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In Vitro Evaluation of Hyper Immunized Chicken Egg Yolk Antibodies Generated Against Dandruff Causing Yeasts *Malassezia globosa*

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Abstract

Aim: *In vitro* evaluation of egg yolk antibodies (IgY) generated against the commensal yeast *Malassezia globosa,* which is more prevalent in dandruff condition. Dandruff is scaling and itching of scalp, that can be treated by controlling the proliferation scalp flora with aid of antigen specific IgY antibodies. **Methods:** The chicken egg yolk antibodies which are generated specifically against the cell wall binding proteins of *M. globosa* from White leghorn chickens were evaluated under *in vitro* condition. IgY was produced from egg yolks obtained from these immunized hens using water dilution, salt precipitation. Purity of IgY was evaluated in the stained SDS-PAGE bands. An enzyme linked immunosorbent assay was performed to indicate high specificity of IgY. **Results:** 46 kDa and 68 kDa antigenic proteins were isolated and characterized from the cell wall of the organism and antigen specific IgY antibodies were generated in hens. NaCl precipitation was done to extract yolk antibodies from eggs of immunized hens. Ion Exchange column purification was performed to acquire purified 180kDa IgY. A range of 10mg/ml to 15 mg/ml of anti *M. globosa* IgY was determined as Growth Inhibitory concentration of *M. globosa*. **Conclusion:** Based on the results observed it was concluded that egg yolk antibodies would serves as a better alternate to synthetic agents in control of dandruff condition.

Keywords
Dandruff, *Malassezia*, Egg yolk antibodies, Antidandruff, *In vitro.*

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INTRODUCTION

Dandruff (D) /Seborrheic Dermatitis (SD) is the most common *Malassezia*-associated dermal disorder, occurring in up to 50% of healthy humans and up to 75%–90% of immunocompromised patients [1]. It results from at least three etiological factors such as *Malassezia* fungi, sebaceous secretions and individual sensitivity. Isolated scaling of the scalp characterized by flaking and pruritis is termed as Dandruff, when accompanied by visible redness and

extension beyond the scalp to other areas of the face it is termed as Seborrheic Dermatitis [2,3]. These yeasts are unique, because they are part of the microbial flora of the human skin, but under optimal growth conditions can become pathogenic and lead to the worsening of certain skin diseases and rarely, to the development fungaemia [4]. Using an advanced molecular technique, terminal fragment length polymorphism, it was previously identified *Malassezia globosa* and *Malassezia restricta* as the predominant species present on the scalp of D/SD sufferers [5].

Malassezia is the most probable target for controlling the dandruff. The exact mechanisms behind the pathogenetic role of *Malassezia* in Seborrheic dermatitis or dandruff remain unclear as there is no evidence that the organisms invade the skin. In most biopsy samples, they are clustered near the hair follicle openings and respond to the treatment that inhibits the growth of them. Specifically, the organisms contain lipases that hydrolyze triglycerides, freeing specific saturated fatty acids that the yeast requires to proliferate [6]. Currently available treatment options for the management of dandruff include therapeutic use of zinc pyrithione, salicylic acid, imidazole derivatives, glycolic acid, steroids, Sulphur and coal tar derivatives. But these drugs are unable to prevent recurrence of dandruff. Recently, molecular work has elucidated the structure of three major allergen components, namely two protein components of 67 and 37 kDa each, and one carbohydrate component of 14 kDa [7].

A range of skin micro environmental factors, such as the bacterial microbiota present, pH, salts, immune responses, biochemistry, and physiology, may play a role in adherence and growth of *Malassezia* species, favoring distinct genotypes depending on the skin sites. In addition, the biochemical composition of the skin selecting genetic populations of *Malassezia* yeasts can indirectly affect their drug susceptibility [8]. Alternative therapeutic protocols, i.e., desensitization to *Malassezia* by immunotherapy or administration of inhibitors of yeast adherence factors, have been proposed to avoid repeated administration of antifungal and the occurrence of drug resistance phenomena [9].

Egg yolk antibodies (IgY) are obtained from egg-laying fowl. These birds have characteristics of transferring the immune component to a chick through passive immunity and antibodies get accumulated in the yolk of egg. Nearly 30-40g of IgY can be produced from a laying hen in a year, probably in which 1 to 10% can be specific to its respective antigen and it is as much as 120 times of that of rabbit. Because of phylogenic difference, fowl IgY does not cross-react with the mammalian IgG [10]. Layer hens are generally used as lab animal for large quantity of antibody production and its easy maintenance. A tentative result shows that a monoclonal antibody against the unique proteins binds to *Malassezia* with binding activity of 90 % or more [11]. Hence, generation of egg yolk antibodies against the scalp binding proteins of *Malassezia* fungi, can be used to control the proliferation of the yeast.

MATERIALS AND METHODS

This experiment was deliberated in order to check the efficacy of antigen specific egg yolk antibodies generated against the dandruff causing *Malassezia globosa*.

Culture Maintenance:

A standard isolate of *Malassezia globosa* (CBS No.7966) procured from NCCPF, Post Graduate Institute of Medical Education and Research, Chandigarh, India was used for the *in vitro* study. The isolate was maintained in Sabouraud's dextrose agar (Himedia) supplemented with olive oil until use.

