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International Journal of Current Research
Vol. 5, Issue, 08, pp.2131-2134, August, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

ERG11 MUTATIONS ASSOCIATED WITH AZOLE RESISTANCE OF *Candida albicans* ISOLATES FROM VAGINAL CANDIDIASIS IN MOROCCO

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ARTICLE INFO

Article History:
Received 24th May, 2013
Received in revised form
02th June, 2013
Accepted 18th July, 2013
Published online 23rd August, 2013

Key words:
Candida albicans,
Azole,
Resistance,
Mutations,
ERG11 gene.

ABSTRACT

Summary: Aim of the study: To determine profile of résistance to *C.albicans* and mutations in ERG 11 gene in strains of *C. albicans* azoles resistant.
Material and Methods: In this study, we isolated and identified the yeast species in the vagina of patients consulted in the Pasteur Institut and tested in vitro activities of antifunguals. We amplified and sequenced the ERG11 gene of C. *albicans* azole resistant.
Résulte: C. *albicans* (69, 1%) was the most frequently identified species followed by *C. glabrata* (21 %), *C.Tropicalis* (6, 2%) and *C. parapsilosis* (3,7 %). Susceptibility testing carried out on 56 representative isolates of *C.albicans* that 12,4 % were resistant witch 1,7 % to 5-fluorocytosine and 10,7 % to itraconazole **,** 3,5% dose dependant to fluconazole and 1,7% to voriconazole. No resistance has reported with amphotercine B. Sequence analysis of *ERG11* gene of selected itraconazole-resistant isolates identified In the 6 isolates, 4 types of amino acid substitutions: K128T, D116E, E266D and D153E.
Conclusion: Our study provides information on antifungal susceptibility of vaginal yeast isolates in a rural community in Morocoo. Since the majority of *C. albicans* isolates were susceptible to fluconazole, its use may be continued for empirical therapy of uncomplicated candidal vulvovaginitis in the Morrocan community. The frequency of *C. albicans* isolates resistant to itraconazole was considerably higher .The relationship between D116E substitution and resistance to itraconazole were confirmed, other mutations such K128T, E266D and D153E are confirmed to not participate in itraconazole resistance. Other mechanisms of resistance, such as overexpression of ERG11 and efflux pumps and mutations in the ERG3 gene should also be investigated.

INTRODUCTION

Mycotic vaginitis is a common mucosal infection caused by the saprophytic, opportunistic yeast of the *Candida* genus. *Candida albicans* is responsible for 85–90% of vulvovaginal candidiasis (Sobel *et al.*, 2003 a). However, other less frequently non-albicans species such as *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis* and *Saccharomyces cerevisiae* may also be involved (Grigoriou O *et al.*, 2006). The majority of women suffer from uncomplicated vaginitis characterized by sporadic attacks without any predisposing factors. In contrast, 5–7% of women experience recurrent infection characterized as at least four microbiologically proven symptomatic vaginal candidosis within a 12 month period (Mårdh *et al.*, 2002). Therapy of vulvovaginal candidiasis frequently fails due to resistance of yeast pathogens to the drugs used. Resistance of *Candida* species to azole antifungals is the most prevalent type of resistance to antimycotics (Cross *et al.*, 2000; Kontoyiannis and Lewis, 2002). In recent reports, 3.6% of *C. albicans* vaginal isolates were found to be resistant to fluconazole (Sobel *et al.*, 2003 b; Richter *et al.*, 2005), whilst resistance to itraconazole was considerably higher (16.2%) (Richter *et al.*, 2005). Azoles inhibit fungal growth by interfering with the synthesis of ergosterol, a necessary component of fungal cell membranes. The ergosterol biosynthetic pathway is interrupted by azoles through inhibition of the enzymatic activity of 14 α -sterol demethylase (also known as ERG16, CYP51A1), the product of the ERG11 gene. Azoles have basic nitrogen that coordinates to the iron atom of the heme group located in the active site of 14- α -sterol demethylase. The active site is thus occupied by the azole, which acts as a non-competitive inhibitor (Sanglard *et al.*, 1998 a; Podust *et al.*, 2001; *et al.*, 2000). Many studies have identified point mutations in the ERG11 gene in azole-resistant *C. albicans* isolates. Such mutations can alter the affinity of CYP51A1 for an azole if the resultant amino acid substitutions lead to changes in the tertiary structure of the enzyme. Mutations in the ERG11 gene encoding more than 160 distinct amino acid substitutions have been reported

