Research Article

Inherent immunological activity of chicken antibodies toward *Anopheles punctipennis* salivary gland antigens as early biomarkers for low-level infestation of malaria

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ABSTRACT

Introduction: Malaria is a major public health concern in most of the Asian and Sub-Saharan Africa, including Kenya, with *Anopheles punctipennis*, *Anopheles dirus, Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* as the principal vectors. Flocks that receive high mosquito exposure will be most effective for surveillance purposes. However, mosquito population indices at the flock sites may only provide an indirect measure of potential exposure. Aim: In this study, we have examined the applicability for IgY obtained from the screening of different kinds of hen eggs, for its capability to interact towards a mosquito antigenic protein. **Methods:** The growing diffusion of monoclonal and polyclonal antibodies in immunotherapy and immuno-diagnostics and the strict standards for animal care have led to a renewed interest in the use of chickens for antibody production, as opposed to mammals. Egg yolk represents an economical source of polyclonal antibodies since the concentration of IgY is similar or higher than in the serum of chickens or rabbits or humans reaching levels ranging from 15 to 25 mg IgY per ml of yolk in the case of hyperimmunized hen. **Results:** Soluble proteins were separated from the lipidic fraction of egg yolk by various methods and loaded onto polyethylene glycol-column. High recovery and purity of IgY were obtained for Guinea fowl eggs by charcoal and sodium alginate methods and for Polish hen eggs by charcoal method. **Conclusion:** Although the difference in egg sources for the IgY is significant and shown extreme interaction towards the antigen, development of antimalarial agents represents technical challenges and immense opportunities for improvement of global health.

KEY WORDS: *Anopheles*, Antigen, Enzyme-linked immunosorbent assay, Guinea fowl, IgY, Lipoprotein

INTRODUCTION

Mosquito saliva contains anti-hemostatic, anti-inflammatory, and immunomodulatory molecules that facilitate the acquisition of a blood meal.[1] Mosquito stage malaria vaccines are designed to induce an immune response in the human host that will block the parasite's growth in the mosquito and consequently block transmission of the parasite. A mosquito membrane-feeding assay is used to test transmission-blocking activity (TBA) but in this technique cannot accommodate many samples.[2] A clear understanding of the relationship between antibody levels and TBA may allow enzyme-linked immunosorbent assay (ELISA) determinations to be used to predict TBA and assist in planning vaccine development. Although domestic animals may not be permissive for *Plasmodium*, they could nevertheless play a role in the epidemiology of malaria by attracting *Anopheles* away from humans.[3] To investigate interactions between domestic animals and mosquitoes, antibodies directed against the salivary proteins *of Anopheles* species are required where malaria is endemic.[4] The current attempts to develop malaria vaccines are primarily focused on Anopheles dirus and Anopheles punctipennis and are directed toward reducing morbidity and mortality.[5] Collectively, studies investigating the effects of mosquito saliva on the vertebrate immune response suggest that high concentrations of salivary proteins are immunosuppressive,[6] whereas lower concentrations modulate the immune response; specifically, TH1 and antiviral cytokines are downregulated, while TH2 cytokines are unaffected or amplified.[7] Many probiotic effects are mediated through immune regulation, particularly through balance control of pro- and anti-inflammatory

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cytokines.[8] Antibodies also play a role in many different compounds of biological origin that is used in the immune response; these are interferons, interleukin 2, and monoclonal antibodies which can be used for the prevention of malaria or cancer.[9] In response to ischemia/reperfusion (I/R), interferon regulatory factor 9 accumulated in neurons, and a transcription factor that regulates innate immune responses has been implicated in neurological pathology.[10]

MATERIALS AND METHODS

Purification Methods for Immunoglobulin Y

Charcoal method

Purification of IgY was carried out as described by Al-Edany.[11] 10 ml of yolk was diluted 1:9 times with distilled water acidified with 1N HCl to reach pH 5.0 and centrifuged at 2800 g for 40 min at 4°C. After centrifugation, the supernatants were separated from the pellets, and 0.1% charcoal was added and adjusted pH 4.0. Then centrifuged at 2800 g for 40 min at 4°C, collected supernatant, and filtered it on filter papers. Crude extract was collected and stored for further usage.

