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Candida nivariensis, an Emerging Pathogenic Fungus with Multidrug Resistance to Antifungal Agents[▽]

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In 2005, *Candida nivariensis*, a yeast species genetically related to *Candida glabrata*, was described following its isolation from three patients in a single Spanish hospital. Between 2005 and 2006, 16 fungal isolates with phenotypic similarities to *C. nivariensis* were submitted to the United Kingdom Mycology Reference Laboratory for identification. The strains originated from various clinical specimens, including deep, usually sterile sites, from patients at 12 different hospitals in the United Kingdom. PCR amplification and sequencing of the D1D2 and internal transcribed spacer 1 (ITS1) regions of the nuclear ribosomal gene cassette confirmed that these isolates from the United Kingdom are genetically identical to *C. nivariensis*. Biochemically, *C. glabrata* and *C. nivariensis* are distinguished by their differential abilities to assimilate trehalose. However, in contrast to the original published findings, we found that *C. glabrata* isolates, but not *C. nivariensis* isolates, are capable of assimilating this substrate. Antifungal susceptibility tests revealed that *C. nivariensis* isolates are less susceptible than *C. glabrata* isolates to itraconazole, fluconazole, and voriconazole and to have significantly higher flucytosine MICs than *C. glabrata* strains. Finally, *C. nivariensis* could be rapidly distinguished from the other common pathogenic fungus species by pyrosequencing of the ITS2 region. In the light of these data, we believe that *C. nivariensis* should be regarded as a clinically important emerging pathogenic fungus.

Invasive fungal infections caused by *Candida* spp. remain major causes of morbidity and mortality in the immunocompromised host (18, 20, 26), and more than 150 species of yeast have now been associated with human pathologies (4, 9). Although *Candida albicans* remains the predominant agent of nosocomial infections, an increasing number of infections have been attributed to non-*Candida albicans* species, with *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. krusei* emerging over recent years as significant opportunistic pathogens (13, 16, 21, 25). Given the inherently variable antifungal susceptibility profiles of different *Candida* spp., correct identification to the species level is often critical for informed therapeutic decisions.

The principal *Candida* spp. associated with human disease are readily identified by conventional mycological methods, which rely upon a combination of morphological features coupled with the abilities of the organisms to ferment selected sugars or assimilate a variety of carbon and nitrogen sources (12). However, an increasing number of less common yeast species that are difficult to identify by phenotypic methods have been reported from human infections (12), and certain *Candida* spp. (e.g., *C. orthopsilosis* and *C. metapsilosis* [22]) can be unambiguously identified only by molecular techniques. In addition, several new potentially pathogenic *Candida* spp. have recently been described on the basis of atypical fermentation and assimilation profiles coupled with unique DNA sequences. One such example, *C. nivariensis*, was described in 2005 following its isolation from three patients in a single Spanish hospital over a 3-year period. *C. nivariensis* was shown to be a close genetic relative of *C. glabrata* and was suggested to be a possible new opportunistic fungus (1). On the basis of the sequence similarities of the *C. nivariensis* strain with the sequence of a strain isolated from flowers in Canada (11), it was suggested that the *C. nivariensis* infection or colonization of the three patients might have been acquired from the hospital garden or potted plants.

In 2005 and 2006, 16 fungal isolates with phenotypic similarities to *C. nivariensis* were submitted to the United Kingdom Mycology Reference Laboratory (MRL) for routine identification. In the study described here we confirmed that these isolates, many of which were cultured from deep, usually sterile body sites, are identical to *C. nivariensis* and show that *C. nivariensis* can be rapidly distinguished from the other common pathogenic fungal species by pyrosequencing of the internal transcribed spacer 2 (ITS2) region. Our results also indicate that *C. nivariensis* isolates often exhibit multidrug resistance to azole antifungal agents and have MICs similar to or even more elevated than those for *C. glabrata*. Thus, we propose that *C. nivariensis* be regarded as a clinically important emerging pathogenic yeast.

