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Evaluation of Immunization Efficacy for Cell Wall Fraction Antigen Separated from Clinical Isolate of *Candida albicans*

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Abstract

Objective: Preparation of cell wall fraction (CWF) antigen from *Candida albicans* and testing immunization efficiency by evaluating the humoral immune response (by measuring IgG level in serum) and cellular immune response (by measuring IFN-γ and IL-4 concentration).

Methods: *C. albicans* were isolated from woman with vaginal candidiasis and identified morphologically and genetically, first cultured on Sabouraud Dextrose Agar (SDA) and CHROMagar Candida media and identified by biochemical test. Then identified by molecular diagnosis by using ITS1 and ITS4 primers. BalB/c mice were immunized subcutaneously and intradermaly with *C. albicans* strain M366B cell wall fraction antigen for 21 days to evaluate humoral and cellular immune response represented by IgG, Th1 and Th2 cytokine level by microtiterplate ELISA.

Results: IgG level appeared in high concentration in immunized mice by comparison with control groups in a high significant differences ($P<0.01$), and immune response represented by (IL-4) concentration in immunized group was higher than those in control mice in a

significant differences (P<0.01); while there was significant decreasing in (IFN-γ) concentration in mice injected subcutaneously and intraderamly by comparing with control groups. So CWF antigen can efficiently stimulate both humoral and cellular immune response by elevation both IgG and IL-4 concentration in sera of immunized mice.

Conclusions: CWF antigen was immunogenic in regarding in inducing IgG and IL-4 secretion.

Introduction:

The commensal yeast *Candida albicans* considers an opportunistic endogenous fungal pathogen which is responsible for candidiasis (candidiosis) in human. It is inhabiting as a part of microflora of oral cavity, skin, the gastrointestinal tract, vaginal canal, and the urinary environment of the human.

Over growth of these organisms, however will lead to infection that are ranging from superficial infection of the skin to life threatening systemic infections, and it usually occur in immunocompermised patients, such as human immunodeficiency virus (HIV), infected victims, transplant recipients, chemotherapy patients, pregnant women, patients with chronic disease and low birth weight babies [(1)] .

Candida spp. reside as a part of the lower genital tract microflora in (20%– 50%) of healthy asymptomatic women *C. albicans* is the most common colonizer, and is incriminated in most cases of VVC. Through the last ten years, research evidence demonstrated an increase in the repeatedly of cases caused by non-*albicans* species, with *C. glabrata* consistently being the leading species [(2, 3, 4)]. About 75% of women will suffer at least one occurrence of VVC through their lifetime. Actually, among healthy adult women about (70%–75%) have at least one episode of VVC during their reproductive lifespan, and half of college women will by the age of 25 years have had one episode of VVC diagnosed by a physician [(5)].

In recent years, a number of publications have confirmed the immunogenicity and efficacy of vaccines against candidiasis in animal models, and even have tested the efficacy

and safety in clinical trials. Fungal cell-wall polysaccharide, proteins, and live attenuated fungi have been investigated as vaccine targets.

Even considering the capital and technical barriers, bringing protective vaccines to the clinic appears promising [6].

Aim of the study:

The present study was aimed to highlights on one of the most important infections like candidiasis and trying to present an immunization models against candidiasis infection and this aim materialized by:

1. Isolation and identification of *C. albicans* from women infected with vaginal candidiasis.
2. Preparation of cell wall fraction antigen (CWF) and evaluating immunization efficiency by determination of:
   A- Humoral immune response (by measuring total IgG in serum).
   B- Cellular immune response (by determining serum level of IFN-γ and IL-4).

MATERIALS AND METHODS

Three of moist vaginal swap specimens were collected by trained physician from women suspected with vaginal candidiasis infection from the-Child and birth hospital in Al-Basra province in February, 2017. Swaps were streaked on Sabouraud dextrose agar and CHROMEagar Candida media and incubated for (24-48) h at 37o C [7], then identified by germ tube test and study microscopical characters of yeasts on skim milk media using API Candid system. In addition by using ITS1 and ITS4 primers [8]; the isolates from *Candida* spp. were identified by sequencing by MEGA.6 program in macrogen company/Korea.