Sodium alginate method

10 ml of yolk was diluted 1:4 with Tris-buffered saline buffer, 6 ml of 10% sodium alginate, and 15 ml of 1M calcium chloride were added, and the mixture was incubated for 20 min at room temperature. After incubation samples were centrifuged at 10000 g for 20 min, the supernatants were separated from the pellets and filtered on filter papers. Crude extract was collected and stored for further usage.[12]

Chloroform method

Ten ml of yolk was diluted with chloroform at 1:1 ratio and incubated for 20 min at room temperature. After incubation samples were centrifuged at 10000 g for 20 min, the supernatants were separated from the pellets and filtered on filter papers. Crude extract was collected and stored at low temperatures for further usage.[13]

Polyethylene glycol (PEG) method

Antibodies were isolated from various eggs with the use of varying concentrations of PEG.[14] This method consists of two successive precipitations in PEG, using 3.5% PEG to remove fatty substances and then 12% PEG to precipitate the IgY and the samples were run on desalting column (Merck Biosciences) to remove salts and/or stored at −20°C until further use.

Diethylaminoethyl (DEAE) Purification of IgY

Sample from the desalting column was further purified using DEAE cellulose ion exchange column (HiMedia, Mumbai). Column was equilibrated with 25 mM phosphate buffer pH 8.0. Sample was loaded, washed with phosphate buffer, and eluted with increasing sodium chloride (0–2 M) buffer.[15] Elutions were collected, and purity was analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined using Bio-Rad protein assay (Merck Biosciences) using bovine serum albumin as a standard.

SDS-PAGE Analysis of Proteins

Purified antibodies were suspended in a ×2 gel sample buffer (2% SDS, 75 mM Tris HCl [pH 6.7], 10% glycerol, 0.2% bromophenol blue, and 2% beta-mercaptoethanol) and subjected to 12% PAGE. Fractions were collected and diluted 1:2 times with ultra-clean milliQ water before SDS-PAGE analysis.[16] Molecular weight markers (Merck Biosciences) were used according to the manufacturers' instructions. Protein samples of ~10 µg/ml were loaded per lane and migrated with a constant voltage at 100 V for 2 h. Gel bands were visualized after staining with direct stainer (HiMedia, Mumbai) for 1 h at room temperature.

Determination of Antigen Binding Toward IgY by ELISA

ELISA plates were coated with 100 µl of antigen dilution with a concentration of 1 µg/ml and incubated for overnight at room temperature or for 2 h at 37°C. 200 µl blocking buffer is added and incubated for 1 h, further with wash buffer (×1 PBST) wells were washed 3 times to remove any unbound or loosely bound antibodies with antigen. The plate was incubated for 1 h at room temperature. IgY extracted from column method was used as primary antibody and horseradish peroxidase conjugated mouse anti-chicken-IgE antibody (Merck Biosciences) was added. Subsequently, 100 µl of the substrate (TMB, HiMedia) were loaded into each well and analyzed in ELISA reader at 450 nm.[17]

RESULTS AND DISCUSSION

Isolation of IgY

Four different types of eggs Hen, Duck, Polish Hen, and Guinea Fowl were collected from Bird sanctuary located in K.L. University, Vijayawada, Andhra Pradesh. IgY is preliminarily isolated from the egg yolk using three different methods, namely, Charcoal, Chloroform, and Sodium Alginate. These samples were then subjected to further purification using ion exchange resin for better yield and to determine the activity toward the mosquito antigens. Pure form of antigens was obtained from Sri Raghavendra Biotechnologies Pvt. Ltd, Bengaluru. The antigens were revived with 2 N NaOH, in the presence of 0.85% sodium chloride solution (NSS) and 20 µl each of 10 mM of the protease inhibitors TPCK and PMSF as recommended for Salivary gland and Larvae stage. While total antigen is not revived as used directly to determine its activity as that described by Boreham and Gill, 1973 [Table 1].[18]

