New Microbiologica, 43, 1, 47-50, 2020, ISSN 1121-7138

CASE REPORT

# *Candida palmioleophila* isolation in Italy from two cases of systemic infection, after a CHROMagar and Vitek system mis-identification as *C. albicans*

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SUMMARY

A correct, fast, reliable identification method is pivotal in nosocomial environments to guide treatment strategies, whereas misidentification might lead to treatment failure. For routine identifications the Vitek system and CHROMagar are widely used but not always reliable, especially now with an increasing number of new emerging fungal pathogens that need careful identification. Here we describe two cases of candidemia, due to *Candida palmioleophila* previously misidentified as *Candida albicans* by using the Vitek2 system and CHROMagar. The first case is a 54-year-old man with an infected ulcer in the lower right limb, treated with a targeted therapy using a central venous catheter (CVC). After two months he developed a CVC-related candidemia MDR identified as C. albicans. The second case is a 2-month-old male baby that was admitted to the neonatal unit with acute respiratory failure due to a severe community-acquired bilateral pneumonia; blood cultures were all positive for *C. albicans MDR.* The isolated strains where re-identified with Maldi-Tof and DNA sequencing as *C. palmioleophila*.
From the identification point of view, CHROMagar can be clearly misleading, especially because CHROMagar types currently available are not designed to discriminate new emerging species, suggesting that systems other than MALDI-TOF and marker sequencing may be inadequate even for routine identification and could contribute to producing misleading identifications and therapeutically wrong practices, leading to failures and patient death.

*Received* July 21, 2019 *Accepted* November 9, 2019

## INTRODUCTION

The ability of new molecular and phenotypic methods to better identify pathogenic species of *Candida* with indistinguishable phenotypic characteristics has enabled the identification of new species such as *C. orthopsilosis, C. metapsilosis* and others (Tavanti *et al.,* 2005). Some new species such as *C. palmioleophila* might be resistant to anti-fungal agents *(Agualimpia et al., 2016)*, therefore a correct identification is pivotal to guide treatment strategies, whereas misidentification might lead to treatment failure (Coignard *et al.,* 2004; Jensen and Arendrup 2011). *C. palmioleophila* was described in 1988 (NAKASE *et al.,* 1988) and rarely reported in clinical setting afterwards (Datta *et al.,* 2015).
This species is relatively well known, with 58 different ITS sequences in Pubmed and 16 related publications, of which 6 are of clinical interest. *C. palmioleophila* was isolated from endogenous endophthalmitis (Datta *et al.,* 2015), from catheter-associated candidemia (Sugita *et al.,* 1999), and from hospital environments in general (Feng *et al.,* 2014; Scapaticci *et al.,* 2018). This fact indicates that the species is not entirely of medical interest and is not yet perceived as an opportunistic pathogen, although its incidence is already around 1% (Scapaticci *et al.,* 2018). In a 6-year nationwide study of fungemia in Denmark, Arendrup et al. described 2820 episodes of fungemia, 9 cases of which were due to *C. palmioleophila* (Arendrup *et al.,* 2011). Similar frequencies were obtained from a large collection of *Candida* clinical isolates in Tunisia (423 strains), in which only two strains were identified as *C. palmioleophila (Eddouzi et al., 2013)*.
*C. palmioleophila* might be misidentified as *C. famata* (*Debaryomyces hansenii*) or as *C. guillermondi* (*Meyerozyma guilliermondii*). Jensen and Arendrup found that 5 strains of *C. famata* and 3 strains of *C. guillermondii* were later re-identified as *C. palmioleophila* (Jensen and Arendrup 2011). Furthermore, it is important to highlight that even *C. auris* can be misidentified as *C. famata* (Kim *et al.,* 2016). These papers indicate that the misidentifica-

*Key words:*
*Candida palmioleophila*, yeast, CHROMagar, MDR, fungicide resistance.

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tion of this species is possible and relatively frequent, if compared with its incidence, raising the problem of using more powerful tools for identification in order to allow the most appropriate therapeutic choices.
The aim of this work is to describe two cases of misidentification of this species due to the use of routine systems that are not always able to discriminate among pathogenic species, even if not particularly close from a phylogenetic viewpoint, and to show how molecular tools can not only lead to a correct identification, but can also shed light on the clonality of the isolates.

