Polish Journal of Microbiology
2020, Vol. 69, No 1, 73–84
https://doi.org/10.33073/pjm-2020-010

ORIGINAL PAPER

Resensitization of Fluconazole-Resistant Urinary *Candida* spp. Isolates by Amikacin through Downregulation of Efflux Pump Genes

EVA A. EDWARD*, NELLY M. MOHAMED and AZZA S. ZAKARIA

Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Egypt

Submitted 12 November 2019, revised 11 January 2020, accepted 6 February 2020

Abstract

The contribution of fluconazole-resistant *Candida* spp. isolates to urinary tract infections in Egypt has become a nationwide problem. A recent approach to overcome such disaster is combining conventional antifungals with non-antifungals. This study investigated the interaction of amikacin with fluconazole against resistant *Candida* strains isolated from the urine culture of patients admitted to Alexandria Main University Hospital. Among the collected *Candida* spp. isolates, 42.9% were resistant to fluconazole with MICs ranging between 128 and 1,024 μg/ml. The resistance-modifying activity of amikacin (4,000 μg/ml) was studied against fluconazole-resistant isolates where amikacin sensitized 91.7 % of resistant *Candida* spp. isolates to fluconazole with a modulation factor ranging between 32 and 256. The rhodamine efflux assay was performed to examine the impact of amikacin on efflux pump activity. After 120 minutes of treatment, amikacin affected the efflux pump activity of the isolates tested with a percentage of reduction in the fluorescence intensity of 8.9%. Quantitative real-time PCR was applied to assess the amikacin effect on the expression of the efflux pump genes *MDR1*, *CDR1*, and *CDR2*. The downregulatory effect of amikacin on the expression of the studied genes caused a percentage of reduction in the expression level ranging between 42.1 and 94%. In conclusion, amikacin resensitized resistant *Candida* spp. isolates to fluconazole and could be used in combination in the management of candiduria with a higher efficiency or at lower administration doses. To the best of our knowledge, this is the first study evaluating the enhancement of fluconazole activity in combination with amikacin against *Candida* spp.

Key words: *Candida* spp., amikacin, fluconazole, rhodamine efflux assay, quantitative real-time PCR

Introduction

The urinary tract infections (UTIs) caused by various *Candida* spp. have been recognized by clinicians to be a growingly extensive and pervasive nosocomial issue (Bukhary 2008). It has been estimated that *Candida* spp. are in charge of about 10–15% of nosocomial UTIs (Alkilani et al. 2017). The presence of *Candida* in urine, a medical condition known as candiduria (Alkilani et al. 2017), if not correctly treated, might result in considerable morbidity and mortality rates (Goyal et al. 2016). Observational studies have particularized *Candida albicans* to be the most prevalent etiologic agent detected in more than 51% of candiduria cases, followed by *Candida glabrata* as well as *Candida tropicalis* (Bukhary 2008). Many risk factors predispose to the occurrence of candiduria, for instance, immunosuppressive therapy, extremes of age, underlying genitourinary abnormality, female sex, prior surgeries, indwelling urinary catheters, diabetes mellitus, recent use of broad-spectrum antibiotics, as well as the prolonged hospital stay (Bukhary 2008; Alhussaini et al. 2013; Hassaneen et al. 2014).

In Egypt, the contribution of *Candida* spp. to UTIs is alarmingly increasing reflecting a problem that might be described as nationwide. In Alexandria, located on the Mediterranean coast in North Egypt, a study measuring the incidence rate of catheter-associated urinary tract infections (CAUTIs) in four intensive care units (ICUs) in Alexandria University hospitals from 2007 to 2008 encountered *Candida* spp. in 51% of the examined urine cultures (Talaat et al. 2010). In the capital and southern governorates, 20% of renal failure patients visiting the University hospitals of Cairo, Assiut and Sohag in 2012 were suffering from candiduria (Alhussaini et al. 2013). In the eastern part of the Nile delta, a study conducted on 300 hospitalized patients in Zagazig University hospitals, from 2012 to 2013, revealed a candiduria prevalence of 14% in these patients (Hassaneen et al. 2014). The situation in Menofia, another Delta

* Corresponding author: E.A. Edward, Department of Microbiology and Immunology, Faculty of Pharmacy, El-Khartoum Square, Azarita, Alexandria, Egypt; **e-mails: eve.farid@alexu.edu.eg, dr.eva_adel@hotmail.com**

governorate, was almost similar where candiduria infection was reported among 19% of catheterized ICU patients in its University hospitals, from 2013 to 2014 (Alkilani et al. 2017). As the resistance of different *Candida* spp. towards different antifungal agents is proceeding to develop and expand, new treatment trends are extensively necessitated (Spampinato and Leonardi 2013). Unfortunately, developing novel antifungal agents is an extremely complicated and difficult task. Thus, an alternative approach nowadays focuses on the combination of conventional antifungal agents with non-antifungals (Lu et al. 2018).

Aminoglycosides are natural or semisynthetic antimicrobials and are one of the first agents to be used for routine clinical practice (Krause et al. 2016). They are bactericidal agents exerting their effect during the translation process through the interference with the correct decoding of the mRNA (Chiem et al. 2016). They are active against Gram-negative bacteria like *Pseudomonas* spp., members of the *Enterobacteriaceae* family, and could be combined with beta-lactams to treat Gram-positives infections (Chiem et al. 2016). Amikacin is the most widely used semisynthetic aminoglycoside that can be used alone or with other antibiotics to treat infections caused by aerobic Gram-negative bacteria, *Nocardia* species, and *Mycobacterium tuberculosis*, and has a particular significance in the treatment of several life-threatening infections disseminated among neonates (Ramirez and Tolmasky 2017).

Over the past decades, substantial attempts have been made to explore novel antimicrobial activities of aminoglycosides, for example, their antifungal effect (Lu et al. 2018). However, until now, the data concerning the enhancement of conventional antifungal agents' activity in combination with amikacin against *Candida* spp. has not been reported.

The current study aimed at the investigation of the activity of amikacin against *Candida* spp. isolates causing UTIs in the patients admitted to the Alexandria Main University Hospital (AMUH) in Egypt. It also focused on elucidating the role of amikacin as a resistance-modifying agent that might help in increasing the susceptibility of resistant strains to one of the most frequently used antifungal agents, fluconazole. Moreover, the underlying mechanisms of amikacin sensitization of *Candida* isolates to fluconazole were studied.

Experimental

Materials and Methods

Clinical isolates and culture conditions. Twenty-eight non-replicated clinical isolates of *Candida* spp. were collected from the urine culture of patients submitted to "AMUH" through the hospital's routine laboratory facility during October 2018. These isolates were numbered from C1 to C28. In addition, the standard strain of *C. albicans* ATCC 231GI was included in this study.

All isolates were preserved as frozen stocks in 15% glycerol at –20°C. A fresh culture was obtained by subculturing the isolates on Sabouraud dextrose agar (SDA) (LAB M, UK) for 48 h at 37°C before use.

Antimicrobial agents. Amikacin (Advomikacin® 500 mg/2 ml, Advocure, Egypt) was purchased from community pharmacies, while fluconazole was obtained from Sigma Aldrich, USA. Stock solutions of both amikacin and fluconazole were prepared in sterile distilled water.