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(Feng *et al.*, 2010; Morio *et al.*, 2010; Manastır *et al.*, 2011). Only few, have been shown to cause azole resistance, such Y132H, T315A, S405F, G464S, R467K and I471T (Lamb *et al.*, 1997; Kamai *et al.*, 2004). The knowledge of further specific point mutations associated with azole resistance could be used to identify resistant strains, to adjust treatments accordingly, and for the rational design of new drugs less prone to resistance. The aim of the present study was to identify mutations in the *C. albicans* ERG11 gene associated with clinical cases of azole-resistant candidiasis. To this end, we amplified and sequenced the ERG11 genes from clinical isolates of *C. albicans* and identified mutations that might be related to azole resistance.

MATERIALS AND METHODS

Isolates

This prospective study was conducted from January 2009 to March 2011. Clinical isolates of *C. albicans* were collected from patients with vulvovaginal candidiasis. The characteristics of these clinical isolates were confirmed by germ tube formation in serum, standard biochemical and microbiological procedures, including assessment of carbohydrate assimilation pattern (API 20C; Biomerieux, Marcy l'Etoile, France) and colony colour in chromogenic medium (Chromagar, France).

Antifungal susceptibility tests

Drug susceptibility testing to determine the susceptibility of the isolates of *C.albicans* to fluconazole (FLZ), itraconazole (ITZ), voriconazole (VOR), 5flucytosine (5FC) and amphotericine B(AMB) was performed using the broth microdilution method according to the standards of the Clinical and Laboratory Standards Institute (CLSI). *Candida albicans* (IPL CIM 86 14 84) susceptible strain, was used as a control in this study.

Amplification and sequencing of *ERG*11 gene

Genomic DNA of cultured *C. albicans* was extracted using the protocol of Harju s *et al.* (Harju S*et al.*, 2004). The coding region of *ERG*11 was amplified by PCR from genomic DNA of the the *C albicans* isolates using the primers:

*ERG*11-F: 5′-ATGGCTATTGTTGAAACTGTCATTGA.
ERG11-R: 5′- TTAAAACATACAAGTTTCTCTTTTTT.
A volume of 25 µl contained 10×PCR buffer (with Mg2+) 2.5 µl, genomic DNA 10 ng, 10 mmol/L of each dNTP 2 µl, 5 pmol/µl each primer 1 µl and 5 U/µl Ex DNA polymerase 0.25 µl. PCR was accomplished by the following condition: denaturing temperature of 95°C for 5 minutes and 35 cycles of denaturing (94°C, 30 seconds), annealing (48°C, 30 seconds) and extension (72°C, 2 minutes), followed by extension of 72°C 10 minutes. After standard PCR and purification of PCR products by incubation with exonuclease I and alkaline phosphatase.ERG 11 sequencing was performed with the Big Dye Terminator V3.1 Cycle Sequencing Ready Reaction kit V3.1 (Applied Biosystems), and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).Analysis of the sequence was carried out with the ABI SeqScape v 2.5 Software by comparaison of subject DNA sequence to SC5314 reference sequence.

RESULTS

Strain identification

In this retrospective study, 311 women were collected .Clinical signs included erythematous vulva, signs of excoriation or characteristic thick white adherent vaginal discharge. 81 identified clinical yeast isolates in patients with symptomatic vulvovaginal candidiasis, *Candida albicans* was the prevailing species (69, 1%). The second most frequently identified Candida species was *C. glabrata* (21%). In the present study other non-*albicans* species were also isolated, *C. tropicalis* (6, 2%), *C. parapsilosis* (3, 7%).

Antifungal susceptibility tests

Antifungal susceptibility testing in our study revealed that on 56 representative isolates of *C.albicans* ,12,4 % were resistant witch 1,7 % to 5-fluorocytosine and 10,7 % to itraconazole. 3, 5% dose dependant to fluconazole and 1, 7% dose dependant to voriconazole. None of the *Candida albicans* isolates tested were resistant to fluconazole and to amphotercine B. (Table 1). Important effect is that the majority of the isolats of *Candida albicans* resistant to itraconazole have a decreased sensitivity to fluconazole and voriconazole (Table 2).

