Prevalence and antifungal susceptibility patterns of new cryptic species inside the species complexes Candida parapsilosis and Candida glabrata among blood isolates from a Spanish tertiary hospital

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Objectives: There is scarce information on the clinical relevance and antifungal susceptibility of Candida bracarensis, Candida nivariensis, Candida orthopsilosis and Candida metapsilosis. The objective of this study was to assess the prevalence and in vitro antifungal susceptibility of these cryptic species among 173 blood isolates previously identified as Candida glabrata or Candida parapsilosis at the Hospital of Cruces (Barakaldo, Spain). The survey was extended to 518 clinical isolates from the culture collection of the Universidad del País Vasco-Euskal Herriko Unibertsitatea (UPV-EHU; Bilbao, Spain).

Methods: In vitro susceptibilities to 5-fluorocytosine, amphotericin B, anidulafungin, caspofungin, fluconazole, itraconazole, micafungin, posaconazole and voriconazole were tested.

Results: All isolates of C. glabrata were identified as C. glabrata sensu stricto. Inside the C. parapsilosis complex, 2.4% of isolates from the Hospital of Cruces and 5.8% from the UPV-EHU were C. metapsilosis or C. orthopsilosis. Of 457 isolates, 435 (95.19%) were C. parapsilosis sensu stricto, 11 (2.41%) C. metapsilosis and 11 (2.41%) C. orthopsilosis. Only seven blood isolates were C. metapsilosis (0.44%) or C. orthopsilosis (1.09%). These cryptic species were also isolated from other relevant clinical specimens. Four C. parapsilosis sensu stricto (5.6%) were susceptible dose-dependent, and one was resistant to both fluconazole and voriconazole (1.4%). Moreover, 19 isolates of C. parapsilosis sensu stricto (26.4%) were intermediately susceptible to itraconazole and higher concentrations of echinocandins were needed to inhibit this species. Most C. orthopsilosis and C. metapsilosis were susceptible to all antifungal agents tested, but one otic isolate of C. metapsilosis was resistant to fluconazole and 5-fluorocytosine.

Conclusions: C. metapsilosis and C. orthopsilosis are associated with human disease and show a different antifungal susceptibility profile compared with C. parapsilosis sensu stricto.

Keywords: antifungal susceptibility, C. bracarensis, C. glabrata, C. metapsilosis, C. nivariensis, C. orthopsilosis, C. parapsilosis, Spain

Introduction

Invasive candidiasis (IC) is one of the most common causes of nosocomial invasive infectious disease. Although Candida albicans remains the predominant aetiologi cal species, >50% of cases of candidaemia and IC can be due to Candida non-albicans species. At present, Candida parapsilosis and Candida glabrata stand out as the second and third aetiologi cal agents, respectively, of candidaemia in many countries of America, Asia and Europe. Candida parapsilosis and C. glabrata are important nosocomial pathogens in neonates, the elderly, transplant recipients and patients who have received prior antifungal therapy. The occurrence of IC is usually associated with invasive procedures, catheters, parenteral hyperalimentation or prosthetic devices.

C. glabrata has grown in medical importance because of its reduced susceptibility to fluconazole and other current antifungal agents. C. glabrata sensu lato is a complex species including C. glabrata sensu stricto and two newly described species, Candida bracarensis and Candida nivariensis. These latter species are considered emerging pathogens and have been associated with multidrug resistance.
C. parapsilosis can be a member of the human microbiota, but possesses the ability to grow in parenteral nutrition solutions, to form biofilms on catheters, to spread by hand carriage and to persist in the nosocomial environment. C. parapsilosis forms a complex composed of three genetically distinct groups that have been recognized as separate species; C. parapsilosis sensu stricto, Candida metapsilosis and Candida orthopsilosis. The exact importance of C. orthopsilosis and C. metapsilosis as human pathogens remains unknown, but studies point to them being significant in human candidiasis. C. parapsilosis is usually susceptible to antifungal agents, but there are reports of isolates with decreased susceptibility to azoles and echinocandins. Moreover, differences in antifungal susceptibility among the three species have been observed.

