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Genetic Variations of *Candida glabrata* Clinical Isolates from Korea using Multi-locus Sequence Typing

Min Ji Kang[1], Kyung Eun Lee[2] and Hyunwoo Jin[2]*

[1]*Department of Microbiology, College of Medicine, Chungnam National University, Daejeon 34134, Korea*
[2]*Department of Clinical Laboratory Science, College of Health Science, Catholic University of Pusan, Busan 46252, Korea*

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Although *Candida albicans* is the major fungal pathogen of candidemia, severe infections by non-*albicans Candida* (NAC) spp. have been increasing in recent years. Among NAC spp., *C. glabrata* has emerged as the second most common pathogen. However, few studies have been conducted to investigate its structure, epidemiology, and basic biology. In the present study, multi-locus sequence typing (MLST) was performed with a total of 102 *C. glabrata* clinical isolates that were isolated from various types of clinical specimen. For MLST, six housekeeping genes－*FKS, LEU2, NMT1, TRP1, UGP1*, and *URA3*－were amplified and sequenced. The results were analyzed using the *C. glabrata* database. Out of a total of 3,345 base-pair DNA sequences, 49 variable nucleotide sites were found, and the results showed that 12 different sequence types (ST) were identified from the 102 clinical isolates. The data also demonstrated that the undetermined ST1 was the most predominant ST in Korea. Further, seven undetermined STs (UST) containing UST2-8 were classified at specific loci. The data from this study may provide a fundamental database for further studies on *C. glabrata*, including its epidemiology and evolution. The data may also contribute to the development of novel antifungal agents and diagnostic tests.

Key words : *Candida glabrata*, non-albicans Candida, candidiasis, genetic variations, multi-locus sequence typing

Introduction

Candida spp. belong to the normal flora of the vaginal tract, the gastrointestinal tract, and the oral cavity in human [8, 18]. However, rarely, serious infections, ranging from mucosal infections to systemic infection have been caused by *Candida* spp. [8, 18]. Fungal infections caused by *Candida* spp. have increased significantly, especially in acquired immune deficiency syndrome (AIDS) and immunocompromised individuals, including intensive care and, elderly patients [3, 8]. Also, candidemia is associated with a high mortality rate approximately 30 to 40% in hospitalized patients and is difficult to treat, thus increasing the cost of medical care [3, 8].

C. glabrata has emerged as the second or third most common *Candida* pathogen after *C. albicans* in the United States, depending on the site [3, 7, 21]. Despite its increased prevalence, there have been relatively few studies on the population structure, epidemiology, and basic biology of *C. glabrata* compared to those conducted on other *Candida* spp. [6-8].

As mentioned above, *C. albicans* has considered to be the major fungal pathogen of candidemia in the past [6, 23]. However, as the number of severe infections caused by non-*albicans Candida* spp. (NAC) have increased, studies have shifted from *C. albicans* to NAC such as *C. glabrata* in recent years [11, 21]. Furthermore, since *C. glabrata* infections are often resistant to azole antifungal drugs, especially fluconazole, it is important to distinguish NAC from *C. albicans* to ensure the appropriate antifungal therapy and clinical management [8, 20, 21]. Thus, the discrimination of subtypes in these species are required for investigating their epidemiology and evolutionary biology [15, 23, 24].

In recent years, there has been substantial progress in the development of several molecular methods for typing subspecies and strains of fungi [26]. For instance, pulsed-field gel electrophoresis (PFGE) compares total DNA band patterns with or without restriction enzyme digestion, while multilocus variable-number tandem-repeat (VNTR) analysis

***Corresponding author**
Tel : +82-51-510-0567, Fax : +82-51-510-0568
E-mail : jjjnhw@cup.ac.kr

Table 1. Details of diverse yeast isolates used in this study

Clinical specimens	No. of samples (%)
Blood	64 (63)
Urine	14 (14)
Bile	9 (9)
Others[1)]	14 (14)
-[2)]	1 (1)
Total	102 (100)

[1)]Others: ascitic fluid, joint fluid, pleural fluid, tissue etc.
[2)]No clinical information.

examines length variations in six to nine PCR-amplified loci that contain polymorphic tandem repeats. Further, the random amplification of polymorphic DNA compares banding patterns following PCR with a nonspecific primer. Finally, multilocus enzyme electrophoresis, studies the different electrophoretic mobility of multiple core metabolic enzymes. These four approaches have some limitations, such as a lower reproducibility and portability [10, 22], and the results obtained in different laboratories are difficult to compare [2, 13].

