Efllux pumps genes of clinical origin are related to those from fluconazole-resistant *Candida albicans* isolates from environmental water

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**ABSTRACT**

Efllux pumps coded for by *CDR1, CDR2, FLU1* and *MDR1* genes could be responsible for the observed resistant phenotypes in azole-resistant *Candida albicans* from environmental water. This was demonstrated for clinical isolates. The aim of this study was to determine the presence and genetic similarity between efllux pump genes from clinical and environmental *C. albicans* isolates.

Yeasts were isolated and identified using 26S rRNA gene sequencing. Disk diffusion tests were conducted. PCR was used to detect the presence of efllux genes. The fragments were sequenced and subjected to BLAST and subsequent phylogenetic analysis. Thirty seven *C. albicans* were identified from five selected rivers; Mooi River (19 isolates), Harts River (9 isolates), Marico River (5 isolates), Crocodile River (3 isolates) and Schoonspruit River (1 isolate). All the isolates were completely resistant to azoles. Efllux pump genes were detected in most (>60%) of the isolates. Phylogenetic analysis showed high sequence similarity between sequences from environmental isolates and clinical isolates. Resistance to the azoles and the detection of efllux pump genes renders these antifungal agents ineffective. This is a major problem, particularly for the immune-compromised sector of the community of the North West Province and warrants further investigation.

**Key words** | *Candida albicans*, efllux pump genes, fluconazole resistance, phylogenetic analysis, yeast infections

**INTRODUCTION**

Yeast species have been isolated from North West Province (NWP) rivers (Van Wyk et al. 2012; Monapathi et al. 2017). Some of these are known human pathogens/opportunistic pathogens (Yamaguchi et al. 2007). *Candida albicans*, the most notable opportunistic pathogen (Ahearn 1998) has been isolated from NWP surface water resources (Van Wyk et al. 2012; Monapathi et al. 2017). In healthy people, it is a commensal, harmless colonizer of mucosal surfaces (Achkar & Fries 2010). However, in immune-compromised people such as cancer and transplant patients, as well as those infected by the human immunodeficiency virus (HIV), it causes superficial as well as life-threatening systemic infections (Fridkin & Jarvis 1996; Morschhäuser 2002; López-Martínez 2010; Mayer et al. 2013). The presence of *C. albicans* in surface water environments has been linked to polluted waters (Cook & Schlitzer 1981; Van Wyk et al. 2012; Monapathi et al. 2017). This is a concern as people in the NWP use surface water for different purposes that involve direct contact such as recreation as well as religious activities (NWDACE, 2008). In such direct contact with water, immune-compromised individuals are at risk of being infected with *C. albicans*. In the North West Province the HIV prevalence rate has been estimated to be at 13% (Shisana et al. 2014).

Antifungal agents are used to treat mycotic infections. These are categorized into different classes based on their action mechanism. The various classes include the azoles, polyenes and pyrimidines. Azoles are mostly preferred in mycotic therapy (Mansfield et al. 2010). Fluconazole, an azole derivative, is the antifungal of choice in South Africa (Truter & Graz 2015) and is part of the prophylactic treatment in HIV-positive patients to prevent increasing infections by *Candida* spp. and *Cryptococcus* spp. (Morschhäuser 2002;
Abrantes et al. 2014). The latter species (Cryptococcus spp.) cause cryptococcosis and causes of death among HIV-infected patients (Srichatrapimuk & Sungkanuparp 2016). Azoles enter the yeast cells by facilitated diffusion (Mansfield et al. 2010) and inhibit lanosterol 14α-demethylase, a critical enzyme in the ergosterol biosynthetic pathway (Akins 2005). Ergosterol is the major sterol in fungal membranes responsible for fungal cell growth and proliferation as well as fluidity and integrity of the cell membrane (Joseph-Horne & Hollomon 1997; White et al. 1998). The enzyme inhibition leads to depletion of ergosterol as well as the production of a toxic methylated sterol. Consequently, the membrane functions are impaired (Morschhäuser 2016).

