GENERATION AND CHARACTERISATION OF CHICKEN EGG YOLK ANTIBODIES (IgY) AGAINST CANDIDA ALBICANS

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ABSTRACT

Candida albicans, an opportunistic pathogen causes candidiasis. IgY have been recognized as an inexpensive alternative for the treatment of candidiasis. 21 weeks old laying hens were immunized with Candida albicans. The anti-Candida albicans IgY purified from egg yolk had a total protein and total IgY concentration of 41.63 and 35.06 mg/ml. SDS-PAGE analysis of IgY showed a single band of 180kDa; titre specific antibody was found to be 1:10000 dilutions at 490nm. The expected outcome of this research will form a platform to formulate products comprising egg yolk derived antibody developed against Candida albicans, an alternative to fungal antimycotics.

Keywords: Candidiasis, Candida albicans, IgY, ELISA.

INTRODUCTION

Candida albicans is a member of the native born microbial flora of the gastrointestinal tract, mucocutaneous membranes, and oral cavity in healthy humans [1]. Although Candida albicans rarely causes infections in healthy human without predisposing factors, immunosuppressed patients can suffer from mucosal, cutaneous, or systemic candidiasis [2]. Oropharyngeal candidiasis is the most common opportunistic infection associated with oral injuries [3] and hyposalivation [4]. Oral thrush is a common form of the oropharyngeal candidiasis and its clinical features include white patches appearing as discrete lesions on the buccal mucosa, throat, tongue, and gum linings that develop into confluent pseudomembranes resembling milk curds [5]. Long-term treatment of oropharyngeal candidiasis with antifungal therapy such as fluconazole, itraconazole, and ketoconazole sometimes leads to the emergence of drug-resistant Candida albicans [6]. Candida albicans expresses several virulence factors that required for the establishment of candidiasis such as adhesion to the host cells, phenotypic switching, and germ tube formation [7]. Adhesion of the organism to mucosal epithelium is a prerequisite for colonization and is, therefore, regarded as the initial step in the process leading to infection. Moreover, adhesion and colonization of the organism to oral epithelium can serve as a reservoir for disseminated infections, such as pneumonia, and gastrointestinal infection [8]. Furthermore, adhesion to endothelium and extracellular matrix (ECM) components are required for dissemination of Candida albicans [9]. Limited antifungal drug choices and the potential risk of the emergence of the drug-resistant Candida albicans strains [10] besides the lack of safe and reliable vaccines to confer protective immunity against fungal infection [2] indicated the need for adjunct therapeutic strategies. On the basis of these previous reports and advantage of IgY over the mammalian antibodies, the present
study focused to develop chicken egg yolk antibodies *Candida albicans* for passive immunization.

**MATERIALS AND METHODS**

**Experimental animal**

Twenty one weeks old egg laying white leghorn chickens in good health was obtained from conventional poultry farm, Palladam, Tamil Nadu, India. The birds were maintained and fed with layer mesh. The birds were injected with different antigen.

**Fungal strain**

Clinically isolated *Candida albicans* was cultivated on Sabourad’s Dextrose Agar (SDA) plates and incubated at room temperature for 24-48 hours. The pure culture was maintained in SDA plates and slants. Sub-culturing was done periodically.

**Germ tube test**

0.5ml of serum was inoculated with a loopful of culture taken from isolated colonies on SDA plates and was incubated at 37°C for 2-3 hours. After incubation, a drop of the suspension was taken and placed on a slide and covered with cover slip. It was then observed under high power objective in a light microscope.

**Preparation of whole cell antigen**

*Candida albicans* used as antigen was cultured for 24hours in YPD (yeast extract 1%, poly peptone 2%, and dextrose 2%) broth at 37°C at 100 rpm in orbital shaker. The fungal cells were harvested by centrifugation at 8000rpm for 10 minutes at 4°C, washed twice with sterile phosphate buffer saline (PBS ; pH 7.2). The cells were re-suspended in PBS and a loop of cells were taken for purity testing and streaked on SDA plate and the suspended cells were heat killed in water bath at 80°C for one hour. A loop full of heat killed suspension was taken for sterility testing on SDA plate. The plates were incubated at room temperature for 24-48 hours. The heat killed cell suspension was stored in tightly packed sterile centrifuge tubes at 4°C for further use.

**Immunization of chickens**

For first immunization, the five month old white leghorn chickens were intra muscally injected at multiple sites of the breast muscles with prepared bacterial antigens. Booster doses were given with two weeks intervals. Blood was sampled at intervals of two weeks of immunization and checked for the presence of antibodies. Further, eggs laid by the chicken under the test were collected regularly and stored at 4°C.

