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Research article

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The investigation of antifungal susceptibilities of *Kluyveromyces marxianus* and *Clavispora lusitaniae* strains isolated from various clinical specimens

M. Altay Atalay, A. Nedret Koç, Nuri Çakır, Fatma Mutlu Sarıgüzel, Pınar Sağıroğlu

Department of Medical Microbiology, Erciyes University School of Medicine, Kayseri, Turkey

ORCID ID of the author(s)
MAA: 0000-0003-4169-0637
ANK: 0000-0002-1736-9707
NC: 0000-0002-9935-7397
FMS: 0000-0003-2747-0208
PS: 0000-0001-6742-0200

Corresponding Author
M. Altay Atalay
Department of Medical Microbiology, Erciyes University School of Medicine, Kayseri, Turkey
E-mail: altayatalay@gmail.com

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Ethics approval and patient consent were not considered necessary since the isolates originated from the clinical samples obtained during routine laboratory activities.
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

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No conflict of interest was declared by the authors.

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Abstract

Background/Aim: *C. albicans* remains the most common pathogen responsible for invasive candidiasis. On the other hand, increased rates of candidiasis have been reported worldwide, caused by other *Candida species* (such as *K. marxianus* and *C. lusitaniae*). Considering these uncommon yeasts may be crucial pathogens in the future, it is preferable to describe the in-vitro activities of antifungal agents as potential options for their treatments. This study aimed to evaluate the in-vitro activity of nine different antifungal agents that are routinely used to contribute to the treatment of the infections caused by *K. marxianus* and *C. lusitaniae*.

Methods: The study included 21 *K. marxianus* and eight *C.lusitaniae* strains isolated from various clinical specimens of patients with suspected invasive fungal infection. Conventional identification was confirmed using the molecular methodology of DNA sequencing analysis. Antifungal susceptibilities of the isolates were tested using the Sensititer Yeast One Test Panel Y06 kit, a colorimetric microdilution test.

Results: For *K. marxianus*, amphotericin B had the highest geometric mean MIC (1 µg/mL) and voriconazole had the lowest geometric mean MIC (0.010 µg/mL). For *C. lusitaniae*, flucytosine had the highest geometric mean MIC (8 µg/mL) and voriconazole had the lowest geometric mean MIC (0.011 µg/mL).

Conclusion: Considering that these two species, rare causes of invasive candidiasis nowadays, may become important pathogens in the future, it is reasonable to investigate the in-vitro activities of antifungal agents that can be used in their treatment.

Keywords: Antifungal susceptibility, Clavisopra lusitaniae, Kluyveromyces marxianus, Sequencing

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Introduction

Although five *Candida* species (*C.albicans*, *C. glabrata*, *C.parapsilosis sensu stricto*, *C.tropicalis*, and *C.krusei*) account for ≥95% of all candidemia or other forms of invasive candidiasis, other less common species (*Kluyveromyces marxianus*, *Clavispora lusitaniae*) may cause problems, particularly in cancer and leukemia patients [1-3]. Although *K. marxianus* is uncommonly documented in the literature, recent reports suggest that it may be an emerging pathogen, especially in patients with hematologic malignancies [4]. The ecology of the *K. marxianus* is not exactly understood, although it appears to grow in different habitats, including dairy products like kefir, fermented milk, cheese and yoghurt [5]. It was isolated from kefir in 1909, named *Saccharomyces fragilis* at first, then *C. pseudotropicalis,* and was reclassified as *K.marxianus* [5]. *C. lusitaniae* was first isolated from the gastrointestinal system of warm-blooded animals in 1959. It was first reported as an opportunistic human pathogen in a patient with acute myelogenous leukemia in 1979 [6]. Recent studies have indicated that the incidence of serious infections, especially blood stream infections caused by *K. marxianus* and *C. lusitaniae* are increasing. The clinical management of systemic infections caused by these organisms is challenging because of high MICs of amphotericin B for *K. marxianus* and the intrinsic amphotericin B resistance in some isolates of *C. lusitaniae* [5, 7]. Considering these uncommon yeasts may be crucial pathogens in the future, it is preferable to describe the in-vitro activities of both new and conventional antifungal agents as potential options for their treatments [2].

This study aimed to evaluate the in-vitro activity of nine different, routinely used antifungal agents to contribute to the treatment of infections caused by *K. marxianus* and *C. lusitaniae*.

