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Molecular Identification and Antifungal Susceptibility Testing of Clinical Isolates of the *Candida rugosa* Species Complex and Proposal of the New Species *Candida neorugosa*

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Candida rugosa is a poorly known fungal species occasionally involved in human infections. A molecular analysis of the sequences of the D1/D2 domains and the internal transcribed spacer (ITS) region of the ribosomal genes of 24 clinical isolates phenotypically identified as *C. rugosa* demonstrated that only 10 (41.6%) isolates belonged to that species. The other isolates were identified as *Candida pararugosa* (41.6%) and *Candida pseudorugosa* (8.3%). The remaining two isolates, from human and equine infections, respectively, were clearly different from the others and represent a new species proposed here as *Candida neorugosa*. The closest species by D1/D2 sequences was the type strain of *C. rugosa*, with only 92.3% similarity. *C. neorugosa* can also be differentiated from all other species of the *C. rugosa* complex by phenotypic features. The eight antifungal drugs tested showed high *in vitro* activity against the 24 isolates included in the study.

The incidence of *Candida* infection has increased in recent years, representing an important cause of morbidity and mortality. Although candidiasis is caused mainly by *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (15), infections caused by rarer species have increased in recent years (7, 18). Among these less common species, *Candida rugosa* has been recognized as an emerging fungal pathogen capable of causing invasive infection in immunocompromised patients (12), mostly related to the use of catheters but also by other modes of nosocomial acquisition (6, 8, 12). This species represents only 0.2% of the *Candida* isolates in the global ARTEMIS DISK Antifungal Surveillance Program (18) but shows a high prevalence in Latin America (18) and India (19). The fungus has decreased susceptibility to fluconazole (FLC) (17, 18), amphotericin B (AMB) (7), and the echinocandins (7). Considering its increasing pathogenic role and the potential development of resistance to antifungals, the reliable identification of *C. rugosa* is an important issue. However, the biochemical systems currently used for yeast identification in clinical laboratories commonly fail to identify the less frequent *Candida* spp. (23). Some genetic heterogeneity in *C. rugosa* has been reported (2, 13, 21), and the novel species *Candida pseudorugosa*, closely related to *C. rugosa*, has been recently proposed (11).

We have analyzed the D1/D2 domains and the intergenic transcribed spacer (ITS) sequences of the rRNA genes of a set of clinical isolates phenotypically identified as *C. rugosa* in order to assess the genetic heterogeneity of the species.

MATERIALS AND METHODS

Fungal isolates. A total of 24 clinical isolates, received as *C. rugosa* by the Fungus Testing Laboratory in the Department of Pathology at the University of Texas Health Science Center (UTHSC) at San Antonio, TX, for identification and/or antifungal susceptibility determination, were included in the study. In addition, several ITS and D1/D2 sequences from type or reference strains, retrieved from GenBank, were also included in the phylogenetic analyses (Table 1).

DNA extraction, amplification, and sequencing. The fungal isolates were grown on potato dextrose agar (PDA) (Pronadisa, Madrid, Spain) at 28°C for 24 h, and DNA was extracted using a PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The DNA was quantified using GeneQuant Pro (Amersham Pharmacia Biotech, Cambridge, England). The D1/D2 domains of the 24 isolates and the ITS regions of two isolates from the different clades obtained in the D1/D2 phylogenetic analysis were amplified with the primer pairs NL1/NL4 and ITS5/ITS4, respectively, following the protocols described by Cano et al. (4) and Gilgado et al. (10). The PCR products were purified and sequenced with the same primers used for amplification at Macrogen Europe Inc. (Amsterdam, The Netherlands) with a 3730XL DNA analyzer (Applied Biosystems). The program SeqMan (Lasergene, Madison, WI) was used to obtain consensus sequences.

Alignment and phylogenetic analysis. The sequences were aligned using the ClustalX (version 1.8) computer program (22) with default parameters, followed by manual adjustments with a text editor. The phylogenetic analysis was performed with the software program MEGA 5.0 (20), using Maximum Likelihood (ML) with General-Time-Reversible (GTR) as a substitution model. Gaps were treated as pairwise deletion. Support for internal branches was assessed by a search of 1,000 bootstrapped sets of data.