Characterization of *Malassezia globosa* [12]

a. *Gram's staining:* The morphology of the lipophilic yeast cells was studied by gram stained smears from Sabouraud's dextrose agar (SDA) after 3-4 days of incubation at 37°C.
b. *Cultural characteristics:* The organism was cultivated on SDA medium supplemented with olive oil and incubated at 37°C for 3-4 days. Further, the culture was grown on selective medium (Himedia) like Modified Dixons agar (mDA) and further maintained on SDA medium.
c. *Biochemical characteristics:* The organism was biochemically analyzed for its nitrate reduction, urease and gelatin hydrolysis ability and the results were recorded.
d. *Physiological characteristics:* The catalase reaction was determined by application of 2-3 drops of 3% hydrogen peroxide onto a portion of a colony on a glass slide and the result was recorded. The ability of the organism to utilize individual Tween was tested by the following procedure called Tween Assimilation Test. Sterile SDA (16 ml) was melted and allowed to cool to about 50°C. The yeast being identified was added (2 ml) to the medium. The suspension was obtained by inoculating 5 ml of sterile distilled water with a loopful of actively growing yeasts and the concentration was adjusted to 10^5cells/ml. The seeded agar was then vigorously mixed and poured into Petri dish. Once the medium had solidified, four wells were

made by means of a 2mm diameter punch and filled with 50µL of Tween 20, 40, 60 and 80 (Himedia) respectively. Since these reagents are water soluble, a concentration gradient formed around each well. The plates were systematically incubated for one week at 32°C and the results were recorded.

e. *Antifungal Sensitivity test:* 0.1ml of $1X10^9$ cells/ml of *M. globosa* was streaked with help of sterile swabs on Muller Hinton Agar + 2% Glucose + 0.5 mcg/ml Methylene Blue Dye agar medium (Himedia M1825) for carrying out susceptibility of antifungal discs. Hexa Antimyco-01 (Himedia) antibiotic disc consists of 6 discs of 6 mm diameter on its projection coated with Amphotericin-B (AP) 100 units, Clotrimazole (CC) 10 mcg, Fluconazole (FLC) 25 mcg, Iitraconazole (IT) 100 mcg, Ketoconazole (KT) 100 mcg and Nystatin (NS) 100 units was placed on the swabbed culture medium and incubated at 37°C and examine after 24 hours. Zone of Inhibition (ZOI) was be calculated after the growth period.

Preparation of cell wall antigen:

M. globosa cells were grown in Sabouraud's dextrose broth (SDB) supplemented with antibiotics (Cycloheximide-200 µg/ml, Chloramphenicol-50 µg/ml- Himedia) and 2 ml of sterilized olive oil. The cells were harvested by centrifugation, rinsed thrice with distilled water and the resultant pellet was treated with 300mM 2-Mercaptoethanol (Himedia) in 100mM EDTA (Himedia) (pH 9.0) for 60 minutes at 20-25ºC with agitation. The suspension was placed in boiling water for 5 minutes and rapidly shifted to ice bath for 2 minutes. The resultant supernatant was dialyzed against distilled water for 48 hrs at 4-6ºC. Dialysates were lyophilized and referred to as 2-ME extract. 400µg/ml of 2-ME extract was prepared in PBS and used as antigen. Purity was checked in 10% reducing SDS-PAGE [13].

Experimental Hen:

19 weeks old white leghorn chickens (BV 300) were segregated into separate groups based on the respective antigens. The chickens were provided with feed and water *ad libitum*. Hens were intramuscularly injected on both sides of their breast muscles. Subsequent booster doses with proper adjuvant were given to elicit high titer of antibodies and maintain antibody production. The eggs were collected daily, cleaned and labeled on the day of collection.

Purification of anti-*Malassezia* IgY Antibodies from the hyper immunized eggs:

The IgY antibodies were purified from the egg yolk of the immunized chicken using precipitation techniques. The water dilution method followed by Sodium Chloride precipitation (NaCl) was employed because of the ease and least loss of antibodies during purification [14]. Egg yolks were separated from the albumin and the yolk content was pooled up, diluted with distilled water in the ration of 1: 9. pH was adjusted to 5.0 with 0.5 M HCl, and the mixture was frozen in polypropylene centrifugation tubes at -20 °C for overnight. The frozen mixture (in a conical shape) was transferred to another filtration funnel (with filtration paper) and allowed to melt freely at the laboratory temperature. The filter paper entirely retains the lipid aggregates formed by freezed yolks and water-soluble (WS) fraction was collected. As a second purification stage, fractionation of proteins from WS fraction with NaCl (Himedia) was done. 8.8% NaCl was added to the WS fraction and pH adjusted to 4.0 using 0.5M HCl and precipitation carried out for 2 hours. This suspension was subjected to centrifugation at 8000rpm for 20 minutes at 4°C. The pellets containing IgY antibodies were resuspended in phosphate buffer and stored at 4°C for further characterization. IgY extract was dialyzed overnight in 0.1% saline and gently stirred by means of magnetic stirrer. Next day, saline is replaced by PBS and dialyzed for another three hours [15]. The dialyzed chicken egg yolk antibodies were purified by DEAE cellulose (Himedia) ion exchange column chromatography.