SDS-PAGE Analysis of Immunoglobulins

SDS-PAGE analysis revealed two major bands of immunoglobulin (10 µg/ml) with molecular weight 50 KDa and 25 KDa [Figure 1] against 5 µl of protein marker (0.1 mg/ml). Various fractions (elution and wash) were collected, and after protein elution, they were diluted 1/2 with milliQ ultrapure water before SDS-PAGE and protein determination.[19]

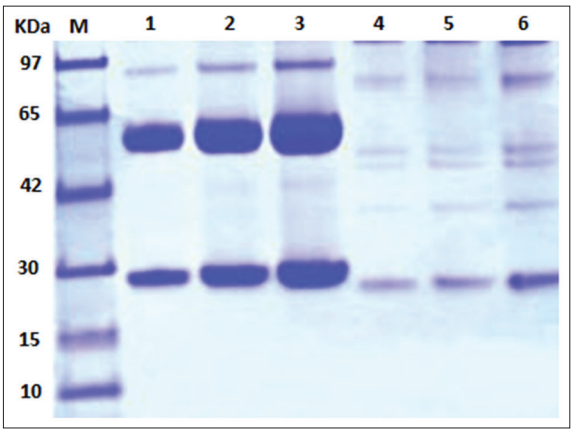

Figure 1: Analysis of IgY using sodium dodecyl sulfate-polyacrylamide gel electrophoresis: Lane 1: Protein marker (Puregene). Lane 2: Guinea fowl - sodium alginate, Lane 3: Guinea fowl - charcoal, Lane 4: Polish hen - charcoal, Lane 5: Polish hen - sodium alginate, Lane 6: Hen - sodium alginate, and Lane 6: Duck - sodium alginate

ELISA for IgY Against *Anopheles* Antigen

ELISA experiment was performed using increasing concentration of antibodies with a constant concentration of antigen.[20] The concentration of antibody increases from 1 µg/ml to 96 µg/ml and antigen at a constant concentration of 1 µg/ml. A complete 96 well ELISA plate was performed, and at 450 nm filter, the readings were noted. The binding assay results clearly show that protein in extract showed specific direct binding to IgY toward various antigens, namely, Antigen A as Salivary gland, Antigen B as Larvae, and Antigen C as total antigen. Similarly, phosphate-buffered saline used as a negative control showed no affinity for IgY [Figures 2-9].

CONCLUSION

Monoclonal antibodies have been applied clinically to the diagnosis and therapy of an array of human disorders, including cancer and infectious diseases, and have been used for the modulation of immune responses. Effective therapy using unmodified monoclonal antibodies has, however, been elusive.

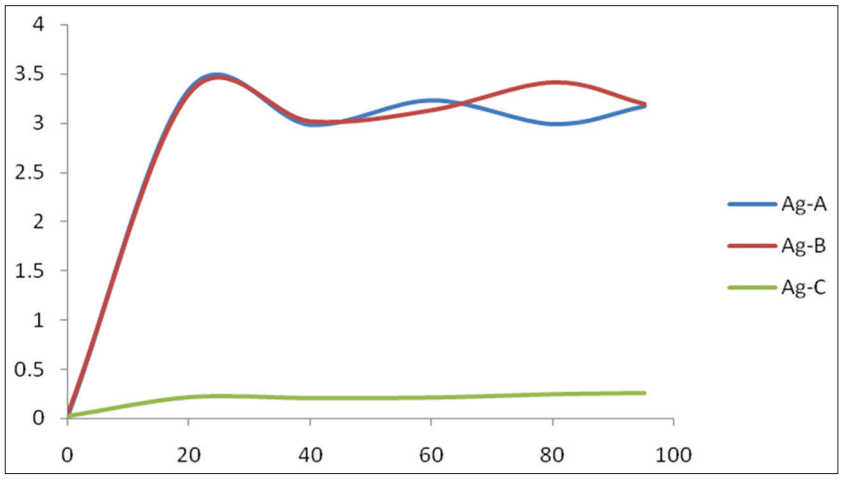

Figure 2: Duck - sodium alginate method - for the Antigen A the optimum result is observed at 20 µg, Antigen B is 80 µg, and for Antigen C it is at 95 µg, respectively