Identification of *Candida* spp. isolates to the species level. The Tween 80 opacity test. For the preparation of the Tween 80 opacity test medium, 10 g of bacteriological peptone (LAB M, UK), 0.1 g of $CaCl_2$, 5 g of NaCl, and 15 g of agar were added to 1,000 ml of distilled water. After autoclaving, the medium was allowed to cool to approximately 50°C; then, 5 ml of previously autoclaved Tween 80 (Guangzhou Jinhuada Chemical Reagent Co., China) was added. Few colonies of an overnight culture of each of the tested isolates grown on SDA were subcultured to the Tween 80 opacity test medium, using sterile cotton swabs, to form an inoculation site of about 10 mm in diameter. The plates were incubated for up to 10 days at 37°C. The isolates tested were inoculated in duplicate. The detection of a halo around the site of the inoculation was recognized as a positive result that indicated the capability of *Candida* isolates to produce an esterase (Aktas et al. 2002).

Germ tube formation test. Two or three colonies of each of the isolates tested were inoculated into 0.5 ml of sterile trypticase soy broth (HIMEDIA, India), dispensed in a sterile Eppendorf, and then, incubated at 37°C for 2–3 hours. After incubation, few drops of the suspension were transferred to a clean microscopic slide for examination under a magnification of 100× for the detection of germ tube (Deorukhkar et al. 2012). A positive control (*C. albicans* ATCC 231GI) was included in the experiment.

Identification of non-albicans *Candida* isolates using the Vitek-2 system. The Vitek-2 system (Kaur et al. 2016) (Vitek® 2 Compact, BioMerieux, France) was used to confirm the identity of the non-albicans *Candida* isolates according to the manufacturer's instructions.

Determination of virulence determinants among *Candida* spp. isolates. Biofilm formation. Biofilm formation was assayed according to Pongracz et al. (2016) with some modifications. *Candida* spp. isolates were grown on SDA plates at 37°C for 24 h. A suspension of each isolate at a density of 2×10^6 cells/ml

was prepared in sterile saline. Then, 100 µl of the cell suspension was placed in each well containing 100 µl of sterile double strength RPMI 1640 medium (Sigma Aldrich, USA) buffered with morpholine propane sulfonic acid (MOPS) (Sigma Aldrich, USA). After 48 h incubation at 37°C, the planktonic cells were discarded and the wells were properly washed twice with saline. Biofilms were stained with 100 µl of crystal violet dye (0.2%) for about 15 min. The excess dye was washed and 100 µl of ethanol was added to each well to solubilize the bound dye (Pongracz et al. 2016). The absorbance was measured at 630 nm. Each isolate was tested in triplicate. The optical density of each strain (ODs) was calculated as the mean value of the absorbance of 3 wells and this value was then compared with the absorbance of negative control (ODnc) (containing 100 µl of saline instead of bacterial inoculum). The results were interpreted as follows: strong biofilm formation (4 ODnc < ODs), moderate biofilm formation (2 ODnc < ODs ≤ 4 ODnc), weak biofilm formation (ODnc < ODs ≤ 2 ODnc) and no biofilm formation (ODs ≤ ODnc) (Rodrigues et al. 2010).

Production of proteinase enzymes. The production of proteinase enzymes was detected using bovine serum albumin medium (2% dextrose, 0.05% $MgSO_4$, 0.1% KH_2PO_4 and agar 2% mixed after autoclaving and cooling to 50°C with bovine serum albumin (MP Biomedicals, USA) at a concentration of 1%). Aliquots of 20 µl of each of the *Candida* isolate's suspension (at a density of 10^8 cells/ml) were inoculated into previously punched cups in the serum medium. The plates were incubated for six days at 37°C. The precipitation zone (Pz) value was calculated as the ratio of the diameter of the cup to the total diameter of the cup plus the precipitation zone. The results expressing the enzymatic activity were interpreted as follows: Pz value = 1 (negative); Pz value = 0.75 – 0.9 (low producers); Pz value = 0.51 – 0.74 (moderate producers); and Pz value = 0.35 – 0.5 (high producers). The test was performed in duplicate and the average of two measurements was recorded (Mohan Das and Ballal 2008; Mattei et al. 2013).

Production of the phospholipase enzyme. Phospholipase activity was tested using egg yolk agar medium consisting of SDA, 0.005 mol $CaCl_2$, 1 mol NaCl and 8% sterile egg yolk emulsion (HIMEDIA, India). The egg yolk emulsion was centrifuged; the supernatant was completed to its initial volume with sterile distilled water and added to the sterilized medium (Fotedar and Al-Hedaithy 2005). Aliquots of 20 µl of each of the *Candida* isolate's suspensions (at a density of 10^6 cells/ml) were inoculated into previously punched wells in the solid medium. The plates were incubated for 48 h at 37°C. The results expressing the enzyme activity were interpreted as follows: Pz = 1 (no enzyme activity), 0.63 < Pz < 1.0 (moderate enzyme activity), and Pz ≤ 0.63 (strong enzymatic activity) (Wiebusch et al. 2017).

Determination of the minimum inhibitory concentrations (MICs) of fluconazole and amikacin against *Candida* spp. isolates. The MICs of both amikacin and fluconazole against all the isolates tested were determined by the broth microdilution method using RPMI buffered with MOPS. For the preparation of a suitable inoculum, each isolate was subcultured on SDA plates and incubated at 37°C, and then, adjusted to 0.5 McFarland standard. A working suspension was prepared by a 1:100 dilution of the stock *Candida* isolate's suspension (Schwalbe et al. 2007). The growth control and sterilized medium control wells were included in each experiment. MICs of fluconazole was defined as the concentration resulting in 50% growth inhibition, while MICs of amikacin was defined as the concentration resulting in 100% growth inhibition. Isolates with fluconazole MICs of ≥ 64 µg/ml were interpreted as resistant (CLSI 2008).

The antibiotic resistance-modifying activity of amikacin against fluconazole-resistant *Candida* clinical isolates. To assess the role of amikacin as a resistance-modifying agent against 12 fluconazole-resistant *Candida* clinical isolates, the MIC of fluconazole alone was compared to its MIC in the presence of 4,000 µg/ml of amikacin to test the capability of amikacin to sensitize the *Candida* isolates resistant to fluconazole. Modulation factor (MF), calculated as $MIC_{\text{fluconazole alone}}/MIC_{\text{fluconazole + amikacin}}$, was used to express the modulating effect of amikacin (Fankam et al. 2015).

Rhodamine efflux assay. A rhodamine efflux assay was performed according to Lu et al. (2018) with minor modifications. This assay was conducted to determine whether amikacin affected the efflux pump activity in three fluconazole-resistant *Candida* spp. isolates: C6, C8, and C21. Briefly, each of the selected isolates was subcultured on SDA plates. A loopful from each isolate culture was subcultured in yeast extract-peptone-dextrose (YPD) broth and incubated overnight at 35°C in a shaking incubator at 200 rpm. The overnight culture was collected into a sterile falcon, washed with glucose-free PBS and the concentration was adjusted to 1×10^7 CFU/ml. Next, the ethanolic rhodamine solution (Rhodamine B, Loba Chemie, India) was added to the cell suspension to reach a final concentration of 10 µM. The culture was incubated with rhodamine at 37°C for 50 min, followed by incubation on an ice water-bath for 10 min. Cells were collected, washed properly with glucose-free PBS and resuspended in glucose/PBS (5%). Amikacin was added to a final concentration of 4,000 µg/ml and, at the same time, the rhodamine-alone group (containing no amikacin) served as a control group. The fluorescence intensity of the extracellular rhodamine was recorded every 30 min, at time intervals

of 0, 30, 60, 90 and 120 min, using a spectrofluorometer (Shimadzu, Japan) with excitation at 485 nm and emission at 530 nm. All results were represented as an average of three biological samples.