Determination of concentration of IgY Antibodies:

The total protein content of the eluted IgY antibody fractions were estimated using Folin-Ciocalteau method with BSA as standard protein. 10mg of Bovine Serum Albumin (Himedia) was dissolved in 5ml of distilled water, from which 1ml was diluted to 10 ml with distilled water. From this working stock 0.2ml to 1ml was taken in separate test tubes and diluted to 1ml with distilled water. 5ml of alkaline solution was added to each tube and incubated at room temperature for 20-30 minutes. To all the tubes 0.5 ml of Folin-Ciocalteau reagent (Himedia) was added and incubated at room temperature for 30 minutes. The color developed was measured with spectrophotometer at 700 nm against the blank [16]. The OD values of standard protein samples and test samples were plotted on a graph. Total IgY content of the sample (mg/mL) was measured spectrophotometrically at 280 nm (1:50 diluted with PBS) and calculated according to the Beer-Lambert's law with an extinction coefficient of 1.33 for IgY ([17]. Protein profiling was done to assess the purity of IgY by SDS- PAGE [18].

Specificity of anti- *M. globosa* IgY determination using Indirect ELISA:

The microtitre plate (Himedia) was coated with 100µl/well with the cell wall antigen solution using

coating buffer (PBS, pH 7.2-7.4) and incubated at 4°C over night for binding. After coating, unbound antigens in the wells were removed by washing with PBS containing 0.05% Tween 20 (PBST) for 3 times. The empty sites were blocked by adding 100µl/well of 1% bovine serum albumin and the plates were incubated at 37°C for 1 hour. Plates were subsequently washed thrice with PBST and incubated with 100µl of egg yolk antibodies (IgY) at appropriate concentrations. Control wells had PBS which served as control. Plates were incubated for one hour at 37°C and subsequently washed thrice with PBST. 100µl of diluted (1:1000) rabbit anti-chicken immunoglobulin (Sigma-Aldrich) coupled to horseradish peroxidase was added to the plates [19].

Binding efficacy of IgY antibody:

Binding efficacy of IgY antibody generated against *M. globosa* was determined by Growth Inhibition Assay. Anti- *M. globosa* IgY solutions were sterilized by using a 0.22-µm membrane filter. 5ml of $1X10^9$ cells/ml of *M. globosa* in SD Broth was poured into 10 ml tubes and 50 mg/ml of anti- *M. globosa* IgY antibodies were added and double dilution was executed. The tubes with 10µg/ml of ketoconazole and inocula were used as positive control and tubes without introduction of antibodies as a growth control. Tubes with 50mg/ml of anti- *M. globosa* IgY alone were used as negative control and sterile SD broth supplemented with olive oil was treated as blank. The tubes were mixed well and cultured at 37°C for 72 hours in rotating shaker. Then, 1 ml of the culture solution was taken at every 24 hours interval, heated at 60 °C for 30 minutes and then kept cold and the turbidity was measured at 660nm. At last, broth solution used for the assay was cultured on the SD agar medium for better visualization of results [20].

RESULTS

Culture Characterization:

a. Colony Morphology: *M. globosa* colonies are pale yellowish precipitate, smooth, wrinkled to ceribriform, shiny or dull with the slightly lobate margin on SDA. Small cream colored colonies on mDA (Figure: 1).
b. Staining: The morphology of the lipophilic yeast cells was studied by gram stained smears of the isolates from SDA. *M. globosa* is a Gram Positive, Spherical shaped cell with monopolar budding on narrow base (Figure: 2).
c. Biochemical and Physiological characterization: The organisms were biochemically analyzed and the results are mentioned in Table 1.
d. Antimicrobial sensitivity testing: Antimicrobial sensitivity (AST) of *M. globosa* was tested by disc diffusion method with help of anti mycotic drugs (Figure: 3). Most of the azole drugs proved to be effective against the organism with zone diameter between 10-20mm (Table. 2). ZOI is the diameters of the zones to the nearest millimeter using a calibrated instrument like zone scales (Himedia PW096/PW297).

Antigen storage:

No growth on the agar medium indicates the complete sonication/killing of the live cells. Finally, the antigenic suspension was stored in aliquots of 0.5ml at 4°C until immunization. Sterility of the prepared antigen was checked by preparing thin smear on a clean grease free glass slide and then the smear was subjected to observation under microscope and plated on a selective medium and screened for any contamination. 46kDa and 67kDa antigenic components of cell wall antigens were observed in 10% reducing SDS-PAGE (Figure: 4).

Generation of Anti *M. globosa* IgY antibodies:

Antibody levels against immunizing antigens were maintained throughout the egg laying stage of the chicken (Table. 3). Collected eggs were stored at 4°C from the first day of immunization and labeled as 0^{th} day. Egg parameters are studied in order to see the variations which would help in determination of parameters affecting the final product as well as the cost of production of IgY (Table. 4). Moreover, it was recorded that immunization or antigen type has no relation with the egg laying capacity of the leghorn.