Fluconazole is also mostly used to treat Candida infections due to its favourable bioavailability and safety outline (Rex et al. 1995; Morschhäuser 2002). However, fluconazole is not completely metabolised in the body and it is excreted in the urine and eventually ends up at wastewater treatment plants (WWTPs). Most WWTPs in the NWP are poorly managed (Greendrop report 2013) and sewage may not be properly treated. Consequently antifungal drugs and other antimicrobial chemicals may not be removed by the WWTP processes and could end up in surface water. Antifungals can also be introduced into water resources through run off from agricultural settings where azoles are used to protect grain crops from fungal diseases (Boyacioglu et al. 1992; Mateo et al. 2013).

Prophylactic and continuous use of fluconazole has resulted in the development of yeast strains that are resistant to this and related drugs (Ruhnke et al. 1994; Monapathi et al. 2017). For clinically relevant C. albicans several molecular mechanisms that explain resistance to fluconazole have been documented. These include the most frequent multidrug transporters encoded by the active efflux pump genes (CDR1 and CDR2, FLU1 and MDR1) (Albertson et al. 1996; White 1997) or the alteration (either by mutation or by overexpression) of the target enzyme, 14α-lanosterol demethylase. The latter enzyme is encoded by the gene ERG11 (Sanglard et al. 2016). On the other hand, the inactivation of the sterol C5.6-desaturase is encoded for by ERG3 gene (Cernica & Subik 2006). The product of the latter gene (ERG3) is also involved in the ergosterol biosynthesis pathway. A series of isolates from individual patients have shown an amalgamation of several of these mechanisms that could result in a stepwise development of clinically relevant fluconazole resistance (White et al. 1998; Morschhäuser 2002).

Efflux pumps mediated resistance is the dominant drug-resistance mechanism seen in yeast from HIV-infected patients (Perea et al. 2001). The resistance mechanism uses transporter protein pumps to transport toxic substances as well as drugs across the fungal plasma membrane to the external environment. The accumulation of drugs in the cells is reduced (Webber & Piddock 2003; Cernica & Subik 2006). Candida drug resistance (CDR1 and CDR2) are genes that encode ATP-binding cassette (ABC) transporters. Multidrug resistance (MDR1) and fluconazole resistance (FLU1) genes encode major facilitator (MF) drug pumps. MFs use a proton gradient across the membrane as the energy force (Morschhäuser 2002).

Studies conducted in the North West Province have reported the presence of the micro-organisms that are resistant to several clinically relevant antimicrobial agents (Mulamattathil et al. 2014; Molale & Bezuidenhout 2016; Monapathi et al. 2017). The emphasis on efflux pumps as resistance tools has mainly been on clinical isolates as these pose a higher public health threat (Dada et al. 2015). Little attention has been brought to environmental isolates. Molale & Bezuidenhout (2016) determined efflux pumps as the resistance mechanism in Enterococcus species isolated from surface water systems. Mechanisms involved in antifungal resistance have not been determined in antifungal-resistant yeast from the NWP. The aim of this study was to determine antifungal susceptibility of environmental C. albicans and to establish whether: (i) efflux pump genes are present in these isolates; and (ii) these are genetically different or similar to clinically isolated strains.

**MATERIALS AND METHODS**

**Study design**

Antifungal-resistant C. albicans was isolated from North West Province rivers during 2015 and 2016. This was a follow-up study to that described by Monapathi et al. (2017). Water samples were collected from the Mooi River, Harts River, Schoonspruit River, Crocodile River as well as the Marico River (Figure 1). The impacts on the rivers are pollution from domestic, agricultural and mining activities (Bezuidenhout 2013). However, the water is also used in industries, mining, agriculture, domestic and religious activities (Molale & Bezuidenhout 2016; Coetzee et al. 2017).

**Samples collection and yeast isolation**

Water samples were collected aseptically using the direct and dip sampling technique (Monapathi et al. 2017).
Membrane filtration was performed to determine the levels of yeasts in water. For this the membranes were incubated on yeast malt (YM) agar (Wickerham 1951; Van Wyk et al. 2012). Incubation was at 37 °C overnight. Successive streak plating on YM was done to obtain pure colonies. The initial staining and biochemical identification processes were conducted as described by Monapathi et al. (2017).