Table 1: Concentration of IgY in various egg sources obtained by purification methods

Source of the egg	Purification method	Concentration (µg/ml)	Absorbance at 280 nm
Duck	Alginate	96.17	0.136
	Charcoal	272.25	0.385
	Chloroform	108.19	0.153
Guinea fowl	Alginate	576.32	0.815
	Charcoal	458.22	0.648
	Chloroform	195.87	0.277
Hen	Alginate	301.95	0.427
	Charcoal	265.17	0.375
	Chloroform	516.21	0.730
Polish hen	Alginate	205.07	0.290
	Charcoal	779.27	1.102
	Chloroform	36.77	0.052

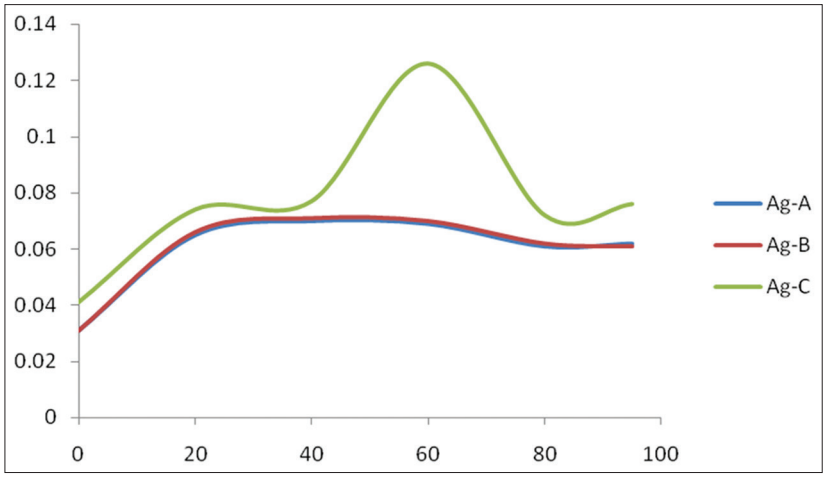

Figure 3: Duck - charcoal method - for the Antigen A the optimum result is observed at 40 µg, Antigen B is 40 µg, and for Antigen C it is at 95 µg, respectively

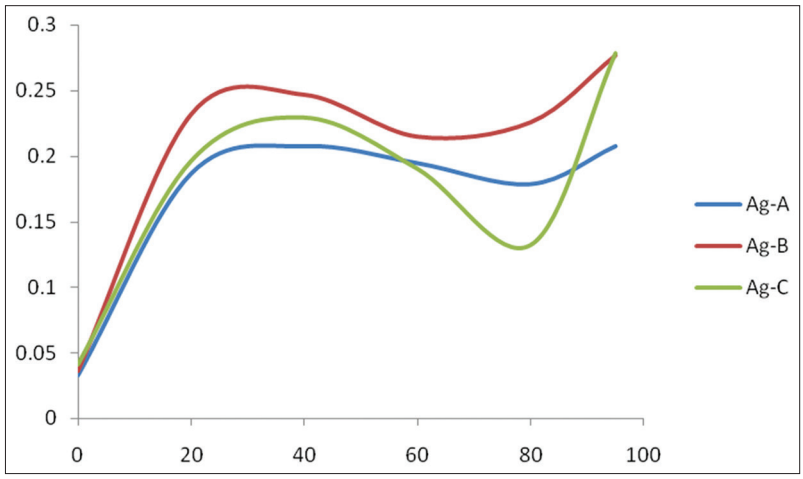

Figure 4: Guinea fowl - sodium alginate method - for the Antigen A the optimum result is observed at 40 µg and 95 µg, Antigen B is 95 µg, and for Antigen C it is at 95 µg, respectively

