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# Identification and Antifungal Susceptibility Profiles of *Candida haemulonii* Species Complex Clinical Isolates from a Multicenter Study in China

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**_Candida haemulonii_ complex (_Candida haemulonii_, _Candida haemulonii_ var. _vulnera_, and _Candida duobushaemulonii_) consists of emerging pathogens. Thirty-one isolates from 14 hospitals in China were studied for their species classification and antifungal susceptibilities. Performances of molecular (i.e., ribosomal DNA [rDNA] internal transcribed spacer [ITS] sequencing, D1/D2 sequencing, and ITS sequencer-based capillary gel electrophoresis [SCGE]) and phenotypic identification methods in species identification were compared. Twenty-six (83.9%) of 31 isolates were identified as _C. haemulonii_ and 5 isolates were identified as _C. duobushaemulonii_ by ITS sequencing as the reference method; results obtained by D1/D2 sequencing and ITS SCGE were concordant with those obtained by ITS sequencing for all (100%) of the isolates. All 31 isolates were identified as _C. haemulonii_ by the Vitek matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) system (bioMérieux, France), whereas the Bruker MS system (Bruker Daltoniks, Germany) correctly provided species identification for 77.4% and 100% of isolates using cutoff scores for species of ≥2.0 and ≥1.70, respectively. The Vitek 2 compact (bioMérieux) only identified 9 (29%) of 31 isolates. All isolates showed high MICs for amphotericin B (range, 2 to >8 μg/ml) and fluconazole (≥128 μg/ml) but low MICs (≤0.5 μg/ml) for the echinocandins. Our results reinforce the need for MALDI-TOF MS and/or molecular differentiation of species within the _C. haemulonii_ complex. The multiresistant antifungal susceptibility profile of these isolates represents a challenge to therapy.**

*Candida* species remain the most common fungal pathogens worldwide, and invasive candidiasis (IC) is associated with high mortality and excess hospital costs, particularly in seriously ill and immunocompromised patients (1). Although four species, namely, *Candida albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* are the most often encountered, previously uncommon or emerging novel *Candida* species are becoming more recognized (2, 3).

One such emerging pathogen is *Candida haemulonii* complex, which has been described to cause human infection, including peritonitis (4); neonatal candidemia, including case clusters (5); catheter-related candidemia (6); and osteitis (7). Members of this species complex can be divided into three genotypically distinguishable species: *C. haemulonii*, *C. haemulonii* var. *vulnera*, and *C. duobushaemulonii* (8). Their differentiation relies on molecular methods with good discrimination to the species and varietal or intraspecies levels, such as sequence analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) genes. The use of ITS sequencing (considered the reference method) together with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) may provide improved discrimination of members of this complex for reference spectra that are contained within the used MS database (8, 9). In contrast, phenotypic identification methods, such as the API 20C and Vitek yeast identification systems (bioMérieux, Marcy l'Etoile, France), typically lead to errors in identification (10, 11). Other molecular methods may also be employed to identify *C. haemulonii*. One such approach is sequencer-based capillary gel electrophoresis (SCGE), also targeting the ITS regions, where one reported study was able to distinguish *C. haemulonii* (cha-1) and *C. duobushaemulonii* (cdh-1) (12).

*C. haemulonii* complex often exhibits antifungal resistance, with clinical failure associated with *in vitro* resistance to amphotericin B and reduced susceptibility to azoles (6, 7, 9, 10, 13–15). Moreover, *C. haemulonii* isolates with resistance to echinocandins have been reported (11).

Despite their growing clinical significance, data on the occurrence and distribution of *C. haemulonii* complex in clinical specimens in China have not been described. Therefore, we studied the epidemiology and antifungal susceptibility of *C. haemulonii* complex clinical isolates collected from multicenter surveillance in China over 5 years.

