Utility of two PCR-RFLP-based techniques for identification of *Candida parapsilosis* complex blood isolates

Running title: Identification of *Candida parapsilosis* infections

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Abstract:

Background: *Candida parapsilosis* is the second or third most frequently isolated *Candida* species related to nosocomial infections, even overtaking *Candida albicans* in some hospitals. *C. parapsilosis* constitutes a complex of closely related species: *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis*. Accurate detection of these species is of importance, as the incidence of *C. orthopsilosis* has been reported to surpass that of *Candida krusei*.

Objective: To evaluate the diagnostic utility of two PCR-RFLP methods targeting the *SADH* and *FKS1* genes and to determine the prevalence of cryptic species in 96 bloodstream isolates of *C. parapsilosis* from 93 patients.

Methods: Restriction patterns of the *SADH* and *FKS1* genes were analysed, and sequencing of the D1/D2 regions of the ribosomal RNA was used to evaluate the reliability of both PCR-RFLP methods.

Results: In our study, 77 *C. parapsilosis sensu stricto*, 13 *C. orthopsilosis* and five *C. metapsilosis* were identified by sequencing. Both PCR-RFLP methods demonstrated strong agreement with D1/D2 sequencing in the identification of *C. parapsilosis* and *C. orthopsilosis*, while both methods were unable to identify the *C. metapsilosis* isolates. Moreover, unexpected restriction patterns were observed for two isolates on *SADH* PCR-RFLP and for four isolates on *FKS1*

PCR-RFLP. Mixed bloodstream infections of *C. parapsilosis sensu stricto* and *C. orthopsilosis* were detected for three patients, for which differential growth characteristics were observed.

Conclusion: The molecular method chosen for identification could have an impact on determination of the real prevalence of *C. metapsilosis* in candidaemia, and mixed fungaemias can remain undetected.

Keywords: *Candida metapsilosis*, *Candida orthopsilosis*, PCR-RFLP, D1/D2 Large-Subunit sequencing, *SADH* and *FKS1* genes.

Introduction

The incidence of invasive candidiasis has increased in recent decades, and *Candida parapsilosis* is a notable species of *Candida*. Depending on the geographical area, *C. parapsilosis* is the second most frequently isolated species, and in some hospitals in Europe, Asia and South America, it has even overtaken *Candida albicans* in incidence.[1-6] *C. parapsilosis* infection predominates in low-weight neonates, critically ill patients or those with cancer and is associated with the disruption of anatomical barriers by invasive procedures, such as central venous catheter placement, surgery and parenteral nutrition administration.[3,5] In 2005, the three distinct clades formerly known as *C. parapsilosis* I, II and III were replaced by a complex of three closely related species: *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis*, respectively.[7] Among them, *C. parapsilosis* is the most frequently isolated species, while 1 to 10% of the isolates belong to the cryptic species *C. orthopsilosis* and *C. metapsilosis.*[8] Whether geographical variation contributes to the high variability observed in the frequency of isolation of cryptic species is not yet clear.[3] Phenotypical methods are unable to differentiate between the species of the complex; therefore, several molecular approaches have been developed for this purpose, including PCR-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), real-time PCR, PCR analyses of intron length polymorphisms, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and sequencing analysis of panfungal markers, among others.[9-11] Among these molecular methods, PCR-RFLP targeting the secondary alcohol dehydrogenase gene (*SADH*) was the first developed and is notable for its wide use since its description due to its simplicity and high interlaboratory reproducibility.[7] Since then, other PCR-RFLP protocols targeting different genes have also been proposed.[8] However, sequencing of panfungal markers, such as the internal transcribed spacer (ITS) regions (ITS1-5.8S-ITS2) or the large subunit (LSU) D1/D2 region of the 26S rRNA gene, remains the gold standard for identification.[12]

Although most candidiasis is caused by a single species of *Candida*, cases of polyfungal infections are also reported, especially in patients in intensive care units (ICUs), and the most frequent associations are *C. albicans* with *C. parapsilosis* and *C. albicans* with *C. glabrata*.[13-15] Moreover, it has been observed that in mixed candidaemias, *C. parapsilosis sensu stricto* predominates along with other species of *Candida*.[2]