A Loopfall of *C. albicans* strain M366B colony were inoculated in Sabouraud dextrose broth and incubated for 24-48 h at 37^{o} C under aeration with reciprocal shaking at 100 rpm. Cells of *C. albicans* were harvested by centrifugation for 10 min at 4^{o}C at 1400 rpm washed twice with sterile 50mM potassium phosphate buffer (pH 7.5) containing 1M NaCl as a

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stabilizer. The cells number were adjusted equal to tube number 10 of McFarland standard ($3*10^9$) in a sterile 50 mM potassium phosphate buffer (pH 7.5) containing 1M NaCl. The suspension was treated with 0.3mg/ml Zymolyase -20T and 1mg/ml Tricoderma lysing enzymes. The cell suspension was incubated for 24hr at 37oC with orpital shaking incubator at 100 rpm. The cell suspension was centrifuged at 15000 rpm at 4°C for 10 min, the supernatant was collected as the cell wall fraction (CWF), the supernatant and the sediment were tested for the digestion of cell wall as described above, the total protein for two antigens were measured by equation as follow:

Concentration of protein mg/ml= (1.55*absorbance at 280)-(0.77*absorbance at 260) [(9)].

Two groups of BalB/c mice were used and each one comprises eighteen animals were immunized subcutaneously and intradermaly with 0.2µl CWF antigen emulsified with complete Freund's adjuvant at the first week, after 7 days with incomplete Freund's adjuvant (sigma USA) and at the third week without adjuvant, in addition the control groups were injected with normal saline at the same volume and the methods of injection (5 animals injected intradermaly and 5 animals subcutaneously), then after 21 days from first injection mice were killed; serum was collected for measuring (IgG, IFN-γ and IL-4 levels by microtiterplate ELISA at OD 450 nm).

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Results

Microscopic Examination

The results of direct examination for vaginal swaps that treated with wet preparation and stained slide smear were showed epithelial cells, blastospore (yeasts budding cells), pseudohyhae and true hyphae.

Culturing data results:

Colony colour on CHROMagar Candida and Brilliance ™ Candida Agar:

Depending on colony colour and morphology (smooth/rough) to distinguish the yeast according to enzymatic activity; *C. albicans* appeared with green colour on CHROMagar Candida and Brilliance ™ Candida Agar plates. While *C. parapsilosis* appeared with off white colour on CHROMagar Candida and brown /beige colour on Brilliance ™ Candida Agar plates

Microscopical characters

Germ tube test of *Candida albicans:*

From 3 yeast isolates only two isolates revealed that they have the ability to germ tube formation figure that appear without constrictions distinguished from other species of *Candida.*

Chlamydospore formation test of *C. albicans* on casein agar:

The results of casein agar revealed that *C. albicans* isolates can produce chlamydospore in addition to blastospore of yeast budding cell figure (1):

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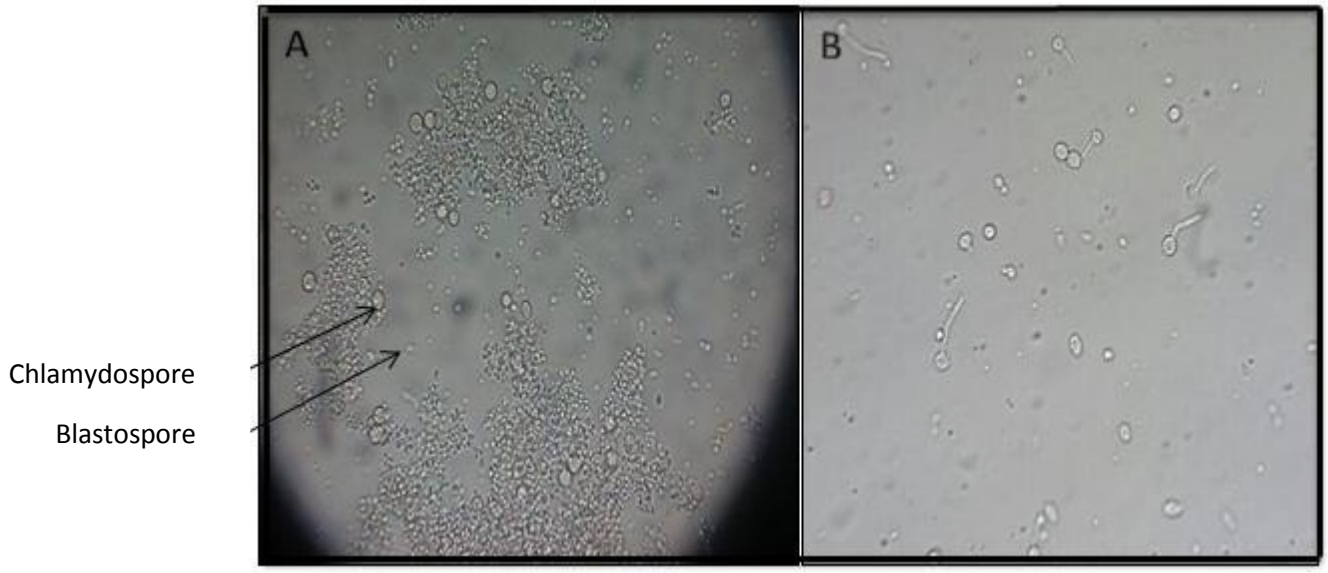

Figure (1): (A) Chlamydospores and blastospores on casein agar (B) germ tube formation of *C. albicans* after 3h incubated at 37o in human serum (40x).