Due to the medical importance of C. parapsilosis and C. glabrata in Spain, and the limited local epidemiological data on these cryptic species, the aim of this study was to assess the prevalence and in vitro antifungal susceptibility of C. bracarensis, C. metapsilosis, C. nivariensis and C. orthopsilosis among 173 blood isolates previously identified as C. glabrata or C. parapsilosis at the tertiary care Hospital of Cruces (Barakaldo, Spain). Moreover, this survey was extended to 518 C. glabrata and C. parapsilosis isolates from different clinical origins belonging to the culture collection of the Universidad del País Vasco-Euskal Herriko Unibertsitatea (UPV-EHU, Bilbao, Spain).

Materials and methods

Microorganisms

One hundred and seventy-three blood isolates previously identified as 45 C. glabrata and 128 C. parapsilosis from 173 patients admitted to different units at the Hospital of Cruces over a 5 year period were studied. Additionally, 518 clinical isolates (189 C. glabrata and 329 C. parapsilosis) conserved in the culture collection of the UPV-EHU, over a 17 year period, were studied. The 189 C. glabrata included 49 isolates from blood and other sterile fluids, 86 from mucosa, 3 from skin and 51 isolates whose clinical origin was not described in the database. The 329 C. parapsilosis included 75 isolates from blood and other sterile fluids, 178 from mucosa, 39 from skin and 37 isolates whose clinical origin was unknown. Moreover, the following type strains were used as quality controls for the correct identification of these species: C. glabrata sensu stricto NCFP 3240 and NCFP 3203; C. bracarensis NCYC 3397 and NCYC 3133; C. nivariensis CECT 11998 and CECT 11999; C. parapsilosis sensu stricto ATCC 22019 and ATCC 90018; C. metapsilosis ATCC 96139 and ATCC 96141; and C. orthopsilosis ATCC 96143 and ATCC 96144.

All isolates were originally identified as C. parapsilosis or C. glabrata by conventional mycological methods, such as colony morphology on Sabouraud dextrose agar (bioMérieux, Marcy l’Étoile, France), ChromID Candida (bioMérieux) and CHROMagar Candida (CHROMagar, Paris, France), microscopic morphology on corn meal agar and carbon source assimilation using the ID 32C system (bioMérieux).

Molecular identification of cryptic species from C. glabrata and C. parapsilosis

The identities of C. glabrata sensu stricto, C. bracarensis and C. nivariensis were confirmed as previously described by Romeo et al. C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis isolates were identified as previously described by Tavanti et al. Isolates were grown on fresh Sabouraud agar (Difco, St Louis, MO, USA) and incubated at 37°C for 24 h. A 3 μL equivalent of yeast was scraped from the plate and resuspended in 20 μL of sterile water. The yeast suspensions were treated by heating to 95°C for 8 min and then placing in a −80°C freezer for 1–2 h.

The identification of C. glabrata sensu stricto, C. bracarensis and C. nivariensis was achieved by performing a multiplex PCR based on the amplification of the ITS1 region, with specific forward primers for C. glabrata sensu stricto (GLA-f (5’-CGTTGTGGTTGTTGTCTTG-3’), C. bracarensis (BRA-f (5’-GGACGTGATAGTTCCTCG-3’)) and C. nivariensis (NIV-f (5’-AGGGAGATTGTTGATCTTTC-3’)), and the reverse primer UNI-5.8S (5’-ACAGAGGCGGCCATGTG-3’), which amplify the 5.8S rDNA region. The amplification procedure consisted of an initial denaturation step at 95°C for 5 min, followed by 34 cycles of 3 s at 94°C, 40 s at 60°C and 50 s at 72°C, and a final extension step of 10 min at 72°C. After thermal cycling, 5 μL of each amplified product was separated by electrophoresis on a 1% agarose gel, stained with GelRed (Biotium, Hayward, CA, USA) and visualized with UV light (Figure 1).