External acquisition by medical devices is considered an important route of *C. parapsilosis* infection, and prolonged catheterization has been demonstrated to be the most important underlying condition, with this factor being of greater relevance to the *C. parapsilosis* species complex than to *C. albicans*.[2,5,16,17] In 2015, Barbedo *et al.* observed that the species identified in the catheter was not always responsible for the bloodstream infection, and different cryptic species of the *C. parapsilosis* complex could be identified in the same patient from both samples.[18]

Therefore, in the present study, we compared both the *SADH* PCR-RFLP method and a PCR-RFLP targeting the β(1,3)-glucan synthase subunit 1 (*FKS1*) gene with the sequencing of the gene fragment encoding the D1/D2 domains of the 26S rRNA to assess their diagnostic utility and to determine the prevalence of cryptic species in a collection of bloodstream isolates of *C. parapsilosis*.

Methods

Microorganisms and growth conditions

Ninety-three blood isolates previously identified as *C. parapsilosis* by the AUXACOLOR (Bio-Rad, Spain) and/or by the Vitek-2 system (BioMeriéux, Spain) were isolated from 93 patients with candidaemia of the Hospital Universitario y Politécnico La Fe of Valencia (Spain). These isolates were sent to the Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU) to perform molecular identification. The isolates were stored in vials containing sterile distilled water at room temperature until use. The reference strains *C. parapsilosis* ATCC

22019, *C. parapsilosis* ATCC 90018, *C. metapsilosis* ATCC 96143, *C. metapsilosis* ATCC 96144, *C. orthopsilosis* ATCC 96139 and *C. orthopsilosis* ATCC 96141 were included. Both clinical isolates and reference strains were grown on Sabouraud dextrose agar (Difco, Becton Dickinson, USA) and incubated at 37 °C for 24 h before use.

DNA extraction

The rapid detection abilities of both PCR-RFLP methods for the *C. parapsilosis* complex were initially tested directly from the colonies without DNA extraction. When no amplification or inconclusive results were obtained, genomic extracted DNA was used, as well as for sequencing analysis for all the isolations.

Genomic DNA extraction was performed with a DNeasy Ultraclean Microbial Kit (QIAGEN, Germany) following the manufacturer's instructions. DNA purity and content were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), and extracts were stored at -20ºC until use.

*PCR-RFLP targeting the secondary alcohol dehydrogenase (*SADH*) gene*

Each isolate was subjected to PCR with the primers S1F (5'-GTT GAT GCT GTT GGA TTG T-3') and S1R (5'-CAA TGC CAA ATC TCC CAA-3'), which amplify a 716 bp fragment of the *SADH* gene, according to the method described by Tavanti *et al.*[7] Subsequent digestion with the *Ban*I restriction endonuclease (New England Biolabs, USA) was performed following the manufacturer's instructions. DNA fragments were separated and visualized on a 1.5% agarose gel stained with Gel Red (Biotium, USA). *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* products contain one restriction site, which produces two bands (521 and 196 bp); three restriction sites, which produce four bands (370, 188, 93, and 60 bp); and zero restriction sites (716 bp), respectively.

When conflicting results among other techniques were present, digestion of the amplified region of the *SADH* gene fragment with the *Nla*III restriction endonuclease (New England

Biolabs) was performed as described before[19], and the digestion patterns visualized on a 1.5% agarose gel stained with Gel Red contained two bands (505 and 131 bp) for *C. parapsilosis*, three bands (74, 288 and 348 bp) for *C. metapsilosis* and three bands (131, 217 and 288 bp) for *C. orthopsilosis*.

*PCR-RFLP targeting the β(1,3)-glucan synthase subunit 1 (*FKS1*) gene*

A 1032 bp fragment of the *FKS1* gene was amplified using the primers REA-F (5'-GAT GAC CAA TTY TCA AGA GT-3') and REA-R (5'-GTC AAC ATA AAT GTA GCA TTC TAG AAA TC-3') as described by Garcia-Effron *et al.*[8] The *Eco*RI restriction endonuclease (New England Biolabs) was added afterwards, following the manufacturer's instructions. Digested DNA fragments were separated and visualized as described above. The digestion patterns expected from *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* were one band (1032 bp), two bands (564 and 474) and three bands (474, 306 and 258 bp), respectively.