Characterization of anti-*Malassezia* IgY:

The high molecular weight protein (180kDa) showed the purity of IgY in 10% non-reducing SDS-PAGE (Figure: 5). Protein fractions obtained from diluted egg yolk were loaded into the gel and their band patterns were studied by Coomassie Brilliant Blue R 250 (Himedia) staining. The total Protein and IgY concentration of egg yolk showed a steady increase from the initial immunization to the 22^{nd} week after first immunization (Figure: 7 and 8).

Antibody titer of IgY:

Specificity and titer of IgY against specific antigen was tested by Indirect ELISA and the titer of IgY antibodies in egg yolk of immunized hens started to increase by 28^{th} day with OD of 1.45 and it goes on increasing up to 1.92 by 110th day and the values remained constant further. The titer of IgY antibodies showed gradual and significant increase from the period of first immunization and at subsequent booster immunizations. Indirect ELISA revealed that there was no significant increase in the titer of specific IgY in the antibody solutions purified from unimmunized egg yolk. It indicates the immunization of chicken with the target antigen influences the

generation of specific IgY against the same antigen (Figure: 9).

Binding Efficacy: Anti *M. globosa* IgY are expected to bind to the surface of unique proteins of 46 kDa and 68 kDa in cell wall of the organism and reduce the proliferation of their growth by decreasing the lipase activity. Hence there is a reduction in count of organism which decreases the severity of the condition. 12.5mg of anti-*M. globosa* IgY was effective in inhibiting the growth of *M. globosa* (Figure: 6and 10). The results stated above were aggregate values of tests performed in triplicates.

Table 1 Biochemical tests for *M.globosa*

Urease	++
Catalase	++
DBB reaction	+
Tween utilisation	-
Pigment Induction	-
Nitrate Reduction	
Carbohydrate Utilization	+

Table 2. Antifungal susceptibility testing

Antibiotics discs	ZOI against *M.globosa*
Amphotericin B 100units	12mm
Clotrimazole 10mcg	11mm
Flucanazole 25mcg	33mm
Itraconazole 10 mcg	10mm
Ketoconazole 10mcg	33mm
Nystatin 100units	22mm

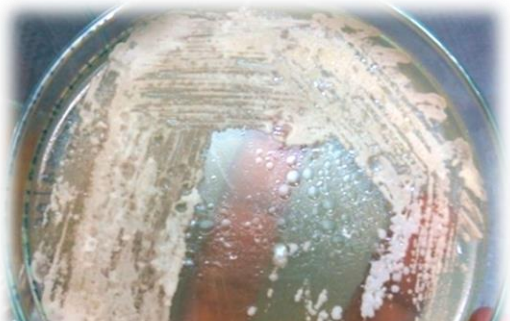
Table 3. Immunization Schedule

Antigen	Antigen dose	Antigen volume	Adjuvant	Site of immunization
***M.globosa* cell wall extract**	400 ug/mL	0.5ml	0.5mL Freunds Complete Adjuvant (FCA) for initial dose & Freunds Incomplete Adjuvant (FIA) for booster doses	Intra-muscular Breast muscle 2 sites 0.5ml per site
Booster doses at every week interval after initial immunization for 4 weeks and when required				

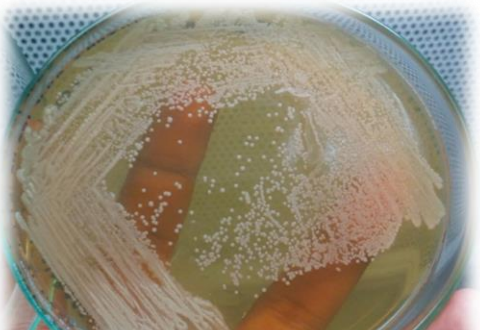
Table 4. Egg Laying Capacity of Hens (n=2 per group)

Antigen	*M.globosa* Whole cell	*M.globosa* cell wall	Control Hen
Egg laying capacity / week	6.9±0.98	6.5±0.73	6.4 ± 0.70
Values obtained are mean of eggs laid by total number of hens per group			

Figure 1: Colony Morphology of *M. globosa*

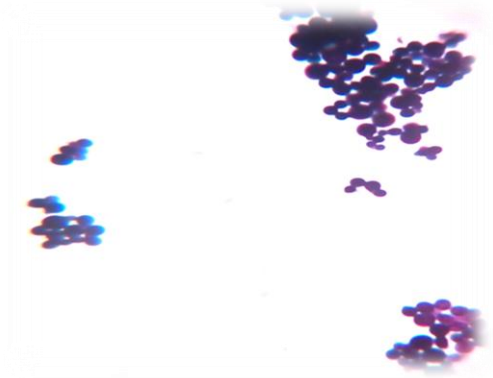


(a) Revival of Standard strain of *M. globosa* in SDA medium.



