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Immunization Protocol for Whole Cell Fraction Antigen Extracted from *Candida albicans* Isolated from Pigeon with Candidiasis

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Abstract | In pigeons (*Columba livia domestica*), Candidiasis is considered one of the most common types of infection, and this leads to the risk of the infection spreading to the digestive system because the infection specifically targets the crop in birds. Therefore, this study aimed to prepare an attenuated antigen that had previously been tested on mice. We aim to apply the approach for the first time on a group of Pigeons to stimulate an immune response represented by the levels of antibodies in the pigeons' serum. *Candida albicans* was isolated from birds infected with Candidiasis using CHROMagar *Candida* and diagnosed microscopically to detect the presence of pseudohyphae and true hyphae. Many microscopic examinations, such as germ tube tests, were performed to accurately diagnose the species of *Candida*. The identification was confirmed using PCR targeting the ITS gene region. Live attenuated antigen was prepared using sonicator device and then injected subcutaneously in healthy pigeons. A group of pigeons at one month old (n=10 birds/group) was immunized with the prepared antigen. Another group, as a control group, was injected with normal saline via the subcutaneous route. The second booster was done seven days later, blood samples have been collected after another seven days after the last immunization. The results showed that the antigen prepared from local isolate induced an immune response that protects or reduces the severity of infection with *Candida albicans* in birds. This study was based on the previous research to produce an antigen from whole-cell fraction (WCF) antigen in mice. The estimation of humoral immune responses and IgG showed that the levels of the antibodies were higher in the immunized group than in the control group. Collectively, the study revealed a high level of IgG antibodies in the serum of pigeons immunized with the attenuated WCF antigen. However, future work is warranted to use a purified vaccines to demonstrate the immune induction potentials in pigeons.

Keywords | Candidiasis, CHROMagar, Immunization

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INTRODUCTION

The endogenous *Candida albicans* is an opportunistic pathogenic yeast that causes illness (called candidiasis or candidosis) especially when immune system is

suppressed (Abass et al., 2018). In birds, fungus infections are often linked to morbidity and death (Radwan et al., 2016). It poses a significant global health risk to humans and animals, particularly thrush (candidiasis). Thrush, moniliasis, sour crop, soar, crop mycosis, and digestive tract

mycosis are synonyms for this illness.

Candida albicans are sporogenesis yeasts that reproduce by chlamydospores and budding, and they have a polymorphic appearance due to the formation of pseudo-hyphae or hyphae (Lim et al., 2012). Usually, the illness develops when the host's immune system is weakened due to different factors like disturbances in microbial biota; the potential pathogenesis of the species of *Candida* appears to depend on several environmental and immunological host factors and strain virulence factors, which include the formation of hyphae and biofilm, drug resistance and production of the extracellular hydrolytic enzymes (Thein and Seneviratne, 2009; Dahlhausen, 2006). About 95% of clinical cases of crop mycosis in pigeons are caused by *Candida albicans*, which also causes the disease in other species such as *C. tropicalis*, *C. galabrata*, *C. cruzi*, and *C. parapsilosis* (Wyatt and Hamilton, 1975). Numerous susceptible animals, such as nearby chickens, geese, pigeons, turkeys, and other birds, can contract *Candida* (Kangogo et al., 2011).

According to Vazquez et al. (2011), *Candida albicans* are the most common species causing human infections transmitted by raw chicken meat (Vazquez et al., 2011). One of the main ways that humans contract the sickness is through eating raw meat. Most illnesses and deaths globally, particularly in developing nations, are caused by food-borne diseases. Foods, notably those derived from animals, are the primary way harmful microorganisms are spread to people.

C. albicans is thought to be a typical component of the commensals on the mucocutaneous surface of the digestive tract in birds, as well as the flora of the mouth, esophagus, and crop. The crop is the target organ of *Candida albicans* growth, resulting in increased mucosal thickness and white discolouration (Cavanagh and Naqi, 2003). The *C. albicans*-infected bird senses a brain abnormality that manifests as a stumbling walk and head jerking. It was seen that the neck flexion of dying crop mycosis-stricken birds resembled an S, and their legs were extended rearward.

Numerous mammalian and avian species are susceptible to animal candidiasis caused by *Candida albicans* (Kaab, 2013). The disease, which affects chickens, chicks, and other animals often and has a mortality rate of 8% to 20%, could have an economic impact. Birds with weakened immune systems are particularly vulnerable to fungal pathogen infections. The majority of their systemic infections were found to be caused by *Candida albicans*, which had 50% to 100% fatality rates (Tsat et al., 1994).