For identification of C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis, the amplification of the SADH gene was performed by PCR with the primers S1F (5’-TGATGCTTGGTGGATGTG-3’) and S1R (5’-CAATGCWAATCACTCTCCAA-3’), which amplify a fragment of 716 bp. PCR mixtures were prepared as suggested by the manufacturer (Bioline, London, UK). Each 25 μL reaction mixture contained 2 μL of the prepared yeast supernatant. The amplification conditions were a first cycle of 5 min at 95°C, followed by 40 cycles at 92°C for 1 min, at 45°C for 1 min and at 68°C for 1 min, with a final extension step of 10 min at 68°C. The PCR product was then digested with BanI enzyme (New England Biolabs, Ipswich, MA, USA) in a 40 μL volume containing 20 μL of the PCR product and 40 U of BanI, and incubated at 37°C for 2 h. The digestion products were separated on a 1% agarose gel, stained with GelRed and visualized with UV light. Isolates of C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis were identified by differences in the restriction sites contained in the SADH amplicons, which numbered one, zero (no restriction site) and three BanI restriction sites, respectively (Figure 2). All isolates identified as C. orthopsilosis or C. metapsilosis were digested a second time for confirmation. Furthermore, the identities of C. orthopsilosis and C. metapsilosis clinical isolates were confirmed by DNA sequencing of the ITS1 and ITS4 regions of the 28S rRNA gene, in order to avoid potential misidentification due to a possible point mutation in C. parapsilosis sensu stricto. The PCR was carried out with panfungal ITS1 and ITS4 primers under the following conditions; a first cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, at 60°C for 30 s and at 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplicons were sequenced and BLAST searches were performed for species identification.

![Figure 1. Agarose gel electrophoresis of PCR products of C. glabrata sensu stricto, C. bracarensis and C. nivariensis. Lanes: 1, C. glabrata sensu stricto; 2, C. bracarensis; and 3, C. nivariensis.](image-url)
YeastOne 9 testing was carried out in accordance with the manufacturer's instructions, including MIC 50, MIC 90, MIC ranges and geometric mean MIC values at 24 h and 48 h. Most of the 72 C. parapsilosis sensu stricto isolates were susceptible to all of the antifungal agents tested. 5-Fluorocytosine, amphotericin B, anidulafungin, caspofungin, micafungin, itraconazole, micafunig, posaconazole or voriconazole with incorporated Alamar blue, which changes from blue to pink in the presence of microorganisms. The drug concentrations ranged from 0.008 to 16 mg/L for amphotericin B, anidulafungin, caspofungin, itraconazole, micafungin, posaconazole and voriconazole, from 0.125 to 256 mg/L for fluconazole and from 0.03 to 64 mg/L for 5-fluorocytosine. Sensitiitre YeastOne 9 testing was carried out in accordance with the manufacturer's instructions, as follows: 20 μL of the inoculum suspension was added to 11 mL of RPMI 1640 broth to obtain a working suspension (1.5–5 × 10^4 cells/mL), of which 100 μL was added to each well. The panels were sealed and incubated in air at 37°C and read at 24 h by comparing the colour of the medium in each well with those of the reading mask provided by the manufacturer. If no growth was noted in the control, a second reading was made after an additional 24 h of incubation. Classification of the isolates in terms of their susceptibilities to these antifungal agents was based on the MIC breakpoints recommended in the M27-S3 supplement (CLSI). For the echinocandins (anidulafungin, caspofungin and micafungin) the MIC for susceptibility was ≤2 mg/L, the MIC for intermediate susceptibility was 4 mg/L and the MIC for resistance was >8 mg/L. For fluconazole, the MIC for susceptibility was ≤2 mg/L, the MIC for susceptible dose-dependent (SSD) was 4 mg/L and the MIC for resistance was >8 mg/L. For itraconazole, posaconazole and voriconazole, the MIC for susceptibility was ≤0.125 mg/L, the MIC for intermediate susceptibility was 0.25–0.5 mg/L and the MIC for resistance was ≥1 mg/L. For 5-fluorocytosine, the MIC for susceptibility was ≤4 mg/L, the MIC for intermediate susceptibility was 8–16 mg/L and the MIC for resistance was >32 mg/L. For amphotericin B, those isolates with MICs ≥1 mg/L were considered susceptible (breakpoints not recommended in the M27-S3 supplement). Quality control was performed using C. albicans ATCC 90028, C. parapsilosis ATCC 22019 and Candida krusei ATCC 6258.