MATERIALS AND METHODS

Antifungal agents. Antifungal drugs were obtained from their respective manufacturers as standard powders. Amphotericin B (Sigma Chemical Co., St Louis, MO) and voriconazole (Pfizer Central Research, Sandwich, United Kingdom) were dissolved in dimethyl sulfoxide. Itraconazole (Janssen Research Foundation, Beerse, Belgium) and posaconazole (Schering Corporation) were prepared in polyethylene glycol 400 with heating to 70°C. Caspofungin (Merck Sharp & Dohme, Hoddlesdon, United Kingdom), flucytosine (Sigma Chemical Co.), and fluconazole (Pfizer Central Research) were resuspended in sterile water. Serial twofold dilutions of the various drugs were prepared in RPMI 1640 medium

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TABLE 1. Fungal strains employed in this study[a]

Organism	NCPF strain no.	Other collection no.	Yr of isolation	Isolation site	Clinical details	Hospital	EMBL accession nos.
C. nivariensis	8842		2006	Mouth	Oral candidosis	Oxford	AM745268, AM745269
C. nivariensis	8843		2006	Pelvic collection	Not stated	Oxford	AM745270, AM745271
C. nivariensis	8844		2005	Blood culture	Neutropenia, AML	UCL	AM745272, AM745273
C. nivariensis	8845		2005	Mouth	Oral candidosis	Plymouth	AM745274, AM745275
C. nivariensis	*C. nivariensis* 5		2005	Not stated	Pneumonia	Leicester	NA
C. nivariensis	8846[b]						AM745276, AM745277
C. nivariensis	*C. nivariensis* 7		2005	Pelvic abscess	Not stated	Weston-s-Mare	NA
C. nivariensis	8847		2005	Ascitic fluid	Malignancy	Cambridge	AM745278, AM745279
C. nivariensis	*C. nivariensis* 9[b]						AM745280, AM745281
C. nivariensis	8848		2006	Mouth	Oral candidosis, neutropenia	Leeds	AM745282, AM745283
C. nivariensis	8849		2005	Exit site swab	CAPD	Sheffield	AM745284, AM745285
C. nivariensis	8850		2006	Peritoneal fluid	Peritonitis	Exeter	AM745286, AM745287
C. nivariensis	8851		2006	Lung biopsy	Not stated	Sheffield	AM745288, AM745289
C. nivariensis	8852		2006	Blood culture	Not stated	Newcastle	AM745290, AM745291
C. nivariensis	8853		2006	Not stated	Pneumonia	Salisbury	AM745292, AM745293
C. nivariensis	*C. nivariensis* 16		2006	Blood culture	Not stated	Barnet	NA
C. glabrata[T]	3309	CBS13, ATCC 2001					AM745294, AM745295
C. glabrata	*C. glabrata* 1		2006	Not stated	Pneumonia	Salisbury	AM745312, AM745313
C. glabrata	*C. glabrata* 2		2006	Blood culture	Not stated	Dublin	AM745314, AM745315
C. glabrata	*C. glabrata* 3		2006	BAL	Not stated	Cumbria	AM745316, AM745317
C. glabrata	*C. glabrata* 4		2006	Blood culture	Febrile	Monmouth	AM745318, AM745319
C. glabrata	*C. glabrata* 5		2006	Not stated	Febrile	Newport	AM745320, AM745321
C. glabrata	*C. glabrata* 6		2006	Blood culture	Not stated	Bristol	AM745322, AM745323
C. glabrata	*C. glabrata* 7		2006	Blood culture	Not stated	Cambridge	AM745324, AM745325
C. glabrata	*C. glabrata* 8		2006	Drain fluid	Not stated	Sheffield	AM745326, AM745327
C. glabrata	*C. glabrata* 9		2006	Blood culture	Pneumonia, breast cancer	Bath	AM745328, AM745329
C. glabrata	*C. glabrata* 10		2006	Blood culture	Not stated	Hull	AM745330, AM745331
C. glabrata	*C. glabrata* 11		2006	Blood culture	Renal dialysis	Cumbria	AM745332, AM745333
C. glabrata	*C. glabrata* 12		2006	Sputum	Not stated	Gloucester	AM745334, AM745335
C. glabrata	*C. glabrata* 13		2006	Urine	Sepsis	Salisbury	AM745336, AM745337
C. albicans	3281		1981	Not stated	Not stated	Bristol	AM745296, AM745297
C. parapsilosis	8334	CBS604, ATCC 22019					AM745298, AM745299
C. norvegensis T	3861	CBS1922, ATCC 22977					AM745300, AM745301
C. inconspicua T	3859	CBS180, ATCC 16783					AM745302, AM745303
C. krusei	3953	CBS573, ATCC 6258					AM745304, AM745305
C. lipolytica	8630[c]						AM745306, AM745307
C. kefyr	8678[c]						AM745308, AM745309
C. zeylanoides	8426[c]						AM745310, AM745311