## MATERIALS AND METHODS

**Ethics.** The study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (no. S-263). Written in-

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**TABLE 1** Information about and ITS sequencing identification of the *C. haemulonii* complex isolates included in this study

| Strain | Source of isolate | Collection date | Sequencing of ITS gene | |
|---|---|---|---|---|
| | | | Species | GenBank accession no. |
| 10H1099 | Ascitic fluid | 2010 | *C. haemulonii* | KU865058 |
| 11TJ209 | Venous catheter | 2011 | *C. haemulonii* | KU865060 |
| 11H1121 | Blood | 2011 | *C. haemulonii* | KU865061 |
| 12HX468 | Blood | 2012 | *C. haemulonii* | KU865062 |
| 13HS048 | Cerebrospinal fluid | 2012 | *C. haemulonii* | KU865064 |
| 12HX416 | Blood | 2012 | *C. haemulonii* | KU865066 |
| 12HX417 | Blood | 2012 | *C. haemulonii* | KU865067 |
| 13J2022 | Blood | 2013 | *C. haemulonii* | KU865069 |
| 12XY117 | Pus | 2013 | *C. haemulonii* | KU865070 |
| 13HS038 | Cerebrospinal fluid | 2013 | *C. haemulonii* | KU865071 |
| 13HS045 | Cerebrospinal fluid | 2013 | *C. haemulonii* | KU865072 |
| 13HS050 | Cerebrospinal fluid | 2013 | *C. haemulonii* | KU865073 |
| 13W3055 | Blood | 2013 | *C. haemulonii* | KU865074 |
| 13XN013 | Venous catheter | 2013 | *C. haemulonii* | KU865075 |
| 13SJ002 | Blood | 2013 | *C. haemulonii* | KU865076 |
| 13SJ004 | Blood | 2013 | *C. haemulonii* | KU865077 |
| 13NJ153 | Blood | 2013 | *C. haemulonii* | KU865078 |
| 13SJ016 | Blood | 2013 | *C. haemulonii* | KU865079 |
| 13SJ012 | Blood | 2013 | *C. haemulonii* | KU865080 |
| 14HS165 | Cerebrospinal fluid | 2014 | *C. haemulonii* | KU865081 |
| 14NJ254 | Blood | 2014 | *C. haemulonii* | KU865082 |
| 14XH138 | Blood | 2014 | *C. haemulonii* | KU865083 |
| 14HS107 | Cerebrospinal fluid | 2014 | *C. haemulonii* | KU865085 |
| 14TJ463 | Blood | 2014 | *C. haemulonii* | KU865086 |
| 14NJ205 | Blood | 2014 | *C. haemulonii* | KU865087 |
| 14XN091 | Venous catheter | 2014 | *C. haemulonii* | KU865088 |
| 10LR033 | Blood | 2010 | *C. duobushaemulonii* | KU865059 |
| 12PU491 | Blood | 2012 | *C. duobushaemulonii* | KU865063 |
| 12HX325 | Blood | 2012 | *C. duobushaemulonii* | KU865065 |
| 12PU411 | Blood | 2012 | *C. duobushaemulonii* | KU865068 |
| 14FS093 | Venous catheter | 2014 | *C. duobushaemulonii* | KU865084 |

formed consent was obtained from patients for the use of the samples in research.

**Yeast isolates.** A total of 31 clinical isolates of *C. haemulonii* collected from 14 different hospitals in 12 provinces, as part of a nationwide surveillance program for invasive fungal diseases (IFDs) in China (the China Hospital Invasive Fungal Surveillance Net [CHIF-NET[), from August 2009 to July 2014 were studied (Table 1). CHIF-NET was a prospective laboratory-based surveillance network established to provide updated information on the epidemiology of IFDs in China as previously described (16). Isolates of the same species and susceptibility profile from the same site of a given patient that were recovered at a different time were excluded. Data on the distribution of isolates by specimen source are summarized in Table 1. Isolates recovered from blood cultures comprised 61.3% (19/31) of the isolates, and those from cerebrospinal fluid (CSF) comprised 19.4% (6/31) of the isolates. Species identification was initially performed using the Vitek 2 compact system (bioMérieux, France).

**Sequence-based identification.** For DNA sequencing, all isolates were subjected to sequencing of the fungal ITS gene and the D1/D2 domain of the 28S rRNA gene as previously described (17, 18). For ITS SCGE, the ITS1 and full-length ITS regions of each isolate were amplified by a duplex PCR as previously described (12).