(b) Small, Cream colored colonies on mDA medium

Figure 2: Microscopic Characterization of *M. globosa*



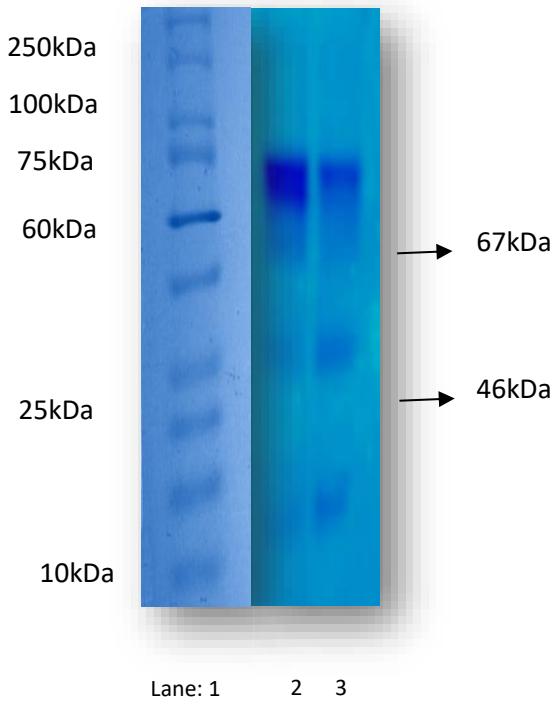
M.globosa: Gram Positive cells. Cells are spherical; budding is monopolar on narrow base with short filament structures was observed under 100x objective

Figure 3: Antimicrobial susceptibility testing against *M. globosa*

Hexa Antimyco 01 rings were used to test the Antifungal susceptibility by Disc diffusion method and their ZOI is mentioned in Table 2.

Figure 4: Characterization of *M.globosa* Cell wall extraction

Antigenic components of *M.globosa* 46kDa and 68kDa of cell wall binding protein was characterize by CBB stain in 10% Polyacrylamide (reducing) gel



