Polymorphism analysis of virulence-related genes among Candida tropicalis isolates

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Abstract
Background: Adhesion, biofilm formation, yeast-hyphal transition, secretion of enzymes, and hemolytic activity are all considered important factors in Candida tropicalis infection. However, DNA sequence data for this pathogen are limited. In this study, the polymorphism and heterogeneity of genes agglutinin-like sequences (ALS)1-4 as well as the relationship between phenotype and genotype were analyzed.

Methods: This study started in August 2013, and ended in July 2017. The complete length of ALS2, LIP1, LIP4, and SAPT1-4 of 68 clinical C. tropicalis isolates was sequenced. Single nucleotide polymorphisms (SNPs) as well as insertions and deletions (indels) were identified within these genes. In addition, phenotypic characteristics of the virulent factors, including adhesion and the secretion of aspartyl proteinases and phospholipases, were determined.

Results: There were 73, 24, 17, 16, 13, and 180 SNPs in the genes LIP1, LIP4, SAPT1, SAPT2, SAPT3, and SAPT4, respectively. Furthermore, 209 SNPs were identified in total for the gene ALS2. Interestingly, large fragment deletions and insertions were also found in ALS2. Isolate FXCT 01 obtained from blood had deletions on all 4 sites and showed the lowest adhesion ability on the poly methylpentene surface. In addition, isolates with deletions in the regions 1697 to 1925 and 2073 to 2272 bp displayed relatively low abilities for adhesion and biofilm formation, and this phenotype correlated with the deletions found in ALS2. LIP1, SAPT4, and ALS2 displayed great heterogeneity among the isolates. Large deletions found in gene ALS2 appeared to be associated with the low ability of adhesion and biofilm formation of C. tropicalis.

Conclusion: This study might be useful for deeper explorations of gene function and studying the virulent mechanisms of C. tropicalis.

Keywords: Candida tropicalis; Virulence-related genes; Phylogenetic analysis; Gene ALS; Gene LIP; Gene SAP

Introduction
Candida species are considered to be the 4th most commonly isolated organisms from blood-stream infections in the United States, and the 6th most common in Europe; it is responsible for the overwhelming majority of urinary tract infections.¹ However, infections caused by non-Candida albicans Candida (NCAC) species, including C. tropicalis, C. glabrata, C. krusei, C. dubliensis, and C. parapsilosis, are increasing. Candida tropicalis accounts for 40% to 70% of mortality caused by blood infections, which is associated with many other factors, such as leukemia, neutropenia, central venous catheters, parenteral nutrition, and extended time in intensive care units.² Candida tropicalis has emerged as the second or third most common agent of candidemia, mainly in oncology patients, and is often associated with nosocomial urinary-tract infections.³ Candida tropicalis is close to C. albicans genetically, and it is able to form germ tubes, pseudohyphae, and hyphae.⁴ Adhesion to host surfaces (epithelial cells and medical devices), biofilm formation, the ability to undergo a morphological switch between yeast and hyphal growth, secretion of enzymes (proteases and phospholipases), and hemolytic activity are all considered important factors in C. tropicalis infection.⁵ ALS1-3, LIP1-10, and SAPT1-4 encode adhesins, lipases, and secreted aspartyl proteinases (Sap) of C. tropicalis, respectively.⁶ However, limited DNA sequence data have been published, and no study has investigated the role of these genes in the virulence of C. tropicalis.

Li-Juan Zhang and Shuan-Bao Yu contributed equally to the study.

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Until recently, little was known about the role of these genes involved in the virulence of *C. tropicalis*. Many studies have been performed for SAPT1-4 genes, including DNA sequencing and protein function studies.[8] However, limited DNA sequence data for ALS1-3 and LIP genes of *C. tropicalis* are available in GenBank. Moreover, there are only 2 whole genomes available for *C. tropicalis*. In this study, we designed primers for and amplified the genes SAPT1-4, ALS1-3, LIP1, and LIP4 based on the whole-genome sequences of *C. tropicalis* available.[4] We intend to study the heterogeneity of each virulence-related gene family and explore the relationship between the genotypes and phenotypes of *C. tropicalis*.