Growth on chromogenic media

The purity of non-amplifying isolates by both PCR-RFLP methods was assessed by studying their growth characteristics on Candida chromogenic agar (Laboratorios Conda, Spain). The plates were incubated for 48 h at 37 °C, and the differentiation between different isolates was made on the basis of colony morphology and colour development. When different colonies were observed in the same culture, one colony of each morphotype was then isolated and subjected to molecular identification by any of the molecular methods mentioned above.

Sequencing and phylogenetic comparison of the 26S rRNA gene encoding the LSU D1/D2 domain

One to ten nanograms of the extracted genomic DNA of each isolate was amplified using the NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') primers. The fragments of 600 bp were purified and sequenced by Macrogen Spain Inc. using the Sanger method with the automated sequencer ABI 3730 XL

(Applied Biosystem, USA). Sequences were assembled manually using the sequence alignment software BioEdit (Ibis BioSciences, USA) and subjected to BLAST analysis to find similarities to sequences deposited in the GenBank and Mycobank databases. For the species assignment, it was necessary to consider the three parameters of "Query cover", "E value" and "Ident" from GenBank. Phylogenetic and molecular evolutionary analyses based on the neighbour-joining and Kimura 2-parameter methods were conducted using MEGA version 4 software.[20] LSU D1/D2 sequences of the following reference strains already available in the GenBank database were used to construct phylogenetic dendrograms*: Candida albicans* ATCC 90028 (KU729160.1), *Candida dubliniensis* ATCC MYA-646 (JQ070170.1), *C. metapsilosis* ATCC 96143 (KJ463415.1) and ATCC 96144 (FJ746055.1), *C. orthopsilosis* ATCC 96139 (FJ746056.1) and ATCC 96141 (KJ463414.1) and *C. parapsilosis* ATCC 90018 (KU729146.1) and ATCC MYA-4646 (KP780474.1). All nucleotide sequences of the 26S rRNA gene D1/D2 domain from the 95 clinical isolates were deposited in the GenBank database under accession numbers MH612971 to MH613064 and MN121341.

Data analysis

All the methods yielding inconsistent or uninterpretable results were repeated at least twice in separate experiments.

The internal validity of both *SADH* PCR-RFLP and *FKS1* PCR-RFLP was evaluated by comparison of the results with those obtained by sequencing of the 26S rRNA gene encoding the LSU D1/D2 domain, considering the latter as the gold standard technique.

The sensitivity and specificity of both PCR-RFLP methods were calculated, as well as their positive and negative predictive values. The diagnostic testing utility of these methods was evaluated through the determination of positive and negative likelihood ratios.[21] Moreover, evaluation of the concordance between both PCR-RFLP techniques was studied by calculating the percent agreement and the kappa statistic, interpreting the level of agreement

158 of the latter as follows: values ≤ 0-0.20 as no agreement, 0.21–0.39 as minimal, 0.40–0.59 as
159 weak, 0.60– 0.79 as moderate, 0.80–0.90 as strong, and 0.91–1.00 as almost perfect
160 agreement.[22] All statistical parameters were evaluated using IBM Statistical Package for the
161 Social Sciences software (23.0 version, IBM SPSS, USA).

162 **Results**

163 *Identification of* the *C. parapsilosis complex by SADH PCR-RFLP*

164 Identification of the 93 isolates yielded three with repeated inconsistent or questionable
165 results by the PCR-RFLP method involving the *SADH* gene, so the purity of these isolates
166 was checked by analysis of the growth characteristics on Candida chromogenic agar for 48 h
167 at 37ºC. Two isolates each produced two distinct morphotypes, with smooth and rough
168 colonies, and the other one produced colonies with distinguishable shades of beige to violet.
169 When these different colonies were subjected to *SADH* PCR-RFLP, different digestion
170 patterns were observed, with *C. parapsilosis* and *C. orthopsilosis* identified in the three cases.
171 Both species presented diverse morphotypes, with smooth or violet colonies belonging to *C.*
172 *orthopsilosis* and rough or beige colonies belonging to *C. parapsilosis*. Of note is that one of
173 these three patients with mixed fungaemia carried a central venous catheter. Thus, the initial
174 93 samples yielded three additional isolates, with 96 different isolates for molecular
175 identification.