Among these genotyping methods, multilocus sequence typing (MLST) is a useful tool to assign single nucleotide polymorphisms as allele numbers, which are stored in a database on line (PubMLST) and determine the differences from between closely related isolates by their geographical origins, sources, and other properties [23]. Also, it is possible that database are accessed by laboratories worldwide [13, 24].

In the present study, MLST was performed with a total of 102 *C. glabrata* clinical isolates from various clinical specimens such as blood, urine, and other body fluids in Korea. The results were analyzed by using the *C. glabrata* MLST database (http://pubmlst.org/cglabrata/). The aim of the study is to discriminate STs in the same *C. glabrata* spp. by using common MLST and investigate the most prevalent ST from the *C. glabrata* in Korea.

Materials and Methods

Clinical strains

A total of 102 *C. glabrata* clinical isolates were provided from Korean Culture Collection of Medical Fungi (KCMF) and those isolates were collected from tertiary hospitals in Korea. Clinical isolates were isolated from a wide variety of clinical samples, including blood, catheterized urine, bile and other body fluids (Table 1).

Genomic DNA extraction from fungal isolates

Genomic DNA of *C. glabrata* clinical isolates was extracted using a I-genomic BYF DNA Extraction Mini Kit (iNtRON Inc., Seongnam, Korea) according to the manufacturer's instructions [5]. The concentration and purity of the genomic DNA were checked by 260/280 optical density using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted genomic DNA was stored at 4℃ until use.

Polymerase chain reaction and sequence analysis of the fungal ITS region for precise identification of *C. glabrata* clinical isolates

The fungal internal transcribed spacer (ITS) region, the conserved region between the 18S and 28S ribosomal RNA (rRNA) was amplified and sequenced using each primer pairs (Table 2). Target amplification was carried out in 20 μl reaction mixture containing 10 μl Prime Taq Primix (Genet Bio Inc., Daejeon, Korea), 5 μl of distilled ultra-pure water, 1 μl of each primer (10 pmol/μl), and 3 μl of genomic DNA template. The PCR condition was: an initial denaturation at 94℃ for 1 min, 30 cycles including subsequent denaturation at 94℃ for 30 sec, annealing at 57℃ for 30 sec and extension at 72℃ for 45 sec followed by final extension at 72℃ for 7 min and holding at 4℃. The amplified products were visualized by gel electrophoresis to confirm the presence of desired product.

The resulting amplicon was purified and sequenced by Macrogen Inc. (Daejeon, Korea). All sequences with low-quality bases in the chromatogram were re-sequenced for the high-quality results. The obtained sequences were aligned with reference sequences in the Genebank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), and percent homology scores were generated to precise identification of

Table 2. Sequences of primer pairs for fungal species identification

Target region	Primer	Nucleotide sequences (5′ to 3′)	Amplicon size (bp)	Tm (℃)	Reference
ITS	pITS-F	GTCCTAACAAGGTTAACCTGCGG	970-980	62.4	[16]
	pITS-R	TCCTCCGCTTATTGATATGC		55.3	

Table 3. List of gene fragments and primer sequences for *C. glabrata* MLST analysis

