

**DEVELOPMENT OF CHICKEN EGG YOLK ANTIBODIES
(IgY) AGAINST *Aeromonas hydrophila* AND ITS EFFECT ON
DISEASE CONTROL IN FARMED FISHES**

*Thesis Submitted to the University of Calicut in partial fulfilment
of the requirement for the award of the degree of*

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

Submitted by,

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NOVEMBER, 2025

**DEVELOPMENT OF CHICKEN EGG YOLK ANTIBODIES (IgY)
AGAINST *Aeromonas hydrophila* AND ITS EFFECT ON DISEASE
CONTROL IN FARMED FISHES**

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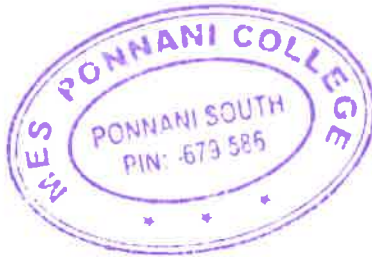
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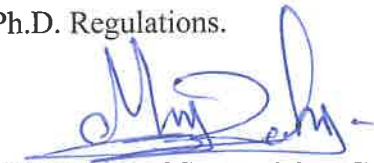
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I hereby declare that the work presented in the thesis entitled “**Development of Chicken Egg Yolk Antibodies (IgY) against *Aeromonas hydrophila* and its Effect on Disease Control in Farmed Fishes**” is based on the original work done by me under the guidance and supervision of **Dr. Mujeeb Rahiman. K.M.**, Associate Professor, School of Industrial Fisheries, Cochin University of Science and Technology, Kochi and under the co-guidance of **Dr. Denoj Sebastian**, Professor, Dept. of Life Sciences, Calicut University, Thenjippalam, and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis have undergone a plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated content.

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Thushara James

DEVELOPMENT OF CHICKEN EGG YOLK ANTIBODIES (IgY) AGAINST *Aeromonas hydrophila* AND ITS EFFECT ON DISEASE CONTROL IN FARMED FISHES

Abstract

Aeromonas hydrophila, the causative agent of Motile *Aeromonas* Septicemia (MAS), has emerged as a major threat to aquaculture because of high fish mortality, economic losses, and the increasing prevalence of antimicrobial resistance resulting from excessive antibiotic use. The present study aimed to develop, characterize, and evaluate chicken egg yolk immunoglobulin Y (IgY) antibodies against *A. hydrophila* and to investigate their prophylactic and therapeutic potential for controlling bacterial infections in cultured fish.

Specific IgY antibodies were produced by immunizing White Leghorn hens with formaldehyde-inactivated *A. hydrophila*. The antibodies were extracted from egg yolks using polyethylene glycol (PEG) and ammonium sulphate precipitation and purified by DEAE cellulose ion-exchange chromatography. SDS-PAGE analysis revealed a distinct protein band at approximately 180 kDa, confirming the purity and structural integrity of IgY. The average IgY yield was 11.88 mg/mL, of which 26% was antigen-specific. Indirect ELISA demonstrated a high antibody titre of 1:10,000, indicating successful production of highly specific antibodies against *A. hydrophila*.

The biological activity of purified IgY was evaluated through a series of *in vitro* assays. IgY effectively inhibited the growth of *A. hydrophila* and exhibited cross-reactivity with *Aeromonas caviae*. In combination with ciprofloxacin and chloramphenicol, IgY showed synergistic activity, with Fractional Inhibitory Concentration Index (FICI) values below 0.5, suggesting enhanced antibacterial efficacy and reduced antibiotic dependence. The antibodies retained substantial antigen-binding activity over a wide pH range (3-10), remained stable at temperatures up to 70°C, and maintained functional activity under different molar concentrations and in the presence of ethanol, methanol, and isopropanol.

The protective efficacy of IgY was further assessed through *in vivo* challenge studies using grass carp (*Ctenopharyngodon idella*) experimentally infected with *A. hydrophila*. Following determination of the pathogen LC₅₀, IgY-coated feed was evaluated under pathogen-neutralization, therapeutic, and prophylactic treatment regimes. Oral administration of IgY significantly improved fish survival, with survival rates of 70%, 60%, and 80% in the neutralization, therapeutic, and prophylactic groups, respectively. Histopathological examination revealed markedly reduced tissue damage, inflammatory cell infiltration, necrosis, and epithelial degeneration in IgY-treated fish. The use of IgY-coated feed offers a practical strategy to enhance fish survival, reduce antibiotic usage, and promote sustainable aquaculture production.

Keywords: IgY antibodies; *A. hydrophila*; *Ctenopharyngodon idella*; *in vitro* studies; *in vivo* studies.

ഡെവലപ്മെന്റ് ഓഫ് ചിക്കൻ എഗ്ഗ് യോക് ആന്റിബോഡിസ് (ഐ.ജി.വൈ) എഗ്ഗെൻസ് എയ്റോമോണാസ് ഹൈഡ്രോഫില ആൻഡ് ഇറ്റ്സ് ഇഫ്ഫെക്ട് ഓൺ ഡിസിസ് കൺട്രോൾ ഇൻ ഫാഡ് ഫിഷസ്

സംഗ്രഹം

ജലകൃഷി മേഖലയിലെ പ്രധാന രോഗകാരികളിലൊന്നായ എയ്റോമോണാസ് ഹൈഡ്രോഫില മൂലമുണ്ടാകുന്ന മോട്ടെൽ എയ്റോമോണാസ് സെപ്റ്റീസീമിയ (MAS) ഉയർന്ന മത്സ്യമരണത്തിനും സാമ്പത്തിക നഷ്ടത്തിനും ആന്റിബയോട്ടിക്കുകളുടെ അമിത ഉപയോഗവുമായി ബന്ധപ്പെട്ട ആന്റിമൈക്രോബിയൽ പ്രതിരോധത്തിനും കാരണമാകുന്ന പ്രധാന വെല്ലുവിളിയാണ്. ഈ സാഹചര്യത്തിൽ, എ. ഹൈഡ്രോഫിലയ്ക്കെതിരായ കോഴിമുട്ടയുടെ മഞ്ഞക്കരുവിൽ നിന്നുള്ള ഇമ്യൂണോഗ്ലോബുലിൻ വൈ (ഐ.ജി.വൈ) ആന്റിബോഡികളുടെ വികസനം, വിശകലനം, രോഗനിയന്ത്രണത്തിലെ പ്രതിരോധപരവും ചികിത്സാപരവുമായ സാധ്യതകൾ ഈ പഠനത്തിൽ വിലയിരുത്തി.

ഫോർമാൽഡിനൈഡ് ഉപയോഗിച്ച് നിർജീവമാക്കിയ എ. ഹൈഡ്രോഫില ആന്റിജൻ ഉപയോഗിച്ച് വെള്ള ലഗോൺ കോഴികളെ പ്രതിരോധവൽക്കരിച്ചാണ് പ്രത്യേക ഐ.ജി.വൈ ആന്റിബോഡികൾ ഉൽപ്പാദിപ്പിച്ചത്. പോളിഎത്തിലീൻ ഗ്ലൈക്കോൾ (PEG), അമോണിയം സൾഫേറ്റ് എന്നിവ ഉപയോഗിച്ച് ആന്റിബോഡികൾ വേർതിരിച്ചെടുത്ത് DEAE സെല്ലുലോസ് അയൺ-എക്സ്ചേഞ്ച് ക്രോമാറ്റോഗ്രാഫി വഴി ശുദ്ധീകരിച്ചു. SDS-PAGE വിശകലനത്തിൽ ഏകദേശം 180 kDa-ൽ വ്യക്തമായ പ്രോട്ടീൻ ബാൻഡ് കണ്ടെത്തി. ശരാശരി 11.88 mg/mL ഐ.ജി.വൈ ലഭിച്ചതിൽ ഏകദേശം 26% രോഗാണുവിനെതിരായ പ്രത്യേക ഐ.ജി.വൈ ആയിരുന്നു. ഇൻഡയറക്ട് ELISA പരിശോധനയിൽ 1:10,000 എന്ന ഉയർന്ന ആന്റിബോഡി ടൈറ്റർ രേഖപ്പെടുത്തി.

ശുദ്ധീകരിച്ച ഐ.ജി.വൈയുടെ ജൈവപ്രവർത്തനം വിവിധ ഇൻ വിട്രോ പരീക്ഷണങ്ങളിലൂടെ വിലയിരുത്തി. ഐ.ജി.വൈ, എ. ഹൈഡ്രോഫിലയുടെ വളർച്ച ഫലപ്രദമായി തടയുകയും എയ്റോമോണാസ് കാവിയേയുമായി ക്രോസ്-റിയാക്ടിവിറ്റി പ്രകടിപ്പിക്കുകയും ചെയ്തു. സിപ്രോഫ്ലോക്സാസിൻ, ക്ലോറാഫെനിക്കോൾ എന്നിവയുമായി സംയോജിപ്പിച്ചപ്പോൾ Fractional Inhibitory Concentration Index (FICI) മൂല്യം 0.5-ൽ താഴെയായിരുന്നതിനാൽ ശക്തമായ സിനർജിസ്റ്റിക് പ്രവർത്തനം തെളിഞ്ഞു. കൂടാതെ pH 3-10 പരിധിയിലും 70°C വരെ താപനിലയിലും വ്യത്യസ്ത മോളാർ സാന്ദ്രതകളിലും ഐ.ജി.വൈ പ്രവർത്തനക്ഷമത നിലനിർത്തുന്നതായി കണ്ടെത്തി.

ടീനോഫാരിംഗോഡോൺ ഇഡെല്ല എന്ന ഗ്രാസ് കാർപ്പിൽ നടത്തിയ ഇൻ വിവോ പഠനങ്ങളിൽ രോഗാണുവിന്റെ LC₅₀ നിർണയിച്ച ശേഷം, ഐ.ജി.വൈ ചേർത്ത മത്സ്യതീറ്റയുടെ പ്രതിരോധപരവും ചികിത്സാപരവുമായ ശേഷി വിലയിരുത്തി. രോഗബാധിത നിയന്ത്രണ ഗ്രൂപ്പിനെ അപേക്ഷിച്ച് ഐ.ജി.വൈ നൽകിയ മത്സ്യങ്ങളിൽ അതിജീവനം ഗണ്യമായി വർദ്ധിച്ചു. പ്രതിരോധ ഗ്രൂപ്പിൽ 80%, ചികിത്സാ ഗ്രൂപ്പിൽ 60%, ന്യൂട്രലൈസേഷൻ ഗ്രൂപ്പിൽ 70% അതിജീവനനിരക്ക് രേഖപ്പെടുത്തി. ഫിസ്റ്റോപാത്തോളജിക്കൽ പരിശോധനയിൽ കോശനാശം, നെക്രോസിസ്, അണുബാധാ കോശങ്ങളുടെ കടന്നുകയറ്റം എന്നിവ ഗണ്യമായി കുറഞ്ഞതായി കണ്ടെത്തി.

ഈ പഠനം എ. ഹൈഡ്രോഫിലയ്ക്കെതിരായ ഐ.ജി.വൈ ആന്റിബോഡികൾ ജലകൃഷിയിലെ ബാക്ടീരിയ രോഗനിയന്ത്രണത്തിന് ഫലപ്രദവും പരിസ്ഥിതി സൗഹൃദവുമായ ഒരു ആന്റിബയോട്ടിക് ബദലാണെന്ന് തെളിയിക്കുന്നു.

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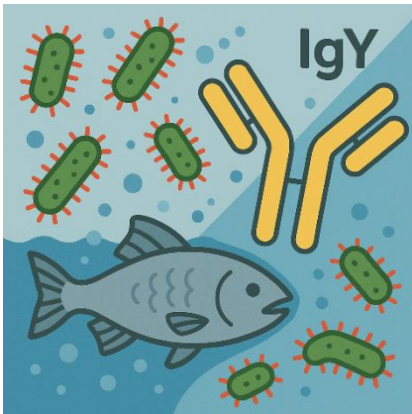
LIST OF ABBREVIATIONS

Abbreviation	Full Form
Σ FIC	Sum of Fractional Inhibitory Concentrations
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
ADE	Antibody-dependent enhancement
AMR	Antimicrobial resistance
ANOVA	Analysis of Variance
ARB	Antibiotic-resistant bacteria
BKD	Bacterial Kidney Disease
BSA	Bovine Serum Albumin
CAA	Coastal Aquaculture Authority
CDSCO	Central Drugs Standard Control Organisation
CFU	Colony Forming Units
CH	Constant domain of heavy chain
COVID-19	Coronavirus Disease 2019
Cv	Variable domain of constant region
Cy	Constant domain of the gamma chain
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DOF	Directorate of Fisheries
ECVAM	European Centre for the Validation of Alternative Method
ED ₅₀	Effective Dose for 50% of the population

ELISA	Enzyme-Linked Immunosorbent Assay
EUS	Epizootic Ulcerative Syndrome
EYA	Egg Yolk Antibodies
FAO	Food and Agriculture Organization
FC	Fragment Crystallizable
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FSSAI	Food Safety and Standards Authority of India
GCAT	Glycerophospholipid: cholesterol acyltransferase
GIA	Growth inhibition assays
GVA	Gross Value Added
H ₂ O ₂	Hydrogen Peroxide
H ₂ SO ₄	Sulphuric acid
HCl	Hydrochloric acid
HRP	Horseradish Peroxidase
IAEC	Institutional Animal Ethical Committee
IBM	International Business Machines
IBSC	Institutional Bio Safety Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IMC	Indian major carp
kDa	KiloDalton
Kg	Kilogram
km	Kilometre

LC ₅₀	Lethal Concentration for 50% of the population
LPS	Lipopolysaccharide
MAI	Motile <i>Aeromonas</i> Infections
MAS	Motile <i>Aeromonas</i> Septicaemia
MAT	Micro agglutination Test
MFU	McFarland's Unit
MIC	Minimum Inhibitory Concentration
mL	Millilitre
mm	Millimetre
mM	Millimolar
MPEDA	Marine Products Export Development Authority
MSAT	Microscopic Slide Agglutination Test
MTCC	Microbial Type Culture Collection
N	Normality/Normal
NFDB	National Fisheries Development Board
Nm	Nanometer
NNV	Nervous Necrosis Virus
OD	Optical Density
OMP	Outer Membrane Protein
OPD	o-Phenylenediamine dihydrochloride
PaIL-1 β	Protein <i>Aeromonas</i> Interleukin-1 beta
PaLECT2	Protein <i>Aeromonas</i> Lectin-2
PaTGF- β	Protein <i>Aeromonas</i> Transforming Growth Factor-beta
PaTNF- α	Protein <i>Aeromonas</i> Tumor Necrosis Factor-alpha
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween-20

PCT	Post-challenge trials
PEG	Polyethylene Glycol
PMMSY	Pradhan Mantri Matsya Sampada Yojana
RPM	Revolutions Per Minute/Rotations Per Minute
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOP	Standard Operating Procedure
SPSS	Statistical Package for the Social Sciences
SUS	Skin Ulceration Syndrome
TiLV	Tilapia Lake Virus
TMB	3,3',5,5'-Tetramethylbenzidine
V	Volt
VH	Variable domain of heavy chain
w/v	Weight per volume
WHO	World Health Organization
WSSV	White Spot Syndrome Virus



Chapter -1

General Introduction

1.1 Strategic Importance of Aquaculture

Farming is a vital activity that sustains human life by providing food, raw materials, and livelihoods. The diverse range of farming practices varies from region to region, directly influenced by geography and climate. It also implies individual or corporate ownership of the stock being cultivated. Aquaculture is a distinct category of farming that focuses on the cultivation of aquatic flora and fauna in controlled environments. According to The State of World Fisheries and Aquaculture 2024, the fisheries and aquaculture sectors have been increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first century (FAO, 2024a). About 164.6 million tonnes (89%) of the 185 million tonnes of aquatic animals produced in 2022 were intended for human consumption, or an estimated 20.7 kg per person (FAO, 2024a). In 2022, aquaculture accounted for more than 57% of aquatic animal foods, consolidating its contribution to human consumption (FAO, 2024a).

Considering the cultivated organism and production method employed, aquaculture can be classified into numerous groups. The notable categories include seaweed aquaculture, shellfish aquaculture and finfish aquaculture. Advanced technological and scientific developments play a vital role in the remarkable enhancement of effective aquaculture production, disease control and environmental sustainability. Production expenses of aquaculture prove to be one of the major economic factors involved in it, which greatly cover all the essential requirements to enhance production, including labour charges, feed, equipment and other inputs.

In order to generate profit, aquaculture producers should maintain a minimum cost of production without compromising on the quality and safety of their products. The market plays a crucial economic role in aquaculture. It is important for the producers to consider consumer preferences, substitute

availability and worldwide consumeristic trends before deciding what to farm and production quantity. According to the FAO, the COVID–19 pandemic results in varied effects on the aquaculture sector (FAO, 2020). Difficulties were encountered by some producers owing to interruptions in supply chains and decreased demand from the restaurants and food catering sectors. At the same time, a surge in need was also observed for seafood from consumers who prioritize a nutritious and sustainable diet. Moreover, consumer demand for aquaculture products can be influenced by government policies and regulatory measures. Thus, the global aquaculture industry is anticipated to thrive in the coming years, fuelled by the growing consumer demand for seafood, coupled with an increasing focus on sustainable and responsible measures.

1.2. Aquaculture scenario

Global Aquaculture production marked a record high of 130.9 million tonnes, in which aquatic animals constitute about 94.4 million tonnes while algae constitute 36.5 million tonnes (Tomasi *et al.*, 2025). For the first time, production of animal species from aquaculture (51%) surpassed that from capture fisheries (FAO, 2024a). India secured the second-largest position in fish production in the world after China, as it accounts for 6.3% of the global fish production. India's economy and food security are significantly reliant on the country's fisheries and aquaculture industry. By virtue of geographical location, India has an extensive area for aquaculture in the form of ponds and tanks distributed in almost all the states of India (DOF, 2025a). The fisheries sector provides livelihoods to an estimated 28 million fishers and fish farmers, and the sector offers immense opportunities for employment generation, especially for youth and women (George and Hossain, 2021). All the major aquaculture species produced in India include freshwater fish

such as carp, catfish, and prawns, as well as brackish water species such as shrimp and crab. However, there is a growing trend towards the adoption of cage and pen culture systems, particularly for marine fish species. Some of the challenges faced by the Indian aquaculture industry include disease outbreaks, poor quality seed, inadequate infrastructure, and limited access to finance and technology. The government has been taking various initiatives to promote the development of the aquaculture sector.

Some of these initiatives include the National Fisheries Development Board (NFDB), the Blue Revolution Scheme, and the Pradhan Mantri Matsya Sampada Yojana (PMMSY). However, addressing the challenges faced by the industry will be crucial for its sustainable growth and development. In aquaculture species, pathogenic microorganisms like bacteria, viruses, fungi and parasites can cause infections. These pathogens can enter the aquatic conditions through polluted water, infected wild or farmed stocks or improperly sanitised personnel and equipment (Shrivastava, 2024). When aquatic species are exposed to environmental stressors such as poor water quality, temperature changes, high stocking densities or low oxygen, they can become highly prone to infections as these stressors impair the immune system of the animals, making them susceptible to various infections. There are several contributing factors leading to infections and financial losses in aquaculture, which include various improper management practices like insufficient feeding, inadequate nutritional management and inferior husbandry methods such as over feeding, unhygienic conditions and improper handling. Also, specific aquaculture species are more vulnerable to infections owing to genetic reasons (Nguyen *et al.*, 2025). Additionally, targeted breeding practised for enhanced growth or other favourable traits can unintentionally increase the vulnerability of animals to certain infections.

Aquaculture operations can interact with wild populations of fish and other aquatic species, which can introduce new pathogens to the farmed stocks or lead to the spread of infections between the wild and farmed populations (Nguyen *et al.*, 2025; Nguyen, 2024).

1.3. Diseases in aquaculture

Fish health can be affected by both infectious and non-infectious diseases. Infectious diseases are caused by pathogenic organisms such as bacteria, fungi and parasites (Sahoo *et al.*, 2020). Severe infections can occur in both internal and external organs of fish. These types of infections are communicable, and treatments and prevention are required to control the outbreak. Non-infectious diseases are caused by physical, chemical or mechanical factors, and the diseases are either genetically transmissible or due to the effect of environmental impacts or due to the lack of nutritional level. Non-infectious diseases are non-communicable; generally, medications are not advisable for them. Bacterial infections are a common problem in aquaculture, particularly in intensive farming systems where large numbers of fish are kept nearby. Bacteria can enter the fish through various means, including contaminated water, infected feed, or *via* wounds on the fish's skin. Some of the common bacterial infections in aquaculture are caused by *Aeromonas* spp., *Vibrio* spp., *Streptococcus* spp., *Pseudomonas* spp., *Edwardsiella* spp., and *Flavobacterium* spp.

1.4. Role of *Aeromonas hydrophila*

A. hydrophila has been recovered from a wide range of freshwater fish species worldwide (Peyghan *et al.*, 2010). Conflicting views have been expressed concerning whether *A. hydrophila* is a primary pathogen of freshwater fish or a secondary opportunistic pathogen of compromised or

stressed hosts (Jeney and Jeney, 1995). *A. hydrophila* is widely recognised as a key pathogen in aquaculture, linked to conditions such as tail and fin rot and haemorrhagic septicaemia (Austin and Adams, 1996; Roberts, 1997). It is the primary causative agent of ‘fish-bacterial-septicaemia’ in freshwater cultured cyprinid fish species (Qian, 1997).

Furthermore, *A. hydrophila* plays a significant role in epizootic ulcerative syndrome (EUS), serving as a primary pathogen responsible for this globally distributed epidemic (Yadav *et al.*, 1992). Along with *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* are identified as the motile aeromonads responsible for causing Motile *Aeromonas* Septicaemia (MAS) (Hanson *et al.*, 2014). The widespread occurrence and the notable impact of *A. hydrophila* infections highlight its crucial significance in aquaculture health management and in the advancement of functional disease minimization methods.

1.5. Antibiotic application in aquaculture practices

Many countries have introduced laws that allow the usage of antibiotics and other antimicrobial medications for combating bacterial diseases in aquaculture (Bondad-Reantaso *et al.*, 2023). Due to the extensive use of antibiotics in aquaculture systems, antibiotic-resistant bacteria have developed, which has led to a rise in the selection pressure (Luthman *et al.*, 2024; Hossain *et al.*, 2022; Preena *et al.*, 2020). Antibiotics such as oxytetracycline, florfenicol and sulfadiazine are widely used in treating fish diseases (Chen *et al.*, 2020; Lulijwa *et al.*, 2020). In order to combat bacterial infections in Indian freshwater aquaculture, several antibiotics such as enrofloxacin, furazolidone, amitrax, ciprofloxacin, doxycycline and cypermethrin are extensively used (Gnaneshwar *et al.*, 2023).

There exists an inconsistency in the implementation of controlling structures regulating the use of antibiotics in freshwater aquaculture in India (Gnaneshwar *et al.*, 2023). The instructions set by the Central Drugs Standard Control Organisation (CDSCO) regarding the use of antibiotics in aquaculture cover a list of authorized medications and usage limitations (Bhushan *et al.*, 2016). Agencies such as the Food Safety and Standards Authority of India (FSSAI), the Marine Products Export Development Authority (MPEDA) and the Coastal Aquaculture Authority (CAA) have prohibited certain antimicrobials, intending to regulate the use of antibiotics. Despite the stringent laws, antibiotic resistance remains a significant issue. According to Patil *et al.* (2022), there are almost 250 registered pharmaceutical companies that manufacture almost 3000 medical and chemical products for aquaculture farming in India.

1.6. Antimicrobial resistance in aquaculture

Antimicrobial Resistance (AMR) is a prominent health crisis on a global basis and according to a predictive statistical models, bacterial AMR has resulted in an estimated 4.95 million deaths in 2019 worldwide (Murray *et al.*, 2022). When antibiotics are widely utilized to combat bacterial infections in aquaculture systems, it leads to the development of antibiotic resistance and further dissemination of resistant strains in aquatic conditions. This resistance can be transferred to clinically relevant bacterial strains through horizontal gene transfer, which ultimately poses a great threat to public health and the ecosystem (Preena *et al.*, 2020). Studies shown that *Ctenopharyngodon idella*, *Labeo rohita* and *Catla catla* have been found to support antibiotic-resistant aeromonads in their gut, which are resistant to ampicillin, oxytetracycline, amoxicillin and novobiocin (Hatha *et al.*, 2005; Preena *et al.*, 2020).

The development of AMR in aquaculture is highly influenced by public health measures, ecological factors and genetic adaptations. Furthermore, the risks associated with antibiotic-resistant pathogens are assimilated when pathogen infected fish are consumed. As a mitigation strategy to this widespread issue, several sustainable solutions such as stringent monitoring, legal enforcement and the implementation of innovative alternatives to antibiotics are crucial (Preena *et al.*, 2020). As AMR poses a grave threat to aquaculture productivity, environmental balance and global public health, a combined effort is essential to tackle this ongoing issue. It is important to maintain a balance with the antibiotic use by taking into account their environmental and health implications while promoting research into substitutes conventional antibiotics.

1.7. New approaches in aquaculture health management

The widespread use of antibiotics in aquaculture has resulted in the emergence of antibiotic-resistant bacteria, which are influenced by intrinsic and acquired factors in aquaculture environments. Considering the limitations of antibiotics, alternative therapeutic practices like probiotics, prebiotics, synbiotics, bacteriocins, immunostimulants, and even phage therapy and herbal products are encouraged by researchers and practitioners in aquaculture (Preena *et al.*, 2025; Nayak *et al.*, 2022). Furthermore, techniques like selective breeding and the execution of effective management strategies are essential in developing disease-resistant strains, which play a central role in maintaining resilient fish stocks. Even though these strategies present viable options, there are a lot of challenges associated with it. For instance, meticulous planning and research is required for vaccination programs, inconsistent effectiveness of probiotics and limited specificity of

bacteriocins, regulated use of immunostimulants, and phage therapy involved in high production cost.

1.8. Use of immunoglobulin Y (IgY) in aquaculture

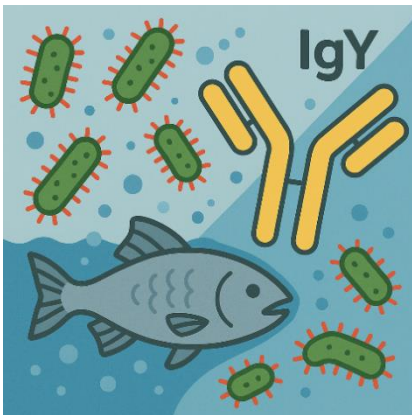
Egg yolk antibodies or IgY are the chief immunoglobulin present in the yolk of birds, reptiles, amphibians and lungfish. The development of IgY antibodies to combat aquatic pathogens can be a promising alternative for preventing and controlling health crises in aquaculture species (Munhoz *et al.*, 2014). The IgY, found in high concentrations in chicken egg yolk, can be used to target a specific pathogen in order to reduce the prevalence of diseases in fish populations. Li *et al.* (2006) have significantly shown that the intraperitoneal administration of IgY antibodies can effectively neutralize *A. hydrophila* infections. The development of IgY antibodies is a potential method for diagnosing, preventing and controlling the disease-causing microorganisms in aquaculture and to maintain the overall health of aquatic animals.

Chicken IgY offers notable advantages as it is cost-effective and an effective substitute for polyclonal and monoclonal antibodies. It can be produced on a large scale with minimal effort. Additionally, IgY does not interact with mammalian FC receptors compared to conventional antibodies, thus ensuring minimal background interference (Carlander *et al.*, 2000). It also possesses several other benefits, including wider recognition of epitopes, higher yield and reduced immune response when used in mammals. Moreover, they are environment-friendly, demonstrate no side effects or host allergies and altogether do not lead to the evolution of resistant pathogens (Schade *et al.*, 2005; Coleman, 1996). Their improved stability in varying conditions highlights their potential in diverse applications.

The chicken egg yolk antibodies have potential as a diagnostic or therapeutic for common pathogenic infections. Our present study aims to develop the IgY-coated fish feed against *A. hydrophila* and evaluate the *in vitro* and *in vivo* activity of IgY and its therapeutic applications in aquatic environments.

1.9. Objectives

- ✓ Immunization of chickens with *A. hydrophila* antigens.
- ✓ Purification of egg yolk IgY from the egg.
- ✓ Characterization and identification of antigen-specific egg yolk IgY.
- ✓ Interaction of antigen and antibody at various environmental parameters.
- ✓ Evaluation and efficacy analysis of egg yolk IgY against *A. hydrophila* by *in vitro*.
- ✓ Evaluating IgY antibody-coated fish feed for motile *Aeromonas* Septicaemia control in *Ctenopharyngodon idella* (Grass Carp) *in vivo*.



Chapter -2

Review of Literature

2.1. Aquaculture scenario

Aquaculture involves the cultivation, breeding, and harvesting of aquatic organisms-both flora and fauna-in organised surroundings such as oceans, rivers, lakes, ponds, and streams. According to the State of World Fisheries and Aquaculture (FAO, 2024a), global aquaculture and fisheries production reached a record 223.2 million tonnes in 2022, comprising 185.4 million tonnes of aquatic animals and 37.8 million tonnes of algae. For the first time in recorded history, aquaculture surpassed capture fisheries as the leading source of aquatic animal production, contributing 130.9 million tonnes, of which 94.4 million tonnes were aquatic animals - accounting for 51% of the global aquatic animal output (FAO, 2024a).

This growth underscores aquaculture's increasing role in addressing food security and malnutrition. Global consumption of aquatic animal-based foods rose to 162.5 million tonnes in 2021, with per capita intake expanding from 9.1 kg in 1961 to 20.7 kg in 2022, nearly doubling population growth rates (FAO, 2024a). However, aquaculture production remains concentrated in a handful of countries. Ten nations-including China, Indonesia, India, Vietnam, and Bangladesh-account for nearly 90% of global aquaculture output, while several low-income countries in Africa and Asia have yet to realize their full potential in this domain.

India, with a coastline stretching 8,118 km and an Exclusive Economic Zone of 2.02 million km², holds immense marine fishery potential estimated at 5.31 million metric tonnes annually (DOF, 2025b). In the 2023–24 year, capture fisheries harvest reached approximately 4.495 million tonnes. Inland fisheries play an equally crucial role in India's fishery economy, offering employment and nutritional support to millions. India ranks as the second-largest fish producer globally, supported by extensive inland water bodies:

0.28 million km of rivers and canals, 1.2 million ha of floodplain lakes, 2.45 million ha of ponds and tanks, and 3.15 million ha of reservoirs (DOF, 2025b).

Fisheries and aquaculture significantly contribute to India's economy by generating foreign exchange, ensuring food security, and supporting livelihoods. In 2022–23, the sector added nearly ₹2,885 billion to the Gross Value Added (GVA), which constituted 7.2% of the agriculture and allied sectors' GVA and 1.3% of the national total (PIB, 2023). Over 16 million people are engaged directly, and many more indirectly, across the fish production and processing value chains (IBEF, 2020). Technological innovations and policy interventions have helped boost India's fish production to over 17 million metric tonnes in 2023, with aquaculture alone contributing nearly 7% of global production (DOF, 2025b).

India's rich ichthyofaunal diversity remains underutilised, with only a fraction of available fish species cultivated commercially. To meet the projected fish demand of 20 million tonnes per year by 2050, aquaculture must account for 80% of total production. Among freshwater species, major carps continue to dominate, contributing close to 80% of India's aquaculture output. Strengthening capacity building among researchers, farmers, and stakeholders is critical to ensuring global competitiveness and national food security (ICAR-CIFA, 2022).

Kerala, located in southwestern India, is distinguished by its 590 km coastline and a continental shelf of 39,139 km² (Sarada, 2013). The state benefits from extensive marine and inland fishery resources, including vast backwaters and rivers that have long supported traditional inland fishing practices. Kerala holds a leading position among Indian maritime states in terms of aquatic biodiversity and fisheries potential (DOF, 2025b).

The presence of diverse freshwater and brackish water ecosystems offers opportunities for further expansion of aquaculture through sustainable utilization (Kerala Fisheries Profile, 2022).