Molecular identification and antifungal susceptibility tests

Ten millilitres overnight YM broth cultures of the yeast isolates were prepared and centrifuged to obtain a pellet of the cells. Extraction of the genomic DNA was done according to the modified method of Hoffman & Winston (1987),
DNA samples were stored at 4 °C for short periods. NanoDrop® 1,000 spectrophotometer (Thermo Scientific, USA) (NanoDrop 2007) and gel electrophoresis were done to determine the quality and quantity of the extracted DNA. Molecular identification was performed as described in Monapathi et al. (2017). All the 26S rRNA sequences were deposited into GenBank. The provided accession numbers were (KM102991-KM102997), (KM103005-KM103007) and (MF042197-MF042198). The sequences were subjected to phylogenetic analysis (Monapathi et al. 2017).

The Kirby–Bauer disk diffusion method was performed according to CLSI standard M44-A2 (CLSI 2009) to determine antifungal susceptibility of the C. albicans isolates. The details of the methods are described in Monapathi et al. (2017).

Detection of efflux-mediated resistance genes

End-point PCR was used to determine the presence of resistance genes: CDR1, CDR2, FLU1 and MDR1 in fluconazole-resistant C. albicans isolates. To amplify these resistance genes, the following primers were used: CDR1 and CDR2fwd (5’TATGTCAGATTCTAAGATGTC-3’) and CDR1 and CDR2rev (5’TGATACCTTCACCTCTG-3’) and FLU1fwd (5’CAAGATTGCTGCTGAG-3’) and FLU1rev (5’TGGCTTCCTCGATAATTCA-3’) and MDR1fwd (5’TTACCTGGAACTTTTGCCAAACA-3’) and MDR1rev (5’ACTTTGTGATTCTGTCTGATCCG-3’) (Mukherjee et al. 2003; Chau et al. 2004, Li et al., 2005). PCR was carried out in a total volume of 25 μl. PCR reagents consisted of: (i) 12.5 μl double strength AmpliTaq Gold® 360 Master Mix (Applied Biosystems, USA) (AmpliTaq Gold DNA Polymerase, 0.05 U/μl, GeneAmp PCR Gold Buffer, 30 mM Tris/HCl, pH 8.05, 100 mM KCl, dNTP, 400 μM each, MgCl2, 5 mM), (ii) 2 μl of (~50 ng) genomic DNA template, (iii) 5.0 μl primer mix (10 μM) and (iv) 7.5 μl nuclease-free water, Thermo Scientific, Life Sciences, USA). PCR conditions consisted of an initial denaturation (600 seconds, 95 °C) followed by 35 cycles of denaturation (30 seconds, 95 °C), annealing 30 seconds at 52 °C (CDR1, CDR2 and MDR1) or 54 °C (FLU1) and extension (90 seconds at 72 °C). This was followed by a final extension step (420 seconds, 72 °C).

Determination PCR successes

Two microliters of amplified DNA was examined in a horizontal agarose gel (1% w/v) containing ethidium bromide (0.1μg/mL) using 1× TAE buffer [(20 mM acetic acid (Merck, USA); 40 mM Tris (Sigma Aldrich, USA); 1 mM EDTA (Merck, USA), pH 8.0)] as the electrophoresis buffer. Electrophoresis was conducted at 80 volts for 45 min and viewed using a ChemiDocTM (BioRad, USA) imager.

Phylogenetic analysis of resistance genes

The sequences of the amplification gene products were determined (Monapathi et al. 2017). These were submitted to GenBank and accession numbers for the genes are: CDR1/CDR2 (KY979111-KY979117), MDR1 (MF042166-MF042169), FLU1 (MF115144-MF115149). A number of clinically representative CDR1, CDR2, FLU1 and MDR1 yeast gene sequences were downloaded from GenBank to compare their phylogenetic relationship with the environmental yeast genes. Multiple-sequence alignment was done using ClustalW version 1.8 (Thompson et al. 1994). DAMBE was used to edit aligned sequences (Xia & Xie 2001). The neighbour-joining method in MEGA version 7.0 software (Kumar et al. 2016) was used to construct a phylogenetic dendrogram.