Phenotypic studies. Morphological, biochemical, and physiological characterization of a representative number of isolates of the different clades obtained in the molecular study was performed using methods and protocols previously described (24). The tests included growth on Sabouraud chloramphenicol agar (Bio-Rad, Marnes-LaCoquette, France) at 30°C, 37°C, and 45°C; growth in liquid culture medium; germ tube tests;

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TABLE 1 Isolates and sequences of *C. rugosa* species complex included in the study

Species	Isolate ^a	Origin	GenBank accession no.	
			ITS	D1/D2
<i>C. rugosa</i>	CBS 613 ^T	Feces of man	AY500374 ^b	GU246244 ^b
<i>C. pseudorugosa</i>	CBS 10433 ^T	Sputum, Tianjin, China	DQ234792 ^b	DQ234791 ^b
<i>C. catenulata</i>	NRRL Y-1508 ^T	Feces of man, Puerto Rico	AY493436 ^b	CCU45714 ^b
<i>Clavispora lusitaniae</i>	CBS 4413 ^T	Cecum of pig, Portugal	EU568907 ^b	AY190538 ^b
<i>C. pararugosa</i>	NRRL Y-17089 ^T	Feces of man	AF421856 ^b	CPU62306 ^b
<i>C. rugosa</i>	LYSM3	Soil from forest, Thailand		AB498988 ^b
<i>C. rugosa</i>	EB2	Water in mangrove forest, Thailand		AB436404 ^b
<i>C. rugosa</i>	EF1	Water in mangrove forest, Thailand		AB436406 ^b
<i>C. rugosa</i>	STC4	Blood, Kuala Lumpur, Malaysia		HQ412590 ^b
<i>C. rugosa</i>	STC1	Blood, Kuala Lumpur, Malaysia		HQ412589 ^b
<i>C. rugosa</i>	L154	Feces of man, Brazil		FJ768915 ^b
<i>C. rugosa</i>	L2683B	Blood, Brazil		FJ768918 ^b
<i>C. rugosa</i>	L387A	Rectal swab, Brazil		FJ768920 ^b
<i>C. rugosa</i>	L412D	Catheter, Brazil		FJ768919 ^b
<i>C. rugosa</i>	L69D	Blood, Brazil		FJ768917 ^b
<i>C. pseudorugosa</i>	MZKI K-259	Coastal Arctic, Norway		EU056285 ^b
<i>C. pseudorugosa</i>	MZKI K-269	Coastal Arctic, Norway		EU056286 ^b
<i>C. pararugosa</i>	CBS 9121	Saliva, sarcoma patient, Japan		AB112430 ^b
<i>C. pararugosa</i>	CBS 9122	Saliva, sarcoma patient, Japan		AB112432 ^b
<i>C. rugosa</i>	UTHSC 01-2568	Toe, USA		HE716177
<i>C. rugosa</i>	UTHSC 05-205	Blood, USA		HE716180
<i>C. rugosa</i>	UTHSC 05-646	Jackson-Pratt drain, USA		HE716182
<i>C. rugosa</i>	UTHSC 05-1919	Ankle, USA		HE716179
<i>C. rugosa</i>	UTHSC 06-3729	Ear, USA	HE716760	HE716180
<i>C. rugosa</i>	UTHSC 06-3931	Stool, USA		HE716181
<i>C. rugosa</i>	UTHSC 06-3976	Sputum, USA		HE716178
<i>C. rugosa</i>	UTHSC 09-1289	Dolphin, USA		HE716183
<i>C. rugosa</i>	UTHSC 09-1402	Blood, USA		HE716176
<i>C. rugosa</i>	UTHSC R-3412	Unknown	HE716759	HE716175
<i>C. pararugosa</i>	UTHSC 03-344	Bronchial wash, USA		HE716167
<i>C. pararugosa</i>	UTHSC 03-1143	Blood, USA		HE716170
<i>C. pararugosa</i>	UTHSC 04-1051	Blood, USA		HE716173
<i>C. pararugosa</i>	UTHSC 05-1693	Bronchial wash, USA		HE716172
<i>C. pararugosa</i>	UTHSC 06-538	Blood, USA		HE716174
<i>C. pararugosa</i>	UTHSC 07-2797	Blood, USA		HE716171
<i>C. pararugosa</i>	UTHSC 07-3133	Blood, USA		HE716169
<i>C. pararugosa</i>	UTHSC 08-442	Urine, USA	HE716757	HE716166
<i>C. pararugosa</i>	UTHSC 09-2953	Vaginal, USA	HE716758	HE716165
<i>C. pararugosa</i>	UTHSC 10-2648	Blood, USA		HE716168
<i>C. pseudorugosa</i>	UTHSC 06-3641	Catheter urine, USA	HE716755	HE716163
<i>C. pseudorugosa</i>	UTHSC 08-707	Knee, USA	HE716756	HE716164
<i>C. neorugosa</i>	SK75	Ulcerated lesion, Brazil	GQ176145 ^b	GQ176145 ^b
<i>C. neorugosa</i>	UTHSC 10-2054 ^T	Leg wound, USA	HE716762	HE716185
<i>C. neorugosa</i>	UTHSC 10-121	Wound on left forelimb of horse, USA	HE716761	HE716186