Lane 1: Protein Marker (10-250 kDa)
Lane 2 and 3: Cell wall extract of M.globosa

Figure 5: Characterization of anti *M.globosa* egg yolk antibodies

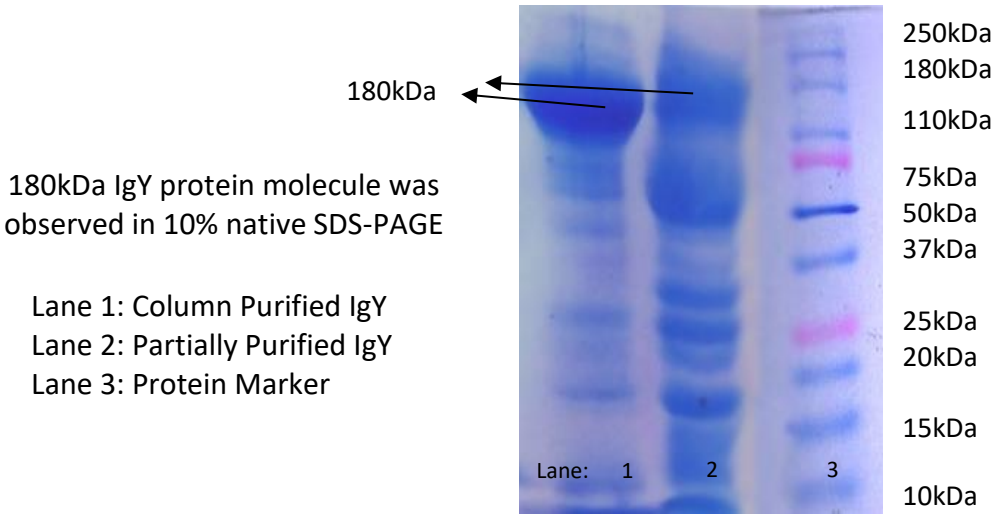
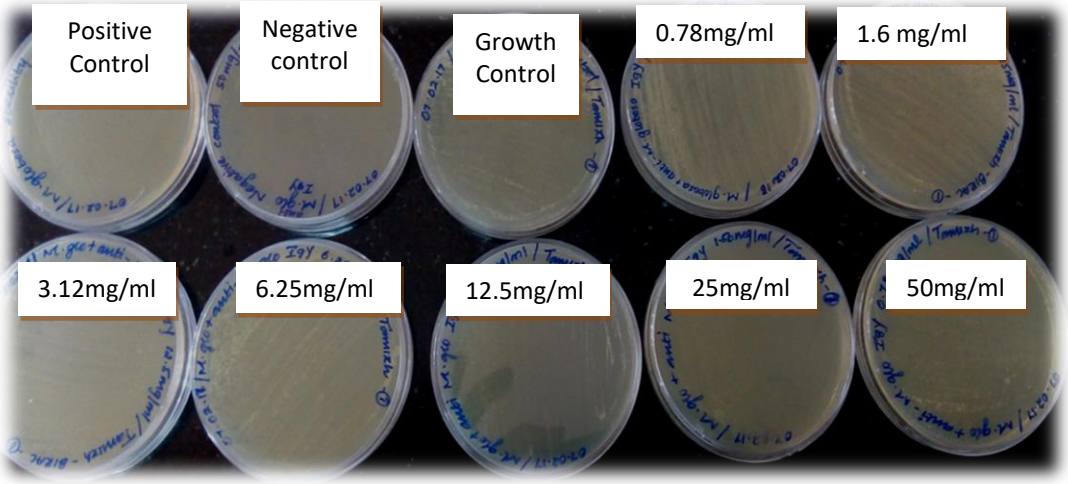
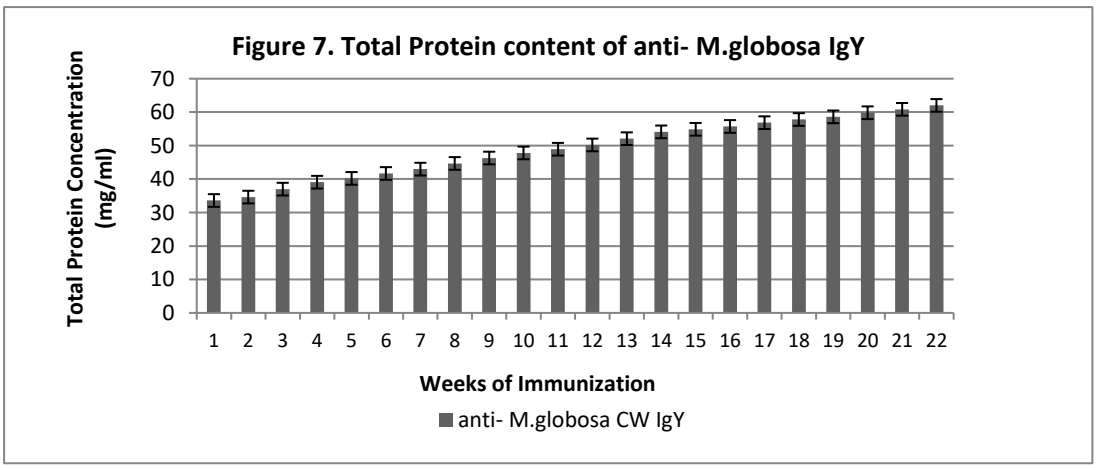
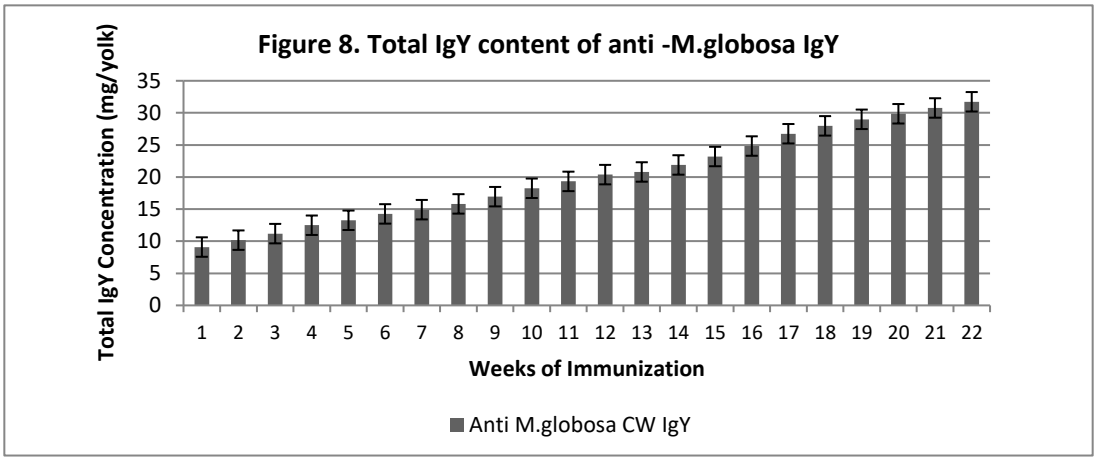


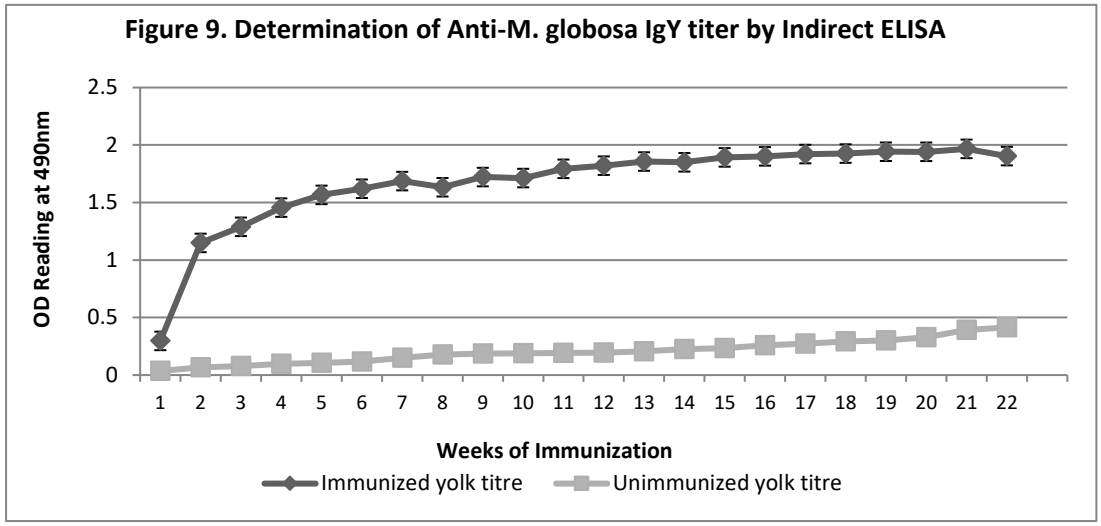
Figure 6: Growth Inhibition Assay of anti *M.globosa* IgY antibodies against standard strains of *M.globosa*