RESULTS

Molecular yeast species identification

Two hundred and thirty five yeasts were isolated and identified from the selected five rivers using 26S rRNA gene sequencing. The PCR amplicon size of these yeast isolates for 26S rRNA was between 600 to 650 base pairs (bp) (Figure 2). Of these isolates, 37 were C. albicans that were obtained from the Mooi River (19 isolates), Harts River (9 isolates), Marico River (5 isolates), Crocodile River (5 isolates) and Schoonspruit River (1 isolate) (Table 1). The
numbers of the isolates varied but have no quantitative relevance.

A constructed phylogenetic tree revealed a high gene sequence similarity between clinical sequences from GenBank and environmental isolates of the 26S rRNA gene. A bootstrap confidence of 82% supported the relationship between the isolates. The bootstrap support is based on 1,000 replicates (Figure 5(a)).

**Antifungal susceptibility**

From the identified yeast isolates, susceptibility testing was done on *C. albicans* using a Kirby–Bauer disk diffusion method. In accordance with the zone breakpoints and interpretative categories for antifungal agents as recommended by CLSI (2009), all *C. albicans* isolates were completely resistant to the following azoles: fluconazole, econazole, ketoconazole, miconazole, and itraconazole. Resistance was also observed to flucytosine, a pyrimidine with a completely different action mechanism. However, all these isolates were intermediately resistant to nystatin.

**Presence of resistance genes in *Candida albicans***

*Candida* drug resistance (CDR1 and CDR2), multidrug resistance (MDR1) and fluconazole resistance (FLU1) genes were amplified and detected in all *C. albicans* isolates from the selected rivers (Table 1). PCR amplicon sizes of these resistance genes from gel electrophoresis are shown in Figure 2. Efflux pump genes were detected in most (≥60%) of the isolates. *C. albicans* isolates had more than one of the genes present. *Candida* drug resistance genes (CDR1 and CDR2) were the most frequently detected genes followed by the fluconazole-resistance gene (FLU1).

**Phylogenetic analysis of the efflux pumps genes**

Phylogenetic analysis reveals high gene sequence similarity between clinical sequences from GenBank and environmental isolates from the present study. There was 99–100% bootstrap confidence support for these relations. The bootstrap support is based on 1,000 replicates (Figure 3(b)–3(d)). Primers that were used in the study amplified both CDR1 and CDR2. The clustering between CDR1/CDR2 genes of this study and clinical CDR1 suggests that the environmental isolates possess the identical CDR1 gene that is found in clinical strains.

**DISCUSSION**

The aim of this study was to determine the antifungal susceptibility of *C. albicans* isolated from NWP rivers as well as to determine efflux-mediated resistance mechanisms in the isolates. A study by Monapathi *et al.* (2017) showed that *C. albicans* and other yeast species isolated from two rivers in the NWP were resistant to several clinically relevant antifungal agents that are used to treat yeast infections in humans. There is limited information on the distribution of these pathogenic yeasts as well as the genotype of antifungal resistance. In the present study, 36 fluconazole-resistant *C. albicans* species were isolated from environmental water and their efflux pumps mediated resistance was investigated. Resistance genes from these environmental species were compared with clinical isolates and similar genes were observed in the two resources. The results from the present study are important when considering that surface water resources in the NWP are used for domestic, mining, recreation and religious purposes as well as agriculture (animal watering and irrigation; NWDACE, 2008; Van Wyk *et al.* 2012; Molale & Bezuidenhout 2016).