Molecular quantification of the *Candida* efflux pump genes *MDR1*, *CDR1*, and *CDR2* using quantitative real-time PCR. Quantitative real-time PCR was applied for two isolates C6 and C21 whose efflux pump activity was more prominently affected by amikacin using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific Inc., USA). This was employed to assess the localized expression of the *MDR1* (multidrug resistance 1), *CDR1* (*Candida* drug-resistant 1), and *CDR2* (*Candida* drug-resistant 2) genes before and after 48-hour treatment with 4,000 µg/ml of amikacin. The data were normalized against the housekeeping gene *ACT1*. Gene-specific primer pairs were synthesized in Willowfort, UK, relying on the previously published sequences (Chau et al. 2004). The primers of the *ACT1, MDR1*, *CDR1*, and *CDR2* genes for the RT-PCR amplification of cDNA are shown in Table I.

RNA isolation and reverse transcription. For both selected isolates, the total RNA was extracted, according to the manufacturer's instructions, from the overnight subculture using the TRIzol® MaxTM Bacterial RNA Isolation Kit (Ambion by Life Technologies). Then, the quantification of RNA was carried out using Jenway Genova Nano, Keison Products, UK.

Using the TOPreal™ One step RT qPCR Kit (Enzynomics), the step of reverse transcription was accomplished. The composition of the real-time PCR mixture was as follows: 1 µl of TOPreal™ One step RT qPCR Enzyme Mix, 10 µl of 2X TOPreal™ One step RT qPCR Reaction Mix, 1 µl of each primer, 1 µl of total RNA, and sterile DNase-free water to reach to a total reaction volume of 20 µl.

After applying a preliminary holding step at 50°C for 30 min, the cycling conditions for the PCR reaction were as follows: an initial denaturation step at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 5 sec, followed by annealing/extension at 60°C for 30 sec. In every RT-PCR run, negative control containing sterile DNase-free water instead of the RNA template was involved. Samples were tested in triplicate.

To ensure the absence of the primer-dimers or any other artifacts, analysis of the melting curves was done in one cycle of 94°C, 53°C and 94°C, one minute each. The amplification curves, as well as the values of the cycle threshold (Ct) were established using the Stratagene MX3005P software.

The levels of the expression of each of the *MDR1*, *CDR1* and *CDR2* genes were normalized to the expression level of the house keeping gene *ACT1* and compared to the corresponding expression levels in the control untreated isolates. For the calculation of the transcripts of each of the target genes, the Pfaffl method or ΔΔCt method was applied (Pfaffl 2001).

Statistical analysis. Data were expressed as means ± S.D. In the case of multi-variable comparisons, one-way ANOVA and Bonferroni testing were performed with the Prism 3 GraphPad software. Differences were recognized to be significant at p-value < 0.05. The included data were the mean of three biological replicates.

Results

Demographic profile of candiduric patients. The demographic characteristics of 28 candiduric patients included in this study are shown in Table II. Infection with *C. albicans* isolates was more prevalent in males (61.1%) when compared to females (38.9%). However, the incidence of non-albicans *Candida* isolates was equally detected in both sexes. The frequency of both *C. albicans* and non-albicans isolates was higher among elderly patients (61 – > 70 years) in comparison with younger adults, and showed a focused predominance in patients reaching extremes of age (61.1, and 50%, respectively).

Identification of *Candida* spp. isolates. The applied phenotypic tests (the germ tube formation and the Tween 80 opacity test), as well as the Vitek-2 system, segregated the *Candida* isolates tested into: 18 *C. albicans*, 7 *C. glabrata*, 2 *C. tropicalis*, and 1 *C. famata* (Table III; Fig. S1).

Table I
Sequences of primers for the genes selected for transcript analysis using quantitative RT-PCR.

Gene	Orientation	Sequence (5'–3')	Reference
ACT1	F	TTGGTGATGAAGCCCAATCC	Chau et al. 2004
	R	CATATCGTCCCAGTTGGAAACA	
MDR1	F	TTACCTGAAACTTTTGGCAAAACA	Chau et al. 2004
	R	ACTTGTGATTCTGTCGTTACCG	
CDR1	F	TTTAGCCAGAACTTTCACTCATGATT	Chau et al. 2004
	R	TATTTATTTCTTCATGTTCATATGGATTGA	
CDR2	F	GGTATTGGCTGGTCCTAATGTGA	Chau et al. 2004
	R	GCTTGAATCAAATAAGTGAATGGATTAC	

Table II
Demographic profile of candiduric patients.

	Demographic variables											
	Sex				Age group (years)							
	Male		Female		30–40		41–50		51–60		61–>70	
	n	%	n	%	n	%	n	%	n	%	n	%
Candida albicans (n = 18)	11	61.1	7	38.9	0	0	6	33.3	1	5.6	11	61.1
Non-albicans *Candida* isolates (n = 10)	5	50	5	50	1	10	3	30	1	10	5	50
Total (n = 28)	16	57.1	12	42.9	1	3.6	9	32.1	2	7.1	16	57.1

Table III
Identification, virulence determinants, and the minimum inhibitory concentration (MIC) of fluconazole against the *Candida* spp. isolates tested.

Isolate code	Candida species	Virulence determinants			MIC of Fluconazole (μg/ml)[c]
		Biofilm formation	Proteases production Pz value[a]	Phospholipase production Pz value[b]	
C1	*C. tropicalis*	Strong	0.26	0.33	128
C2	*C. albicans*	Strong	0.4	0.48	16
C3	*C. albicans*	Strong	0.16	0.29	8
C4	*C. albicans*	Strong	0.19	0.29	32
C5	*C. albicans*	Strong	0.2	1	8
C6	*C. albicans*	Strong	0.2	0.3	1024
C7	*C. tropicalis*	Strong	0.22	0.23	128
C8	*C. glabrata*	Weak	0.18	0.21	1024
C9	*C. glabrata*	Weak	0.67	0.22	1024
C10	*C. glabrata*	Moderate	0.5	0.2	32
C11	*C. albicans*	Strong	0.26	0.26	16
C12	*C. glabrata*	Moderate	0.43	0.23	32
C13	*C. albicans*	Strong	0.4	0.2	8
C14	*C. albicans*	Strong	0.26	0.3	256
C15	*C. albicans*	Strong	0.24	0.24	16
C16	*C. albicans*	Strong	0.24	0.24	8
C17	*C. albicans*	Strong	0.15	0.27	1024
C18	*C. glabrata*	Moderate	0.25	0.21	8
C19	*C. albicans*	Weak	0.15	0.67	2
C20	*C. albicans*	Strong	0.14	0.31	1024
C21	*C. albicans*	Strong	0.17	0.4	1024
C22	*C. albicans*	Weak	0.15	0.39	1
C23	*C. albicans*	Strong	0.18	0.32	1024
C24	*C. albicans*	Strong	0.17	0.41	8
C25	*C. glabrata*	Weak	0.17	0.23	8
C26	*C. glabrata*	Moderate	0.2	0.16	256
C27	*C. albicans*	Strong	0.16	0.32	16
C28	*C. famata*	Strong	0.16	0.4	128

[a] The Pz (precipitation zone) value: 1 (negative), 0.75–0.9 (low producers), 0.51–0.74 (moderate producers) and 0.35–0.5 (high producers)
[b] The Pz value: 1 (negative), < 1 – > 0.63 (moderate), and < 0.63 (strong)
[c] CLSI breakpoint for fluconazole is 64 μg/ml

Table IV
Fluconazole susceptibility of different *Candida* spp.