^a CBS, Centraalbureau voor Schimmelmcultures, Utrecht, The Netherlands; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, TX; NRRL, National ARS Culture Collection, Peoria, IL.
^b Sequences retrieved from GenBank database.

assessment of chlamydospore and ascospore production; hydrolysis of esculin; and the urease test. The ability of the isolates to assimilate carbohydrate source compounds was determined for glucose, D-xylose, melibiose, L-arabinose, D-ribose, L-sorbose, galactose, salicin, raffinose, sucrose, D-mannitol, trehalose, glycerol, 2-ceto-D-gluconate, ribitol, xylitol, inositol, sorbitol, α-metil-D-glucoside, N-acetyl-D-glucosamine, cellobiose, lactose, maltose, melezitose, and citric acid. Chromogenic testing of the colonies was performed on Chromagar *Candida* (Chromagar Company, Paris, France). The API ID 20C yeast identification kit (bioMérieux SA, Lyon, France) was also used for identification according to the manufacturer's instructions.

In vitro studies. We evaluated the antifungal susceptibility of the fungal isolates to AMB, FLC, itraconazole (ITC), posaconazole (PSC), voriconazole

(VRC), caspofungin (CAS), micafungin (MCF), and anidulafungin (AND). The tests were performed in duplicate using a broth microdilution method according to the M27-A3 guidelines for yeasts (5). MIC results for echinocandins and FLC were read after 24 h and the others after 48 h of incubation. Two reference strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were included as quality controls for all testing.

Nucleotide sequence accession numbers. The new DNA sequences generated in this study were deposited in the GenBank database (Table 1).

RESULTS

Molecular analysis. The phylogenetic tree inferred from the ML analysis of the D1/D2 sequences revealed that the 24 isolates tested