**In vitro antifungal susceptibility testing**

All isolates identified as C. metapsilosis or C. orthopsilosis were evaluated for their susceptibility to antifungals in vitro, and the same was done with 72 randomly selected isolates of C. parapsilosis sensu stricto. The study of the in vitro antifungal susceptibility of C. metapsilosis, C. orthopsilosis and C. parapsilosis sensu stricto was performed using the Sensitiitre YeastOne 9 broth microdilution test (Trek Diagnostic Systems, East Grinstead, UK). Briefly, this test consists of a 96-well microtitre plate containing dried C. parapsilosis sensu stricto after digestion with BanI enzyme. Lanes: 1, 2 and 3, respectively. The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively).

**Results**

All tested C. glabrata sensu lato isolates were identified as C. glabrata sensu stricto. One hundred and twenty-five out of 128 blood isolates (97.7%) from the tertiary Hospital of Cruces were identified as C. parapsilosis sensu stricto, 1 (0.8%) as C. metapsilosis and 2 (1.6%) as C. orthopsilosis. Among the culture collection of the UPV-EHU isolates, 310 out of 329 (94.2%) were identified as C. parapsilosis sensu stricto, 10 (3.0%) as C. metapsilosis and 9 (2.8%) as C. orthopsilosis. C. metapsilosis and C. orthopsilosis were found in biological products other than blood. Of note, in this collection, C. metapsilosis was isolated from three otic specimens and one wound specimen (10.3% of all superficial isolates), from vagina (three isolates from three women with vaginitis; 10% of all vaginal isolates), from blood (one isolate; 1.7% of blood isolates) and from anus and sputum (1.1% of isolates from mucosa different from vagina). C. orthopsilosis was isolated from blood (three isolates; 5.1% of blood isolates), from the teats and oral mucosa of three babies, from oral and respiratory specimens from adult patients (3.4% of total oral and respiratory isolates) and from vagina (one isolate; 3.3% of all isolates from vagina). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively). The mean MIC of amphotericin B was significantly higher for C. metapsilosis (0.5 mg/L) compared with those for C. parapsilosis sensu stricto (1, 2 and 1.201 mg/L, respectively) and for C. orthopsilosis (1, 2 and 0.878 mg/L, respectively).
The prevalence of *C. parapsilosis* and *C. glabrata* as causative agents of candidaemia and IC has risen in many areas, to become the second and third most common aetiological agents of these infections after *C. albicans*. During the period 1999–2004, the average incidence of candidaemia at the Hospital of Cruces was 1.3 episodes/10,000 patient-days of stay in hospital (range 1.1–1.4 episodes); the average incidence was significantly higher in children (incidence 1.97 episodes). In the last 5 year period (2005–09), the average incidence of candidaemia in Cruces has doubled (2.63 episodes/10,000 patient-days of stay in hospital, range 66–90 episodes; José L. Hernández-Almaraz and Leyre M. López-Soria, unpublished data). These incidence rates are higher than those reported in Europe by the European Confederation of Medical Mycology Societies survey (0.31–0.44 episodes), but lower than those reported in the USA (1.5 episodes). The rate of candidaemia appears to increase with the number of hospital beds. In our hospital, non-*albicans* species of *Candida* were more frequently isolated than *C. albicans* (59.6% versus 40.4% of isolates).