Compared to older birds, it is more common in younger birds. A bird's susceptibility to candidiasis increases after

three weeks of age (Tsat et al., 1994). Poultry affected by the disease exhibit poor and stunted growth, depression, diarrhea, and dehydration, all of which contribute to direct death (Al-Akeel et al., 2013). Several circumstances, including living in crowded quarters, eating food contaminated by chicken excrement, being overly humid, malnourished, and having intestinal coccidiosis, enhance the chance of developing a *Candida albicans* infection (Tsat et al., 1994).

The current study aims to develop an antigen against the local isolate of *C. albicans*. After identification by PCR, this isolation was obtained from pigeons and subjected to the development of an antigen to induce the humoral immune response in pigeons as a model for other bird species.

MATERIALS AND METHODS

ISOLATION OF *CANDIDA ALBICANS*

Ten yeasts sample were isolated from pigeons infected with candidiasis from a private clinic in Al-Najaf city from November 2020 to February 2021. The first culture and isolation was attempted on broth medium (Salucea Dutch Technology) followed by growth in nutrient broth (Netherland-Holland) at 37 °C for 24 h. The broth was prepared by dissolving media in distilled water by heating on a stirrer and adding antibiotics (chloramphenicol) with concentration of 5% (Al-Akeel et al., 2013) and then sub-cultured on CHROMagar Candida (Pronadisa/ Canada), and lactobacillus agar (HIMEDIA Lactobacillus MRS Agar – M641I-500G made in India). The growth on yeast extract agar for further purification at 37° C for 48 h was performed. A small colony portion from each isolate has been transferred to new agar plates of CHROMagar for further purification and incubation at 37 °C 24 h. After the period of incubation, the yeast was differentiated and identified according to the shape and color of the colonies.

IDENTIFICATION OF *CANDIDA ALBICANS*

GERM TUBE FORMATION TEST

The test was performed according to the instruction from Sheppard et al. (2008). Briefly, a few drops of fresh chicken serum were dispensed into labelled test tubes. A portion of yeast colony has been added to the serum using a sterile culture loop, thoroughly mixed, and incubated for approximately three hours at 37°C. A drop of the suspension has been removed from the test tube using a Pasteur pipette, dried, and covered with a clean cover glass. The sample has then been viewed under a microscope with a 10x, 40x objective lens to see the creation of the germ tube. A cylindrical filament originating from the blastoconidium without any constriction at the point of origin and without noticeable swelling along the filaments' length indicates a germ tube-positive yeast (Sheppard et al., 2008).

Table 1: Primers used in the PCR.

Primer	Sequencing	Length	Tm	Ta
ITS1-F	5'-TCC GTA GGT GAA CCT GCG G-3'	19	62	57
ITS4-R	5'-TCC TCC GCT TAT TGA TAT GC-3'	20	58	53

DNA EXTRACTION

Total DNA was extracted from pure culture by the use of the gSYNCTM biotechnology kit (Tiwan) according to the manufacturer's protocol (Talazadeh et al., 2023).

POLYMERASE CHAIN REACTION (PCR)

A universal PCR for confirmation of fungi was applied using primers, ITS1 (forward) and ITS4 (reverse) (Table 1), as outlined earlier (Mirhendi et al., 2006). A 10µM/µl concentration was prepared from stock primers to use as a working primer. The PCR thermal cycle conditions were set at 94 °C, 56°C, and 72°C.

ANALYTICAL AGAROSE GEL ELECTROPHORESIS

The agarose gel was prepared according to protocol that has been described previously by Sambrook et al. (1989), with a 1% concentration. Following loading of Safe-Green 100bp Opti-DNA Marker (Safe-Green 100 bp Opti-DNA Marker and samples, the system of the electrophoresis has been set as follows: 90 Volt, constant current, 45 min. Finally, the gel has been transferred into the UVP system in order to visualize PCR products under a 320 nm ultra-violet light source.

PREPARATION OF WCF ANTIGEN

The selected samples were subjected to further processing, cultured in nutrient broth and then after 24 h, the cells were lysed by the use of vibra-sonicator set at 30 sec bursts for 10min in an ice bath. Samples were then centrifuged to separate the pellet from the supernatant. Both pellet and supernatant were tested by culturing them on CHROMagar Candida to ensure no live yeast remained after sonication. Then, the pellet was diluted to obtain a dilution equivalent to the dilution tube number ten of the MacFarland tubes, which equals (3×10^9). According to a previous study by Abass et al. (2018), the dose was calculated as 1ul of the concentration (3×10^9) of the pellet was diluted in 249µl phosphate buffer saline. The same volume of adjuvant oil was added to the total volume of one single vaccine dose, which is 0.5 ml/bird. The second dose was seven days later, and the blood samples were collected after seven days after the administration of the second dose.