176 Of the 96 isolates analysed, 80 were identified by *SADH* PCR-RFLP as *C. parapsilosis* (80/96,
177 83.3%), 13 as *C. orthopsilosis* (13/96, 13.5%) and none as *C. metapsilosis*. Three isolates (3/96,
178 3.1%) gave questionable results: two of them yielded restriction patterns compatible with the
179 presence of two species (Table 1), and the other one showed one band of lesser size than the
180 expected for *C. orthopsilosis* (<700 bp). However, no differences were found in their growth
181 pattern on Candida chromogenic agar or with the random selection of isolated colony-
182 forming units (CFU) to perform PCR-RFLP.

Identification of the C. parapsilosis complex by FKS1 *PCR-RFLP*

This technique identified 77 isolates as *C. parapsilosis* (77/96, 80.2%), 10 as *C. orthopsilosis* (10/96, 10.4%) and two as *C. metapsilosis* (2/96, 2.1%). Moreover, four isolates (4/96, 4.2%) gave questionable restriction patterns compatible with two species, *C. parapsilosis* and *C. metapsilosis*, and another three isolates did not amplify (3/96, 3.1%). In these last isolates, no differences were found either in their growth pattern on Candida chromogenic agar or with the random selection of isolated CFU to perform PCR-RFLP. Of note is the fact that the restriction patterns expected for reference strains and clinical isolates of *C. metapsilosis* did not agree with those described by other authors [8,23], as two additional bands of approximate sizes of 306 and 258 bp, in addition to 564 and 474 bp, could be seen for the *C. metapsilosis* restriction pattern (Figure 1). This pattern was consistent and reproducible for all repeated PCR experiments. Moreover, by sequencing the amplicon of the *FKS1* gene, we verified the presence of two peaks of equal intensity for T and C in the base 780 of the amplified region. This result revealed that all *C. metapsilosis* strains hold a heterozygous point mutation in the base 4423 of the complete *FKS1* gene sequence (ref: EU221325). Then, when the nucleotide was C, it matched with the specific restriction sequence site of the *Eco*RI enzyme (GAATTC), resulting in two additional restriction bands (306 and 258 bp) (Figure 2). Moreover, the three *C. metapsilosis* clinical isolates that gave questionable restriction patterns yielded this pattern when *FKS1* PCR-RFLP was performed with extracted DNA.

Sequencing of the 26S rRNA *gene D1/D2 domain*

All isolates except one for which no PCR amplification was obtained produced a fragment of approximately 600 bp. After purification and sequencing, consensus sequences of sizes ranging between 555 and 575 bp were constructed. Comparison with the sequences deposited in GenBank and Mycobank showed the same identification for both databases. For some isolates, the homology ranged from 98 to 100% with the three species of the *C.*

parapsilosis complex. A phylogenetic tree constructed with these sequences confirmed the identification and allowed us to clarify the questionable identification of some isolates obtained by either of the two PCR-RFLP techniques (Figure 3). The dendrogram grouped all the isolates into three separate clusters with their reference strain sequences of *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, except for one *C. parapsilosis* isolate (accession number MN121341) that did not group along with the other *C. parapsilosis* isolates (Figure 3). As a result, 77 isolates were identified as *C. parapsilosis* (77/95, 81%), 13 as *C. orthopsilosis* (13/95, 13.7%) and five as *C. metapsilosis* (5/95, 5.3%).

Comparison of the identification results between both PCR-RFLP methods and sequencing

The identification results obtained by both PCR-RFLP methods were compared and were found to be concordant for 87 of the 96 isolates (90.6%).

When both methods were compared with sequencing of the D1/D2 domain, it was found that the method involving the *SADH* gene misidentified four isolates of *C. metapsilosis,* three of *C. orthopsilosis* and one of *C. parapsilosis*. Although the method involving the *FKS1* gene gave fewer misidentifications, questionable or null results were observed for three isolates of *C. orthopsilosis*, three of *C. parapsilosis* and one of *C. metapsilosis* (Table 1). Of note, one isolate (15-083) was concordant by both PCR-RFLP methods being identified as *C. orthopsilosis*; however, sequencing of the 26S rRNA gene D1/D2 domain identified this isolate as *C. parapsilosis*, although it did not group with the other *C. parapsilosis* isolates (accession number MN121341) (Figure 3).