## MATERIALS AND METHODS

The susceptibility of *Candida Palmioleophila* strains, isolated from both cases, to anidulafingin, caspofungin, micafungin, itraconazole, posaconazole, fluconazole, voriconazole and amphotericin B was investigated using Etest with RPMI-2% glucose medium (Biomérieux S.p.A., Italy), following the manufacturer's instructions.
The isolates were subjected to MALDI-TOF MS (Bruker, Germany) for molecular identification. Prior to sample preparation, all yeasts were grown on CHROMagar for 2 days at 37°C. A colony was then subjected to 70% ethanol fixation, acetonitrile and 70% formic acid were added after the complete evaporation of ethanol, and 1µl of the supernatant was deposited on the suitable support. The dried spots were covered with the Matrix HCCA (α-Ciano-4-idrossicinnamic acid) (Bruker, Germany) and let dry at room temperature. Samples were finally submitted to MALDI-TOF analysis. Spectra were calibrated using Escherichia coli ribosomal proteins and evaluated against the Bio-typer spectrum database by the default pattern-matching algorithm.
A further identification was obtained by sequencing of the ITS1 (Internal Transcribed Spacer), 5.8S, ITS2 rDNA gene cluster regions and the D1/D2 domain of the LSU (Large Subunit) gene. Genomic DNA was extracted as indicated by Cardinali *et al.* (Cardinali *et al.,* 2001). The ITS1, 5.8S, ITS2 rDNA gene cluster regions and the D1/D2 domain of the LSU gene were amplified with FIREPole® Taq DNA Polymerase (Solis BioDyne, Estonia), using the ITS1 (5′-TCCGTAGGTGAACCTGCGG)-NL4 (GGTCCGTGTTTCAAGACGG) primers pair. The amplification protocol was carried out as follows: initial denaturation at 94°C for 3 min, 30 amplification cycles (94°C for 1 min, 54°C for 1 min and 72°C for 1 min) and final extension at 72°C for 5 min. Amplicons were sequenced in both directions with ABI PRISM technology by MACROGEN (www.macrogen.com) with the same primers used for the generation of the amplicons. The reads of each strain, contained in FASTQ file, were analysed with Geneious R9 (Kearse *et al.,* 2012).
For the biofilm forming ability test, the cultures were grown in YPD medium (Yeast Extract 1%, Peptone 1%, dextrose 1%) incubated at 37°C for 24 hrs, at 120 rpm. The cellular suspension was calibrated, in properly modified RPMI medium (Pierce *et al.,* 2008) to $OD_{600}$= 0.1 and 100 µl of the standardized cell suspensions were seeded in each selected well of a 96-well microtiter plate. Three biological replicates for each strain were seeded, each one in triple replicates. The plate was incubated for 2 hours at 37°C. After this first phase of biofilm priming, the inoculum in each well was carefully removed with a multichannel pipette and each well was washed three times with PBS. 100 µl of fresh RPMI medium was then added to each well. After 24 hours of growth at 37°C, the plate was collected, and the same washing procedure described above was applied. For the same strain we obtained 3 different measures of the biofilm, at 405 nm of the washed biofilm, at 570 nm after crystal violet staining procedure and at 492 nm after XTT Reduction Menadione Assay (XRMA), with TECAN Infinite F200 plate reader (Tecan Trading AG, Mannedorf, Switzerland).

