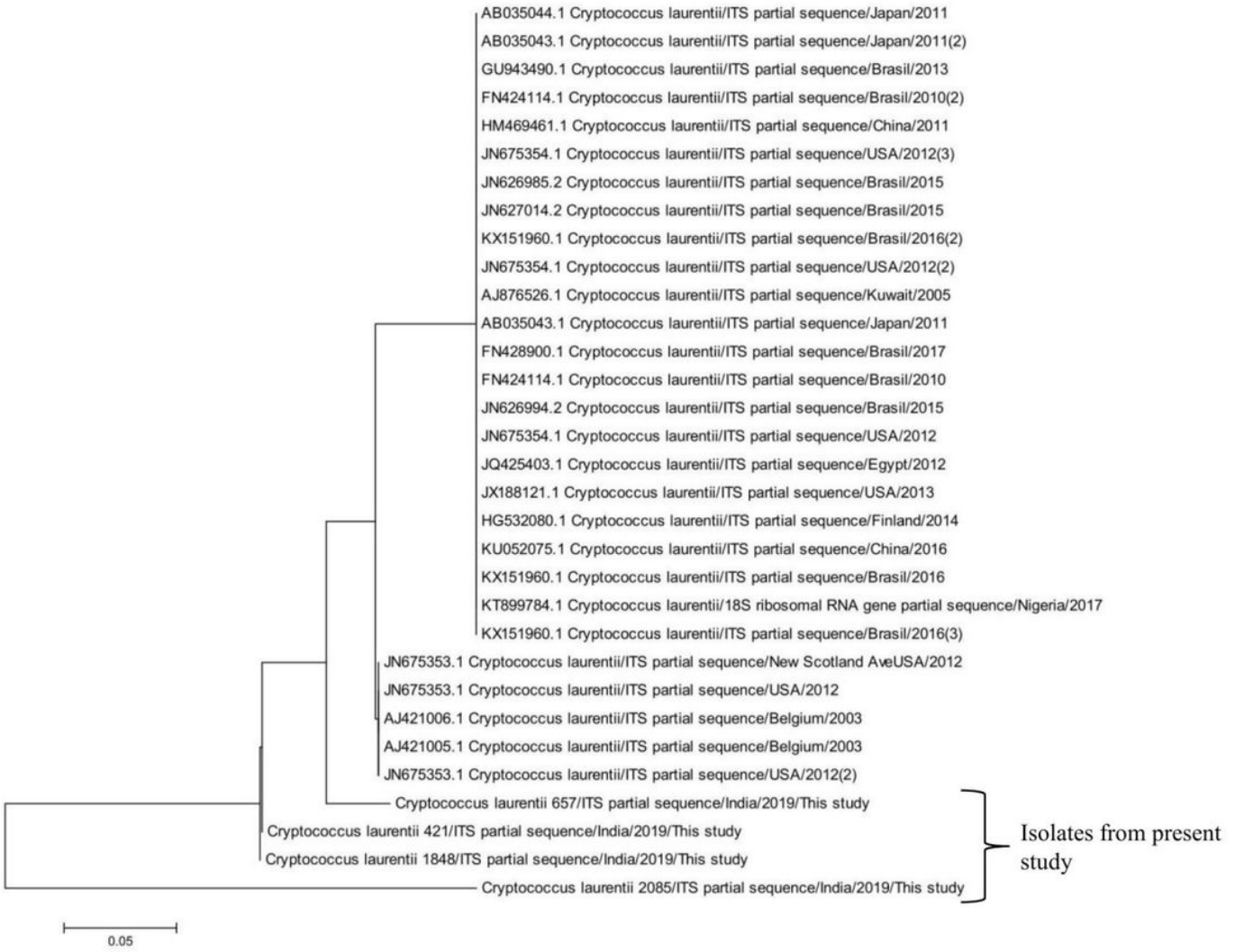


Figure 3: Dendrogram generated by sequence analysis of 18S rRNA internal transcribed sequence (ITS)

Figure 3

Phylogenetic tree (UPGMA) constructed by sequence analysis of 18S rRNA internal transcribed sequence (ITS)