Biochemical test:

By using API candida kit to identify and confirm identification of yeast to the species level, two isolates were identified as *Candida albicans* and the other were identified as *C. parapsilosis*, figure (2)

Figure (2): Results of (Analytical profile index) API C test B=*C. albicans*,

Molecular diagnosis:

PCR for identification of *C. albicans*:

The three isolates of *Candida* spp. has been identified by molecular analysis. According to PCR assay of ITS1 and ITS4 primers the DNA band in figure (3) confirmed the size of gene was approximately 500bp.

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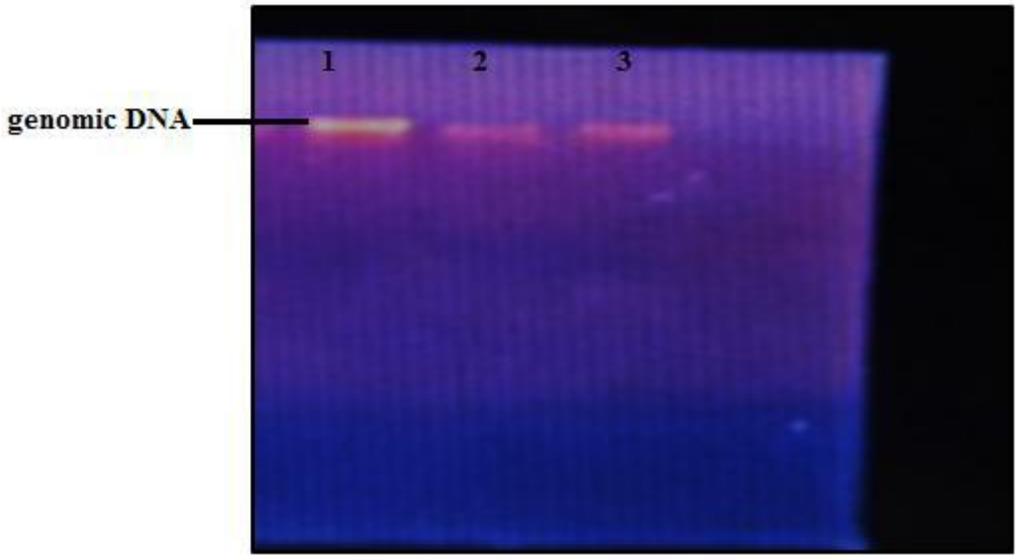

Figure (3): 0.8% agarose gel electrophoresis analysis for genomic DNA extracted from *Candida* spp. Isolates Lanes:1-3.

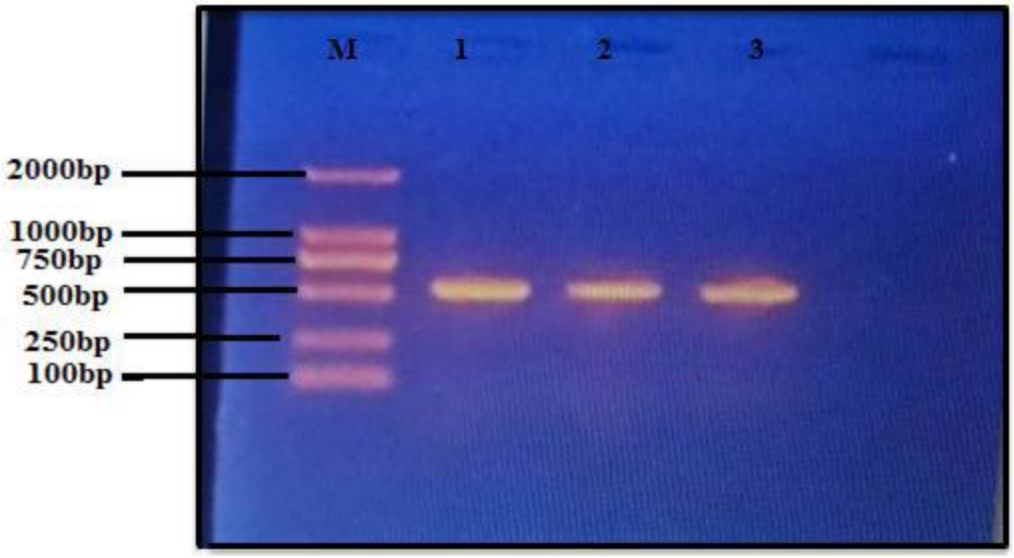

Figure (4): 2% agarose gel electrophoresis analysis for PCR assay with two primers ITS1, ITS4 M=DNA marker Lanes:1-3 gen of *Candida* spp.

