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Detection of azole resistance and *ERG11* point mutations in *Candida albicans* isolates from tertiary hospitals in the Philippines

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Abstract

Candida albicans is commonly isolated from nosocomial fungal infections, and controlling these infections depends on the immune status of the patient, gravity of the infection, and the choice of the administered antifungal drug. Increasing worldwide reports of strains that are resistant to the azole drugs, which are commonly used for treatment of diseases caused by *C. albicans*, warrant the conduct of drug susceptibility testing of clinical isolates, which is not routinely done in the Philippines. Twenty-six local *C. albicans* clinical isolates were tested for their susceptibility to the azole drugs fluconazole and voriconazole using the standard disc agar diffusion method. Likewise, the *ERG11* gene coding for lanosterol-14-α-demethylase involved in ergosterol synthesis, which is the target of the azole drugs, was studied for the occurrence of point mutations. Results of the assay showed phenotypic resistance patterns to both drugs in 19 isolates (or 73.08%). Six isolates were determined susceptible to both drugs, while one isolate was susceptible-dose dependent also to both antifungals. Detection of *ERG11* mutations following nucleotide sequencing revealed the presence of point mutations A369C, T462C and C558T. Mutations A369C and T462C have been identified as possible factors associated with the resistance to azole agents in previous studies. The results imply that it is imperative to continuously perform susceptibility testing on clinical isolates of *C. albicans* for effective treatment management and for the surveillance of antifungal resistance in the organism.

Key words – Fluconazole – Voriconazole – Antifungal resistance – Susceptibility testing

Introduction

Candida albicans is an opportunistic fungal pathogen, which is reported to cause a wide spectrum of diseases such as vagino-mucosal, oral, and systemic infections (Mayer et al. 2013). Among the three classes of antifungal agents (polyenes, echinocandins and azoles) that are available for treatment of mucosal and invasive fungal infections, azole drugs exhibit a high *in vitro* and *in vivo* activity against *C. albicans*. Azoles like fluconazole are commonly used due to their safety and tolerance profile (Johnson & Perfect 2010). However, prolonged exposures to this drug can result in the emergence of acquired resistant isolates and treatment failures (Ostrosky-Zeichner et al. 2011). The increase in the prevalence of fungal infections in the recent years and the concomitant widespread use of antifungal agents have resulted in the increase in drug resistant

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strains (Dagi et al. 2016, Pfaller 2012). Thus, antifungal susceptibility becomes essential since *C. albicans* has already evolved a multitude of mechanisms as a means of surviving against exposure to antifungal drugs (Whaley et al. 2016).

Mechanisms of resistance to azole drugs of *C. albicans* include the overexpression of the drug efflux pump–encoding genes *CDR1* and *MDR1*. The *CDR1* gene is involved in resistance to all azole agents, while the *MDR1* gene encodes for a fluconazole-specific efflux pump (Gulat & Doluca 2014). In addition, mutations in the *ERG11,* which codes for lanosterol-14-α-demethylase involved in ergosterol synthesis, have also been identified and found to be associated with fluconazole resistance (Flowers et al. 2012). Studies have shown that spontaneous point mutations in *ERG11* in fluconazole resistant clinical isolates have resulted to an over expression of *ERG11* (Hoot et al. 2011, Sasse et al. 2012).

The study aimed to investigate the susceptibility of *C. albicans* isolated from two tertiary hospitals in the Philippines to fluconazole and voriconazole. It also determined the occurrence of point mutations in the *ERG11* gene of *C. albicans* which may be associated with the resistance of the isolates to the azole drugs. We deemed this study essential since antifungal susceptibility testing is not routinely conducted in clinical laboratories in the Philippines. Literature search likewise showed that the most recent published report on antifungal susceptibility of *C. albicans* in the country was the study of Tan et al. (2016), which reported 17 isolates that were all susceptible to fluconazole and voriconazole.

Materials & Methods

Candida albicans isolates and drug susceptibility testing

Candida albicans isolated from clinical specimens from two tertiary hospitals in Metro Manila, Philippines from November 2016 to January 2017 were included in the study. The 26 clinical isolates were cultured and maintained on Sabouraud dextrose agar (SDA, Merck). These were identified using phenotypic and genotypic assays, which included germ tube and chlamydospore production, carbon assimilation, and internal transcribed spacer (ITS) sequencing. Susceptibility of the *C. albicans* isolates to the azole drugs fluconazole and voriconazole (Liofilchem, Italy) was determined using the standard disc diffusion of the Clinical and Laboratory Standards Institute (CLSI M44–A2 2009). A fluconazole-susceptible strain *C. abicans* ATCC 14053 was used as a reference. Zones of inhibition were compared with CLSI interpretative guidelines on antifungal susceptibility testing as follows: Resistant (R): $\leq$ 14 mm; Susceptible-Dose Dependent (S–DD): 15–18 mm, and Susceptible (S): $\geq$ 19 mm.

