Plant Archives Volume 20 No. 1, 2020 pp. 2711-2716 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

STUDY OF THE EFFECT OF ANTIFUNGAL ON *CANDIDA ALBICANS* ISOLATED FROM DIFFERENT CASES

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Abstract

Candidiasis is an acute or chronic infection produced by the *Candida* that is normally present in the mucous membranes of oral healthy individuals.

During the period from September to November 2019, a total of 150 clinical samples were collected from AL_Zahraa Teaching Hospital and Middle Euphrates Cancer Center in AL_Najaf Governorate, which included swabs from the mouth through the swab (88 cancer samples, 62 thalassemia samples). The present study was conducted to isolation and identification *Candida albicans* from different clinical cases patients by different methods including direct examination, laboratory culture, finally, identification by PCR and determine virulence factors (adhesion and biofilm formation), determine biofilm by two method (in tube and in microtiter plate). Study the effect of antifungal, alternative material (isomethoxide) on growth *C. albicans* and their virulence factors. This research seems to be, Trbinafine and Fluconazole and isomethoxide are affected on culture and virulence factors of *Candida albicans*

Key words : Candidiasis, Fluconazole, *Candida ablicans*, biofilm.

Introduction

Candidiasis is an acute or chronic infection produced by the Candida that is normally present in the mucous membranes of oral healthy individuals, upper respiratory tracts, gastrointestinal tracts and genital tracts of women, can produce serious systemic disease and it is developed to infection usually in vagina, mouth, on the skin that causes itching, red patches or white patches and irritation (Dabas, 2013). *Candida* is not harmful in healthy hosts, but may cause opportunistic infections in immune-compromised hosts, such as patients suffering from AIDS, leukemia and diabetes (Batool *et al.,* 2011).

The ability of *Candida albicans* to infect such diverse host niches is supported by a wide range of virulence factors and fitness attributes. A number of attributes, including the morphological transition between yeast and hyphal forms (the reversible transition between unicellular yeast cells and filamentous, growth forms), the expression of adhesins and invasins on the cell surface, the formation of biofilms and germ tube (Pankhurst, 2009).*C. albicans* varied in their susceptibility to the most commonly used antifungal agents, and the intrinsic resistance to antifungal therapy seen in *C. albicans*, along with the development of acquired resistance then seen in other species, that could be form a major problem in the management of *Candida* infection. A better understanding of the mechanisms and clinical impact of antifungal drug resistance is required for efficient treatment of patients that infected with *Candida* and for improving treatment outcomes (Alenzi, 2016).

Materials and Methods

Specimen's collection and Identification

The samples were then transported to the laboratory for culture on Sabouraud's dextrose agar and Chrom agar. Then incubated at 37C° for 24-48 hr. for visible growth of *Candida* spp. colonies for diagnosis and study (Sayyada *et al.,* 2010)

Identification by PCR using primer is (*ERG*11) with the following sequences: *ERG*11- F**:** 5'-CAAGAAGAT

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CATAACTCAAT-3'and *ERG*11- R: 5'-AGAACACTG AATCGAAAG-3'.

Biofilm formation by microtiter plate

The microtiter plate test are the most frequently used techniques for quantifying biofilm formation, an important indicator for the pathogenicity of *Candida* colonies. Biofilm was formed on pre-sterilized 96 well flat bottom polystyrene microtitre plates in triplicates as described elsewhere. Briefly, A 10 µl of cell suspension was inoculated in 180 µl SDB medium (contain sucrose) in each well. Then microtitre plate was incubated for 48 h at 37C. Then, crystal violet solution was added to all wells. After 15 min, the excess crystal violet was removed and plates were washed twice by distilled water and air dried. Finally, measuring using micro plate reader (Li *et al.,* 2003 ; Peeters *et al.,* 2008).

Adherence assay

The candidal adhesion assay was performed as follow : an overnight culture of candida isolates on SDA was harvested by using a loop and suspended in PBS in a turbidity adjusted to match a 0.5 McFarland density standard resulting in a suspension containing $5*10^6$ yeast cells/ml. Then 1ml from this suspension was mixed with 1 ml of suspension containing buccal mucosa and suspended in PBS. The mixture was inoculated in a shaking incubator operated at 80rpm at 37C for 1 hr. A drop of this mixture was mounted on glass slide, air dried, heated fixed and stained with safranin for 1 min and adherence assayed under microscpy at 40 lens (Henriques *et al.,* 2007).