Table 1. In vitro susceptibilities of *C. albicans* isolates of antifungal

Antifungal	*Candida albicans* (n=56)					
	S		SD D		R	
	n	%	n	%	n	%
Fluorocytosine	55	98,2	-	-	1	1,7
Amphotericine B	56	100	-	-	-	-
Fluconazole	54	96,4	2	3,5	-	-
Itraconazole	50	89,2	-	-	6	10,7
Voriconazole	55	98,2	1	1,7	-	-

Table 2. The MICs of 3 azoles for the 6 *C. albicans* isolates resistant to itraconazole and 2 *C.albicans* sensitive to itraconazole with decreased sensitivity to fluconazole

No. of isolate	MIC(mg/l)		
	Fluconazole	Itraconazole	Voriconazole
58	16	4	0,5
65	8	1	0,5
71	4	1	0,25
26	16	> 4	2
69	8	1	0,5
73	8	2	0,5
31	2	0	0
34	4	0	0

The interpretative minimal inhibitory concentration (MCI) breakpoints used for different antimycotics were proposed by CLS/ NCCLS (National Committee for Clinical Laboratory Standards) for Candida sp (CLSI, 2005).

S : Sensitive for the isolats with MCI ≤ 4 mg/l to 5FC, MCI ≤ 8 mg/l to FCA, MCI ≤ 0 ,125 mg/l to ITR, MCI ≤ 1 mg/l to VRC , MCI ≤ 2 mg/l to AMB.
R : resistant for the isolats with MCI ≥ 32 mg/l to 5FC, MCI ≥ 64 mg/l to FCA, MCI ≥ 1 mg/l to ITR, MCI ≥ 4mg/l to VRC , MCI ≥ 2mg/l to AMB.
SDD: susceptibility dose dependent for the isolats with MCI between 8 -16 mg/l to 5 FC,16-32 mg/l to FCA, 0, 25 -0, 5 mg /l to ITR and 2 mg /l to VRC.

Table 2: The MICs of 3 azoles for the 6 *C. albicans* isolates resistant to itraconazole and 2 *C.albicans* sensitive to itraconazole with decreased sensitivity to fluconazole.

Mutations in *ERG*11 gene

The size of coding region of *ERG*11 amplified by PCR was 1587 bp. From the sequencing results of *ERG*11 in the 6 itraconazole resistant *C. albicans* isolates, we detected 4 missense mutations. The Table 3 shows mutations in *ERG*11 and the resultant amino acid changes in the 6 *C. albicans* isolates itraconazole resistant. All the mutations had been previously described. (CA31 and CA 34) à ajouter devant Two isolates sensitive of itraconazole, showed decreased levels of susceptibility to Fluconazole had two mutations (Table 3).

Table 3. Mutations in *ERG*11 gene and the resultant amino acid substitutions in the 6 *C. albicans* itraconazole resistant

Isolates	Amino acid substitutions	Missense mutations
58	K128T	A383C
65	D116 E, K128T	T348A, A383C
71	E266D	A798C
26	D116E, K128T	T348A, A383C
69	D153E	T459 A
73	K128T	A383 C
31	K128T	A383C
34	D153E	T459A

DISCUSSION

In this study, the overall prevalence of vulvovaginal candidiasis in a community setting was found to be 26 per cent which is similar to other studies (Goswami *et al.,* 2000). *C. albicans* was identified as a dominant species of pathogenic yeast isolated from patients with symptomatic vulvovaginal candidiasis. A similar frequency has been also reported in other studies (Monika Sojakova *et al.*, 2004; Saporiti *et al.*, 2001). Thus in recent years, there has been a significant increase in infections caused by non-albicans species of *Candida*, particularly, *C. glabrata* and *C. tropicalis*. This is demonstrated in this study. We speculate this increasing detection of non-albicans Candida species is probably related to the widespread and inappropriate use of antimycotic treatments (self-medication, longterm maintenance treatments and repeated treatments for candidosis episodes) (Ferrer, 2000). *C. albicans* eradication by these means causes a selection of species (such as *C. glabrata*) that are resistant to commonly used agents ((Ferrer, 2000). Clinical resistance is defined as a failure of an antifungal agent to cure a fungal infection and correlates with the susceptibility of clinical isolates to antimycotics determined in vitro (Espinel-Ingroff *et al.*, 1998). Resistance of *Candida* species to azole derivatives is the most prevalent type of antifungal resistance (Cross *et al.*, 2000; Kontoyiannis and Lewis, 2002). In this study. The frequency of *C. albicans* isolates resistant to itraconazole was considerably higher than that reported in other studies (Kelen F*et al.*, 2011; Richter *et al.*, 2005). Our study provides information on antifungal susceptibility of vaginal yeast isolates in a rural community in Morocco. Since the majority of *C. albicans* isolates were susceptible to fluconazole, its use may be continued for empirical therapy of uncomplicated candidal vulvovaginitis in the Moroccan community. Use of alternative agents (like boric acid, flucytosine) (Sobel, *et al.*, 2003b) may be considered when treating vulvovaginitis caused by non-albicans species (especially *C. glabrata* and *C. krusei*). As only a limited number of *Candida* isolates could be tested in this study, further clinical studies need to be performed involving more number of isolates to confirm the findings. Several molecular mechanisms may be involved in the resistance to azole antifungal agents in *C. albicans*. These include upregulation or mutation of the target enzyme of azole antifungals (Franz *et al.*, 1998; White, 1997a; Maebashi *et al.*, 2002) and overexpression of the genes encoding efflux pumps CDR1, CDR2 and MDR (Maebashi *et al.*, 2002; Perea *et al.*, 2001). Decreasing the affinity between Erg11p and azole antifungals induced by mutations in *ERG*11 gene are frequent in azole resistant *C. albicans*, but upregulation of *ERG*11 causes resistance only in a small number of the strains. In addition, multiple resistant mechanisms, including *ERG*11 mutation and overexpression of efflux, occurred in one azole resistant *C. albicans* (Perea *et al.*, 2001).