DNA extraction

Genomic DNA from nine azole resistant *C. albicans* isolates was extracted using InstaGene matrix solution (Bio–Rad) according to the manufacturer's instructions.

PCR amplification and *ERG11* sequencing

A region of the *ERG11* gene from the selected resistant isolates was amplified through PCR using the following primer pair: forward *ERG11* (5'– GCAGCTTCATCATGGTCAACAACC-3') and reverse *ERG11* (5'– TAACATTGGCAACCCCATGAG-3') (Favre et al. 1999, Strzelczyk et al. 2013). These primers amplify a 325bp region in the *ERG11* gene where missense point mutations have been previously reported and commonly detected. The reaction mixture consisted of 1x PCR buffer, 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 0.05 unit/ µl of DNA polymerase, 0.5 µM of primers, and approximately 10 ng of template DNA. PCR run was conducted as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 sec, and 72 °C for 30 sec, and a final extension at 72 °C for 4 min. The amplicons were sent to First Base Laboratories (Malaysia) for sequencing. The amplified *ERG11* sequences were compared with a previously reported *ERG11* sequence (GenBank accession number X13296) retrieved from a fluconazole-susceptible *Candida* strain (Lai & Kirsch 1989).

Results

This study investigated a total of 26 clinical *C. albicans* isolates from two hospitals collected from November 2016 to January 2017. The results of the *in vitro* susceptibility testing are shown in Tables 1, 2, whereas Fig. 1 shows some of the results on the test plates. All the isolates that showed resistance to both antifungals did not show any zone of inhibition on the test plates following the appropriate incubation (Table 1). These observed phenotypic results imply that the 19 *C. albicans* were highly resistant to both voriconazole and fluconazole. Among the 26 isolates, a high percentage of 73.08% showed resistance to both fluconazole and voriconazole, in which 19 *C. albicans* isolates were resistant to both antifungals (Table 2). Six of the isolates (23.08%) on the other hand, were susceptible to both antibiotics. One isolate (3.84%) was identified as susceptible-dose dependent, which indicates that susceptibility of this isolate is dependent on achieving maximal blood levels of the azole drug.

Table 1 Antifungal susceptibility phenotypic patterns of clinical *Candida albicans* used in this study.

Isolate Code	Fluconazole (diameter of ZOI, mm)	Voriconazole (diameter of ZOI, mm)	Phenotypic Susceptibility
JRP11	--	--	R
JRP41	--	--	R
JRP51	--	--	R
JRP52	--	--	R
JRP53	--	--	R
JRP61	--	--	R
JRP62	--	--	R
JRP72	--	--	R
JRP91	--	--	R
CGP21	20	23	S
CGP22	32	30	S
CGP23	32	32	S
CGP41	--	--	R
CGP61	15	17	SDD
CGP62	24	24	S
CGP73	--	--	R
CGP74	--	--	R
CGP81	25	25	S
CGP82	--	--	R
CGP83	--	--	R
CGP101	22	24	S
CGP102	--	--	R
CGP103	--	--	R
CGP104	--	--	R
CGP121	--	--	R
CGP122	--	--	R
C. albicans ATCC 14053	32	32	S

(--) - no zone of inhibition (ZOI); (R) - resistant; (SDD) - susceptible dose dependent; (S) – susceptible.

Table 2 *In vitro* susceptibility patterns of *Candida albicans* to azole drugs.

	R	SDD	S
Azole drug	**no. of isolates (%)**	**no. of isolates (%)**	**no. of isolates (%)**
Fluconazole (25 μg)	19 (73.08)	1 (3.84)	6 (23.08)
Voriconazole (1μg)	19 (73.08)	1 (3.84)	6 (23.08)

(R)- resistant; (SDD)-susceptible-dose dependent; (S)- susceptible.
N= 26 clinical isolates.

