Production, Purification and Characterization of Chicken Egg Yolk Immunoglobulin against Cryptococcus neoformans Capsular Antigen

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ABSTRACT

Cryptococcus neoformans is an encapsulated yeast that can live in both plants and animals which cause Cryptococcal meningitis in human. Objectives: The aim of the present investigation is to produce anti-capsular egg yolk antibody in adult White Leghorn hen to promote immunodiagnosis and immunotherapy. Methods: The capsular antigen was prepared and administered to chickens. After immunization, the egg yolk antibodies were purified by Polyethylene and Ammonium sulphate precipitate method. IgY produced were titrated against killed whole cell antigen by micro-titer plate agglutination method and Growth inhibition assay was also carried out against live C. neoformans. Results: After 35 days of immunization, the antibody titer raised at high level. The intramuscular route of inoculation stimulates better IgY production than any other routes tried. The growth inhibition assay was directly proportional to the concentration of IgY added to the test medium. Conclusion: These findings suggest that the IgY is less invasive and not harm the experimental animals. It could be an alternate to IgG serum antibodies for both diagnosis as well as therapy.

Keyword: Cryptococcus neoformans capsular antigen, anticapsular IgY antibodies, Diagnosis, Growth inhibition.

INTRODUCTION

Birds transmit maternal antibodies to their offspring through their eggs [1]. Three classes of antibodies are present in chicken eggs, viz. IgY, IgA and IgM. In which IgA and IgM are similar to mammalian IgA and IgM respectively in terms of their molecular weight, structure and immunoelectrophoretic mobility [2]. In eggs, IgY is present predominantly in the yellow yolk, whereas IgA and IgM are present in the white yolk as a result of mucosal secretion in the oviduct [3]. Serum IgG antibodies of immunized chickens were efficiently transported and accumulated in the egg yolk. High levels of antibody activity were maintained in egg yolk for several months by periodic immunization [4]. The egg yolk antibody has also an advantage over the serum antibody because of its compatibility with modern animal protection regulation [5]. Chicken antibodies do not react with anti-mammalian antibodies in human serum, such as rheumatoid factors and human anti-mammalian anti-IgG. In immunological assays, the interference by anti-mammalian IgG antibodies is eliminated [6]. Chicken antibodies recognize more epitopes on a mammalian protein than the corresponding rabbit does, making it advantageous to use immunoglobulin IgY in immunological assays of mammalian protein. This is especially true with antigens which have highly conserver protein, such as hormone [7]. Chicken egg yolk antibodies are cheaper, simple to produce in larger amount; where as other polyclonal and monoclonal antibodies are expensive and difficult to produce. Chicken antibodies do not bind to Fc receptors of human immune cells. Chicken is an attractive and alternative to mammals as antibody producers [8].

Cryptococcus neoformans is a pathogenic yeast that causes cryptococcosis, it migrates from the lungs to the central nervous system where they cause meningo encephalitis. It occurs in immunocompetent persons but more often in patients with HIV/AIDS, hematogenous malignancies, and other immunosuppressive conditions. All Cryptococcus species are encapsulated. The capsular polysaccharides have a similar structure in all species. They are long, unbranched polymers consisting of a 1, 3-linked polymannose backbone with -l- linked monomeric branches of xylose and glucuronic acid. During infection, the capsular polysaccharide is getting solubilised into spinal fluid, serum, or urine and can be detected by immunological
techniques. The capsular antigen has high pathogenic potential [9]. It inhibits the phagocytosis of mammalian immune system [10]. Based on the above background, this work deliberate to produce IgY against Cryptococcal capsular antigen and its diagnostic significance.

**MATERIALS AND METHODS**

White Leghorn Chickens were purchased from a poultry farm located at Sivakasi, Tamil Nadu, and India. The chickens were maintained in an animal room with 12-12hr light dark cycle and good air circulation. The capsular antigen of C. neoformans was enriched by growing strain in Czapekdox broth with 20% sucrose. [11]. In brief, capsular Polysaccharide (CPS) was precipitated by the addition of 95% ethanol to the culture supernatant and the flask was stored at 4°C overnight. CPS was collected by centrifugation, transferred to a Buchner funnel with 80% ethanol, washed with 95% ethanol, triturated with acetone and then ether. After lyophilization, CPS was stored at 4°C. Whole CPS is used as capsular antigen to immunize chickens.

**Development of anti C. neoformans antibodies in chicken**

C. neoformans capsular antigen was dissolved in 0.9% phosphate buffered saline [PBS] and mixed with Freund’s Complete Antigen (FCA) to get 1mg/ml final concentration of CPC [12]. Antigen was injected through different modes, like intramuscular (group I), intravenous (group II) and subcutaneous (group III) to 24week-old White Leghorn chickens. Subsequent booster doses were given with increasing concentration of antigens at 15 days interval by the same route of administration (Table 1). Test bleedings were made frequently to check the presence of anti C. neoformans antibodies in the serum. Eggs were collected from day 1 to the end of the experiment and stored at 4°C until testing.

