

RESEARCH ARTICLE

Bioregulatory Role of *Wickerhamomyces anomalus* Killer Toxin against Pathogenic Members of the Genus *Candida*

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ABSTRACT

Objectives: *Candida* species are endogenous fungus causing both mucosal and invasive infections. With the increasing rate of non-*albicans* *Candida* infections with varying susceptibilities, early and accurate species identification would help the clinician in befitting therapeutic management. So the interest in natural products secreted by a fungus *Wickerhamomyces anomalus* itself as an antimicrobial agent against pathogenic *Candida* species has evaluated in the present study.

Methods: A total of 336 *Candida* species isolated from various clinical samples were included in the study. *Candida* identification, antifungal susceptibility was done by standard procedures. The environmental yeast killer toxin *Wickerhamomyces anomalus* was purified and the killer toxin assay against *Candida* species was done by standard procedures.

Results: The predominant *Candida* species were *Candida albicans* (31%), but the emergence of non-*albicans* *Candida* also noted with 69%. Total antifungal resistance was 117 (35%) with fluconazole resistance in *Candida albicans* 13 (13%) and in non-*albicans* *Candida* were 50 (21%). The characterization of *W. anomalus* toxin has revealed that it is a glycoprotein of having 42.6 kDa active at pH 5.5 and temperatures 250 C. *Candida* isolates showed 292 (87%) sensitivity to the killer toxin.

Conclusions: The study emphasizes the need for rapid and precise speciation and antifungal susceptibility of *Candida* isolates for effective treatment and management strategies. Our study proved the antifungal ability of *W. anomalus* and this resource can be used for alternative treatment of candidiasis with further assessments. *J Microbiol Infect Dis* 2019; 9(3):155-160.

Keywords: *Candida albicans*, Non-*albicans* *Candida*, Yeast killer toxin, *Wickerhamomyces anomalus*

INTRODUCTION

Candidiasis is an important opportunistic fungal infection of immunocompromised hosts with changing epidemiological trends [1]. The increased rate of immunosuppressive conditions during the last few decades, as a result of chemotherapy led to a parallel increase in the incidence of *Candida albicans* species and non-*albicans* *Candida* species causing mucosal and systemic infections [2]. The emergence of antifungal resistant non-*albicans* *Candida* and the irrational use of antibiotics are also complicated the antifungal therapy of candidiasis. So the change in epidemiology and antifungal resistance has created the need of its

speciation and susceptibility testing in selecting the antifungal agent for the treatment of *Candida* infections [3,4].

Natural antifungal proteins are produced by a diverse group of organisms including bacteria, fungi, insects, vertebrates, invertebrates as well as plants. Yeast killer toxins are considered in this group and have been shown to have a broad spectrum of killing activity against various plant and human pathogens [5]. It has been reported that killer phenomenon is widely distributed and almost 100 yeast species belonging to more than 20 genera are killer toxin producers [6]. Among the killer yeasts *W. anomalus* has been found to have a potential

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killing effect on pathogenic and non-pathogenic fungus. The strong therapeutic activity exerted against different experimental mucosal and systemic mycoses by monoclonal and recombinant microbicidal killer toxin antibodies as well as by a synthetic killer peptide suggested new potential strategies for therapy [7,8]. So the need of a novel antifungal agent with less toxicity and wide spectrum of action from a natural source also pointing towards the use of killer toxins from yeasts. Indian studies on yeast killer toxin activity against pathogenic *Candida* species are scanty. Hence the present study was chosen to evaluate the anticandidial effect of yeast killer toxin produced by *W. anomalus* from environmental isolates.

METHODS

A total of (n= 336) *Candida* strains were isolated from patients attending both outpatient and inpatient departments of Travancore Medical College, Kollam. It was conducted over a period starting from August 2011 to August 2016. The study protocol was approved by the Institutional Ethical Committee of the Travancore Medical College Hospital, Kerala registered IEC. No:-001/11.