Results of immunization protocol

Protein concentration of prepared antigen

The protein concentration of prepared antigen was approximately 0.085 mg/ml.

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Determination of humoral immune response:

The results indicated that the mean level of IgG in subcutaneously (Sc.) and intradermaly (Id.) immunized groups was higher than those of control groups table (1). There were high significant statistical differences between immunized and control groups (P<0.01).

Table (1): IgG level in mice immunized subcutaneously and intradermaly with CWF Ag and control group.

Groups	Route of immunization	No.	Mean	Range	SD	SE
immunized	Sc.	18	20.84	14.06-22.15	2.215	0.522
	Id.	18	20.29	12.4-31.08	5.469	1.2890
Control	Sc.	5	10.44	9.18-11.11	0.95	0.4248
	Id.	5	10.15	8.9-11.8	1.104	0.4937

Determination of cellular immune response:

A- Determination of IFN-γ concentration:

Cell wall fraction antigen there was significance decreasing (P<0.05) in IFN-γ levels, comparing with control group. As well as there was high significant decreasing (P<0.01) in concentration of IFN-γ in the group of mice which immunized intradermaly with CWF antigen comparing with control group, table (2).

Table (2): IFN-γ level in intradermaly and subcutaneously immunized group with CWF antigen and control groups

Groups	Route of immunization	No.	Mean	Range	SD	SE
CWF	Sc.	18	218.14	154.20-393.34	59.63	14.054
	Id.	18	152.54	72.005-228.93	61.01	14.380
Control	Sc.	5	302.17	184.1-348.5	72.14	32.261
	Id.	5	296.22	176.62-355.98	75.46	33746

B- Determination of IL-4 concentration:

Recent data represented that subcutaneously (Sc.) immunized group with CWF antigen the concentration of IL-4 was higher than control group with high significant differences (P<0.01). As well as, the level of IL-4 in mice injected intradermaly (Id.) with CWF antigen was higher than control group. There was a high significant differences between them (P<0.01). table (3)

Table (3): IL-4 level in mice immunized with CWF antigen and control groups.

Groups	Route of immunization	No.	Mean	Range	SD	SE
Immunized	Sc.	18	204.69	190.49-236.27	10.16	2.395
	Id.	18	176.03	139.56-195.03	18.20	4.289
Control	Sc.	5	119.22	111.82-130.31	8.40	2.236
	Id	5	108.12	84.08-125.69	15.81	6.7082

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Discussion

Candida albicans.is the major cause for vaginal candidiasis in child bearing women [(10)]. It can infect approximately about (75%) of women. Misdiagnosing of vaginal candidiasis is resulted by usually diagnosis without laboratory examination.

The reasons make *C. albicans* colonization increase during the pregnancy is that the hormonal environment of the vagina during pregnancy can enhance *Candida* colonization and serve as risk factors, where Progesterone has suppressive effect on the anti-candida activity of neutrophils while estrogen has been found to reduce the ability of vaginal epithelial cells to inhibit the growth of *Candida albicans* [(11)].

Conventional laboratory tests for identification *Candida* to species level involving the phonotypical tests, germ tube tests and carbohydrates utilization. CHROMagar Candida is an important method for identifying certain yeast, for example *C. albicans* and other *Candida* spp. In current study *C. albicans* appeared with green colors, while *C. parapsilosis*, revealed off (white/beige), these results agreed with Kalia *et al*., (2015) , also Paritpokee *et al*., (2005) revealed comparable results in *C. parapsilosis* and *C. glabrata* growth colors [(12 ,13)].

Using of the modern commercial *Candida* agar (CCA) (Brilliance *Candida* Agar) combines X-NAG (5-bromo-chloro-3-indolyl/N-acetyl-β-D-glucosamide) and BCIP (5-bromo-6-chloro-3-indolyl/N-acetyl-β-toluidine salt) as a chromogenic substrates which described by Baixench *et al*., (2006) use of this has revealed that (CCA) is highly selective medium for yeast and

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allows presumptive identification of *C. albicans*[14]. In the present study colonies that exhibited light green colors after incubation at 37o C for (24-48) h identified as *C. albicans* according to manufactures instruction. Recorded investigation was acceptable with previous studies like this done by Ghelardi *et al*., (2007) who estimated the Brilliance Candida agar for presumptive identification of 521 yeast isolates with specificity and sensitivity of this medium exceeded 99.4%[15] and Arirachakaran *et al*., (2009) who identified clinical yeast isolates by culturing on the same medium and noting specific colony features of species[16]. Al-Rubayae *et al* (2013) in Iraq mentioned that Brilliance Candida agar supported the growth of all the clinical isolates, *C. albicans* showed green colour [17].