Antifungal susceptibility

Three antifungal: Terbinafine, Flucanazole and alternative material (isomethoxide). Use *in vitro* against of *Candida albicans* was investigated, using well diffusion method in Muller Hinton agar, well was made at the four well in Petri dish and filled with four concentrations for each one. The antifungal were diluted by DMSO 10%. 50 ml of each antifungal agent and placed into each well and triplicates with four concentrations and incubated at 37 C for 2-4 days. The diameter of inhibition was measured in millimeters after 48 hours of incubation.

Results and Discussion

Morphological identification

Identification *Candida albicans* on Sabouraud dextrose agar

All collected samples were cultured on Sabouraud dextrose agar (SDA); the colonies of *Candida albicans* were cream colored to yellowish, grow rapidly mature in 24-48 hr., the texture of the colony smooth, glistening or

Fig. 1: Showing growth of *Candida* spp. on SDA at 37°C for 48 hours.

dry depending on the species. These results were agreed with (Bhavan*et al.,* 2010) as show in Fig. 1.

Identification of *Candida albicans* on Chrom agar medium

Chrom agar is a selective medium for the isolation of yeast that simultaneously provides direct differentiation and identification of several C*andida* spp. (Sayyada *et al.,* 2010). This study has showed that using Chrom agar Candida which is considered a differential agar the colonies appear *C. albicans* characterized by leaf-green

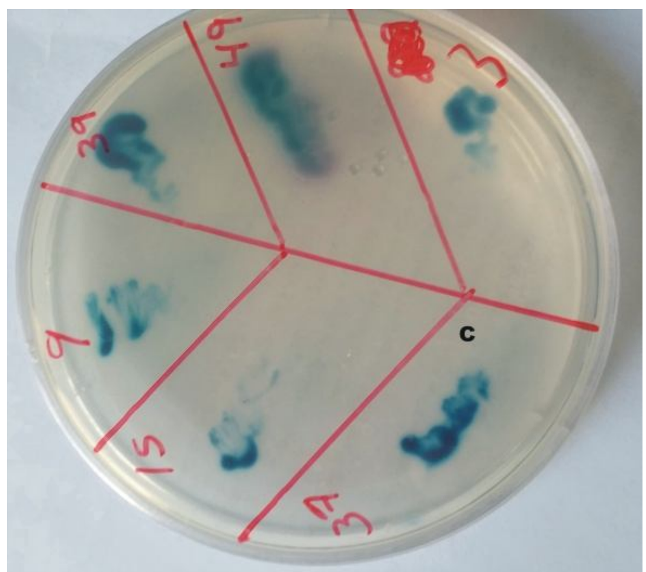

Fig. 2: Show colonies of *C. albicans*, on Chrom agar medium at 37C for 48 hours.

colored colonies Fig. 2.

Chromogenic media are effective and rapid testing in the diagnosis of *Candida* at the species level of the resulting color after inoculation and incubation compared with other culture traditional methods, change in color produced by reactions of species-specific enzymes with a proprietary chromogenic substrate, the medium greatly facilitates the detection of specimens containing mixtures of yeast species. (Iyampillia *et al.,* 2004).

Molecular identification PCR assay

The results showed that our different molecular sizes of *ERG11*Primer of *Candida* spp. (this primer considered

Fig. 3: Agarose gel electrophoresis of PCR products for *Candida* spp. isolates amplified by *ERG*11 Primer (1.5 g agarose gel 80 volts for1 hour).

as universal primer for detection *Candida* spp.). In, it offered PCR products of these isolates in Fig. 3.