Various clinical specimens like high vaginal swabs, blood, urine, aspirates, catheter devices, cerebrospinal fluid, oral scrapings, sputum and nail clippings received at the Microbiology laboratory for culture were included in the study. Samples from drainage tubes or catheter bags and *Candida* colony counts below 10^2 CFU/mL of urine and sputum specimens were excluded.

Phenotypic Identification of *Candida* isolates

The Standard protocol for yeast identification included the following tests - KOH mount, Gram's stain, culture on Sabouraud's dextrose agar with chloramphenicol, germ tube test, urea hydrolysis, cornmeal agar morphology, sugar fermentation test, sugar assimilation test (Auxanographic Plate Method), CHROM agar morphology, and VITEK.

Antifungal Susceptibility Testing

Antifungal susceptibility testing of the *Candida* isolates was performed by micro broth dilution method in accordance with Clinical and Laboratory Standard institute (CLSI) guidelines M27-A3 [9].

Antifungal agents

Antifungal agents were used in powder forms amphotericin B (AMB- Sigma) fluconazole (FCZ- Pfizer NY), 5-flucytosine (Sigma), voriconazole (VCZ- Pfizer NY), caspofungin (Merck & Co., Inc) E strips were used only for blood isolates.

Wickerhamomyces anomalus killer toxin assay

W. anomalus was the environmental yeast killer toxin strain originally isolated from cashew leaves was used in the study. The strains were maintained on YEPD agar slants at 20 °C. For the killer toxin production and purification *W. anomalus* strains were cultured in YEPD medium. YEPD broth medium supplemented 5% glycerol buffered with citrate phosphate to pH 4.5 was used for killer toxin production [10,11].

Purification of killer toxin

The crude toxin of *W. anomalus* was subjected to ammonium sulphate precipitation (30-100%), and each of the precipitated fraction was dissolved in citrate phosphate buffer (50 mM, pH 5), and examined for killer activity and protein content by Lowry's method [12]. The eluted fraction after ammonium precipitation which showed killer activity were pooled, dialyzed, and subjected to gel chromatography using sephadex gel filtration G-10 (5.5 cm x 100 cm) (Sigma Aldrich) [13].

SDS-PAGE

The active fraction obtained from HPLC was subjected to 5 %-20 % linear gradient SDS-PAGE gel in a discontinuous buffer system under denaturing conditions. Sodium dodecyl sulphate (SDS)-PAGE analysis of purified killer toxin protein was done for determining its molecular weight [14].

Agar Well Diffusion Assay

W. anomalus killer yeast isolate was grown in yeast extract peptone dextrose medium (YEPD) for 72 hours [15]. Crude toxin was prepared and poured into wells of diameter 7 mm (at 50 µl per well) cut on yeast extract peptone dextrose medium with methylene blue. The plates pre-spread with *Candida* species. Plates were incubated at 30 °C for 24 hours, and zone of inhibition was measured. The killing activity of

each sample was measured and defined as the mean of inhibition of three replicate wells.

Optimum temperature and pH for *Wickerhamomyces anomalous* killer toxin

The purified toxin was poured in the wells of YEPD plates pre-spread with sensitive *Candida* species and incubated at different temperatures (20, 25, 30 and 37 °C), for 24-72 hours and at different pH (3-7). The activity was determined by measuring the inhibition zone size [15].

Statistical analysis

Descriptive statistics were used for analysis. Chi-square test used for analysis of virulence factors, antifungal susceptibility and killer toxin assay (p value <0.05 was considered to be statistically significant). Statistical software SPSS (ver. 16, IL, Chicago) was used for data analysis. Measure of agreement between the virulence tests and killer toxin assay tests was estimated using cross tabulations. The significance level was set at a p value <0.05.