Candida species	Fluconazole susceptibility n (%)		MIC_{50}	MIC_{90}	MIC range (μg/ml)
	S	R			
Candida albicans (n = 18)	12 (66.7%)	6 (33.3%)	16	1024	1–1024
Non-albicans *Candida* isolates (n = 10)	4 (40%)	6 (60%)	128	1024	8–1024
Candida glabrata (n = 7)	4 (57.1%)[a]	3 (42.9%)	32	1024	8–1024
Candida tropicalis (n = 2)	0 (0%)[a]	2 (100%)	128[b]	ND[c]	ND
Candida famata (n = 1)	0 (0%)[a]	1 (100%)	ND	ND	ND

[a] The percentage was calculated relative to the total number of non-albicans *Candida* isolates collected in this study
[b] MIC_{50} is calculated here as the arithmetic mean of the MIC values for both *Candida tropicalis* strains
[c] ND – not determined

Determination of the MIC of fluconazole against *Candida* spp. isolates. Among *Candida* isolates, 12 isolates (42.9%) were resistant to fluconazole with MICs ranging from 128 to 1,024 μg/ml. The percentage of the resistant non-albicans *Candida* spp. isolates reached about 60% when compared to fluconazole-resistant *C.* albicans (33%). About 43% of *C. glabrata* isolates were resistant to fluconazole. Both isolated strains of *C. tropicalis* were resistant to fluconazole, as well as a single *C. famata* isolated strain in this study (Tables III and IV).

Relationship between the virulence determinants in *Candida* spp. isolates and their susceptibility to fluconazole. Out of the 28 tested isolates, 19 isolates (67.9%) were strong biofilm formers. Among these, three isolates (15.8%) were non-albicans *Candida* isolates (2 *C. tropicalis*, and 1 *C. famata*) that showed resistance to fluconazole (MIC = 128 μg/ml). The remaining 16 strong biofilm formers (84.2%) were *C. albicans*, out of which six isolates were resistant to fluconazole (MICs ranging from 256 to 1,024 μg/ml). The moderate biofilm formation was detected among four *C. glabrata* isolates, where only one of these was resistant to fluconazole (MIC = 256 μg/ml). Five isolates (17.9% of the total number of the isolates tested) were defined as weak biofilm formers, and this group comprised two fluconazole-susceptible *C. albicans*, one fluconazole-susceptible *C. glabrata*, and two fluconazole-resistant *C. glabrata* isolates (MIC = 1,024 μg/ml) (Table III).

Production of proteinase enzymes was high among 27 isolates (96.4% of the tested isolates), among which 11 isolates (40.7%) were resistant to fluconazole. These high producers of proteinases were classified as 18 *C. albicans* and nine non-albicans *Candida* isolates. Only one *C. glabrata* isolate showed moderate production of proteinases, however, it was resistant to fluconazole (MIC = 1,024 μg/ml) (Table III).

A total of 26/28 isolates (92.9%) were strong producers of phospholipase, out of which 12 isolates (46.2%) were resistant to fluconazole. One isolate was a moderate producer, while the second showed a negative result for phospholipase production. Both isolates were fluconazole-susceptible *C. albicans* (Table III; Fig. S2).

Determination of the MIC of amikacin against *Candida* spp. Isolates. The MIC of amikacin determined by the broth microtiter dilution method was > 4,096 against 27 isolates (96.4 % of the tested isolates), while isolate C28 had a MIC of 2,048 μg/ml.

The antibiotic-resistance modifying activity of amikacin against fluconazole-resistant *Candida* clinical isolates. The antibiotic-resistance modifying activity of amikacin (4,000 μg/ml) was tested against 12 fluconazole-resistant *Candida* clinical isolates. As illustrated in Table V, amikacin potentially sensitized the antimicrobial activity of fluconazole against 11 tested isolates with an MF ranging between 32 and 256. The MF calculated for the majority of fluconazole-resistant *C. albicans* isolates (with exception of one strain showing an MF of 64) reached very high values equivalent to 256. The MIC of fluconazole was reduced by 32-fold in the presence of 4,000 μg/ml of amikacin when tested against fluconazole-resistant isolates of *C. tropicalis*, *C. glabrata*, and *C. famata*. A single isolate of *C. glabrata*, C26, was not sensitized to fluconazole in the presence of amikacin.

Rhodamine efflux assay. To illustrate the mechanism of amikacin sensitization to fluconazole, the efflux pump activity of two *C. albicans* isolates C6 and C21, as well as one *C. glabrata*, C8, was studied in the absence and presence of amikacin (4,000 μg/ml) using the rhodamine efflux assay (Fig. 1–3). Both *C. albicans* isolates, C6 and C21, showed a decline in the fluorescence intensity with percentages of reduction varying over time. The fluorescence intensity dropped promptly during the first 30 and 60 minutes of contact of isolate C6 with amikacin with percentages of reduction in the fluorescence intensity reaching 13.4 and 12.9%, respectively. After 90 and 120 minutes of treatment, those percentages began to decrease although still being considerable (Fig. 1). The pattern of fluorescence intensity reduction for isolate C21 displayed dissimilarity over time, where the effect of amikacin on the efflux pump activity for

Table V
Resistance-modulating effect of amikacin (4000 µg/ml) on fluconazole-resistant *Candida* clinical isolates.

Isolate code	Candida species	MIC of fluconazole alone (µg/ml)	MIC of fluconazole (µg/ml) in the presence of amikacin	Modulation factor (MF)[a]
C1	*C. tropicalis*	128	4	32
C6	*C. albicans*	1024	4	256
C7	*C. tropicalis*	128	4	32
C8	*C. glabrata*	1024	32	32
C9	*C. glabrata*	1024	32	32
C14	*C. albicans*	256	4	64
C17	*C. albicans*	1024	4	256
C20	*C. albicans*	1024	4	256
C21	*C. albicans*	1024	4	256
C23	*C. albicans*	1024	4	256
C26	*C. glabrata*	256	256	1
C28	*C. famata*	128	4	32

[a] Modulation factor (MF) was calculated as $MIC_{\text{fluconazole alone}}/MIC_{\text{fluconazole + amikacin}}$.

the first 30 minutes was weak resulting in a percentage of reduction of merely 3.7%. However, a time-gradient remarkable effect was established and percentages of reduction in the fluorescence intensity reached 7.3, 8.5, and 8.9% at 60, 90, and 120 minutes, respectively (Fig. 3). The isolate of *C. glabrata*, C8, didn't show any prominent decrease in the fluorescence intensity for the amikacin-treated cells when compared to the control where the percentages of reduction in the fluorescence intensity over the tested time intervals ranged from 0.4 to 1.5% (Fig. 3).

Comparing the effect of amikacin (4,000 µg/ml) on the efflux of rhodamine among the three tested isolates C6, C8, and C21 after 120 minutes of treatment, a statistically significant effect (p-value < 0.05) was observed for C6 and C21 with percentage of reduction in the fluorescence intensity of 8.9% for both isolates. However, the effect of amikacin treatment was statistically non-significant (p-value > 0.05) for isolate C8 where the percentage of reduction in the fluorescence intensity reached hardly 1.5% after 120 minutes of treatment (Fig. 4).

