Research Paper

Defending health: chicken egg igy antibodies targeting infectious diseases caused by vibrio harveyi

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ABSTRACT

This study investigates the potential use of chicken egg yolk-derived immunoglobulin Y (IgY) as an alternative to mammalian antibodies for passive immunization against Vibrio harveyi infections in Fenneropenaeus indicus. The research assesses the effectiveness of an immunogen derived from inactivated V. harveyi, with and without the immunoadjuvant Glycine max saponin. Purified IgY antibodies are prepared and characterized for their molecular weight, physicochemical parameters, and binding activity. The study aims to provide an alternative approach to combat Vibrio infections in aquatic species, offering a potential substitute for current antibiotic and synthetic drug delivery methods.

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1. INTRODUCTION

Advancements in vaccine technology have yielded vaccines featuring highly purified antigens, offering improved safety and tolerability. However, these vaccines often exhibit suboptimal immune responses without the assistance of adjuvants. Soybean meal, known for its abundance of saponins, particularly soyasaponins, has demonstrated potent adjuvant activity when of high purity. This makes them a cost-effective and safe option for practical use in vaccine adjuvants [1]. Traditional vaccination methods, while effective in preventing disease outbreaks, may not be as impactful for crustaceans due to their lack of a true adaptive immune response [2]. Nonetheless, passive immunization using pathogen-specific antibodies produced in hens emerges as a promising approach to address diseases, offering crustaceans a specific antibody response despite their inherent immune limitations [3].

Chicken egg yolk has been recognized as a cost-effective source of antibodies, and hyper-immunized hens offer a convenient and economical means of obtaining immunoglobulins in their egg yolk, known as IgY. IgY exhibits notable heat stability, with most antibody activity retained after 15 minutes at 70°C. While incubation at pH levels above 4 is well-tolerated, a decline in activity is observed at pH 2 and 37°C, likely due to conformational changes rather than peptide breakdown, as indicated by SDS-PAGE analysis. IgY fractions have demonstrated long-term stability when stored in 0.9% NaCl, 0.02% NaN3 at +4°C for over a decade, showing no significant loss of antibody titer. Furthermore, eggs can be stored at +4°C with only minimal loss of IgY activity for at least six months [4]. The objectives of this study were to assess the physicochemical properties and efficiency of anti-Vibrio harveyi IgY. In vitro studies from this research unmistakably demonstrated the efficient protection of shrimps from V. harveyi using egg yolk antibodies from laying hens (Gallus gallus domesticus)

2. METHODOLOGY

Anti-Vibrio harveyi IgY samples (ABC - Antibody from Control egg, ABG - Antibody with Glycine max adjuvant, ABWO - Antibody without adjuvant) were previously obtained from our laboratory and stored at 4-6 ºC as shown in Figure 1. The process involved careful separation of egg yolk from the egg white, followed by a 10-fold dilution in distilled water. The solution was then acidified with 0.1 N HCl to achieve a pH of 5.2 at 4ºC overnight. Subsequently, the egg suspension underwent centrifugation at 16000 x g for 30 minutes at 4ºC. The resulting filtrate, containing the water-soluble fraction (WSF) of egg yolk, was utilized as a source of IgY for freeze-dried yolk, following the procedure outlined by Akita and Nakai [5]. The pH was studied by varying the (4 -7.5) of the Control IgY, Antibody with and without Glycine max Adjuvant. Of these different NaCl concentrations (0.50, 1.00, and 1.50 etc) added. After the incubation period 10ml of taken in the beaker and the values obtained from the pH meter. The turbidity was determined by reading the absorbance of sample solutions using a spectrophotometer at 600 nm. The estimation of lipid a known amount of IgY was taken and grinds it in chloroform methanol (2:1 ratio). [6] Then add 2ml of 0.9% NaCl and keep it overnight. Two layers are developed. Take the layer and transfer it is another test tube. Then evaporate it an oven or boiling water bath. The dried lipid content is dissolved in 0.5 ml concentrated H_2SO_4. From this take 0.2 ml then add 5ml of vanellin reagent. A red colour was developed and read at 520 nm.

The quantification of immunoglobulin involves the precipitation of proteins by adding 40% APS, and the supernatant is utilized for protein estimation. Aromatic amino acids, such as tyrosine and tryptophan present in the protein, react with APS, producing a dark blue color that can be measured colorimetrically at 650 nm. Additionally, the crude IgY concentration was determined by measuring the absorbance at 280 nm, following the method outlined by Tenenhouse and Deutsch in 1966. The specific IgY concentration was calculated using the following formula or method, which unfortunately is not provided in the current context [7].

$$Specific\ IgY\ (\%) = \frac{Absorbance\ without\ antigen - absorbance\ with\ antigen}{Absorbance\ without\ antigen} * 100$$

The growth inhibition assay, as described by Saxena [8], was employed to assess the binding activity of anti-V. harveyi IgY, demonstrating its capability to inhibit V. harveyi growth in a liquid medium. The antigen utilized for immunizing chickens consisted of the same strain of V. harveyi, subcultured on tryptic soy agar plates supplemented with 1.5% NaCl and suspended in TSB. A 2 ml volume of the prepared bacterial culture was mixed with 2 ml of TSB and incubated at 37ºC with shaking. The turbidity of the culture, measured as optical density at 600 nm, was recorded using a spectrophotometer at 1-hour intervals, and the growth curve was plotted until reaching the stationary phase. Statistical analysis of all data obtained from experiments was conducted using one-way ANOVA, with significance set at $P < 0.05$, utilizing the Statistica 6.0 computer package (Statsoft, UK). Means were further compared using the SNK test.