2.2. Outbreak of diseases in aquaculture

The fish are commonly encountered with different groups of pathogens, including viruses, bacteria, fungi and parasites (Pan, 2024; Petty *et al.*, 2022). Mohan and Bhatta (2002) described the extent of social and economic impact of two important fish diseases, namely epizootic ulcerative syndrome (EUS) and white-spot disease, on rural aquaculture. Gopal *et al.* (1992) recorded parasitic disease incidences as the most prevalent among all other diseases in India. A study by Sahoo *et al.* in 2020 found that bacterial diseases were the second-most common cause of fish mortality, accounting for 12.8% of cases. The Aeromonads group among all the bacterial pathogens was found to be a major pathogen causing bacterial fish diseases (66.66%) (Sahoo *et al.*, 2020). Strict supervision and careful inspection have recently led to the discovery and documentation of several fish viral diseases within the country, such as cyprinid herpes virus 2, carp oedema virus, tilapia lake virus, and ranavirus, which had not been identified before (Sahoo *et al.*, 2020). As aquaculture expands and intensifies, the economic loss due to diseases is likely to increase manifold (Mohan and Bhatta, 2002).

Indian major carp (IMC) cultivation accounted for the majority of cases, with Catla being the most susceptible species, followed by rohu and mrigal (Sahoo *et al.*, 2020). Vineetha (2009) also noticed infections in *C. catla* and *L. rohita*, as they are mostly cultured fish species. The cause lies in their surface-nurturing habits, susceptibility to temperature variations of water, followed by immunity compromises (Sahoo *et al.*, 2020). Further, disease incidences in *Channa striata* farming, ornamental fish and wild seabass seeds

collected for stocking in freshwater ponds were also recorded (Sahoo *et al.*, 2020). Sahoo *et al.* (2020) conducted a study on seasonality variation and how it will affect the prevalence of diseases in the aquaculture sector. He concluded that the winter season increased the infections in fish more than the summer and monsoon seasons (Sahoo *et al.*, 2020). Dash *et al.* (2015) also mentioned winter as the advantageous season for disease occurrence, as in winter, fish become immunologically weak due to low temperature stress and other deteriorated water quality-related problems. Such data enlightened the importance of various conclusive studies and parameters for different disease conditions (Sahoo *et al.*, 2020).

Several significant disease outbreaks in aquaculture have been documented in peer-reviewed studies and scientific reports since 2020. In South Korea, a marked increase in juvenile chum salmon mortality in 2021 was linked to *Renibacterium salmoninarum*, marking the first recorded Bacterial Kidney Disease (BKD) outbreak in *Oncorhynchus keta* under mariculture conditions (Kong *et al.*, 2024). In Southeast Asia, Nervous Necrosis Virus (NNV) continues to wreak havoc in farmed Asian seabass, especially among larvae and juveniles, where mortality frequently reaches 90%; NNV remains highly prevalent in countries such as Thailand, Vietnam, Indonesia, and Malaysia (Yue and Guo, 2025). In Zambia, a preprint study reported a localized but severe outbreak of lactococcosis in Nile tilapia at Siavonga, with a prevalence rate of 68.4% in affected farms, while no cases of Tilapia Lake Virus (TiLV) were detected (Songe *et al.*, 2025). Elgendy *et al.* (2022) made a report of *V. mimicus* outbreaks associated with mass mortality in Egyptian farmed Nile tilapia (*O. niloticus*). Additionally, small-scale cage culture on Lake Kariba revealed frequent disease occurrences in Nile tilapia (*O. niloticus*) associated with *Aeromonas* spp., *Lactococcus* spp., and

Streptococcus spp., highlighting stress-related opportunistic infections common in such systems (Siamujompa *et al.*, 2023).

2.3. Major bacterial pathogens in aquaculture

Considering marine aquaculture, freshwater aquaculture fishes are highly susceptible to bacterial infections, which susceptibility is also influenced by climatic conditions and geographic locations (Pekala-Safinska, 2018). Alterations to freshwater ecosystems seem to be central to the development of diseases, including emerging ones. The aquatic environment is home to various organisms in addition to fish, such as saprophytic bacteria living in sediments and on plants, as well as phyto- and zooplankton. Some of these organisms form colonies on the skin, gills, and digestive tracts of fish, where they live as commensals. These organisms assist with digestion and have a beneficial effect on the fish's immune system (Pekala-Safinska, 2018). Some of these microorganisms could also threaten fish health and, therefore, are referred to as conditionally pathogenic (Pekala-Safinska, 2018).

Gram-negative bacteria represent a significant threat to fish health, with species such as *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp., *Edwardsiella* spp., *Vibrio* spp., *Acinetobacter* spp., and *Plesiomonas shigelloides* frequently implicated in aquatic disease outbreaks (Dissasa *et al.*, 2022). Additionally, emerging bacterial pathogens, including *Klebsiella pneumoniae*, *Edwardsiella tarda*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Morganella morganii*, and *Serratia marcescens*, have been increasingly associated with fish mortality (Cortes-Sanchez *et al.*, 2025). Fish displaying clinical symptoms of infection commonly harbour opportunistic microorganisms, including *Aeromonas* spp., *Acinetobacter* spp., *Kocuria* spp., *Plesiomonas shigelloides*, *Sphingomonas paucimobilis*, and *Stenotrophomonas maltophilia* (Pekala-Safinska, 2018).

2.4. *Aeromonas* infections in aquaculture

Aeromonads are known to be of great importance both economically and medically. Members of this genus are mainly distributed in freshwater and sewage, and in association with aquatic animals are sometimes predominant components (Stecchini and Domenis, 1994; Cahill and MacRae, 1992). Additionally, they are known to cause a diverse spectrum of diseases in both warm- and cold-blooded animals (Janda, 1991). The presence of *Aeromonas* species can be found in the physiological microflora of the fish intestine (Austin and Austin, 2016) and it results in considerable loss in aquaculture. Even though the role of aeromonads in gastrointestinal illness is widely debated, the developing research indicates that there is a close link between certain strains and diarrheal infections (Sugita *et al.*, 1995). Hence it is evident that the ecological study of *Aeromonas* in aquatic systems is inevitable for the effective prevention of diseases caused by these organisms (Janda, 1991).

It is observed recently that, the species which was previously referred to as *Vibrio* has exhibited the addition of at least one new strain every decade (Fernandez-Bravo and Figueras, 2020; Figueras *et al.*, 2011). The motile *Aeromonas*, which include *A. hydrophila*, *A. sobria*, and *A. caviae*, is the major cause of bacterial infections (Nokhwal *et al.*, 2025; Hanson *et al.*, 2014). The medical symptoms of health disorders are highly dependent on the type of disease.

For Motile *Aeromonas* Infections (MAI), skin ulceration and gill and fin lesions are the commonly recorded symptoms (Pekala-Safinska, 2018). When the infection becomes systemic, it is referred to as Motile *Aeromonas* Septicaemia (MAS) (Pekala-Safinska, 2018). Another psychrophilic *Aeromonas*, *A. salmonicida* subsp. *salmonicida* causes furunculosis in salmonids, which is characterized by skin ulcers (Pekala-Safinska, 2018). These infections can

lead to high mortality rates, sometimes affecting up to 80% of the fish population (Pekala-Safinska, 2018). Interaction with bacteria is especially dangerous under conditions of stress, which include unfavorable environmental conditions as well as human intervention in catching, sorting, and transporting the fish (Austin and Austin, 2016).

Within the aeromonads group, *A. hydrophila* (52.94%) was found to be involved in the maximum number of disease cases, followed by *A. sobria* (26.47%), *A. veronii* (14.70%), *A. jandei* (2.94%) and *A. caviae* (2.94%) (Sahoo *et al.*, 2020). Karunasagar *et al.* (1986) reported motile aeromonad infections causing mortalities in IMC farms in India and marked it as one of the major bacterial pathogens of fish. Aeromoniasis is also described as one of the major threats to IMC farming in India (Sahoo *et al.*, 2020).

The involvement of aeromonads in fish diseases has been recognized for decades, predating their acknowledged role in causing systemic infections in humans. Broadly, fish diseases associated with these bacteria are classified into two major groups (Janda and Abbot, 2010). *A. salmonicida* causes fish furunculosis, particularly in salmonids. The disease has several presentations, ranging from an acute form characterized by septicemia with accompanying haemorrhages at the bases of fins, inappetence, and melanosis to a subacute to chronic variety in older fish, consisting of lethargy, slight exophthalmia, and haemorrhaging in muscle and internal organs (Austin and Austin, 2016). Mesophilic species (*A. hydrophila* and *A. veronii*) cause a similar assortment of diseases in fish, including motile *Aeromonas* septicemia (hemorrhagic septicemia) in carp, tilapia, perch, catfish, and salmon, red sore disease in bass and carp, and ulcerative infections in catfish, cod, carp, and goby (Martin-Carnahan and Joseph, 2005). Mesophilic *Aeromonas* species, most notably *A. hydrophila*, have been linked to major die-offs and fish kills around the globe over the past decade, resulting in enormous economic losses.

According to Patil *et al.* (2025), diseases place a substantial economic burden on Indian aquaculture, amounting to 14.95% of the sector's annual production value, or approximately \$2.48 billion. In Assam, India, from November 2020 to January 2021, a fish farm experienced a major outbreak among *Channa punctata* due to dual infection of *A. veronii* and *A. hydrophila*. (Das *et al.*, 2023). Similar outbreaks were found in Malaysia, where farmed red hybrid tilapia experienced high mortality owing to co-infection of Tilapia Lake Virus (TiLV) and *A. hydrophila* in 2020 (Basri *et al.*, 2020). In 2019, cultured Nile tilapia were observed to be co-infected with *Streptococcus iniae*, which resulted in a large-scale mortality event (Puneeth *et al.*, 2022). Mostly, *Aeromonas* species were the only or co-pathogens creating secondary infections in immunosuppressed fish, which are triggered by environmental factors like spawning, high temperature and low water levels (Janda and Abbot, 2010).

These bacteria, which are naturally present in the natural microbiota of healthy fish, are prevalent in aquatic conditions. They are present in significant amounts in waters with organic contamination, where infected carrier aquatic species act as reservoirs. Transmission generally occurs either directly between fish or through polluted water (Meyers *et al.*, 2019). Water temperatures at or above 10°C are considered suitable for the multiplication of these opportunistic pathogens. Data show that both humans and aquatic animals are commonly unaffected by enteric *Aeromonas*, as these bacteria are frequently found in the gut naturally, either transient or part of the long-term microbiota. There's a range of capacities of *Aeromonas* spp. to cause disease. This capacity is influenced by multiple factors such as the age of the host, the immune status of the host, dosage of infection, any underlying health problems and the expression of virulence determinants by the pathogen (Nichols *et al.*, 1996).

2.5. Virulence factors and toxins of *Aeromonas* spp.

The mechanisms of pathogenesis in *Aeromonas* infections are complicated, given that its various virulence mechanisms, including attachment mechanisms and toxins, are still being researched. Various studies have shown that strains of *A. hydrophila* have lectins and adhesins that permit them to adhere to epithelial surfaces and gut mucosa (Gosling, 1996). Additionally, two types of pili have been characterized from Hep-2-adherent *A. hydrophila* (Gosling, 1996; Carrello *et al.*, 1988), and invasion of Hep-2 cells by facially derived *A. hydrophila* has also been reported (Lawson *et al.*, 1985).

Species of *Aeromonas* are capable of expressing several extracellular toxins and enzymes (Gosling, 1996; Howard *et al.*, 1996). The primary toxins produced are haemolysins, of which the most significant is aerolysin, expressed by many strains of *A. hydrophila* and *A. sobria* (Howard *et al.*, 1996; Janda, 1991). This is a heat-labile b-haemolysin, which exhibits phospholipase A and C activity. It is a pore-forming cytotoxin able to insert into the cell membrane bilayer, causing leakage of cytoplasmic contents. Haemolytic enterotoxins have been reported by some authors (Gosling, 1996; Chopra *et al.*, 1991). A weak haemolysin, glycerophospholipid: cholesterol acyltransferase (GCAT), has been characterized from *A. hydrophila* and *A. salmonicida* (Howard *et al.*, 1996). Other haemolysins may also exist, but need to be isolated and purified before haemolytic activity can be confirmed. In addition, at least one cytotoxic enterotoxin with similar activity to cholera toxin has been demonstrated (Gosling, 1996; Ljungh *et al.*, 1982), and there may be several. Evidence for plasmid-encoded expression by *A. hydrophila* and *A. caviae* of a cytotoxin similar to Shiga-like toxin 1 has been reported (Haque *et al.*, 1996). Species of *Aeromonas* also produce a range of cell-surface and secreted proteases, which probably enhance virulence (Gosling, 1996). Expression of virulence

factors, including haemolysins and proteases, by aeromonads is influenced by environmental temperature (Mateos *et al.*, 1993).

2.6. Use of antibiotics in aquaculture

In aquaculture, antibiotics are employed as preventive or curative treatments or as feed additives. They can enter the pond environment through the use of human and animal waste or *via* integrated farming systems (Aly and Albutti, 2014). There has been a surge in the number of foodborne infections caused by antibiotic-resistant bacteria (ARB) (Foodborne Antimicrobial Resistance, 2022). Similar to other areas of animal production, aquaculture uses the same strategies to manage infectious diseases (FAO, 2005). Any use of antibiotics should be strictly regulated under the same code of practice as other veterinary medicines (FAO, 2005). Because no antibiotics are made exclusively for aquaculture, the industry relies on authorized products that were originally developed for other fields of veterinary medicine (FAO, 2005).

Antibiotics frequently used in human medicine, such as oxytetracycline, sulfamerazine, and ormetoprim, are also administered to treat bacterial infections in commercially farmed fish like salmon, catfish, and trout (FAO, 2005). These drugs are primarily used to combat common fish infections, including skin ulcers, diarrhoea, and blood sepsis (FAO, 2005). The concern with this practice is that the microorganisms responsible for these fish diseases belong to the same bacterial families that can infect humans, creating a high risk of transferring antibiotic resistance (FAO, 2005). This transmission of resistance can occur even after treatment has ended and before the fish are sold for consumption (FAO, 2005). Recognizing these environmental and public health risks, many countries have implemented veterinary medicine controls over the use of antibiotics in aquaculture

(Reily *et al.*, 1997). The main method of administration involves medicated food pellets. As a result, uneaten food and excreted drugs accumulate in the pond environment, with previous studies indicating that 70-80% of the antibiotics can end up in the surrounding ecosystem (FAO, 2005). Drug concentrations with antibacterial activity were found in the sediment underneath the fish farm (Samuelsen *et al.*, 1992). The effectiveness of a therapeutic antibiotic on a bacterial strain in fish is influenced by several factors (FAO, 2005). These include the method of drug administration, the distribution of the antibiotic relative to the pathogen's location within the fish, and the physicochemical properties of the aquatic environment (FAO, 2005). Ultimately, the application of antibiotics in aquaculture may contribute to an increased frequency of resistance in the local microbial communities (FAO, 2005).

There is also a chance for direct bacterial transmission to humans through contact with fish. (Alderman and Hastings, 1998). In the same way, a great number of people who handled aquaculture live tilapia produced were affected with severe infections caused by a biotype of *V. vulnificus* in Israel (Bisharat and Raz, 1996). Moreover, consuming farmed products that are cross-contaminated by bacteria from fish can lead to the transmission of bacteria to humans. For instance, in Japan, the consumption of farmed fish has resulted in the occurrence of the food-borne disease named *V. parahaemolyticus* (WHO-FAO-UN, 2011).

The advancement and spread of antimicrobial resistance, which has been aggravated by both human and non-human antimicrobial usage, have become a global health concern recently. When antimicrobials are used in aquaculture, they lead to the development and transmission of antimicrobial resistance, which can potentially impact human health through horizontal gene transfer and environmental exposure.

Today, the development and spread of antimicrobial resistance has become a global public health problem that is impacted by both human and non-human antimicrobial usage (Okocha *et al.*, 2018; WHO, 2023). One way that antibiotic resistance may affect human health is *via* horizontal gene transfer and environmental exposure. Resistance developed in one region or ecological niche could spread around the world through connected ecosystems and trade networks (Preena *et al.*, 2020). Bacteria in water can also be a pathway for infection in humans, and some illnesses require more and more expensive treatment due to antibiotic resistance (Aly and Albutti, 2014). The transmission of antibiotic resistance from water to humans could involve the transmission of resistant pathogens like *V. cholerae* or *Salmonella* spp, as well as opportunistic pathogens such as *A. hydrophila* (Aly and Albutti, 2014). Essentially, the resistant bacteria can be transmitted to humans by intake of contaminated water, direct contact with water or aquatic life, and consumption of seafood (Aly and Albutti, 2014). A large part of this issue is that 80% of all antibiotics added to aquaculture end up in the environment. The selection of resistant bacteria leads to the transfer of the resistance to other bacteria, by means of mobile genetic elements (Aly and Albutti, 2014). This disturbs the natural balance of aquatic ecosystems that rely on healthy bacteria in fish and shellfish (Aly and Albutti, 2014).

Aquatic environments also provide an avenue for a genetic transfer of resistance between bacteria from aquatic and terrestrial niches (Aly and Albutti, 2014). There is increasing evidence that some of the genetic mechanisms for antibiotic resistance are transferred between aquatic bacteria, bacteria that infect fish, and bacteria that cause human infections (Aly and Albutti, 2014). The implications of antibiotic resistance in human infections can be severe and widespread, and can include more infections and more

severe infections, more treatment failures, and greater costs associated with healthcare (Aly and Albutti, 2014). More severe infections imply longer illnesses, more bloodstream infections, more hospitalizations, and greater mortality (Aly and Albutti, 2014; Samuelsen *et al.*, 1992).

The use of antibiotics in aquaculture is beneficial, but the continued presence of residues in fish and environments and the transfer of resistant organisms (and their resistance genes) that result from the use of antibiotics can result in pathogens developing with resistant genes. Traces of antibiotics are indirectly transferred from fish pathogens to human pathogens through horizontal gene transfer, leading to resistance by negative assortative mating to hospital-acquired strains that are resistant to multiple types of antibiotics. Resistance can emerge in non-pathogenic bacteria, which then transfer antibiotic resistance to pathogenic human intestinal bacteria, which then develop diseases or become ill from one or more of these antibiotic-resistant bacteria, which are treatable for humans by antibiotics (Hossain *et al.*, 2022).

Health problems from eating fish with antibiotic residues are, in part, due to, if not completely due to, the residue, and to people's potential allergens/reactions to these antibiotics. All epidemiological research also shows that patients get sick from foods they eat, whether or not they were raised with antibiotics. Allergies to antibiotic residues exist, particularly in individuals with or without a history of allergies to these antibiotics. An allergic individual would need to carefully avoid eating seafood if even minuscule amounts of an antibiotic are in the product. In addition to allergic reactions to antibiotics, which put them at risk for potentially severe reactions, individuals can become ill due to other effects on their immune system. This can occur, for example, when penicillin used to treat various fish types and aquatic invertebrates is consumed, or when fish contain even trace amounts of antibiotics in their cells.

A significant challenge is that it can be difficult to accurately diagnose the cause of the allergic reaction, since the patient may not be aware of which antibiotic they ingested or what food triggered the response. Allergies are most common in those who administer the antibiotics and work with large concentrations of them (Cabello, 2006).

Open-water aquaculture systems, particularly salmon farming, pose notable environmental risks due to the accumulation of waste, pathogens, and veterinary drugs in surrounding ecosystems. Antibiotic-laced feed, used to prevent or treat infections in cultured fish, can be consumed by wild fish populations near farming sites, leading to the detection of antibiotic residues such as tetracyclines and quinolones in their tissues. Studies from the Mediterranean region and elsewhere have confirmed that both wild fish and benthic scavengers like crabs near aquaculture zones may contain antimicrobial residue levels exceeding permissible consumption limits (Pelic *et al.*, 2024; Angel, 2013). These residues not only pose potential food safety risks to consumers but also contribute to the broader dissemination of antimicrobial resistance in marine environments (Kendall, 2024; Nixon, 2024). It is estimated that up to 70–80% of antibiotics administered in aquaculture are excreted unmetabolized, entering the surrounding environment primarily through fish urine and faeces (Bojarski *et al.*, 2020). Due to their chemical stability, many of these antimicrobials are poorly biodegradable and persist in aquatic ecosystems, where they contribute to long-term environmental contamination and selective pressure for antimicrobial resistance (Akhter *et al.*, 2024).

2.7. New trends in aquaculture health management

Probiotics are defined as mono or mixed cultures of live microorganisms that, when administered in adequate amounts, confer health benefits to the host (Fachri *et al.*, 2024; Sihag and Sharma, 2012). In aquaculture, probiotics enhance disease resistance by modulating gut microbiota, producing antimicrobial compounds, and competitively excluding pathogens through nutrient and space competition, thereby supporting digestion and immune function (Hasan and Banerjee, 2020; Suguna, 2020). In parallel, bacteriophages - viruses that specifically infect and lyse bacterial pathogens - are emerging as a targeted and eco-friendly alternative to antibiotics. Unlike broad-spectrum antimicrobials, phages selectively eliminate harmful bacteria without disturbing beneficial microbiota, making them a promising tool for disease control in aquaculture systems (Gangwar *et al.*, 2025; Ninawe *et al.*, 2020).

In recent years, vaccination has emerged as a cornerstone strategy for the prevention of infectious diseases in aquaculture, driven by advances in fish immunology and biotechnology. Although fish immunology is relatively younger than its human and veterinary counterparts, it employs many of the same immunological principles (Makesh and Rajendran, 2022; Anderson, 1974). Early vaccine development efforts in the 1970s led to the production of bacterins targeting key salmonid pathogens such as *Yersinia ruckeri* (enteric red mouth), *V. anguillarum* (vibriosis), and *A. salmonicida* (furunculosis), all of which were licensed by the U.S. Department of Agriculture/Animal and Plant Health Inspection Service (Souter, 1983).

The first commercial bacterial vaccines for fish were introduced in the U.S. approximately five decades ago (Dadar *et al.*, 2016; Gudding and Muiswinkel, 2013; Sommerset *et al.*, 2005). Fish vaccination methods vary depending on species, pathogen, water temperature, and production system,

with immersion, spray-shower, and intraperitoneal injection being the most widely used delivery routes (Makesh and Rajendran, 2022). While immersion and spray-shower techniques are cost-effective and suitable for small fish, they are prone to reduced efficacy due to improper vaccine dilution or overuse of the same vaccine bath.

Conversely, injection vaccination is considered more stable and immunologically robust, although it requires careful manipulation to achieve the suggested delivery without tissue damage, necrosis, or deterioration due to improper suspension conditions (Abbas *et al.*, 2024; Anderson, 1974). Vaccination is presently considered the best method for controlling infectious disease in fish (Hossain, 2018) as it involves putting 'non-pathogenic' antigens from disease-causing organisms to force fish to produce a protective immune response (Hossain, 2018). Although we have made great strides toward effective vaccine development, there are still few available commercial vaccines available to fish farmers to treat key bacterial and viral diseases. Vaccines are presently commercially available only for some fish species and for the treatment of economically important bacterial and viral-related fish diseases (Hossain, 2018; Dadar *et al.*, 2016).

Current commercial fish vaccines can offer protection following a single administration, often lasting until harvest; however, the underlying immunological mechanisms remain insufficiently characterized (Rathor and Swain, 2024). Multivalent vaccines, comprising two or more antigens-are increasingly used to target multiple pathogens simultaneously, yet not all included antigens elicit a robust protective response, making it challenging to formulate safe and effective combinations (Abbas *et al.*, 2024). Additionally, many fish species exhibit heightened sensitivity to handling stress during

vaccination, which can lead to adverse post-vaccination effects and reduced welfare (Martos-Sitcha *et al.*, 2020).

Despite growing interest, much of the vaccine development has been driven by industry, with limited publicly available data on efficacy and immunological outcomes (Kumar *et al.*, 2024). In several species, critical disease outbreaks occur during early life stages- larval or fry- when fish are too small or immunologically immature for vaccination, and the lack of maternal immunity further restricts the potential for parental immunization strategies (Nuryati *et al.*, 2024). Developing vaccines against intracellular pathogens remains a major challenge, but DNA vaccines offer promise due to their ability to induce both humoral and cellular immune responses (Rathor and Swain, 2024; Bedekar and Kole, 2021). To address these gaps, there is a pressing need for innovative vaccine platforms, expanded aquaculture immunology research, and dedicated disease surveillance infrastructure (Abbas *et al.*, 2024).

2.8. IgY technology

More than 100 years ago, Klemperer (1893) first demonstrated that avian maternal antibodies are transferred from serum to egg yolk in order to protect the developing embryo from potential pathogens, but at that time, there was no scientific application for this knowledge. However, when animal welfare became a matter of serious ethical concern for the scientific community, the results of Klemperer began to receive more interest, particularly since the 1980s (Janda and Abbot, 2010). Since 1996, IgY technology (i.e. the production and use of IgY) has become an internationally accepted practice (Schade *et al.*, 1996). The use of chicken egg yolk antibodies (IgY) has gained international recognition as a humane and effective alternative to mammalian antibodies. In 1996, the European Centre for the Validation of Alternative Methods (ECVAM) strongly recommended IgY

technology as a refinement and replacement strategy to reduce animal suffering in antibody production. This recommendation was followed by formal approval in 1999 by the Swiss Veterinary Office, which recognized IgY production as an animal welfare supportive method.

IgY antibodies come from hen's eggs, with layers of chicken antibodies found in the egg yolk created by immunizing laying hens with specific antigens, resulting in the production of high-titer antibodies in the hen's blood serum that are transferred to and deposited into the egg yolk as part of the egg. To facilitate the transfer process, hens are usually given some type of booster immunization to continue the transfer of antibodies from serum to yolk. The antibodies are extracted and purified afterwards using simple methods (for example, polyethylene glycol (PEG) precipitation) to produce sufficient amounts of functional IgY for use in either oral or topical applications (Li *et al.*, 2015; Calzado *et al.*, 2010). The concept of antibody transfer to egg yolk has a long history, originating from Klemperer's 1893 report that characterized the ability of immunized hens to provide passive immunity to chickens after consumption of their eggs. Interest in the production and use of IgY antibodies was renewed in the 1980s, spurred by ethical concerns over the use of animals in experimentation, which has been a strong justification for using IgY as a much better option than antibodies derived from blood.

The phylogenetic distance between birds and mammals enhances epitope recognition, while the lack of cross-reactivity with mammalian Fc receptors and rheumatoid factors improves assay specificity (Eriksson and Larsson, 2025; Behn *et al.*, 2001). IgY technology now supports a wide range of applications in diagnostics, therapeutics, and food safety, offering scalable, species-specific, and ethically sound antibody production.

Three major classes of immunoglobulins in birds are IgY, IgA, and IgM have been well characterized in avian species such as chickens and ducks (Taylor *et al.*, 2009; Warr *et al.*, 1995). Historically, IgY was often misclassified as IgG due to its functional similarity and abundance in serum, but structural studies have since clarified that IgY is distinct from mammalian IgG (Leslie and Clem, 1969). Notably, IgY shares evolutionary ancestry with mammalian IgE, and structural comparisons have revealed conserved domains between these immunoglobulins (Taylor *et al.*, 2009).

In chickens, IgY is the major antibody in both serum and egg yolk, where it is maternally transferred, thus providing passive immunity to offspring (Hamal *et al.*, 2006; Narat, 2003). The term “IgY” was officially devised by Leslie and Clem. (1969) to describe this avian antibody separately from the mammalian IgG. While IgG and IgY are functionally similar, IgY is larger in molecular weight and does not possess the hinge region, which has some unique biochemical properties that have been reviewed (Schade *et al.*, 2005; Warr *et al.*, 1995). IgY concentrations in serum and yolk typically are 10-20 mg/ml and comprise around 75% of total immunoglobulins in chickens (Warr *et al.*, 1995). Immunological studies have indicated that IgA and IgM serum levels are much lower (Gebriel *et al.*, 2018; Hamal *et al.*, 2006; Leslie and Clem, 1973; Rose *et al.*, 1974).

Antibodies are transferred from hen to chick during embryonic development *via* the egg, providing essential passive immunity to the immunologically immature chick. Serum IgY is selectively transported from the hen’s bloodstream across the oolemma into the developing oocyte within the ovarian follicle, a process mediated by specific IgY receptors (Kovacs-Nolan and Mine, 2012; Rose *et al.*, 1981).

Although the precise mechanisms by which IgY neutralizes pathogens are still under investigation, several functional pathways have been proposed. These include bacterial agglutination, inhibition of adhesion, opsonization followed by phagocytosis, and toxin neutralization (Li *et al.*, 2015; Xu *et al.*, 2011). Among these, inhibition of microbial adhesion to intestinal epithelial surfaces is considered the primary protective mechanism. IgY antibodies specific to enteric pathogens can prevent colonization by blocking mucosal receptors, interfering with mucin binding, or neutralizing colonization factors such as fimbriae, pili, flagella, lipopolysaccharides, and outer membrane proteins (Esmaeili *et al.*, 2024; Wang *et al.*, 2019).

2.9. Structure of IgY

Chicken IgA and IgM are structurally and functionally analogous to mammalian IgA and IgM, while IgY differs from mammalian IgG despite their similar function. IgY is the predominant serum antibody in birds, reptiles, amphibians, and lungfish, and its serum concentrations far exceed those of IgA (Carlander *et al.*, 1999). IgYs are concentrated in the egg yolk, while IgA and IgM are found in the egg white (Warr *et al.*, 1995). Although functional similarity exists between IgY and IgG, their structures are markedly different, as shown in Figure 2.1. In the differences, IgYs with a heavy (H) chain have one variable region (VH) linked to four constant regions (CH) while mammalian IgGs only have three CH regions (Aly and Albutty, 2014). In addition, IgY does not have a hinge region between the CH1 and CH2 domains, which limits the flexibility of IgY compared to IgG (Shimizu *et al.*, 1992). IgG with heavy chain (y) has a total of four domains, the variable domain (VH) and three constant domains (Cy1, Cy2 and Cy3). The Cy1 domain is separated from Cy2 by a hinge region, providing flexibility of the Fab fragments. The heavy chain of IgY (v) is comprised of a single

heavy chain having a molecular weight of 65,000 Da, four constant domains (Cv1-Cv4) and a variable domain, which, unlike IgG, does not have a hinge region. Findings of similarities in sequence data between IgG and IgY show that the Cy2 and Cy3 of IgG are homologous to Cv3 and Cv4, and the Cv2 equivalent domain in IgG is replaced by a hinge region (Warr *et al.*, 1995). The safety aspect of chicken antibodies (IgY) has already been well documented and is deemed safe (Abbas *et al.*, 2018; Kubickova *et al.*, 2014).

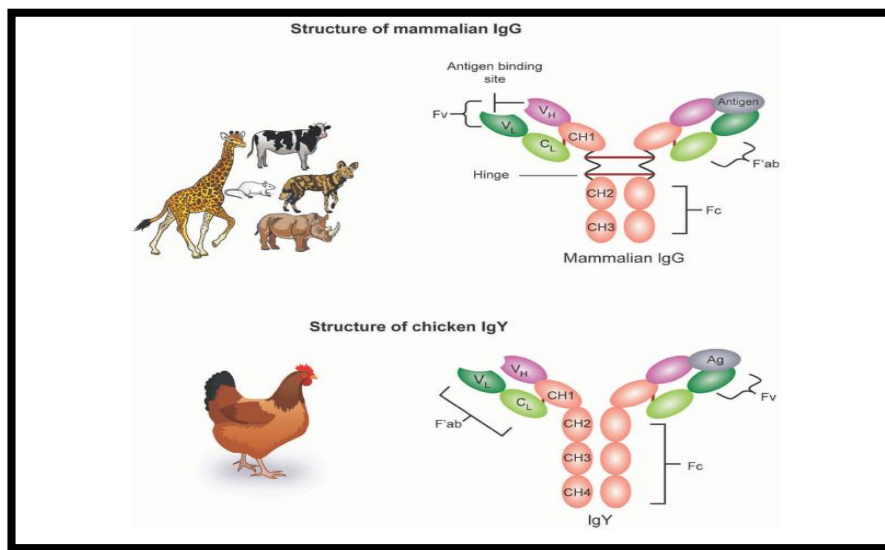


Figure 2.1. Structural difference between mammalian IgG and chicken IgY (Shimizu *et al.*, 1992)

2.10. Advantages of Chicken Antibodies (IgY)

Research on chicken immunology has advanced for years, and the research findings made available have helped shape and illuminate basic immunology concepts and awareness of the developmental trajectories for different immunoglobulin classes (Carlander *et al.*, 1999). The use of avian antibodies instead of mammalian antibodies has many more practical advantages. Now, small amounts of avian antibodies are only used as a result

of limitations of familiarity and handling practices when working with chickens. Up to now, commercially available polyclonal antibodies have been produced in mammals, including mice, rats, rabbits, sheep, goats and horses, typically obtained from sera following immunization with the relevant target (Schade *et al.*, 1996). The resultant antibodies cannot be produced on an industrial level since the acquisition of large amounts of blood is cumbersome, as well as for ethical reasons (Mezhatsu *et al.*, 2010). Monoclonal antibodies can be produced by hybridoma technology, but they are quite often expensive (Mezhatsu *et al.*, 2010).