RESULTS

Species distribution of *Candida* species from clinical specimens

A total of 336 *Candida* isolates were obtained from various clinical specimens during the period of study from 2011 to 2015. A higher rate of *C. albicans* was found in the high vaginal swabs 41 (31%) collected from women of reproductive age, urine 25 (24%) and blood 11 (11%) out of total 103 isolates. The predominant non-*albicans* *Candida* were *C. tropicalis* 87 (26%) followed by *C. krusei* 71 (21%), *C. glabrata* (12%). A total of 40 (12%) isolates were from blood, in which *C. albicans* (28%) were the major isolate followed by *C. tropicalis* (23%). Invasive device related *Candida* infections were 26 (8%), which is associated with urinary catheters and central lines. The major isolate was *C. tropicalis* 7 (27%).

Antifungal Susceptibility by Micro-dilution Method

Total antifungal resistance strains were 117 (35%) out of 336 *Candida* isolates. Fluconazole resistance in *C. albicans* were 13 (13%) and 50 (21%) in non-*albicans* *Candida* isolates, except *C. krusei* because it is not tested for fluconazole as it is inherently resistant. Amphotericin-B and

flucytosine resistance in *C. albicans* were 5 (5%) and 7 (7%). *C. tropicalis* got 27 (31%) fluconazole resistances, 5 (6%) resistance to flucytosine, 4 (5%) resistance to voriconazole, and 3 (3%) resistance to amphotericin B.

Purification of killer toxin

SDS PAGE- Determination of molecular weight

The killer toxin fraction obtained from HPLC was homogeneous on a discontinuous gradient SDS-PAGE with a wide range of molecular mass marker, the toxin moved in a single band near the ovalbumin. The molecular mass of the toxin was 42.6 kDa (Figure 1).

***W. anomalous* killer toxin assay**

A total of 336 *Candida* isolates were screened for killer toxin assay against *Wickerhamomyces anomalous* along with standard strain of *Saccharomyces cerevisiae*. *Candida* isolates 292 (87%) were sensitive to the *Wickerhamomyces anomalous* killer toxin (Figure 2).

Temperature and pH stability of *W. anomalous* killer toxin

The killer activity of *W. anomalous* was higher in low temperature and at low pH but as temperature and pH increased killer activity decreased. Killer activity was highest at pH 5.5 with temperatures 25 °C and lowest at pH 6 and 30 °C.

Cell killing kinetics of *W. anomalous* killer toxin against *Candida* species

Cell killing activity was started with in the first 2 hours for all of the tested *Candida* strain. Complete cell death was observed after 6 hours, in all species except *C. tropicalis* at 4 hours.

DISCUSSION

Candidiasis is the most common opportunistic fungal infection. In the past three decades with the use of potent antibacterial, immune-suppressive drugs and advancement in medical practices increased the frequency of invasive candidiasis [16,17]. The treatment of fluconazole resistance is extremely difficult with limited options of antifungals [18]. Prolonged therapy and increased use of antifungals for recurrent candidiasis are the most common risk factors for

azoles resistance in vulvo-vaginitis candidiasis [19-21]. The advancement of antifungal resistance does not give off an impression of being an issue amid their fleeting use, yet is a chief concern for long term treatments [22].

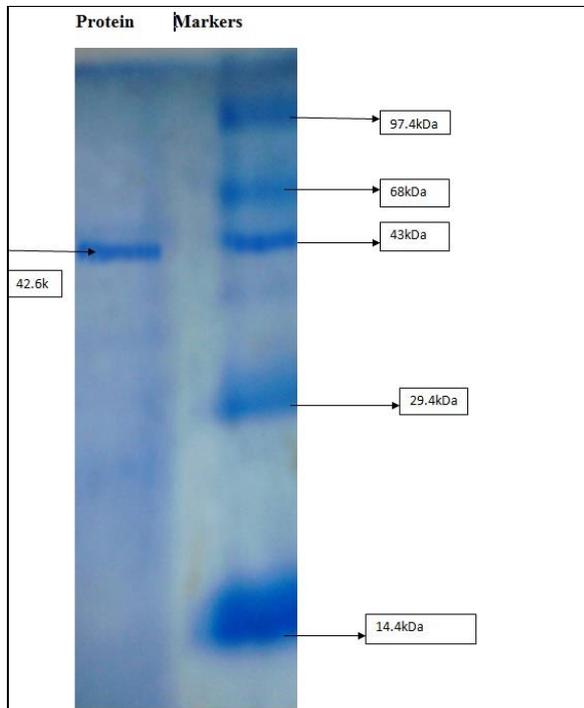


Figure 1. SDS –PAGE analysis of *W. anomalus* Killer toxin protein.