**MALDI-TOF MS analysis.** All isolates were studied by both the Vitek MS system (IVD knowledgebase version 2.0; bioMérieux) and the Bruker autoflex speed TOF/TOF MS system (Biotyper version 3.1 software; Bruker Daltonics, Billerica, MA). For the Vitek MS system, the results were scored in one of three ways (17). *C. haemulonii* var. *vulnera and C. duobushaemulonii* reference spectra are not included in the Vitek MS v2.0 database. With the Bruker system, identification was provided according to manufacturer-determined criteria: a spectral score of ≥2 was considered to provide identification at the species level (19). The current Bruker Daltonic database contains 5,989 main spectra (MSP), which includes *C. haemulonii*, *C. haemulonii* var. *vulnera*, and *C. duobushaemulonii* reference spectra.

**Antifungal susceptibility testing.** The *in vitro* susceptibility to nine antifungal drugs was determined by the broth microdilution Sensititre YeastOne YO10 methodology (Thermo Scientific, Cleveland, OH) following the manufacturer's instructions. MIC values were interpreted according to CLSI document M27-S3 (20).

**Nucleotide sequence accession numbers.** The ITS region and D1/D2 domain sequences of strains found in this study were deposited in GenBank with accession numbers KU865058 to KU865088 and KU883328 to KU883358, respectively (Table 1).

## RESULTS

**Species identification of *C. haemulonii* complex by DNA sequencing.** The ITS sequences of the study isolates exhibited 100% sequence identity to the corresponding ITS sequences from reference *C. haemulonii* complex isolates in GenBank (*C. haemulonii* CBS 5149 and *C. duobushaemulonii* CBS 7798). Of 31 clinical isolates, 26 (83.9%) were identified as *C. haemulonii* and 5 (16.1%) as *C. duobushaemulonii* (Table 1).

Analysis of the D1/D2 gene region sequences and those obtained by ITS SCGE identified all 31 clinical isolates with 100% concordance with ITS sequencing results. Results of ITS SCGE identified SCGE lengths of ITS1 and ITS for *C. haemulonii* and *C.*

**TABLE 2** Epidemiological cutoff values (ECV) of nine antifungal agents based on aggregated MIC distributions of *C. haemulonii* and *C. duobushaemulonii*

| Antifungal agent | Species | No. of isolates | MIC (μg/ml) | | ECV (μg/ml)[b] at: | | |
|---|---|---|---|---|---|---|---|
| | | | Range | Mode[a] | 95% | 97.5% | 99% |
| Fluconazole | *C. haemulonii* | 26 | 2 to >256 | >256 | >256 | >256 | >256 |
| | *C. duobushaemulonii* | 5 | 128 to >256 | >256 | >256 | >256 | >256 |
| Voriconazole | *C. haemulonii* | 26 | 0.06 to >8 | >8 | >8 | >8 | >8 |
| | *C. duobushaemulonii* | 5 | 2 to >8 | >8 | >8 | >8 | >8 |
| Itraconazole | *C. haemulonii* | 26 | 0.12 to >16 | >16 | >16 | >16 | >16 |
| | *C. duobushaemulonii* | 5 | 1 to >16 | 1 | >16 | >16 | >16 |
| Posaconazole | *C. haemulonii* | 26 | 0.03 to >8 | >8 | >8 | >8 | >8 |
| | *C. duobushaemulonii* | 5 | 0.5 to >8 | >8 | >8 | >8 | >8 |
| Caspofungin | *C. haemulonii* | 26 | 0.03 to 0.5 | 0.06 | 0.5 | 0.5 | 0.5 |
| | *C. duobushaemulonii* | 5 | 0.03 to 0.12 | 0.03 | 0.12 | 0.12 | 0.12 |
| Micafungin | *C. haemulonii* | 26 | 0.06 to 0.5 | 0.06 | 0.5 | 0.5 | 0.5 |
| | *C. duobushaemulonii* | 5 | 0.06 to 0.12 | 0.12 | 0.12 | 0.12 | 0.12 |
| Anidulafungin | *C. haemulonii* | 26 | 0.015 to 0.5 | 0.06 | 0.5 | 0.5 | 0.5 |
| | *C. duobushaemulonii* | 5 | 0.06 to 0.25 | 0.12 | 0.25 | 0.25 | 0.25 |
| 5-Flucytosine | *C. haemulonii* | 26 | 0.06 to >64 | 0.06 | >64 | >64 | >64 |
| | *C. duobushaemulonii* | 5 | ≤0.06 to >64 | 0.12 | >64 | >64 | >64 |
| Amphotericin B | *C. haemulonii* | 26 | 2 to >8 | 2 | >8 | >8 | >8 |
| | *C. duobushaemulonii* | 5 | 4 to >8 | >8 | >8 | >8 | >8 |

[a] Most frequent MIC.
[b] Calculated ECVs comprising ≥95%, ≥97.5%, or ≥99% of the statistically modeled MIC population.