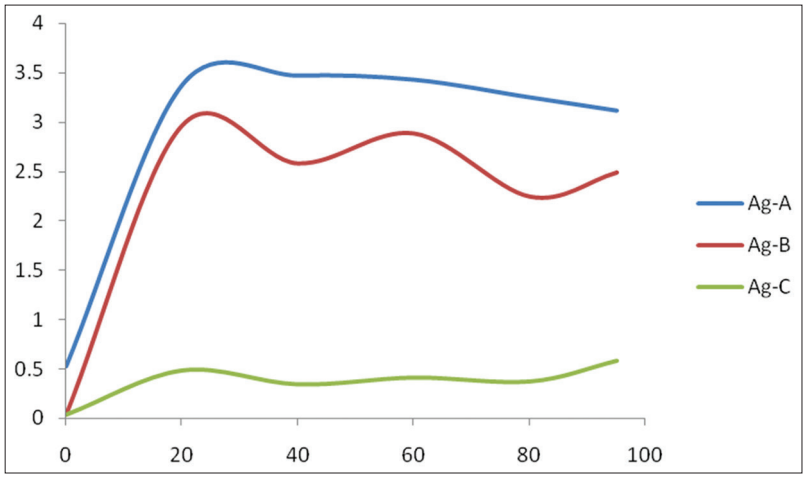

Figure 5: Guinea fowl - charcoal method - for the Antigen A the optimum result is observed at 40 µg, Antigen B is 20 µg, and for Antigen C it is at 95 µg, respectively

Recently, monoclonal antibody-mediated therapy has been revolutionized by advances such as the definition of cell-surface structures on abnormal cells as targets for effective monoclonal antibody action, genetic engineering to create less immunogenic and more effective monoclonal antibodies, and the arming of

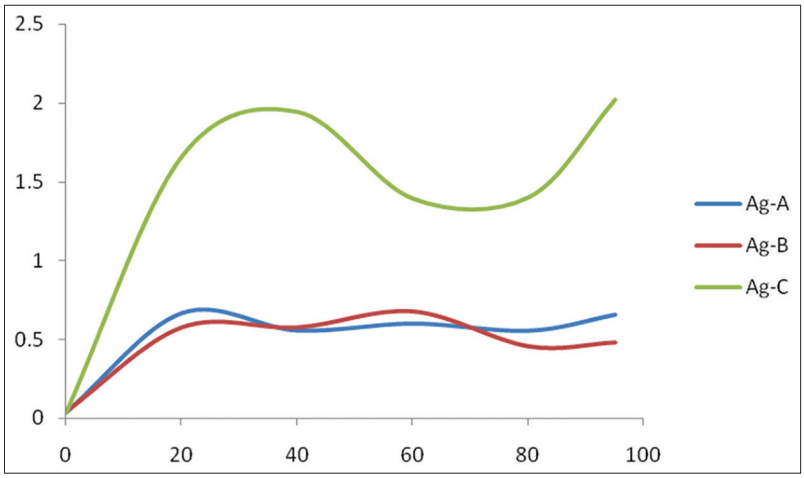

Figure 6: Hen - sodium alginate method - for the Antigen A the optimum result is observed at 20 µg, Antigen B is 60 µg, and for Antigen C it is at 95 µg, respectively