**Table 1.** Immunization schedule in Chickens

<table>
<thead>
<tr>
<th>Days of immunization</th>
<th>Test Antigen</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>250 µl capsular antigen+250 µl FCA</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>15th day</td>
<td>500 µl capsular antigen+500 µl FCA</td>
<td>Intra venous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subcutaneous</td>
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**Purification and characterization of anti C. neoformans antibodies from egg yolk**

The antibodies were separated from egg yolk by using Polyethylene and Ammonium sulphate precipitate method [13]. Briefly, the yolk was divided from egg white and rinsed with distilled water. The membrane was pierced and the yolk without the membrane was permitted to flow into a graduated cylinder. An equal amount of buffer S (10 mM phosphate, 100mM NaCl, pH 7.5, containing 0.01% Sodium Azide) was added to the yolk and stirred. To this mixture 10.5% PEG 8000 in buffer S was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature and centrifuged at 11000 rpm for 20 min. The supernatant was filtered through double-layered cheesecloth. Then 42% PEG in buffer S was added to make final concentration of 12% PEG. The mixture was mixed thoroughly and centrifuged at 11000 rpm for 20 min. The pellet was re-dissolved in buffer S to the original yolk volume and an equal volume of 4M Ammonium sulphate (pH7) was added and the precipitate was centrifuged at 11,000 rpm for 20 min, the pellet was re-dissolved in 1ml of buffer S (without NaCl) over night. The content was desalted by dialysis process. The crude fraction of IgY thus obtained was further purified by DEAE cellulose ion exchange column chromatography. The IgY fraction was then concentrated with Poly Vinyl Pyrolidone (PVP) at room temperature. The titration of antibodies was carried out by Micro Agglutination Test (MAT).

**Agglutination test and Growth inhibition assay**

For estimating anti-capsular antibodies, antibody titration was performed in 96 well "V" bottom micro titre plate by agglutination assay [14]. Fifty micro litres of serum/IgY preparation was added to the first well and two fold serial dilutions were made with physiological saline (PS). A volume of 50µl of C. neoformans suspension (1%) was added to each well. The microtitre plate was gently shaken for efficient mixing of the reagents and was incubated at 37°C for 1h. The highest dilution of sample that showed detectable macroscopic agglutination was recorded and expressed as log_2 antibody titre of the serum.

**Growth Inhibition Assay** was carried out by novel methods described by Guimaraes et al. [15] and Bruatto et al. [16] with some modifications. The experiment is to check whether the anti C. neoformans IgY could inhibit C. neoformans growth in liquid medium. C. neoformans was inoculated into 5ml Sabouraud Dextrose Broth (SDB), and into another 5 sets of 5 ml of SDB containing increasing concentrations of chicken egg yolk antibodies plus equal volume of serum from unimmunized chicken (as a source of complement) (100µl to 500µl of IgY) and incubated 48 hrs at 37°C. Amphotericin B 1µg/ml in SDB was act as positive control. The blank was set by medium with serum. The optical density of the tubes was checked at 600nm to measure the growth inhibition of C. neoformans by chicken IgY antibodies.
Fig. 1 shows the level of serum antibody titre after immunization with cryptococcal CPS. Each point represents Mean ± SD of three samples.

Fig. 2 shows the level of Egg Yolk antibody titre after immunization with cryptococcal CPS. Each point represents Mean ± SD of three samples.

DISCUSSION

Cryptococcal diseases like Cryptococcosis and Cryptococcal meningitis are treated with antifungal agents such as fluconazole, amphotericin B, flucytosine and ambismine. Sometimes, use of antimicrobial agents stimulates adverse side effect in human body. To avoid such side effects, and use IgY as a passive immunizing agent is under intensive research. It is highly recommended to use chicken egg yolk antibody (IgY) for diagnosis purpose. IgY antibodies have been successfully applied in a variety of assay such as RIA, ELISA, Indirect Haemagglutination assay and Immunodiffusion [17, 18]. IgY antibodies were used as a primary antibody in Western Blotting and also as secondary antibodies. Avian yolk antibodies are used in diagnosis of bacterial polysaccharides, alginate and Hepatitis B surface antigen [19].

Immunotherapy is one of the greatest opportunities for use of antibodies raise in laying birds and isolated from their eggs. IgY can specifically recognize gastrointestinal cancer. A small and efficient target carrier is the key component for anti-cancer drugs. For this reason, it may become an important carrier for anti-tumorigenic drugs [20]. Chicken egg yolk anti-venom antibodies can able to neutralize cobra and krait venoms [21].

Polson et al. [13] observed that the agglutinating types of antibodies were experimental even after 1 month of immunization. The concentration of protein in egg yolk is an indicator of the level of agglutinating antibodies. Additionally, he stated that the egg yolk laid by immunized chicken has been recognized as an excellent source of polyclonal antibodies. A single egg contains as much polyclonal antibodies as an average bleed from a rabbit would yield. Typically each egg may contain 90-100 mg of IgY. Our study revealed that egg yolk antibody have acceleration and specificity towards Cryptococcal antigens.

The present study showed that the chicken egg yolk antibodies can be used for the control of C. neoformans infections. Chicken antibodies can be prepared in large scale since chicken IgY is a continuous source from immunized chickens, which makes it possible to produce in large amount. Hence chicken egg yolk antibodies may serve as an alternative source to neutralize the C. neoformans by Kuncorojakti and Suwarno [22] used egg yolk derived Anti Hemaglutinin antibody as Immunotherapeutic Agent on the Chicken Infected by Avian Influenza Virus. Silva et al. [23] considered IgY as a promising antibody in immunodiagnostic assay and in immunotherapy.

Much more studies are required to evaluate the potency of C. neoformans antibodies. Chicken egg yolk antibodies will play an increasing role in research, diagnostics and immunotherapy in future.

CONCLUSION

With regard to animal welfare (i.e. bleeding), antibodies purified from the egg yolk of immunized chickens are a good alternate source for antibody raised from serum of mammals. Since the antibodies are extracted from the yolks of laid eggs, the method of antibody production is non-invasive. Thus, no blood must be taken from the animals for the extraction of blood serum. The amount of IgY antibody produced is also very high when compared with serum antibody. IgY produced against C. neoformans capsular antigen can be employed in the field of immunodiagnostics and therapy as well.

REFERENCE:


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