Target gene	Gene product	GenBank accession no.	Primer	Nucleotide sequences (5′-3′)	Sequenced fragment size (bp)	Tm (℃)	Reference
FKS	1,3-Beta-glucan synthase	AF229171	FKSF FKSR	GTCAAATGCCACAACAACAACCT AGCACTTCAGCAGCGTCTTCAG	589	55.0	[6]
LEU2	3-Isopropylmalate dehydrogenase	U90626	LEU2F LEU2R	TTTCTTGTATCCTCCCATTGTTCA ATAGGTAAAGGTGGGTTGTGTTGC	512	54.0	
NMT1	Myristoyl-CoA, protein N-myristoyltransferase	AF073886	NT1F NMT1R	GCCGGTGTGGTGTTGCCTGCTC CGTTACTGCGGTGCTCGGTGTCG	607	59.0	
TRP1	Phosphoribosyl-anthranilate isomerase	U31471	TRP1F TRP1R	AATTGTTCCAGCGTTTTTGT GACCAGTCCAGCTCTTTCAC	419	50.0	
UGP1	UTP-glucose-1-phosphate uridylyltransferase	AB037186	UGP1F UGP1R	TTTCAACACCGACAAGGACACAGA TCGGACTTCACTAGCAGCAAATCA	616	57.0	
URA3	Orotidine-5′-phosphate decarboxylase	L13661	URA3F URA3R	AGCGAATTGTTGAAGTTGGTTGA AATTCGGTTGTAAGATGATGTTGC	602	53.0	

C. glabrata clinical isolates.

MLST analysis for identifying sequence type of *C. glabrata* clinical isolates

Table 3 shows primers for the amplification and sequence analysis of *C. glabrata* six housekeeping gene fragments including *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3*. For the PCR amplification, 20 μl of final mixture contained 10 μl of Prime Taq Premix, 5 μl of distilled ultra-pure water, 1 μl of each forward and reverse primer (10 pmol/μl), and 3 μl of genomic DNA template. To amplify each six gene, the PCR reaction conditions were as follows: 7 min at 94℃, 30 cycles of 1 min at the relevant annealing temperature (Table 3), and 1 min at 74℃, followed by 10 min at 74℃.

The resulting sample was analyzed by gel electrophoresis. The PCR product of all loci were purified and sequenced using reverse sequence primer at Macrogen Inc.. The obtained sequences were analyzed by using the *C. glabrata* MLST database (http://pubmlst.org/cglabrata/). Each unique sequence at a locus defined an allele number, and unique combinations of alleles assigned as a ST.

Data analysis

The alignment of combined six target gene sequences and loci (3,345 bp) was performed using the Molecular Evolutionary Genetics Analysis (MEGA) v. 7.0 software [12]. For relatedness of the same species, the phylogenetic tree was drawn with the Unweighted Pair Group Method using Arithmetic algorithm (UPGMA) with randomized 1,000 bootstrapping. And then the eBURST package (http://eburst.mlst.net/) was used to determine that all related isolates were grouped into clonal complexes.

Results

PCR and sequence analysis of the fungal ITS region for species identification

1.5% TBE agarose gel DNA electrophoresis data showed that the size of amplified fungal ITS region was 978 bp, and amplicons have shown one clear band (data not shown). As a analysis result of comparison by Genebank BLAST tool for verifying the amplified PCR products, all clinical isolates used in this study were identified as *C. glabrata* with high concordance rate (97.8%±2.9).

PCR, sequence analysis, obtaining allele number of six housekeeping genes for the MLST analysis

In order to perform the MLST analysis, six housekeeping genes of 102 *C. glabrata* clinical isolates were amplified by PCR. The size of amplified fragments of *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP*1, and *URA3* were 589 bp, 512 bp, 607 bp, 419 bp, 616 bp, and 602 bp, respectively as the expected size and they represented clear band (data not shown).

Amplified six housekeeping gene fragments were sequenced and then sequenced data was trimmed as each length manually by using Chromas software. Trimmed sequence of six housekeeping gene fragments was analyzed at the *C. glabrata* PubMLST database then allele number of each gene was obtained. Obtained allele number of *FKS* gene were 3, 5, 7, 8, and 10. Those of *LEU2* gene were 5, 6, 16,

Table 4. The number of polymorphic site and different alleles, and sequence variable position of six housekeeping gene fragments in 102 *C. glabrata* clinical isolates