[a] Abbreviations: NA, not analyzed; AML, acute myeloid leukemia; CAPD, continuous ambulatory peritoneal dialysis; UCL, University College Hospital, London, United Kingdom.
[b] The isolate was from the same patient from whom isolate *C. nivariensis* 5 was recovered.
[c] New York State Department of Health Proficiency testing program isolate.

(with L-glutamine, without bicarbonate; Sigma Chemical Co) and buffered to pH 7.0 by using a 0.165 M solution of morpholinepropanesulfonic acid (Sigma Chemical Co).

Fungal isolates. Clinical isolates with phenotypic similarities to *C. nivariensis* and recent clinical isolates of *C. glabrata* that were included for comparison had been submitted to the MRL for routine identification and were stored in sterile water at room temperature. Reference isolates of various *Candida* species were from the National Collection of Pathogenic Fungi (NCPF) and had been preserved in liquid nitrogen. All isolates were subcultured twice on plates of Oxoid Sabouraud dextrose agar containing 0.5% (wt/vol) chloramphenicol (Unipath Limited, Basingstoke, England). The cultures were incubated for 24 h at 35°C before they were tested.

Conventional yeast identification methods. The clinical isolates included in this study were all subjected to the conventional identification methods employed by the MRL. These methods were as follows: after initial germ-tube testing, the isolates were subjected to testing with the Auxacolor2 identification kit (Sanofi Diagnostics Pasteur, Paris, France) exactly as described previously (6). The isolates were also cultured on Dalmau plates (Oxoid cornmeal agar supplemented with 1% Tween 80, with a sterile coverslip placed over a single streak of the organism) to establish the additional morphological characteristics required to obtain complete Auxacolor2 profiles. All *C. nivariensis* isolates were then also tested in the API 20C system (bioMerieux, Marcy l'Etoile, France), again, exactly as described previously (7).

Molecular methods. Genomic DNA was prepared from the yeast cultures after 2 days of incubation on Sabouraud agar by use of the Whatman FTA filter paper technology exactly as described previously (5, 12). Amplification of a region of the large-subunit gene (LSU) and the ITS1 region was performed with the primers described in references 10 and 24, respectively. Amplification of a fragment of the ITS2 region prior to pyrosequencing was performed with the primers supplied with the PyroMark fungi ASR kit (Biotage, Sweden). In all cases, PCR amplification (100-μl reaction volumes) was performed in the presence of 200 μM of each deoxynucleoside triphosphate, 250 nM of the appropriate primers, 2 U of HotStar *Taq* polymerase (Qiagen, Valencia, CA), and a single FTA filter punch sample. Following enzyme activation at 94°C for 15 min, the reaction