Overall, 41.2% of bloodstream fungal infection episodes were due to *C. parapsilosis*, followed by *C. albicans* (40.4%), *Candida tropicalis* (7.2%), *C. glabrata* (5.1%), *C. krusei* (2.7%) and other *Candida* spp. (3.4%). At our institution, the rates of isolation from blood of *C. parapsilosis* (41.2%) and *C. glabrata* (5.1%) were very similar to those reported in other studies in America and Europe. *C. glabrata* and *C. parapsilosis* have been considered for many years as complex species with different biotypes or varieties. These biotypes are now delineated as new cryptic species: *C. bracarensis* and *C. nivariensis* from *C. glabrata*; and *C. metapsilosis* and *C. orthopsilosis* from *C. parapsilosis*. Among clinical isolates, *C. glabrata* and *C. parapsilosis* are much more common than

### Table 1. *In vitro* activity of current and new antifungal agents against isolates of *C. metapsilosis*, *C. orthopsilosis* and *C. parapsilosis sensu stricto*

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<td>24 h</td>
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<td><strong>C. orthopsilosis (n=11)</strong></td>
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<td>voriconazole</td>
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### Discussion

The prevalence of *C. parapsilosis* and *C. glabrata* as causative agents of candidaemia and IC has risen in many areas, to become the second and third most common aetiological agents of these infections after *C. albicans*. During the period 1999–2004, the average incidence of candidaemia at the Hospital of Cruces was 1.3 episodes/10,000 patient-days of stay in hospital (range 1.1–1.4 episodes); the average incidence was significantly higher in children (incidence 1.97 episodes). In the last 5 year period (2005–09), the average incidence of candidaemia in Cruces has doubled (2.63 episodes/10,000 patient-days of stay in hospital, range 66–90 episodes; José L. Hernández-Almaraz and Leyre M. López-Soria, unpublished data). These incidence rates are higher than those reported in Europe by the European Confederation of Medical Mycology Societies survey (0.31–0.44 episodes), but lower than those reported in the USA (1.5 episodes). The rate of candidaemia appears to increase with the number of hospital beds. In our hospital, non-*albicans* species of *Candida* were more frequently isolated than *C. albicans* (59.6% versus 40.4% of isolates). Overall, 41.2% of bloodstream fungal infection episodes were due to *C. parapsilosis*, followed by *C. albicans* (40.4%), *Candida tropicalis* (7.2%), *C. glabrata* (5.1%), *C. krusei* (2.7%) and other *Candida* spp. (3.4%). At our institution, the rates of isolation from blood of *C. parapsilosis* (41.2%) and *C. glabrata* (5.1%) were very similar to those reported in other studies in America and Europe. *C. glabrata* and *C. parapsilosis* have been considered for many years as complex species with different biotypes or varieties. These biotypes are now delineated as new cryptic species: *C. bracarensis* and *C. nivariensis* from *C. glabrata*; and *C. metapsilosis* and *C. orthopsilosis* from *C. parapsilosis*. Among clinical isolates, *C. glabrata* and *C. parapsilosis* are much more common that...
these new cryptic species. However, data are scarce and it is difficult to define their true clinical significance.

*C. glabrata* is clearly associated with more severe infections in elderly or severely immunodeficient patients and isolates can show a reduced susceptibility to common azoles.\(^3,19\) Lockhart *et al.*,\(^22\) in their analysis of 1598 *C. glabrata* isolates from 29 countries, observed that *C. bracarensis* and *C. nivariensis* isolates constituted a very small percentage (0.2%) of the *C. glabrata* clinical isolates. However, these cryptic species could be more prevalent in specific regions. The first description of *C. nivariensis* isolates was reported from the Canary Islands (Spain) in 2005, as three atypical *C. glabrata* isolates from a pulmonary abscess, blood and urine.\(^5\) Then there have been reports of new isolates from the oral cavity, blood, ascitic fluid, pleural fluid and peritoneal fluid in different countries in Asia, Europe and Australia.\(^7,22\) Reports of *C. bracarensis* are even scarcer. Since the first two isolates were described in Portugal from a patient suffering from vaginal candidiasis and in the UK from a candidaemia, in 2006,\(^6\) there have been a few reports of isolates from throat, stool, a pelvic abscess, sputum and blood in America. Most reports have underlined the lower susceptibility of *C. bracarensis* and *C. nivariensis* to the most commonly used azoles.\(^5,22\) In the current study, none of the 234 *C. glabrata* isolates was reidentified by molecular methods as *C. bracarensis* or *C. nivariensis*, and the collection strains were not tested for antifungal susceptibility.