**Methods**

**Ethical approval**

The study (from August 2013 to July 2017) was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. As retrospective study and data analysis were performed anonymously, this study was exempt from the requirement of informed consent from patients.

**C. tropicalis isolates used in this study**

A total of 68 *C. tropicalis* isolates were obtained from 3 different general hospitals during the period of August 2013–July 2014 in Beijing, China. These strains belong to the archive collection of the Chinese Centre for Control and Prevention. The origins of these strains were diverse, including 31 from sputum, 10 from urea, 7 from feces, 4 from vaginal secretion, 3 from blood, 3 from drainage, 2 from throat swab, 1 from prostatic secretion, 1 from prostatic secretion, 1 from prostatic secretion, and 6 from other unknown. All isolates were identified by internal transcribed spacer (ITS) sequencing and AUX 20C (BioMérieux, Lyon, SA, France) in our lab. The universal primers ITS1 and ITS4 were used to amplify and sequence the ITS fragment in both directions.[8] The strains were stored at 280°C in brain-heart infusion (Oxoid, Basingstoke, UK). The isolates were maintained on Sabouraud agar (Oxoid) during the study. *In vitro* enzymatic activities (aspartyl protease, phospholipase, and hemolytic activities), adhesion, and biofilm formation were analyzed for all *C. tropicalis* isolates.

**Primer design, amplification of target genes, and sequence assembly**

Primers were designed to amplify the genes ALS2, SAPT1, SAPT4, LIP1, and LIP4 based on the related DNA sequences and whole genomes obtained from GenBank. Primers reported previously were used to amplify genes SAPT2 and SAPT3.[7] The total genomic DNA of the isolates was extracted using a Yeast DNA purification kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The concentration of the genomic DNA samples was estimated using a spectrophotometer by reading the absorbance at 260 nm. DNA extracts were stored at –20°C until use. SAPT1-3 and ALS2 were amplified through regular polymerase chain reaction (PCR), while SAPT4, LIP1, and LIP4 were amplified through long and accurate PCR (LA-PCR). Amplification was carried out in a final reaction volume of 50 μl that consisted of 25 μl of Premix Taq or Premix LA Taq (TAKARA, Tokyo, Japan), 20 μl of dH2O, 3 μl of template DNA, and 1 μl of the forward and reverse primers each. Amplification conditions are shown in Table 1. The amplified fragments were purified using a PCR purification kit (Qiagen, Düsseldorf, Germany) according to the manufacturer’s instructions. Both strands of the purified fragments were sequenced using the same primers as those used in the initial amplification. DNA sequencing was performed with an ABI 3730 DNA analyzer (Applied Biosystems, Beverly, MA, USA).

For some isolates, it was difficult to get high-quality sequences using PCR products for direct sequencing; for these isolates, the genes were 1st cloned to obtain the whole-gene sequences. Here, pGEM®-T Easy vector systems II (Promega, Fitchburg, WI) was applied as the cloning vector. The details of constructing the cloned vector and selecting positive clones to be sequenced were in accordance with the methods described earlier.

To get the whole length of each gene, primer walking strategy was applied for all genes except ALS2. Based on the obtained sequences from the 1st primers, the 2nd pair of primers was designed, and then PCR products were sent for sequencing. Then, the following pair of primers was designed based on the newly acquired sequences until the full length of the gene was obtained. Five pairs of primers were designed to amplify the whole length of gene ALS2 [Table 1]. Software DNA Star (http://www.dnastar.com) was used to assemble DNA sequences. Primers that were designed are shown in Table 1.