3. RESULTS AND DISCUSSION

The egg yolk powder serves as a rich source of antibodies for Vibrio infection prevention through passive immunization, containing approximately 100-150 mg of antibodies per egg. Notably, the antibody content in the antibody powder is approximately ten times higher than that in the yolk powder alone. Incubating a diluted egg yolk solution at freezing (-20°C) or refrigeration temperature (4°C) proves beneficial in eliminating lipoproteins from the water-soluble fraction. Additionally, the quantitative aspects of the immunoadjuvant significantly influence the enhancement of antibody production. The physicochemical properties of antibodies with Glycine max adjuvant (AB_G) and without adjuvant (AB_{WO}) were investigated, and the results are summarized as follows. The pH stability of Control IgY, AB_G, and AB_{WO} was assessed at different NaCl concentrations, revealing optimal stability within the pH range of 6.15 to 6.78. The stability was further enhanced at pH levels of 6.78 and 7.0 for both AB_G and AB_{WO}. Turbidity of Anti-Vibrio harveyi IgY was measured at 0.972 for 0.50% NaCl in the control group, with an increase to 1.345 for AB_{WO} and 1.083 for AB_G, signifying significant differences ($P < 0.05$) among the groups

Table 1. The utilization of herbal immunoadjuvant resulted in a notable enhancement of lipid production. The lipid levels exhibited a gradual and significant increase ($P < 0.01$) compared to the control IgY in both Antibody With and Without Adjuvant. Subsequently, lipid levels were elevated across all Anti-Vibrio harveyi IgY antibodies Table 2. Specifically, the IgY concentration without Adjuvant was measured at 2.284 mg/ml, surpassing that of the control IgY. Furthermore, IgY determination at 650 nm across different concentrations (ranging from 0.20% to 0.80%) showed an increasing trend Table 3. The maximum IgY determination for ABG was recorded at 2.128, displaying significant differences ($P < 0.001$). The growth of V. harveyi, when incubated with antibody, specifically with Glycine max adjuvant IgY, demonstrated a significant reduction in bacterial growth after 4 hours of incubation. In contrast, control IgY exhibited a limited effect on bacterial growth. The effectiveness of anti-V. harveyi IgY in inhibiting bacterial growth was notably observed in IgY with Glycine max adjuvant, while IgY without adjuvant showed a lower impact. The effectiveness was determined by the concentration of IgY powder, as outlined in

Table *4*.

Discussion

The potential therapeutic application of egg yolk immunoglobulin (IgY) through passive immunization therapy via oral ingestion has been evaluated. [9] Reported stability of IgY over a prolonged period (5 years) at cold temperatures (4°C) without affecting antibody activities, our findings suggest that some IgY experiences precipitation during freezing and subsequent loss during storage at cold temperatures. This phenomenon is likely attributed to irreversible aggregation under these specific storage conditions. The effect of Anti- V. harveyi IgY was more evident at p H values close to or higher than the IgY isoelectric point (5.7). Rapid decrease of the IgY activity at low pH indicated conformational changes and damage in the Fab portion including the antigen-binding site. Under alkaline conditions, the activity of IgY did not change until the pH increased to 11. However, it was markedly diminished at p H 12 or higher. Similar results about pH effect were presented by Lee [4].

This study evaluates the efficacy of vaccines containing inactivated V. harveyi, with and without an immunoadjuvant, in generating Anti-V. harveyi IgY. In contrast, various in vitro studies have demonstrated the inhibitory effect of specific IgY on the bacterial growth of Salmonella spp [10]. To be effective in shrimp immunization and protection, these antibodies must withstand the gastrointestinal environment and retain their intact biological properties upon reaching their target areas. [11], [12], [13] highlighted the numerous benefits of IgY technology, emphasizing its universal application in research and medicine. It is anticipated that IgY will play an increasingly significant role in future research, diagnostics, and immunotherapy due to its versatile nature.

Table 1. Evaluating Egg Yolk Properties: Ph and Turbidity with Anti-Vibrio Harveyi Igy

IgY Antibody	pH						Turbidity				
	0.50 %	1.50 %	1%	2%	2.50 %	3%	0.10 %	0.20 %	0.30 %	0.40 %	0.50 %
AB_C	5.92	5.72	5.81	6.0	6.05	6.01	0.217^{a} ± 0.04	0.349^{a} ± 0.06	0.841^{a} ± 0.07	0.825^{a} ± 0.01	0.292^{a} ± 0.02
AB_G	6.62	6.64	6.6	6.67	6.69	6.78	0.634^{b} ± 0.07	0.868^{b} ± 0.01	0.899^{a} ± 0.05	0.766^{a} ± 0.07	1.083^{b} ± 0.08
AB_{WO}	6.48	6.62	6.45	6.70	6.85	7.0	0.169^{c} ± 0.06	0.149^{c} ± 0.09	1.011^{b} ± 0.05	1.344^{b} ± 0.05	0.345^{c} ± 0.06

Means with the same superscripts do not differ from each other ($P < 0.05$).