Molecular techniques are targeted to detect *Candida* species in a short period of time, with a high sensitivity and specificity. Such as PCR technique (Maaroufi *et al.,* 2004; Innings *et al.,* 2007. In order to overcome the limitations of conventional diagnostic tests, DNA-based methods have been developed for the detection of *Candida* species and offer a potentially more sensitive means of diagnosing candidiasis (Reiss*et al.,* 1998). These results were in disagreement with Bin *et al.,* (2015). The use of PCR-based tests to detect *Candida* DNA has produced encouraging results (Khan *et al*., 2012). DNA amplification with universal fungal primers followed by detection using species-specific probes greatly improved the sensitivity of *Candida* detection (Posteraro *et al.,* 2000).

Virulence factors of *C. albicans*

Biofilm growth in tubes and microtiter plate

The results in this study that shown the ability of all *C. albicans* isolates was positive for biofilm formation, as show in Figs. 4, 5. This result was similar with (Emily *et al.,* 2011). Biofilms have been considered as an important virulence factor in the pathogenesis of infections, biofilm is extracellular matrix (ECM) composed of exopolymeric substances in which the ratio of all macromolecules varies with the environment, the recurrent infections caused by *Candida* spp. are difficult to treat because of their ability to form biofilm, a three-dimensional, complex architecture of surface-adhered cells encased into (ECM) where microbes afford protected environment, *C. albicans* forms highly structured biofilms composed of multiple cell types (round budding yeast-form cells, oval pseudohyphal cells, and elongated hyphal cells) encased in an extracellular matrix (Nett, *et al.,* 2011). giving the biofilm a thick and structured appearance as well as providing protection from chemical and physical injury (Donlan, 2002). A mature biofilm typically forms by 24 hours, and can be

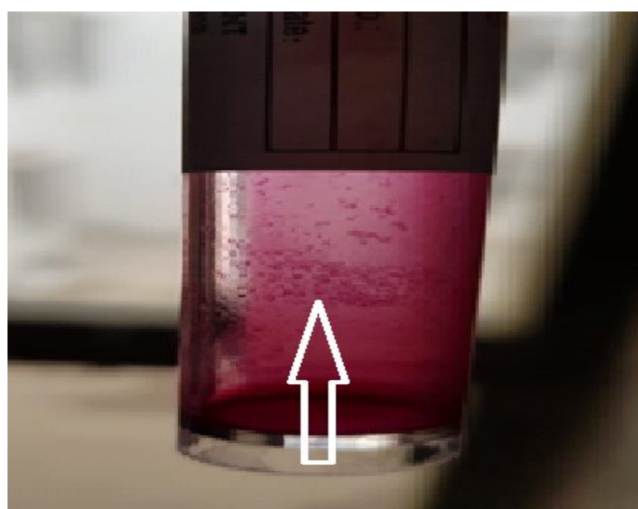

Fig. 4: Biofilm formation of *C. albicans* (positive).

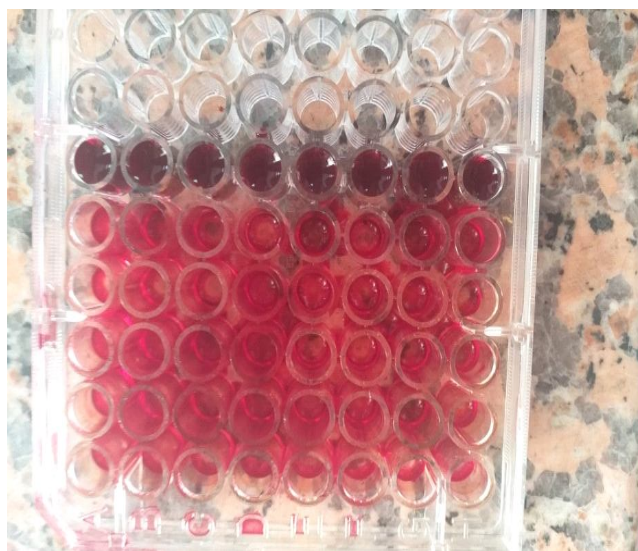

Fig. 5: Biofilm formation by microtiter plate.

visualized by eye as a cloudy surface structure on top of the solid surface, and under a microscope, as an organized collection of different cell types. The ability of *C. albicans* to form biofilms on abiotic or biotic surfaces is an important virulence factor (Achkar and Fries, 2010).