The chicken has proven to be a useful alternative source for producing antibodies, mainly due to the large quantity of immunoglobulin Y (IgY) found in egg yolk. Individual eggs can have 50-100 mg of IgY for a feasible, high-yield polyclonal antibody source (Redwan *et al.*, 2021). IgY is easily and quickly extracted using simple precipitation techniques (such as polyethylene glycol or caprylic acid precipitation), providing a low-cost method of production appropriate for both laboratory and industrial applications (Morgan *et al.*, 2021; Gassmann *et al.*, 1990).

From both an animal welfare perspective and a bioethics standpoint, IgY technology provides benefits over classic mammalian antibody production methods (Figure 2.2). Antibodies can be collected non-invasively from eggs, meaning there is no bleeding (or repeated bleeding) and no other terminal procedure (Schade *et al.*, 2005). Collectively, chickens are more tolerant of immunizations and less likely to form abscesses or have systemic problems, which can happen with a rabbit (Carlander *et al.*, 1999). Chickens do produce high-titer antibodies, even from immunization with conserved mammal proteins, while rabbits struggle to produce high-titer antibodies and immune responses. Since birds are evolutionarily distant from mammals,

IgY antibodies often recognize many epitopes on mammalian antigens (Tini *et al.*, 2002; Cakir-Koc, 2016). Additionally, chickens cost much less to house and maintain compared to housing and caring for mammals like rabbits, which makes for a lower cost of IgY production (Carlander *et al.*, 1999).

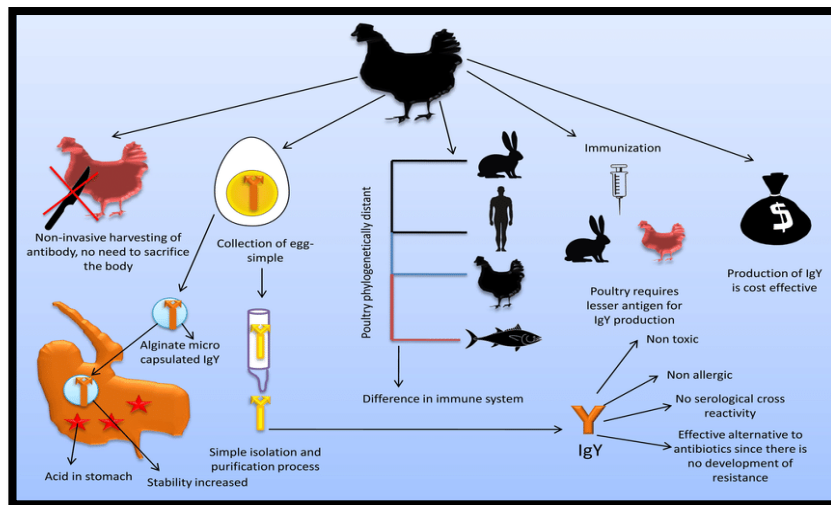


Figure 2.2. Advantages of chicken egg yolk antibodies (Zorriehzah *et al.*, 2016)

Chickens, therefore provide a more hygienic, cost-efficient, convenient, and plentiful source of antibodies, as compared to the traditional method of obtaining antibodies from mammalian serum (Carlander *et al.*, 2000; Gassmann *et al.*, 1990). Nakai *et al.* (1994) estimated that the productivity of antibodies in hens is nearly 18 times greater than that of rabbits, based on the weight of antibody produced per animal. Due to the high concentration of IgY in egg yolk, over 100 mg of IgY can be extracted from a single egg (Akita and Nakai, 1992). A chicken can produce about 20 eggs in a month, and when rearing a prolific producer, more than 2 grams of IgY can be provided each month from those eggs (Carlander *et al.*, 1999). After IgY is laid into the egg, it remains in a stable form for many months, and it could be stored in purified form for years under refrigeration with no notable change in activity

(Larsson *et al.*, 1993). Basically, with current technology and methods for the mass collection and separation of eggs, the possibilities for more industrial-scale production of specific IgY antibodies for immune-therapeutics are now both possible and economically feasible (Cotterill and McBee, 1995). In fact, vaccinating poultry flocks has been a common practice in many areas pertaining to avian disease; therefore, the work to immunize chickens for production of IgY can be both practical and harnessed for mass production. In fact, Ganapathy and Parthiban (2023) published a review on the topic and provided additional support for a continued focus on flock vaccination in commercial poultry health management.

2.11. Use of IgY in aquaculture

A considerable effectiveness has been observed in Immunoglobulin Y (IgY) antibodies against a great variety of bacterial and viral pathogens in aquatic species. A notable study by Gutierrez *et al.* (1993) signifies that oral administration of *Edwardsiella tarda* IgY remarkably enhanced survival and controlled disease symptoms in Japanese eels (*Anguilla Japonica*), which clearly marks the potential of IgY in preventing edwardsiellosis.

Likewise, environmental yeast antagonists have been effective against pathogens such as White Spot Syndrome Virus (WSSV) in shrimp and crayfish, *V. anguillarum* and *Yersinia ruckeri* in rainbow trout and *A. hydrophila* in gibel carp, *Carassius auratus gibelio* (Zorriehzah *et al.*, 2016; Xu *et al.*, 2011). Of these, passive immunization against *Y. ruckeri*, the pathogen responsible for enteric red mouth disease in trout, was the most promising. Fish treated with pathogen-specific IgY exhibited notably lower mortality rates and reduced bacterial loads- an outcome attributed to enhanced mucosal protection and pathogen neutralization (Chelliappan *et al.*, 2021; Lee *et al.*, 2000).

Li *et al.* (2006) demonstrated that specific IgY antibodies, derived from egg yolks of White Leghorn hens immunized with formalin-inactivated *A. hydrophila*, conferred 60–70% protection in polyploid gibel carp challenged with the pathogen. The study confirmed that IgY could inhibit bacterial growth and reduce mortality when administered either before or after infection. Lu *et al.* (2009) further explored the antiviral potential of IgY in crustaceans. In crayfish (*Procambarus clarkii*) challenged with WSSV, passive immunization using WSSV IgY- delivered *via* intramuscular injection, oral administration, or immersion- significantly reduced mortality, suggesting a promising immunotherapeutic strategy for shrimp aquaculture. In another study, Li *et al.* (2014) reported that IgY antibodies raised against formalin-inactivated *V. anguillarum* not only enhanced macrophage phagocytic activity in ayu (*Plecoglossus altivelis*), but also downregulated pro-inflammatory cytokines such as PaTNF- α , PaIL-1 β , PaTGF- β , and PaLECT2. These immunomodulatory effects contributed to reduced bacterial load and improved survival in infected fish.

Piscirickettsiosis, a systemic disease caused by the intracellular bacterium *Piscirickettsia salmonis*, poses a major threat to salmonid aquaculture. In a pivotal study, specific IgY antibodies were produced by immunizing hens with *P. salmonis* proteins and purifying the antibodies from egg yolks using ammonium sulfate precipitation. These IgY antibodies demonstrated strong *in vitro* reactivity against *P. salmonis* proteins and effectively inhibited bacterial growth through agglutination, likely *via* interaction with surface components of the pathogen. *In vivo* trials in Atlantic salmon confirmed a protective effect, with IgY detectable in serum post-oral administration, indicating successful mucosal uptake (Oliver *et al.*, 2015). To enhance gastrointestinal stability, alginate encapsulation of IgY was employed, improving delivery efficiency and bioavailability in the fish gut.

The capability of encapsulated IgY as a passive immunization method is reinforced through the Post-challenge trials (PCT), which exhibited 65% - 70% survivability in IgY-treated groups after 14 days. In another supporting study, Sotomayor-Gerding *et al.* (2022) optimized the microencapsulation of *P. salmonis* antigens in alginate for oral applications, demonstrating that their optimized approach not only had a high rate of encapsulation efficiency but also maintained antigen viability in gastrointestinal conditions. Many researchers have also outlined the potential for IgY to be utilized on a wide spectrum in aquaculture, indicating its potential effect on multiple pathogens as well as acting as a non-antibiotic immunotherapeutic tool (Parmar, 2020; Xu *et al.*, 2011; Zorriehzah *et al.*, 2016).

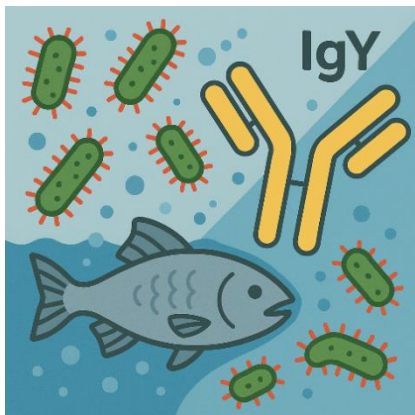
V. splendidus is a significant cause of Skin Ulceration Syndrome (SUS) affecting the sea cucumber *Apostichopus japonicus*, which is a serious concern for aquaculture. Previously, IgY from chicken egg yolk was prepared against *V. splendidus*, then intraperitoneally injected into the infected cucumbers. The research study found an 80% survival in infected sea cucumbers that were treated with IgY, compared to an untreated control. The treated sea cucumbers also had a much lower bacterial load in their respiratory trees and intestines, indicating the potential of specific IgY antibodies to provide protective effects against *V. splendidus* infections (Li *et al.*, 2015). Positive oral administration of an IgY product in fish against *A. salmonicida* has been documented, which indicates the long-standing viability of IgY in aquaculture immune-prophylaxis (Radhakrishnan *et al.*, 2023; Gan *et al.*, 2015). The oral route of administering IgY has received international attention as a targeted method for controlling fish bacterial and viral diseases. The specificity and compatibility of IgY adherence to mucosa offers a potential solution to reduce bacterial and viral diseases in fish (Paul *et*

al., 2019). The fish sector continues to progress in oral vaccines and in developing encapsulation technologies. One recent example was the use of nanoparticles to deliver antigens in fish gut. Also, live feeds are being used to develop bio-encapsulation of antigens. Another model of protecting antigens is using polymeric materials. Encapsulation methods may offer protection, some could improve mucosal uptake and sensitization of cells (Meng-Han, 2024; Radhakrishnan *et al.*, 2023).

2.12. Gap area of study

Even though IgY-based immunotherapy has turned up as a favourable alternative to antibiotics in aquaculture, various unsolved challenges exist as a severe constraint to its functional implementation. Even though delivering orally *via* functionalized feed presents an adaptable and non-invasive route for passive immunization, enhancing the stability, bioavailability and antigen-specific efficacy of IgY under aquatic systems continues to be an ongoing project. Additionally, it is yet to completely explicate the immunological mechanisms that regulate the mucosal uptake and systemic protection in fish, specifically regarding the various farming environments.

The existing knowledge regarding the long protective effects of IgY, its cross-reactivity among pathogen strains, and its potential role in diagnostic applications and post-harvest preservation is insufficient to a great extent. However, we do not know if *A. hydrophila*, has broadly *in vivo* assessments and translational pathways for wide-scale commercial utility. Closing these research gaps is critical for achieving the maximum therapeutic and preventative potential of IgY in sustainable aquaculture health management.



Chapter -3

Production, Extraction and Purification of IgY Antibodies from Egg Yolks

3.1. Introduction

In aquaculture, bacterial infections have the potential to spread deadly illnesses that impair fish survival, growth, and health. Several bacterial infections in fish species, including *Aeromonas* septicemia (Miryala and Swain, 2025), Edwardsiellosis (Ogunleye *et al.*, 2022), Columnaris (Hoai *et al.*, 2025), Streptococcosis (Phuoc *et al.*, 2021), and Vibriosis (Shafiee *et al.*, 2024), have been reported in the aquaculture sector. Nevertheless, a few of these pathogens are found to be highly responsible for the majority of global economic losses in aquaculture production (Maldonado-Miranda *et al.*, 2022). *Aeromonas* spp. are among the most common types of bacterial pathogens in numerous fish species that occur in freshwater and tropical environments (Irshath *et al.*, 2023). For instance, diseases caused by *Aeromonas* spp. in fish are haemorrhagic septicaemia, ulcer disease, and motile MAS (Chen *et al.*, 2019; Zhao *et al.*, 2019). In fish, the susceptible organs and tissues include skin, gills, fins, liver, kidney, spleen, and intestine (Wassif and Mohammed, 2022). For disease establishment, *Aeromonas* produces a variety of virulence factors, such as toxins, enzymes, and adhesins that contribute to their pathogenicity and host damage (Tomas, 2012). Species that are transmitted from infected fish to humans can cause gastroenteritis and septicaemia in immunocompromised individuals (Janda and Abbott, 2010).

Aquaculture industries across the world have been decimated by epidemics of a hyper-virulent pathotype of *A. hydrophila* (vAh) (Nhin *et al.*, 2021; Abdel-Latif and Khafaga, 2020; Hemstreet, 2010; Nielsen *et al.*, 2001) and predominantly cause disease outbreaks in cultured freshwater fish (Mzula, 2020; Mzula *et al.*, 2019; Rasmussen-Ivey *et al.*, 2016). *A. hydrophila* infections are associated with various symptoms like haemorrhagic septicaemia, oedema, epizootic ulcerative syndrome (EUS), haemorrhagic enteritis, and red body

disease and affect different cultivable finfish species, including common carps, goldfish, eel, catfish, and tilapia (Irshath *et al.*, 2023). *A. hydrophila* is ubiquitous within warm-water environments and has a diverse host range (i.e., amphibians, birds, fishes, reptiles, and mammals) with equally diverse diseases that include MAS in fish (Irshath *et al.*, 2023). The MAS produces significant internal and external haemorrhage and exophthalmia, often followed by mortality within several hours of manifestation of the disease (Beaz-Hidalgo and Figueras, 2013).

Among the different control measures that can be applied in the fish farms are antibiotics, vaccines, probiotics, and the application of biosecurity measures (Assefa and Abunna, 2018). The escalating challenge of antibiotic resistance, along with the diversity of *Aeromonas* strains, hinders effective control measures to limit the ailments they invoke (Sherif and Kassab, 2023). In aquaculture, if *A. hydrophila* isolates are often resistant to tetracycline antibiotics such as oxytetracycline and doxycycline usually used as preventative and treatment measures for disease, this could have lasting repercussions for veterinary knowledge (Abdella *et al.*, 2024; Goudarztalejerdi *et al.*, 2022). Other antibiotic classes continue to show an increase in resistance to those antibiotics which typically govern good animal husbandry and veterinary knowledge. *A. hydrophila*, for example, has already demonstrated increased resistance to fluoroquinolones, including ciprofloxacin and enrofloxacin (Nasser *et al.*, 2022). There are also reports of resistance in *A. hydrophila* to combinations of sulfonamides and trimethoprim previously isolated from aquaculture (Thaotumpitak *et al.*, 2023). Some *A. hydrophila* strains are being reported to be resistant to some beta-lactam antibiotics (ampicillin and cephalosporins) and subsequently prescribed routinely by human and other animal veterinarians (Abdella *et al.*, 2021; Saengsitthisak *et al.*, 2020). Further examples of resistance in some

A. hydrophila isolates have also been documented in macrolide antibiotics, specifically, erythromycin and azithromycin (Saengsitthisak *et al.*, 2020).

The use of IgY in aquaculture is beneficial through its ability to neutralize antigens and enhance the immune response in aquatic animals. There are a few studies directly related to IgY antibodies in aquaculture with a focus on reducing infection rates. Some studies utilizing IgY antibodies from egg yolk for *A. hydrophila* pre-primed with polyploid gibel carps from specific IgY achieved a 60% protection rate, when other groups had 0% survivors and mortality was reduced by 30% after the challenge with specific IgY for post-challenge treatment (Li *et al.*, 2006). A study using powdered egg yolk antibody may provide some protection against *Vibrio* infections in white shrimp (*Litopenaeus vannamei*). Using IgY powder may also have fewer total bacteria present in seed ponds (Gao *et al.*, 2016). Another study showed that specific IgY antibodies utilized against *Aeromonas* infections, in blunt snout bream (*Megalobrama amblycephala*) could directly bind to *A. hydrophila*, and agglutinated and inhibited bacterial growth in a dose-dependent manner (Qin *et al.*, 2018). Recently, studies have also demonstrated the IgY antibodies can be used as potential polyvalent passive vaccines in aquaculture.

Live and inactivated *V. fluvialis* IgY antibodies provide effective passive protection against *V. fluvialis* and *A. hydrophila* in gold fish, *C. auratus* (Liu *et al.*, 2024). In addition, the Outer Membrane Proteins (OMPs) of *A. hydrophila* were expressed and purified, and the corresponding IgY antibodies provided effective immune protection, activated innate and inflammatory responses, maintained the integrity of visceral tissue structure, and reduced apoptosis and DNA damage in kidney cells infected by *A. hydrophila* and *Pseudomonas fluorescens* in goldfish (Liu *et al.*, 2024). Another study demonstrated that IgY antibodies against *Streptococcus agalactiae*

enhance immune regulation and provide significant immune protection in Nile tilapia, *Oreochromis niloticus* (Zhang *et al.*, 2024; Eto *et al.*, 2018).

A. hydrophila is a significant bacterial pathogen in aquaculture. Historically, it has been recognized as one of the earliest pathogens affecting fish farming. However, currently, it remains prevalent and continues to impact fish health and production (Abdella *et al.*, 2024). Given the frequent and damaging outbreaks of *A. hydrophila* in aquaculture, as seen in Egypt in 2022 (Khursheed *et al.*, 2023; Vankara *et al.*, 2022), there's a critical need for new solutions to protect the industry (Abdella *et al.*, 2024). With growing antibiotic resistance, alternative treatments are urgently required. One promising option is the use of IgY antibodies from chickens, which have unique properties that can help fight disease. This study concentrated on developing IgY antibodies to combat *Aeromonas* species, and this chapter with a specific focus on developing antibodies against *A. hydrophila* and evaluating their effectiveness in laboratory settings.

However, we still don't know much about how stable and effective these antibodies are, especially when combined with antibiotics. Therefore, this research aims to fill those knowledge gaps and help to create better treatment plans for *A. hydrophila* infections. To achieve this, we focused on the production, extraction and purification of IgY, and then confirming its ability to specifically target and neutralize *A. hydrophila* through tests like ELISA and agglutination.

Objectives of the Chapter

- ✓ Immunization of White Leghorn chickens with formaldehyde-inactivated *A. hydrophila* antigens.
- ✓ Extraction and purification of egg yolk IgY antibodies.
- ✓ Estimation of IgY protein concentration.
- ✓ Characterization of antigen-specific egg yolk IgY antibodies.

3.2. Materials and Methods

3.2.1. Source of poultry and bacteria

A. hydrophila (MTCC–1739) strains were obtained in lyophilized form from the MTCC, Chandigarh, India. Strains of *A. hydrophila* were brought to pure culture by transferring lyophilized forms into double-strength nutrient broth and incubated at 37 °C for 24 hours. The organisms were sub-cultured on nutrient agar plates (MTCC Media-3). The Bio-safety institutional ethical clearance for the immunization with killed strains of *A. hydrophila* in the experimental animals were obtained from the Institutional Bio Safety Committee (IBSC- Approval No: IBSC-21-13) of the College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India as per the Standard Operating Procedure (SOP).

Single comb white leghorn egg laying chickens (*Gallus gallus domesticus*) aged 22-24 weeks old, were collected and maintained in a small animal breeding station, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India. The Institutional Animal Ethical Committee, CVAS, Mannuthy, sanctioned the approval for the use of chickens as experimental animals (IAEC- Approval No: IAEC/CVASMTY16/2021).

3.2.2. *A. hydrophila* antigen preparation

A. hydrophila cells were allowed to grow overnight in the nutrient broth. Cells were harvested by centrifugation, washed three times, and re-suspended in phosphate-buffered saline (PBS) at a density of 10^6 cells/mL. Formaldehyde (4:1) was added to a final concentration of 3% (v/v), and the suspension was held overnight at 4°C (Uma *et al.*, 2012). Cells were washed twice with PBS to remove the formaldehyde and re-suspended in sterile PBS (pH 7.2). Complete killing of the cells was confirmed by sterility check. Bacterial suspensions were stored at 4°C for further use (Dinesh *et al.*, 2013; Uma *et al.*, 2012).

3.2.3. Immunization with bacterial antigens

Formaldehyde-killed antigens of *A. hydrophila*, re-suspended in PBS, were prepared for immunization in chickens using the McFarland standards, with turbidity ranging from 0.5 to 8 MFU (McFarland's Unit). Where McFarland standards were made by mixing barium chloride and sulfuric acid to create a barium sulphate precipitate, which was kept for comparison of the turbidity of the test sample. Three millilitres of inactivated antigens were administered *via* intramuscular injection into the breast muscles of White Leghorn chickens commencing with an initial dose equivalent to 0.5 MFU (Figure 3.1A). Chickens received subsequent doses with increasing bacterial loads up to 8 MFU at 7-day intervals *via* the same route, and booster injections were administered similarly (Schade *et al.*, 1996) for up to 180 days (Table 3.1).

Table 3.1. Schedule of Immunization in chicken

Weeks	Dose (in MFU)
week 1	0.05
week 2	1
week 3	2
week 4	3
week 5	4
week 6	5
week 7	6
week 8	7
week 9	8
week 10	Booster dose
week 11	Booster dose
week 12	Booster dose
Week 13 (up to 24 th week)	Booster dose

3.2.4. Detection of antibody titer from serum

Test bleedings were made to check the presence of *Aeromonas* IgY antibodies in the serum in the second week of the first immunization. Blood samples were collected from the wing vein of immunized chickens and subjected to centrifugation. Serum collected after centrifugation was used to check the presence of specific antibodies *via* the microscopic agglutination method (Dinesh *et al.*, 2013; Narat, 2003; Tini *et al.*, 2002). In the microscopic slide agglutination test, one drop each of *A. hydrophila* antigens and specific antibodies from serum was mixed thoroughly in a microscopic slide and observed for agglutination.

3.2.5. Egg collection and storage

Eggs from immunized chickens were collected daily after agglutination was confirmed through microscopic slide tests. The egg shell surfaces were washed with distilled water and labelled by day order. The collected eggs were properly marked and stored (Figure 3.1B) at 4°C for purification (Uma *et al.*, 2012).

3.2.6. Extraction of IgY antibodies from egg yolk

The IgY antibodies were extracted from the egg yolk by polyethylene glycol (PEG) and ammonium sulphate precipitation method (Polson *et al.*, 1980). Egg shells were sterilized with 70% ethanol to prevent contamination (Shahein and Sedeek, 2014). Egg yolk was separated from egg white (Figure 3.1C), washed with distilled water to remove as much albumin as possible (Figure 3.1D), and rolled on a paper towel to remove adhering egg white (Figure 3.1E). The yolk membrane was punctured, and the yolk was allowed to flow into a graduated cylinder (Figure 3.1F).

An equal volume of Buffer S (10 mM phosphate, 100 mM NaCl, pH 7.5, containing 0.01% sodium azide) was added to the separated egg yolk and mixed thoroughly. To this mixture, 10.5% of PEG 6000 was prepared in Buffer S and added to achieve a final concentration of 3.5% (w/v). The mixture was stirred for 30 minutes at room temperature, followed by centrifugation at 11,000 rpm for 20 minutes. The resulting supernatant was filtered through a double layer of cheesecloth to remove precipitated lipoproteins and debris. Subsequently, PEG 6000 (prepared as a 42% solution in Buffer S) was added to the supernatant to reach a final concentration of 12% (w/v). After thorough mixing, the solution was centrifuged again at 11,000 rpm for 20 minutes. The pellet was re-dissolved in Buffer S to the original yolk volume and an equal volume of 4M ammonium sulphate (pH 7) solution and stored overnight at 4°C for the precipitation reaction (Figure 3.1G). The precipitate was obtained through centrifugation at 11,000 rpm for 20 minutes. The pellet was collected and re-dissolved in 1mL of Buffer S (without NaCl) for storage.

3.2.7. Removal of salts by dialysis

The precipitated IgY was transferred to the activated dialysis bag tied after including some air and twisting the open end of the dialysis bag (Mahenthiran *et al.*, 2021). The bags containing IgY antibodies were dialysed against buffer S without NaCl, pH 7.4 (Figure 3.1H) for up until no colour change for Nessler's reagent. The contents were dispensed into micro centrifuge tubes after dialysis and centrifuged (11,000 rpm for 20 minutes) to remove the remaining impurities (Pauly *et al.*, 2011).

3.2.8. Purification of IgY by DEAE cellulose - ion exchange column chromatography

The IgY was further purified by ion exchange column chromatography (Jensenius *et al.*, 1981). For making activated DEAE cellulose (Diethylaminoethyl

cellulose), 25-30 grams of pre-swollen (with distilled water) DEAE cellulose was added to a beaker containing an equal volume of 0.1 N NaOH, stirred well in such a way that no air bubbles were formed, and left at room temperature for 30 minutes. The supernatant was decanted, and the sediment was treated with double-distilled water, stirred well, and left at room temperature for 30 minutes. This was repeated several times until the pH of the double-distilled water was neutral. Further, the sediment was mixed with an equal volume of (initial volume of DEAE cellulose) 0.1N HCl and was left at room temperature with intermittent stirring for 30 minutes. This was further washed several times with double-distilled water until the pH of double-distilled water was neutral.

The column (Sigma, India) was packed with activated DEAE cellulose and equilibrated with 25 mM phosphate buffer, pH 8.0 (Uma *et al.*, 2012). The IgY antibodies were loaded and washed with 25 mM phosphate buffer, pH 8.0 (Uma *et al.*, 2012). The column was eluted by passing linearly increasing sodium chloride through the column. Three millilitre fractions were collected at the flow rate of 0.6 mL/min (Figure 1.I). Collected IgY fractions were further purified by dialysis to remove the sodium chloride.

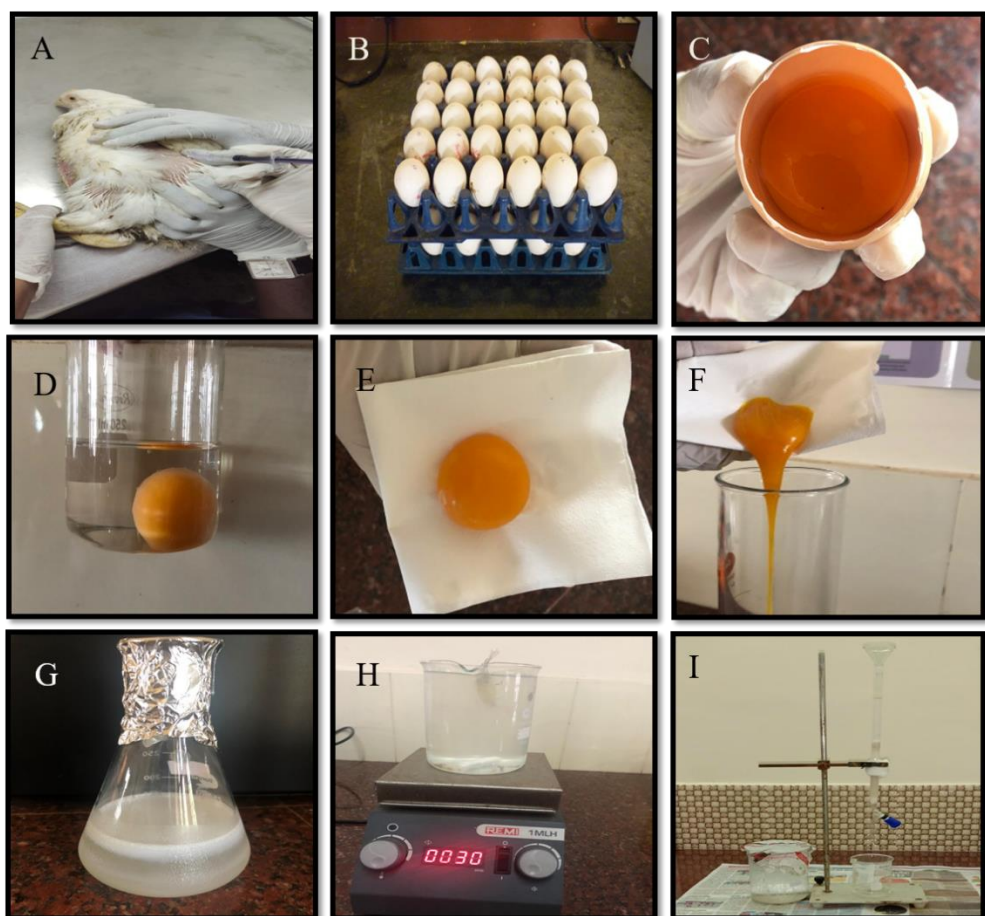


Figure 3.1. Various stages of the extraction of IgY antibodies. A: Immunization in chicken, B: Collected eggs, C: Yolk, D: Yolk in distilled water, E: Yolk on tissue paper for external lipid removal, F: Yolk separation without yolk sac, G: Ammonium sulphate precipitation, H: Dialysis, I: Column chromatography

3.2.9. Determination of total protein

The protein content of the column-eluted fractions was determined by using the conventional method (Lowry *et al.*, 1951). A working standard solution was prepared by diluting 1 mL of 0.1% Bovine Serum Albumin (BSA) with 9 mL of distilled water. From this standard solution, increasing

volumes (0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1.0 mL) were pipetted into separate test tubes, and each was brought up to 1 mL with distilled water. A separate test tube containing 1 mL of distilled water served as the blank.

For the test samples, 0.1 mL of each sample was diluted to 1 mL with distilled water. To each tube (standards and samples), 5 mL of alkaline copper reagent was added, and the mixture was allowed to stand at room temperature for 10 minutes. Then, 0.5 mL of Folin–Ciocalteu reagent was added, and the tubes were incubated for 30 minutes at room temperature. Absorbance was measured at 750 nm against the blank. A standard graph was constructed by plotting OD values against known protein concentrations, and the protein content of test samples was extrapolated from this graph.

3.2.10. Determination of specific protein

The specific IgY concentration in total protein was determined by adding 0.5 mL of the crude protein solution to 1 mL of a 0.01% antigen solution in PBS (pH 7.2) and incubating overnight at 37°C. The supernatant was separated by centrifugation, and the absorbance was measured at 280 nm (Lee *et al.*, 2002a).

The specific IgY concentration was calculated as follows:

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

3.2.11. Protein profile of IgY antibodies

The purity of chicken egg yolk antibodies was assessed using Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE), with a 4% polyacrylamide stacking gel and a 12% polyacrylamide separating gel (Laemmli, 1970). The IgY sample was mixed with an equal volume of sample

treatment buffer containing 5% mercaptoethanol, 2% SDS, 0.005% bromophenol blue, and 0.05 M Tris buffer (pH 8.0). The test sample and broad-range molecular weight markers (Genei Pvt. Ltd., Bangalore) were loaded into separate wells of the gel. Electrophoresis was performed at a constant voltage of 100 V using a Tris–glycine running buffer.

Electrophoresis was stopped when the bromophenol blue dye front reached the bottom of the gel. The gel was then carefully removed and stained with Coomassie Brilliant Blue R-250 for 30 minutes. Excess stain was removed by rinsing the gel with distilled water, followed by de-staining using 7% acetic acid, which was changed frequently until the gel background became clear. The purity of IgY was confirmed by comparing its band with those of the molecular weight marker.