Locus	No. of polymorphic sites (%)	No. of alleles defined	Variable positions
FKS	5 (0.9)	5	403, 352, 154, 118, 43
LEU2	6 (1.2)	4	392, 384, 336, 290, 111, 54
NMT1	12 (2.0)	8	575, 551, 512, 480, 434, 396, 341, 328, 305, 301, 243, 155
TRP1	13 (3.1)	8	387, 378, 357, 352, 333, 309, 272, 268, 255, 229, 176, 162, 158
UGP1	4 (0.7)	4	585, 435, 413, 195, 99
URA3	9 (1.5)	7	581, 574, 556, 549, 440, 380, 257, 164, 44
Total	49		

and 17. Those of *NMT1* gene were 3, 4, 6, 8, 11, 14, 19, and 22. Those of *TRP1* gene were 2, 3, 5, 7, 10, 12, 19, and 50. Those of *UGP1* gene were 1, 3, 5, and 51. Those of *URA3* gene were 2, 4, 6, 8, 9, 17, and 20 (Table 4). A total of 49 (1.5%) polymorphic sites were found among six housekeeping gene fragments. The number of polymorphic site in each gene fragment were as follows: *FKS* (5, 0.9%); *LEU2* (4, 1.2%); *NMT1* (8, 2.0%); *TRP1* (8, 3.1%); *UGP1* (4, 0.7%); and *URA3* (7, 1.5%), as shown in Table 4. Additionally, insertions, deletions, or heterozygosity were only detected in the *NMT1* fragments.

Sequence type and cluster of *C. glabrata* clinical isolates

The MLST scheme revealed a high diversity of *C. glabrata* isolates with a total of 12 STs, 8 of which were identified as undetermined STs (USTs) that were not discovered in the previous studies.

The data demonstrates that the UST1 was the most predominant ST in this study as a total of 54 clinical isolates (52.9%) were contained in this ST, and the following most predominant ST was the ST63 as a total of 24 clinical isolates (23.5%) were contained in this ST. In addition, this study obtained the ST55, ST22, and ST43 were as a total of 3 (2.9%), 6 (5.9%), and 2 (1.2%) clinical isolates were contained in respective ST and the UST2 was identified in 6 isolates (5.9%).

Table 5. Unique sequence types determined with a combination of six loci

C. glabrata sequence types	no. of isolates (%)
ST63	24 (23.5)
ST22	6 (5.9)
ST55	3 (2.9)
ST43	2 (1.2)
undetermined ST1	54 (52.9)
undetermined ST2	6 (59)
undetermined ST3	2 (1.2)
undetermined ST4	1 (1)
undetermined ST5	1 (1)
undetermined ST6	1 (1)
undetermined ST7	1 (1)
undetermined ST8	1 (1)
Total	102 (100)

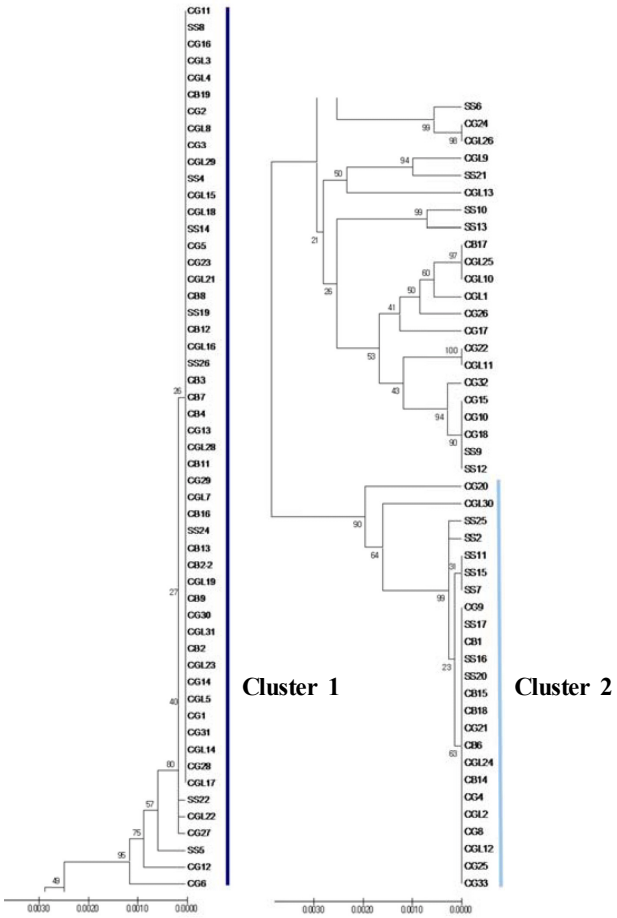

Fig. 1. Evolutionary relationships of distinct sequence types. UPGMA dendrogram used to determine the pairwise differences of the concatenated sequence of *C. glabrata* clinical isolates.