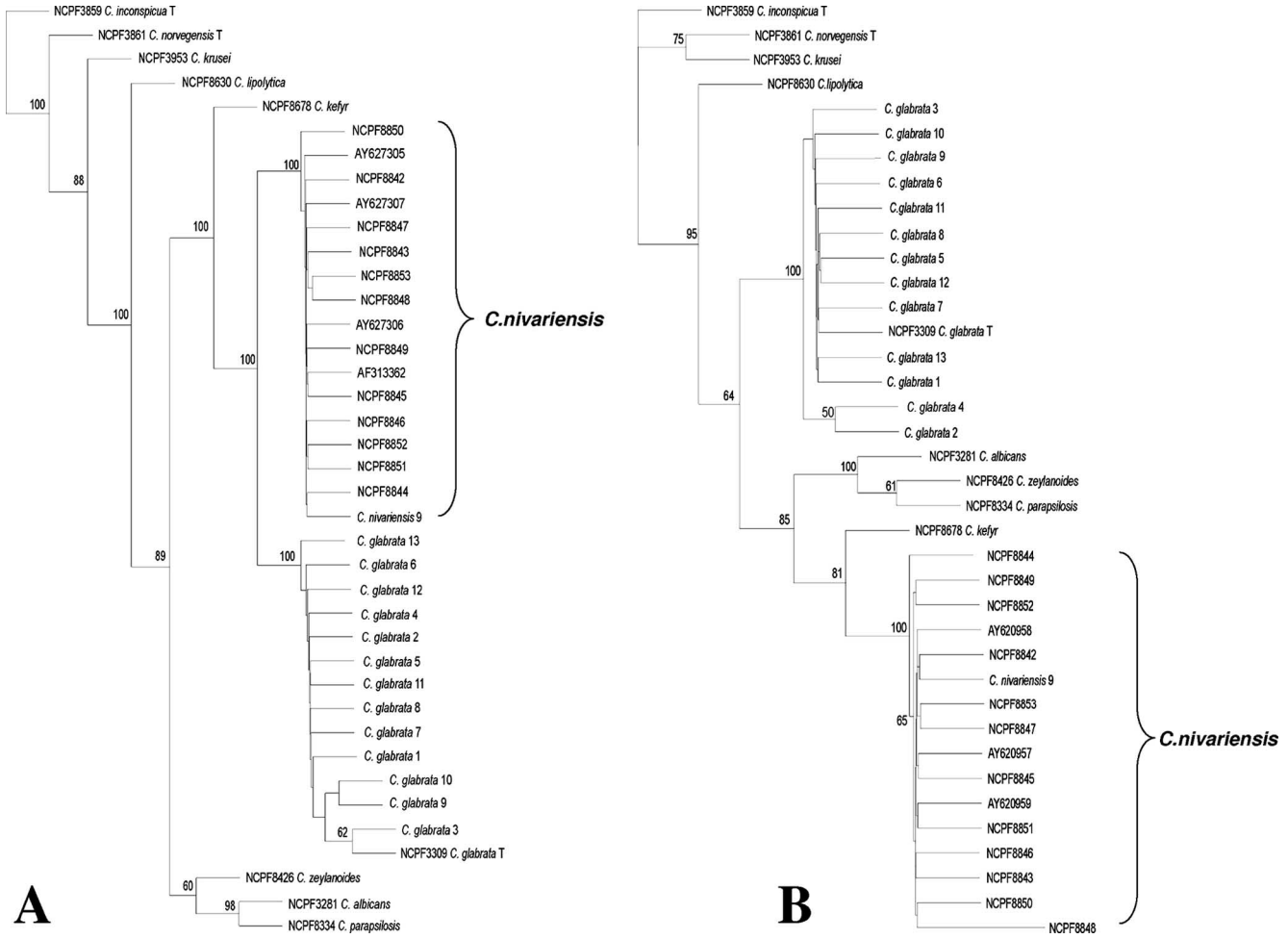

FIG. 1. Phylogenetic analysis of the LSU (A) and ITS1 (B) data sets. Unrooted neighbor-joining consensus trees are drawn. Bootstrap values above 50% are indicated. The EMBL accession and NCPF numbers are listed in Table 1. The sequences with EMBL accession numbers AY627305, AY927306, and AY627307 correspond to the D1D2 sequences of the three original *C. nivariensis* isolates described previously (1), and EMBL accession numbers AY620957, AY620958, and AY620959 correspond to the ITS1 sequences of the three original *C. nivariensis* isolates described previously (1). EMBL accession number AF313362 corresponds to the D1D2 sequence of a potential *C. nivariensis* isolate from flowers in Canada (11).

mixtures were subjected to 40 thermal cycles of 94°C (15 s), 55°C (15 s), and 72°C (90 s) on a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA). The success of the amplification was evaluated by electrophoresis of a fraction of the total amplification products in 1.2% (wt/vol) agarose gels run for 45 min at 120 V in Tris-borate buffer.