**Purification and concentration of egg yolk antibodies**

The egg yolk was separated from white, washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured and the yolk without the membrane was allowed to flow into a graduated cylinder. The egg yolk antibodies were purified by the method of Polson et al. (1980). To the 20ml of egg yolk, an equal amount of buffer “S” (10mM phosphate, 100mM NaCl, pH 7.4 containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture 10.5% PEG 6000 in buffer “S” was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature. The stirred mixture was centrifuged at 11,000 rpm for 20 minutes. The supernatant was filtered through double-layered cheese cloth. The 42% PEG in buffer-“S” was added to make final concentration of 12.5% PEG. The mixture was stirred thoroughly for 30 minutes at room temperature and centrifuged at 11,000 rpm for 20 minutes. The pellet was redissolved in buffer “S” to the original yolk volume and equal volume of 4M Ammonium sulphase (pH 7) was added and incubated at 0°C.
for 30 minutes. The solution was centrifuged at 11,000 rpm for 20 minutes. The precipitate was re-dissolved in buffer-“S” (without NaCl) and was desalted by dialysis to remove ammonium sulphate.

**Estimation of total protein content of *Candida albicans* antigen**

The total protein content of the *Candida albicans* antigens were compared and estimated by Lowry *et al.* (1951) \(^{12}\) method using Folin Ciocalteu reagents. An aliquot (1 ml) of antigen (stock solution) was mixed with 5 ml of solution C and left for 10 minutes in dark condition. After which 0.5 ml of solution D was added, mixed and incubated for 30 minutes. The absorbance of the coloured solution was read at 750nm using colorimeter against a reagent blank. Bovine serum albumin was used as the standard.

**UV-Vis Spectrophotometer**

The total protein content was estimated by UV-Vis Spectrophotometer. An aliquot of (300 µl) of IgY samples were made up to 3ml with distilled water and the OD was read at 260nm and 280nm against the reagent blank. The concentration of anti-*Candida albicans* antibody and IgY was calculated using the formula,

\[
\text{Concentration (mg/ml) = } A_{280} \times \text{dilution factor/ 1.36}
\]

The protein profile of IgY samples were analyzed by SDS-PAGE, the proteins were resolved with 10% polyacrylamide gel at 100 V and 10mA as described by Laemmli (1970) \(^{13}\).

**Protein profile of anti *Candida albicans* antibody**

The protein profile of *Candida albicans* samples were compared and analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970) \(^{13}\). According to Laemmli (1970) \(^{13}\) the proteins are resolved with 10% (W/V) polyacrylamide separating gel and 4% (W/V) polyacrylamide stacking gel at 250 V and 10mA. Equal ratio of *Candida albicans* (20 µl) and sample treating buffer (20 µl) were mixed well and loaded into sample wells. A wide range molecular weight (6.5 – 205 kDa) marker was also run along with the proteins. The samples were run until they reach the bottom of the gel. The characteristic protein pattern for anti-*Candida albicans* can be visualized after Commassie brilliant blue. Even minute quantities of proteins can be predicted by Commassie brilliant blue method, since it is highly sensitive.

**Titration of antibodies ELISA**

The antibody titre potency of each sera and IgY fractions obtained above was determined by the following modified ELISA (Enzyme-Linked Immuno Sorbent Assay) as described by Voller *et al.* (1976) \(^{14}\). The particulate *Candida albicans* antigen was dispensed at a concentration of 5µg/100µl in 0.05M Carbonate bicarbonate (buffer pH 9.6). The resultant dispersion was coated into the individual wells of a 96-well plate (Polyvinyl ELISA plates), at a rate of 100µl per well and incubated at 4°C over night. The solution was then discarded and unbound antigens in the wells were removed by washing with PBS-T (PBS containing 0.05% tween 20) for 3 times. The nonspecific binding sites were blocked by adding 200µl per well of 1% bovine serum albumin in PBS and incubating the plates at 37°C for 1 hour. Plates were subsequently washed with PBST and the individual wells of each plate were then added with 100µl aliquots of Egg yolk antibodies (IgY) at appropriate dilutions, followed by reaction at 37°C for 1 hour. For control wells PBS were served as respective control. After the reaction the plates were washed three times with PBS-T.

As a secondary antibody biotinated rabbit anti-chicken IgY coupled to horseradish peroxidase (Genei Pvt. Ltd, Bangalore) was added at the
rate of 100µl per well and the plates were incubated for 1 hour at 37°C. The plates were then washed three times with PBST. The antibody titre were determined by adding 100µl of freshly prepared substrate solution (4mg of O-phenylene diamine dissolved in 10ml of 50mM citrate buffer, pH 5.0 containing 10µl of hydrogen peroxide) to wells of each plate and followed by reaction at room temperature in dark for 15 min. The reaction was terminated by adding 50µl of terminating solution (4N H₂SO₄). The absorbance of the well was measured in an ELISA reader at OD₄₉₀.