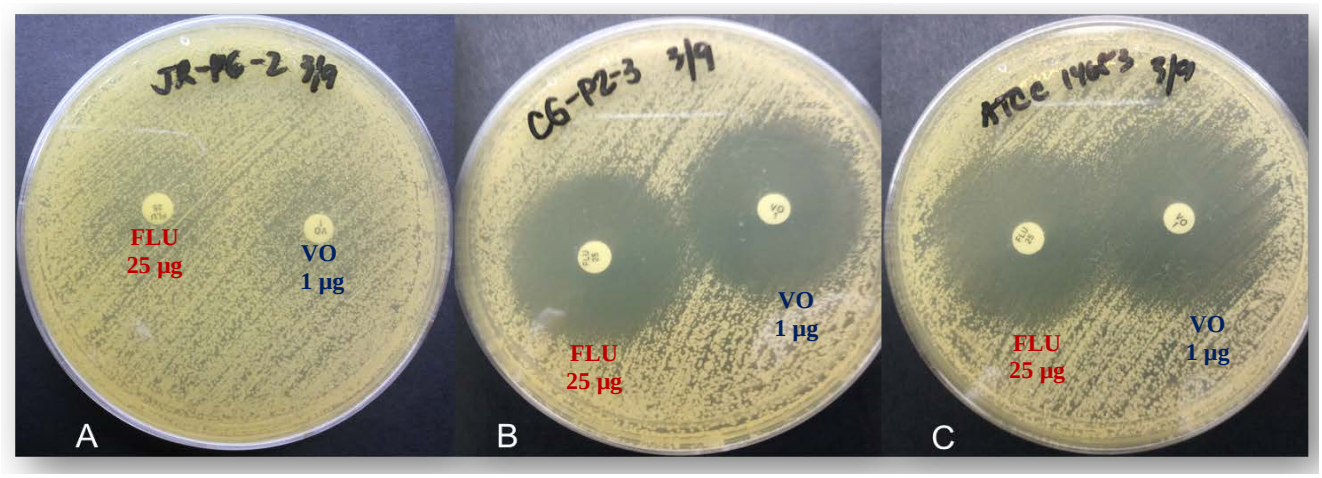

Fig. 1 – Antifungal susceptibility patterns of *Candida albicans* isolates to azoles. A *C. albicans* clinical isolate resistant to fluconazole (FLU) and voriconazole (VO). B *C. albicans* clinical isolate susceptible to fluconazole and voriconazole. C *C. albicans* ATCC 14053 exhibiting susceptible phenotypic patterns to fluconazole and voriconazole.

This study also detected the presence of *ERG11* mutations in the azole-resistant clinical *C. albicans* isolates, which could be a potential mechanism for resistance among the isolates. Fig. 2 shows the amplicons of a region of the *ERG11* gene that were sequenced. Three point mutations were detected following sequence analyses of the selected resistant isolates: A369C, T462C and C558T (Fig. 3). Mutations T462C and C558T were observed in seven azole-resistant strains, while point mutation A369C was observed in one resistant isolate.