Alternative digestion of the *SADH* gene with the *Nla*III restriction endonuclease was performed for the nine isolates for which the identification between any of the techniques did not match. The identification of six isolates was concordant with that obtained by sequencing. However, the restriction patterns from the other three isolates continued to be compatible with the presence of two species (Table 1). These three isolates were clustered in

the dendrogram of the 26S rRNA gene D1/D2 domain inside the group of *C. metapsilosis* or *C. orthopsilosis*. The diagnostic utility of both PCR-RFLP methods was assessed through analysis of the sensitivity, specificity, level of agreement and kappa statistic (Table 2). For the identification of *C. parapsilosis*, both PCR-RFLP methods reached high sensitivity (0.99 and 0.97 for the *SADH* and *FKS1* genes, respectively), although the method involving the *FKS1* gene was more specific than the *SADH* gene method (1 and 0.78, respectively). Equal specificity and sensitivity values of both methods in the detection of *C. orthopsilosis* were reached. In general, the level of agreement for the detection of these species by both PCR-RFLP techniques with sequencing was strong, except for the detection of *C. parapsilosis* by the *FKS1* gene method, for which the agreement was almost perfect; however, the detection of *C. metapsilosis* scored the lowest values of sensitivity and specificity, and only *FKS1* PCR-RFLP was able to detect two of the five isolates, reaching a weak level of agreement with sequencing.

Discussion

The incidence of nosocomial infections caused by *Candida* has risen significantly in recent decades. Among this genus, *C. parapsilosis* is the species that has most notably increased in incidence, causing 10-25% of candidaemias in neonates and patients in intensive care units.[5,17,24] *Candida parapsilosis* infections are associated with the use of medical devices, such as central venous catheters, parenteral nutrition administration and health care worker contact.[5,25-28] Moreover, *C. parapsilosis* is a complex of three species, with *C. parapsilosis* being the most frequently isolated (80 to 90% of the isolates), followed by the cryptic species *C. orthopsilosis* and *C. metapsilosis*. These species show differences in virulence and in vitro susceptibility to antifungal agents.[11] In this context, the emergence of azole resistance in *C. parapsilosis* reported recently highlights the necessity to perform an accurate identification to choose the most appropriate treatment.[29]

In our study, compared with sequencing identification, the *SADH* PCR-RFLP method misidentified ten isolates. It has been suggested that the misidentification of *C. orthopsilosis* and *C. metapsilosis* could be due to the loss of *BanI* restriction sites;[19,23] therefore, an improvement in the procedure consisting of the use of the *Nla*III restriction endonuclease has been proposed by Mirhendi *et al.*[19] This method allowed the conclusive identification of two isolates that had shown hybrid digestion patterns but yielded three additional isolates with hybrid patterns that were not shown previously with the method involving *BanI*.

Interestingly, a low number of discrepancies were observed when comparing both PCR-RFLP methods with each other, as only one isolate yielded clear discrepant identification results between the two PCR-RFLP techniques. However, the method involving the *FKS1* gene gave numerous inconsistent identifications, as it yielded restriction patterns that were distinct from those expected or failed to amplify some clinical isolates. One possible explanation for this could be the presence of additional copies of the *FKS1* gene.[8] Several mutations of the *FKS1* gene are described elsewhere[30], and since the gene may be subject to pressure through the use of echinocandins, *FKS1* could generate few conserved sequences that yield inconsistent restriction patterns, as was found in the case of our *C. metapsilosis* isolates.

Overall, in certain isolates, the analysis of both the *SADH* and *FKS1* genes by PCR-RFLP techniques identified two species, but the sequencing revealed only one of them. This finding seems to argue in favour of allelic differences and emphasize the risk of trying to distinguish closely related species by active gene analysis.

When these PCR-RFLP methods were compared with the sequencing of the D1/D2 domains of the 26S rRNA gene, the number of *C. metapsilosis* in our study was underestimated. Consistency or agreement between an identification method and another one taken as a gold standard is desirable to measure the effectiveness of such a method;

283 sensitivity, specificity and predictive values are extensively used in the assessment of
284 diagnostic testing but are influenced by prevalence.[21,22,31] As such, both PCR-RFLP methods
285 reached strong agreement with the reference technique in the identification of *C. parapsilosis*
286 and *C. orthopsilosis*, indicating that 64 to 81% of the data are reliable.[22] However, in the
287 identification of *C. metapsilosis*, only the *FKS1*-based PCR-RFLP achieved weak agreement.