RESULTS AND DISCUSSION

CLINICAL SIGNS AND PM FINDINGS

Clinical signs and symptoms showed that the infected birds were suffering from lethargy, loss of appetite and

aggregation of crop contents. We observed crop lesions, which include thickness and sour liquid, and case history, which provides for mortality, fever, loss of appetite, vomiting, and watery diarrhea (Figure 1). In addition, there were nervous signs, including shriveling of the neck and dullness. There were clear signs in the birds upon visual inspection including shriveling of the neck and sleepy birds with contaminated tails resulting from watery diarrhea.

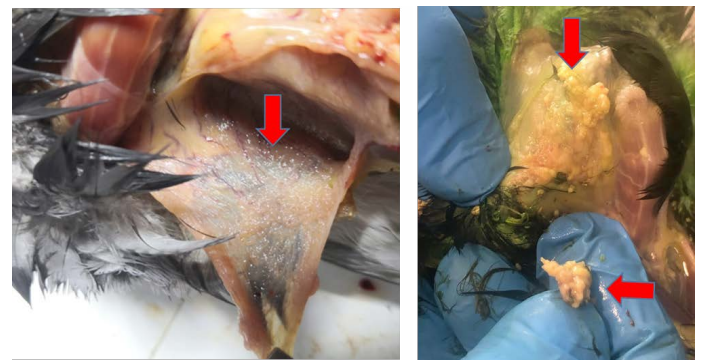


Figure 1: Crop lesions in pigeon infected with *Candida albicans*.



Figure 2: Green colour of *C. albicans* colonies on CHROMagar Candida.

CULTURE RESULTS

Culture results on CHROMagar Candida media showed *C. albicans* a light green color and smooth colonies. On YPDA, the culture showed white creamy, smooth, and little elevated colonies with distinct odor (Figures 3).

GERM TUBE TEST

The growth of *C. albicans* in the serum of pigeons for 3 hours showed the formation of germ tubes without constriction as examined under the light microscope (40x) (Figures 3).

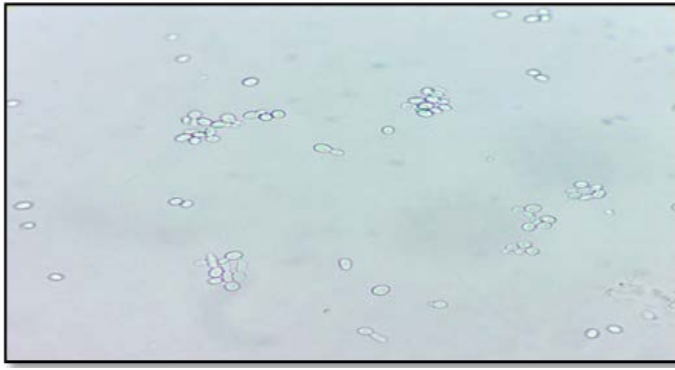


Figure 3: Germ tube test visualization of *C. albicans* in serum of pigeons 40X (left picture).

PCR AMPLIFICATION RESULTS

The PCR was run on ten samples and a total of two samples were loaded on the gel to determine the molecular weight of bands in electrophoresis. The gel analysis showed a specific band of the *Candida albicans*. The molecular weight of the *C. albicans* bands was 535 bp (red arrow) as expected (Figure 4).

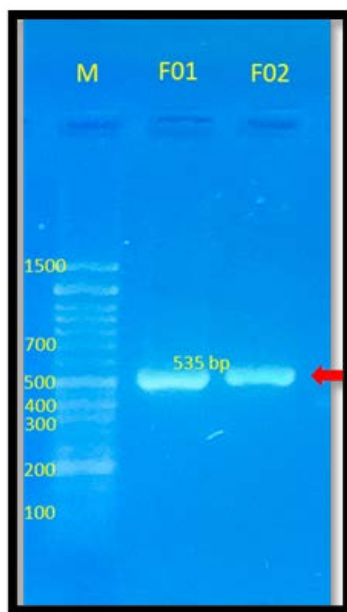


Figure 4: Gel electrophoresis showing the bands of *C. albicans*. The red arrow indicates the bands.