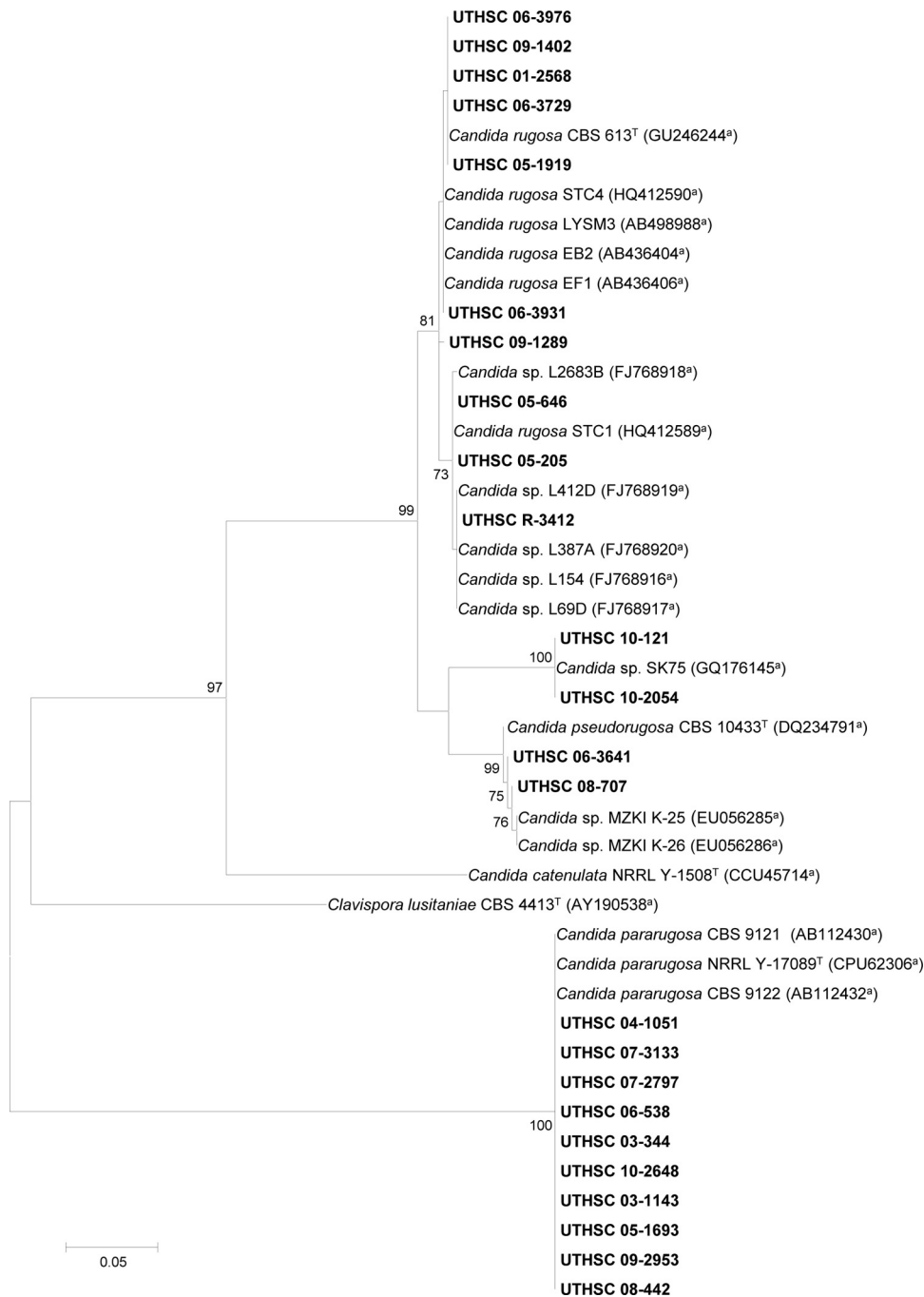


FIG 1 ML tree obtained from the D1/D2 domains of the 28S rRNA gene sequences of the strains of *C. rugosa* and related taxa. Bootstrap support values above 70% are indicated at the nodes. Clinical isolates from UTHSC are indicated in boldface. The bar indicates genetic distance. Sequences retrieved from GenBank are marked with a superscript "a."

were distributed in four statistically well-supported groups, each representing a phylogenetic species (Fig. 1). The first group (bootstrap support [bs], 81%) was formed by 10 clinical isolates, the sequence of the type strain of *C. rugosa*, and several sequences of clinical and environmental strains retrieved from GenBank, some of which were previously reported as atypical isolates of *C. rugosa* (21). The isolates' sequence similarities ranged from 99.3 to 100%. The second group (bs, 100%) was comprised of three identical

sequences; two belonged to our clinical isolates (UTHSC 10-2054 and UTHSC 10-121) and the third (SK75; GQ176145), retrieved from GenBank, was from an ulcerated lesion in Brazil. The members of that group showed low similarity to the type strains of the species *C. rugosa* (92.3%), *C. pseudorugosa* (92%), and *C. pararugosa* (67.3%). The third group (bs, 99%) consisted of two clinical isolates, the type strain of *C. pseudorugosa*, and sequences of two environmental isolates retrieved from GenBank. The sequence

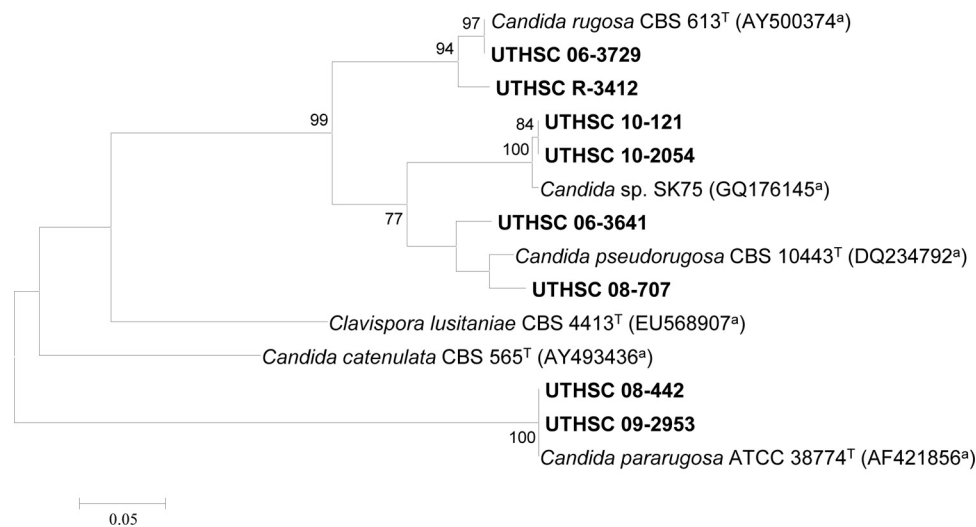


FIG 2 ML tree obtained from the ribosomal ITS sequences of several isolates of *C. rugosa* and related taxa. Bootstrap support values above 70% are indicated at the nodes. Clinical isolates from UTHSC are indicated in boldface. The bar indicates genetic distance. Sequences retrieved from GenBank are marked with a superscript “a.”

similarity of the members of this group ranged from 99.2 to 100%. Finally, 10 clinical isolates were nested with the type strain of *C. pararugosa*, along with two sequences from clinical isolates deposited in GenBank (bs, 100%).

The phylogenetic tree inferred from the ML analysis of the ITS sequences (Fig. 2) showed topology and genetic relationships similar to those seen in the D1/D2 tree.