*duobushaemulonii*, which were identified as cha-1 and cdh-1, as previously described (12).

**MALDI-TOF identification.** All 26 *C. haemulonii* isolates were identified as *C. haemulonii* by the Vitek MS system (confidence value, 99% to 99.9%). However, all 5 *C. duobushaemulonii* were also identified as *C. haemulonii* (confidence value, 99% to 99.9%). The Bruker system correctly identified most *C. haemulonii* to the species level (23/26, 88.5%). The remaining 3 isolates were identified as *C. haemulonii* but with an MS score of <2.00 but ≥1.70. For *C. duobushaemulonii*, 1 isolate was identified as *C. duobushaemulonii* with an MS score of >2.00, and 4 isolates were identified as *C. duobushaemulonii* but with MS scores of <2.00 but ≥1.70.

**Performance of Vitek 2 compact system.** Compared with ITS sequencing, of 9 (34.6%) of 26 *C. haemulonii* isolates were identified as *C. haemulonii*, with a probability of identity ranging from 93% to 97%. The other 17 isolates were identified with a low confidence score of *C. haemulonii/Kodamaea ohmeri* (50%/50%). Four *C. duobushaemulonii* isolates were identified as *C. haemulonii*, with a probability of identity ranging from 97% to 98%. The remaining isolate was identified as *Candida pelliculosa/C. haemulonii* (51%/49%).

**Antifungal susceptibility profiles.** Aggregated MIC distributions to nine antifungal agents of *C. haemulonii* and *C. duobushaemulonii* isolates are shown in Table 2. All 31 *C. haemulonii* complex isolates showed low MICs for echinocandins (≤0.5 μg/ml) and high MICs for amphotericin B (range, 2 to >8 μg/ml) and fluconazole (≥128 μg/ml) (Table 2). Moreover, 96.2% (25/26) of *C. haemulonii* and 80% (4/5) of *C. duobushaemulonii* isolates were resistant to voriconazole (MIC, >8 μg/ml). For itraconazole, 96.2% (25/26) of *C. haemulonii* and all 100% (5/5) of *C. duobushaemulonii* isolates were resistant (MIC, ≥1 μg/ml). A total of 69.2% (18/26) of *C. haemulonii* and 40% (2/5) of *C. duobushaemulonii* isolates had MICs of ≥2 μg/ml to posaconazole. A total of 26.9% (7/26) of *C. haemulonii* and 20% (1/5,) of *C. duobushaemulonii* isolates were resistant to 5-flucytosine (MIC, >64 μg/ml).

## DISCUSSION

Members of the *C. haemulonii* complex are uncommon pathogenic yeasts that cause human infection. In the ARTEMIS DISK Global Antifungal Surveillance Study during 1997 to 2007, the isolation rate of *C. haemulonii* was very low (<0.01%), and during that period, 11.1% of the isolates were classified as resistant to fluconazole and voriconazole (21). In the CHIF-NET 2010-2011 study, IFDs caused by *C. haemulonii* were also infrequent (0.1% to 0.2%), but susceptibility testing by disk diffusion methods indicated that all isolates were susceptible to fluconazole and voriconazole (16, 17). In 2012, Cendejas-Bueno et al. (8) proposed a reclassification of the *C. haemulonii* complex based on molecular methods; this taxonomic reclassification has prompted many laboratories to reexamine their culture collections for these species. To date, most of the *C. haemulonii* complex isolates identified by ITS sequencing were reported in Brazil (5, 8, 9, 22), including 12 isolates and 31 isolates from two reports of a local national epidemiology study (9, 22). We therefore determined the proportion of

*C. haemulonii* complex isolates among clinical strains cultured from patients with IFDs in China and their antifungal susceptibilities by the broth microdilution method. Overall, during the 5-year CHIF-NET study, *C. haemulonii* complex isolates represented 0.3% (31/9,673) of all yeast isolates, which was higher than the rate noted in the ARTEMIS study but similar to the rate found in Brazil (21, 22). The identification and differentiation of *C. haemulonii* complex isolates relies on molecular methods, so it is difficult to compare the proportions because laboratories differ in the extent to which they identify uncommon *Candida* species to the species level; of laboratories that do identify to the species level, different methodologies are often used (8, 9). In our study, we have reinforced that molecular methods are the preferred approach for definitive identification of members within the species complex.