Materials and methods

Identification

This study included 21 *K. marxianus* and eight *C.lusitaniae* strains isolated from various clinical specimens of patients with suspected invasive fungal infection, which were sent to Mycology Laboratory of Erciyes University Hospital, Faculty of Medicine. All yeast isolates were taken from different patients. Cultures were performed on Sabouraud dextrose agar (SDA) (Oxoid; United Kingdom), chloramphenicol SDA containing or not containing cycloheximide, and chromogenic media (CHROMagar *Candida*, Becton Dickinson, USA) in accordance with the standard identification procedure for fungal isolates. The Germ Tube Test (GTT) was performed on colonies grown on agar plates after 24 hours of incubation. Yeast isolates negative for GTT were cultured on corn meal-Tween 80 agar medium to identify their morphological characteristics and species. Twenty-one isolates identified as *K. marxianus* and eight isolates identified as *C.lusitaniae* based on the color they formed in chromogenic medium, colony morphology, and microscopic appearance in Corn Meal-Tween 80 Agar were confirmed with API 20 C AUX (Biomerieux, France). This initial identification was affirmed using the molecular methodology of DNA sequencing analysis. The sequences were compared with those available in the GenBank database using the BLASTN tool for species identification of each sequence.

Antifungal susceptibility test

Isolates were tested for their antifungal susceptibility with Sensititre Yeast One Test Panel Y06 kit (Trek Diagnostic Systems Inc., USA), a colorimetric microdilution test. A yeast suspension of 1.5-8 x 10^3 cells/mL was prepared in the Sensititre Yeast One broth, which was then added to the wells containing a certain amount of antifungal agent and incubated at 35°C for 24-48 hours. The value of the first well, where no red color was observed in the positive growth well, was considered the minimum inhibitory concentration (MIC).

Statistical analysis

Microsoft Excel 2010 software was used to determine the MIC ranges, MIC_{50} and MIC_{90} values of the isolates.

Results

K. marxianus species was isolated from the bronchoalveolar lavage (BAL) fluid (n=12), urine (n=4), peritoneal fluid (n=3) and blood (n=2) cultures and *C.lusitaniae* species was isolated from the urine (n=3), BAL fluid (n=1), peritoneal fluid (n=3) and blood (n=1) cultures of the patients who were hospitalized at the Erciyes University Hospital, Faculty of Medicine, in Kayseri. The ranges of minimum inhibitory concentrations (MICs), geometric mean MICs, MIC_{50} and MIC_{90} values (expressed in μg/ml) of the 21 *K. marxianus* and six *C. lusitaniae* isolates were detailed in Table 1. For *K. marxianus*, amphotericin B had the highest geometric mean MIC (1 μg/mL) and voriconazole had the lowest (0.010 μg/mL). For *C. lusitaniae*, flucytosine had the highest geometric mean MIC (8 μg/mL) and voriconazole had the lowest (0.011 μg/mL).

Table 1: The ranges of minimum inhibitory concentrations (MICs), geometric mean MICs and MIC_{50} and MIC_{90} values

Antifungal agent		Incubation time (24 hour)				Incubation time (48 hour)			
	Candida species	MIC range	GM	MIC_{50}	MIC_{90}	MIC range	GM	MIC_{50}	MIC_{90}
Amphotericin B									
(n: 21)	*C. kefyr*	0.5-2	1	1	1	1-2	1.935	2	2
(n: 6)	*C. lusitaniae*	0.12-0.5	0.246	0.25	0.5	0.25-1	0.629	0.5	1
Fluconazole									
(n: 21)	*C. kefyr*	0.12-32	0.238	0.25	0.25	0.12-64	0.423	0.25	0.5
(n: 6)	*C. lusitaniae*	0.12-4	0.442	0.25	0.5	0.12-4	0.702	0.5	1
Voriconazole									
(n: 21)	*C. kefyr*	0.008-0.5	0.010	0.008	0.008	0.008-2	0.014	0.008	0.015
(n: 6)	*C. lusitaniae*	0.008-0.06	0.011	0.008	0.008	0.008-0.06	0.015	0.015	0.015
Posaconazole									
(n: 21)	*C. kefyr*	0.015-2	0.039	0.03	0.06	0.03-2	0.064	0.06	0.06
(n: 6)	*C. lusitaniae*	0.008-0.25	0.021	0.015	0.015	0.015-0.25	0.042	0.03	0.06
Itraconazole									
(n: 21)	*C. kefyr*	0.03-1	0.062	0.06	0.06	0.03-4	0.070	0.06	0.06
(n: 6)	*C. lusitaniae*	0.03-0.25	0.060	0.06	0.06	0.012-0.25	0.135	0.12	0.12
Caspofungin									
(n: 21)	*C. kefyr*	0.03-0.06	0.046	0.06	0.06	0.03-0.12	0.052	0.06	0.06
(n: 6)	*C. lusitaniae*	0.015-0.12	0.06	0.06	0.12	0.03-0.5	0.246	0.5	0.5
Anidulafungin									
(n: 21)	*C. kefyr*	0.03-0.25	0.116	0.12	0.12	0.03-0.25	0.133	0.12	0.25
(n: 6)	*C. lusitaniae*	0.12-0.12	0.12	0.12	0.12	0.12-0.5	0.172	0.12	0.25
Micafungin									
(n: 21)	*C. kefyr*	0.06-0.12	0.062	0.06	0.06	0.06-0.25	0.105	0.12	0.12
(n: 6)	*C. lusitaniae*	0.03-0.25	0.067	0.06	0.06	0.12-0.25	0.153	0.12	0.25
Flucytosine									
(n: 21)	*C. kefyr*	0.06-4	0.180	0.12	2	0.06-4	0.287	0.12	4
(n: 6)	*C. lusitaniae*	0.5-64	8	16	64	1-64	17.95	64	64