**Analysis of virulence phenotype and the corresponding genes**

The characteristic phenotypes of virulence factors, including adhesion and the secretion of aspartyl proteases and phospholipases, were determined as described previously.[9] In brief, the hydrolytic activity was determined on plates containing specific substrates by observing precipitation or the formation of a translucent halo. Adhesion was analyzed on both abiotic (polystyrene) and biotic (human urinary bladder epithelial cell) surfaces.[10] The complete lengths of the genes SAPT1-4, LIP1, LIP4, and ALS2 from all 68 *C. tropicalis* isolates were aligned and further analyzed using MEGA 6 separately. Single nucleotide polymorphisms (SNPs) in SAPT1-4, LIP1, and LIP4 were also determined using MEGA 6, and the results were displayed by Origin Viewer software (OriginLab Corporation, Northampton, MA, USA).

**Phylogenetic analysis of C. tropicalis isolates based on their virulence-related genes**

The genetic relationships among the 68 strains were determined using the neighbor-joining (NJ) method in MEGA 6 software based on each virulence gene family
independently. In addition, data of every single gene from each virulence gene family of *C. tropicalis* were conjoined into a single sequence (ie, the sequences of LIP1 and LIP4 were combined into LIP, the sequences of SAPT1-4 were combined into SAP), and then each base in the sequence was rewritten twice for a homozygous (A, C, G, or T) datum or as the 2 component bases for a heterozygous (K, M, R, S, W, Y) datum. These revised sequences were then used to generate the genetic distance matrices.

**Analysis**

The DNA sequences of all tested genes were assembled using Software DNA Star (http://www.dnastar.com). And then they were further aligned by MEGA 6. In addition, SNPs of these genes were also determined by MEGA 6 and results were displayed by Origin Viewer software. The genetic relationship among the 68 strains was determined using NJ tree in MEGA 6 software based on each virulent gene family.

**Results**

We successfully amplified the whole length of each gene from all 68 *C. tropicalis* isolates. Primers used in this study are displayed in Table 1. The length of ALS2 in the *C. tropicalis* isolates was 4071 bp, LIP1 was 1398 bp, LIP4 was 1392 bp, SAPT1 was 1185 bp, SAPT2 was 1820 bp, SAPT3 was 1200 bp, and SAPT4 was 1920 bp. All 68 *C. tropicalis* isolates were divided into 60, 66, 36, 21, 30, 35, and 64 genotypes according to ALS2, LIP1, LIP4, SAPT1, SAPT2, SAPT3, and SAPT4, respectively. The A+T content (60–65%) was much higher than the G+C content (35–40%) in all these genes. All these DNA sequences have been submitted to GenBank and their accession numbers are shown in Table 2. Furthermore, point mutations and SNPs were discovered in all gene sequences. There were 73, 24, 17, 16, 13, and 180 SNPs found in LIP1, LIP4, SAPT1, SAPT2, SAPT3, and SAPT4, respectively. The locations of the SNPs within each gene and the number of isolates with each SNP site are displayed in Figure 1.

Furthermore, point mutations and SNPs were discovered in all gene sequences. There were 73, 24, 17, 16, 13, and 180 SNPs found in LIP1, LIP4, SAPT1, SAPT2, SAPT3, and SAPT4, respectively. The locations of the SNPs within each gene and the number of isolates with each SNP site are displayed in Figure 1. In the SAPT gene family, there were several suspected SNPs that were identical among the 68 tested isolates but were distinct from the reference strain; there were 11 SNPs in SAPT1, 11 in SAPT2, 2 in SAPT3, and 2 in SAPT4 [Figure 1]. Furthermore, 209 SNPs in total were identified in ALS2. Interestingly, ALS2 had large fragment deletions and insertions [Figure 2]. There were 2 fragments of insertion located in the 1731 to 1841 and 2163 to 2273 bp regions. Isolate CYCT02 had insertion on both sites, while ZRCT45, 03, 06, 07, and 09 only had 1 insertion on either of the 2 sites [Figure 2]. Four fragments of deletion with lengths of 107, 228, 110, and 199 bp were found in the 1731 to 1841, 2163 to 2273, 2274 to 2374, and 2375 to 2475 bp regions, respectively.