*C. albicans*, the most common human fungal pathogen with an infectious mortality rate of ~40% (Gunsalus *et al.* 2015), has been isolated from some of NWP water resources (Van Wyk *et al.* 2012; Monapathi *et al.* 2017). The presence of *C. albicans* in water has been linked to faecal pollution of water (Cook & Schlitzer 1981). The origin of this species in the surface water of the NWP could potentially be due to pollution from WWTPs. In many previous studies, WWTPs are implicated in the dissemination of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes to environmental waters (Bouki *et al.* 2013; Rizzo *et al.* 2013). The present study is thus contributing to understanding...
the role of urban WWTPs in the dissemination of the broader resistome to environmental waters. According to the Greendrop report (2013), the majority of WWTPs in the NWP do not work effectively or are poorly managed. The presence of fluconazole-resistant yeasts in our rivers confirms this.

In immune-compromised people, such as those with HIV, *C. albicans* can cause severe infections when these individuals come into direct contact with the water. This is a concern as the province has an HIV prevalence rate of 13% (Shisana et al. 2014). Many members of urban, peri-urban and rural communities use the available surface water for recreation and religious purposes that involves direct contact (Coetzee et al. 2017). Infections by antifungal-resistant yeast could have devastating effects on the susceptible sector of communities in the North West Province. Similar scenarios could be evident in other provinces or in other developing countries.

In *Candida* infections, antifungal agents especially azoles are used (Rex et al. 1995). A study by Truter & Graz (2015) demonstrated that fluconazole and nystatin are the most commonly prescribed antifungals in South Africa. This drug is also used as the preferred antifungal agent in the prophylactic treatment of HIV-positive individuals to

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Figure 3 | (a) A neighbour-joining tree showing phylogenetic relationships between *C. albicans* 26S rRNA gene between environmental and clinical isolates (**bold**). A bootstrap test (1,000 replicates) was conducted and the cluster percentage of trees supporting the cluster is provided. (b) A neighbour-joining tree showing phylogenetic relationships between *C. albicans* resistance genes *CDR1* and *CDR2* between environmental and clinical isolates (**bold**). A bootstrap test (1,000 replicates) was conducted and the cluster percentage of trees supporting the cluster is provided. (c) A neighbour-joining tree showing phylogenetic relationships between *C. albicans* resistance gene *FLU1* between environmental and clinical isolates (**bold**). A bootstrap test (1,000 replicates) was conducted and the cluster percentage of trees supporting the cluster is provided. (d) A neighbour-joining tree showing phylogenetic relationships between *C. albicans* resistance gene *MDR1* between environmental and clinical isolates (**bold**). A bootstrap test (1,000 replicates) was conducted and the cluster percentage of trees supporting the cluster is provided.
prevent Candida and Cryptococcus infections (Morschhäuser 2002; Abrantes et al. 2014). The findings of this and a previous study on the resistance to azole antifungals demonstrated general resistance to azoles in environmental C. albicans strains. Furthermore, the present study also shows that the genetic elements responsible for the resistance mechanism is similar to what is observed in the clinical settings. This is of great concern. There is also the potential of the genetic elements to be selected for and maintained in the surface water environments when the selection pressure is maintained. This could be so because fluconazole that is excreted by humans may not be removed by urban WWTPs (Ebele et al. 2017). Such systems were not designed to perform such functions. These residues could end up in water resources. Furthermore, runoff from agricultural activities may also be responsible for residues of this drug finding its way into the water sources. Such residues, even in very low sub-therapeutic concentrations, would be a sufficient selective pressure for the maintenance of antifungal-resistant yeast strains and associated resistance genes. Similar observations have been made in clinical scenarios where the overly long use of these drugs has been responsible for the selection of drug-resistant yeast strains (Luque et al. 2009; Abrantes et al. 2014). Fluconazole resistance is a major problem as it has been routinely administered to treat candidiasis in healthcare facilities in the African continent for an extended period (Powderly 1999; Abrantes et al. 2014).

In the present study, susceptibility test screening was performed on C. albicans isolated from South Africa’s North West Province. The various yeast species from the different river systems presented the same susceptibility pattern to the antifungal drugs. They were resistant to azoles and flucytosine and were intermediately resistant to nystatin. In the treatment and prophylaxis of oro-oesophageal candidiasis in the early 1990s, fluconazole became the antifungal of choice (Maenza et al. 1997). However, in the years following the introduction of fluconazole, resistance was reported in 41% of the patients (Canuto & Rodero 2002). The data reported in this study on C. albicans conform to previously published data regarding its susceptibility to fluconazole (Hazen et al. 2003; Pfaller & Diekema 2004). Multiple antifungal agents can be substrates for efflux-mediated ABC transporters and their expression may lead to cross resistance to different drugs (Ramage et al. 2002). This could explain C. albicans resistance to various antifungal agents (White 1997).