Descriptions:

AB_C= Antibody from control egg yolk
AB_G = Antibody from V. harveyi with Glycine max
AB_A = Antibody from V. harveyi without Adjuvant

Table 2. Lipid Content in Extracted Egg Yolk: A Comparative Analysis of Control, with, and Without Anti-Vibrio harveyi IgY

Sl.No	IgY Antibody	NaCl concentration (%)		
		0.1	0.2	0.3
1	Blank control	1.15^{a} ± 0.09	1.53^{a} ± 0.10	1.82^{a} ± 0.15
2	Control (AB_C)	3.52^{b} ± 0.18	4.73^{b} ± 0.21	5.10^{b} ± 0.46
3	AB_G	2.58^{b} ± 0.19	3.48^{c} ± 0.08	3.95^{c} ± 0.17
4	AB_{WO}	1.45^{a} ± 0.17	1.71^{a} ± 0.19	1.97^{a} ± 0.20

Means with the same superscripts do not differ from each other ($P < 0.01$).

Table 3. Characterisation of Igy Powder Prepared from the Water Soluble Fraction (WSF) Containing V. Harveyi with and Without Adjuvant

IgY Antibody Powder	Total IgY (mg/ml)	Purity (%)	Concentration (mg/ml)
Blank control	1.00^{a}	4.05^{a}	0.923^{a}
AB_C	1.15^{a}	18.5^{b}	1.452^{a}

ABG	2.86 b	23.4 c	1.734 b
ABWO	2.13 b	21.12 c	2.284 c

Means with the same superscripts do not differ from each other ($P < 0.01$).

Table 4. Growth Inhibition Assay Performed between V. Harveyi and Anti - V. Harveyi Igy Produced by with and Without Adjuvant

IgY Antibody		**Incubation Time (Hours)**				
		0	**2**	**4**	**6**	**8**
100mg	ABC	0.274	0.778	0.945	0.734	0.654
	ABG	0.183	0.465	0.215	0.074	0.00
	ABWO	0.167	0.397	0.324	0.021	0.00
200mg	ABC	1.098	0.786	0.857	0.654	0.579
	ABG	1.318	0.854	0.156	0.015	0.00
	ABWO	1.316	0.991	0.244	0.047	0.00

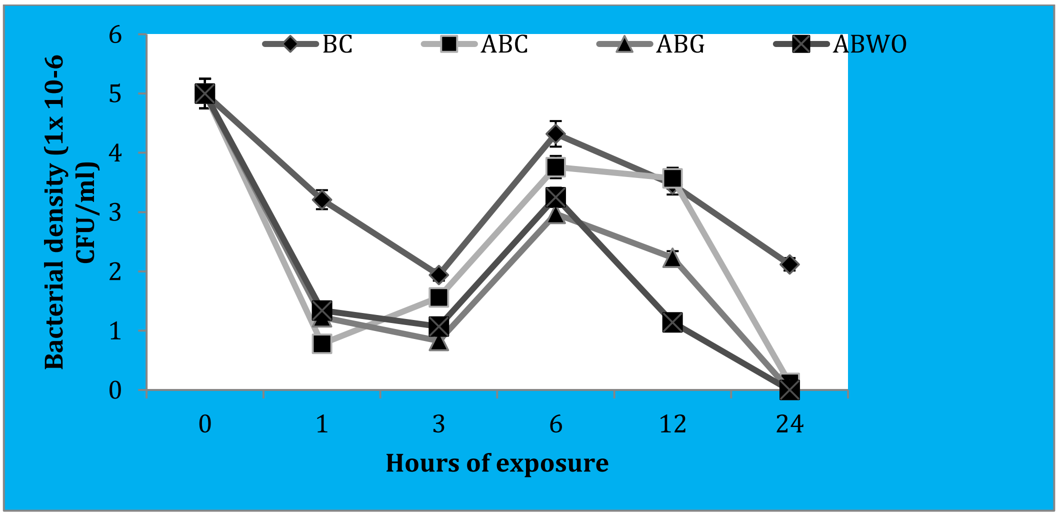

Figure 1. In Vitro Neutralizing Antibody Experiment: Evaluating IgY containing V. harveyi with and without Adjuvant

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Conflict of Interest Statement

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Dr. T. Kumaran	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Prathika M	✓			✓	✓			✓	✓		✓		✓	
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C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

Fo : **Fo**rmal analysis E : Writing - Review & **E**diting

Informed Consent

All participants were informed about the purpose of the study, and their voluntary consent was obtained prior to data collection.

Ethical Approval

The study was conducted in compliance with the ethical principles outlined in the Declaration of Helsinki and approved by the relevant institutional authorities.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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