Molecular quantification of the *Candida* efflux pump genes *MDR1*, *CDR1*, and *CDR2* using real-time PCR. For two *C. albicans* isolates, C6 and C21, the effects of 48-hour treatment with 4,000 µg/ml amikacin on the expression of the *Candida* efflux pump genes *MDR1*, *CDR1* and *CDR2* was studied using the quantitative RT-PCR. Representative amplification curves and melting curve analysis for each of the housekeeping gene *ACT1* and the three efflux pump genes *MDR1*,

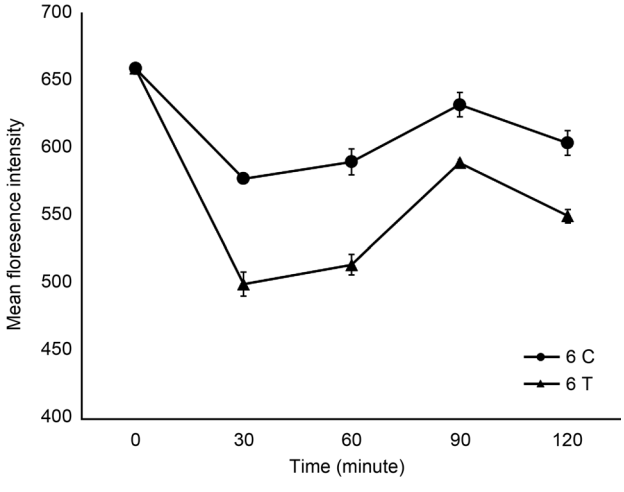

Fig. 1. Inhibitory effect of amikacin (4,000 µg/ml) on the efflux of rhodamine in fluconazole-resistant *Candida albicans* (C6).

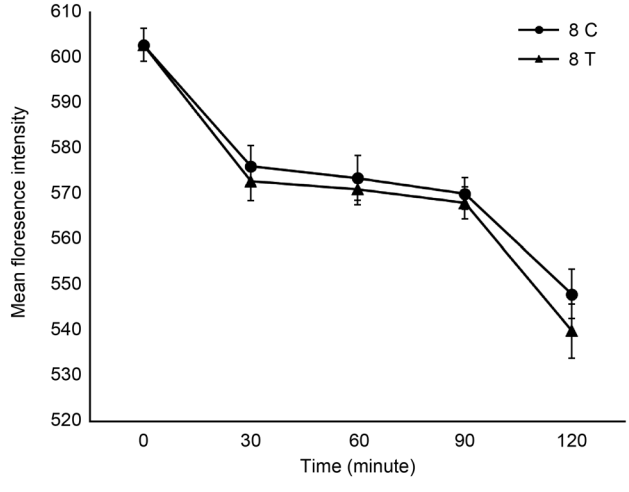

Fig. 2. Inhibitory effect of amikacin (4,000 µg/ml) on the efflux of rhodamine in fluconazole-resistant *Candida glabrata* (C8).

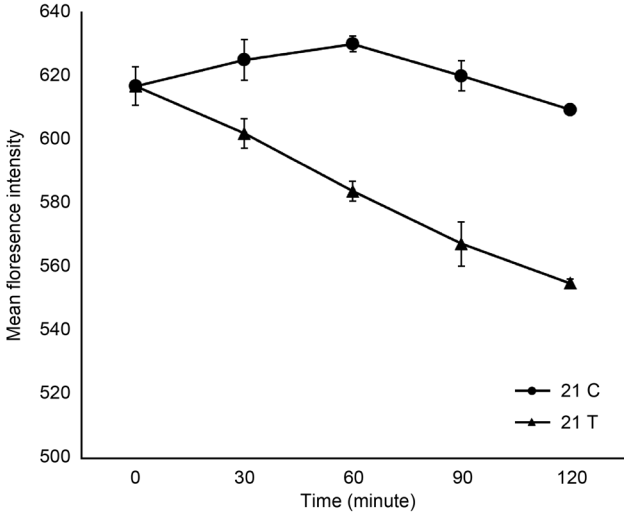

Fig. 3. Inhibitory effect of amikacin (4,000 µg/ml) on the efflux of rhodamine in fluconazole-resistant *Candida albicans* (C21).

CDR1, and *CDR2* for C6 isolate are provided in the supplementary material (Fig. S3A and S3B).

The downregulatory effect of amikacin on the expression of the efflux pump genes of isolate C6 was exerted most prominently on the gene *CDR1*, followed by the gene *MDR1*, and lastly on the gene *CDR2* with percentages of reduction in the level of expression equivalent to 89.4, 61, and 42.1%, respectively. Amikacin treatment of isolate C21 resulted in a profound downregulatory effect of the gene *CDR2* with a percentage of reduction in the level of expression equivalent to 94%. This was observed to a lower extent for the gene *MDR1*, the expression of which was reduced by 43.5%. Concerning the gene *CDR1*, it has not been originally detected in the control untreated isolate C21. A highly significant reduction in the expression levels of the target genes, with a p-value of <0.0001 compared to the control, was detected for both tested isolates C6 and C21. The relative gene expression levels of the efflux pump genes *MDR1*, *CDR1*, and *CDR2* compared to control cells for isolates C6 and C21 are illustrated in Figures 5A and 5B, respectively.

Discussion

Candida spp. are responsible for 10–15% of UTIs worldwide and are categorized as the fourth most common cause of UTIs, especially in hospitalized and ICU patients (Yashavanth et al. 2013). In Egypt, a study carried out in Alexandria University Students Hospital, a hospital providing services to more than 50,000 people per year, identified *C. albicans* to be the causative organism of 36.7% of UTI cases in 2005 (Sallam et al. 2005). In AMUH in 2008, *Candida* spp. were isolated from 63 out of 161 patients with UTIs (Talaat et al. 2010). In the current study, 28 *Candida* spp. isolates from the urine culture of patients admitted to AMUH with UTIs were subjected to species characterization using two phenotypic tests; the germ tube formation and the Tween 80 opacity tests. Non-albicans *Candida* spp., the identity of which was confirmed by the automated Vitek-2 system (Fig. S1), were less prevalent (35.7%) than *C. albicans* that were encountered in 18 (64.3%) of total isolates. Among non-albicans *Candida* isolates, *C. glabrata* was the most predominant (25%), followed by *C. tropicalis* (7.1%), and *C. famata* (3.6%), respectively (Table III). Hassaneen et al. reported similar incidence rates of *C. glabrata* (21.4%) and *C. tropicalis* (7.1%) isolated from the urine of patients admitted with UTIs to the Zagazig University Hospitals in Egypt (Hassaneen et al. 2014). The situation in the Middle East seems to be analogous, and *C. albicans* was the most encountered in diabetic patients with candiduria in Arar, the northern area of Saudi Arabia, followed by *C. glabrata*, and *C. tropicalis* (Alenezy 2014). In Kuwait, *C. albicans* was the most prominent species recovered from urine cultures from candiduric patients in a tertiary care hospital (Alfouzan 2015). Although non-albicans *Candida* spp. are emerging nowadays as potential pathogens responsible for candiduria (Kauffman 2005), *C. albicans* is still being reported as the dominant species infecting the urinary tract of not only Egyptian patients but also patients in several Arabic countries (Sallam et al. 2005; Bukhary 2008; Omar et al. 2008; Alhussaini et al. 2013; Awad and Mohamad 2014; Alkilani et al. 2017). Demographically, in this study, a higher prevalence of candiduria was recorded in males (57.1%) as compared to females (42.9%) (Table II). Although females are known to be at a higher risk of developing candiduria owing to the frequent colonization of their vulvo-vestibular area with *Candida* spp. (Bukhary 2008), other observers found that it was more common in males (Jain et al. 2011). This could be attributed to the involvement of other risk factors not approached in the current study. Older age is a classical risk factor for candiduria and the high incidence of cases observed at this age group (57%) (Table II) is explained by the attenuated host defense mechanisms in patients reaching extremes of age. This finding is supported by the results of other researchers (Jain et al. 2011).