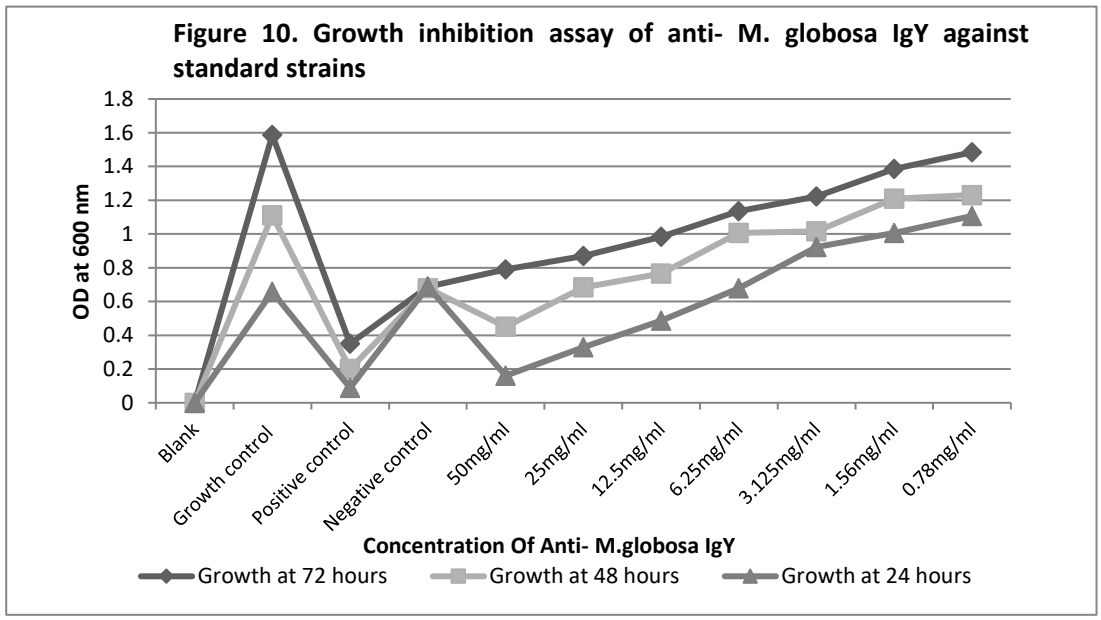


Growth of *Malassezia globosa* was inhibited by specific IgY compared with the blank control, and the inhibitory effect was dependent on the concentration of the specific IgY added









DISCUSSION

Dandruff is an abnormal skin condition characterized by detachment and itching of scalp. Genus *Malassezia* was widely observed in dandruff way back in 1846. In India, still mechanical techniques, antifungal agents and antidandruff shampoo were

used to address this problem. But these means do not provide a proper solution and same condition arises after few days of treatment with major secondary effects like hair fall, loss of suppleness, smoothness. They do not control the growth of commensal; instead they eradicate the microbiome, which is a threat to species diversity. Antibodies remove antigens from the host by inactivating them; use of antibodies for research, diagnosis, and therapeutic purposes has increased. Hence, therapeutic antibodies can be used as an alternate mode to control the proliferation of yeast microflora in scalp. *Malassezia globosa* and *Malassezia restricta* are the most commonly isolated species from human scalp with dandruff and other dermal infections.

Twenty-one-week old white leghorn chickens were immunized with *Malassezia globosa* antigen for generation of antibodies followed by booster dosages. The eggs were obtained and the antibodies were purified from egg yolk using NaCl precipitation method described by Hodek *et al*., (1980). The protein profile of anti- *M. globosa* IgY (i.e) 180kDa was analyzed by SDS-PAGE with CBB staining of Laemmli *et al.*, (1970). The titre of antibodies was estimated using Indirect ELISA as mentioned by Voller *et al.*, (1976). The specific antibodies for cell wall of the fungal components started to appear in serum and egg yolk two weeks after immunization began and reached high titre on 45 to 60 days later which remained stable. Binding efficacy of anti- *M. globosa* IgY was examined by Growth Inhibition test by broth dilution method. Later, the broth culture used for inhibitory assay was plated on SDA supplemented with olive oil and incubated at 37°C for 4-5 days. It gives better observation of growth control.