For conventional sequencing of the LSU and ITS1 PCR products, the contents of the PCR mixtures were adjusted so that the final concentration of polyethylene glycol 8000 was 10% (wt/vol) and that of $MgCl_2$ was 10 mM and were centrifuged at 12,000 rpm for 10 min in a benchtop centrifuge. The resulting DNA pellets were washed in 75% ethanol, air dried, resuspended in sterile water, and subjected to automatic sequencing by use of the commercial service available at the Advanced Biotechnology Centre (Imperial College, London). The ITS2 amplification products were subjected to pyrosequencing with the reagents supplied with the Pyrogold SQA kit by using a PyroMark ID pyrosequencer (Biotage, Sweden).

The organisms were identified by using BLAST searches of their sequences against the fungal sequences in existing DNA databases (3) and multiple-sequence alignments (Clustal W, version 1.82) (23) by using a database of formally identified organisms compiled by the MRL. For phylogenetic analyses, sequence alignments were performed with the Clustal W program (version 1.82), and the final alignments were edited by hand. Phylogenetic inferences were made from distance trees constructed by using the Kimura two-parameter measure and neighbor joining obtained by use of the Phylip package (version 3.5) (8). The trees were unrooted and were not scaled. The final consensus trees generated from each data set were drawn by using the TreeView program (17), and bootstrap values (1,000 repetitions) greater than 50% are indicated.

Broth microdilution determination of yeast MICs. MICs were determined according to CLSI (formerly NCCLS) methodologies (15) in round-bottomed

TABLE 2. Species-specific ITS2 signature sequences obtained by pyrosequencing

Organism	Sequence[a]
C. nivariensis	GTCAAACTTA AAGGTTCCTG
C. glabrata	**GTCAAACTTA AAG**acgt**CTG**
C. kefyr	**GTCAAACTTT** g**AG**ag**T**tt**TG**
C. lipolytica	**GTCAAACTTA AA**a**G**aa**C**aac
C. albicans	**GTCAAA**g**TT**t g**A**a**G**a**T**atac
C. parapsilosis	**GTC**g**AA**t**TT**g g**A**a**G**aagt**T**t
C. krusei	**GTC**g**AG**t**TT**t tt**G**ttgt**CT**c
C. inconspicua	**GTC**g**AG**t**TT**g **A**ttaaagt**T**c
Consensus sequence	GTC.A..TT. . . . .

[a] Conserved bases between a given *Candida* sp. and *C. nivariensis* are given in uppercase and boldface.

TABLE 3. MIC and MFC ranges for *C. nivariensis* and *C. glabrata*

Organism	Amphotericin B				Itraconazole				Voriconazole			
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)
C. nivariensis (n = 13)	0.5	0.5	0.5	2	**8**[a]	**>16**	>16	>16	0.5	**4**	>16	>16
C. glabrata (n = 13)	0.5	1	0.5	2	1	1	>16	>16	0.5	0.5	>16	>16
C. glabrata (azole-resistant strains[b])	0.5	1	NA[c]	NA	>16	>16	NA	NA	2	8	NA	NA

[a] Values in boldface indicate significant differences in MIC or MFC values between *C. nivariensis* and *C. glabrata*.
[b] Data were compiled from unpublished results of routine MIC testing by the MRL of nine clinical isolates of *C. glabrata* that exhibited the most significant resistance to azole antifungal agents.
[c] NA, MFC data are not available for these isolates.

96-well plates with fungus blastospore suspensions prepared in saline and then diluted into RPMI 1640 and adjusted to final concentrations of 2.5×10^3 CFU/ml. The inoculated plates were incubated for 48 h at 35°C. The MICs were read at 48 h as the concentration of drug that elicited 100% inhibition of growth (for amphotericin B) or significant (approximately 80%) inhibition of growth compared with the growth of a drug-free control (for itraconazole, fluconazole, flucytosine, voriconazole, posaconazole, and caspofungin). It should be noted that use of 50% inhibition as an endpoint for the reading of the MICs of itraconazole, fluconazole, flucytosine, voriconazole, posaconazole, and caspofungin, as suggested in CLSI document M27-A2, did not significantly affect either the final recorded MICs or the interpretation of the resistance or susceptibility of the various test organisms.