3.2.12. Determination of antibody titer by indirect ELISA

The antibody titer of the antibodies generated against *A. hydrophila* was determined by indirect Enzyme-Linked Immunosorbent Assay (ELISA) (Voller *et al.*, 1976). Nuncpolysorp ELISA plates were coated with respective antigens using coating buffer (0.05M carbonate bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. After coating, the plates were washed with Phosphate Buffered Saline with Tween-20 (PBST) 3 times. The empty sites were blocked by 1% BSA (200µL/well) and incubated at 37°C for 1 hour. Plates were subsequently washed and incubated with antibodies (100µL/well). PBST and non-specific IgY served as controls. Wells were washed thrice with PBST, and 100µL of diluted (1:1000) rabbit anti-chicken immunoglobulin coupled to Horseradish peroxidase (Genei Pvt Ltd, Bangalore) was added and incubated at 37°C for 1 hour. Then the plates were washed and 100µL of freshly prepared 3,3',5,5'-Tetramethylbenzidine/ Hydrogen Peroxide (TMB/H₂O₂) solution (Genei Pvt Ltd, Bangalore) was added. The plates were allowed to

remain at room temperature in the dark for 20 minutes. The reaction was stopped by adding 50µL of 4N sulphuric acid (4N H₂SO₄), and plates were read at 490 nm in an ELISA reader (Thermo Scientific, Multiskan FC; Software: SkanIt 6.1.1). The samples were tested separately in triplicate.

3.2.13. Inhibition ELISA

Chicken egg yolk antibodies (IgY), raised against *A. hydrophila* and prepared at varying concentrations from 0 to 32 µg/mL, were initially pre-incubated for 1 hour at 37°C with overnight cultures of *A. hydrophila* (1.0 x 10⁷ cells/mL). This pre-incubation allowed the IgY to neutralize *A. hydrophila* antigens in solution. Following this, these mixtures were transferred to microtiter plate wells coated with *A. hydrophila* antigens. In this competitive binding setup, any IgY not bound to *A. hydrophila* in solution would then bind to the immobilized antigens on the plate. After washing with PBST, the amount of IgY successfully bound to the coated wells was detected using rabbit anti-chicken immunoglobulin conjugated to horseradish peroxidase. The plates were subsequently developed with OPD (o-Phenylenediamine dihydrochloride) and H₂O₂ (Hydrogen peroxide), the reaction stopped with 50 µL of 4N sulfuric acid, and absorbance read at 490 nm using an ELISA reader (Thermo Scientific, Multiskan FC; Software: SkanIt 6.1.1) (Meenatchisundaram *et al.*, 2011).

3.2.14. Statistical analysis

The results were analyzed statistically using SPSS (Statistical Product and Service Solutions) version 28, manufactured by IBM (International Business Machines) Corporation, Armonk, New York, United States, and the graphs were plotted using Origin Pro 8.1, developed by Origin Lab Corporation, Northampton, Massachusetts, United States.

Welch's t-test was used for column chromatography, a post-hoc test for protein concentration, and One-way ANOVA for inhibition ELISA.

3.3. Results

3.3.1. Detection of antibody titre from serum

Microscopic slide agglutination test confirmed the presence of specific *A. hydrophila* IgY antibodies in the serum collected during the second week following the first immunization. Clear and visible agglutination reactions were observed upon mixing the antigen and antibody drops in a 1:1 ratio on the microscopic slide, indicating the formation of antigen-antibody complexes (Figure 3.2).



Figure 3.2. Agglutination Assay

3.3.2. IgY elution by DEAE cellulose - ion exchange column chromatography

High yield of IgY was eluted between 23rd and 27th fractions by DEAE cellulose ion exchange column chromatography (Figure 3.3, Welch's t-test, $p < 0.05$, mean $\pm$ SD, $n=3$).

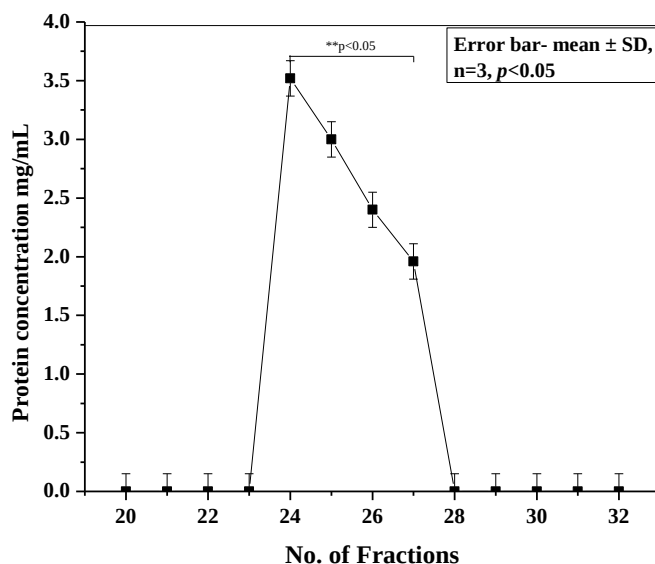


Figure 3.3. Purification of IgY by DEAE Cellulose Ion Exchange Column Chromatography

3.3.3. Determination of protein concentration

The protein concentration of purified IgY fractions of *A. hydrophila* IgY was in a range between 1 to 14.8 mg/mL with an average yield of 11.88 mg/mL (Figure 3.4, post-hoc test, $p < 0.05$ mean $\pm$ SD, $n=3$), and the specific IgY was 26%.

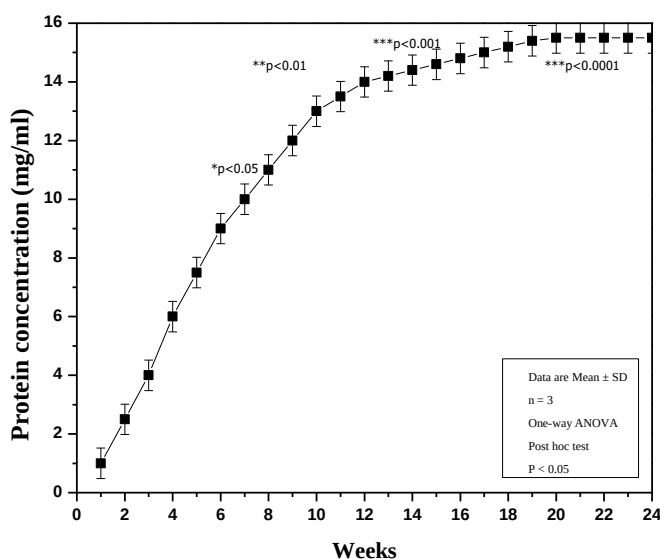


Figure 3.4. Protein concentration of IgY antibodies against *A. hydrophila* over 24 weeks

3.3.4. Purity of IgY analysis by SDS-PAGE and titration by ELISA

The 180 kDa IgY antibodies were confirmed via SDS-PAGE analysis, showing 95% purity. The results (Figure 3.5) were compared with the broad-range molecular marker (Genei, Pvt Limited, Bangalore). Additionally, a high titer was observed at a 1:10,000 dilution, confirming specificity to *A. hydrophila* antigens via ELISA.

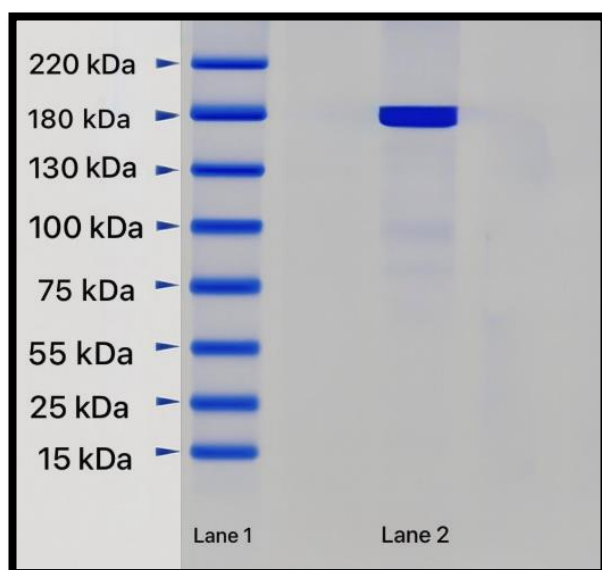


Figure 3.5. SDS-PAGE analysis of IgY antibodies. Where, Lane 1 is molecular marker band and Lane 2 is IgY

The inhibition ELISA showed a decrease in absorbance with increasing concentrations of IgY antibodies. This indicates that the chicken egg yolk antibodies (IgY) effectively neutralized the *A. hydrophila* antigens during the pre-incubation period. The results of the linear regression analysis support the conclusion that increasing IgY concentration effectively neutralizes *A. hydrophila* antigens, as indicated by the decrease in absorbance (Figure 3.6, One-way ANOVA - Post-hoc Dunnett's test, $p < 0.05$. Error bars represent mean $\pm$ SD, $n = 3$).

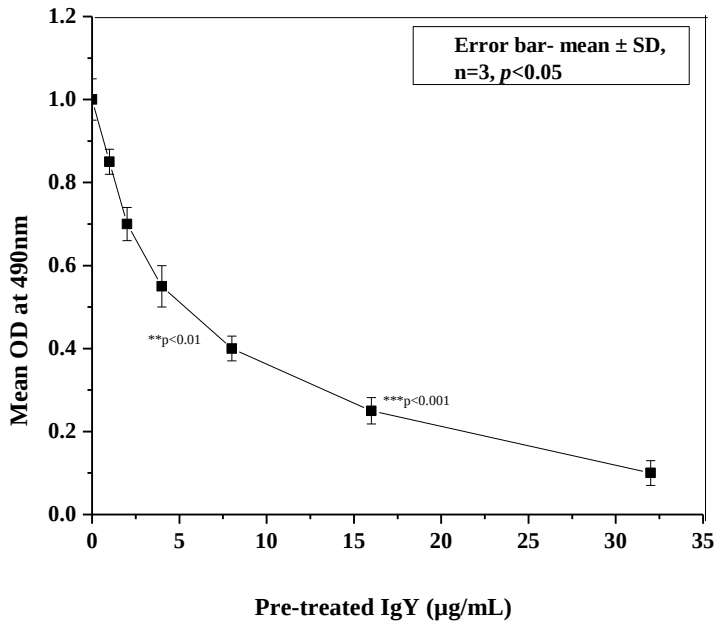


Figure 3.6. Inhibition ELISA

3.4. Discussion

The rising concerns with antibiotic resistance in aquaculture are mainly attributed to common practice of treating sick fish with medicated food (Pridgeon and Klesius, 2012). The medicated food has encouraged bacterial pathogens to adapt and resist treatment. As evidence from literature cited (Pridgeon and Klesius, 2012), producers are being forced to double the dose and with the same effect. As noted in several studies (Moffo *et al.*, 2024; Minich *et al.*, 2018), this public outcry that is forcing public concern contributes to the increased need for non-antibiotic alternatives of similar or potentially better protection for aquatic animals.

Vaccination is recognized as one of the most promising alternative for controlling diseases, and there have been continuous development of new

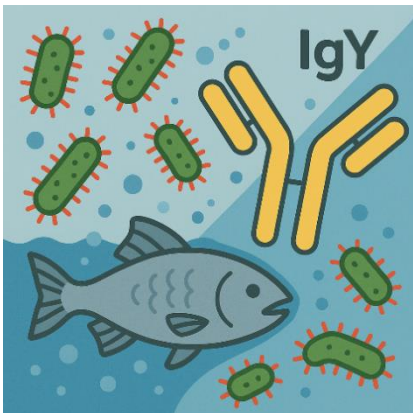
vaccines to combat bacterial disease in fish farming (Rathor and Swain, 2024; Tammas *et al.*, 2024). The salmon industry has been the most visible example of vaccine use, for approximately 30 years, which has led to a significant decrease in antibiotic use for salmon production (Rahim *et al.*, 2023; Adams, 2019; Gudding and Goodrich, 2014; McLoughlin, 2014). The effectiveness of the vaccines to control fish farm disease has been well documented in Europe, America and Japan (Chinabut and Puttinaowarat, 2005). The present study developed a vaccine composed of formaldehyde-killed *A. hydrophila* cells and injected the vaccine into the breast muscles of 22-24 week-old White Leghorn chickens. We found IgY antibodies appeared in the egg yolks within four to seven days of appearing in the blood, and that after each booster dose during the immunization period, their concentration in the yolks increased.

We used a two-step approach with PEG to extract IgY. In the first step, a 3.5% PEG solution was used to remove lipid substances; in step two, IgY was precipitated with a 12% PEG solution. The process is very efficient, and it is possible for one laying hen to produce about 20 eggs in one month, which can provide approximately 2 grams of antibody per month (Shimizu *et al.*, 1994). Another method that consisted of both poloxamer and PEG was even more efficient, with total concentrations of protein at 30 mg/mL and an IgY purity at 92.71% (Li *et al.*, 2017). The therapeutic possibilities of the PEG method were demonstrated with recent studies on honey bees with *Nosema* disease, where researchers used the PEG precipitation technique to extract specific IgY antibodies and were still able to significantly reduce the amount of *Nosema* spore counts as well as reduce the numbers of infected bees (Acik *et al.*, 2024).

Both prior and current studies have shown that PEG-based IgY purification will produce a functional IgY in high yield and purity. IgY titration in this study was seen at dilutions greater than 1:10,000 over the 180-day observation period. Another study, Meenatchisundaram *et al.* (2011), noted the greatest titer of 1:10,000 on the 150th day of observation. Many studies, including Redwan *et al.* (2021), Rezaeifard *et al.* (2021), and Acik *et al.* (2024) used SDS-PAGE to test the purity and activity of IgY antibodies. Our study results of high molecular weight proteins (180 kDa) are consistent with the studies identified.

Our study yielded an IgY quantity of 11.88 mg/mL, with 26% specificity, which was consistent with Diaz *et al.* (2024), Bizanov (2017) and Chelliappan *et al.* (2017). Therefore, we can conclude that our PEG-based extraction method works as an extraction method. The chromatogram results matched the work of Hatta *et al.* (1990), who reported a nearly pure yield (98%) of IgY of 70–100 mg per egg, using a different purification method.

These conclusions confirm that IgY is a viable financial and biological alternative to antibiotics and can be produced and scaled enough to control bacterial infections in aquaculture. This research provides vital insight into how IgY antibody effects can be used and scaled, supporting IgY's role in contributing to advances in sustainable blue growth methods of managing diseases. Our future work will investigate the efficacy of IgY in a lab and then assess whether IgY-coated feed can control disease in live fish.



Chapter -4

**Comprehensive *in-vitro* Efficacy
Evaluation and Stability of IgY against
*Aeromonas hydrophila***

4.1. Introduction

Avian immunoglobulin Y, or IgY, corresponds structurally and functionally to the familiar mammalian antibody IgG, but it differs in a number of structural and functional characteristics (Pereira *et al.*, 2019). IgY has a distinct evolutionary path (Eriksson and Larsson, 2025) and a "hinge" region that is somewhat unique and rigid with a differing amino acid sequence than that of IgG (Warr *et al.*, 1995). In addition, the hinge may very well influence how the antibodies bind to the cervical antigen. One important aspect of indirect or passive immunity conferred by IgY compared to the mammalian antibody IgG is that IgY can never be responsible for antibody-dependent enhancement (ADE), in which antibodies can simultaneously enhance infectivity and worsen an infection. That, in combination with fewer side effects, gives IgY particular advantages for therapeutic use (Esmaeili *et al.*, 2024; Fink *et al.*, 2017).

In 1996, the European Centre for the Validation of Alternative Method (ECVAM) declared IgY technology to be an ethical alternative to existing methods (Schade *et al.*, 1996). Since then, both patents and clinical trials have occurred, as well as products in the diagnostics and bio-therapeutics space for humans and animals. Research has not been limited, entertaining all different avenues of application, particularly in the agricultural arena, aquaculture and also as a substitute for antibiotics (Bondad-Reantaso *et al.*, 2023). When presented in powder form, IgY is convenient and cost-effective for oral delivery, especially when combined with stabilizers (Ge *et al.*, 2020; Rahman *et al.*, 2013). Other routes of delivery, such as nasal spray, are also underway (Frumkin *et al.*, 2022; Carlander *et al.*, 1999). The stability of IgY in different conditions is a factor in its applicability (Zhang *et al.*, 2023a; Yakhkeshi *et al.*, 2022; Rahman *et al.*, 2013; Dias da Silva and Tambourgi, 2010).

IgY enhances immune responses in aquatic organisms and reduces infection in farm animal aquaculture. Egg yolk antibodies in powdered form have been shown to protect against *Vibrio* infections in shrimp and lessen the bacterial load in ponds (Gao *et al.*, 2016). The antibacterial protection from IgY antibodies were examined *in vitro* with the use of *Campylobacter jejuni*, *Salmonella typhimurium*, *S. enteritidis*, and *Escherichia coli* specified sustainable, effective, ethical, available, and inexpensive replacements for antibiotics in livestock management (Czoska *et al.*, 2025). Liu *et al.* (2023) showed that these IgY antibodies were effective against red crucian carp infections that were caused by *P. fluorescens* and *A. hydrophila* and supported IgY antibodies as strong candidates for multivalent passive vaccines.

A. hydrophila causes significant economic losses in aquaculture, and its virulence and antibiotic resistance complicate the incidences (Miryala and Swain, 2025). Increased multi-antibiotic resistance and toxic side effects of antibiotics on fish metabolic processes have triggered the pursuit of alternative vaccine methods (Karnwal *et al.*, 2025). Vaccination and antibiotics are essential, but practical issues, reduced effectiveness, and the rise of antibiotic resistance pose significant challenges. Specific IgY antibodies have shown a lot of promise, successfully protecting fish and reducing mortality rates in infected populations (Zhang *et al.*, 2023b). IgY antibodies are notable for having high specificity by attaching to designated target sites and, thus, perform as well as prevailing industry standards (Lee *et al.*, 2021; Ge *et al.*, 2020). Not only do these antibodies remain consistent in numerous conditions, but also possess high specificity and limited cross-recognition with other molecules (Lee *et al.*, 2021). Interestingly, cross-recognition of such antibodies with pathogens of the same species signals they could yield broad-spectrum therapies and diagnostics (Saputri *et al.*, 2024; Wallach *et al.*, 2011). The addition of IgY to other therapies can also

enhance their efficiency and afford excellent defences against infections (El-Kafrawy *et al.*, 2023; Sanches *et al.*, 2022; Suzuki *et al.*, 2004).

As aquaculture provides such economic value in so many countries, it's essential to control and prevent infections from *A. hydrophila*. Recognizing this urgent need, our study is examining the therapeutic potential of IgY *via* extremely rigorous laboratory testing. This chapter addresses the evaluation and analysis of the effects of IgY on *A. hydrophila* using various methods, specifically growth inhibition assay, tests for cross-reactivity, tests for synergism, and tests for stability under different environmental conditions, such as temperature and pH.

Objectives of the Chapter

- ✓ Evaluation of the growth inhibitory activity of IgY antibodies against *A. hydrophila*.
- ✓ Investigation of the cross-reactivity of IgY antibodies with related *Aeromonas* species.
- ✓ Evaluation of the combinational (synergistic) effect of IgY antibodies with antibiotics against *A. hydrophila*.
- ✓ Assessment of the interaction and stability of IgY antibodies under different physicochemical conditions.

4.2. Materials and Methods

4.2.1. Source of poultry and microbes

This study used single-comb White Leghorn chickens (*Gallus gallus domesticus*) and *A. hydrophila* (MTCC-1739) strains (details given in section 3.2.1). We also utilized *A. caviae* strains (MTCC-6541) for cross-reactivity. It is also purchased from MTCC, Chandigarh, India. The procedures for *A. caviae* antigen preparation, IgY antibody production, extraction, purification, and protein estimation were carried out similarly to those described for *A. hydrophila* in section 3.2.

4.2.2. Growth inhibition assay

4.2.2.a. Growth inhibition assay by dilution method

To perform the growth inhibition assay by the dilution method, log-phase bacterial cultures were prepared and adjusted to the 10^8 - 10^9 CFU/mL desired concentration. About 10-fold serial dilutions of the *A. hydrophila* IgY antibody were then prepared in separate sterile test tubes. About 1 mL of the bacterial culture *A. hydrophila* was then added to each test tube containing different dilutions of the IgY antibody, mixed well, and incubated at 37°C for 24 hours (Lee *et al.*, 2002b). The results were compared with a non-specific IgY control. The results were further confirmed by streaking the cultures from each tube onto nutrient agar plates and again incubated overnight at 37°C for 18-24 hours, checked the results and compared them with the controls.

4.2.2.b. Growth inhibition assay by the well diffusion method

The growth inhibition assay was also performed on nutrient agar plates by the well diffusion method (Kavanagh, 2014) to assess the inhibition properties of *A. hydrophila* IgY antibodies against *A. hydrophila* cells. The log culture of *A. hydrophila* was swabbed onto the agar plates to get even growth of the pathogen. Then the agar plates were left to set, and in each of these plates 2 wells, 10 mm in diameter, were made using a sterile cork borer No. 4 and the agar cut-outs were removed. The wells were filled with *A. hydrophila* antibodies of 1:1000 and 1:10000 dilution and allowed for diffusion of IgY antibodies into the medium for 30 minutes and kept in an incubator at 37°C. After 18-24 hours, the agar plates were examined for inhibition zones around the wells, and the diameter of the zones was measured in millimetres (Juni *et al.*, 2016).

4.2.3. Cross-reactivity studies

4.2.3.a. Microscopic Slide Agglutination Test (MSAT)

For the microscopic slide agglutination test, *A. caviae* cell suspension was mixed with two-fold serial dilutions of *A. hydrophila* IgY antibodies.

Equal drops of each mixture were placed on microscopic slides and observed for agglutination at room temperature. This test was also performed *vice versa*, where the drops of *A. hydrophila* cell suspension and *A. caviae* IgY antibodies were mixed and observed for agglutination.

4.2.3.b. Micro agglutination Test (MAT)

For the micro agglutination test, a bacterial cell suspension of *A. caviae* was adjusted to an optical density of 0.42 at 620 nm. In a microtiter plate, two columns were designated for the test. The first well of each column was filled with 80 μ L of sterile saline, while the remaining wells received 50 μ L each. Subsequently, 20 μ L of *A. hydrophila* IgY antibodies were added to the first well of the first column, followed by a doubling dilution by transferring 50 μ L from one well to the next along the row. The same procedure was repeated for the second row (control), except that 20 μ L of a non-specific IgY antibody solution was used instead of *A. hydrophila* IgY antibodies. Finally, 0.05 mL of *A. caviae* bacterial cell suspension was added to all wells in each column and incubated overnight at 25°C. After incubation, the plate was observed for agglutination, and the results were compared with the controls. The same experiment was conducted *vice versa* to study the reactivity of *A. caviae* IgY antibodies to *A. hydrophila* antigens.

4.2.3.c. Tube agglutination test

For the tube agglutination test, purified IgY antibodies of *A. hydrophila* were serially diluted with sterile phosphate-buffered saline in Eppendorf tubes at dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . To each dilution, 50 μ L of *A. caviae* cell suspension was added. The tubes were thoroughly mixed and left to stand until agglutinates appeared. The obtained results were compared with controls and *vice versa* to assess the reactivity of *A. caviae* IgY antibodies to the *A. hydrophila* antigen.

4.2.3.d. Indirect ELISA method

To determine if the IgY antibodies from *A. caviae* would cross-react with *A. hydrophila*, we followed a conventional indirect ELISA procedure from Voller *et al.* (1976). The assays began by coating microtiter plates with *A. hydrophila* antigens and incubated overnight at 4°C, followed the next day by multiple washes to remove non-adhering antigen. The non-specific binding was reduced by a one hour incubation with a 1% solution of bovine serum albumin (BSA), again followed by rinse washes. We added the *A. caviae* IgY antibodies to the wells.

Following a one-hour incubation we washed the wells again and added a rabbit anti-chicken immunoglobulin conjugated to horseradish peroxidase (HRP) to bind to our IgY antibodies, and incubated for another hour, followed by additional rinse washes. The TMB and H₂O₂ solutions were added, giving rise to a color reaction which we allowed to develop for 20 minutes in the dark, and then stopped with sulfuric acid. We used an optical density measurement at 490 nm with an ELISA reader to quantify our measure of cross-reactivity (Thermo Scientific, Multiskan FC; Software: SkanIt 6.1.1). The results were analyzed by comparing the optical density (OD) values from the interaction of antigens with non-specific IgY antibodies. Similarly, the procedure was performed *vice versa* using *A. hydrophila* antibodies and *A. caviae* antigens to assess the cross-reactivity in both directions.

4.2.3.e. Growth inhibition assay

Growth inhibition assay was performed using *Aeromonas* IgY antibodies against *Aeromonas* pathogens on nutrient agar plates by the well diffusion method (Artur *et al.*, 1999). The cell suspension of *A. caviae* and *A. hydrophila* was evenly spread onto nutrient agar plates using a sterile swab to make a lawn culture. Well with 10 mm in diameter, were created in the agar

using a sterile cork borer No. 4, and the agar cuts were removed. Nutrient agar plates with wells containing *A. hydrophila* IgY antibodies were loaded with antigens from *A. caviae*. Likewise, nutrient agar plates swabbed with *A. caviae* were infused with IgY antibodies targeting *A. hydrophila*. The result was compared with their respective antigen, too. The plates were left for 30 minutes to allow the IgY antibodies to diffuse into the medium before being incubated at 37°C. After 24 hours of incubation, the plates were examined for inhibition zones, and the diameters of these zones were measured in millimetres.

4.2.4. Combinational effect of IgY antibodies with antibiotics against *A. hydrophila*

On a "U" bottom 96-well microtiter plate, the Minimum Inhibitory Concentration (MIC) tests (NCCLS, 1993) for the two broad-spectrum antibiotics, chloramphenicol and ciprofloxacin, as well as the particular IgY antibodies, were conducted. Initially, three rows of a 96-well plate were poured with 100µL of Muller-Hinton broth. A 200µL of the appropriate test sample, antibiotic or IgY from a stock solution containing 1 mg/mL was added to the first well of each row. The surplus sample was then poured off after 100µL of the test sample had been serially diluted from each row's first well to its tenth well. The 12th column received antibiotic or IgY treatment, serving as the negative control, as opposed to the 11th column, which acted as the positive control. Each well was then filled with 100µL of inoculum containing 5×10^5 CFU/ml, covered with the lid, and left to incubate for 12 hours at 37°C. Triplicates of each plate were examined directly or using an ELISA reader (Thermo Scientific, Multiskan FC; Software: SkanIt 6.1.1).

The concentration of the test sample with no growth that took place served as a confirmation of the MIC for each of the test samples. The Fractional Inhibitory Concentration Index (FICI or $\sum$ FIC) was examined

using the 1/16 to 8 times MIC of both IgY and antibiotics *via* the checkerboard method (Jain *et al.*, 2011). It is argued that a pharmacological combination is synergistic if $\sum FIC$ is less than or equal to 0.5. It is claimed that a medicine combination is indifferent or additive if the $\sum FIC$ ranges from 0.5 to 4 and it is considered antagonistic if $\sum FIC$ is ≥ 4 (Odds, 2003).

$$\sum FIC = FIC_A + FIC_B$$

Where $FIC_A = \text{MIC of drug A in combination} / \text{MIC of drug A alone}$

And $FIC_B = \text{MIC of drug B in combination} / \text{MIC of drug B alone}$

(Orhan *et al.*, 2005)

4.2.5. IgY interaction and its stability

To determine the stability of IgY, 50 μL of specific IgY was incubated with 950 μL of PBS. The binding activity of this IgY against the antigen was subsequently evaluated by indirect ELISA (Voller *et al.*, 1976) across a range of conditions, including pH (2 to 12), temperatures (-20°C to 100°C), molarities (0 mM to 100 mM), and varying percentages (0% to 100% v/v) of ethanol, methanol, and isopropanol.

4.2.6. Statistical analysis

The results were analyzed statistically using SPSS (Statistical Product and Service Solutions) version 28, manufactured by IBM (International Business Machines) Corporation, Armonk, New York, United States and the graphs were plotted using Origin Pro 8.1, developed by OriginLab Corporation, Northampton, Massachusetts, United States. Chi-square test was performed for the MAT study of cross-reactivity; One-Way ANOVA for indirect ELISA of cross-reactivity and the stability studies; and Two-Way ANOVA for synergistic analysis.

4.3. Results

Following the extraction and purification process, the concentrations of the obtained IgY antibodies were quantified. The average protein concentrations of the IgY antibodies produced were 11.8 mg/mL against *A. hydrophila* (from a 180-day IgY extraction study) and 5.2 mg/mL against *A. caviae* (from a 30-day IgY extraction study), respectively.

4.3.1. Growth inhibition assay

The growth inhibition assay result showed that an increase in bacterial concentration was proportional to the dilution of IgY antibodies in the test tubes. The result showed that specific *A. hydrophila* IgY up to the 10^{-4} dilution was able to completely inhibit the growth of *A. hydrophila* in both test tubes and its corresponding nutrient agar plates (Figure 4.1 A & B), and the MIC of specific IgY antibodies was determined as 10^{-4} dilution. In the well diffusion method of the growth inhibition assay, 1:10000 and 1:1000 dilutions of *A. hydrophila* IgY showed zones of 15mm and 25mm diameter, respectively, after 24 hours of incubation at 37°C, confirmed that IgY can actively inhibit the growth of *A. hydrophila* (Figure 4.2).

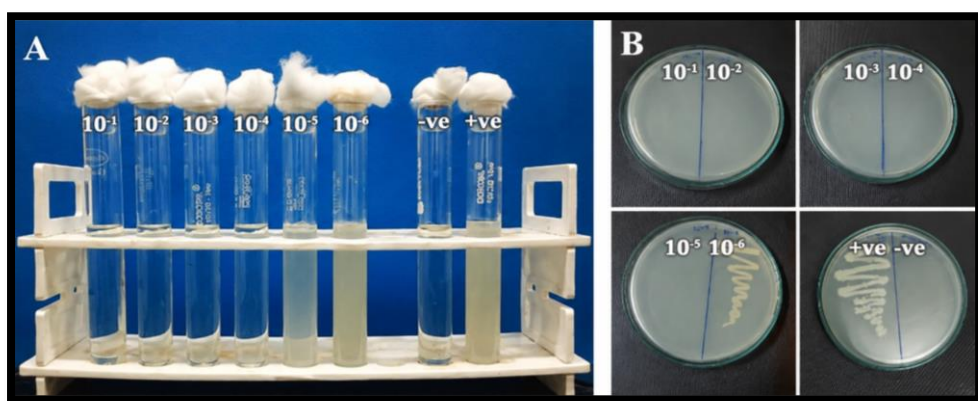


Figure 4.1A. Growth inhibition assay by dilution method in tubes and
4.1B. Corresponding plate results

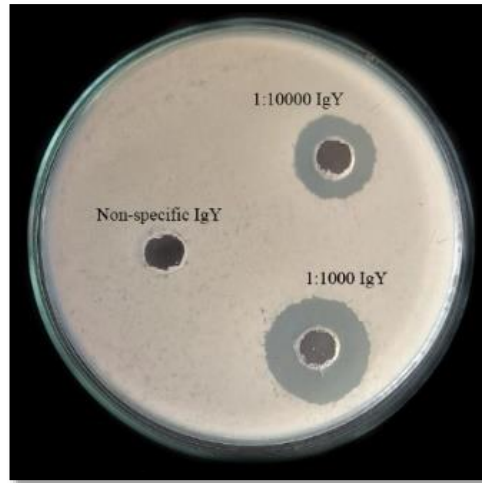


Figure 4.2. Growth inhibition assay by the well diffusion method

4.3.2. Cross-reactivity studies

In MSAT, agglutination was observed in both tests, indicating that *A. hydrophila* IgY antibodies can agglutinate *A. caviae* cells, and *A. caviae* IgY antibodies can agglutinate *A. hydrophila* cells, demonstrating cross-reactivity. The observed agglutination occurred up to a dilution of 1:32, while no agglutination was seen with non-specific IgY antibodies, confirming the specificity of the reactions. Agglutination scores are defined as follows: 4 denotes strong agglutination, 3 denotes medium agglutination, 2 denotes low agglutination, 1 denotes weak agglutination, and 0 denotes no agglutination.

The results of the cross-reactivity study using the MAT showed that specific *A. hydrophila* IgY antibodies reacted with *A. caviae* antigens and specific *A. caviae* IgY antibodies reacted with *A. hydrophila* antigens, both demonstrating agglutination up to a dilution of 1:64 (Figure 4.3, Chi-square test, $p < 0.01$, Error bars represent mean $\pm$ SD, $n = 3$). Conversely, non-specific IgY antibodies did not exhibit any agglutination with either antigen.