Invasive infections and candidaemia caused by *C. parapsilosis* are increasing, and this species is the first or second most frequently isolated species of *Candida* in many European and American hospitals. Its prevalence is even higher in neonates suffering from candidaemia in the neonatal intensive care unit (ICU). Moreover, *C. parapsilosis* is observed causing infections of skin, nail and mucosa in a wide range of patients. Isolates of this species are commonly susceptible to current antifungal agents; however, there are reports of clinical isolates with decreased susceptibility and resistance to fluconazole and the echinocandins.\(^11\) The exact prevalence of *C. orthopsilosis* and *C. metapsilosis* as aetiological agents of superficial and deep-seated candidiasis is not known, and there is an apparently high variability. However, published reports are gradually elucidating their genetics, virulence and pathogenicity, epidemiology and antifungal susceptibility.\(^23,24\)

Table 2 summarizes the studies that have been published on the distribution of *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis*. It is remarkable that in 5 of the 15 previously published studies, *C. metapsilosis* was not isolated from clinical specimens. Something similar is reported in the worldwide study published by Lockhart *et al.*,\(^8\) where *C. metapsilosis* was not present among the isolates of 14 out of 29 participant countries. Conversely, *C. orthopsilosis* was frequently isolated and the number of isolates was higher for *C. orthopsilosis* than for *C. metapsilosis* (8 out of 15 studies and 19 out of 29 participant countries).

The prevalence of *C. metapsilosis* and *C. orthopsilosis* in Spain is highly variable. Two Spanish studies have been published showing prevalences of 0.8%–7.7% for *C. metapsilosis* and of 6.4%–8.2% for *C. orthopsilosis* among *C. parapsilosis sensu lato* blood isolates.\(^9,10\) In the current study, the prevalence of *C. metapsilosis* and *C. orthopsilosis* among blood isolates was at the lower limit (0.8% and 1.6%, respectively), and both species were sporadically isolated during the 5 year study period. Differences with the two other Spanish studies could be explained by the geographical locations; the previous studies

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**Table 2. Summary of published studies on distribution of *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis***

<table>
<thead>
<tr>
<th>No. of isolates tested</th>
<th><em>C. parapsilosis sensu stricto</em></th>
<th><em>C. metapsilosis</em></th>
<th><em>C. orthopsilosis</em></th>
<th>Country/region</th>
<th>Reference</th>
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<tbody>
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<td>27</td>
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<td>0 (0)</td>
<td>7 (25.9)</td>
<td>Italy</td>
<td>15</td>
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<tr>
<td>9</td>
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<td>1929(^a)</td>
<td>1762 (91.3)</td>
<td>34 (1.8)</td>
<td>117 (6.1)</td>
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</table>

\(^a\)Sixteen isolates were identified as the close species Lodderomyces elongisporus.

\(^b\)One hundred and twenty-eight blood isolates from the Hospital of Cruces (Barakaldo).