The UST3 was identified in 2 isolates (1.2%). The remaining 5 USTs (UST4, -5, -6, -7, and -8) were classified only once each (1%) (Table 5).

Combined sequence (3,345 bp) of six housekeeping genes was used for the phylogenetic tree analysis. Apart from 3 outliers, the isolates were divided into 2 major clusters: cluster 1 and cluster 2 (Fig. 1). Cluster 1 consisted of the UST1 and cluster 2 consisted of the ST63 (Fig. 2, Fig. 3).

Discussion

C. glabrata is a highly opportunistic pathogen of the urogenital tract and the bloodstream in humans [17]. It is especially prevalent in the elderly and within the human immunodeficiency virus positive population [19]. Although candidiasis is frequently treated with azole antifungal agents, treatment failure has become a serious concern with azole-resistant clinical isolates due to widespread and long-term use of these agents. Nevertheless, few studies have been conducted on the structure, epidemiology, and basic biology of *C. glabrata.*

Healthcare-associated infections may be endogenous in origin or nosocomially transmitted, and the only way to distinguish them is through strain typing. Recently, MLST directly investigated the DNA sequence variations in a set of housekeeping genes and characterized the strains by their unique allelic profiles. The principle of MLST is simple, involving PCR amplification followed by DNA sequence analysis. Nucleotide differences between strains can be verified at a variable number of genes depending on the desired degree of discrimination. MLST schemes now exist for several important bacterial pathogens including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Campylobacter jejuni*. The technique has also been used to assess genetic relatedness among strains of *Candida spp*. including *C. glabrata*, *C. albicans*, *C. tropicalis*, and *C. krusei*. However, MLST scheme for *C. glabrata* has only been used by Dodgson, A. R. (2003) and Katiyar, S. (2016).

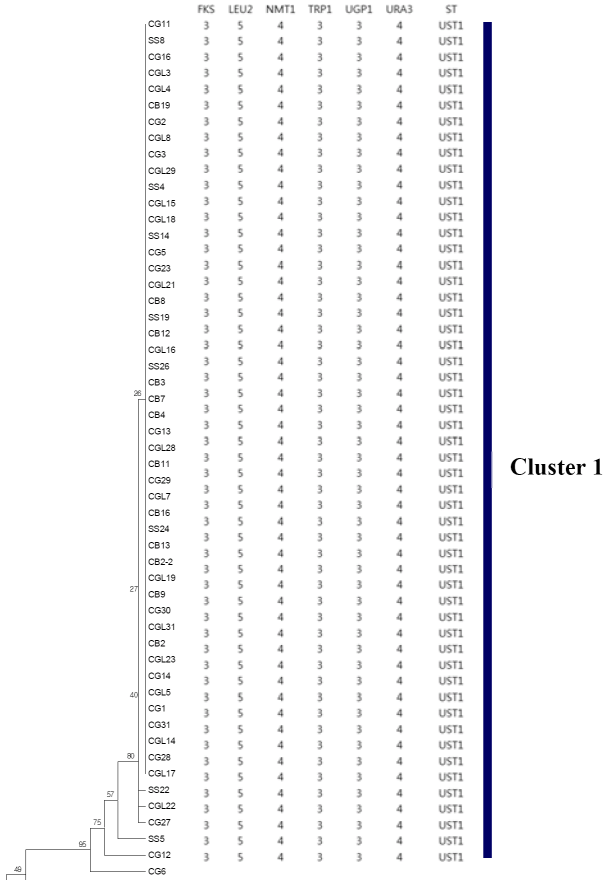

Fig. 2. Phylogenetic tree generated from each isolate. The MLST allele profile, sequence type (ST), and eBURST group (Cluster 1) are listed.