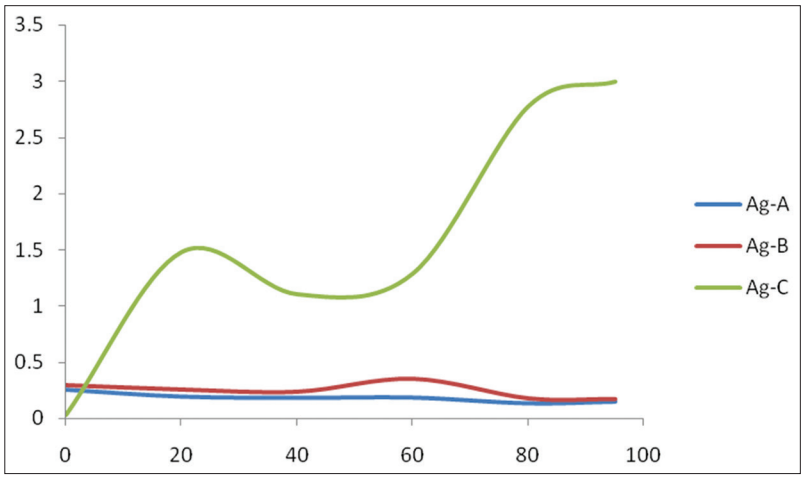

Figure 7: Hen - charcoal method - for the Antigen A the optimum result is observed at 0 µg, Antigen B is 60 µg, and for Antigen C it is at 95 µg, respectively

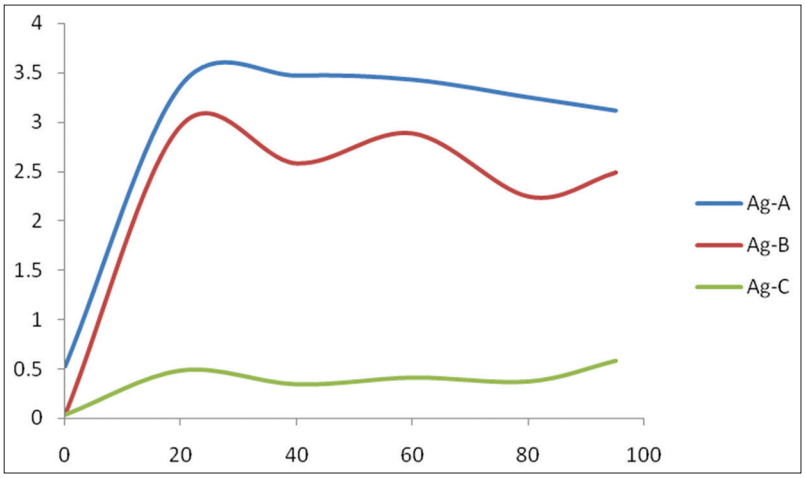

Figure 8: Polish hen - sodium alginate method - for the Antigen A the optimum result is observed at 40 µg, Antigen B is 20 µg, and for Antigen C it is at 95 µg, respectively

such antibodies with toxins to enhance their effector function. There is a pressing need for safe and effective monoclonal antibodies that have minimal side effects. Long-used standard treatments, including hybridoma technology and steroid drugs, have well-known and significant toxic side effects. However,

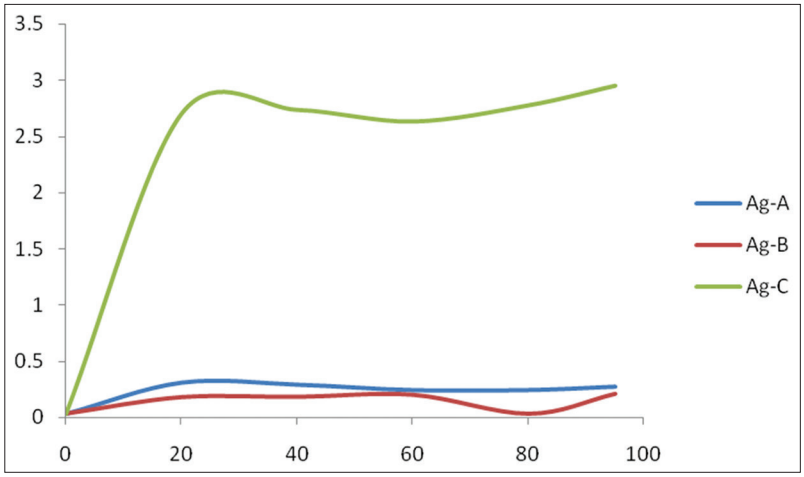

Figure 9: Polish hen - charcoal method - for the Antigen A the optimum result is observed at 20 µg, Antigen B is 95 µg, and for Antigen C it is at 95 µg, respectively

natural monoclonal antibodies production is highly recommended to overcome the significant side effects produced by the immune system.

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