ORIGINAL ARTICLE

INFLUENCE OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON ANTIFUNGAL RESISTANCE OF *CANDIDA* STRAINS ISOLATED FROM VULVOVAGINAL INFECTIONS

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Abstract

In the last few years, it has been noticed an increasing number of infections caused by opportunistic yeasts, due to the development of new therapies and the use of antimicrobial drugs with large spectrum. The main goal of our investigation regards the antifungal susceptibility of pathogenic yeast strains and the effect of non-steroidal anti-inflammatory drugs (NSAIDs) in combination with azole compounds against *C. albicans* and *C. krusei* cell viability. Antifungal susceptibility test was performed using Bio-Rad's Fungitest Kit. The most resistant two strains, *C. albicans* B18 and *C. krusei* Y8, were selected for further analyses. The influence of anti-inflammatory compounds (aspirin, sodium diclofenac and ibuprofen) on fluconazole and ketoconazole cell susceptibility was assessed. Sodium diclofenac and ibuprofen combined with two selected azole compounds increased the susceptibility of the two *Candida* strains.

Rezumat

În ultimii ani, din cauza dezvoltării de noi terapii şi a utilizării unor compuşi antimicrobieni cu spectru larg de acţiune, s-a observat o creştere a numărului de infecţii oportuniste cauzate de tulpini de drojdii patogene. Scopul acestui studiu a fost de a evalua sensibilitatea la compuşi antifungici a unor tulpini de drojdii patogene şi de a evidenţia efectul unor compuşi din clasa anti-inflamatoare nonsteroidiene, în combinaţie cu substanţele din clasa azolilor, asupra viabilităţii a două tulpini aparţinând genului *Candida*. Sensibilitatea la substanţe antifungice s-a determinat utilizând kitul Fungitest Bio-Rad. Cele mai rezistente tulpini au fost selectate pentru analiza influenţei substanţelor anti-inflamatoarelor asupra susceptibilităţii tulpinilor de drojdii la fluconazol şi ketoconazol. Diclofenacul sodic şi ibuprofenul în asociere cu cele două antifungice selectate au indus o creştere semnificativă a sensibilităţii celulelor.

Keywords: *Candida* sp., azole resistance, NSAIDs, cell viability

Introduction

Infections caused by *Candida* species represent an important clinical problem, especially for immune-suppressed patients, who are frequently affected by drug resistant strains [3, 5]. Yeast infections chemo-therapy includes compounds from azole class, which are the most used antifungal drugs, because are easily absorbed and have a good pharmaco-kinetics [7, 10]. Fungistatic rather than fungicidal activity of azole drugs leads to the frequent emergence infections caused by azole-resistant *Candida* sp. [2, 9, 11].

The main goal of our study was to evaluate anti-fungal susceptibility of yeast strains isolated from vaginal infections and the effect of the association between non-steroidal anti-inflammatory drugs like aspirin, sodium diclofenac, ibuprofen and anti-fungal drugs (fluconazole and ketoconazole) on *C. albicans* and *C. krusei* cell viability.

Materials and Methods

Strains taxonomical identification

The 13 analysed strains were isolated in 2010 from vaginal swabs taken from female patients with vulvo-vaginal infections and isolated on Sabouraud + cloramphenicol agar (samples were provided by "Matei Basarab" Medical Center). The yeast strains were grown in YPG (Yeast Peptone Glucose - g/L yeast extract 10, peptone 10 g/L, glucose 20 g/L, agar-agar 20 g/L) broth for 24 h, in aerobic conditions (150 rpm) at 37ºC. The strains were taxonomically identified according to their

morphological and biochemical characteristics. The macroscopic/microscopic characteristics including colony and cell morphology have been analysed. Biochemical characterization of yeast strains was carried out using the API *Candida* system (bio-Merieux) and by the Biolog YT MicroPlate (Biolog System, USA), following the manufacturer's recommendations. The API *Candida* results were integrated using the API Web software (bioMerieux).

Antifungal susceptibility test

Fungitest was performed using Bio-Rad's Fungitest Kit according to manufacturer's recommendation (Bio-Rad, SUA). Bio-Rad's Fungitest Kit is a 16-well microplate designed to determine the yeasts susceptibility to six antifungal compounds: 5-fluoro cytosine (5FC), amphotericin B (AB), miconazole (MCZ), ketoconazole (KET), itraconazole (ITR) and fluconazole (FLU), at two different concentrations, in modified RPMI 1640 medium by a rapid colorimetric test.