Aggravating the problem is the fact of the dissemination of resistance among urinary *Candida* spp. isolates to different antifungal agents, especially to the azole group. Azoles, other than fluconazole, are poorly excreted in urine and, thus, are less effective in the treatment of candiduria (Alfouzan 2015). Therefore, the present study focused on determining the susceptibility of the tested isolates to fluconazole. Totally, 42.9% of the isolates tested were resistant to fluconazole. About 67%

and 40% of *C. albicans* and non-albicans *Candida* spp., respectively, were susceptible to fluconazole (Tables III and IV). With the increased use of fluconazole, numerous studies have become concerned with the emergence of fluconazole-resistant non-albicans *Candida* spp., especially *C. tropicalis*, which showed not only a better adaptation to the kidney but also possessed a reduced susceptibility to this azole (Kashid et al. 2012; Toner et al. 2016). Although the *C. tropicalis* prevalence was not high in this study (n = 2), both of the isolates were resistant to fluconazole (Tables III and IV).

The observed fluconazole resistance in *Candida* spp. and its relationship to the organism's ability to shift from commensalism to pathogenesis has drawn an increasing attention in recent years because of resultant serious infections and failure of treatment (Mayer et al. 2013). This capability is attributable to various virulence determinants; adhesion to surfaces and secretion of extracellular hydrolases, proteinases and phospholipases are among these mechanisms of pathogenesis (Höfs et al. 2016). The majority of *Candida* strains in this study were capable of producing proteinases and phospholipase (about 96 and 93%, respectively) (Table III; Fig. S2). A high rate of proteinases (91.4%) and a moderately high rate of phospholipase (68.6%) production amongst *C. albicans* clinical isolates were reported earlier in the study conducted on 200 candiduric patients admitted to the ICU of the Theodor Bilharz Research Institute Hospital in Egypt (Ashour et al. 2015). When the extracellular enzyme production potential of *Candida* isolates investigated in this study was related to their fluconazole resistance pattern, it was noted that roughly half of these enzyme-producing isolates (40–46%) were resistant to fluconazole (Table III). The previous reports demonstrated that fluconazole resistance was associated with the acquisition of superior virulence traits by *Candida* spp., phospholipase and proteinases secretion being among these traits (Fekete-Forgacs et al. 2000; Ying and Chunyang 2012). Biofilms are complex communities of surface-aggregated microorganisms, trapped in an exopolysaccharide matrix, and growing on surfaces such as medical devices (Jabra-Rizk et al. 2004). Almost 68% of the isolates tested in the current study were strong biofilm producers, the majority of which belonged to *C. albicans* species (84.2%) (Table III). Kuhn et al. and Hasan et al. showed that *C. albicans* produces quantitatively more biofilms compared with the non-albicans *Candida* isolates (Kuhn et al. 2002; Hasan et al. 2009). Scanning electron microscopy studies on biofilm architectures succeeded in relating the strength and integrity of these biofilms to the higher number of hyphal elements in *C. albicans* than in the other species (Tellapragada et al. 2014). The fluconazole resistance was detected in 47.4% of the strong biofilm producers in this study but, no statistical correlation between the biofilm formation and fluconazole susceptibility was established (p-value > 0.05) (data not shown).

Since the treatment of candiduric patients infected with fluconazole-resistant *Candida* spp. with fluconazole alone is not an available option for health practitioners anymore, the combination of fluconazole with non-antifungal agents has been explored to increase the susceptibility of fluconazole-resistant *Candida* spp. (da Silva et al. 2013; Li et al. 2015; Jia et al. 2016). Some of the antibiotic derivatives, such as aminoglycoside analogs obtained by structural modifications of kanamycin A and B, tobramycin, and gentamicin were reported to have antifungal activities and to synergize with azoles against *Candida* spp. (Lu et al. 2018). However, studies emphasizing the sensitizing effect of amikacin on fluconazole against drug-resistant *Candida* spp. are lacking in the literature. In the present study, we investigated the antibiotic resistance-modifying activity of amikacin against 12 fluconazole-resistant *Candida* spp. isolates. Although amikacin showed no antifungal activity (it didn't cause any inhibition of the growth of *Candida* isolates at the concentrations exceeding 4,000 μg/ml), it potentially sensitized 91.7% of the isolates. The MIC of fluconazole in the presence of amikacin (4,000 μg/ml) against the majority of resistant *C. albicans* decreased from 1,024 μg/ml to 4 μg/ml, against *C. glabrata* from 1,024 μg/ml to 32 μg/ml, and against *C. tropicalis* as well as *C. famata* the value of the MIC against fluconazole dropped from 128 μg/ml to 4 μg/ml (Table V). In their study, Lu et al. speculated that gentamicin/fluconazole synergism could be mediated by suppressing the efflux pumps of *C. albicans* (Lu et al. 2018). To test this hypothesis and to give an insight into the mechanism of amikacin sensitization of *Candida* to fluconazole, the rhodamine efflux assay in the absence and presence of amikacin (4,000 μg/ml), was performed on the three

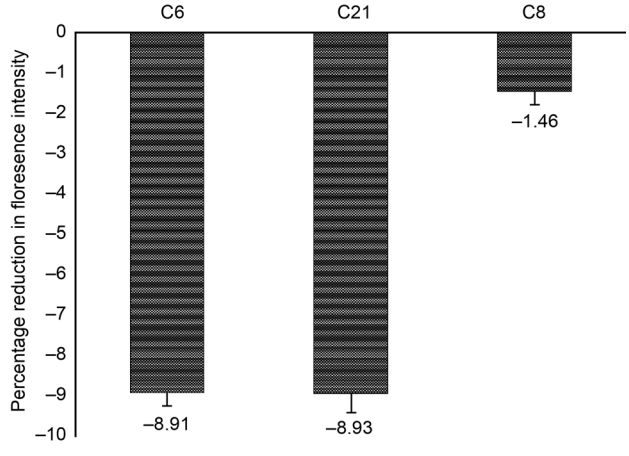

Fig. 4. Percentage of reduction in the rhodamine fluorescence intensity after 120 minutes of amikacin (4,000 μg/ml) treatment of C6, C21 and C8 *Candida* isolates.

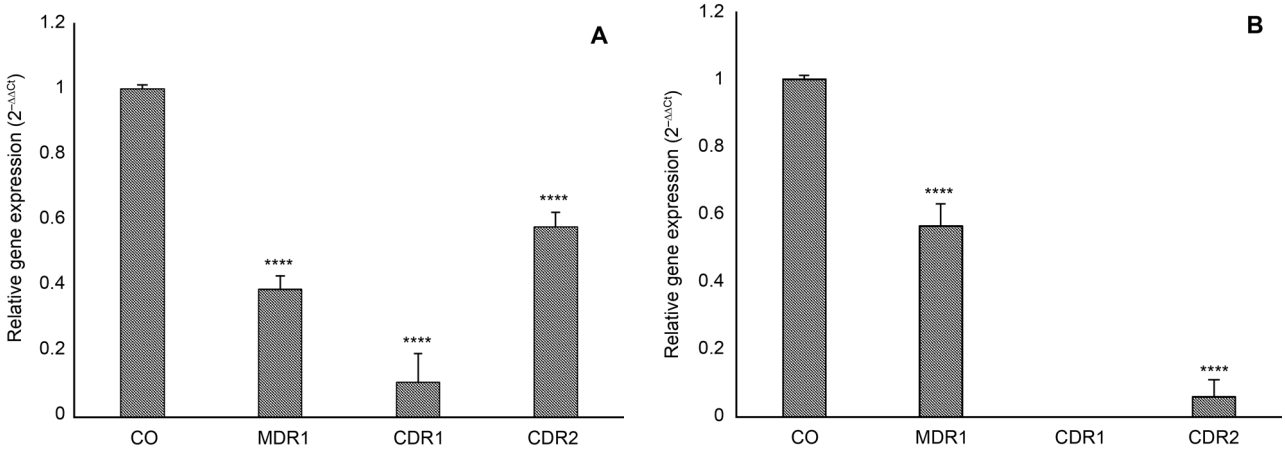

Fig. 5. Relative gene expression levels of the efflux pump mediated genes *MDR1*, *CDR1*, and *CDR2* in (A) *Candida albicans* C6 and (B) *Candida albicans* C21 as measured by the quantitative real-time PCR. Total RNAs were prepared from both selected isolates after 48-hour treatment with 4,000 µg/ml amikacin. The levels of transcripts were normalized to the level of the *ACT1* gene expression and then compared to the level of expression in the untreated control cells. The level of gene transcript in control untreated cells (CO) was set as 1. Results were expressed as the means and standard deviations of three independent determinations. The error bars represent SDs.