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**Table 1: Primers used in this study.**

| Genes | Sequence 5’-3’ | Length of products (bp) | PCR conditions |
|-------|----------------|-------------------------|----------------|
| **SAPT1** | F: TGTTGTTATTGTAGATGGAGGAC | 1734 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R: TTTGGTCCATTATTGTTTCAT | 1530 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
| **SAPT2** | F: TCCGGTATTTTATTCCAGA | 1325 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R: TGAGGGCTAGTACCAATCGT | 1325 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
| **SAPT3** | F: AATTGGAATATAAATACGCT | 936 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R: GGGGGTGAAACTACAATTTA | 936 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
| **SAPT4** | F: GTCAAATGTCGCTGCTGGAGGA | 1970 | Same with LIP1,4 |
|        | R: ATCGTGGTGATGGATACGA | 1970 | Same with LIP1,4 |
| **ALS2** | F1: GGTGAAAGACGATCGAGCAT | 1214 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R1: CTTATTGGACCAAGTACCA | 1214 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | F2: CAGAATGTCGCTGCTGGAGGA | 1426 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R2: GGGGGTGAAACTACAATTTA | 1426 | 94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | F3: AGCATGTCGCTGCTGGAGGA | 936 | Same with primer 1,2 |
|        | R3: GGGGGTGAAACTACAATTTA | 936 | Same with primer 1,2 |
| **LIP1** | F: CCAGGAGCTATGGGTGAGT | 1945 | 94°C, 1 min; 98°C, 10 s; 50°C, 10 min; 35 cycles 72°C, 10 min; 4°C, ∞ |
|        | R: TAAGTGTAAAGTTGCGGTAGT | 1945 | 94°C, 1 min; 98°C, 10 s; 50°C, 10 min; 35 cycles 72°C, 10 min; 4°C, ∞ |
| **LIP4** | F: AAACACGCGACACCAACCTACA | 2277 | Same with primer 3 |
|        | R: TGGGCTGAGATCCACTAC | 2277 | Same with primer 3 |

*Primers reported in reference. † Five primer pairs were designed to cover the whole length of the gene. ALS: Agglutinin-like sequences; LIP: Lipase; SAPT: Secretory aspartyl proteinase tropicalis.*
| No. of strains | ZRCT01 | ZRCT02 | ZRCT03 | ZRCT04 | ZRCT05 | ZRCT06 | ZRCT07 | ZRCT08 | ZRCT09 | ZRCT10 | ZRCT11 | ZRCT12 | ZRCT13 | ZRCT14 | ZRCT15 | ZRCT16 | ZRCT17 | ZRCT18 | ZRCT19 | ZRCT20 | ZRCT21 | ZRCT22 | ZRCT23 | ZRCT24 | ZRCT25 | ZRCT26 | ZRCT27 | ZRCT28 | ZRCT29 | ZRCT30 | ZRCT31 | ZRCT32 | ZRCT33 | ZRCT34 | ZRCT35 | ZRCT36 | ZRCT37 | ZRCT38 | ZRCT39 | ZRCT40 | ZRCT41 | ZRCT42 | ZRCT43 | ZRCT44 | ZRCT45 | ZRCT46 | ZRCT47 | ZRCT48 | ZRCT49 | ZRCT50 | ZRCT51 | ZRCT52 | ZRCT53 | ZRCT54 | ZRCT55 | ZRCT56 | ZRCT57 | ZRCT58 | ZRCT59 | ZRCT60 | ZRCT61 | ZRCT62 | ZRCT63 | ZRCT64 | CYCT1 |
|---------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
Figure 1: The number and location of SNPs present in LIP1, LIP4, and SAPT1-4. (A) The details of the SNPs in LIP1 and LIP4. (B) The details of the SNPs in SAPT1-4. The horizontal axis represents the length of the genes. The vertical axis refers to the number of SNPs on each site and the location of SNPs on each gene. SNPs were analyzed to be either non-synonymous or synonymous mutations. LIP: Lipase, SAPT: Secretory aspartyl proteinase tropicalis, SNPs: Single nucleotide polymorphisms.