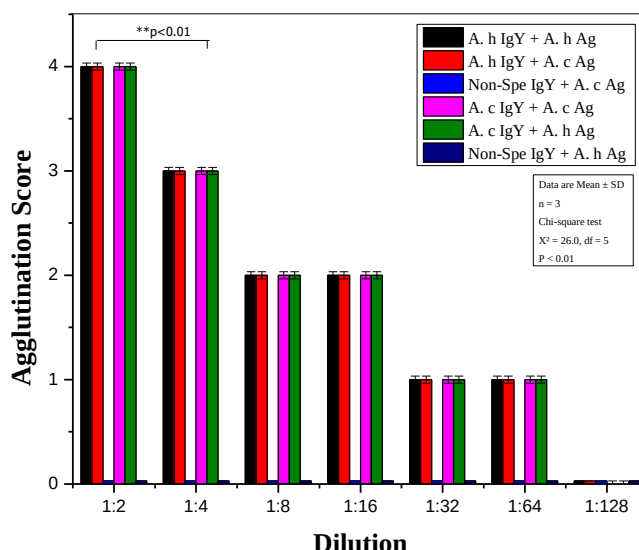


Figure 4.3. Micro agglutination assay. Here, A.h represents *A. hydrophila*, A.c represents *A. caviae* and Ag represents antigen. Statistical analysis using the Chi-square test revealed significant differences among groups ($\chi^2 = 26.0$, $df = 5$, $p < 0.01$).

In tube agglutination assay, agglutination appeared in tubes containing *A. hydrophila* IgY antibodies and *A. caviae* antigens, with increasing agglutination observed at higher IgY concentrations. Agglutination was still visible even at a 10^{-5} dilution. Similar results were observed *vice versa*, indicating cross-reactivity between the antibodies and antigens of both bacteria.

The indirect ELISA results confirmed cross-reactivity between *A. caviae* IgY antibodies and *A. hydrophila* antigens, as well as between *A. hydrophila* antibodies and *A. caviae* antigens. The OD values from the indirect ELISA tests were significantly higher in the test wells up to a dilution of 1:10000 compared to the control wells with nonspecific IgY antibodies (Figure 4.4, One-Way ANOVA, Post-hoc analysis using Tukey's HSD indicated significant differences in groups with a p -value of < 0.01 , Error bars

represent mean $\pm$ SD, n = 3). This indicates a significant degree of cross-reactivity between *A. caviae* and *A. hydrophila*.

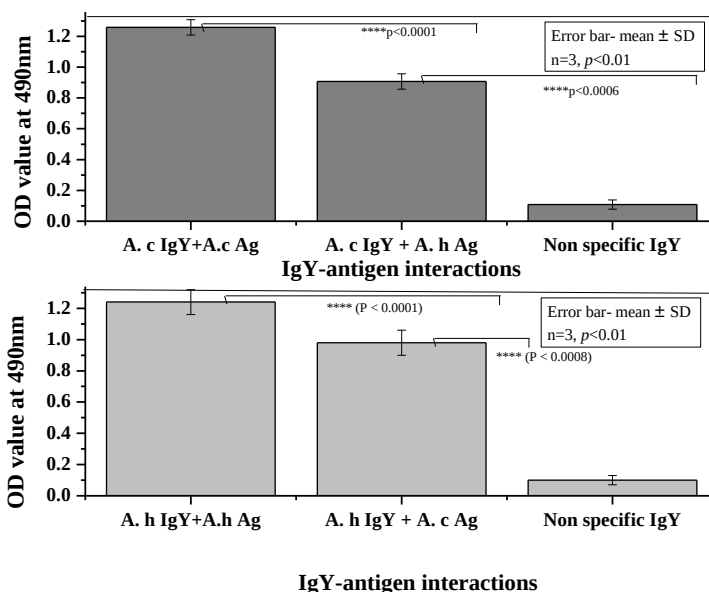


Figure 4.4. The cross-reactivity of the IgY antibodies with *Aeromonas* antigens by indirect ELISA. Where, A.c IgY+A.c Ag- *A. caviae* IgY + *A. caviae* Antigen, A.c IgY+A.c Ag- *A. caviae* IgY + *A. hydrophila* Antigen, A.h IgY+A.h Ag- *A. hydrophila* IgY + *A. hydrophila* Antigen, A.h IgY+A.c Ag- *A. hydrophila* IgY + *A. caviae* Antigen

The growth inhibition study results demonstrated species-specific inhibitory effects of IgY antibodies. *A. hydrophila* IgY antibodies inhibited the growth of *A. hydrophila*, forming a zone of inhibition with a diameter of 28 mm. Similarly, *A. caviae* IgY antibodies inhibited the growth of *A. caviae*, resulting in a 25 mm zone of inhibition. Furthermore, cross-inhibition was observed. *A. hydrophila* IgY antibodies inhibited the growth of *A. caviae* with a 23 mm zone, and *A. caviae* IgY antibodies inhibited the growth of *A. hydrophila*, forming a 21 mm zone (Figure 4.5).

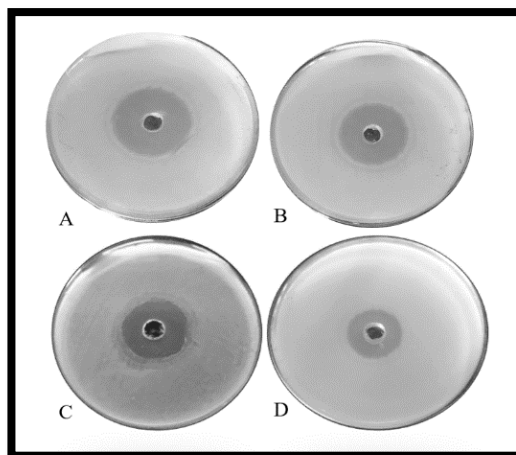


Figure 4.5. Growth inhibition assay. A: *A. hydrophila* IgY antibodies inhibit the growth of *A. hydrophila* (zone 28 mm), B: *A. caviae* IgY antibodies inhibit the growth of *A. caviae* (zone 25 mm), C: *A. hydrophila* IgY antibodies inhibit the growth of *A. caviae* (zone 23 mm), D: *A. caviae* IgY antibodies inhibit the growth of *A. hydrophila* (zone 21 mm).

4.3.3. Combinational effect of IgY antibodies with antibiotics against *A. hydrophila*

In synergism analysis, the MICs of antibody IgY and antibiotics ciprofloxacin and chloramphenicol were determined to be 12.5 $\mu\text{g/mL}$, 0.77 $\mu\text{g/mL}$ and 3.1 $\mu\text{g/mL}$, respectively. The combined effect of IgY antibodies with ciprofloxacin and chloramphenicol demonstrated FICI values of 0.24 and 0.37, respectively, indicating synergism (Table 4.1, Two-Way ANOVA, $p < 0.01$).

Table 4.1. The combinational effect of IgY with ciprofloxacin and chloramphenicol

Component	MIC against <i>A. hydrophila</i> (µg/mL)			ΣFIC	Combi-national effect
	Individual component	IgY in combination	Antibiotic in combination		
IgY	12.5±0.5				
Ciprofloxacin	0.77± 0.05	1.56± 0.10	0.09± 0.01	0.24± 0.02	Synergistic
Chloramphenicol	3.1± 0.2	3.12± 0.15	0.38± 0.02	0.37± 0.02	Synergistic

4.3.4. IgY interaction and its stability

The IgY showed consistent interaction within a pH range of 3 to 10 (Figure 4.6A, One-Way ANOVA followed by Tukey's HSD, $p \leq 0.05$), retained stability at temperatures up to 70°C (Figure 4.6B, One-Way ANOVA followed by Tukey's HSD, $p \leq 0.01$), and maintained effective interaction at molar concentrations as high as 50 mM (Figure 4.6C, One-Way ANOVA followed by Tukey's HSD, $p \leq 0.05$). Additionally, the antigen-antibody interaction remained unaffected by solvents such as ethanol, methanol, and isopropanol, even at concentrations reaching 30% (Figure 4.6D, One-way ANOVA followed by a Post-hoc test, $p \leq 0.01$).

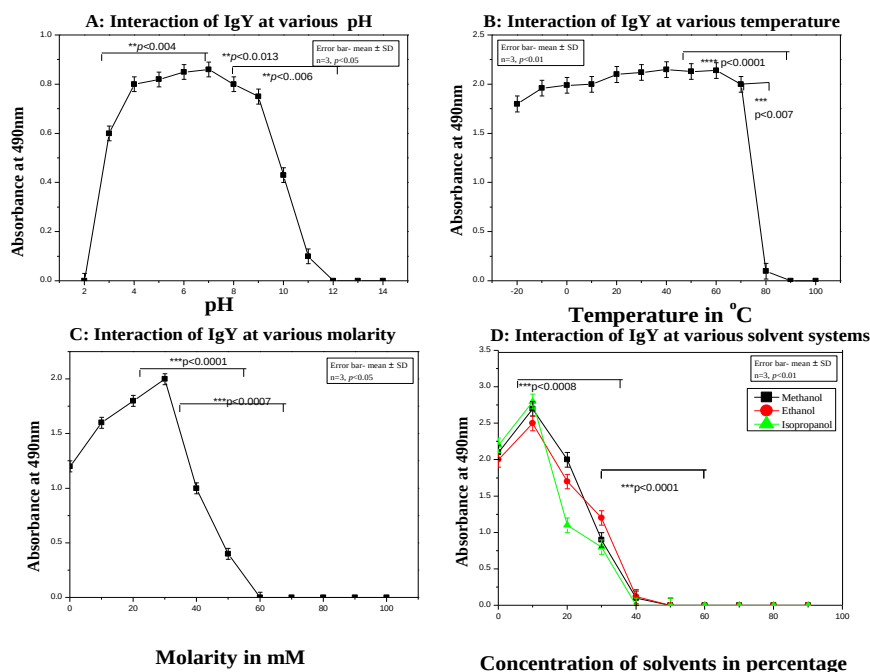


Figure 4.6. Interaction of IgY on various parameters A: at various pH, B- at various temperature, C- at various molarity D- at varying percentage of solvent systems

4.4. Discussion

Feeding infected fish with antibiotic-medicated food has led to food safety issues, antibiotic resistance, necessitating higher doses for effective control, and unscientific approaches, which is a public concern. Fish vaccination began in 1942 with a vaccine for *A. salmonicida* in *Oncorhynchus clarkii* trout (Adams, 2019). A number of studies and innovations have been made to formulate an alternative to antibiotics (Zhang *et al.*, 2023a). Vaccines have proven effective in controlling diseases in aquaculture, particularly in the aquaculture industry, reducing antibiotic use significantly. Despite advancements in vaccine technology, injection vaccination remains the

preferred method due to its effectiveness. However, modern aquaculture demands a cost-effective, feasible vaccination strategy that can be applied at the field level with minimal stress and maximum protection, achievable through oral immunization (Kilic and Gultekin, 2024; Du *et al.*, 2022). Nanotechnology offers potential solutions, but current studies focus on DNA vaccines, which are not permitted in fish farms in many countries. Future research should aim to develop novel immunization strategies that are safe for fish and humans (Tammam *et al.*, 2024). Passive immunization through oral, nasal, and topical administration of egg yolk-derived IgY antibodies has shown effectiveness in treating bacterial infections in both animals and humans. The WHO recognizes the potential of IgY antibodies (Kilic and Gultekin, 2024).

In the previous chapter, the studies involved raising specific polyclonal hyper-immune IgY antibodies in chickens against the fish pathogen *A. hydrophila*. IgY antibodies were detected in egg yolk and extracted using PEG and ammonium sulphate. ELISA, dialysis, SDS-PAGE, and column chromatography confirmed the presence and purity of the antibodies, with an average yield of 11.8 mg/mL. The high production of IgY antibodies (Li *et al.*, 2023) increases the demand for IgY over other mammalian antibodies. The yield of IgY can vary according to the age of the chicken (Pauly *et al.*, 2009, 2011). In one of the recent studies, the IgY extraction procedure described here was successfully applied for therapeutics to *Streptococcal* infection in tilapia aquaculture (Rizkiantino *et al.*, 2023).

4.4.1. Growth inhibition assay of IgY

In this study, the growth inhibition assay conducted through various methods confirmed the inhibitory properties of *A. hydrophila* IgY antibodies against *A. hydrophila* antigens. Similar to our findings, many other researchers

have shown the neutralizing capacity of IgY against a range of bacterial pathogens, such as the broad range of *Salmonella* serotypes, and dental caries caused by *Streptococcus mitis*, *S. mutans*, and *S. gordonii* (Bustos *et al.*, 2021; Dinesh *et al.*, 2020; Meenatchisundaram *et al.*, 2011). Recent research elucidated the *in vitro* growth inhibitory activity of IgY on the growth rate and viability of three major foodborne pathogens: *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia coli* (Czoska *et al.*, 2025). These studies highlight the myriad of potential uses for IgY antibodies in aquaculture, livestock management, the food industry, therapeutics, and environmental management.

The effective inhibition of bacterial growth by IgY antibodies in aquaculture, especially with respect to *A. hydrophila*, is generally attributed to the fact that IgY can bind to various components of the bacterial surface, which may be lipopolysaccharide (LPS), outer membrane proteins, and fimbriae (Sunwoo *et al.*, 2002). By binding these components, IgY may block other functions essential to the bacterium's mobility and replication (Smani *et al.*, 2012). More recently, *in vitro* work on *H. pylori* has shown that IgY antibodies exert their protective capacity by a variety of means, including agglutination, enhanced phagocytosis, neutralization, and blocking bacterial adherence (Zhang *et al.*, 2021). Likewise, IgYs against *S. typhimurium* and *S. enteritidis* were shown to have impressive agglutination (Terzolo *et al.*, 1998; Mine, 1997). These insights into IgY's immunological characteristics contribute to our understanding of its potential for various immunological applications within aquaculture.

4.4.2. Cross reactivity of IgY

The cross-reactivity observed in various bacteria and viruses has advanced research in passive immunization for humans, livestock, and

aquaculture (Lee *et al.*, 2021). These findings suggest that IgY antibodies could be considered as polyvalent vaccine candidates for passive immunization in aquaculture (Liu *et al.*, 2024). In our cross-reactivity study, we assessed the neutralization effectiveness of *A. hydrophila* IgY antibodies against *A. caviae* using indirect ELISA methods. Another *in vitro* study demonstrated that *S. enteritidis* IgY cross-reacted with *S. typhimurium*. Similarly, *S. typhimurium* IgY cross-reacted with *S. enteritidis* (Lee *et al.*, 2002b). Our findings align with these previous results. Additionally, high titers observed using indirect ELISA emphasize the specificity of both IgY. Similarly, *A. caviae* IgY antibodies were effective in neutralizing the *A. hydrophila* antigen. This is applicable in broad-spectrum activity of IgY antibodies against bacterial pathogens in aquaculture. While, studies unrelated to *A. hydrophila* have also shown the potential of IgY, such as the demonstration of protective effects and high specificity against *E. coli* K88 in weaned piglets (Wang *et al.*, 2019) and against *A. veronii* in *S. grahami* (Li *et al.*, 2023).

One of the recent studies has documented, the passive protection efficiency of IgY antibodies against *P. fluorescens* and their cross-protective action against *A. hydrophila* in *C. auratus*, where both of the antigens share the same epitopes (Liu *et al.*, 2024). Although for different pathogens, these findings highlight the potential of IgY in preventing bacterial adhesion and infection.

4.4.3. Combinational properties of IgY

The Σ FIC values for the IgY-ciprofloxacin and IgY-chloramphenicol combinations were 0.24 and 0.37, respectively, indicating significant synergistic effects. Combining IgY antibodies with antibiotics enhances therapeutic efficacy and can potentially lower the necessary antibiotic dosage,

thereby promoting sustainable antibiotic use (El-Kafrawy *et al.*, 2023; Vieira-Pires *et al.*, 2021). Our findings are in alignment with these studies, emphasizing the complementary effects of IgY and antibiotics.

Through the use of IgY in aquaculture production, the possibility of using fewer antibiotics, less selective pressure on bacteria, and slowing down the evolution of antibiotic-resistance genes is possible. Unlike antibiotics, which have selective target bacteria, IgY antibodies have the potential for mass production against a broad spectrum of pathogens and give wider coverage. This simultaneous approach effectively regulates bacteria-based infections and reduces the environmental impact of the conventional use of antibiotic-based remedies. Synergism entails overcoming more than one therapeutic mechanism simultaneously for the pathogen, and for such reasons, bacteria find it hard to become resistant to combination technologies. The multi-target approach ensures bacteria face numerous obstacles at the same time, significantly reducing the likelihood of accumulating resistance (Taheri-Araghi, 2024).

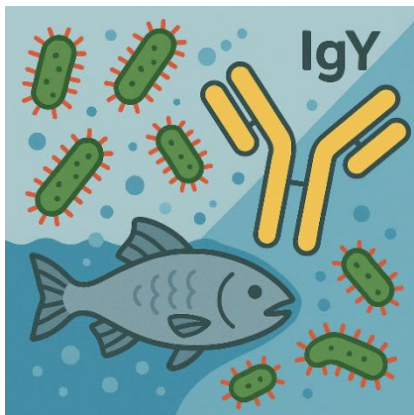
4.4.4. Stability of IgY

Stability of IgY antibodies under various conditions enhances the efficiency and reliability of IgY antibodies for application in industries and renders them a useful asset for both human and veterinary medicine and for food safety and biotechnology. The antibodies have been used as animal feed supplements, in veterinary medicine (Ge *et al.*, 2020), for food safety, biotechnology and diagnosis, and human therapeutics (Yakhkeshi *et al.*, 2022). The IgY antibodies considered in our analysis have favourable interactions at pH ranging from 3 to 10, but remain inactive at pH below 3. There are various reports to support our results demonstrating the loss of function of IgY at pH below 3 to 3.5 or above 10 (Lee *et al.*, 2002a; Hatta *et al.*, 1993;

Shimizu *et al.*, 1993; Losch *et al.*, 1986). Additionally, using sorbitol increases the stability of IgY and hampers its acid-induced inactivation, more specifically in the intestinal segment (Lee *et al.*, 2002a). Ge *et al.* (2020) studied various protocols of IgY preparation and stored stability and concluded that IgY antibodies remained relatively unchanged at pH ranging from 4 to 10 and at 70°C. Nilsson and Larsson (2007) came to similar conclusions and showed maximum stability at 37°C by more than eight weeks using stabilizers in the form of disaccharides. The retention of stability of IgY is of paramount importance for maintaining functional activity and efficiency of maintaining disease prevention and control, food safety (Zorriehzah *et al.*, 2016), improved immunity, oral vaccine, and reduced stress in aquaculture (Paul *et al.*, 2019).

By verifying that IgY maintains its function under various conditions, stability analysis underpins the production of efficacious treatments against aquaculture bacterial infections. The preservation of IgY stability further minimizes environmental impact, reduces preservative use of chemicals, and facilitates sustainable disease control measures. Studies and analysis emphasize the importance of maintaining the stability of IgY antibodies in maintaining their functionality and inform future research and real-world applications in practice (El-Kafrawy *et al.*, 2023; Rizkiantino *et al.*, 2023).

Based on the *in vitro* analysis, IgY antibodies against *A. hydrophila* demonstrated effective neutralization of the infection-causing ability in aquatic farmed fish. The subsequent chapter will assess the *in vivo* neutralization efficacy of IgY antibodies in fish models.



Chapter -5

**Evaluating IgY Antibody-Coated Fish
Feed for Motile *Aeromonas*
Septicaemia Control in
*Ctenopharyngodon idella***

5.1. Introduction

Ctenopharyngodon idella, commonly known as grass carp or white amur, has been cultivated in China for centuries. Historical records indicate that the Chinese royal society once forbade the cultivation and sale of common carp, prompting farmers to adopt grass carp alongside other carp species for aquaculture. In 2023, grass carp production in China alone reached 5.9 million tons, representing one-fifth of the total freshwater aquaculture output (Mengmeng *et al.*, 2024). Globally, India ranks second in grass carp production, trailing only China. At the species level, white-leg shrimp (*Penaeus vannamei*) led global production in 2022 with 6.8 million tonnes, closely followed by cupped oysters (*Crassostrea* spp.) and grass carp (*C. idella*), each at 6.2 million tonnes (FAO, 2024b).

Grass carp farming faces significant challenges due to disease, especially in intensive systems and poorly maintained aquaculture ponds. Contributing factors include a lack of expertise among fish farmers and limited access to high-quality seed, feed, and medicine, which often leads to farmer indebtedness. Rather than relying on antibiotics and chemicals to treat illnesses, maintaining optimal water quality and parameters proves to be a more cost-effective approach. In grass carp, *A. hydrophila* induces severe infections that result in intestinal inflammation, lesions, and heavy infiltration of inflammatory cells. This bacterium can seriously damage the intestinal mucosa barrier's structure and function, affecting the fish's health and survival (Wei-Guang *et al.*, 2017). Although the immune system of grass carp activates innate defence mechanisms in response to *A. hydrophila* infection, severe infections can overwhelm these defences and transmission routes include direct contact with contaminated water or infected fish (Yu *et al.*, 2024). *A. hydrophila* poses a zoonotic risk, as it can be transmitted from fish to humans through contact with the mucus and tissues of

infected fish, especially if there are existing cuts, abrasions, or puncture wounds on the hands. Infection of humans by ingesting contaminated water and fish is another route of transmission (Pal *et al.*, 2020).

Treatment therapies for *A. hydrophila* infection of *C. idella* have remained few and limited to various antibiotics, promoting the swift evolution of drug resistance. Enrofloxacin and florfenicol have been common aquaculture antimicrobials, particularly on grass carp farms, has been used extensively throughout the globe for more than a decade now (Shi *et al.*, 2022; Zhang *et al.*, 2022; Rozas and Enriquez, 2013). Some strains of *A. hydrophila* have shown resistance among macrolide antibiotics such as erythromycin, amoxicillin, and oxytetracycline (Saengsitthisak *et al.*, 2020). Understanding the relationship between environmental factors, host susceptibility, and bacterial virulence is crucial for effectively managing and mitigating the risks associated with *A. hydrophila* in aquaculture systems (Abdella *et al.*, 2024). Notably, some strains of *A. hydrophila* isolated from the gills of healthy freshwater food fish harbour several important virulence genes/factors, which could trigger disease in farmed fish under stress (Muduli *et al.*, 2021).

This chapter emphasises the importance of developing innovative methods for effective disease control in aquaculture settings, focusing on environmental variables. The infectivity pattern of *A. hydrophila* infection in *C. idella* and the effectiveness of neutralization by IgY antibody-coated fish meal are the primary focal points of this study.

Objectives of the Chapter

- ✓ Determination of the **LC₅₀** of *A. hydrophila* in grass carp (*Ctenopharyngodon idella*).
- ✓ Development of **IgY-coated fish feed**.

- ✓ Evaluation of the protective efficacy of IgY-coated fish feed through *in vivo* challenge studies.
- ✓ Histopathological evaluation of tissues from experimental fish.

5.2. Materials and Methods

5.2.1. Bacterial pathogen, bird and fish

The strains of *A. hydrophila* (MTCC – 1739), 22 to 24-week-old White Leghorn chickens (*Gallus gallus domesticus*) (described in Chapter 3), and juvenile grass carp (*Ctenopharyngodon idella*) were used as experimental subjects to investigate the *in vivo* antigen-antibody reaction. The experimental fish, collected from the local market, Wayanad, Kerala, weighing 12-15 grams and measuring 8±2 cm in length, were housed in an aquarium with a volume of 1 cubic foot, accommodating 10 fish per tank (Figure 5.1A). After a 7-day acclimatization period, the fish were transferred to the experimental tanks, where they were maintained in clean, aerated water using air filters, with a temperature range of 26–28°C and a pH of 7.0, and were fed commercially available pellets (Taiyo grow, Tamil Nadu, India) once in a day (5% of body weight of fish). We followed the Prevention of Cruelty to Animals (PCA) Act 1960, Breeding and Experimentation Rules 1998, and other amendments of the Government of India. All these experiments were reviewed and approved by the Institutional Bio-Safety Committee (IBSC- Approval No: IBSC-21-13) and the Institutional Animal Ethics Committee, (IAEC- Approval No: IAEC/CVASMTY16/2021).

5.2.2. Determination of LC₅₀

To determine the median lethal concentration (LC₅₀), *A. hydrophila* was cultured and the bacterial load was adjusted to concentrations of 5×10⁶, 10×10⁶, 15×10⁶, 20×10⁶, 25×10⁶ and 30×10⁶ CFU/mL. These varying concentrations were then used to infect the fish, with each experimental unit containing fish exposed in 1000 ml of the respective bacterial suspension (OECD, 2019).

The fish were observed for 96 hours following treatment, where 10 fish/tank were considered as a single unit. Data was meticulously recorded throughout this period and compared with the control group of fish free of pathogens. The concentration at which 50% of the fish population succumbed to the bacterial infection was identified as the LC₅₀ value (Saganuwan, 2020).

5.2.3. Development of antibody-coated fish feed

Samples of fish feed, commercially available in nearby markets (Taiyogrow, Tamil Nadu, India), each weighing 10 grams, were separately placed in different containers (Xu *et al.*, 2020). Corresponding solutions of the specific IgY (2.5% to 5% of 11.88 mg/mL) were prepared in saline, and their volumes were adjusted to 24 mL. These solutions were then individually poured over each fish feed sample, ensuring comprehensive soaking of the feed with the IgY solution. After soaking, the feed was spread out to dry (Figure 5.1B). We also assessed the impact of different IgY concentrations in feed on fish exposed to the LC₅₀ pathogen concentration for 14 days. It aimed to determine the minimum IgY dose in the feed required to protect more than 50% of the fish from mortality.

5.2.4. Experimental setup for *in-vivo* studies

After acclimatisation and observation for a week, the fish were transferred into the experimental tank and categorized into three groups (Table 5.1), and the impact of varying IgY concentrations with different modes of application was examined. Group 1 was considered as a control and kept free from bacterial pathogens. Group 2, serving as the infection control, was challenged with *A. hydrophila* to study infectivity and mortality rate (see 5.2.2. Determination of LC₅₀). Whereas in the survival study (Group 3), fish were treated in three different phases. In phase 1, fish were treated with antigen and antibody-coated fish feed simultaneously for 14 days by the immersion method to study the neutralization efficacy *in vivo*. In phase 2, fish were

treated initially with antigen for 2 days, then provided antibody-coated fish feed by immersion method up to the 14th day to analyse the therapeutic nature of IgY. In phase 3, fish were allowed to contact with IgY antibodies through the coated fish feed for 7 days and then treated with the live bacterial pathogen by immersion method for up to the 14th days to examine the prophylactic nature of IgY. The fish were observed and documented for their health status, behaviour, and any signs of disease during the experiment.

Table 5.1. Grouping of experimental animals for *in vivo* studies

Group		Mode of application	Fish treated as
Control		Nil	Fish without exposure to bacterial pathogens
Infectivity study		Immersion method	Fish exposed to live culture of bacterial pathogens [#]
Survival study	Phase 1 (Neutralization)	Immersion method and feeding method	Fish was treated with antigens and simultaneously fed with antibody-coated fish feed for 14 days*
	Phase 2 (Curing/therapeutic)	Immersion method and feeding method	Fish was treated with antigen for 2 days, then provided antibody-coated fish feed up to 14 th day*
	Phase 3 (vaccination/prophylactic)	Immersion method and feeding method	Fish fed with antibody-coated fish feed for 7 days and then treated with the live bacterial pathogen for 7 days*

[#]15×10⁶ CFU/mL; *3.5% of antibody/g of feed

5.2.5. Histopathology analysis

Euthanasia was carried out for surviving fish after the completion of the *in vivo* studies (Acts, Rules and Guidelines, 2020). For this procedure, fish

were transferred to separate 500 mL tanks, and euthanasia was performed rapidly and painlessly using 0.20 mL of clove oil (prepared from pure clove oil, 90-95% eugenol, as a 1:9 stock solution in 92.8% ethyl alcohol) (Anderson *et al.*, 1997). Individual fish that didn't show opercular pumping and pectoral fin beating were immediately transferred into another container in which water was constantly renewed for 30 minutes (Figure 5.1C). The tissues of fish that died during euthanasia or due to infection were fixed in 10% formaldehyde solution for the preservation and transported to histopathological analysis (Figure 5.1D and 5.1E).

The histopathology of skin, gills, small intestine and large intestine was studied by adopting the routine paraffin method (Hossain *et al.*, 2007). The smear was fixed with Bowins fluid composition and kept 24 hrs for the complete fixation of the smear. The smear was then washed with tap water to remove the picric acid of Bowins fluid composition. Then it was dehydrated with different concentrations of ethanol (30%, 50%, 70%, 90% and 100%). The smear was then cleaned with xylene for 5 to 10 minutes for 2 to 3 times. The smear was taken out from xylene and kept in molten paraffin wax for the impregnation of the tissue, at 58-60°C for 20 minutes. Thin and uniform sections of embedded smear with thickness 8 µm were obtained using rotary microtome (The Western Electric and Scientific Works (Weswox), model FAMT-2020A, India). Excess paraffin was slashed by trimming. The tissue was floated in water bath to detach the smear from the knife hair line brush for the flattening and spreading of the smear. Slides were coated with egg albumin and kept at room temperature. Paraffin was removed by washing with xylene for 2 to 3 times at an interval of 10 minutes. Then the smear was dehydrated with descending grades of alcohol, followed by flaming with Heamtoxyline for 15 minutes. The smear was rinsed with distilled water and stained with Eosin and dehydrated with ascending grades of alcohol. The smear was then

cleaned with xylene, two times at an interval of 15 minutes. The smear was covered with cover glass slide that makes the permanent slides. The outcomes of phase 1, 2 and 3 experiments were compared to the control (Group 1) and infectivity study group (Group 2) after being scrutinized under the microscope (Labomed-OPTIcx, India).

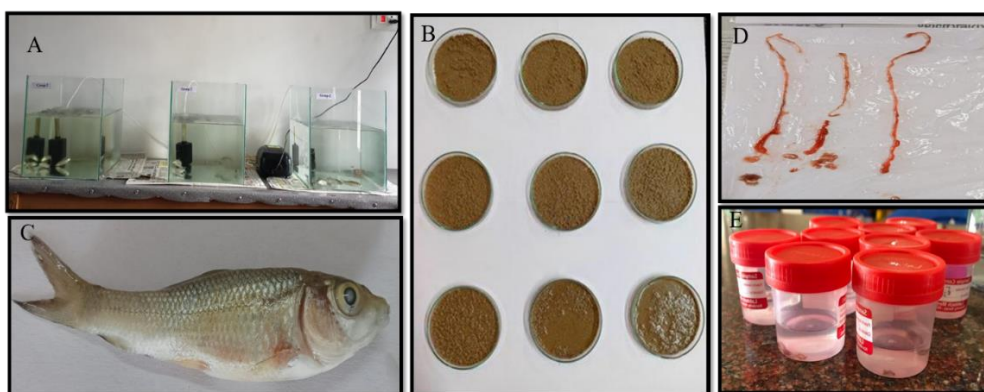


Figure 5.1. A- Experimental fish in aquarium, B- Fish feed embedding with IgY, C- Diseased fish, D- Separated tissues from fish, E- Tissues in 10% formaldehyde solution

5.2.6. Statistical analysis

The results were analyzed statistically using SPSS (Statistical Product and Service Solutions) version 28, manufactured by IBM (International Business Machines) Corporation, Armonk, New York, United States and the graphs were plotted using Origin Pro 8.1, developed by OriginLab Corporation, Northampton, Massachusetts, United States.

For LC_{50} analysis, Welch's ANOVA was performed. The Kaplan–Meier method and Chi-square test were used for evaluating the effect of IgY-coated feed on the survival of pathogen-treated fish. Additionally, the Chi-square test was applied for survival percentage analysis across various groups of fish and for histopathology comparison.

5.3. Results

5.3.1. *In-vitro* and *in-vivo* analysis

The LC_{50} of the pathogen *A. hydrophila* was 15×10^6 CFU/mL at 48 hours (Figure 5.2, Welch's ANOVA, $p < 0.05$, Error bars represent mean $\pm$ SD, $n = 4$). Furthermore, survival analysis revealed a clear dose-dependent protective effect, with 3.5% IgY-coated feed conferring approximately 50% survival by day 10, while higher doses (4.5% and 5%) provided even greater protection (Figure 5.3). Statistical analysis using the Kaplan–Meier method test confirmed that the differences among treatment groups were highly significant ($p < 0.01$), indicating that the observed effects were not due to random variation. Additionally, survival proportions at day 10 were compared using the Chi-square test ($p < 0.001$, $\chi^2 = 67.64$, $df = 6$), which also showed significant differences among groups.