isolates; two isolates of *C. albicans* and one *C. glabrata* (Fig. 1–3). Amikacin suppressed the efflux pumps of resistant *C. albicans* isolates, C6 and C21, with percentages of reduction in the fluorescence intensity of 8.9% after 120 minutes of contact (Fig. 4). The inhibiting effect of amikacin on the function of efflux pumps of *C. glabrata* isolate was not noticeable when compared to the control samples indicating that the impact of amikacin might be strain-dependent (Fig. 4).

Further confirmation on the sensitizing effect of amikacin was investigated by quantifying the gene expression level of three genes responsible for the fluconazole efflux which are CDR1, CDR2 (belonging to ATP-binding cassette, ABC transporter), and MDR1 (a member of major facilitator superfamily, MFS). It was done using the quantitative RT-PCR for two selected fluconazole-resistant *C. albicans* isolates, C6 and C21 (Fig. 5A and 5B). The levels of expression of the housekeeping gene *ACT1* did not change after treatment of the cells with 4,000 µg/ml of amikacin. It indicated that vital cellular functions were not impaired by amikacin treatment. However, amikacin exerted a significant inhibitory effect (p-value < 0.0001) on the genes encoding the multidrug efflux pumps. The expression of the *CDR1*, *MDR1* and *CDR2* genes was downregulated in *C. albicans* isolate C6 with reduction equivalent to 89.4, 61.0, and 42.1%, respectively (Fig. 5A). While in *C. albicans* isolate C21, amikacin downregulated the expression of *CDR2* and *MDR1* by 94% and 43.5%, respectively (Fig. 5B). In conclusion, amikacin restored the fluconazole antifungal activity against resistant *Candida* spp. isolates. The putative mechanism of this sensitizing effect of amikacin may be the suppression of multidrug efflux pumps. Taken together, these findings indicate that amikacin may be regarded as a potential sensitizer of *Candida* isolates to fluconazole that can be used in combination in the treatment of candiduria with a higher efficiency or at lower administration doses. Further *in vivo* and clinical studies are necessitated to consolidate these conclusions.

ORCID
Eva A. Edward https://orcid.org/0000-0001-9426-5074
Nelly M. Mohamed https://orcid.org/0000-0001-9073-5032
Azza S. Zakaria https://orcid.org/0000-0003-3772-1381

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Aktas E, Yigit N, Ayyildiz A. Esterase activity in various *Candida* species. J Int Med Res. 2002 Jun;30(3):322–324. https://doi.org/10.1177/147323000203000315

Alenezy AK. Candiduria in diabetic patients in Arar Northern Area, Saudi Arabia. Life Sci J. 2014;11(1):366–370.

Alfouzan WAM. Epidemiological study on species identification and susceptibility profile of *Candida* in urine. Fungal Genom Biol. 2015;05(02):124. https://doi.org/10.4172/2165-8056.1000124

Alhussaini MS, El-Tahtawi NF, Moharram AM. Phenotypic and molecular characterization of *Candida* species in urine samples from renal failure patients. Sci J Clin Med. 2013;2(1):14–25. https://doi.org/10.11648/j.sjcm.20130201.13

Alkilani AA, El Shalakany AH, Saif WYM. Candiduria in catheterized Menoufia patients: emerging microbiological trends. Menoufia Med J. 2017;30:892–898.

Ashour SM, Kheiralla ZM, Maklad SS, Ameen MR, Zaki SS. Relationship between virulence factors of *Candida* species with candiduria and myeloperoxidase concentrations. Int J Curr Microbiol Appl Sci. 2015;4(1):108–123.

Awad ET, Mohamad EA. The use of chrom agar for detection of *Candida albicans* and non-*albicans* nosocomial infections of immunocompromised patients in Shebin El-Kom teaching hospital: risk factors and an analysis of microbiological data. Egypt J Med Microbiol. 2014 Jul;23(3):37–45. https://doi.org/10.12816/0024350

Bukhary ZA. Candiduria: a review of clinical significance and management. Saudi J Kidney Dis Transpl. 2008 May;19(3):350–360.

Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, McNicholas PM. Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. Antimicrob Agents Chemother. 2004 Jun 01;48(6):2124–2131. https://doi.org/10.1128/AAC.48.6.2124-2131.2004

Chiem K, Jani S, Fuentes B, Lin DL, Rasche ME, Tolmasky ME. Identification of an inhibitor of the aminoglycoside 6'-*N*-acetyltransferase type Ib [AAC(6')-Ib] by glide molecular docking. Med Chem Comm. 2016;7(1):184–189. https://doi.org/10.1039/C5MD00316D

CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved guideline M27-A3. Wayne (USA): Clinical and Laboratory Standards Institute; 2008.

da Silva CR, de Andrade Neto JB, Sidrim JJC, Ângelo MRF, Magalhães HIF, Cavalcanti BC, Brilhante RSN, Macedo DS, de Moraes MO, Lobo MDP, et al. Synergistic effects of amiodarone and fluconazole on *Candida tropicalis* resistant to fluconazole. Antimicrob Agents Chemother. 2013 Apr;57(4):1691–1700. https://doi.org/10.1128/AAC.00966-12

Deorukhkar SC, Saini S, Jadhav PA. Evaluation of different media for germ tube production of *Candida albicans* and *Candida dubliniensis.* Int J Biol Adv Res. 2012 Sep 26;3(9):704–707. https://doi.org/10.7439/ijbar.v3i9.732

Fankam AG, Kuiate JR, Kuete V. Antibacterial and antibiotic resistance modifying activity of the extracts from *Allanblackia gabonensis, Combretum molle* and *Gladiolus quartinianus* against Gram-negative bacteria including multi-drug resistant phenotypes. BMC Complement Altern Med. 2015 Dec;15(1):206. https://doi.org/10.1186/s12906-015-0726-0

Fekete-Forgács K, Gyüre L, Lenkey B. Changes of virulence factors accompanying the phenomenon of induced fluconazole resistance in *Candida albicans.* Mycoses. 2000 Sep;43(7–8):273–279. https://doi.org/10.1046/j.1439-0507.2000.00587.x

Fotedar R, Al-Hedaithy SSA. Comparison of phospholipase and proteinase activity in *Candida albicans* and *C. dubliniensis.* Mycoses. 2005 Jan; 48(1):62–67. https://doi.org/10.1111/j.1439-0507.2004.01057.x

Goyal R, Sami H, Mishra V, Bareja R, Behara R. Non-albicans candiduria: an emerging threat. J Appl Pharm Sci. 2016;6(3):048–050. https://doi.org/10.7324/JAPS.2016.60308

Hasan F, Xess I, Wang X, Jain N, Fries BC. Biofilm formation in clinical *Candida* isolates and its association with virulence. Microbes Infect. 2009 Jul;11(8–9):753–761. https://doi.org/10.1016/j.micinf.2009.04.018

Hassaneen AM, Ghonaim RA, Hassanin HM, Salama NA, El Gohary T. Different aspects of candiduria as an important nosocomial infection. Med J Cairo Univ. 2014;82(1):199–204.