Adhesion test

All the isolates of *C.albicans* showed a positive results to adhesion test after incubation with buccal cavity cells for 1 hour at 37C and then examined under microscope (40x) with add safranin dye, this result show in Fig. 6.

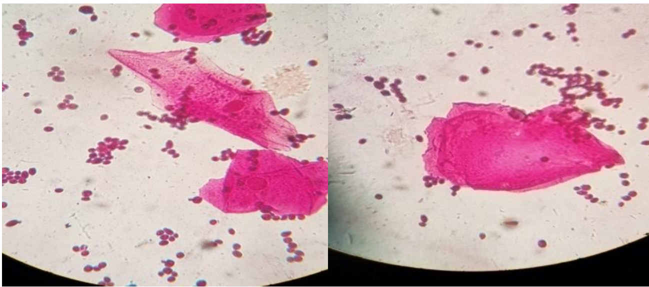

Fig. 6: Adhesion of *C.albicans* to epithelial cells.

The adhesion activity of the protein (peptide) and the mannan moieties of the manno protein in the outer surface of the *Candida albicans* cell wall. A macromolecule of the manno protein located on the outermost surface is undoubtedly a strong adhesin comprising several adhesion molecules including protein and mannan. *Candida* adhesion to host cells (especially epithelial cells) represents the onset of infection, which is next followed by invasion, the strategies of *C. albicans* to attach and invade into the host cells, obtain nutrients, and evade the host immune response show significant differences (Bandara *et al.,* 2009).

Antifungal susceptibility

The result of this study for the antifungal activity for (terbinafine, fluconazole and alternative material (isomethoxide), explaining the effects against *Candida albicans* by using well diffusion method according to statistical results as show in Fig. 7, Fig. 8 and Fig. 9 the concentration of (100µg/ml) for both antifungal drug (fluconazole and terbinafine) the inhibition zone for *C. albicans* 1.4mm and 2.3mm respectively i.e. the meaning of *Candida albicans* is more sensitive. The concentration of (200µg/ml) for both antifungal drug (terbinafine and fluconazole), in this study find that the showed inhibition zone of *C.albicans* 1.8mm for (fluconazole) show, while for the terbinafine the inhibition zone *C.albicans* 2.73 mm. find that the *C.albicans* more sensitive to terbinafine and fluconazole. Alternative material (isomethoxide) within concentration 100mg/ml gave 1.5mm inhibition zone against *C. albicans* while concentration 200mg/ml of alternative material inhibition zone against *C. albicans* was 2.3mm. Antifungals can be grouped into three classes based on their site of action: azoles, which inhibit the synthesis of ergosterol (the main fungal sterol); polyenes, which interact with fungal membrane sterols physicochemically; and 5-fluorocytosine, which inhibits macromolecular synthesis. Antifungal drugs including azoles such as fluconazole, polyenes, allylamines such as terbinafine and flucytosine are mostly commonly used to treat *Candida* infections. Most of these antifungal agents are phenolic structured. The phenolic compounds show the highest anti-Candida bioactivity (Yang, 2003). Both fluconazole and terbinafine decreased growth of *Candida* after incubation period. *C. albicans* in the human body treated by antifungal agents such as azoles (like fluconazole) and allylamines (like terbinafine) as the common antifungal drugs. The primary target of azoles may be the heme protein, which co-catalyzes cytochrome-P450-dependent 14a-demethylation of lanosterol in the last stage of ergosterol biosynthesis, while allylamines act by inhibiting the early stages. Indeed, the inhibition of squalene epoxidase by terbinafine (early step) or 14a-demethylase by fluconazole (last step) of ergosteroln's biosynthesis has principal role in a play of depletion of ergosterol and agglomeration of sterol precursors, resulting some alteration in the structure and function of cell membrane in *Candida* cells (Leonardi *et al*,. 2013).