Physiology. Four of the physiological tests used were useful to distinguish the three phylogenetically most closely related species, i.e., *C. rugosa*, *C. pseudorugosa*, and the unidentified species represented by the isolates UTHSC 10-2054 and UTHSC 10-121. The two isolates of each species tested showed identical results (Table 2). *C. rugosa* was differentiated from *C. pseudorugosa* by the utilization of D-xylose, glycerol, and sorbitol. Clinical isolates UTHSC 10-2054 and UTHSC 10-121 differ from *C. rugosa* by their ability to assimilate ribitol and from *C. pseudorugosa* by their ability to assimilate D-xylose, glycerol, ribitol, and sorbitol. API ID 20C results confirmed those findings. On Chromagar, *C. pseudorugosa* and *C. rugosa* yielded dark blue-green and from white to light-blue colonies, respectively, while the isolates UTHSC 10-2054 and UTHSC 10-121 showed white to dark-blue colonies.

TABLE 2 Key physiological features useful to distinguish the species of the *C. rugosa* complex

Species	Isolate ^a	Assimilation test ^b			
		D-Xylose	Glycerol	Ribitol	Sorbitol
<i>C. pseudorugosa</i>	UTHSC 06-3641	–	–	–	–
	UTHSC 08-707	–	–	–	–
<i>C. rugosa</i>	UTHSC 06-3729	+	+	–	+
	UTHSC R-3412	+	+	–	+
<i>C. neorugosa</i> sp. nov.	UTHSC 10-121	+	+	+	+
	UTHSC 10-2054	+	+	+	+

^a UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, Texas, USA.

^b +, positive; –, negative.

Antifungal susceptibility. The eight drugs tested showed high *in vitro* activity against all the isolates, with no significant differences noted among the different species. MIC ranges were <0.03 to 2 µg/ml for the azoles, 0.25 to 1 µg/ml for AMB, and 0.03 to 0.5 µg/ml for the echinocandins (Table 3).

Origins and clinical data of the isolates UTHSC 10-2054 and UTHSC 10-121. UTHSC 10-2054 was originally recovered from a female patient who sustained leg injuries while in Africa. A culture from the initial wound debridement there yielded yeast that was identified as *C. rugosa*. The patient was subsequently transferred to Johns Hopkins Hospital, Baltimore, MD, where an isolate identified as *C. rugosa* using the API ID 20C kit (profile 2442104, 97.1%) was also recovered from a surgical wound. The wound infection was finally resolved without antifungal therapy.

UTHSC 10-121 was isolated from a 22-month-old male Arabian horse that was presented to the Veterinary Medical Teaching Hospital at Texas A&M University, College Station, TX, for evaluation of a laceration and puncture wound on the palmar medial aspect of the left forelimb. The lesion was surgically debrided, and cultures collected at 24 days of hospitalization grew *Pseudomonas aeruginosa* and a yeast identified as *C. tropicalis* using a commercial identification system (Vitek II YBC; bioMérieux, Durham, NC). The organism was also cultured from the wound 1 week later, along with *Enterococcus* spp. and *Stenotrophomonas maltophilia*. At 56 days of hospitalization, yeast was again isolated from the site and identified as *C. rugosa* (Vitek II YBC; bioMérieux, Durham, NC). The patient was taken to surgery for ankylosis of the joint and started with a daily oral dose of FLC (4 mg/kg of body weight) and broad-spectrum antibiotic therapy. After 79 days of hospitalization, the horse was discharged. *C. tropicalis* was not recovered again.

TAXONOMY

Based on molecular and phenotypic data, it is concluded that the isolates UTHSC 10-2054 and UTHSC 10-121 represent a novel species of the genus *Candida*, for which the name *Candida neorugosa* sp. nov. is proposed.

TABLE 3 Results of *in vitro* antifungal susceptibility testing

Species (no. of isolates)	Antifungal agents	MIC ($\mu\text{g/ml}$) ^a		
		Range	GM	90
<i>C. pseudorugosa</i> (2)	Amphotericin B	0.5–1	0.71	–
	Fluconazole	1	1	–
	Posaconazole	0.06	0.06	–
	Voriconazole	<0.03–0.03	0.04	–
	Itraconazole	0.12	0.12	–
	Caspofungin	0.06–0.5	0.17	–
	Micafungin	0.12–0.25	0.17	–
	Anidulafungin	0.03–0.06	0.04	–
<i>C. rugosa</i> (10)	Amphotericin B	0.25–1	0.66	1
	Fluconazole	0.25–2	0.81	2
	Posaconazole	0.03–0.12	0.07	0.12
	Voriconazole	<0.03–0.03	0.02	0.03
	Itraconazole	0.03–0.5	0.06	0.12
	Caspofungin	0.06–1	0.38	1
	Micafungin	0.06–0.5	0.17	0.5
	Anidulafungin	0.03–0.5	0.12	0.25
<i>C. pararugosa</i> (10)	Amphotericin B	0.25–1	0.47	1
	Fluconazole	0.25–2	0.76	2
	Posaconazole	0.03–0.12	0.08	0.12
	Voriconazole	<0.03–0.06	0.03	0.06
	Itraconazole	0.03–0.25	0.07	0.12
	Caspofungin	0.03–0.25	0.13	0.25
	Micafungin	0.06–0.12	0.1	0.12
	Anidulafungin	0.03–0.12	0.06	0.12
<i>C. neurugosa</i> sp. nov. (2)	Amphotericin B	0.25–1	0.5	–
	Fluconazole	1–2	1.41	–
	Posaconazole	0.12	0.12	–
	Voriconazole	0.06	0.06	–
	Itraconazole	0.25	0.25	–
	Caspofungin	0.12–0.25	0.17	–
	Micafungin	0.06	0.06	–
	Anidulafungin	0.03–0.06	0.04	–