Discussion

C. albicans remains the most common pathogen responsible for invasive candidiasis. On the other hand, increased rates of candidiasis are reported worldwide, caused by other *Candida species* (such as *K. marxianus* and *C. lusitaniae*) [8, 9]. *K. marxianus* is an increasingly important yeast. Studies report that a significant proportion of patients with hematologic malignancies, especially acute myelogenous leukemia patients,

are colonized with *C. kefyr* and there is a significant risk for subsequent bloodstream infection [5]. *C. lusitaniae* (anamorph: *Candida lusitaniae*) is an opportunistic yeast that can be isolated from plants, animals, industrial wastes and humans [10]. In the recent years, it has become increasingly recognized as an emerging nosocomial pathogen with a high mortality rate [11].

So far, it has been noted that amphotericin B has good sensitivity to most non-albicans *Candida* species, but there may be country-specific differences [2,12]. In a surveillance study on fungemia in adults in Germany, it was shown that the MIC of AMB was increased in 9% of all *K. marxianus* isolates [13], On the other hand, a study conducted in Spain reported that all *K. marxianus* strains were susceptible to AMB in-vitro (geometric mean [GM], 0.21 mg / L and MIC range, 0.03-1 mg / L) [8]. A study conducted in our country stated that amphotericin B had had MICs ≤1 µg / mL for all *Candida* species isolated from bloodstream infections except *K. marxianus* [14]. Due to the high MICs of amphotericin B for some *K. marxianus* strains, it can be predicted that the use of empirical therapeutics alongside antifungal prophylaxis may induce the selection of *K. marxianus* in the gastrointestinal flora, particularly in hemato-oncological patients [15]. Similar to the study of Gomez-Lopez A et al. [10], all azole compounds showed a great deal of activity against *K. marxianus* isolates with GM values of 0.238, 0.062, 0.015, 0.010 and 0.039 for FLC, ITC, VRC and POS, respectively, in our study. All *K. marxianus* strains revealed a similar susceptibility pattern against echinocandins, MIC_{50}, MIC_{90}, and the range of the measured MICs were ≤0.25 mg/L for caspofungin, anidulafungin and micofungin.

Due to the possibility that some *C. lusitaniae* species will not respond to chemotherapy, common candidiasis caused by this strain can have serious consequences [12]. *C. lusitaniae* infections respond poorly to amphotericin B despite low MIC values in in-vitro tests [16]. There are no CLSI/EUCAST antifungal susceptibility breakpoints for *C. lusitaniae*. However, several studies have established epidemiological breakpoints for *C. lusitaniae* to distinguish between wild type and non-wild type strains [17]. Investigating the caspofungin susceptibility of 105 *C.lusitaniae* isolates obtained from 91 institutions under the global surveillance program between 2001 and 2004, Pfaller et al. [18] reported that all isolates were inhibited at a concentration of 4 µg/mL. Caspofungin, anidulafungin, or micafungin did not have MIC values >0.25 µg/mL for any our isolates. In a case report, Desnos-Ollivier M et al. [9] reported that increased echinocandin MICs appeared in *C. lusitaniae* isolates 2 weeks after the initiation of caspofungin treatment and these isolates exhibited missense mutation S645F in the HS1 region. In our study, the MIC range for flucytosine was between 05-64 µg/mL and flucytosine had the highest geometric mean MIC. In their antifungal susceptibility study with 80 *C. lusitaniae* strains isolated from various clinical specimens, Favela et al. [19] reported that the MIC range for flucytosine was between 0.004-> 32 mg/L. These extremely high MIC values for flucytosine are important because flucytosine is often used in combination with amphotericin B or azoles to treat patients infected with this species.

Limitations

The small number of clinical samples and the antifungal susceptibility testing with a commercial colorimetric microdilution method, which is not the gold standard, are the limitations of the study.

Conclusions

Considering that these two species, rare causes of invasive candidiasis nowadays, may emerge as important pathogens in the future, it is reasonable to investigate the in-vitro activities of antifungal agents that can be used in their treatment. Further studies are required by testing large panels of geographically diverse clinical isolates.

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