Determination of MFCs. Minimum fungicidal concentrations (MFCs) were determined after 48 h of incubation by removing 10 μl of the contents from wells showing no visible growth and spreading them onto Sabouraud dextrose agar plates. The plates were then incubated for 48 h, and the MFCs were determined as the lowest drug concentrations which killed 95% of the inoculum. MIC and MFC ranges and the drug concentrations required to inhibit or kill 50% of the isolates (MIC_{50}s and MFC_{50}s, respectively) and 90% of the isolates (MIC_{90}s and MFC_{90}s, respectively) were determined.

RESULTS

Isolation and identification of *C. nivariensis* strains from clinical samples in the United Kingdom. Between 2005 and 2006, 16 primary isolates of yeast (including three sequential isolates from a single patient) were submitted to the MRL for identification (Table 1). While these yeast strains had been isolated from a variety of samples and specimen types, more than 50% of the isolates (9/16) had originated from deep, usually sterile body sites, suggesting that they were responsible for invasive infections. In agreement with the published description of *C. nivariensis* (1), the isolates from the United Kingdom were unable to produce germ tubes, pseudohyphae, chlamydospores, or ascospores in culture and yielded white colonies, in contrast to the purple colonies usually exhibited by *C. glabrata* strains, on CHROMagar (data not shown). All 16 isolates of *C. nivariensis* shared identical carbohydrate assimilation profiles by both the Auxacolor2 and API 20C system tests, which demonstrated that they had the ability to assimilate only glucose. This is in marked contrast to the published description of *C. nivariensis*, which stated that while both *C. nivariensis* and *C. glabrata* could assimilate trehalose, *C. nivariensis* strains but not *C. glabrata* strains were able to ferment trehalose (1). It seems likely that this discrepancy reflects an error in the published description, since all available published data, including those in the databases supplied with commercial yeast identification kits, agree that *C. glabrata* isolates are capable of assimilating and fermenting trehalose (see, for example, reference 4).

Phylogenetic analyses of sequences corresponding to the D1D2 and the ITS1 portions of the nuclear ribosomal repeat region confirmed that these isolates from the United Kingdom are genetically indistinguishable from previously described *C. nivariensis* strains (Fig. 1). All of the *C. nivariensis* strains from the United Kingdom clustered with good bootstrap support with the previously described isolates as a single monospecific clade that was separate from *C. glabrata* and from the other *Candida* species tested.

Rapid identification of *C. nivariensis* by pyrosequencing. Although the absolute numbers of isolates for a given species received at the United Kingdom MRL are likely to be biased due to the reference nature of its activities (isolates submitted to the MRL for identification are often those that have failed to be identified by the referring laboratories), over the period of 2005 and 2006, *C. nivariensis* was the 17th most common yeast species identified at the MRL (12) (data not shown). In the light of the relative prevalence of this organism, especially from clinical material suggestive of invasive candidosis (Table 1), we sought to establish a rapid molecular identification system that would distinguish *C. nivariensis* from other *Candida* spp. that are largely unreactive in carbohydrate assimilation-based identification strategies.

To this end, various *Candida* spp., including the *C. nivariensis* strains from the United Kingdom, were submitted to pyrosequencing by use of the PyroMark fungi ASR kit (Biotage), which analyzes a short region of ITS2. All *C. nivariensis* isolates shared an identical 20-bp signature sequence in this highly discriminatory region that was distinct from the sequences of all other *Candida* spp. tested and that is unique among the fungal species represented in the currently available ITS2 databases (Table 2; data not shown).