Development of resistance to fluconazole by C. albicans is a genetically generated micro-evolutionary change during antimycotic therapy (Morschhäuser 2016). In the present study, the resistance was determined to potentially link it to the presence of specific efflux pumps that could be intrinsic but these can also be acquired from external sources (Nikaido & Zgurskaya 1999). In yeast genomes there are more than 30 reputed efflux-mediated genes. In the present study multidrug-associated resistance (MDR1 and FLU1) and the Candida drug resistance (CDR 1 and CDR2) genes were determined in fluconazole-resistant C. albicans. These genes were present in the genomes all isolates from geographically different sampling sites. The expression of these genes could either be individually or simultaneously mediated (Franz et al. 1999; Perea et al. 2001).

Efflux-mediated genes were the cause of resistance to fluconazole by clinical isolates from HIV-infected patients (White 1997; Lopez-Ribot et al. 1998). The present study shows that the same genes were also present in C. albicans isolated from environmental water resources. High bootstrap confidence values indicate close phylogenetic relationships of the environmental isolates to the genes of clinical ones, suggesting that the isolates could be of clinical origin or that the genes were disseminated to the environmental isolates (Jones et al. 2004; Ogunsentean 2005; Holmes et al. 2006; Hall 2013). There are limited studies on the sequences of resistance genes in yeast and no sequences for environmental origin could be found in GenBank. Two scenarios could be speculated on for the detection of these phylogenetic poorly distinguishable gene sequences: (i) the genes were disseminated from clinical strains to environmental ones and that these are now widespread in the aquatic environment of the North West Province; and (ii) that the strains isolated were from clinical sources and ended up in the water sources, due to pollution. Whichever of these scenarios is true, it is with apprehension that the antifungal resistance patterns and presence of these genes in the surface waters of the North West Province are noted.

Most of the C. albicans in the present study was isolated from the Mooi River. The river is used for irrigation and recreational activities such as swimming and angling (Van Der Walt et al. 2002; le Roux 2005) as well as for religious purposes (Molale & Bezuidenhout 2016). The other rivers are used in similar ways. Contamination of these rivers with the pathogenic/opportunistic yeast such as C. albicans and direct exposure of these could result in major health problems, especially for the immune compromised.

Globally, only a small percentage of rivers is minimally affected by anthropogenic activities. These are rivers in
CONCLUSION

Antimicrobial resistance is a growing worldwide health risk and major threat to public health. In the present study, antifungal resistance patterns were demonstrated for a human pathogenic yeast species, *C. albicans* that was isolated from five NWP rivers. All the isolates were resistant to fluconazole and other azole-containing antifungal agents. This is a cause for concern because fluconazole is the most prescribed drug in South Africa. It is used as part of the prophylactic treatment of immune-compromised individuals such as those who are HIV positive. Overexpression of efflux pumps has been correlated with antifungal resistance in *C. albicans*. In the current study, ABC transporter genes (*CDR1* and *CDR2*) and major facilitator gene (*FLU1* and *MDR1*) were present in all the fluconazole-resistant isolates. Findings that more than one of these efflux mechanisms in the *C. albicans* environmental isolates are alarming. It could result in cross resistance to various drugs, resulting in multiple antifungal resistance. The presence of resistance genes in clinical isolates is a public health concern and requires further investigation since it is known that the environmental water systems are used for direct contact activities. The results from the present study calls for investigation into the mechanism of antifungal resistance of yeasts from environmental resources using carefully designed experiments to determine the expression profiles of these genes when present in environmental and clinical isolates. The study on efflux pump resistance mechanisms could help in the development of new strategies to combat the resistance problem.

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