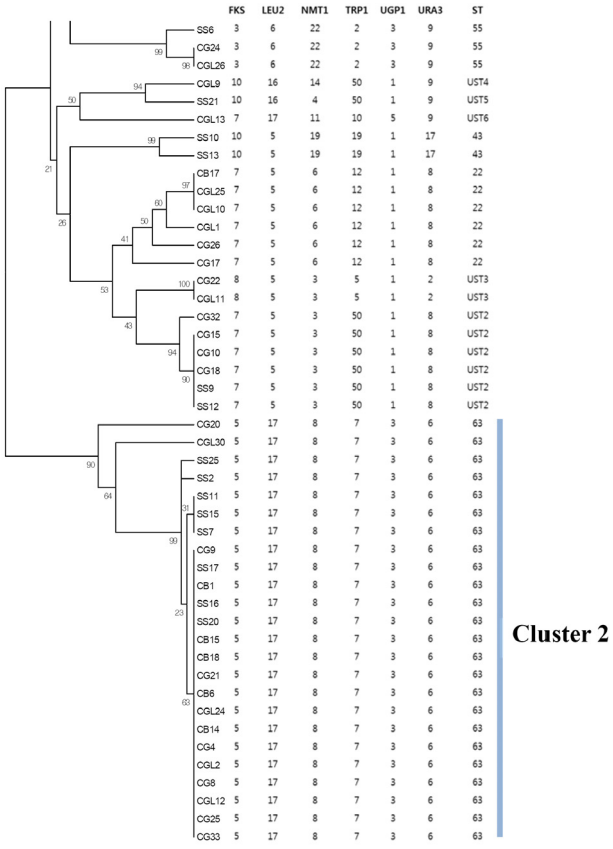

Fig. 3. Phylogenetic tree generated from each isolate. The MLST allele profile, sequence type (ST), and eBURST group (Cluster 2) are listed.

Hence, in this study, the first MLST analysis with the yeast pathogen *C. glabrata* was performed and evaluated in Korea. 6 loci were selected for this study, as recommended by previous studies. While a ST3 was defined as prevalent ST in Dodgson, A. R. (2003), the data in this study demonstrates that the UST1 was the most predominant ST. Additionally, the data defined a total of 12 STs among the 102 clinical isolates, and found 8 USTs as a result.

In conclusion, prevalent and novel *C. glabrata* STs were found in the present study. The data might provide a fundamental database for further studies on *C. glabrata*, including its epidemiology and evolution. Furthermore, these data might also contribute to the development of novel antifungal agents and diagnostic tests. It might even be possible to discover the virulence factors associated with disease, which population genetic studies currently struggle to monitor.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Multi-locus sequence typing을 이용한 한국에서 분리한 *Candida glabrata* 임상균주의 유전자 유형 분석

강민지[1] · 이경은[2] · 진현우[2]*
([1]충남대학교 의과대학 미생물학교실, [2]부산가톨릭대학교 보건과학대학 임상병리학과)

*Candida albicans*가 칸디다 혈증의 주요 원인 균으로 알려져 있지만, 최근에는 non-albicans Candida (NAC)에 의한 심각한 감염이 증가하고 있다. NAC 중에서 *C. glabrata*는 두 번째로 병원성을 나타내는 원인 균이지만 *C. glabrata*의 구조, 역학 등의 기본적인 연구는 거의 없는 실정이다. 따라서 본 연구에서는 다양한 유형의 임상 표본으로부터 분리된 총 102개의 *C. glabrata*균주로 multi-locus sequence typing (MLST)을 수행하였다. *FKS, LEU2, NMT1, TRP1, UGP1* 및 *URA3*을 포함한 6개의 하우스키핑 유전자를 증폭하여 염기서열을 분석하였다. 총 3,345개의 DNA 염기서열 중 49개의 가변 염기서열 부위가 발견되었으며, 그 결과 102개의 균주에서 총 12개의 상이한 서열 유형을 확인하였고, 알려지지 않은 서열 유형(USTs) 중 UST1이 가장 많이 나타났다. 이 연구의 결과는 국내 *C. glabrata* 항생제 처방에 도움이 될 것으로 사료되며, 한국에서 유행하는 *C. glabrata*에 대한 추가 연구를 위한 기본 역학자료로 사용될 것으로 기대한다.