^a 90, MIC₉₀, the minimal concentration of drug capable of inhibiting the growth of 90% of assayed isolates; GM, geometric mean; –, value not calculated.

Candida neurugosa Paredes, D. A. Sutton, Cano, Guarro, sp. nov. (Fig. 3).

MycoBank MB 564807. In medio liquido YPD post dies 3 ad 25°C sedimentum ad annulus formatur, cellulae elipsoideae, ovoidae, 5 to 7 by 3 to 4 μm , singulae, binae et adhaerents aut in racemis brevibus. Per gemmationem multipolarem reproducent. In agar PDA post dies 2 ad 25°C, coloniae convexa, involvit, album ad crema. Germina tubulata non formantur. Chlamydosporas et pseudohyphas non fert. Ascospores non fiunt. Assimilat glucosum, trehalosum, galactosum, D-xylosum, ribitolum, sorbitolum et glycerolum. Fermentatio nulla. Ureum et aesculinum non hydrolysat. Holotypus ex humana crus vulnus in collectione CBS deposita est, CBS H-20946 (cultura viva UTHSC 10-2054, CBS 12627).

Etymology: Refers to its similarity to *C. rugosa*.

***Candida neurugosa* Paredes, D. A. Sutton, Cano, Guarro, sp. nov.** On YPD broth, after 3 days at 25°C, sediment is formed and turbidity of the medium is visible; the cells are ellipsoidal or ovoid, 5 to 7 μm by 3 to 4 μm , single, in pairs, chains or small groups, with multilateral budding. On PDA, the colonies are convex,

folded, and white to cream colored. Germ tubes and chlamydospores are not formed. Abundant pseudohyphae are formed on rice agar. Ascospores are not detected in acetate agar or Gorodkova medium incubated at 25°C for up to 4 weeks. Assimilation of trehalose, glucose, D-xylose, galactose, ribitol, sorbitol, and glycerol is produced. No sugars are fermented. Susceptible to cycloheximide. Unable to hydrolyze esculin and urea.

The type strain CBS 12627 (= UTHSC 10-2054) was isolated in 2010 in the Johns Hopkins University School of Medicine, Baltimore, MD, from a human leg wound. The strain CBS 12628 (= UTHSC 10-121) was isolated in 2010 in the Veterinary Medical Teaching Hospital at Texas A&M University, College Station, TX, from the left forelimb of an Arabian horse.

DISCUSSION

Our molecular analysis revealed that the 24 isolates initially identified as *C. rugosa* belonged to at least four species, three of which were phylogenetically close to each other, i.e., *C. rugosa*, *C. pseudorugosa*, and the undescribed species, while a fourth species, *C. pararugosa*, was very distant from the other three. The lack of agreement between phenotypic and molecular identification of the isolates included in this study can be explained, as indicated above, by the limitations of the commercial identification systems used in clinical laboratories, which do not allow the identification of uncommon yeasts (1, 23). This can lead to an overestimation of the incidence of some species to the detriment of others that are less common.