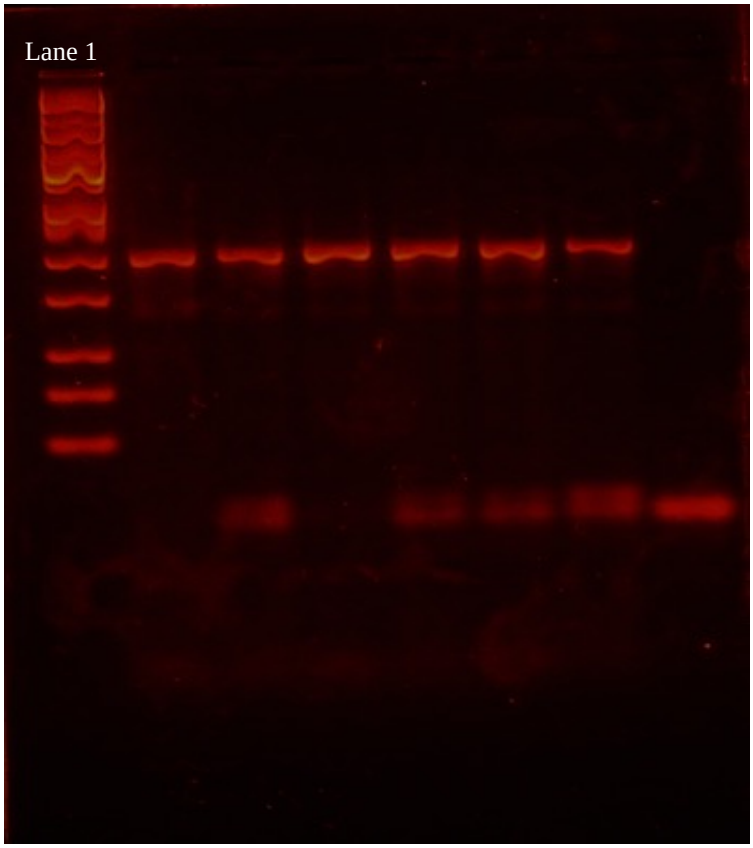

Fig. 2 – DNA bands following PCR amplification using *ERG11* primers. Lane 1 – 1 kb DNA ladder; lane 2 – positive control (*C. albicans* ATCC 14053 strain); lane 3–7 test isolates in the study, lane 8: negative control (DEPC water).

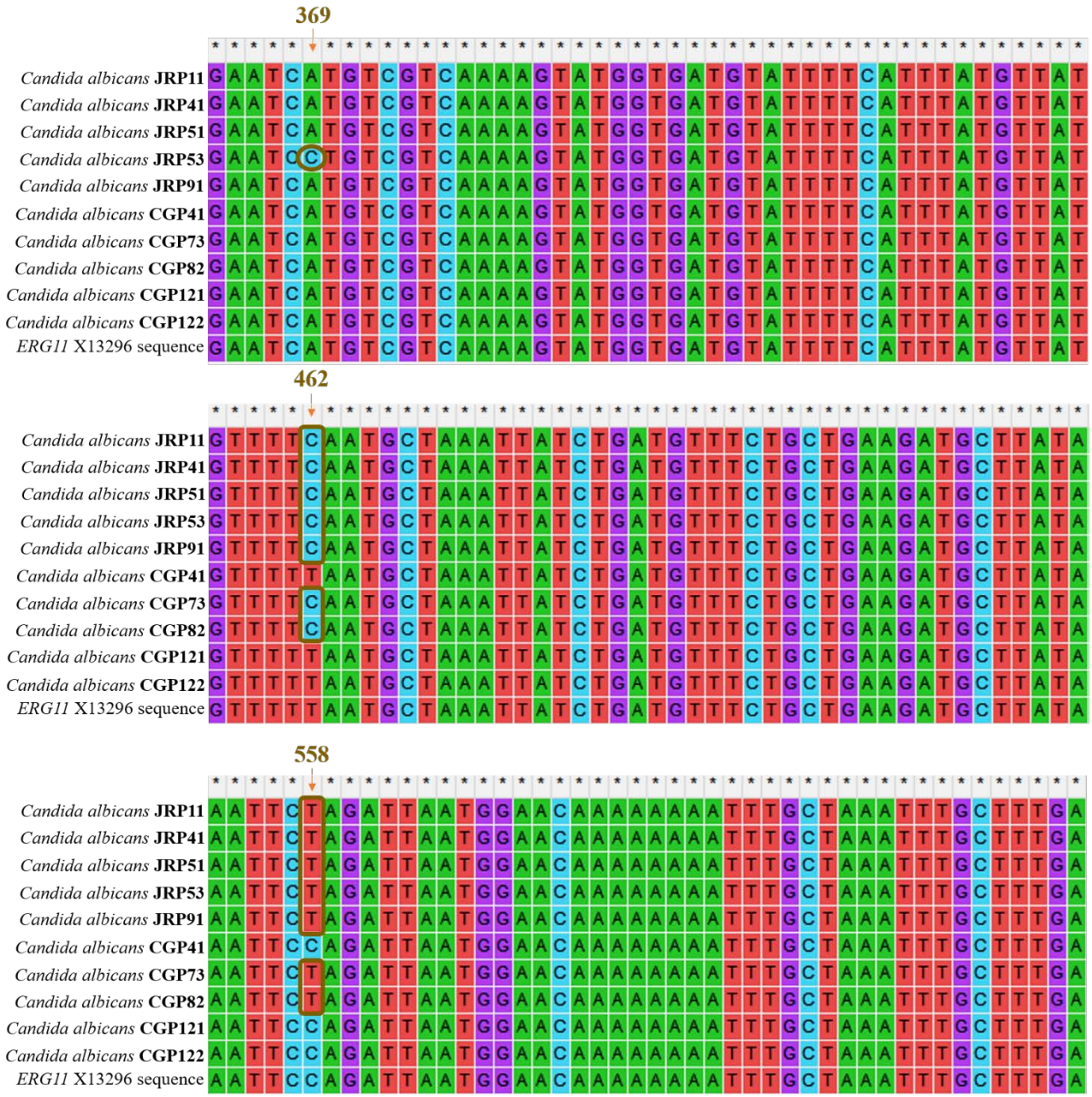

Fig. 3 – Sequences alignment of specific *ERG11* regions showing point mutations in nucleotide positions 369, 462, and 558.