Table 2 (continued).

| No. of strains | SAPT1     | SAPT2     | SAPT3     | SAPT4     | LIP1     | LIP4     | ALS2     | DST  |
|----------------|-----------|-----------|-----------|-----------|----------|----------|----------|------|
| CYCT2          | MF924999  | MF925067  | MF925135  | MF925139  | MF924799 | MF924867 | MF924795 | 414  |
| FXCT01         | MF925000  | MF925068  | MF925136  | MF925140  | MF924800 | MF924868 | MF924796 | 415  |
| FXCT02         | MF925001  | MF925069  | MF925137  | MF925141  | MF924801 | MF924869 | MF924797 | 279  |

DST: Diploid sequence type based on multilocus sequence type analysis, SAPT: Secretory aspartyl proteinase tropicalis, LIP: Lipase.
Isolates with deletions in the 1697 to 1925 and 2073 to 2272 bp regions, respectively [Figure 2]. There were more isolates with deletions than isolates with insertions. For isolates ZRCT01, 28, 38, 41, 42, 44, and 46, and FXCT01 and 02, deletions were detected in all 4 sites [Figure 2].

Three phylogenetic trees were constructed for each gene family, and they displayed specific evolutionary features and distinct gene diversities among individual isolates [Figure 3]. The results showed great heterogeneity in the SAP gene family and in ALS2, indicating possible microevolution among the isolates that correlate with strain-dependent characteristics. The LIP gene family was the most conserved as they almost diverged from the same origin at the same time, indicating no occurrence of microevolution among the tested isolates. Interestingly, no lipase activity was observed for the C. tropicalis isolates in our previous study.

Analysis of the hydrolytic enzymes (proteases, phospholipases, and hemolysins) of all isolates was published in our previous study. All isolates produced protease and hemolytic activity, but none produced phospholipase and lipase activity. All isolates were divided into low, medium, and high activity groups. One strain each displayed high protease (ZRCT28), high hemolytic (ZRCT47), low protease (ZRCT64), and low hemolytic (ZRCT41) activities; the other isolates showed medium enzymes activities. Adhesion and biofilm formation of these 68 isolates on polymethylpentene (PMP) and TCC-SUP cells were also performed in our previous study. Strain ZRCT47 displayed the strongest adhesion and biofilm formation ability on both PMP and TCC-SUP surface as observed through crystal violet assay. However, ZRCT45 exhibited the highest biofilm formation ability on TCC-SUP cells when XTT assay was performed. Isolate FXCT01 obtained from blood had deletions on all 4 sites showed the lowest adhesion ability on PMP, and exhibited medium adhesion ability on TCC-SUP cells. Isolates with deletions in the 1697 to 1925 and 2073 to 2272 bp regions displayed relatively low abilities for adhesion and biofilm formation.

Discussion

Adhesion, biofilm formation, and hydrolytic enzyme activity were recognized as the key pathogenic elements of Candida species. In our previous study, the virulent phenotype of C. tropicalis was analyzed; we showed its strain-dependent features and the corresponding relationships with distinct genotypes. In this study, the complete sequences of virulence-related genes were obtained and their correlations with phenotype were analyzed.