Of the 24 isolates investigated, only 10 that nested with the type strain of *C. rugosa* were identified as that species. An identical number of isolates were identified as *C. pararugosa*. The latter species was first isolated from human feces and, after the initial description, has been recovered from the oral cavity of a denture wearer (9) and the saliva of a sarcoma patient (14), suggesting its contribution to the oral microbiota. In both cases, the isolates were misidentified by using phenotypic methods, and their true pathogenic role was not established. Our study has expanded the host range of *C. pararugosa*, with several additional isolates recovered from various anatomical sites. The present study also identified two isolates, one recovered from a urine catheter and the other from a wound infection of the knee, as *C. pseudorugosa*. This is the first report of this species from clinical specimens, as the only two other isolates are from subglacial ice from arctic coastal environments (3). It is also remarkable that two of the isolates tested, UTHSC 10-2054 and UTHSC 10-121, were clearly genetically different from known species of *Candida* whose sequences have been deposited in GenBank, Centraalbureau voor Schimmelcultures (CBS), or National Institute of Technology and Evaluation Biological Resource Center (NITE) databases. The D1/D2 sequences of these two isolates clustered in the D1/D2 phylogenetic tree with a third sequence of a *Candida* sp. from a Brazilian patient with an ulcerated lesion. The three sequences were identical and formed a clearly separated branch. Unfortunately, the last isolate is not available for comparative studies. The ITS sequence analysis confirmed those results. Previously, divergent ITS2 sequences (30 to 35 bp), obtained by pyrosequencing, were reported in some clinical isolates of *C. rugosa* (2, 13). Our studies confirmed such variability and showed that *C. pseudorugosa* also displays divergence in that fragment, i.e., a different sequence for each isolate (CBS 613^T, GTCAAAAGTGGTTAGTCGGCGA CTTACTTGA; UTHSC 06-3729, GTCGATATTGGTTAGTCTG

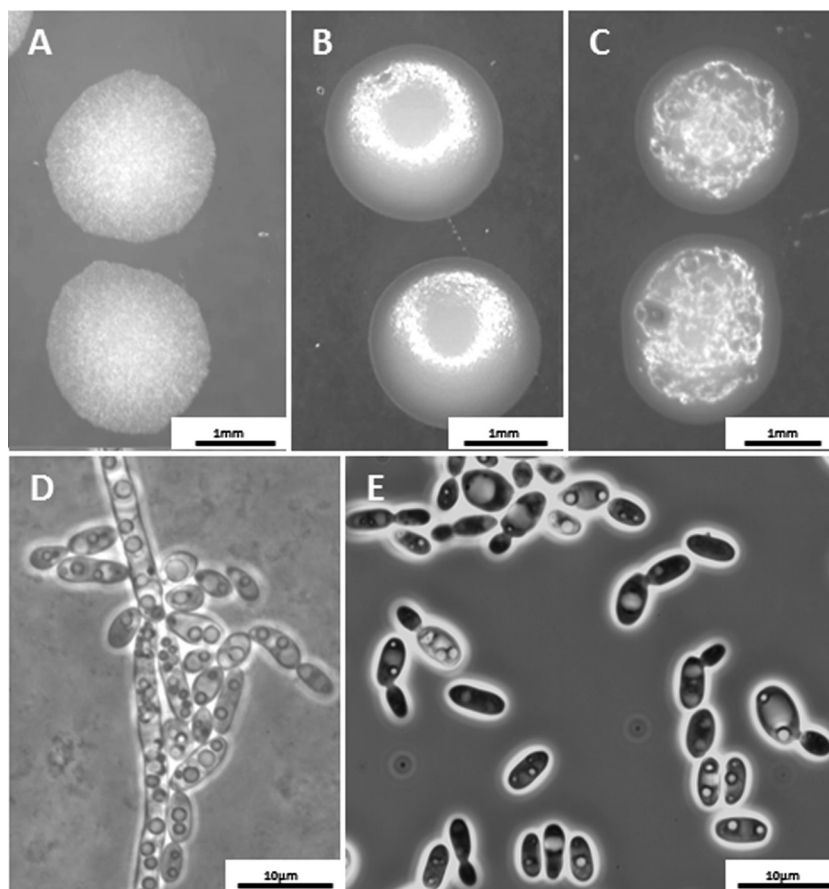


FIG 3 (A) *C. rugosa* UTHSC 06-3729. (B) *C. pseudorugosa* UTHSC 06-3641. (C to E) *C. neorugosa* sp. nov. UTHSC 10-2054. (A, B, and C) Colonies on PDA after 2 days of incubation at 25°C. (D) Pseudohyphae on rice Tween agar after 3 days of incubation at 28°C. (E) Yeast cells on YPD broth after 3 days of incubation at 37°C.

CGACTTACTTGA; and UTHSC R-3412, GTCAACATCTAAAA GTCGGCGACTTACTTGA), and all were different from those of *C. rugosa* and *C. neorugosa*. The three strains of *C. neorugosa* showed the same sequence (GTTCGACGTTCAAAAAGCCGGCGA CTACACTAA) for the referred fragment, which suggests that this new species could be identified by pyrosequencing.

While there are no data on the *in vitro* antifungal susceptibility of *C. pseudorugosa* and *C. pararugosa*, several *in vitro* susceptibility studies have reported reduced susceptibility of *C. rugosa* to AMB (6, 7), FLC and VRC (17, 18), and the echinocandins (7). However, in our case, all the antifungal drugs tested, with the exception of CAS against *C. rugosa*, were active against the 24 isolates. Although *C. rugosa* is not included in the proposed CLSI breakpoints for antifungal susceptibility testing, its GM MIC (0.38 mg/liter) and MIC₉₀ (1 mg/liter) would be interpreted as inferring resistance to CAS (16), as opposed to the other species of the *C. rugosa* complex, which exhibit lower CAS MICs.