\(^c\)Three hundred and twenty-nine clinical isolates from the culture collection of the UPV-EHU (Bilbao).
were conducted in Barcelona (a city bordering the Mediterranean Sea) and Seville (southwestern Spain), which have very different climatic and socio-economic conditions from Barakaldo (a city of the Biscay Gulf, Atlantic Ocean coast, northern Spain), the location of the present study. In the evaluation of the prevalence of C. metapsilosis and C. orthopsilosis among the isolates from the ARTEMIS global surveillance study reported by Lockhart et al., there were 49 Spanish isolates of C. parapsilosis sensu lato [46 C. parapsilosis sensu stricto (93.9%), 1 C. metapsilosis (2.0%) and 2 C. orthopsilosis (4.1%)]. These isolates were sent from two hospitals in Madrid, in the middle of the Iberian Peninsula. Similar rates of C. metapsilosis and C. orthopsilosis were observed in the isolates from other European studies, but some reports do not include both cryptic species, and the prevalence of C. orthopsilosis was apparently higher in warmer Mediterranean countries than in the cooler countries of the Atlantic, Central and North European areas. However, other factors could be responsible for local specificities, such as differences in hospital services (presence or absence of ICU or surgical wards) and the patient population (transplant recipients and other immunodeﬁcient patients). This variability in prevalence has been reported in other parts of the world, with the prevalence of C. orthopsilosis being higher in those countries with hot and humid climates, such as Brazil (9.1%), Malaysia (24.4%) and Taiwan (8.5%) (Table 2).

Among the clinical isolates stocked in the UPV-EHU collection, C. metapsilosis represented 3.0% of clinical isolates. Interestingly, C. metapsilosis was isolated not only from blood but from other specimens (anus, otic, surgical wound, sputum and vagina). C. orthopsilosis represented 2.7% of the C. parapsilosis sensu lato isolates, and this species was isolated from blood, oral and vaginal specimens. It should be noted that our collection includes isolates from many parts of Spain, not only from the Basque country, and this could inﬂuence the higher prevalence of cryptic species compared with blood isolates from the Hospital of Cruces. Other authors have described isolates from other non-mentioned anatomical sites. The presence of both cryptic species in the skin and mucosae, such as oral and genitourinary sites, shows their ability to adapt to different ecological niches of the human body and their association with the aetiology of oral and vaginal candidiasis. However, C. metapsilosis appears to be the least virulent of the three species, as has been shown in vitro.

An important issue is the association of these cryptic species with candidiasis in paediatric patients. Tay et al. observed a higher frequency of C. orthopsilosis among blood isolates from paediatric patients and the absence of C. metapsilosis in blood isolates from adult patients. These authors found C. parapsilosis, C. orthopsilosis and C. metapsilosis in seven (58.3%), three (25.0%) and two (16.7%) isolates from paediatric patients. However, the distribution of these species was different in adult patients; 22 (73.3%) of the isolates were C. parapsilosis and 7 (23.3%) isolates were C. orthopsilosis, with C. metapsilosis not being isolated in this setting. In the current study, C. orthopsilosis was also isolated from teat and mouth samples from paediatric patients, and it is apparent that this species can cause superficial infections in children and can survive on silicone and other inert materials used for making teats and other biosanitary tools. This survival can be associated with the ability of C. orthopsilosis to develop biofilms on different biomaterials.

Whether the differences observed in the distribution of these cryptic species has any association with the treatment and management of candidiasis caused by them is another important issue. Differences in antifungal susceptibility patterns have been reported among C. parapsilosis sensu stricto, and C. metapsilosis and/or C. orthopsilosis, with the former being less susceptible to the echinocandins, amphotericin B and ﬂuconaﬂuzole.

The lower susceptibility of C. parapsilosis sensu stricto to the echinocandins has been widely described, but the number of isolates with MICs >2 mg/L is low. In the current study, one C. parapsilosis sensu stricto isolated from blood was immediately susceptible to micafungin (MIC=4 mg/L). Also, higher MICs of anidulafungin, micafungin and, to a lower degree, caspofungin were observed for C. parapsilosis sensu stricto isolates compared with for C. metapsilosis and C. orthopsilosis isolates. One possible explanation could be the observation made by Chen et al. that mutations from proline to alanine were identified on the hot spot 1 of FKS1 in C. parapsilosis isolates while isoleucine to valine substitutions on the hot spot 2 region of FKS1 were observed in isolates of C. metapsilosis and C. orthopsilosis. The amino acid variations on hot spot 2 did not correspond to the difference observed by these authors in echinocandin MICs for these isolates. However, these naturally occurring mutations from proline to alanine in FKS1 have been associated with a reduced susceptibility to the echinocandins.