***Candida nivariensis* exhibits significant resistance to azole antifungal agents.** To further evaluate the clinical significance of *C. nivariensis* as a potential human pathogen, the MICs of a range of antifungal agents currently employed for the treatment of invasive fungal infections were determined for *C. nivariensis* and *C. glabrata* (Table 3) by using methodologies accepted by the CLSI (15). In addition, in the light of the contradictory nature of the data concerning the relevance of MIC data versus that of MFC data in predicting clinical outcome (19), endpoint plating of MIC assays was also performed to allow the establishment of MFC ranges (see Materials and Methods). The 13 randomly selected recent clinical isolates of *C. glabrata* were used for comparison (Table 1, *C. glabrata* 1 to 13). However, recent antifungal data indicate that between 5 and 10% of *C.*

TABLE 3—*Continued*

Fluconazole				Posaconazole				Flucytosine				Caspofungin			
MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MFC_{50} (μg/ml)	MFC_{90} (μg/ml)
64	>64	>64	>64	1	2	>16	>16	**0.25**	**0.5**	**2**	**32**	0.5	1	2	2
8	32	>64	>64	0.5	4	>16	>16	<0.125	<0.125	<0.125	<0.125	1	2	2	>**64**
64	>64	NA	NA	4	>16	NA	NA	<0.125	<0.125	NA	NA	1	1	NA	NA

glabrata isolates exhibit very high MICs with respect to the some of the azole antifungals, at least in vitro (www.hpa.org.uk/publications/PublicationDisplay.asp?PublicationID=65; MRL, unpublished data; see Discussion). Thus, MIC data were also compiled for nine such highly resistant *C. glabrata* isolates that had previously undergone antifungal susceptibility testing as part of the routine activities of the MRL (*C. glabrata* azole-resistant strains; Table 3). It should be noted that these azole-resistant strains had previously been confirmed to be true *C. glabrata* isolates by a combination of Auxacolor2 and API 20C system testing, conventional sequencing of the D1D2 region, and pyrosequencing of ITS2 (data not shown).

C. nivariensis exhibited significant in vitro resistance to itraconazole, voriconazole, and fluconazole (as assessed by both the MIC and the MFC ranges; Table 3). This resistance was slightly (for fluconazole and voriconazole) or significantly (for itraconazole) greater with *C. nivariensis* than with representative isolates of *C. glabrata*. Indeed, the *C. nivariensis* MIC/MFC ranges with fluconazole, itraconazole, and voriconazole were very similar to those observed with the highly resistant subset of *C. glabrata* strains that were specifically selected for comparison (Table 3). Interestingly, azole cross-resistance (as judged by the MICs) extended to include posaconazole with the subset of azole-resistant *C. glabrata* isolates, but the same was not found for the *C. nivariensis* strains. Other notable differences in the respective antifungal susceptibility profiles were observed with flucytosine, for which *C. nivariensis* but not *C. glabrata* exhibited significantly elevated MIC and MFC values, and caspofungin, for which a minority of *C. glabrata* strains demonstrated very high MFC values (see the MFC_{90} of caspofungin in Table 3).

DISCUSSION

We have presented here a detailed characterization of 16 isolates of *C. nivariensis* received at the United Kingdom MRL over a 12-month period. These strains from the United Kingdom had been isolated from a variety of clinical specimens (including deep, usually sterile body sites) at 12 different hospitals in the United Kingdom. These data indicate that *C. nivariensis* is potentially clinically widespread and is probably significantly more relevant in terms of human disease than was suggested when the species was originally described (1). Our own epidemiological data indicate that it was the 17th most common yeast species referred to the MRL between 2005 and 2006 (12) (data not shown).

The potential clinical significance of *C. nivariensis* was further underscored by antifungal susceptibility testing, which revealed the *C. nivariensis* isolates from the United Kingdom to have elevated itraconazole, voriconazole, and fluconazole MICs (Table 3). Indeed, the MICs of the azole antifungal agents for *C. nivariensis* were significantly higher than those for standard *C. glabrata* isolates tested with the same antifungal agents and were equivalent to the MICs observed for a subset of the most azole-resistant *C. glabrata* strains (Table 3). Furthermore, when the MICs observed here are correlated with the established breakpoints for resistance for the various antifungal agents, *C. nivariensis* isolates are at least as resistant as *C. glabrata* isolates to itraconazole and are more resistant than *C. glabrata* to fluconazole and voriconazole (Table 4) (www.hpa.org.uk/publications/PublicationDisplay.asp?PublicationID=65).