Influence of anti-inflammatory against yeast susceptibility to azoles

Influence of non-steroidal anti-inflammatory drugs against *C. albicans* and *C. krusei* susceptibility to fluconazole and ketoconazole was performed by two methods: disk diffusion and microdilution.

Disk diffusion method: Fresh cultures of pathogenic yeast strains *C. albicans* B18 and *C. krusei* Y8, grown in Sabouraud Dextrose Agar **(**SDA) broth for 18 h at 37ºC, were centrifuged 6 min at 10000 rpm. The pellet was resuspended in phosphate buffer saline (PBS; pH 7.2) to a density of 0.5 McFarland, and plated on SDA agar broth. A volume of 5 µL fluconazole (Diflazon®, KRKA) and ketoconazole (Antibiotice, Iaşi) of 1 mg/mL stock solution and 5 µL of anti-inflammatory compounds: aspirin (Medchim™), sodium diclofenac (Medchim™), ibuprofen (Brufen®, Abbot Laboratories) of 10 mg/mL stock solution were placed on blank disk (Sigma). Plates were incubated for 24 - 48 h at 37ºC. The influence of anti-inflammatory drugs was assessed by measuring the diameter of the inhibition zone (in centimetres) [1, 4].

Microdilution method: The assay was performed in 96 wells polystyrene plates (Corning). A volume of 200 µL of cell suspension (10^3 cells/mL) in RPMI 1640 (Gibco) supplied with 0.165 M morpholino-propanesulfonic acid (MOPS) and 0.3 g/L L-glutamine, in the presence of 1 µg/mL antifungal agent and different concentrations of NSAIDs (50, 25 and 12.5 µg/mL), were incubated for 48 h at 37ºC, without shaking. Cell growth was estimated by determining the absorbance at 660 nm using microtiter plate reader Apollo LB 911 (Berthold Technologies).

Results and Discussion

Strains taxonomical identification

The morphological and biochemical characteristics of the 13 newly isolated strains were compared with the control strains *C. albicans* ATCC 10231, *C. parapsilosis* CBS 604 and *C. krusei* CMGB 59.

According to the results obtained with the API *Candida* test and MicroLog (Biolog, USA), *C. albicans* was the most frequent (8 strains) notated Y1, Y2, Y4, Y6, Y9, Y10, Y12, B18, two strains were identified as *C. krusei* (Y5, Y8), one strain as *C. parapsilopsis* (Y3), one strain as *C. catenulata* (Y7) and one strain as *C. kefyr* (B5).

Susceptibility of Candida strains to antimicrobial compounds

Almost all newly isolated strains presented susceptibility to antifungal drugs with few exceptions. Most of *C. albicans* newly isolated strains were susceptible to all tested antifungal and only some of them presented resistance to itraconazole (Table I).

Table I

Susceptibility of *Candida* strains to the antimicrobial compounds included in Fungitest gallery

Strains	5FC	AB	MCZ	KET	ITR	FLU
C. albicans ATCC 10231	S	S	S	S	S	S
C. albicans Y1	S	S	SDD	SDD	SDD	S
C. albicans Y2	S	S	SDD	SDD	R	S
C. albicans Y4	S	S	S	S	SDD	S
C. albicans Y6	S	S	S	S	SDD	S
C. albicans Y9	S	SDD	S	S	SDD	S
C. albicans Y10	S	S	S	S	SDD	S
C. albicans Y12	SDD	S	S	S	R	S
C. albicans B18	R	S	SDD	R	R	R
C. krusei Y5	SDD	SDD	SDD	SDD	SDD	SDD
C. krusei Y8	SDD	R	R	R	R	R
*C. parapsilosis*Y3	S	S	S	S	S	S
*C. catenulata*Y7	S	S	SDD	S	SDD	S
C. kefyr B5	S	R	S	S	SDD	S

SDD susceptible-dose dependent; R resistant; S susceptible; 5-fluoro cytosine (5FC), amphotericin B (AB), miconazole (MCZ), ketoconazole (KET), itraconazole (ITR) and fluconazole (FLU)

C. albicans B18 and *C. krusei* strains were the most resistant, especially to azole compounds. *C. krusei* Y8 presented a multi-resistant phenotype, being resistant/susceptible dose dependent to commonly clinical used antifungal agents.