## CASE REPORT

**Case 1.** A 54-year-old man with infected ulcer in the lower right limb was admitted to Cotugno Hospital in January 2016. The patient was a drug addict, in therapy with methadone 90 mg per diem, with chronic HCV hepatitis and lower limb polyneuropathy. He was affected by a chronic ulcer of the left leg infected with *Pseudomonas aeruginosa* MDR and had been treated with several antibiotic regimens in the last two years. The patient was treated with a targeted therapy with IV-Meropenem 1gr t.i.d. plus colistin 4.5 bid after loading dose, using a central venous catheter (CVC). After two months he developed a CVC-related candidemia. A presumptive strain of *Candida* was isolated from blood cultures, subsequently identified as *C. albicans* and characterized as susceptible to all antifungal agents: fluconazole 4 mg/L (S), itroconazole 0.50 mg/L (S), anidulafungin 0.008 mg/L (S), micafungin 0.064 mg/L (S), caspofungin 0.064 mg/L (S) and voriconazole 0.094 mg/L (S). This presumptive *C. albicans* strain grew bluish on CHROMagar. The first identification was performed with the Vitek 2 system (BioMerieux, Marcy I' Etoile, France). The patient was treated with IV-Anidulafungin 100 mg o.i.d for two weeks, leading to resolution of the candidemia.
**Case 2.** A 2-month-old male baby was admitted in June 2016 to the neonatal UTI of Monaldi Hospital in Naples (belonging to the same network as Cotugno hospital in case 1, and spatially very close to each other), reference center for ECMO (Extra Corporeal Membrane Oxygenation) in South Italy, with acute respiratory failure due to a severe community-acquired bilateral pneumonia, transferred from another hospital. He was affected by congenital Clippers Syndrome. In the previous hospital, blood cultures resulted positive for *C. albicans*. The baby also had pneumothorax and was treated immediately with ECMO. Blood cultures were all positive for *C. albicans* (Vitek 2 system and CHROMagar), therefore a therapy with micafungin (2 mg/kg every 24 hours) and voriconazole (4 mg/kg every 12 hours) was administered. Subsequently, therapy was changed to ambisome (3 mg/kg) and caspofungin (2 mg/kg every 24 hours), but even with the new therapy we were unable to obtain negativization of blood cultures. Although the first isolates resulted susceptible to fluconazole, the second resulted resistant to all azoles. The baby died of candidemia after 14 days of the last antifungal regimen.
All *Candida* strains isolated from the blood in 2016 were then re-identified in Cotugno hospital when the MALDI-TOF MS (BioMerieux, Marcy I' Etoile, France) system was made available at the end of 2016. These two strains were identified as *C. palmioleophila*.

## DISCUSSION

Here we describe two cases of candidemia due to *C. palmioleophila*, previously misidentified as *C. albicans* by using

CHROMagar plates and Vitek 2 procedures. What presented represents, to the best of our knowledge, one of the first isolations of *C. palmioleophila* in Italy (Scapaticci *et al.,* 2018) and some of the relatively few worldwide.

Both strains, hereinafter designated as CMC2116 and CMC2112 from case 1 and case 2 respectively, were subject to Maldi-Tof analysis in order to test the preliminary identification with the CHROMagar and Vitek 2 systems. In both cases, the Maldi-Tof output indicated that the strains belong to the species *C. palmioleophila*, suggesting that the previous identification with the CHROMagar and Vitek 2 systems was incorrect. In order to further confirm the Maldi-Tof results, DNA extracted from CMC2112 and CMC2116 was subjected to sequencing of the ITS and LSU taxonomic markers (Schoch *et al.,* 2012), finding that CMC2112 and CMC2116 show, respectively, 0.87% and 0.70% distance from the type strain with the ITS and 0.92% and 0.74% with the LSU marker (*Figure S1*, Supplementary material). These figures are below the threshold for species identification, suggested as 1.4% and 1% for ITS and LSU, respectively (Vu *et al.,* 2016), and confirm the identification as *C. palmioleophila* obtained with the Maldi-Tof. These data suggested that the two strains are not identical and in fact an alignment of the sequences produced 0.55% and 0.35% distance between the two isolates for LSU and ITS respectively (*Figure S1*, Supplementary material).

The fact that two markers used for identification find a significant distance between strains (Vu *et al.,* 2016) rules out the hypothesis that the two strains form a clone, although they leave the possibility that a rapid microevolution is occurring in the very harsh nosocomial environment (Blasi *et al.,* 2001).