288 In our study, sequence analysis of the D1/D2 region was the most reproducible method that
289 performed consistently, as observed by other authors.[32] ITS region sequencing is considered
290 the most accurate method to differentiate the species within the complex, although some
291 authors have observed that the sequence analysis of the D1/D2 region failed to differentiate
292 cryptic species even for the reference strains.[19,33] Moreover, ITS sequencing has limitations,
293 such as the reduced discriminatory power in a study of early diverging or high-divergence
294 lineages that could reflect the presence of multiple cryptic species.[12]

295 Cryptic species prevalence and distribution show high variability and appear to be related to
296 multiple factors, such as geographical area, underlying conditions of the patients and local
297 hospital epidemiology.[3,18] In this study, we report an overall prevalence of 13.7% for *C.*
298 *orthopsilosis* and 5.3% for *C. metapsilosis*, which are higher values than those described
299 previously.[2,16,34,35] Some recent studies have reported an increased prevalence of cryptic
300 species,[18,36] and on the other hand, a concurrent downward trend in the prevalence of *C.*
301 *parapsilosis* was observed in our study, as in the cited studies. When analysing the literature
302 about the epidemiology and prevalence of the *C. parapsilosis* complex, it is noteworthy that
303 there is a great diversity of molecular methods used to differentiate these species. Among the
304 various approaches, PCR-RFLP-based methods are notable due to their versatility, and the
305 protocol proposed by Tavanti based on the amplification of the *SADH* gene and subsequent
306 *Ban*I digestion is the most widely used since its description.[7,33] The hypothesis of a time- or
307 geographically dependent epidemiological shift could be drawn from the data, but

interestingly, some recent studies report similar frequencies of isolation that appear to be influenced by the method chosen for the identification.[11,37-39]

The genetic diversity of cryptic species is increasingly recognized, especially for *C. orthopsilosis,* as it has been suggested by using AFLP and ITS sequencing techniques that there are at least two subgroups with highly heterozygous genomes.[40-43] *C. metapsilosis* also has highly heterozygous genomes, while *C. parapsilosis* displays the lowest levels of heterozygosity of the complex of species. Heterozygosity as the byproduct of mating is associated with increased virulence, although *C. metapsilosis* is described as the least virulent species and *C. parapsilosis* as the most virulent.[44-46]

On the other hand, it is hypothesized that the variability in prevalence reflects differences in virulence. However, it should be outlined that many studies about virulence are based on small sample sizes, thus limiting inferences about the influence of virulence or host response.[47]

As an important remark on the prevalence variability, although sequencing still remains costly, PCR-RFLP methods can be quite time consuming, as inaccuracies can be found. Furthermore, the ongoing evolutionary processes within the cryptic species of the complex may make it difficult to select suitable markers for correct identification. Moreover, both PCR-RFLP techniques required repeated amplifications to obtain a conclusive result. Although the performance was not evaluated in this study, this aspect should be taken into account as it decreases the practicality of both PCR-RFLP methods to efficiently detect *C. parapsilosis* infections.

Mixed candidaemias due to *C. parapsilosis* and *C. orthopsilosis* had been reported previously[18] and according to the present study, in which we found three patients with mixed candidaemia involving these species. Although phenotypical methods are regarded as unable to differentiate between the species of the complex, in our study, they provided useful support

when the molecular methods failed to explain the inconsistencies found. To our knowledge, this report is the first study reporting mixed fungaemia due to *C. parapsilosis* and *C. orthopsilosis* together in the bloodstream, as in a preceding study, one species was in the catheter, and another was in the bloodstream.[18] Although the detection of more than one species in the bloodstream is infrequent (2-10%), mixed candidaemia is associated with increased mortality. The impact of mixed bloodstream infections due to species of the same complex remains to be investigated, as the most frequently recognized associations are between *C. albicans* with *C. parapsilosis* and *C. albicans* with *C. glabrata,*[13-15,48] and the role of environmental acquisition of infections should also be considered.

In conclusion, our results prove that frequencies of cryptic species in candidaemia may be underestimated depending on the method chosen for identification, and given that, in some hospitals, the incidence of *C. orthopsilosis* infections exceeds that of even *C. krusei*, the accuracy of the identification method is of great relevance. Many molecular methods fail in the identification of polyfungal infections and to know the specific aetiology could be crucial to elucidate epidemiological associations even within the same species complex and their role in outbreaks.

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