Effect of NSAIDs - azoles combination on yeast strains cell viability

The most resistant strains identified as *C. albicans* and *C. krusei* were selected for testing the cell susceptibility to NSAIDs - antifungal drugs combinations. Two methods were applied: disk diffusion and microdilution, according to NCCLS, M27-A2 document. NSAIDs did not affect the cell viability of yeast strains.

Disk diffusion

Results, obtained using the disk diffusion method, have shown that sodium diclofenac and ibuprofen in association with fluconazole and ketoconazole induced growth inhibition of *C. albicans* B18, and had no effect against *C. krusei* Y8. Aspirin had no effect in combination with azole compounds (Table II).

Table II

Growth inhibition of *Candida* sp. in presence of azoles and non-steroidal anti-inflammatory drugs using the disk diffusion method

	***C. albicans* B18**			***C. krusei* Y8**		
NSAIDs/Antifungal	Aspirin (cm)	Sodium diclofenac (cm)	Ibuprofen (cm)	Aspirin (cm)	Sodium diclofenac (cm)	Ibuprofen (cm)
FLU	0	0.5	0.4	0	0	0
KET	0	0.3	0.6	0	0	0

ketoconazole (KET), fluconazole (FLU)

Microdilution method allowed us to determine the minimal inhibitory concentration (MIC) for different compounds by measuring the $OD_{620\ nm}$ (optical density).

The combination of fluconazole and ketoconazole with either sodium diclofenac or ibuprofen presented a synergic effect against *C. albicans* B18 cell viability (Figure 1), inducing a decrease of cellular viability with 20 - 30% (Figure 1).

A synergic effect against cell viability of *C. krusei* Y8 was observed for ibuprofen in association with ketoconazole, using the microdilution method. Aspirin and sodium diclofenac did not exert any inhibitory effect on *C. krusei* Y8 strain (Figure 2).

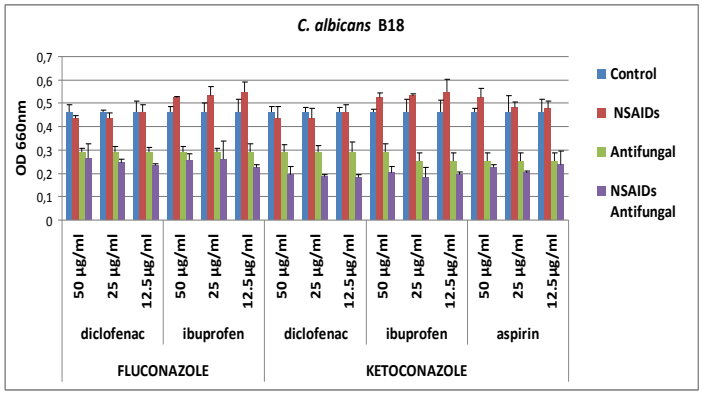

Figure 1.

C. albicans B18 growth in presence of NSAIDs and antifungal drugs

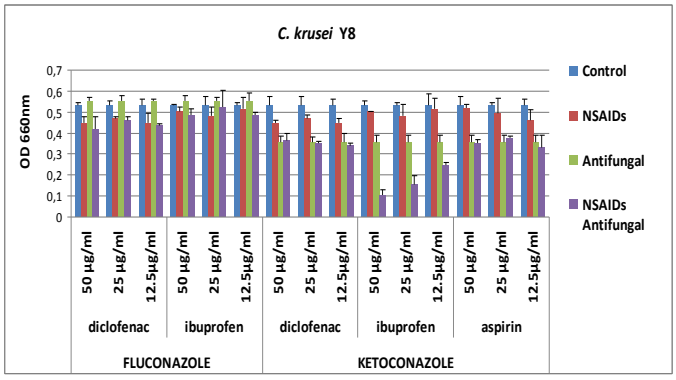

Figure 2.

C. krusei Y8 growth in presence of NSAIDs and antifungal drugs

Ibuprofen in association with ketoconazole led to the decrease of cellular viability of *C. krusei* Y8 to ~ 70%, and in the case of *C. albicans* B18 to ~ 30% (Figure 1, 2).

Conclusions

Anti-inflammatory drugs in association with azole compounds exhibited a strain specific effect. *C. albicans* B18 became more sensitive to fluconazole and ketoconazole in the presence of sodium diclofenac and ibuprofen. Ibuprofen in association with ketoconazole induced a higher effect in the case of *C. krusei* Y8 strain compared with *C. albicans* B18 strain.

The results presented in this paper are in accordance with reported data regarding the increased sensitivity of yeast strains to compounds with antifungal activity in the presence of NSAIDs [6, 8].

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