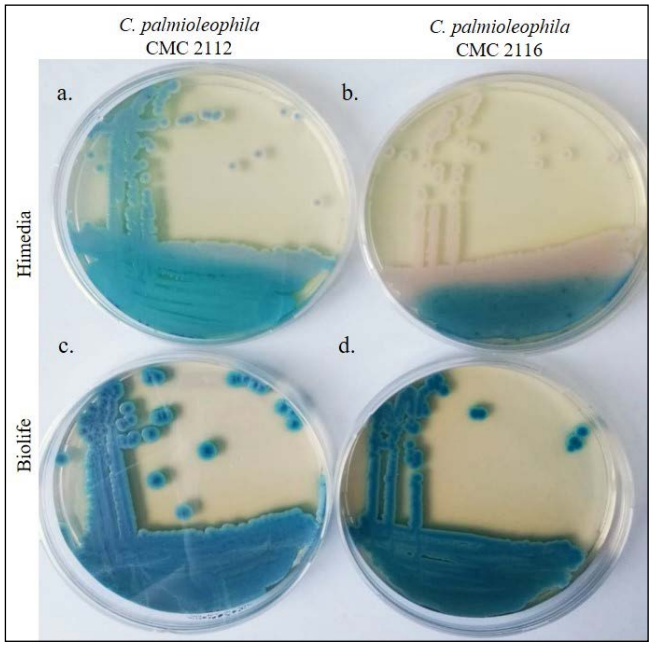


**Figure 1** - *Candida palmioleophila (strains CMC2112 and CMC2116) was grown on Candida CHROMagar obtained from two different suppliers, Biolife and HiMedia, respectively. Panels c.-d. and a.-b. C. palmioleophila CMC2112 developed the typical Candida albicans color on both the media, panels a. and c. while strain CMC2116 developed a pink pigmentation on one of the two media tested (panel b.).*

Finally, because biofilm forming ability is an important virulence factor for candidemia, the two strains studied were tested for their ability to form biofilm. The two strains have little (CMC 2016) or no ability to develop biofilm (CMC 2012), suggesting that this character may be variable, as in the other *Candida* species of medical interest (Corte *et al.,* 2016).

The taxonomic data rule out the hypothesis that the two isolates may be two identical copies of the same strain. Furthermore, these data indicate that either different strains dwell in the hospital environment or that a rapid microevolution is taking place, although the second hypothesis is less likely and requires more evidence. Whatever the explanation for the non-identity of the two isolates, the fact remains that two strains of this relatively rare species were isolated at short time distance, practically in the same place, suggesting that this species could be more frequent than expected either in the nosocomial or in the outer environment. Another point that could not be fully addressed is the origin of the strains, in order to understand whether *C. palmioleophila* should be considered a nosocomial pathogen of an environmental species adapted to the medical environment, as suggested by the current taxonomic knowledge on this species.

From the point of view of identification, it is clear that CHROMagar can be misleading, especially because CHROMagar types currently available are not designed to discriminate this species.

Furthermore, the color developed can be confused with the turquoise color typical of *C. albicans (Jensen and Arendrup 2011)*. Using two different brands of CHROMagar, we found that a single strain developed a pink pigmentation in only one of the two media tested (*Figure 1b*). Furthermore, the pink color is restricted to the part of the cell lawn exhibiting less growth, in agreement with the observations and the pictures presented by Jensen et al. (Jensen and Arendrup 2011), who found similar color variations.

## CONCLUSION

The DNA markers of the two strains are quite similar, but not identical, to each other, ruling out the hypothesis that they are indeed the same isolate. Moreover, the sequences of these strains are quite similar to those of the other sequences available, suggesting little variability among the clinical isolates of this species. Whether this aspect means a clonal structure of the species or a specialization of the clinical isolates in comparison to those of environmental origin, as in *C. guilliermondii (Corte et al., 2015)* (*M. guilliermondii*) is one of the questions to investigate in the future, when more isolates of both origins will be available.

Our study indicates that the CHROMagar and Vitek systems are inadequate even for routine identification and could contribute to producing misleading identifications, as also suggested by other authors (Castanheira *et al.,* 2013), in a period characterized by an increasing number of emerging fungal pathogens (Johnson 2009) that need careful identification.

### Conflict of interest

C. Tascini received funds for speaking at symposia organized on behalf of Pfizer, Novartis, Zambon, Correvio, Biotest, Merck, Thermofisher, Gilead, Angelini and Astellas. All the other authors declare no conflict of interest.

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