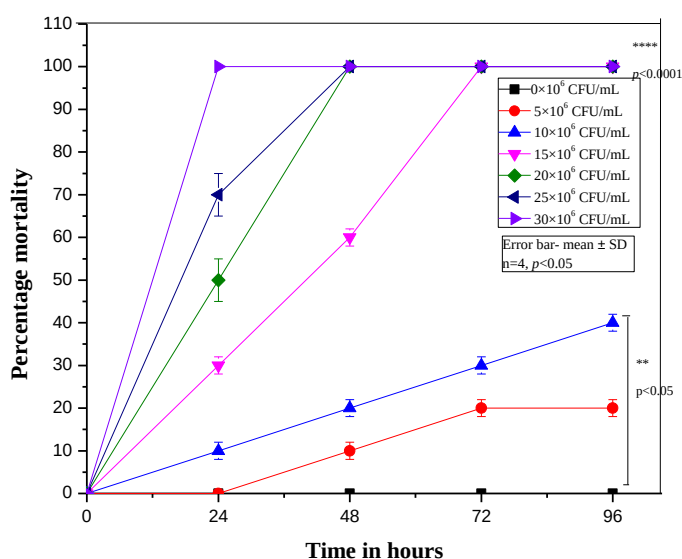


Figure 5.2. LC_{50} of *A. hydrophila* against fish *C. idella*

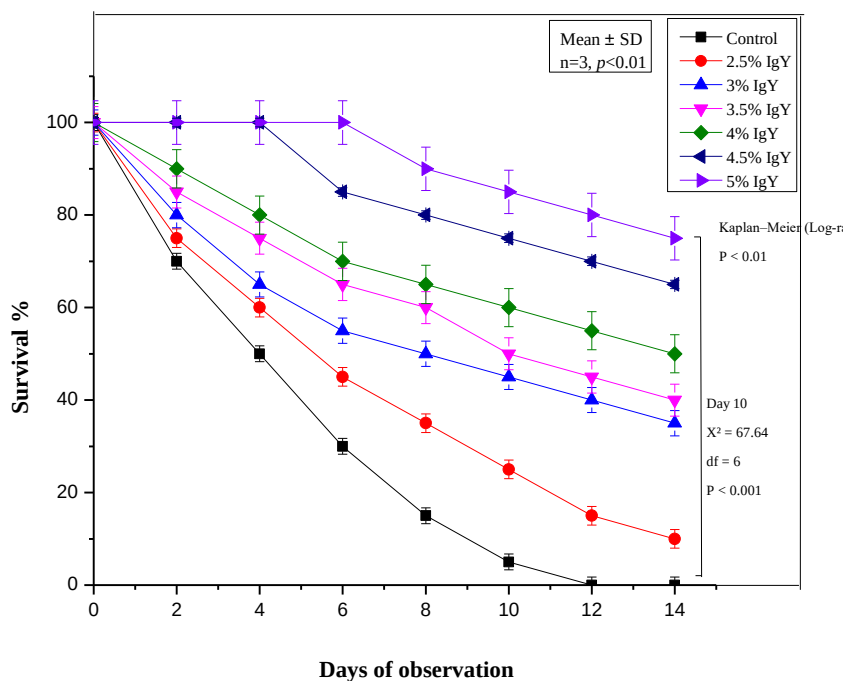


Figure 5.3. Effect of different concentrations of IgY-coated feed on the survival of pathogen-treated fish, *C. idella* post LC₅₀ challenge

Phase 1 of the survival study (neutralization study) showed excellent neutralization capacity of IgY antibodies, where 70% recovery was noted. In phase 2 (curing/ therapeutic study), the curing capacity of IgY antibodies was studied and a 60% survival rate was noted after 14 days of the experiment. In phase 3 (prophylactic study), IgY was used as a prophylactic agent and showed an 80% survival rate after 7 days of challenge with *A. hydrophila* pathogen. The Chi-Square test revealed significant differences in survival rates across the groups (Figure 5.4, $p < 0.05$, Error bars represent mean $\pm$ SD, $n = 4$), indicating the effectiveness of IgY treatment.

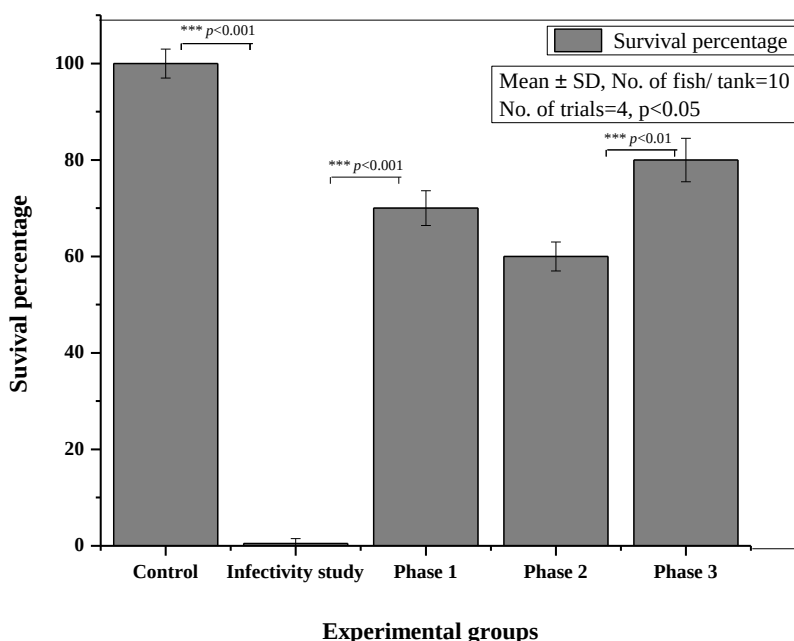


Figure 5.4: Survival outcomes of *C. idella* for 14 days with *A. hydrophila* under different IgY treatment strategies. Where, Phase 1- neutralization study, Phase 2- therapeutic, and Phase 3- prophylactic.

5.3.2. Histopathological analysis of fish specimens

The histopathological analysis of tissue samples from skin, gills, small intestine, and large intestine under control, infected, and IgY-treated conditions revealed notable differences in infection status (Chi-Square, $p < 0.05$, $n = 4$). In the control group, all samples remained non-infected, confirming the absence of pathogen exposure. In the infected group, all samples exhibited infection, verifying the pathogenic effect. The IgY treated group displayed a significant number of cured tissue samples across all types, indicating the efficacy of the IgY antibodies in treating the infection.

The uninfected fish in this study exhibit continuous, rigid skin features without any signs of infection (Figure 5.5A). However, the histopathology analysis of the infected fish revealed ruptured skin epithelium, oedematous with collagenous and inflammatory infiltrates (Figure 5.5B&C). Whereas the IgY-treated fish skin showed a structure similar to uninfected fish (Figure 5.5D).

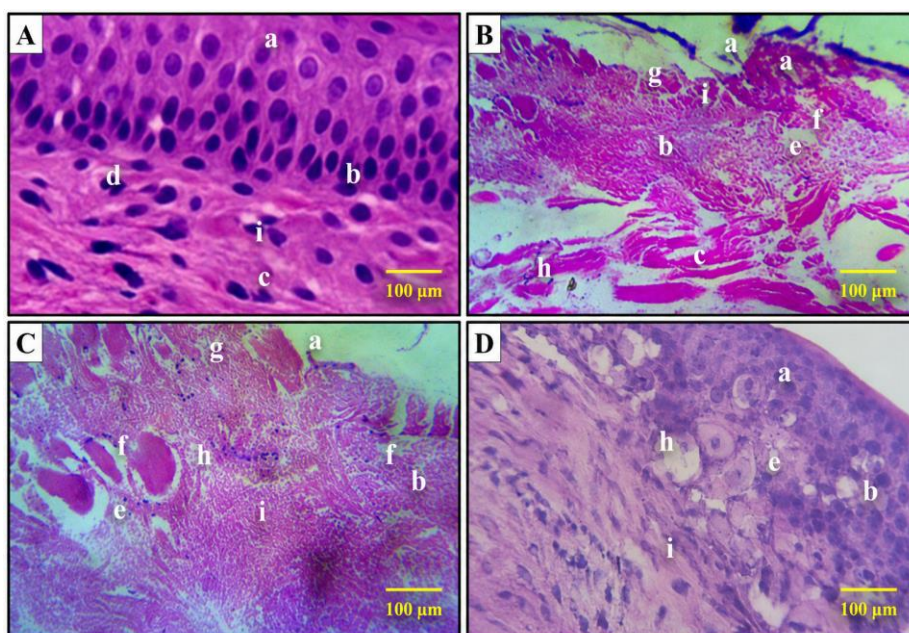


Figure 5.5. Histopathology of fish skin. A- control fish, B and C- diseased/dead fish D- IgY treated fish. a- Epidermis, b-Dermis, c-Scale, d-Mucus cells, e-Edema, f-Necrosis, g-Loss of architecture, h- Inflammatory infiltrate, i-Complete architecture, j- Hemorrhage. H&E stain, 8µm thickness

The fish from the control group had a normal rigid structure of gills (Figure 5.6A), while the infected gills showed necrosis, inflammation and complete loss of architecture (Figure 5.6B&C), whereas, the IgY-treated fish showed mild symptoms of infection (Figure 5.6D).

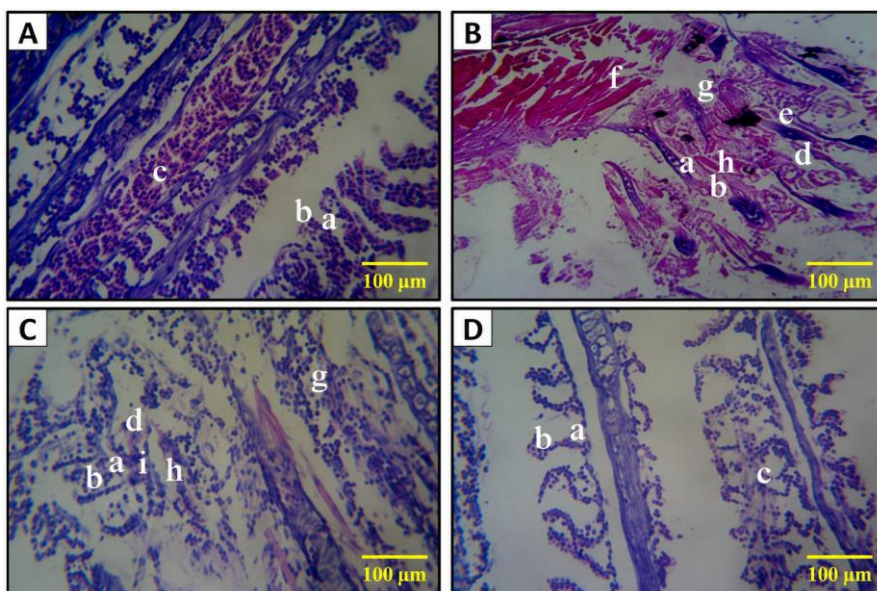


Figure 5.6. Histopathology of fish gills. A- control fish, B and C- diseased/ dead fish D- IgY treated fish. a- Primary lamellae, b-Secondary lamellae, c-Blood vessels, d-Edema, e-Necrosis, f-Haemorrhage, g-Inflammatory infiltrate, h-Disrupted epithelium, i-Sedimentateous lamellae. H&E stain, 8µm thickness

The small intestine of fish from the control group showed normal well-packed structures (Figure 5.7A). In contrast, the infected fish showed necrosis and gangrene in their small intestine (Figure 5.7B & C), and the IgY-treated fish showed a normal small intestine with lamina propria showing mild inflammatory infiltrates (Figure 5.7D).

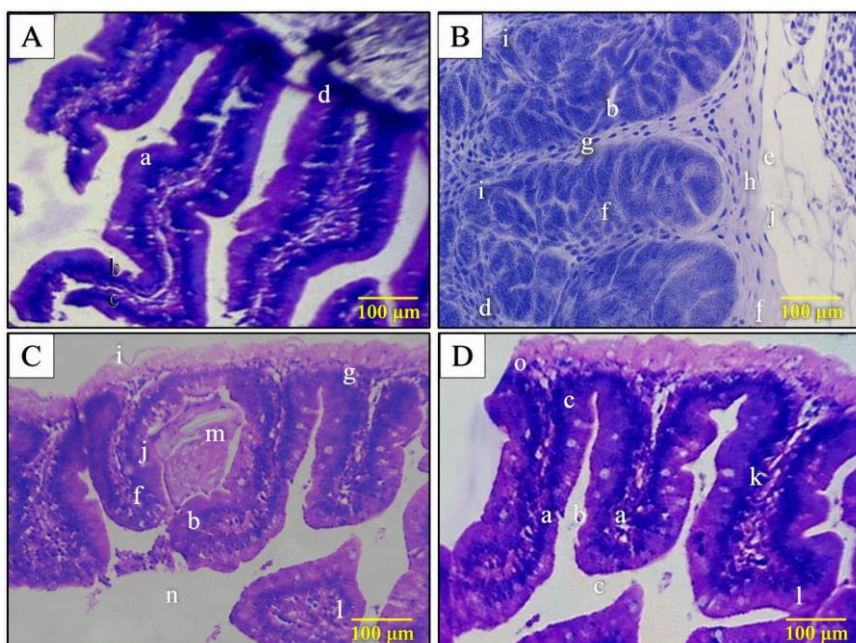


Figure 5.7. Histopathology of fish small intestine. A- control fish, B and C- diseased/ dead fish D- IgY treated fish. The proximal small intestinal section shows: a-Villi, b-Epithelium, c-Lamina propria, d-Crypts (crypts of Lieberkühn), e-Lumen, f-Distorted, blunted villi, g-Edema, h-Inflammatory infiltrate, i-Lamina propria expanded with inflammatory cells, j-Disrupted epithelium, k-Vacuole, l-Necrosis, m-Crypt hyperplasia, n-Distorted intestinal crypts, o-Inflammatory infiltrate. H&E stain, 8µm thickness

The normal untreated fish from the control group showed a fine structure of the large intestine (Figure 5.8A), while the pathogen-treated fish showed necrosis with inflammation and ulceration in the epithelial cells (Figures 5.8B&C). Whereas, the IgY-treated fish showed mild inflammatory infiltrates only (Figure 5.8D).

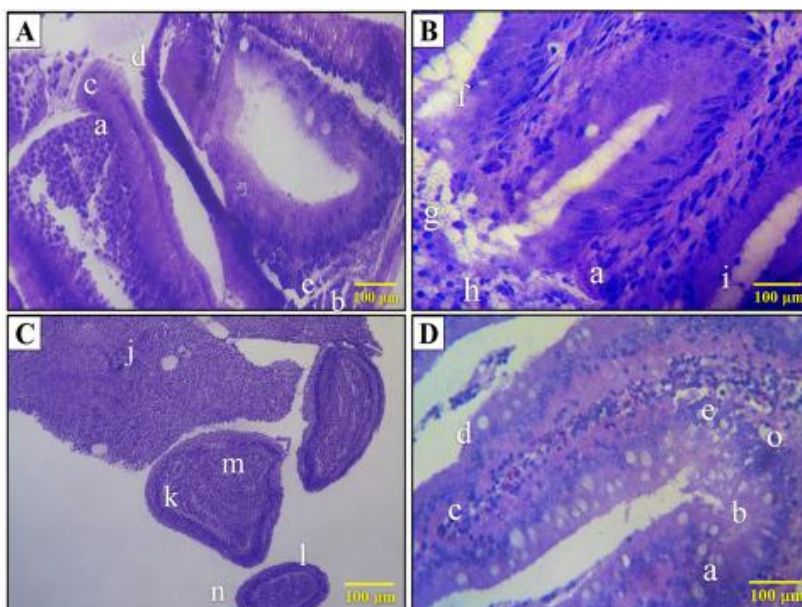


Figure 5.8. Histopathology of fish large intestine. A- control fish, B and C- diseased/ dead fish D- IgY treated fish. The distal large intestinal sections shows: a-Crypts of Lieberkühn, b-Mucosa, c-Goblet cells/mucus, d-Surface of epithelium, e-Lamina propria, f-Necrosis, g-Aggregate, h-Inflammation, i-Gas gangrene, j-Ulceration, k-Inflammatory cell infiltration, l-Vascular congestion, m-Mucosal erosion, n-Submucosal edema, o-Scattered inflammatory infiltrate. H&E stain, 8µm thickness

5.4. Discussion

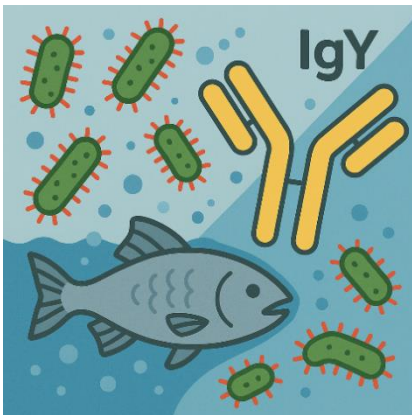
Our previous chapters underscore the effectiveness of IgY antibodies in inhibiting *A. hydrophila* growth, with significant inhibition observed at dilutions up to 10^{-4} . This highlights IgY's potential in preventing bacterial colonization in aquaculture settings. Well diffusion assays confirmed IgY's inhibitory properties, and cross-reactivity studies revealed broad-spectrum activity against related pathogens such as *A. caviae*. High titers from indirect ELISA emphasised IgY's specificity. Combining IgY with antibiotics like chloramphenicol and ciprofloxacin demonstrated synergistic effects, reducing antibiotic use and promoting eco-friendly practices. IgY antibodies exhibited stability under various environmental conditions, increasing its demand for aquaculture.

The studies of this chapter provide insights into the multifaceted role of IgY antibodies in aquaculture for disease management. LC₅₀ of *A. hydrophila* (15×10^6 CFU/mL) provides a critical threshold for assessing pathogen virulence. Inhibition of bacterial growth by IgY antibodies at optimal dosage is efficient. Hen *et al.* (2021) compared the LC₅₀ of various fish pathogens, and assessing lethal concentrations remains central for intended intervention.

Phase 1 demonstrated that IgY antibodies have a great potential to neutralize pathogens, achieving a 70% test animal recovery level. The bright prospect of such a finding may owe its success to the innate benefits of these bird-source antibodies, such as the evolutionary gap between birds and mammals, the distinctiveness of birds' immunity, and the effective deposition of IgY in egg yolks. We have information that IgY antibodies have a remarkably favorable capability of adhering and neutralizing infectious pathogens (Zorriehzah *et al.*, 2016; Narat, 2003). In Phase 2, the potential of the IgY antibodies to cure infection was considered. We achieved a 60% survival level at 14 days, and such results reveal that the antibodies manifest prolonged defence- a critical component for successful disease control. Our findings align with those of other researchers, for instance, *Carassius auratus Gibelio*'s therapeutic defense against *A. hydrophila* by Li *et al.* (2006) and applying IgY to treat streptococcal infections by Rizkiantino *et al.* (2023). Phase 3 of our study, utilized IgY as a preventative treatment. The outcomes were very promising, as seventy percent of the fish survived, after being exposed to *A. hydrophila*, seven days later. Other studies, including the studies by Rizkiantino *et al.* (2023) and Gao *et al.* (2016), support our findings. They showed that white shrimp fed food with certain IgY antibodies gave good protection against infections with *V. harveyi* and *V. parahaemolyticus* resulting in reduced mortality and a reduced bacteria count within the shrimp.

Although various biochemical works have investigated the impact of disease on fish, histopathology offers more valid and reliable data by demonstrating cellular destruction. We have paid some attention in this chapter to applying histopathology in investigating the influence of various experimental conditions on the various organs of *C. idella*. Our *in vivo* experiments identified that the administration of *A. hydrophila* orally with IgY antibodies into the organism is effective in both curing and prophylaxis of *A. hydrophila* tissue loss. Histopathological analysis of the fish confirmed this and revealed that there was a great reduction in the damage, inflammation, necrosis and cellular disruption in the groups which were treated with IgY. Other studies concur with our findings. In a case study, Xiao-Liang *et al.* (2006) demonstrated that goldfish injected with certain IgY antibodies were immunized against infection by *A. hydrophila*. Moreover, the application of IgY, its benefits, and the mechanism of action in aquatic animals were thoroughly reviewed by Paul *et al.* (2019), focusing on its use in the prevention of infectious disease. Likewise, Zorriehzahra *et al.* (2016) have emphasized the possibility of using IgY antibodies as an alternative to treating fish for infectious diseases, such as those induced by *A. hydrophila*, and it is non-toxic and relatively inexpensive.

IgY antibodies have played a crucial role in aquaculture and have offered global protection against pathogens in addition to therapeutic and preventive features. The results of our work, which examined the histopathological modifications in fish receiving IgY under the condition of bacterial infections, contributed to a better understanding of how tissues are guarded by these antibodies. Future studies could examine ways in which the use of IgY in conjunction with other immunotherapies may be especially more effective. Our study, in brief, makes a great contribution towards the relationship issues of IgY in fish farming. These results will provide a way forward in the pursuit of a more sustainable and robust aquaculture process as we continue to battle bacterial infections.



Chapter -6

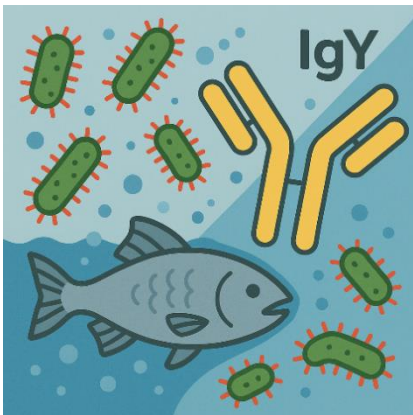
Summary and Conclusion

The present study focused on the production of IgY antibodies against the fish pathogen, *A. hydrophila* and evaluated their efficacy through both *in vitro* and *in vivo*. The first experiment focused on the production of IgY, its extraction, purification, specificity analysis, ELISA titration, and agglutination tests to ensure IgY antibodies that are specifically directed against *A. hydrophila*. The extracted and purified IgY was further tested against *A. hydrophila in vitro* through growth inhibition assays, cross-reactivity studies, synergism analysis, and stability under various environmental conditions. The study additionally conducted the infectivity pattern of *A. hydrophila* infection in *C. idella*, and the effectiveness of IgY-coated fish feed was evaluated for its neutralization capacity. The summary of the findings is provided below.

- IgY antibodies against specific pathogen *A. hydrophila* and *A. caviae* were produced, extracted and purified.
- Vigorous clumping in agglutination assays confirmed IgY specificity.
- High IgY yields were eluted with 95% purity using DEAE ion exchange chromatography.
- IgY titer reached a dilution of 1:10,000, with specificity confirmed *via* ELISA.
- Average IgY yield of 11.88 mg/mL obtained for *A. hydrophila* with specific IgY content of 26% and average IgY yield of 5.2 mg/mL obtained for *A. caviae*.
- Growth inhibition observed up to a 10^{-4} dilution; well diffusion assays confirmed IgY's effectiveness.

- Cross-reactivity demonstrated between IgY for *A. hydrophila* and *A. caviae*, validated through agglutination, ELISA methods and well diffusion methods. Where the visible agglutinates in every agglutination study, significant ELISA results and clear zones around the wells of IgY in respective studies in both directions confirmed the cross-reactivity properties of both IgY.
- Synergistic effects of IgY with antibiotics (ciprofloxacin and chloramphenicol) were significant, reducing required individual dosages.
- Stability of IgY confirmed across pH 3–10, temperatures up to 70°C, molarity up to 30mM and in solvents like ethanol, methanol and isopropanol (up to 30%).
- LC₅₀ of the pathogen at 15×10⁶ CFU/mL at 48 hours; ED₅₀ IgY concentration at 3.5%.
- IgY-coated feed significantly improved fish survival rates under *A. hydrophila* exposure:
 - Neutralization: 70% recovery in 14-day study.
 - Therapeutic use: 60% survival rate after 14 days.
 - Prophylactic use: 80% survival rate after 7 days.
- Histopathological analysis showed reduced tissue damage in IgY-treated fish, proving its efficacy in curing and preventing infections.
- IgY antibodies are sustainable preventive measures of bacterial infections in fish.
- This specificity of IgY antibodies reduces the risk of off-target effects and makes them a safe treatment option for fish.

- IgY antibodies are also orally administered, thus conveniently allowing fish farmers to use them. One can add these antibodies to the fish feed, which is then fed to the fish.
- Controlling the emergence of antibiotic resistance is one of the major advantages of using IgY antibodies in fish farming. By reducing the reliance on antibiotics, IgY-based immunotherapy not only improves fish health but also helps preserve the effectiveness of antibiotics in human medicine.
- The issue with the economic benefits of using IgY: In this field, we must consider the high production costs of IgY and the long-term disadvantage of using antibiotics.
- One of the enormous benefits of IgY production is that it is both humane and sustainable. The chickens involved can lead normal lives after the experiments, and their eggs are not harmful to people.
- Last but not least, the antibodies themselves are highly stable and dependable. IgY antibodies are well-behaved in a wide range of environmental areas, such as pH, temperatures, and solvents, which is why they are a valuable and reliable treatment method in aquaculture and other applications.

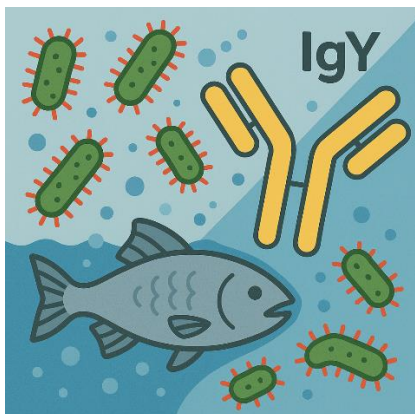


Chapter -7

Recommendations

Based on the results and coverage of the current research study, the subsequent recommendations can be put forward to improve future research and implementations in this sphere of aquaculture.

- Extend studies to include the efficacy of IgY antibodies against different fish pathogens in marine and freshwater aquaculture.
- Investigate the synergistic effects of IgY antibodies in combination with other therapeutic agents, like probiotics or alternative natural remedies, for enhanced disease management.
- Research innovative delivery mechanisms, such as microencapsulation of IgY antibodies, to improve stability, efficacy, and uptake in fish feed.
- Evaluate the economic feasibility and scalability of IgY usage in large-scale fish farming operations, comparing its cost-effectiveness with existing treatments.
- Develop a roadmap for integrating IgY-based therapies into aquaculture industry standards and regulatory frameworks to ensure widespread adoption.



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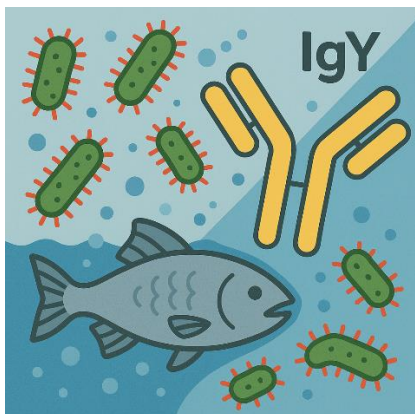
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Appendix

Articles published

- Thushara James, Mujeeb Rahiman K.M, Denoj Sebastian, Suja Rani S, Dinesh M.D. (2025). The combinational effect of avian IgY antibodies and broad-spectrum antibiotics (ciprofloxacin and chloramphenicol) *in vitro* reduces the incidence of *Aeromonas hydrophila*. *Journal of Applied Aquaculture* 1-16. <https://doi.org/10.1080/10454438.2025.2478842>
- Thushara James, Mujeeb Rahiman K.M, Denoj Sebastian, Suja Rani S, Dinesh M.D. (2023). Growth inhibitory properties of specific anti-*A. hydrophila* IgY antibodies: A promising approach for the prevention of *Aeromonas* infection in farmed fish. *Indian Hydrobiology*. 22(2): 211-220. [https://indianhydrobiology.yolasite.com/resources/IH_22/IH%2022\(2\)%20Abstracts.docx](https://indianhydrobiology.yolasite.com/resources/IH_22/IH%2022(2)%20Abstracts.docx)
- Thushara James, Mujeeb Rahiman K.M, Denoj Sebastian, Suja Rani S, Dinesh M.D. (2026). *In-vitro* evaluation of IgY Antibodies against *Aeromonas caviae* and Cross-reactivity with *Aeromonas hydrophila*. *The Microbe*. 1-6. <https://doi.org/10.1016/j.microb.2025.100652>

Book chapter published

- Thushara James, Mujeeb Rahiman K.M, Denoj Sebastian, Suja Rani S, Dinesh M.D (2024). Importance, Production and Use of Egg Yolk Antibody (IgY) in Aquaculture. *Reproductive Physiology and Aquaculture*. 145-167.



The combinational effect of avian IgY antibodies and broad-spectrum antibiotics (ciprofloxacin and chloramphenicol) *in vitro* reduces the incidence of *Aeromonas hydrophila*

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ABSTRACT

The frequent use of antibiotics in aquaculture to control bacterial diseases has led to the accumulation of antibiotic residues in the environment and biomagnification across various food chain trophic levels. This study examines the sustainable application of IgY antibodies to manage *Aeromonas hydrophila* infections. Formaldehyde-killed *A. hydrophila* were intramuscularly immunized in 22- to 24-week-old White Leghorn (*Gallus gallus domesticus*) chickens for the production of IgY antibodies. The purified egg yolk antibodies were analyzed, and a concentration of 11.88 mg/ml was found, with a specific protein concentration of 24%. High titers were observed using indirect enzyme-linked immunosorbent assay (ELISA) at dilutions greater than 1:10,000, demonstrating the efficacy of the IgY antibodies. The results confirmed that IgY remains stable under various conditions, including pH values ranging from 3 to 10, temperatures from freezing up to 70°C, molarity up to 50 mM using phosphate buffered saline, and solvent systems up to 30%. Using the serial dilution method, the minimum inhibitory concentrations (MICs) of IgY, ciprofloxacin, and chloramphenicol were 12.5 µg/ml, 0.77 µg/ml, and 3.1 µg/ml, respectively. The checkerboard method demonstrated synergistic effects between IgY and the antibiotics, with fractional inhibitory concentration (FIC) indices of 0.24 for ciprofloxacin and 0.37 for chloramphenicol. Combining IgY antibodies with these antibiotics shows synergism, significantly reducing the excessive use of antibiotics in fish farms and offering a sustainable solution to bacterial infections in aquaculture.

KEYWORDS

Aquaculture; *A. hydrophila*; IgY; stability of IgY; MIC; synergism

Introduction

Fish are vulnerable to various diseases, significantly impacting mortality, especially among juveniles. *Streptococcus* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Vibrio* spp. are primary bacterial infections in aquaculture (Nasr-Eldahan et al. 2021). *Aeromonas hydrophila*, a Gram-negative bacterium, is known to cause motile *Aeromonas* septicemia (MAS) in fish and is prevalent in bottled water, chlorinated water, well water, and polluted water systems used in aquaculture (Plumb and Hanson 2010; Nayak 2020). The genetic, metabolic, and antigenic variability of members of this group has caused difficulty in determining the etiology of disorders involving aeromonad infections (Sudheesh et al. 2012). This pathogen is a significant concern due to its severe impact on farmed and wild fish populations. Antibiotics like ciprofloxacin and chloramphenicol are broad-spectrum antibiotics effective against a wide range of bacterial infections, making them valuable when other antibiotics fail (Ahmed et al. 2020). Even though antibiotics are often the only treatment option for certain bacterial diseases in aquaculture (Okeke et al. 2022), the indiscriminate use of antibiotics for disease prevention, treatment, control, and growth enhancement (Pepi and Focardi 2021) leads to antimicrobial resistance (AMR), posing significant threats to human health and the environment over time (Preena et al. 2020; Yuan et al. 2023).

Despite regulations and controls on antibiotic use, their application continues in aquaculture, resulting in the rejection of consignment due to antibiotic residues from major aquaculture producing countries (FAO 2024). In India, although there is a banned list of antibiotics, the absence of an allowed list creates further controversy (Coastal Aquaculture Authority CAA 2022). However, major developed countries have strict regulations to use antibiotics in aquaculture and livestock (Aidara-Kane et al. 2018; Casewell 2003; European Commission 2005; Food and Drug Administration FDA 2024; WHO 2021). The urgent need for alternative methods to reduce dependence on these drugs (Luthman et al. 2024) is essential for sustainable fish production.