RESULTS AND DISCUSSION

Characterization of Candida albicans

The Candida albicans was grown on SDA for 24-48 hours in room temperature. The purity of the culture was checked by Gram’s reaction and germ tube test. It was confirmed that the strain was Gram positive, ovoid yeast budding cells. The cultural characteristics were smooth, round, shiny and creamy colonies. The protruding hyphae was found by germ tube test and confirmed as Candida albicans. Candida albicans was cultured for 24 hours in YPD (yeast extract 1%, poly peptone 2%, and dextrose 2%). The heat killed cell suspension was mixed well and was adjusted to be 1500×10⁶ CFU, which was mixed with adjuvant for immunizing twenty one weeks old white leghorn chicken for the production of IgY.

Protein estimation of Candida albicans antigen

Protein concentration of the antigen was analyzed by the method of Lowry et al. (1951) using folin cioclateau reagent. The protein concentrations of Candida albicans antigen were found to be 0.576mg/ml.

Generation of anti-Candida albicans antibodies in white leghorns

The twenty one week old white leghorn hens were immunized intramuscularly with Candida albicans antigen to generate anti-Candida albicans antibodies with two weeks interval. The pre-immune sera and hyper immune sera were collected at specified time intervals during and after the various immunization schedules. The antibody was obtained through chicken serum and egg yolk.

Isolation and purification of anti-Candida albicans antibodies

The method used for purification of chicken egg yolk antibodies were PEG method as described by Polson et al. (1980). The precipitate was desalted by dialysis.

Estimation of total protein and IgY concentration
Generation and Characterisation of Chicken Egg Yolk Antibodies (IgY) Against Candida Albicans

The total protein and IgY concentration from the generated antibody were determined by UV-Visible Spectrophotometer. The total protein concentration of the antibody was found to be 41.63 mg/ml. And the concentration of total IgY was found to be 35.06 mg/ml.

SDS PAGE for purified IgY fraction

Electrophoresis (SDS-PAGE) using 10% polyacrylamide gel at 100 V and 10mA according to the method of Laemmli (1970) \[13\].

The SDS PAGE shows a single dark band with a molecular weight of 180 kDa in both lane 1 and 2. A standard molecular protein marker was also run in parallel lane 3 along with the IgY fraction.

**Estimation of antibody titre by ELISA**

The antibody titre potency of specific IgY fractions obtained above was determined by the following modified ELISA as described by Voller et al. (1976) \[14\]. The antibody titre increases at the time of booster injection, even a minute increase in antibody titre can be traced by this assay. The comparative result shows that the antibody titre potencies changes in the course of the immunization. The rate of dilution of antibodies gives the titre till 1:10000 dilutions at 490nm.
The present investigation was aimed to generate and characterize anti-\textit{Candida albicans}. \textit{Candida albicans} infections have been increased significantly ranging from relatively trivial mucosal candidiasis to life threatening disseminated candidiasis and deeply invasive candidiasis in patients with severe underlying disease. However, limited antifungal drugs, their high toxicity, and the emergence of resistant \textit{Candida albicans} strains indicate the need for novel therapeutic strategies \cite{15}. The heat killed antigen was prepared as per Ibrahim \textit{et al.}, 2008 \cite{16} and the protein content of the antigen was estimated by the method of Lowry \textit{et al.}, 1951 \cite{12} and it was found to be 0.576mg/ml.

Anti- \textit{Candida albicans} antibodies were generated in hen using FCA coated adjuvant. The yolk derived anti- \textit{Candida albicans} antibodies were isolated and purified \cite{11}. The total protein content and total IgY from the generated antibody were determined by UV-Visible Spectrophotometer as described by Laemmli (1970) \cite{13}. The total protein concentration of the antibody was found to be 41.63 mg/ml and the concentration of total IgY was found to be 35.06 mg/ml. The purity of the harvested antibodies was checked by SDS PAGE and Coomassiee brilliant blue staining. The titer potency of antibody was determined using microtitre ELISA plates.

**CONCLUSION**

There is an increasing interest in the use of chicken egg yolk for scientific, diagnostic, prophylactic and therapeutic purposes \cite{17}. Chicken egg yolk was recognized as an inexpensive, alternative antibody source, and the usefulness of egg yolk immunoglobulin Y has been assessed for the therapeutic application by passive immunization therapy through oral ingestion of IgY, as in fortified food products for prevention or control of infections. Taken together, chicken antibody collection and isolation can be described as non invasive, rapid and economical.

**REFERENCES**