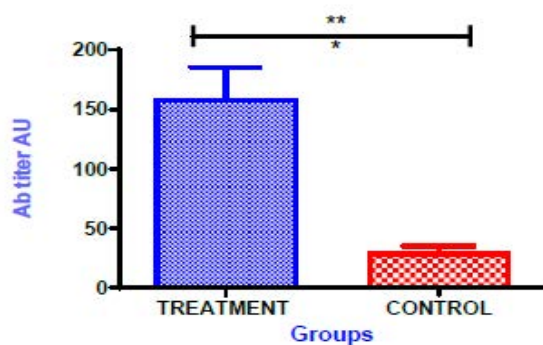


Figure 5: Antibody titre in treated and control group.

IMMUNE RESPONSE AGAINST FUNGAL AG

Immunoglobulin G (IgG) levels in serum from vaccinated and control pigeons were measured. The results summarized revealed that the control group had a higher antibody level titer than the control group (Table 2, Figure 5). The IgG level in the immunized group with subcutaneous injection for the WCF antigen has been higher than the control group with high significant differences $P < 0.01$.

Table 2: IgG concentration in immunized pigeons with WCF antigen and control group.

Group	No.	Mean	Range	SD
Treatment	10	159.41	170.4-101.5	82.33
Control	10	30	30.08-12.021	16.33

Infections of homing pigeons with *Candida* yeast cause significant losses because they target the gastrointestinal tract (Hasenclever and Kocan, 1975) and may lead to tissue injuries in various systems and organs of the pigeon's body that may reach the nervous system, which is called thrush. As a result, there are economic losses and environmental contamination due to *Candida* cells that are excreted from pigeon with watery diarrhea (Nualmalang et al., 2023; Liu et al., 2012). It is well known that the treatment of fungal diseases takes a long period and is hard to eradicate (Talazadeh et al., 2022). *Candida* infection, called thrush, is a common disease in pigeons and other captive birds. This disease causes sour crops and enlargement of the crop (Liu et al., 2012). The current study has focused on preparing an antigen that stimulates the humoral immune responses, which could help protect or reduce the severity of infection with *C. albicans*. In a previous study for the preparation antigen against *Candida albicans* in mice, it was concluded that the best results of immune stimulation were obtained with the subcutaneous route and whole cell fraction antigen with two doses (Abass et al., 2018). This study was a guideline for the current project in many aspects. First, the preparation method of the vaccine candidate antigen. Second, the calculated vaccine candidate antigen dose per body weight. The concentration of the vaccine per dose in the current study is one μ l of 3×10^9 of the sonicated yeast pellet for each 250-gm body weight (when the pigeons are 10 days old). Third, the level of humoral immune response was estimated by measuring total IgG using a specific ELISA kit for pigeons. The PCR results of this study confirmed the strain level of *C. albicans*. The double dose of the WCF prepared vaccine candidate antigen induced strong immunogenicity. The antibody titer level has been higher in the treated group than the control group, with significant importance ($P < 0.01$). This antibody titer level could help protect against Candidiasis infection in pigeons.

However, to confirm the prepared antigen's efficacy and effectiveness, it must experience challenging infection to

estimate the antigen efficiency as a vaccine by measuring antibody survival in serum. The current study is the first local trial to induce immunogenicity by preparing an immunogen against *C. albicans* in pigeons. In addition, the cross-reaction of the immunity formed by *C. albicans* with other types of *Candida* should be studied. Further studies on whole cell fraction by protein band identification is required using Nanoflow HPLC Electrospray Tandem Mass Spectrometry to detect the proteins that induce the immune responses.

CONCLUSIONS AND RECOMMENDATIONS

Finding of the study revealed a high level of IgG antibodies in the serum of pigeons immunized with the attenuated WCF antigen. However, future work is warranted to use a purified vaccines to demonstrate the immune induction potentials in pigeons. It is also suggested to evaluate the antigen strength using challenge infections with high virulent and viable *Candida* yeast strains, monitoring the antibody percentage after the infection, and following up on the animal's health status.

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NOVELTY STATEMENT

Whole cell fraction Ag was firstly prepared locally by Abass, N.F. (2013) and was tested on pigeons for the first time by the research group in this study. So, this study aspires to prepare a vaccine from this Ag in the future for the purpose of reducing candidiasis infections as they are common in birds.

AUTHOR'S CONTRIBUTION

The idea and methods of work were suggested by Abass N.F. and applied practically in the laboratory by Jaafer Haider and Farah Ali under the supervision of Dr. Haider Tuma. The article was written by Nada Fadhil. All authors were read and agreed to the published version of the manuscript.

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ETHICAL APPROVAL

The experiments have been conducted in accordance with

the principles of professional ethics

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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