Discussion

Azoles, particularly fluconazole, are currently the commonly administered antifungals in the clinical setting, and the occurrence of azole resistant strains from clinical samples is an utmost concern (Vandeputte et al. 2012). The results of our *in vitro* susceptibility testing are in marked contrast with the data obtained by Bulmer et al. in 1999 and the study of Tan et al. in 2016 on the fluconazole susceptibility of yeasts from the Philippines. Bulmer et al. reported that all of the 287 (or 100%) *C. albicans* isolates studied were susceptible to fluconazole, which was different from the higher levels of resistance occurring in developing countries at that time. Fluconazole was then still newly introduced to the Philippines. Likewise, the very recent study of Tan et al. (2016) on the antifungal susceptibility of invasive *Candida* bloodstream isolates from 13 centers in seven countries in the Asia-Pacific region collected in a two-year period (2013–2015) showed that 100% of the 17 *C. albicans* isolates from the Philippines were susceptible to both fluconazole and voriconazole, which were consistent with the overall 99.7% fluconazole susceptible and 100%

voriconazole susceptible *C. albicans* isolates from the region. The results of our study now indicate the occurrence of a high 73.08% fluconazole and voriconazole resistant *C. albicans* strains. The results were unexpected especially when compared to the aforementioned results of Tan et al. in 2016. The results reinforce the very rapid emergence and presence of azole-resistant strains of *C. albicans* in the Philippines.

It should be noted that all strains resistant to fluconazole were the same strains exhibiting phenotypic resistance to voriconazole. This shows the cross resistance of the isolates to similar azole drugs, since voriconazole is an expanded-spectrum triazole derivative structurally derived from fluconazole (Kofla & Ruhnke 2005). It has been previously established that *Candida* isolates showing resistance to an azole drug also exhibit cross resistance to other azole antifungals (Odds 1993, Müller et al. 2000, Wang et al. 2017). In addition, cross resistance can also be attributed to pre-exposure to other agents, which have regulated azole resistance by inducing expression of drug efflux pump-encoding genes (Ben-Ami et al. 2012). Similar results were also noted in other studies (Haddadi et al. 2014, Pfaller et al. 2007), which have previously documented that resistance to fluconazole also predicted resistance to other types of azole drugs. For recently developed azoles such as voriconazole and posaconazole, specific mechanisms of resistance have not yet been reported in detail, though in general, azoles inhibit biosynthesis of ergosterol (Shapiro et al. 2011). Lyon et al. (2010) suggested that perhaps mechanisms of resistance to voriconazole are the same as those for fluconazole. These mechanisms of resistance involve the mutations and alterations in the *ERG11* and upregulation of the CDR and MDR efflux pumaps (Manastir et al. 2011, Salari et al. 2016).

Mutation in the *ERG11* gene coding for lanosterol-14-α-demethylase is an important mechanism of resistance against fluconazole. Point mutations in the *ERG11* that results in the overexpression of this gene are reported to be involved in the development of resistance to azole drugs (Ge et al. 2010, White et al. 2002). The presence of point mutations T462C and A369C is related to the occurrence of resistance to the azole drugs, and is significantly associated with *ERG11* expression (Gołabek et al. 2015). On the other hand, the C558T mutation is reported to be present in both susceptible and resistant strains of *C. albicans* (Marr et al. 1998). Several studies have previously indicated that some *ERG11* point mutations may occur in both azole susceptible and resistant strains (Perea et al. 2001, White et al. 2002). Thus, it is reasonable to conclude that the C558T point mutation is not associated with the resistance exhibited by the test isolates. For the azole resistant strains that did not show the T462C and A369C mutations, other mutations may be present in the regions of the *ERG*11 gene that were not studied, or the resistance may be due to other mechanisms.

Overall, this study detected the existence of azole-resistant *C. albicans* strains obtained from different clinical specimens and the presence of point mutations in the resistant isolates as potential mechanism of resistance. It is suggested that further monitoring and continuous surveillance of emerging azole-resistant strains must be conducted as these protocols are not commonly done in the country.

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