ITS sequencing identified all 31 isolates, and concordance of results with ITS sequencing was 100% with those obtained by D1/D2 sequencing and ITS SCGE. Currently, the ITS region is considered a universal DNA barcode marker for fungi (16, 23, 24). The D1/D2 region is also useful and may be required for strain separation (25). ITS SCGE assay has shown promise as a potential reference method, since it is simple to use and adaptable for rapid identification (12). According to the present study, compared to the ITS sequencing method, D1/D2 sequencing and ITS SCGE have the ability to identify *C. haemulonii* and *C. duobushaemulonii*. D1/D2 sequencing cannot distinguish *C. haemulonii* and *C. haemulonii* var. *vulnera* (8). We did not encounter *C. haemulonii* var. *vulnera* infections here. The reasons for its absence are uncertain, but further national surveillance for uncommon yeast pathogens is ongoing.

Conversely, identification of *C. haemulonii* complex isolates using traditional phenotypic methods leads to misidentifications (8, 11). In recent studies, the identification results of the API 32C and Vitek 2 compact systems failed to identify *C. haemulonii* and misidentified these pathogens as *K. ohmeri*; these two species groups are genetically closely related (10, 11, 13). In this study, 17 (65.4%) of 26 *C. haemulonii* were identified with a low confidence score of *C. haemulonii/K. ohmeri* (50%/50%). Hence, any unusual identification result provided by these systems should be confirmed by another more discriminatory method.

MALDI-TOF MS is increasingly used to identify *Candida* species in clinical laboratories (17, 26, 27). However, the database of Vitek MS v2.0 is limited by the absence of reference spectra for *C. duobushaemulonii* and *C. haemulonii* var. *vulnera*, so it was not able to distinguish within the *C. haemulonii* complex as found in our study. In comparison, the Bruker MS system identified 88.5% of *C. haemulonii* and 20% of *C. duobushaemulonii* correctly to the species level; however, when the cutoff score for species was lowered to ≥1.70, all *C. haemulonii and C. duobushaemulonii* were correctly assigned as such. Lower scores that produce "correct" identifications of uncommon yeasts have been reported by other studies, which have not affected the specificity of MALDI-TOF MS (26, 27).

Although uncommon in frequency, the antifungal susceptibility profiles of members of the *C. haemulonii* complex is of concern. *In vitro* antifungal susceptibility results in the present study showed that all *C. haemulonii* complex isolates had high MICs for fluconazole and amphotericin B and that *C. duobushaemulonii* isolates had 2-fold higher amphotericin B MICs than those of the *C. haemulonii* species, as previously reported (22). *In vitro* resistance to these agents has been associated with clinical treatment failure and fatal outcome (6, 7, 10, 13–15). More than 90% of the isolates were cross-resistant to azoles. High MICs to fluconazole may predict high MICs to voriconazole and, to some extent, other azoles. We recommend that *C. haemulonii* complex isolates with high MICs to fluconazole be assessed for susceptibility to the other azoles. Echinocandins demonstrated excellent *in vitro* potency against the isolates (9, 22). In the present study, our *in vitro* antifungal susceptibility data showed that MICs to echinocandins were low, indicating that there may be better treatment options. However, echinocandin resistance in *C. haemulonii* isolates from hemocultures has been reported (11), which highlights the resistance of *C. haemulonii* isolates to antifungals and illustrates the importance of correctly identifying *Candida* species.

Because of considerable regional variability, local epidemiological knowledge is critical in the effective management of IC. This systemic multicenter report of the identification of *C. haemulonii* complex in clinical isolates from patients attending Chinese hospitals will inform antifungal practices.

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