IgY antibodies, derived from egg-laying chickens, offer a promising alternative to antibiotics due to their stability, cost-effectiveness, and high titration values (Lee et al. 2021). The global production of IgY antibodies is substantial, with the market size reaching USD 7 million in 2021 and projected to touch USD 20.79 million by 2031 (Anonymous 2023). The detection of microorganisms, the identification of causal proteins, and food preservation are the other uses of IgY. The antibacterial nature of IgY antibodies was tested in a variety of aquatic animals, including Pacific white shrimp (*Litopenaeus vannamei*) (Keetanon and Chuchird 2020), Japanese pufferfish (*Takifugu rubripes*) (Zhang et al. 2021a), Nile tilapia (*Oreochromis niloticus*) (Fernandes et al. 2019), Koi carp (*Cyprinus carpio*) (Gan et al. 2015) and Chinese mitten crab

(*Eriocheir sinensis*) (Guo et al. 2023), and found that they had a stable and therapeutic action that was beneficial to aquatic health. IgY-based vaccines, oral supplements, and immunostimulants have proven efficacy against various microbes. The unique genetic makeup of IgY enables it to neutralize a diverse array of antigens (Warr, Magor, and Higgins 1996). IgY can specifically bind to antigens, including pathogenic microorganisms, toxins, and other foreign substances. Through agglutination, precipitation, opsonization, and blocking of antigen binding sites, IgY effectively neutralizes these antigens, preventing their interaction with host cells. The variable domain of IgY plays a key role in this neutralizing ability, allowing it to interact with and neutralize a broad range of targets (Zhang et al. 2017).

This study focuses on developing avian IgY antibodies against *A. hydrophila* and evaluating their neutralization efficacy *in vitro*. Taheri-Araghi (2024) reported that rotating or combining antimicrobials can reduce selective pressure on bacteria, slow the development of resistance, and preserve the effectiveness of current antibiotics. The stability and effectiveness of IgY antibodies, particularly in combination with antibiotics, remain largely unexplored. To address these gaps, we performed stability studies on IgY antibodies under various conditions, emphasizing the importance of proper storage to increase shelf life. By developing more effective treatment strategies against *A. hydrophila* infections, this study aims to reduce dependence on traditional antibiotics and mitigate the risk of antimicrobial resistance.

Materials and methods

Source of poultry and *A. hydrophila*

The single-comb white leghorn (*Gallus gallus domesticus*) egg-laying chickens (22 to 24 weeks old) were collected from a small animal breeding station, according to the guidelines of the Institutional Animal Ethical Committee, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India. The fish pathogen *A. hydrophila* (MTCC – 1739) strains were obtained in lyophilized form from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. Strains of *A. hydrophila* were brought to pure culture by transferring lyophilized forms into double strength nutrient broth and incubated at 37°C for 24 h. The organisms were sub-cultured on Nutrient agar plates (MTCC Media-3).

Production of IgY antibodies in chicken against *A. hydrophila*

For the production of IgY antibodies against *A. hydrophila*, the bacterial cells were killed using 3% formaldehyde (vol/vol) and kept overnight at 4°C. After confirming the sterility, formaldehyde-killed *A. hydrophila* (1.5×10^8 to $24 \times$

10^8 cells) was administered intramuscularly in booster doses of immunization at 7-day intervals *via* the same route of administration (Horton et al. 1985; Schade and Hlinak 1996). The IgY antibody production in the chicken was confirmed by collecting the blood from their wing veins and testing the presence of specific antibodies with the serum using the microscopic slide agglutination (Narat 2003).

Extraction and purification of IgY from the eggs

The IgY antibodies for the study were collected from the eggs after the first week of immunization. The collected eggs were sterilized with ethanol, labeled, and stored at 4°C for further studies. The eggs were carefully cracked, and the yolk was separated from the white and washed with distilled water to remove any excess albumin. The yolk was rolled over to tissue paper to remove any remaining egg white, and the yolk sac was pierced and transferred into a graduated cylinder. The yolk was then mixed with an equal volume of buffer S (10 mM phosphate, 100 mM NaCl, and 0.01% sodium azide, pH 7.5). The mixture was treated with polyethylene glycol (PEG) at different concentrations (3.5% and 12%) with proper stirring and centrifugation (11000 rpm for 20 min). After centrifugation, the pellet was collected and made up into the initial yolk volume in buffer S. The mixture was submerged with 4 M ammonium sulfate solutions and stored overnight at 4°C to precipitate IgY. After incubation, the precipitant obtained from the pellet *via* centrifugation was re-suspended with buffer S without NaCl (Polson, von Wechmar, and van Regenmortel 1980). Following ammonium sulfate precipitation, the precipitated IgY was treated with dialysis to eliminate the salt content. After dialysis, the contents of the bags were dispensed into microcentrifuge tubes and centrifuged to remove any remaining contaminants. Diethylaminoethyl Cellulose Ion Exchange Column Chromatography was employed to further purify the IgY, attached to the matrix by passing 250 mM phosphate buffer and eluting by passing 25 mM phosphate buffer. The Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis technique was used to test the purity of the IgY (Laemmli 1970). The total protein concentration of the purified IgY was calculated calorimetrically, and the absorbance was measured against a blank (0.1% BSA) at 750 nm (Lowry et al. 1951).

Specific IgY (anti *A. hydrophila* IgY) analysis

The specific IgY concentration was calculated from the purified IgY by mixing 1 ml of a 0.01% *A. hydrophila* antigen solution from 5×10^5 CFU/ml broth in phosphate-buffered saline (PBS) with 0.5 ml of a 1 mg/ml purified IgY solution (Lee et al. 2002). The purpose of this step is to allow the antigen and IgY to interact and form immune complexes. The mixture was then incubated at

37°C overnight to facilitate the binding between the antigen and IgY. During the incubation, the immune complexes formed due to the specific interaction between the antigen and IgY. After the overnight incubation, the mixture was separated using centrifugation at 3,000 rpm for 30 min, and the absorbance of the supernatant was assessed at 280 nm. The absorbance of a control sample without antigen was also measured to calculate the percentage of specific IgY using the formula:

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

Detection of IgY antibodies from egg yolk by microscopic slide agglutination assay

Purified IgY was serially diluted up to 10^{-5} with sterile phosphate-buffered saline in Eppendorf tubes. Fifty microliters of IgY antibodies were added to the first tube and mixed well, then 50 µl was transferred sequentially from the first tube to the second tube, from the second to the third, and so on. From each dilution, 50 µl of IgY antibodies were taken and applied to microscopic slides containing the same concentration of bacterial antigens. Antigens with ciprofloxacin served as the positive control, while antigens with nonspecific IgY from unvaccinated eggs served as the negative control. The slides were thoroughly mixed and left for a few moments until agglutinates appeared (Narat 2003).

Determination of antibody titer by indirect ELISA

The titration of specific IgY antibodies was quantified by a standard titration method using indirect ELISA (Voller et al. 1976). The experiments were done one after the other in triplicates. A 96-well polystyrene microplate with a flat-bottom sterile ELISA plate was initially coated with formaldehyde-killed antigens of *A. hydrophila* using a coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Then, the plates were washed to remove the unbound antigens from the wells, and empty sites were filled with 1% BSA (200 µl/well) and incubated at 37°C for an hour. Following three more PBST washes, wells were coated with primary antibodies (100 µl/well of serially diluted anti-*A. hydrophila* IgY antibodies). After washing the wells, 100 µl of diluted (1:1000) rabbit anti-chicken immunoglobulin coupled to horseradish peroxidase (Genei Pvt Ltd, Bangalore) was added as secondary antibodies, and the plates were incubated at 4°C for an hour. After further washing, the wells were filled with 100 µl of freshly prepared TMB/H₂O₂ solution (Genei Pvt Ltd, Bangalore) and kept dark for 20 min. The plates were then read at 490 nm

using an ELISA reader (Thermo Scientific, Multiskan FC; Software: SkanIt 6.1.1) after adding 50 µl of 4 N sulfuric acid to each well to terminate the reaction.

Combinational effect of IgY with chloramphenicol and ciprofloxacin against A. hydrophila

The Minimum Inhibitory Concentration (MIC) tests (Clinical and Laboratory Standards Institute CLSI 2024) were conducted using a U-bottom 96-well microtiter plate to determine the combinational effect of two broad-spectrum antibiotics, chloramphenicol and ciprofloxacin, and the specific IgY antibodies. Initially, three rows of the plate were filled with 100 µl of Muller-Hinton broth. A 200 µl aliquot of the test sample, either antibiotic or IgY, from a 1 mg/ml stock solution was added to each row's first well. Then, 100 µl of the test sample was serially diluted from the first well to the tenth well. The 11th column served as the positive control, while the 12th column served as the negative control. Each well was then added with 100 µl of an inoculum containing 5×10^5 CFU/ml, covered with a lid, and incubated for 12 h at 37°C. Triplicates of each plate were examined either directly or using an ELISA reader.

The concentration of the test sample with no observed growth was taken as the MIC for each test sample. The Fractional Inhibitory Concentration Index (FICI or Σ FIC) was determined using the checkerboard method, with concentrations ranging from 1/16 to 8 times the MIC of both IgY and antibiotics (Jain et al. 2011). A pharmacological combination is synergistic if Σ FIC is less than or equal to 0.5. If the Σ FIC ranges from 0.5 to 4, the combination is considered indifferent or additive; if Σ FIC is ≥ 4 , it is considered antagonistic (Odds 2003).

$$\sum FIC = FIC_A + FIC_B$$

Where, FIC_A = MIC of drug A in combination/MIC of drug A alone

And, FIC_B = MIC of drug B in combination/MIC of drug B alone

IgY interaction and its stability

Approximately 50 µl of specific IgY were incubated with 950 µl of PBS and subsequently tested for their binding activity against the antigen under various conditions using the indirect ELISA method (Voller et al. 1976). These conditions included a range of pH values from 2 to 12, temperatures from −20°C to 90°C, different molarities with PBS as the solvent ranging from 0 mM to 100 mM, and various solvent systems of ethanol, methanol, and isopropanol ranging from 20% to 100% vol/vol.

Data analysis

The results were analyzed statistically using SPSS (Statistical Product and Service Solutions) version 28, manufactured by IBM (International Business Machines) Corporation, Armonk, New York, United States, and the graphs were plotted using Origin Pro 8.1, developed by OriginLab Corporation, Northampton, Massachusetts, United States. The Shapiro–Wilk test was used to verify the normality of the data, confirming that all datasets were normally distributed ($p > .05$). Levene's test assessed the equality of variances, confirming that the assumption of homogeneity of variances was met ($p > .05$).

Results

Detection of IgY antibodies from the immunised chicken

The production of IgY in the immunized chicken was estimated by the concentration of proteins in the extracted IgY by ammonium sulfate precipitation, dialysis, and DEAE cellulose ion exchange column chromatography. The antibody concentration in the egg yolk increased with booster doses and stabilized after an exponential phase (Figure 1). SDS-PAGE confirmed the purity of developed IgY antibodies by showing a single band, indicating they were pure and close in size to the 180 kDa standard molecular marker (Genei, Bangalore). Column-purified IgY fractions had an average protein concentration of 11.88 mg/ml, with specific IgY determined as 24% ($p < .01$).

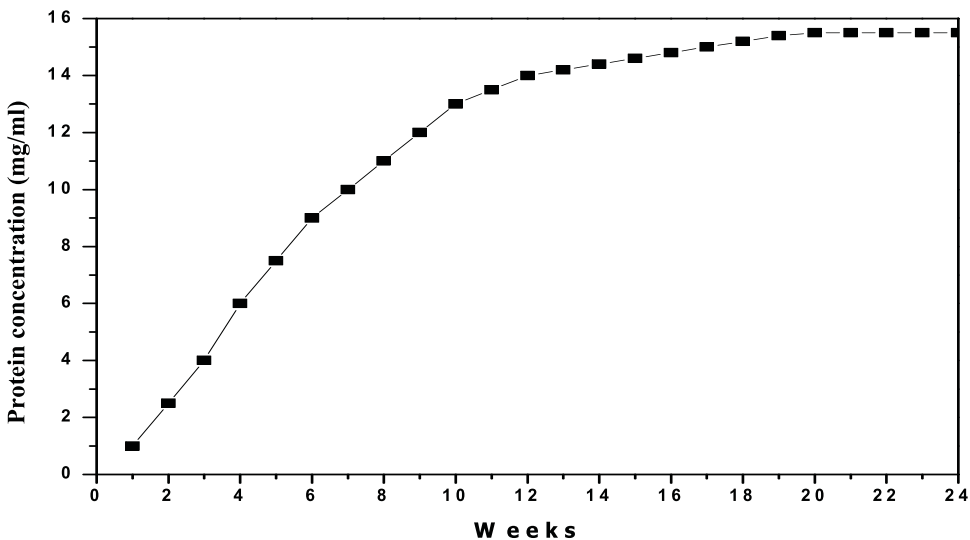


Figure 1. Protein concentration in egg yolk IgY.

Effect of antibody against *A. hydrophila*

Vigorous clumping observed in the microscopic slide agglutination test confirmed the presence of antibodies against *A. hydrophila* antigens (Figure 2). The result showed that the agglutination was observed up to 1:100,000 dilutions. A high titer value of greater than 1:10,000 dilutions (t-test, $p < .05$) in the ELISA result further confirmed the specificity of the antibody produced against *A. hydrophila* (Figure 3).

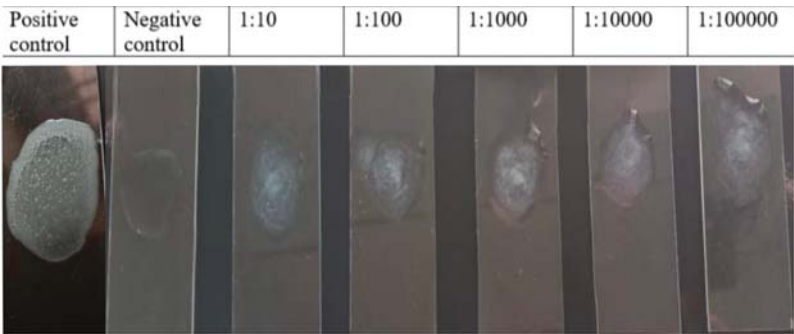


Figure 2. Agglutination assay test.

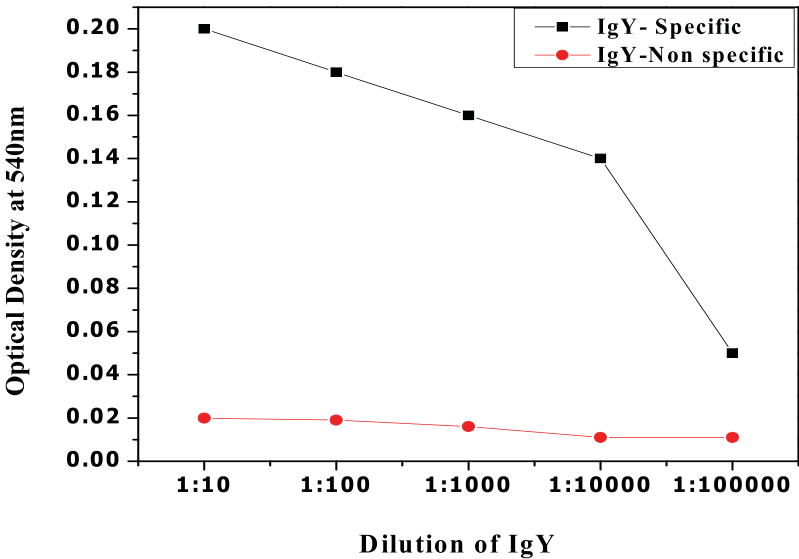


Figure 3. Analysis of antigen-antibody reaction by ELISA.

Table 1. The combinational effect of IgY with ciprofloxacin and chloramphenicol.

Component	MIC against <i>A. hydrophila</i> (µg/ml)				Combinational effect
	Individual component	IgY in combination	Antibiotic in combination	ΣFIC	
IgY	12.5				
Ciprofloxacin	0.77	1.56	0.09	0.24	Synergistic
Chloramphenicol	3.1	3.12	0.38	0.37	Synergistic

Combinational effect of IgY and antibiotics

The MIC of IgY, ciprofloxacin, and chloramphenicol individually against *A. hydrophila* was found to be 12.5 µg/ml, 0.77 µg/ml, and 3.1 µg/ml, respectively (Table 1). The result of the ΣFIC of drug combinations showed synergistic interactions between IgY and antibiotics ($p < .01$).

Stability of IgY at various parameters

IgY demonstrated better stability and interaction across a wide pH range, temperature, and molarity. The stability of IgY was maintained within the range of pH 3 to pH 10 ($p < .05$) (Figure 4(a)). It showed good interaction up to

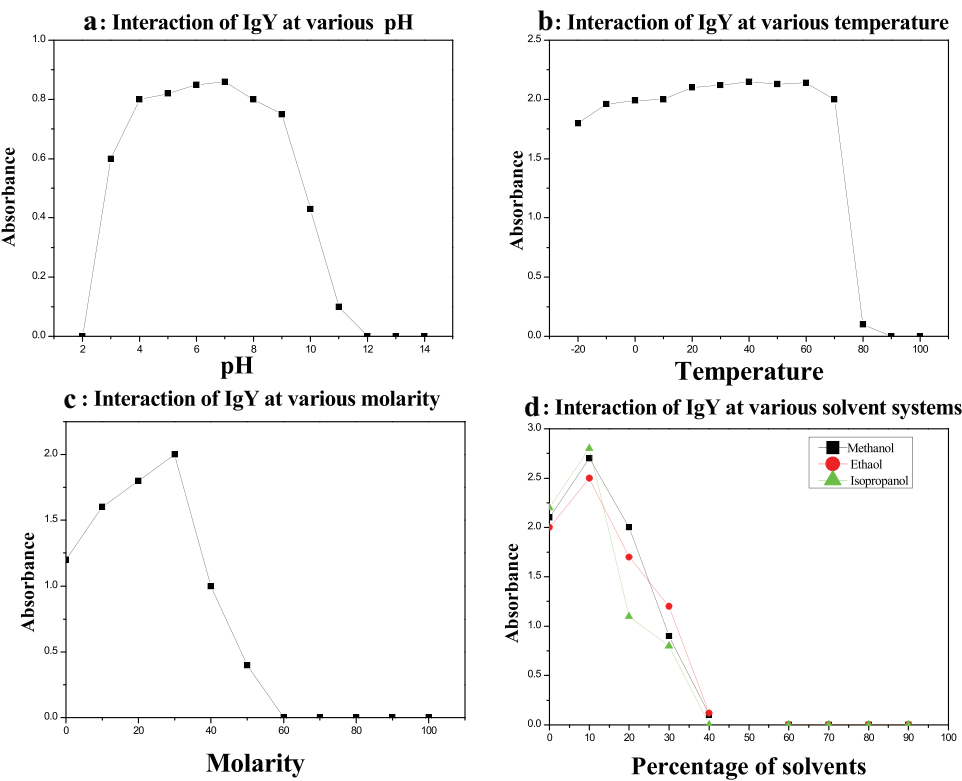


Figure 4. Interaction of IgY on various parameters.

70°C, beyond which it became inactivated ($p < .01$) (Figure 4(b)). Effective interaction of IgY was observed when tested up to 50 mM molarities using phosphate-buffered saline (PBS) ($p < .05$) (Figure 4(c)). The antigen–antibody interaction demonstrated optimal performance around 30 mM molarity. The antigen–antibody interaction was unaffected by solvent concentrations of ethanol, methanol, and isopropanol up to 30% ($p < .01$) (Figure 4(d)) supporting the stability of IgY in these solvents within the given concentration range.

Discussion

The present study focused on developing avian IgY antibodies against *A. hydrophila* and evaluating their neutralization efficacy, aiming to treat diseases caused by *A. hydrophila* in aquaculture systems potentially. Our study successfully demonstrated the production and purification of *A. hydrophila* from the egg yolk of immunized chicken. The result of the IgY production achieved a total protein concentration of 11.88 mg/ml, with 24% specific IgY, using a dual-step purification method involving polyethylene glycol (PEG) and ammonium sulfate. These findings align with previous studies demonstrating successful multistep purification of IgY from egg yolk (Ge et al. 2020). The dual-step method, which includes two rounds of centrifugation with varying PEG concentrations, produced a highly pure form of IgY with superior antibody titration compared to other purification systems (Morgan et al. 2021).

The slide agglutination and ELISA tests confirmed the activity of specific IgY antibodies against *A. hydrophila*. Recent studies have highlighted the potential of IgY antibodies as a promising alternative to disease treatment (Liu et al. 2024a, 2024b; Seixas, Sousa, and Leitão 2022; Wang, Zhong, and Lin 2023). The MIC of IgY against *A. hydrophila* was 12.5 µg/ml, demonstrating IgY's effectiveness in controlling *A. hydrophila* infection. Fernandes et al. (2019) support our findings and found that IgY concentrations ranging from 5 to 20 µg/ml efficiently detected membrane and cytoplasmic proteins of *A. hydrophila* in Nile tilapia (*Oreochromis niloticus*). They emphasized that IgY antibodies can effectively treat bacterial infections, particularly those caused by antibiotic-resistant bacteria. The result of the present study supported by Zhang, Lin, and Qin (2023) documented the role of IgY in combating bacterial diseases and mentioned bacterial agglutination and adhesion are the primary properties of IgY, which inhibits the growth and reproduction of pathogens in the host animal. Zhang et al. (2021b) and El-Kafrawy et al. (2023) also reported that IgY from immunized chickens is effective against bacterial infections in animals and humans.

The Σ FIC values obtained for IgY-ciprofloxacin and IgY-chloramphenicol combinations were 0.24 and 0.37, respectively, indicating synergistic action. According to El-Kafrawy et al. (2023) and Vieira-Pires et al. (2021), combining

IgY antibodies with antibiotics enhances treatment efficacy and can potentially reduce the required antibiotic dosage, promoting sustainable antibiotic use. Our findings align with these results, highlighting the combinational effects of IgY and antibiotics. Integrating IgY into aquaculture could reduce antibiotic reliance, decrease selective pressure on bacteria, and slow the development of resistance genes. Unlike antibiotics that target specific bacteria, IgY can be generated against various pathogens, providing broader protection. This combination effectively controls bacterial infections and offers an alternative to conventional treatments, reducing the environmental impact of antibiotics.

The environmental impact can be minimized by incorporating IgY antibodies into aquaculture practices. IgY offers an alternative treatment for bacterial infections, particularly where antibiotic treatments have become less effective due to resistance or regulatory restrictions. This diversification in treatment approaches can help maintain a healthier and more sustainable aquatic ecosystem (Paul et al. 2019). The use of IgY opens opportunities for further research in developing specific antibodies against different pathogens, advancing our understanding of host-pathogen interactions and antibody-based therapies in aquaculture (Zorriehzahra et al. 2016).

The stability of IgY antibodies under various conditions enhances their versatility and reliability in industrial applications, making them an indispensable resource in both veterinary and human medicine, as well as in food safety and biotechnology. They are utilized as animal feed supplements, in veterinary medicine (Ge et al. 2020), food safety, biotechnology, and diagnostics, and human therapeutics (Yakhkeshi et al. 2022). By confirming that IgY retains its activity under diverse conditions, stability studies support the development of robust and reliable treatments for bacterial infections in aquaculture. Additionally, maintaining IgY stability helps reduce environmental impact, decreases the need for chemical preservatives, and ensures sustainable disease management practices. Recent studies by El-Kafrawy et al. (2023) and Zhang et al. (2021a), emphasize the importance of stability in maintaining the effectiveness of IgY antibodies, guiding future research and applications in the field.

Our study demonstrated that IgY maintains stability under various temperatures, molarities, and solvent systems. And the IgY antibodies exhibit strong interactions at pH levels ranging from 3 to 10 but become inactive at pH levels below 3. The result aligns with findings that IgY loses function at pH 3.5 or below (Hatta et al. 1993; Lee et al. 2002; Löscher et al. 1986; Shimizu et al. 1993). Lee et al. (2002) suggested using sorbitol to improve IgY stability and prevent acid-induced inactivation, especially in the intestinal region. A study by Ge et al. (2020) evaluated different IgY preparation methods and storage stability, finding that IgY antibodies were relatively stable in the pH range of 4–10 and at temperatures up to 70°C. Nilsson and Larsson (2007) found similar results,

showing maximum stability at 37°C for over 8 weeks with disaccharides as stabilizing agents. Ensuring IgY stability is crucial for maintaining functional activity and efficacy, allowing disease prevention and control, food safety (Zorriezhahra et al. 2016), enhanced immune response, oral vaccination, and reduced stress (Paul et al. 2019) in aquaculture.

Conclusion

This study highlights the potential of IgY antibodies and the advantages of using IgY antibodies in conjunction with antibiotics to enhance therapeutic efficacy against bacterial infections. The synergistic interaction between IgY and antibiotics enhances bacterial eradication and allows for lower antibiotic doses, thereby reducing the risk of selective pressure by the pathogens. IgY from chicken egg yolks offers an eco-friendly alternative to antibiotics, thereby reducing environmental pollution and minimizing the ecological footprint of aquaculture practices. IgY's stability under various environmental conditions ensures its functionality under diverse aquaculture practices. These findings have significant implications for the industry, offering a sustainable approach to maintaining aquatic health and advancing aquaculture practices.

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Ethical clearance

For this experiment, we followed the Prevention of Cruelty to Animals (PCA) Act 1960 and Breeding and Experimentation Rules 1998, and other amendments. IBSC and IAEC committees, CVAS, Mannuthy, Thrissur reviewed and approved all these experiments. Institutional Biosafety Committee Approval No: IBSC-21-13

Institutional Animal Ethical Committee Approval No: IAEC/CVASMTY16/2021

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Growth inhibitory properties of specific anti-*A. hydrophila* IgY antibodies: A promising approach for the prevention of *Aeromonas* infection in farmed fish

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Abstract

The avian egg yolk IgY antibodies were produced in chickens that were 22–24 weeks old and the antibodies were collected and stored using the standard extraction and purification processes. Here, we obtained IgY antibodies with an antibody titration of 1:10000 and a protein concentration of 42.8 mg/ml. Here, we used both the well diffusion method and the dilution assay method to perform a growth inhibition assay using specific IgY. According to the results of the studies using growth inhibition assays, it was found that anti-*Aeromonas* IgY antibodies significantly impacted the ability of bacteria to grow. Non-specific IgY, in contrast, did not exhibit any inhibitory effect on *A. hydrophila*. These results indicate that passive immunization with *A. hydrophila*-specific IgY could prevent *A. hydrophila* infection in farmed fish. Such novel concepts can permanently replace antibiotics with eco-friendly, bio-antimicrobial products.

Key words: Aquaculture; *A. hydrophila*; avian IgY antibodies; GIA; passive immunization.

Introduction

Aquaculture is an important industry that provides a significant portion of the world's seafood supply and it is one of the promising industries that support the world economy. However, the high density and stress of the fish-rearing environment make them susceptible to bacterial infections, which can lead to significant economic losses. One of the autochthonous bacterial pathogens affecting fish is *Aeromonas hydrophila*. *A. hydrophila* is a gram-negative bacterium that is widely distributed in aquatic environments and is known to cause mortality in aquaculture (Dien *et al.*, 2023). Antibiotics have usually been used to control *A. hydrophila* infections, but their overuse can lead to the development of antibiotic-resistant strains,

which makes the development of alternative strategies necessary (Dien *et al.*, 2023). One such strategy is the use of immunoglobulin Y (IgY) antibodies (El-Kafrawy *et al.*, 2023), which are produced by birds in response to infection (Li *et al.*, 2023).

Despite having a history of more than a century, IgY technology is still developing (Zhang *et al.*, 2021b). The IgY antibodies are effective in preventing and treating a variety of viral and bacterial infections in aquaculture (Baloch *et al.*, 2015; Zorriehzah *et al.*, 2016), animals (El-Kafrawy *et al.*, 2021, 2023) and humans (Abbas *et al.*, 2019; Constantin *et al.*, 2020). The bacterial fish pathogen *A. hydrophila* is present everywhere with a highly adapting nature. It causes severe infections and leads to massive death of fish on farms, especially in carp, mrigal, catla, rohu,

tilapia etc., (Harikrishnan and Balasundaram, 2005). Our current research characterised the IgY production, extraction, specificity study, ELISA titration, agglutination, and growth inhibition assay (GIA) to develop IgY antibodies specifically against *A. hydrophila*.

Methodology

Experimental set-up

Lyophilized *A. hydrophila* (MTCC-1739) strains were obtained from the MTCC, Chandigarh, India. Pure cultures of *A. hydrophila* strains were obtained by rehydrating lyophilized forms and incubating them in double-strength nutrient broth at 37°C for 24 h. These cultures were then subcultured onto nutrient agar plates (MTCC Media-3). Six 22–24 week old single comb white leghorn egg-laying chickens were selected and housed at the College of Veterinary and Animal Sciences small animal breeding station in Mannuthy, Kerala, with the approval of the Institutional Animal Ethical Committee (IAEC). The Institutional Bio-Safety Committee (IBSC) of CVAS Mannuthy, Thrissur provided ethical clearance for immunization using killed strains of *A. hydrophila* in the experimental animals, following the Standard Operating Procedure (SOP).

Extraction and purification of IgY antibodies from chicken egg yolk

The formaldehyde-killed *A. hydrophila* (MTCC-1739) antigens were immunized intramuscularly to the young egg-laying poultry. After a couple of weeks, the eggs were collected and the developed antibodies were extracted from the egg yolk by dual-step extraction method (Polson *et al.*, 1980). The anti-*A. hydrophila* antibodies were purified by dialysis and DEAE-cellulose ion exchange column chromatography and the purity of IgY was confirmed by SDS-PAGE with a 180 KDa reference marker (Genei Pvt Ltd). Then the concentration of protein in IgY antibodies was confirmed by Lowry's method (Lowry *et al.*, 1951). To confirm the specificity and titration of anti-*A. hydrophila* IgY antibodies against the *A. hydrophila* pathogen, an indirect ELISA was carried out (Voller *et al.*, 1976).

Detection of IgY antibodies

Microscopic slide agglutination assay using serum

Microscopic slide agglutination assay using serum was performed before collecting eggs to confirm the presence of specific antibodies against *A. hydrophila* in blood serum. The serum from the immunized chicken was separated and its 1:1000 dilution was used in this study to reduce any non-specific binding. The formaldehyde-killed bacterial antigen was taken from $1-2 \times 10^8$ CFU/mL of *A. hydrophila* culture and diluted in phosphate-buffered saline (PBS). One drop of both antigen and antibody was allowed to react on a microscope for 30 min and then observed for agglutination under a microscope (Meenatchisundaram *et al.*, 2011). The degree of agglutination was graded based on a "grading scale for agglutination", a scale ranging from 0–4, where 0 indicates no agglutination and 4 indicates strong agglutination. A positive result was defined as a score of 2 or higher, while a negative result was defined as a score of 0 or 1. The results were compared with nonspecific IgY antibodies.

Tube agglutination assay using egg yolk antibodies

Following the microscopic slide agglutination test, the IgY from day 14 were used for the tube agglutination assay. In this method (Banerjee *et al.*, 1978), two-fold dilutions of the IgY sample were prepared and then mixed with 50 µl of formaldehyde-killed bacterial antigen in all test tubes. The tubes were then incubated at room temperature for 30 min and observed for the presence of agglutination or clumping of bacterial cells. Agglutination indicates the presence of specific IgY antibodies against bacteria in the sample as discussed earlier. The level of agglutination can also provide information about the relative amount of IgY antibodies present in the sample. The final dilution tube showing agglutination represents the titer of the sample, indicating the highest dilution of IgY antibodies that can still agglutinate to bacterial cells.

Micro agglutination test (MAT)

In a micro-titre plate, two rows were assigned for the test, and 50 µl of sterile saline was

added into two rows. Then 50 µl of egg yolk antibodies (IgY) were added in the first well of the first row and doubling dilution was done by transferring 50 µl from the first well to the second well, the second to the third and so on. The same procedures were repeated in the second row using non-specific antibodies. Finally, 50 µl of active bacterial cells were added to each well and incubated at 25°C overnight. After incubation, the plate was observed for agglutination by comparing it with the control (Thangapandian *et al.*, 2014).