CONCLUSION

Current experiment paves an alternate way for efficiently controlling the dandruff using antigen specific antibodies rather than using hazardous antidandruff products. Limited drug choice and recurrence over the usage indicates the need for adjunct therapeutic strategy. Antibody isolated from the edible egg is an organic fragment and does not possess harmful effects compared with the chemical anti-fungal agents. Chicken egg yolk is a safe material for preventing dandruff induced by opportunistic microbiome and has long lasting properties in preventing hair damage. However, further *in vitro* and *in vivo* studies in binding mechanism and stability of these antibodies have to be studied in detail to further the prospect of using IgY based cosmetic formulation.

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REFERENCE

1. Schwartz JR. DeAngelis and Dawson, Jr. Dandruff and Seborrheic Dermatitis: A Head Scratcher. Textbook of Practical Modern Hair Science, 12:1-26, (2012).
2. Farthing CF., Staughton RCD., Rowland Payne CME., Skin disease in homosexual patients with Acquired Immune Deficiency Syndrome (AIDS) and lesser forms of human T cell Leukaemia Virus (HTLV III) disease, *Clinical and Experimental Dermatology* 10: 3-12, (1985).
3. Warner RR., Schwartz JR., Boissy Y., Dawson TL., Dandruff has an altered stratum corneum ultra structure that is improved with zinc pyrithione shampoo, *Journal of American Academy of Dermatol*ogy 45: 897-903, (2001).
4. Ashbee RH., Glyn E., and Evans V., Immunology of Diseases Associated with *Malassezia* Species. *American Society for microbiology*, 21–57, (2002).
5. Gemmer C., DeAngelis M., Theelen B., Boekhout T., Dawson Jr., Fast, noninvasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology, *Journal of Clinical Microbiology*, 40(9):3350-7, (2002).
6. DeAngelis., Gemmer M., Joseph R., Dianna C., James R. Dawson., Jr. Three Etiologic Facets of Dandruff and Seborrheic Dermatitis: *Malassezia* Fungi, Sebaceous Lipids, and Individual Sensitivity. *J Investig Dermatol Symp Proc*, 10:295 –297, (2005).
7. Lee JH., Sakong J., Jang D., Yu C., Jang KH., Jun JH. Anti *Pityrosporum ovale* IgY and its uses, European Patent EP 1 379 556 B1, (2006).
8. Rojas.F, Sosa.M, Fernandez. M, Cattana.M, Cordoba.S, Guisiano.G. Antifungal susceptibility of *Malassezia furfur*, *Malassezia sympodialis*, and *Malassezia globosa* to azole drugs and amphotericin B evaluated using a broth microdilution method. *Medical Mycology*, 52: 641–646, (2014).
9. Nojiri N, Naito S, Takahashi H, Fujiki M, Kim M. Yolk antibody-containing hair care product, U S Patent US005976519A, (1991).
10. Michael A., Meenatchisundaram S., Parameswari G., Subbraj T., Selvakumaran R., and Ramalingam S., Chicken egg yolk antibodies (IgY) as an alternative to mammalian antibodies. *Indian Journal of Science and Technology*, 3(4): 468-474, (2010).
11. Zargari A., Emilson A., Hallden G., A Cell Surface Expression of two Major Yeast Allergens in the

Pityrosporum genus, *Clinical & Experimental Allergy*. 27:584–592, (1997).

12. Khosravi A.R., Eidi S., Katiraee F., Ziglari T., Bayat M., Nissiani M., Identification of Different *Malassezia* Species Isolated from Patients with *Malassezia* Infections, *World Journal of Zoology,* 4 (2): 85-89, (2009).
13. Koyama T., Kanbe T., Ishiguro A., Kikuchi A., Tomita Y., Antigenic components of Malassezia species for immunoglobulin E antibodies in sera of patients with atopic dermatitis, *Journal of Dermatological Science*, 26:201–208, (2001).
14. Hodek P., Trefil P., Simunek J., Hudecek J., Optimized Protocol of Chicken Antibody (IgY) Purification Providing Electrophoretically Homogenous Preparations, *International journal of electrochemical science,* 8(1):113-124, (2013).
15. Pauly D., Chacana PA., Calzado EG., Brembs B., and Schade R., IgY Technology: Extraction of Chicken Antibodies from Egg Yolk by Polyethylene Glycol (PEG) Precipitation, *Journal of Visualized Experiment*. 51: 1-5, (2011).
16. Lowry OH., Rosebrough NJ., Farr AL., and Randall RJ., Protein measurements with the Folin-Phenol reagent, *Journal Biological Chemistry*. 193: 265-275, (1951).
17. Dean Goldring JP and Theresa HT. Coetzer. Isolation of chicken immunoglobulins (IgY) from egg yolk. Available online at http://www.bambed.org; 31(3): 185–187, (2003).
18. Laemmli UK., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London). 227:680-685, (1970).
19. Voller A., Ann Bartlett., Bidwell DE., The Detection of Viruses by Enzyme-Linked Immunosorbent Assay (ELISA). *Journal of general Virology,* 33: 165 – 167, (1976).
20. Wan XZ., Fan B., Liu LG., Hu XY., Li RY., Wei Y., Wan Z., Deng XL., *In vitro* inhibition of oral *Candida albicans* by Chicken egg yolk antibody, *Mycopatholgia.* 168: 381- 387, (2008).