It is known that Candida species have several different adhesins (special cell wall proteins) that allow adhesion to specific substrates. Agglutinin-like sequence (ALS) proteins are an important family of proteins involved in the process of adhesion by mediating attachment to different epithelial cells and functioning as an adhesin. Furthermore, Southern blot analysis with ALS-specific probes suggested at least three ALS-encoding genes in C. tropicalis, but no further work has been undertaken in this area. Limited information regarding the DNA sequences of ALS was found in GenBank. Moreover, the whole-genome sequence of C. tropicalis revealed 16 ALS-like sequences. From the sequences deposited in GenBank, we successfully amplified the whole length of the gene ALS2. The heterogeneity of ALS2 was the strongest among the 16 ALS-like sequences. Furthermore, deletions occurred more frequently than insertions in ALS2. For the isolates obtained from blood (FXCT01 and 02), deletions were found in all four sites [Figure 2], and this might explain their low adhesion abilities. Interestingly, isolates with deletions located in the 1697 to 1925 and 2073 to 2272 bp regions showed lower adhesion and biofilm formation abilities on PMP. Functional analysis was needed to more deeply explore the relationship between ALS2 and the adhesion ability of C. tropicalis.

The Saps of Candida have been intensively investigated. The secretion of Sap1-10 by C. albicans is recognized as an

Figure 2: Insertions and deletions identified in ALS2. I: insertions on the pattern of ALS2. D: deletions on the pattern of ALS2. Orange refers to the location of insertions. Blue refers to the location of deletions. Names of isolates in the orange and blue square indicate the isolates with insertions and deletions, respectively. The orange and blue numbers in each square refer to the isolates with insertions and deletions in all sites. ALS Agglutinin-like sequences.
Saps facilitate the colonization and invasion of host tissues through the disruption of host mucosal membranes, as well as by degrading important immunological and structural defense proteins.\(^{[17]}\) A total of 4 SAPT gene families of *C. tropicalis* were identified; however, SAPT1p is the only 1 that has been purified from culture supernatant, biochemically characterized, and crystallized.\(^{[18]}\) Sap secretion by *C. tropicalis* has also been detected when they penetrate tissues during disseminated infection, as well as on macrophages following phagocytosis of yeast cells.\(^{[19]}\) The SAP genes of *C. tropicalis* have been studied widely; however, complete sequences available on GenBank were limited. Here, we acquired the whole length of SAPT1-4 and found that genes SAPT1-3 were relatively conserved, while SAPT4 had great diversity. It is known that SAPT2 and SAPT4 were included in the multilocus sequence typing scheme of *C. tropicalis*. No significant relationship between gene sequence and activity of Saps was found in this study.

In addition, lipases (LIPs), which are involved in both the hydrolysis and synthesis of triacylglycerols, are often considered to be involved in *C. tropicalis* pathogenicity and contributes to host cell membrane damage.\(^{[20]}\) Ten genes encoding LIPs (LIP1-10) have been identified in *C. albicans*, and 5 similar lipase-encoding genes were also detected in *C. tropicalis*.\(^{[21]}\) Based on partial sequences and

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**Figure 3:** Neighbor-joining phylogenetic trees based on concatenated LIP, SAPT, and ALS2 nucleotide sequences. (A) Phylogenetic tree based on ALS2. (B) Phylogenetic tree based on LIP. (C) Phylogenetic tree based on SAPT. Arrows refer to isolates with insertions within gene ALS2. ALS: Agglutinin-like sequences; LIP: Lipase; SAPT: Secretory aspartyl proteinase tropicalis.
the whole genome of *C. tropicalis* obtained from GenBank, LIPI and LIPI4 were successfully amplified. Although LIPI1 displayed more diversity than LIPI4, no lipase activity was found in all 68 *C. tropicalis* isolates.[22-25]

In conclusion, we amplified and sequenced the complete lengths of ALS2, LIPI1, LIPI4, and SAPT1-4 of 68 clinical *C. tropicalis* isolates. SNPs and indels were found in these genes, and these were phenotypically analyzed; deletions found in ALS2 were associated with low adhesion ability. This study might be useful for further exploration of gene function in *C. tropicalis*. These findings should be verified using more isolates and the function of these genes should be studied further.

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**Conflicts of interest**

None.

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