In conclusion, the present study confirmed the value of molecular tools for the identification of cryptic species within the *C. rugosa* species complex. Prior to this evaluation, only *C. rugosa* had been documented as a human etiologic agent. This study confirms that a significant number of isolates of other species have also been recovered from clinical samples. Now that reliable methods for the identification of these species are available, an

important objective for future studies would be to elucidate the true prevalences, susceptibilities, and clinical roles of these species.

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REFERENCES

1. Alcoba-Flórez J, Méndez-Álvarez S, Cano J, Guarro J, Pérez-Roth E, Arévalo MP. 2005. Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus. *J. Clin. Microbiol.* 43:4107–4111.
2. Borman AM, et al. 2010. Rapid molecular identification of pathogenic yeasts by pyrosequencing analysis of 35 nucleotides of internal transcribed spacer 2. *J. Clin. Microbiol.* 48:3648–3653.
3. Butinar L, Strmole T, Gunde-Cimerman N. 2011. Relative incidence of ascomycetous yeasts in arctic coastal environments. *Microb. Ecol.* 61:832–843.
4. Cano J, Guarro J, Gené J. 2004. Molecular and morphological identification of *Colletotrichum* species of clinical interest. *J. Clin. Microbiol.* 42:2450–2454.
5. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard, 3rd ed, document M27-A3. CLSI, Wayne, PA.
6. Colombo AL, et al. 2003. Outbreak of *Candida rugosa* candidemia: an emerging pathogen that may be refractory to amphotericin B therapy. *Diagn. Microbiol. Infect. Dis.* 46:253–257.

7. Diekema DJ, et al. 2009. *In vitro* activity of seven systemically active antifungal agents against a large global collection of rare *Candida* species as determined by CLSI broth microdilution methods. *J. Clin. Microbiol.* 47:3170–3177.
8. Dubé MP, Heseltine PN, Rinaldi MG, Evans S, Zawacki B. 1994. Fungemia and colonization with nystatin-resistant *Candida rugosa* in a burn unit. *Clin. Infect. Dis.* 18:77–82.
9. Giammanco GM, Melilli D, Pizzo G. 2004. *Candida pararugosa* isolation from the oral cavity of an Italian denture wearer. *Res. Microbiol.* 155:571–574.
10. Gilgado F, Cano J, Gené J, Guarro J. 2005. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. *J. Clin. Microbiol.* 43:4930–4942.
11. Li J, Xu J, Bai F. 2006. *Candida pseudorugosa* sp. nov., a novel yeast species from sputum. *J. Clin. Microbiol.* 44:4486–4490.
12. Minces LR, Ho KS, Veldkamp PJ, Clancy CJ. 2009. *Candida rugosa*: a distinctive emerging cause of candidaemia. A case report and review of the literature. *Scand. J. Infect. Dis.* 41:892–897.
13. Montero CI, et al. 2008. Evaluation of pyrosequencing technology for the identification of clinically relevant non-dematiaceous yeasts and related species. *Eur. J. Clin. Microbiol. Infect. Dis.* 27:821–830.
14. Nakagawa Y, et al. 2004. Recurrent isolation of an uncommon yeast, *Candida pararugosa*, from a sarcoma patient. *Med. Mycol.* 42:267–271.
15. Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20:133–163.
16. Pfaller MA, et al. 2011. Clinical breakpoints for the echinocandins and *Candida* revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. *Drug Resist. Updat.* 14:164–176.
17. Pfaller MA, et al. 2006. *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program. *J. Clin. Microbiol.* 44:3578–3582.
18. Pfaller MA, et al. 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* 48:1366–1377.
19. Singh RI, et al. 2011. Epidemiology of candidemia in critically ill trauma patients: experiences of a level I trauma centre in North India. *J. Med. Microbiol.* 60:342–348.
20. Tamura K, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739.
21. Tay ST, Tan HW, Na SL, Lim SL. 2011. Phenotypic and genotypic characterization of two closely related subgroups of *Candida rugosa* in clinical specimens. *J. Med. Microbiol.* 60:1591–1597.
22. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876–4882.
23. Vijgen S, et al. 2011. Comparison of Vitek identification and antifungal susceptibility testing methods to DNA sequencing and Sensititre Yeast-One antifungal testing. *Med. Mycol.* 49:107–110.
24. Yarrow D. 1998. Methods for isolation, maintenance and classification of yeasts. p 77–100. *In* Kurtzman C. P. Fell J. W. (ed), *The yeasts, a taxonomic study*, 4th ed. Elsevier Science BV, Amsterdam, Netherlands.