Differences have been reported in the in vitro activity of anidulafungin, caspofungin and micafungin. In the present study, the mean MICs of anidulafungin (1.427, 0.182 and 0.354 mg/L, respectively) and micafungin (1.177, 0.182 and 0.193 mg/L, respectively) were higher for C. parapsilosis isolates than for C. metapsilosis and C. orthopsilosis isolates than the mean MICs of caspofungin (0.467, 0.170 and 0.249 mg/L, respectively). Apparently, micafungin is the least active of them in vitro, but published results are controversial and further studies are required.

Differences in the in vitro susceptibilities of other antifungal agents are not so clear. Lockhart et al. reported that 15% of C. parapsilosis isolates had an amphotericin B MIC of >4 mg/L compared with 8% of C. orthopsilosis isolates and 3% of C. metapsilosis isolates. The presence of this in vitro resistance to amphotericin B has been observed by Gómez-López et al.

In the present study, there were no isolates with amphotericin B MICs >1 mg/L, but one C. metapsilosis blood isolate had an amphotericin B MIC of 1 mg/L. Moreover, the MIC mean of amphotericin B was significantly higher (P<0.05) for C. metapsilosis (0.5 mg/L) than for C. parapsilosis sensu stricto (0.310 mg/L) and C. orthopsilosis (0.218 mg/L). This lower susceptibility of C. metapsilosis to amphotericin B has not been observed in other studies.

Resistance to 5-ﬂuorocytosine was uncommon among C. parapsilosis sensu lato in a previous study in our region, but 6% of isolates had MICs of 5-ﬂuorocytosine >32 mg/L. In the current study, one C. metapsilosis isolated from a patient with otitis externa was resistant to 5-ﬂuorocytosine and to ﬂuconaﬂuzole, but the rest of the isolates of this species and most isolates of C. parapsilosis and C. orthopsilosis were susceptible. Conversely, Gonçalves et al. reported the presence of 3 out of 13 (23.1%) C. orthopsilosis isolates with in vitro resistance to 5-ﬂuorocytosine. This fact should be taken into account, as C. orthopsilosis represented 9.1% of candidaemia caused by C. parapsilosis sensu lato in this Brazilian study.
The in vitro activities of fluconazole and other azoles against *C. parapsilosis sensu lato* are also excellent, but some isolates showing multiazole resistance have been described.\(^{11}\) However, *C. metapsilosis* isolates need higher fluconazole MICs to be inhibited, as has been reported in some studies\(^{8,31}\) and in the present study. Moreover, Chen et al.\(^{25}\) found three *C. metapsilosis* isolates from a patient with candidaemia that had decreased susceptibility to fluconazole. In their study, *C. metapsilosis* represented 5.6% of *C. parapsilosis sensu lato* blood isolates. In the present study, we have observed an unusually high percentage of *C. parapsilosis* sensu stricto blood isolates (26.4%) showing intermediate susceptibility to itraconazole, which has not been reported previously. This should be given consideration, as itraconazole has irregular pharmacokinetic/pharmacodynamic patterns and this could complicate the successful outcome of itraconazole-treated IC caused by *C. parapsilosis*. Finally, posaconazole and voriconazole have been very active against most tested isolates of the *C. parapsilosis* complex, and in vitro resistance has not been described for *C. metapsilosis* and *C. orthopsilosis*.\(^{9}\)

In conclusion, there is an increasing presence of these new cryptic species in important clinical infections and in relevant ecological niches with clear geographical differences. There is also disparity in the antifungal susceptibility of these species, with *C. parapsilosis* being the least and *C. orthopsilosis* the most susceptible. These facts emphasize the necessity for further studies monitoring the virulence, epidemiology and antifungal susceptibility patterns of *C. metapsilosis*, *C. orthopsilosis* and *C. parapsilosis*, and the need to assess the clinical relevance of the differences among them.

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None to declare.

**References**