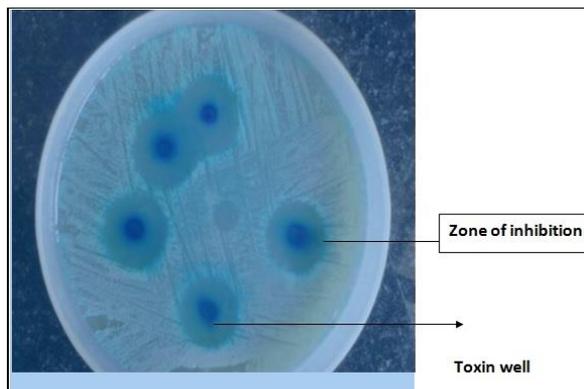


Figure 2. Agar well diffusion assay of Killer toxin.

Killer toxins are reported to have the potential for treatment of fungal infections, which are distributed in several environmental sources. In this study, we aimed to isolate and characterize the yeast killer protein secreted by the yeast *W. anomalus*. Several species of environmental

yeasts *Wickerhamomyces* species have been reported to have the killer character [23]. In our study, we determined the optimum culture conditions for the production of *W. anomalus* killer toxin protein with molecular weight 42.6 kDa with a single band, estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This appears to be another rare finding of our study that a new killer toxin is identified from *W. anomalus* species which is susceptible to pathogenic *Candida* species. These findings are in agreement with other studies characterized different types of killer yeasts having molecular mass K1 (20 kDa), K2 (21 kDa), K3 (42 kDa), PaKT (105 kDa) type killer proteins [24,25]. The present study demonstrated a different killer toxin from *W. anomalus* species rather than the previous discoveries. The diversity of killer proteins of *W. anomalus* with molecular masses ranging from 8 kDa to more than 300 kDa is already established [26,27], however, this wide range in protein size may arise mainly from different levels of glycosylation.

The protein isolated from *W. anomalus* exhibited basic characteristics of mycocin with pH 5.5 and temperature ranging from 4 to 25 °C. One of the most important aspects of this study is the good activity (87%) of killer toxin against the pathogenic strains of *Candida* species including both clinical and standard isolates tested which correlates with the antagonistic action of *W. anomalus* (WC65, NCYC 432, 434, YF07b) killer toxin against other fungus [28].

This paper validates the observation that different killer toxins are produced by different isolates of the same species. The susceptibility of pathogenic yeasts to killer toxin produced by yeasts, belonging to the genera *Wickerhamomyces* could lead to the design of synthetic derivatives to be used as antifungal agents and further studies.

The limited number of antifungal drugs with fewer side effects has increased the incidence of candidial infection. So there is a need to search for new products with the effective antifungal abilities without adverse side effects. Environmental yeast killer toxin *W. anomalus* possessed broad antimycotic activity against pathogenic *Candida* and non-albicans *Candida* species and can be proposed as a novel

antimycotic agent for candidiasis. Purified *W. anomalus* killer toxin is identified as a protein having a molecular weight of 42.6 kDa. It has a wide range of pH (3.5-5.5) and temperature (4-25 °C). The pH 5.5 and temperature (25°C) were appropriate for the use of this toxin in medical and fermentation industry.

Further studies are needed to elaborate an easy-to-use technique for killer toxin which could be used in medicine and which will open its use as an antimicrobial agent and powerful bio control agents in food preservation and fermentation industry. However, the identification of killer characteristics and strain maintenance are labor and cost consuming because the killer characteristics are often controlled by plasmids. Therefore, thorough studies are needed in future regarding the relative stability of purified killer toxins after long term storage, including the establishment of the actual time course of their possible degradation or loss of effectiveness.

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