Our own records indicate that *C. nivariensis* was not identified in clinical specimens prior to 2005, lending support for the proposal that it might be considered an emerging pathogen. Certainly, from the elevated MICs observed here for *C. nivariensis* with the azoles (see above), it is plausible that prophylactic azole antifungal use might have contributed to the proliferation of this species, as has been suggested for other azole-resistant *Candida* species, including *C. glabrata* (13, 14, 16, 21).

However, we do not believe that *C. nivariensis* has emerged as a pathogen only over the last 3 years. Effectively, all *C. nivariensis* isolates studied to date demonstrate the ability to assimilate only glucose among the carbohydrate sources

TABLE 4. Percentage of *Candida* species intermediate or resistant to amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and caspofungin tested at the MRL in 2005 (or 2005 to 2006 for *C. nivariensis*)[a]

Organism	Amphotericin B		Flucytosine			Fluconazole			Itraconazole			Voriconazole			Caspofungin		
	n	% R	*n*	% I	% R	*n*	% I	% R	*n*	% I	% R	*n*	% I	% R	*n*	% I	% R
C. albicans	1,425	0	127	0	2	1,816	1	1	1,727	1	1	1,375	0	0	908	0	0
C. krusei	74	0	15	47	0	82	**77**	**23**	74	**82**	**10**	74	3	3	52	0	0
C. glabrata	726	0	116	0	2	883	**32**	**12**	751	**41**	**56**	751	5	7	500	0	0
C. nivariensis	13	0	13	0	0	13	**8**	**54**	13	**31**	**62**	12	**50**	**0**	13	0	0

[a] Data are adapted from MRL unpublished records and reference 4. Values in boldface indicate organism-antifungal agent combinations in which significant resistance is apparent. Abbreviations: *n*, number of isolates tested; R, resistant; I, intermediate.

present in most commercially available yeast identification kits. Several other pathogenic yeast species, including *C. norvegensis*, *C. lipolytica*, *C. krusei*, *C. inconspicua*, and *C. zeylanoides*, share identical assimilation profiles in Auxacolor2 tests. *C. norvegensis*, *C. lipolytica*, *C. krusei*, and *C. zeylanoides* can potentially be distinguished from *C. nivariensis* by their abilities to produce pseudohyphae under the appropriate growth conditions, which form a part of the full Auxacolor2 profile. *C. inconspicua*, however, like *C. nivariensis*, is unable to form pseudohyphae or true hyphae and can be distinguished from the latter only with further specialized tests. Thus, we believe that *C. nivariensis* is likely to have been confused with some of these other largely unreactive fungi (or, indeed, *C. glabrata*), explaining in part why the emergence of this organism has closely followed the recent introduction of molecular methods for yeast identification. Nevertheless, it is still likely that the azole cross-resistance of *C. nivariensis* will ensure that the relative prevalence of this species in clinical specimens will continue to increase.

Future studies will involve the retrospective molecular analysis of unreactive yeast isolates that have been submitted to the MRL in order to establish when *C. nivariensis* isolates first became associated in significant numbers with clinical specimens and in an attempt to assess how rapidly this organism may be emerging as a leading pathogenic yeast. Toward this aim, we have demonstrated that *C. nivariensis* can be rapidly identified by pyrosequencing of a short region of ITS2. We believe that pyrosequencing has distinct advantages over the recently described *C. nivariensis*-specific PCR (2) for the rapid identification and epidemiological surveillance of this organism. Since all *Candida* species tested to date yielded unique species-specific pyrosequencing sequence signatures over the 20 bp of the ITS2 region analyzed (this study; MRL, unpublished data), this method will potentially identify any of the unreactive yeast species with biochemical profiles similar to the profile of *C. nivariensis* rather than specifically detect only this organism.

In conclusion, in the light of the potential clinical significance of *C. nivariensis* coupled with the patterns of azole cross-resistance observed here, we believe that *C. nivariensis* should be added to the increasing number of pathogenic yeast species that require molecular identification.

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