Effect of antifungal drug on virulence factors (biofilm formation and on adhesion)

The result of this study for effect of fluconazole and

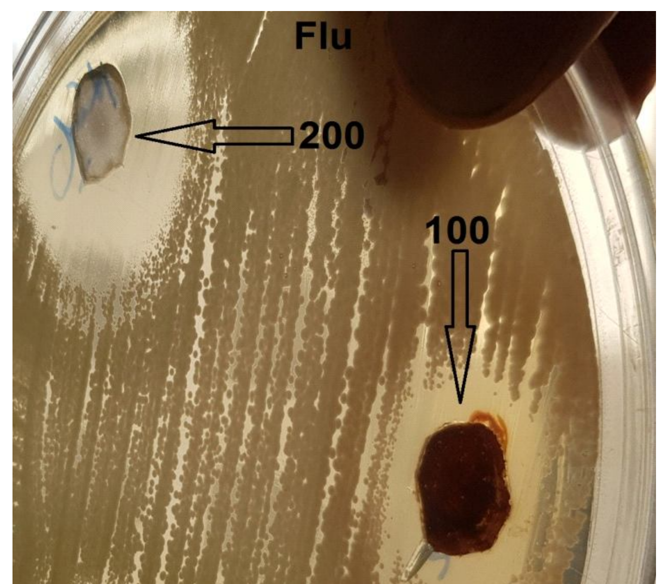

Fig. 7: The sensitivity of *C. albicans* to fluconazole in concentration of (100-200 µg/ml).

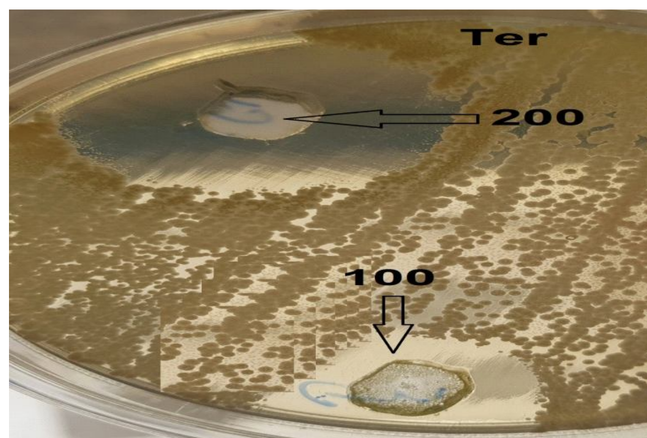

Fig. 8: The sensitivity of *C. albicans* to terbinafine in concentration of (100-200 µg/ml).

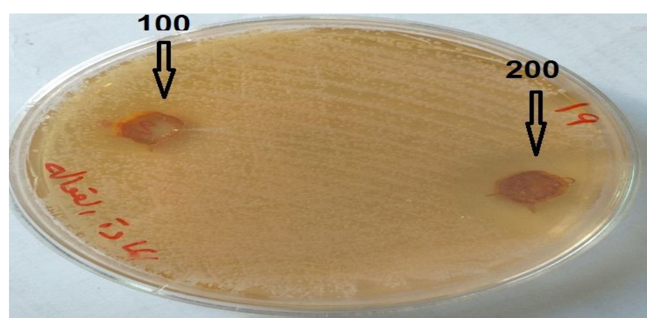

Fig. 9: Effect of alternative material (isomethoxide) against *C. albicans* isolated from oral the patients.

terbinafine on biofilm formation of *C. albicans*, no biofilm formed in both tubes and microtiter plate a results were in agreement with Zahra, (2016). In the presence of fluconazole and terbinafine, clear effect was observed on biofilm formation or viable cells within the biofilm layer (konopka *et al.,* 2010). Also the alternative martial (isomethoxide) Affected or prevented formation of Adhesion of *C. albicans* to epithelial cells the formation of biofilms when the compounds/drugs were added to the medium at the same time as the cells. If an agent is added at the beginning of the experiment, the agent might act before the biofilm is formed and inhibit its development; this could be of interest for combating recalcitrant infections involving *Candida* biofilms. (Vu, *et al.,* 2019). *C. albicans* rely upon the use of antifungal drugs designed to kill the yeast or arrest its growth. An alternative approach, aimed at disrupting the adherence of the yeast to host tissue in cases of superficial infection, may have potential for controlling disease, particularly in situations where the unattached fungal cell can be removed from the affected site, either by the flushing action of the oropharynx or by the production of mucus in the vagina (Teo *et al*., 2019).

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