In this study, we searched for mutations in *ERG*11 gene in isolates of itraconazole resistant *C. albicans* using the methods of PCR amplification and sequencing. We found 4 missense mutations reported previously ; K128T (Kallakuri, *et al.*, 1996; Manavathu, *et al.*, 1996 ; Löffler *et al*., 1997) , D116E (Kallakuri, *et al.*, 1996; Manavathu, *et al.*, 1996 ; Ryder and Favre, 1997; Marr, *et al.*, 1998), E266D (Kallakuri, *et al.*, 1996; Löffler *et al.*, 1997 ; Ryder and Favre, 1997; Favre, *et al.*, 1999) , and D153E (Marichal *et al.*,1999) The mutations K128T and D153E were detected also in two isolates sensisitive of itraconazole who showed decreased levels of susceptibility to Fluconazole, this could be explained by the fact that fluconazole shows properties similar to those of itraconazole with respect to its capacity to be a substrate for multidrug efflux transporters and to respond to *ERG11* mutations, as has recently been shown by (Sanglard, *et al.*, 2000 b). (Marichal *et al.*, 1999) have identified three hot spots within the amino acid sequence of the *ERG11* gene based on a compilation of *ERG11* mutations reported to be associated with azole resistance. These hot spots include amino acid regions 105–165, 266–287 and 405–488. The mutation E266D could be detected in azole antifungal susceptible strains; therefore, it may not cause drug resistance (Loffler *et al.*, 1997; Marichal *et al.*, 1999).

The isolate with the mutation D116E is itraconazole resistant and have a decreased sensitivity to fluconazole and voriconazole. This is in agreement with previous studies witch demonstrated the relationship between D116E substitution and resistance to azole antifungal, (White *et al.*, 2002 b; Perea *et al.*, 2001). Especially the cross resistance to fluconazole and voriconazole. D153E can be found in strains susceptible to azoles and is unrelated to the resistance, however. In this study, D153E occurred in two isolates, resistant and sensitive to itraconazole. K128T were isolated in this study in itraconazole susceptible, this mutation confirmed to not participate in azole resistance, in agreement with previous reports (Favre, *et al.*, 1999; Richter *et al.*, 2005). However, the association between the two mutation K128T and D116E can be explained the resistance of itraconozole. In after this study, the mutation K128T was isolated in susceptible and resistant strains to itraconazole and the E266D isolated in itraconazole resistant have described in the previous study non contrinung to resistance, this suggested that other mechanisms of resistance, such as overexpression of the ERG11 gene and efflux pumps and mutations in the ERG3 gene should also be investigated. (Perea *et al.*, 2001) investigated the molecular mechanisms of C. albicans isolates and concluded that multiple resistance mechanisms existed in approximately 75% of isolates and ERG11 mutations existed in 65% of them. In summary, we demonstrated that in a series of clinical *C. albicans* isolates, the majority of *C. albicans* isolates were susceptible to fluconazole, its use may be continued for

empirical therapy of uncomplicated candidal vulvovaginitis in the Moroccan community.

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