The identification of possible protective fungal antigens is rather complicated due to the extensiveness of fungal genomes. Current data indicated, that the immunized group with cell wall fraction antigen antigens had a significant elevation in IL-4 and IgG levels, Whereas Th1 cytokine IFN-γ level was low by comparing with control groups. This results give an evidence that CWF antigen may act as immunogens which have the ability to induce both humoral immune response by production antibodies and stimulate the cellular immune response by elevation levels in Th2 cytokine IL-4.

Present study indicated that IgG level increases after immunization with prepared antigen (CWF) which was also documented by De Bernardis *et al*., (2002) whom showed that IgG and IgA level was the most relevant protective isotype after immunization of rats by intravaginal and intranasal routes with an extract of mannoproteins (Mp) or secreted aspartyl proteinase

(Sap) with or without cholera toxin as a mucosal adjuvant [18]. Recorded data related to elevate IgG titers were in agreement with Vilanova *et al*., (2004) who have been studied the potential vaccination with Sap2 as virulence factor of *C. albicans* in mice. Intradermal injection with highly purified Sap2 mixed with alum adjuvant provided immune protection against systemic candidiasis. This protection was associated with anti-Sap2 IgG antibodies in serum [19].

In agreement with the present work of IFN-γ levels, Stuehler *et al*., (2011) in their study proved that variable antigens from *A. fumigatus* or another fungi induced only weak IFN-γ, such as superoxide dismutase, catalase, the major allergen and cytotoxin AspF1 and 1,3-β-glucanosyl-transferase Gel1. By contrast, glycosyl phosphatidylinositol anchored extracellular cell wall glucanase Crf1 and secreted protein peptidase which elicited high IFN-γ [20].

Li *et al*., in (2011a) in their study they were immunized C57BL/6J mice subcutaneously with recombinant enolase as an important glycolytic enzyme antigen. Mice were immunized subcutaneously every two weeks, and titers of specific antibodies to enolase in serum were evaluated the protective effect against systemic challenge, and by levels of Th1/2 cytokines in serum. The titers of enolase specific IgG1 and IgG2a in the immunized mice serum was elevated. Moreover, Concentration of Th1 (IL-12 and IL-8) and Th2 (IL-10) in the immune serum were significantly increased in immunized mice in comparing to the control group. They were concluded that recombinant enolase efficiently protected mice against disseminated candidiasis, also it may be a promising target as a vaccine against variable strains of *C. albicans*. So these data is in consistency with recent documented

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data which also referred to increasing in humoral immune response (IgG level) and Th2 cytokine (IL-4 level) after immunization[21]. Murciano *et al*., in (2006) recorded comparable results in humoral immune response where the diversity of antigens recognized by *C. albicans*-specific antibodies.in sera was obviously diminished in aged C57BL/6 mice which were experimentally injected intravenously with cells and hyphae of *C. albicans* compared with young mice. And there was decreasing in level of IFN-γ concentration in sera of aged mice comparing with young mice. The decreasing of IFN-γ level which recorded in the resent results may be recorded to the fact that the production of TNF-α and IFN-γ by macrophage in aged mice lower than in young mice [22].

An increasing of IL-4 level as a Th2 response which recorded in present work indicated that the prepared antigen give polyclonal activation as a T-dependent antigen interference with MHC class II which simulate the production of IL-4 that recorded a higher concentration in immunized mice[23,24]. Paulovićova´ *et al*., (2013) injected two groups of mice subcutaneously by using two structure of oligomannoside antigens (M5 & M6) and conducted that the two antigens were stimulated cytokine production where show the differences in structure of oligomannoside antigens or conjugate constructs that were reflected in the increase of co-stimulatory molecules CD80 and CD86 expression on neutrophils, and in induced cytokine response. M5 BSA conjugate antigen induced only a slight increase in CD80 expression but a significant increase in IFN-γ, TNF-a, and IL-10. While M6 BSA conjugate antigen induced a significant increase of CD80 expression and increase of TNF-a, IL-4, and IL-10. Data represented

by Th2 immune response in similar to the current results when IL-4 concentration appeared with a significant elevation [25].

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