Höfs S, Mogavero S, Hube B. Interaction of *Candida albicans* with host cells: virulence factors, host defense, escape strategies, and the microbiota. J Microbiol. 2016 Mar;54(3):149–169. https://doi.org/10.1007/s12275-016-5514-0

Jabra-Rizk MA, Falkler WA, Meiller TF. Fungal biofilms and drug resistance. Emerg Infect Dis. 2004 Jan;10(1):14–19. https://doi.org/10.3201/eid1001.030119

Jain M, Dogra V, Mishra B, Thakur A, Loomba P, Bhargava A. Candiduria in catheterized intensive care unit patients: emerging microbiological trends. Indian J Pathol Microbiol. 2011;54(3):552–555. https://doi.org/10.4103/0377-4929.85091

Jia W, Zhang H, Li C, Li G, Liu X, Wei J. The calcineruin inhibitor cyclosporine a synergistically enhances the susceptibility of *Candida albicans* biofilms to fluconazole by multiple mechanisms. BMC Microbiol. 2016 Dec;16(1):113. https://doi.org/10.1186/s12866-016-0728-1

Kashid RA, Belawadi S, Devi G, Indumati I. Incidence of *non-Candida albicans* in patients with urinary tract infection with special reference to speciation and antifungal susceptibility. J Evol Med Dental Sci. 2012 Oct 29;1(4):572–577. https://doi.org/10.14260/jemds/89

Kauffman CA. Candiduria. Clin Infect Dis. 2005 Sep 15;41(6) Supplement_6:S371–S376. https://doi.org/10.1086/430918

Kaur R, Dhakad M, Goyal R, Haque A, Mukhopadhyay G. Identification and antifungal susceptibility testing of *Candida* species: A comparison of Vitek-2 system with conventional and molecular methods. J Glob Infect Dis. 2016;8(4):139–146. https://doi.org/10.4103/0974-777X.192969

Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: an Overview. Cold Spring Harb Perspect Med. 2016 Jun;6(6):a027029. https://doi.org/10.1101/cshperspect.a027029

Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect Immun. 2002 Feb 1;70(2):878–888. https://doi.org/10.1128/IAI.70.2.878-888.2002

Li H, Zhang C, Liu P, Liu W, Gao Y, Sun S. *In vitro* interactions between fluconazole and minocycline against mixed cultures of *Candida albicans* and *Staphylococcus aureus.* J Microbiol Immunol Infect. 2015 Dec;48(6):655–661. https://doi.org/10.1016/j.jmii.2014.03.010

Lu M, Yu C, Cui X, Shi J, Yuan L, Sun S. Gentamicin synergises with azoles against drug-resistant *Candida albicans.* Int J Antimicrob Agents. 2018 Jan;51(1):107–114. https://doi.org/10.1016/j.ijantimicag.2017.09.012

Mattei AS, Alves SH, Severo CB, Guazzelli LS, Oliveira FM, Severo LC. Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of *Candida albicans.* Rev Soc Bras Med Trop. 2013 Jun;46(3):340–342. https://doi.org/10.1590/0037-8682-0045-2013

Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. Virulence. 2013 Feb 15;4(2):119–128. https://doi.org/10.4161/viru.22913

Mohan Das V, Ballal M. Proteinase and phospholipase activity as virulence factors in *Candida* species isolated from blood. Rev Iberoam Micol. 2008;25(4):208–210. https://doi.org/10.1016/S1130-1406(08)70050-0

Omar M, Fam N, El-Leithy T, El-Said M, El-Seidi E, El-Etreby T. Virulence factors and susceptibility patterns of *Candida* species isolated from patients with obstructive uropathy and bladder cancer. Egypt J Med Microbiol. 2008;17:317–326.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 May 1;29(9):45e–45. https://doi.org/10.1093/nar/29.9.e45

Pongrácz J, Benedek K, Juhász E, Iván M, Kristóf K. *In vitro* biofilm production of *Candida* bloodstream isolates: any association with clinical characteristics? J Med Microbiol. 2016 Apr 01;65(4):272–277. https://doi.org/10.1099/jmm.0.000207

Ramirez M, Tolmasky M. Amikacin: uses, resistance, and prospects for inhibition. Molecules. 2017 Dec 19;22(12):2267. https://doi.org/10.3390/molecules22122267

Rodrigues LB, Santos LR, Tagliari VZ, Rizzo NN, Trenhago G, Oliveira AP, Goetz F, Nascimento VP. Quantification of biofilm production on polystyrene by *Listeria*, *Escherichia coli* and *Staphylococcus aureus* isolated from a poultry slaughterhouse. Braz J Microbiol. 2010 Dec;41(4):1082–1085. https://doi.org/10.1590/S1517-83822010000400029

Sallam SA, Arafa MA, Razek AA, Naga M, Hamid MA. Device-related nosocomial infection in intensive care units of Alexandria University Students Hospital. East Mediterr Health J. 2005 Jan-Mar; 11(1–2):52–61.

Schwalbe R, Steele-Moore L, Goodwin AC. Antimicrobial susceptibility testing protocols. Antifungal susceptibility testing of yeasts. New York (USA): CRC Press; 2007. p. 173–208.

Spampinato C, Leonardi D. Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. BioMed Res Int. 2013;2013:1–13. https://doi.org/10.1155/2013/204237

Talaat M, Hafez S, Saied T, Elfeky R, El-Shoubary W, Pimentel G. Surveillance of catheter-associated urinary tract infection in 4 intensive care units at Alexandria university hospitals in Egypt. Am J Infect Control. 2010 Apr;38(3):222–228. https://doi.org/10.1016/j.ajic.2009.06.011

Tellapragada C, Eshwara VK, Johar R, Shaw T, Malik N, Bhat PV, Kamath A, Mukhopadhyay C. Antifungal susceptibility patterns, *in vitro* production of virulence factors, and evaluation of diagnostic modalities for the speciation of pathogenic Candida from blood stream infections and vulvovaginal candidiasis. J Pathogens. 2014;2014:1–8. https://doi.org/10.1155/2014/142864

Toner L, Papa N, Aliyu SH, Dev H, Lawrentschuk N, Al-Hayek S. *Candida* growth in urine cultures: a contemporary analysis of species and antifungal susceptibility profiles. QJM. 2016 May;109(5): 325–329. https://doi.org/10.1093/qjmed/hcv202

Wiebusch L, de Almeida-Apolonio AA, Rodrigues LMC, de Paula Bicudo B, dos Santos Silva DB, Lonchiati DF, de Araujo RP, Grisolia AB, de Oliveira KMP. *Candida albicans* isolated from urine: phenotypic and molecular identification, virulence factors and antifungal susceptibility. Asian Pac J Trop Biomed. 2017 Jul; 7(7):624–628. https://doi.org/10.1016/j.apjtb.2017.06.006

Yashavanth R, Shiju MP, Bhaskar UA, Ronald R, Anita KB. Candiduria: prevalence and trends in antifungal susceptibility in a Tertiary Care Hospital of Mangalore. J Clin Diagn Res. 2013 Nov; 7(11):2459–2461. https://doi.org/10.7860/JCDR/2013/6298.3578

Ying S, Chunyang L. Correlation between phospholipase of *Candida albicans* and resistance to fluconazole. Mycoses. 2012 Jan;55(1): 50–55. https://doi.org/10.1111/j.1439-0507.2011.02024.x

Supplementary materials are available on the journal's website.