Growth inhibition assay

Growth inhibition assay by dilution method

To perform the assay, log-phase bacterial cultures were prepared and adjusted to the desired concentration, typically 10^8 – 10^9 CFU/ml. 10-fold serial dilutions of the IgY antibody were then prepared in separate sterile test tubes. 1 ml of the bacterial culture *A. hydrophila* was then added to each test tube containing different dilutions of the IgY antibody, mixed well, and incubated at 37°C for 24 h (Lee *et al.*, 2002). The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the IgY antibody that completely inhibits the visible growth of the microorganism. The results were further confirmed by streaking cultures from each tube onto nutrient agar plates and again incubated overnight at 37°C for 18–24 h, checked the results and compared them with the controls.

Growth inhibition assay by well diffusion method

The growth inhibition assay was performed on nutrient agar plates by the well diffusion method (Kavanagh, 2014) to assess the inhibition properties of anti-*A. hydrophila* antibodies against *A. hydrophila* cells. The principle of this method involves the diffusion of the antimicrobial agent from the well into the surrounding agar, resulting in a concentration gradient of the agent.

The log culture of *A. hydrophila* was swabbed onto the agar plates to get even growth of the pathogen. Then the agar plates were left to set and in each of these plates 2 cups, 10 mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed.

The wells were filled with anti-*A. hydrophila* antibodies of 1:1000 and 1:10000 dilution and allowed for diffusion of IgY antibodies into the medium for 30 minutes and kept in an incubator at 37°C. After 18–24 h, the agar plates were examined for inhibition zones, and the zones were measured in millimetres in diameter (Juni *et al.*, 2016). The growth of the bacteria was inhibited in areas where the concentration of the antimicrobial agent exceeded a critical threshold, resulting in the formation of clear zones around the well.

Statistical analysis

The results were statistically analysed by using Microsoft Excel and SPSS version 25.

Results

The specific IgY antibodies were developed and purified successfully, where the concentration of antibodies increased in the egg yolk with subsequent booster dose administration and maintained the antibody level in the egg yolk. Column purified IgY fractions had a total protein concentration of 42.8 mg/ml. Vigorous clumping showed the presence of antibodies against *A. hydrophila* antigens in the microscopic slide agglutination test (Fig. 1). In the tube agglutination test, the agglutination was observed up to 1/128th dilutions (Table 1). It exhibits a high titre using the indirect ELISA method at dilutions of 1:10000 at 490 nm. The highest titre of specific IgY was observed at 1:512th dilution by micro-agglutination assay (Table 2). These results suggest that specific and high titres of antibodies were generated against *A. hydrophila*.

In the dilution method, the absence of growth when particular egg yolk antibodies were added to the culture demonstrated the specific neutralization activity of anti-*A. hydrophila* egg yolk antibodies (IgY) over its antigen. The result showed that specific IgY up to the 10^{-4} dilution was able to completely inhibit the growth in both test tubes and its corresponding nutrient agar plates (Fig. 2(a)–(b)) and the MIC of specific IgY antibodies was determined as 10^{-4} dilution.



Fig. 1: Agglutination of IgY antibodies (The agglutination formed as the result of antigen-antibody reactions, where antibodies were extracted from serum).

Growth inhibition assay performed by well diffusion method to assess the inhibition properties of anti-*A. hydrophila* antibodies against *A. hydrophila* cells. The zones 15 and 25 mm diameter were formed for 1:10000 and 1:1000 dilution of IgY respectively formed around the IgY wells after 24 h of incubation at 37°C confirming that the IgY can actively inhibit the growth of *A. hydrophila* (Fig. 3).

Discussion

Bacterial infections pose a significant challenge in aquaculture, often leading to substantial financial losses for farmers. While antibiotics are commonly employed in modern

medicine to combat these infections, they are not without their drawbacks. Although often effective, antibiotic use can result in environmental harm due to residual presence. Moreover, the use of such infection control strategies carries risks, including the possibility of overdosing and the emergence of antibiotic-resistant pathogens.

In light of these issues, antibodies present a promising alternative to traditional antibiotic therapy. They offer several advantages, including lower production costs, ease of production, and simple extraction procedures. Additionally, they boast high productivity and are easy to handle for experimental animals. Importantly, after experiments, birds can return to their normal

Table 1: Tube agglutination assay.

Antibody dilution	Agglutination
1/2	++++
1/4	++++
1/8	++++
1/16	++++
1/32	+++
1/64	++
1/128	++

The agglutination formed as the result of antigen-antibody reactions, where the antibodies were extracted from the egg yolk. ++++ – very high, +++ – high, ++ – moderate, + – low/no agglutination.

Table 2: Micro agglutination test.

Dilution of the sample	Agglutination	
	Specific IgY	Non-specific IgY
1:2	++++	+
1:4	++++	+
1:8	+++	–
1:16	+++	–
1:32	++	–
1:64	++	–
1:128	+	–
1:256	+	–
1:512	+	–
1:1024	–	–

The agglutination formed as the result of antigen-antibody reactions, where the antibodies were extracted from the egg yolk. ++++ – very high, +++ – high, ++ – moderate, + – low/no agglutination.

lives, and there is no risk of resistant microbes emerging. This makes antibodies a potentially more sustainable and effective solution for managing bacterial infections in aquaculture (Dias da Silva and Tambourgi, 2010). So IgY technology offers a possible plan for the creation of substitute therapies for *A. hydrophila* infections in aquaculture and perhaps other industries as well.

The production of a high amount of antibodies in poultry in response to an immune stimulation accumulated in egg yolk has the ability of passive immunization. Klemperer in 1893 identified these immunoglobulins successfully (Klemperer, 1893) and the term “IgY” (Leslie and Clem, 1969) was coined by Leslie and Clem in 1969. There was a revolutionary growth

in the extraction, diagnostic, prophylactic/therapeutic application in this area during the last 40 years (Wu *et al.*, 2022).

To evaluate the *in vitro* neutralization capacity of the IgY antibodies produced, we performed both well diffusion and tube agglutination assays against bacterial pathogen *A. hydrophila* and examined its antibacterial properties, including the quantity of IgY. Our results confirmed that these antibodies have the efficacy to neutralize the pathogen successfully. Here, the IgY antibodies were developed in 22–24 weeks old white leghorn chickens by immunizing them with formaldehyde-killed antigens and the extracted IgY from egg yolk was purified by a series of techniques. Certain studies by researchers on both *in vitro* and *in vivo* neutralization ability of IgY antibodies on various bacterial pathogens like *Propionibacterium acnes*, *E. coli*, *Streptococcus mitis*, Streptococcal dental caries (*Streptococcus mitis*, *S. mutans* and *S. gordonii*) and *Salmonella* serotypes (Revathy *et al.*, 2014; Meenatchisundaram *et al.*, 2011; Dinesh *et al.*, 2020; Bustos *et al.*, 2021) supports our results.

The primary step required to cause the inhibition of bacterial growth may be the binding of the antibody to the bacteria. Inhibition of bacterial growth may therefore result from IgY's binding activities against components of the bacterial surface such as LPS, outer membrane protein, and fimbriae (pili) (Sunwoo *et al.*, 2002). The function of certain components that may be essential for bacterial motility and growth may be blocked by antibodies that are bound to specific components on the bacterial surface (Smani *et al.*, 2012). One of the recent *in-vitro* studies on the mechanism of prevention of *H. pylori* by IgY antibodies confirmed that the agglutination fastened phagocytosis, or neutralization as well as blocking the adherence of the bacteria (Zhang *et al.*, 2021a). In other studies, IgYs were generated against *S. typhimurium* and *S. enteritidis* and were found to exhibit significant agglutination (Mine, 1997; Terzolo *et al.*, 1998). These studies may advance our understanding of IgY's immunological characteristics and provide evidence in favour of its potential use in immunological applications. There have

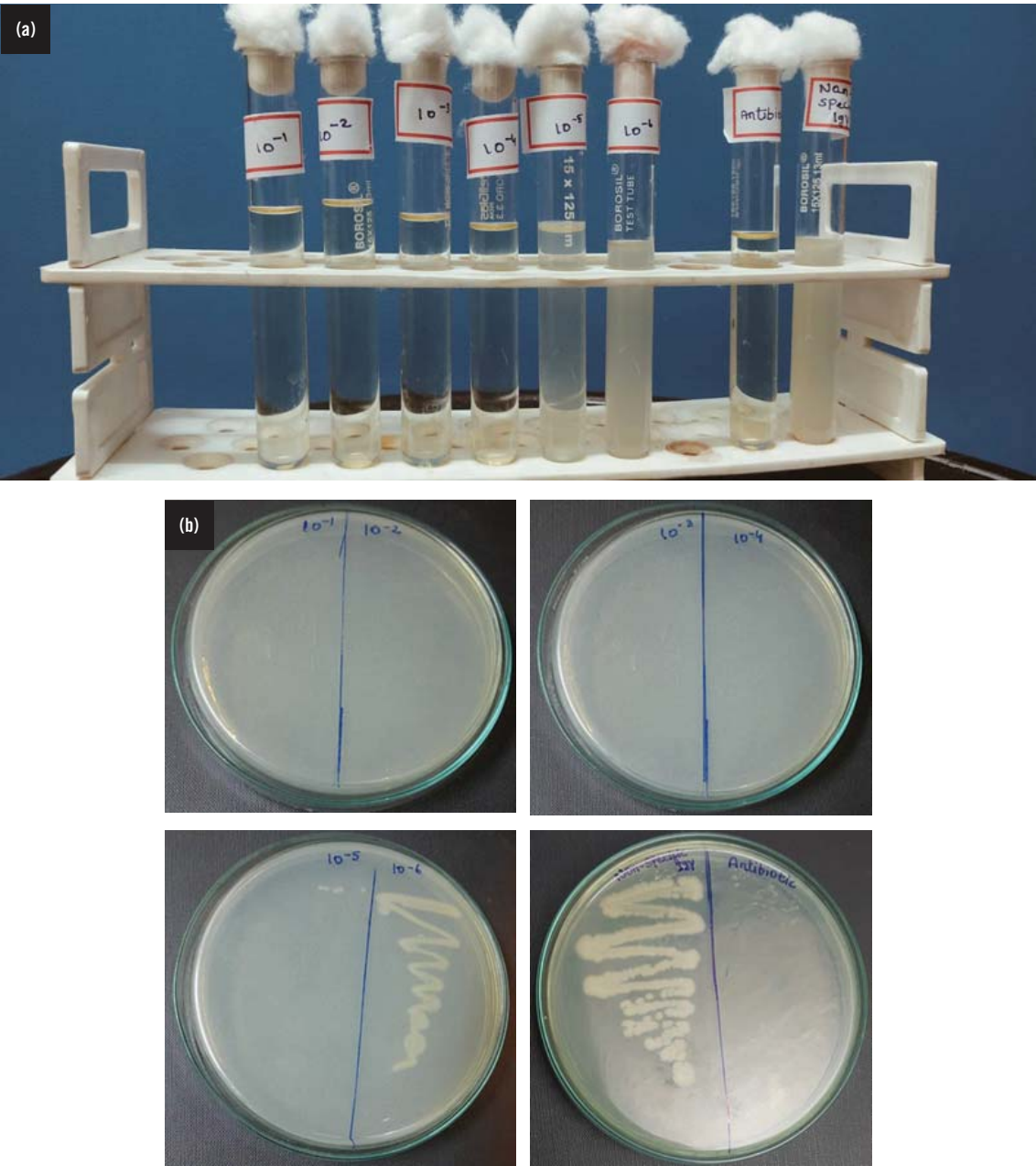


Fig. 2: Growth Inhibition Assay by dilution method: (a) As the dilution of the antibody increases the growth inhibition decreases. The test tubes 10^{-1} – 10^{-4} show the lack of growth of bacteria, whereas 10^{-5} and 10^{-6} show bacterial growth. The terminal two test tubes served as both positive and negative controls; (b) The corresponding plates of the test tubes (2a) showed the same results.

been numerous recent successful studies on aquaculture to control bacterial diseases in aquaculture to control *A. hydrophila* infections

in-vitro (Fernandes *et al.*, 2019; Jin *et al.*, 2013; Li *et al.*, 2006, 2023), necessitating further study in the area.

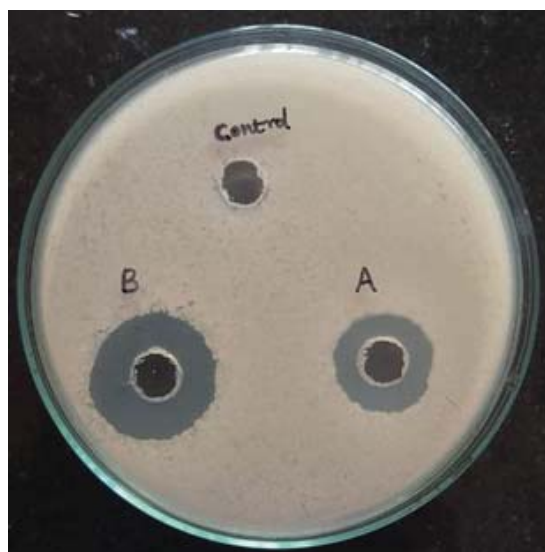


Fig. 3: Growth inhibition assay by well diffusion method (The zones formed around the well of IgY showed the neutralization of the pathogen. Whereas the nonspecific IgY doesn't show any inhibitory action towards pathogen *A. hydrophila*).

Non-piscine antibodies are antibodies derived from non-fish sources, such as chicken egg yolk (IgY), mammalian serum (IgG), or recombinant technologies. These antibodies can be used in fish for various purposes, such as immunotherapy, immunodiagnosis, immunomodulation, immunogenicity and immunotoxicology. Non-piscine antibodies have some advantages over piscine antibodies, such as higher specificity, lower cross-reactivity, easier production, and lower ethical concerns. The use of non-piscine antibodies in fish requires careful optimization and validation of the antibody source, quality, dose, route, and timing of administration, as well as the evaluation of the efficacy, safety, and environmental impact of the antibody treatment. In addition to the species, age, sex, and health state of the fish, environmental factors can also affect the *in vivo* effects of non-piscine antibodies in fish (Segner *et al.*, 2022; Segner *et al.*, 2012).

Passive immunization with IgY as a disease control measure presents several opportunities and challenges that need to be considered. The general stability of IgY refers to its ability

to maintain its structure and function under different environmental conditions, such as temperature, pH, and salinity. It is generally observed that IgY remains active even after multiple freeze-thaw cycles, operating within a temperature range of -20 – 60°C . IgY can become denatured at very low or very high pH values, but it is also stable at neutral or slightly acidic pH values. IgY is unaffected by salinity and is capable of remaining active in seawater or saline solutions. The intestinal stability of IgY refers to its ability to resist degradation by digestive enzymes and acids in the gastrointestinal (GI) tract of animals. IgY is resistant to proteolytic enzymes like pepsin and trypsin because of its special structure, which also keeps it from attaching to immune cells' Fc receptors. Since IgY has a high isoelectric point and is not soluble at low pH levels, it can withstand the acidic environment of the stomach. However, the species, age, diet, and overall health of the animal, in addition to the amount, mode, and timing of IgY administration, can all affect how stable IgY is in the GI tract. The stability of IgY is crucial for its oral delivery and absorption in the GI tract, which can provide passive immunity against enteric pathogens. Nevertheless, additional optimisation and validation are necessary to fully understand the *in vivo* stability and bioavailability of IgY. Among the strategies to improve IgY's stability and defence are encapsulation, coating, and conjugation with other molecules (Wang *et al.*, 2021).

In conclusion, these studies suggest that anti-*A. hydrophila* IgY antibodies have the potential to be used for controlling bacterial infections in aquaculture. However, further research is needed to optimize the assay conditions and to evaluate its efficacy *in-vivo*.

Conclusion

It is possible to successfully extract the IgY from the eggs that hens produced in response to a particular pathogen, and this can then be used to neutralise the pathogen efficiently and affordably. It is the best alternative to antibiotics and produces highly concentrated IgY with

high productivity using a clear and simple technique and without any residue-related problems. Non-invasive methods enable the experimental animal to live naturally after all experiments. Additionally, the fish to which we apply IgY as a remedy to pathogens will be safe for consumers and we can reduce the use of antibiotics in fish farms in future.

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Conflict of interest

The authors have no conflict of interest.

Ethical clearance

For this experiment, we followed the Prevention of Cruelty to Animals (PCA) Act 1960 and Breeding and Experimentation Rules 1998, and other amendments. IBSC and IAEC committees, CVAS, Mannuthy, Thrissur reviewed and approved all these experiments.

Institutional Biosafety Committee Approval No: IBSC-21-13.

Institutional Animal Ethical Committee Approval No: IAEC/CVASMTY16/2021.

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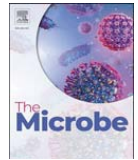
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In-vitro evaluation of IgY antibodies against *Aeromonas caviae* and cross-reactivity with *Aeromonas hydrophila*

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ABSTRACT

Aeromonas caviae, one of the most prevalent *Aeromonas* species, remains understudied compared to *Aeromonas hydrophila*. *A. caviae* is known to infect a wide range of aquatic organisms including fish, prawns, and crabs, leading to opportunistic diseases. The frequent use of antibiotics to combat these infections has resulted in the emergence of multidrug-resistant bacteria, causing significant residue-related issues in both biotic and abiotic environments. In this study, we propose the use of IgY antibodies as an innovative solution to prevent and treat bacterial infections. Formaldehyde-inactivated *A. caviae* and *A. hydrophila* strains were administered into the breast muscles of two different groups of 22–24 week old white leghorn hens on a weekly basis. *A. caviae* and *A. hydrophila* IgY antibodies were subsequently extracted and purified from the egg yolks, yielding an average protein concentration of 5.2 mg/mL and 11.88 mg/mL respectively. Our findings demonstrate that, IgY antibodies generated against *A. caviae* inhibit the growth of *A. hydrophila* at a dilution of 1:10,000. Furthermore, IgY antibodies specific to *A. hydrophila* exhibit cross-reactivity with *A. caviae* at the same dilution. In conclusion, IgY antibodies demonstrated significant *in vitro* inhibition and cross-reactivity offering a sustainable alternative to conventional antibiotic treatments.

1. Introduction

Due to their widespread presence and remarkable adaptability, aeromonads are found globally, with their virulent traits becoming increasingly pronounced with each generation (Janda and Abbott, 2010; Chen et al., 2019; Abdella et al., 2024). *Aeromonads* are responsible for severe opportunistic infections, targeting organisms in both freshwater and brackish water environments (Van Der Marel et al., 2008). Motile *Aeromonas* Septicemia (MAS), caused by organisms such as *A. hydrophila*, *A. caviae*, *A. veronii*, and *A. sobria*, primarily manifests as hemorrhagic septicemia, characterized by fin rot, dropsy, and hemorrhages (Syrova et al., 2018; Hossain et al., 2019; Saengsitthisak et al., 2020). The pathogenicity is primarily driven by virulence factors such as surface polysaccharides, exotoxins, extracellular enzymes, and secretion systems, which enable the bacteria to colonize, invade, and overcome the host immune response (Tomás, 2012; Fernández-Bravo and Figueras, 2020). Despite *A. caviae* being a common aquatic pathogen that

severely impacts aquaculture (Thomas et al., 2013; Hossain et al., 2019), studies focusing on this species and the development of targeted therapeutic agents are limited compared to those on *A. hydrophila*, presenting a clear research gap.

The traditional use of different groups of antibiotics to combat *Aeromonas* infections has led to widespread drug resistance (Sen and Rodgers, 2004; Igbinosa et al., 2012; Piotrowska and Popowska, 2014). This excessive use has alarmingly increased multidrug-resistant strains and caused environmental hazards from residue accumulation in aquatic ecosystems. This growing resistance diminishes the effectiveness of traditional treatment strategies, necessitating alternative approaches to managing bacterial infections sustainably. Given rising antibiotic resistance, immunotherapeutic approaches like IgY offer an alternative.

In this context, Immunoglobulin Y (IgY) technology, involving antibodies derived from chicken egg yolk, has emerged as a promising solution in immunotherapy (Kovacs-Nolan and Mine, 2012; Eriksson and Larsson, 2025). IgY offers several advantages: compared to

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mammalian antibodies, IgY demonstrates lower cross-reactivity with mammalian proteins and higher antigen-binding specificity (Schade et al., 2005). Furthermore, unlike conventional vaccine-based prophylaxis in aquaculture which may take weeks to induce a protective immune response, IgY is known to offer rapid passive immunity, which is crucial for quick intervention during disease outbreaks (Lu et al., 2009; Qiu, 2025a).

Antibodies that cross-react within and between species are frequently generated against common microbial epitopes. Studies have established that cross-reactive antigens play a significant role in the natural epidemiology of specific infections and that cross-protection can impact the progression and outcome of such infections (Augustyniak et al., 2017). Due to structural similarities in the surface antigens of related pathogens, IgY antibodies developed against one species may exhibit cross-reactivity against other related bacterial pathogens, such as *A. hydrophila* (Chen et al., 2025). This characteristic highlights the potential of using a single IgY product to manage multi-intra-species infections.

Based on these gaps and the advantages of IgY technology, this study investigates the *in vitro* neutralization potential of *A. caviae* IgY and its cross-reactivity with *A. hydrophila*. This research aims to assess the feasibility of utilizing cross-protective IgY antibodies as a sustainable, non-invasive strategy for managing bacterial infections in aquaculture.

2. Materials and methods

2.1. Experimental organism and experimental bird

The experimental organisms, *A. caviae* (MTCC-6541) and *A. hydrophila* (MTCC – 1739), were obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India, and sub-cultured on media-3 (Nutrient agar).

The experimental birds, White Leghorn Chickens (*Gallus gallus domesticus*) aged 22–24 weeks, were purchased from the Small Animal Breeding Station at the College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur, Kerala, India. All animal experimentation protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) constituted at CVAS, Mannuthy, Thrissur, Kerala, India.

2.2. Experimental bird management

Chickens were housed in individual cages under standard environmental conditions (25 ± 2 °C temperature, 12-hour light/dark cycle, etc.) and provided with commercial feed and water, kept them with ensuring minimal stress. Daily health monitoring was performed to assess the physical condition and behaviour of the birds. Humane endpoints were strictly implemented to minimize distress, including, but not limited to, body weight loss exceeding 20 %, severe localized inflammation, or inability to access food or water. Any bird reaching a humane endpoint was immediately euthanized under veterinary supervision, adhering strictly to the IAEC guidelines.

2.3. Immunization protocol and collection of eggs

Aeromonas cells were allowed to grow overnight in the nutrient broth. Cells were harvested by centrifugation, washed three times, and re-suspended in phosphate-buffered saline (PBS) at a density of 10⁶ cells/mL. Formaldehyde was added to a final concentration of 3 % (v/v), and the suspension was held overnight at 4 °C (Uma et al., 2012). Cells were washed twice with PBS to remove the formaldehyde and re-suspended in sterile PBS (pH 7.2). Complete killing of the cells was confirmed by sterility check by plating 100 µL of the final antigen suspension onto Nutrient Agar media and incubating at 37 °C for 48 h. Only suspensions showing no bacterial growth were approved for use in immunization. Bacterial suspensions were stored at 4 °C for further use

(Uma et al., 2012; Dinesh et al., 2013).

The antigens were standardized for immunization into the experimental birds according to McFarland's standard for the production of specific antibodies against both antigens (McFarland, 1907; Sudjarwo et al., 2017). Formaldehyde-killed antigens of *A. hydrophila*, re-suspended in PBS, were prepared for immunization in chickens using the McFarland standards, with turbidity ranging from 0.5 to 8 MFU (McFarland's Unit). Where McFarland standards were made by mixing barium chloride and sulfuric acid to create a barium sulphate precipitate, which was kept for comparison of the turbidity of the test sample. Three millilitres of inactivated antigens were administered via intramuscular injection into the breast muscles of White Leghorn chickens with PBS commencing with an initial dose equivalent to 0.5 MFU. Antigen suspensions were prepared in sterile PBS and administered intramuscularly to hens without adjuvant (adjuvant-free immunization) (Meenatchisundaram et al., 2011; Uma et al., 2012).

After two weeks of first immunization, test bleeding was done to determine the presence of specific antibodies in the serum. The eggs were collected, surface-sterilized with 70 % ethanol, and stored for the purification of specific IgY antibodies.

2.4. Purification of IgY antibodies from the egg yolk

The egg yolk and white were separated, and the yolk was treated with 3.5 % and 12 % polyethylene glycol solution. Protein precipitation from the egg yolk was achieved using the ammonium sulphate precipitation method (Polson et al., 1980), and dialysis was used to purify specific IgY antibodies. The IgY was further purified by DEAE Cellulose-Ion Exchange column Chromatography. The purity of chicken egg yolk antibodies was checked by SDS-PAGE analysis (Laemmli, 1970). The total protein from the egg yolk was calculated using Folin-Ciocalteu reagents by Lowry's method (Lowry et al., 1951). The antibody titer of the antibodies generated against both antigens was determined by indirect ELISA (Voller et al., 1976).

2.5. Detection of IgY antibodies against *Aeromonas* spp

2.5.1. Microscopic slide agglutination test (MSAT)

For the microscopic slide agglutination test, drops of 1–2 × 10⁸ CFU/mL of *Aeromonas* cell suspension (*A. caviae* and *A. hydrophila*) were mixed with corresponding IgY antibodies at two-fold serial dilutions on microscopic slides. The mixture was briefly incubated to allow agglutination, and the results were compared with those obtained using non-specific IgY antibodies (Meenatchisundaram, et al., 2011).

2.5.2. Tube agglutination test

For the tube agglutination test, purified IgY antibodies of specific pathogens *A. hydrophila* and *A. caviae* were serially diluted with sterile PBS in Eppendorf tubes at dilutions of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵. To each dilution, 50 µL of *Aeromonas* cell suspension was added. The tubes were thoroughly mixed and left to stand until agglutinates appeared. The results were compared with controls (Banerjee et al., 1978).

2.6. Cross reactivity of IgY antibodies

2.6.1. Micro agglutination test (MAT)

For the micro agglutination test, 1–2 × 10⁸ CFU/mL bacterial cell suspension of *A. caviae* was adjusted to an optical density of 0.42 at 620 nm. In a microtiter plate, two columns were designated for the test. The first well of each column was filled with 80 µL of sterile saline, while the remaining wells received 50 µL each. Subsequently, 20 µL of *A. hydrophila* IgY antibodies were added to the first well of the first column, followed by a doubling dilution by transferring 50 µL from one well to the next along the row. The same procedure was repeated for the second row (control), except that 20 µL of a non-specific IgY antibody

solution was used instead of *A. hydrophila* IgY antibodies. Finally, 0.05 mL of *A. caviae* bacterial cell suspension was added to all wells in each column and incubated overnight at 25°C (Thangapandian et al., 2014). After incubation, the plate was observed for agglutination, and the results were compared with the controls. The same experiment was conducted *vice versa* to study the reactivity of *A. caviae* IgY antibodies to *A. hydrophila* antigens.

The Microscopic Slide Agglutination Test (MSAT) and the tube agglutination test were also performed for confirmatory purposes. Each assay was performed in triplicate.

2.6.2. Cross-reactivity of IgY by indirect ELISA method

The cross-reactivity of *A. caviae* IgY antibodies with *A. hydrophila* and *A. hydrophila* with *A. caviae* was assessed using the indirect ELISA method (Voller et al., 1976). Microtiter plate wells were coated overnight at 4°C with 100 µL of *A. hydrophila* antigens in carbonate-bicarbonate buffer (pH 9.6). The wells were then washed three times with PBST (phosphate-buffered saline with Tween 20) to remove any unbound antigens. To block non-specific binding sites, 200 µL of 1 % bovine serum albumin (BSA) was added to each well and incubated at 37°C for one hour.

After the blocking step, the wells were washed again three times with PBST. Subsequently, 100 µL of *A. caviae* IgY antibodies were added to each well to allow interaction with the coated antigens. After incubation, the wells were washed three more times with PBST, followed by the addition of 100 µL of a 1:1000 dilution of rabbit anti-chicken immunoglobulin conjugated to horseradish peroxidase (HRP) (Genei Pvt Ltd, Bangalore). The plates were incubated at 37°C for one hour.

Following this, the wells were washed and 100 µL of freshly prepared TMB (3,3',5,5'-tetramethylbenzidine) and H₂O₂ solution (Genei Pvt Ltd, Bangalore) was added. The reaction was allowed to develop at room temperature in the dark for 20 min. The enzymatic reaction was terminated by adding 50 µL of 4 N sulfuric acid, and the optical density (OD) was measured at 490 nm using an ELISA reader. The results were analyzed by comparing the OD values from the interaction of *A. caviae* IgY antibodies with *A. hydrophila* antigens to controls with nonspecific IgY antibodies.

2.6.3. Detection of cross reactivity of IgY antibodies to *Aeromonas* spp by growth inhibition assay

Growth inhibition assay was performed using *Aeromonas* IgY antibodies against *Aeromonas* pathogens on nutrient agar plates by well diffusion method (Smania et al., 1999). The cell suspension of *A. caviae* and *A. hydrophila* were evenly spread onto nutrient agar plates using a sterile swab. Well with 10 mm in diameter, were created in the agar using a sterile cork borer No. 4, and the agar discs were removed. Nutrient agar plates with wells containing *A. hydrophila* IgY antibodies were loaded with antigens from *A. caviae*. Likewise, nutrient agar plates swabbed with *A. caviae* were infused with IgY antibodies targeting *A. hydrophila*. The plates were left for 30 min to allow the IgY antibodies to diffuse into the medium before being incubated at 37°C. After 24 h of incubation, the plates were examined for inhibition zones, and the diameters of these zones were measured in millimeters. The results were compared with positive control using antibiotic chloramphenicol.

2.7. Statistical analysis

The results were analyzed statistically using SPSS (Statistical Product and Service Solutions) version 28, manufactured by IBM (International Business Machines) Corporation, Armonk, New York, United States and the graphs were plotted using Origin Pro 8.1, developed by OriginLab Corporation, Northampton, Massachusetts, United States.

3. Results

3.1. Purification of IgY antibodies from the egg yolk

The purity of IgY was confirmed by SDS-PAGE analysis. It was roughly 70–80 % pure when compared to Genei private limited Bangalore's optimum molecular marker. The concentration of IgY was 5.2 ± 0.3 mg/mL and 11.88 ± 0.4 mg/mL for *A. caviae* and *A. hydrophila* IgY antibodies respectively. In antibody titer determined by indirect ELISA, 1:10000 dilution of IgY also gave positive results. The specificity of *A. caviae* IgY antibodies were detected by micro agglutination test (MAT) and microscopic slide agglutination test (MAST).

3.2. Cross reactivity analysis

The results of the cross-reactivity study using the MAT showed that specific *A. hydrophila* IgY antibodies reacted with *A. caviae* antigens and specific *A. caviae* IgY antibodies reacted with *A. hydrophila* antigens, both demonstrating agglutination up to a dilution of 1:64 (Fig. 1). Conversely, non-specific IgY antibodies did not exhibit any agglutination with either antigen. Non-parametric statistical analysis using the Kruskal–Wallis test revealed a significant difference in agglutination scores among groups ($H = 17.33$, $df = 2$, $p = 0.01$), confirming the specificity and cross-reactivity of the IgY preparations.

In MSAT, agglutination was observed in both directions, indicating that *A. hydrophila* IgY antibodies can agglutinate *A. caviae* cells, and *A. caviae* IgY antibodies can agglutinate *A. hydrophila* cells, demonstrating cross-reactivity. The observed agglutination occurred up to a dilution of 1:32, while no agglutination was seen with non-specific IgY antibodies, confirming the specificity of the reactions. Agglutination scores are defined as follows: 4 denotes strong agglutination, 3 denotes medium agglutination, 2 denotes low agglutination, 1 denotes weak agglutination, and 0 denotes no agglutination.

In tube agglutination assay, agglutination appeared in tubes containing *A. hydrophila* IgY antibodies and *A. caviae* antigens, with increasing agglutination observed at higher IgY concentrations. Agglutination was still visible even at a 10^{-5} dilution. Similar results were observed *vice versa*, indicating cross-reactivity between the antibodies and antigens of both bacteria.

The indirect ELISA results confirmed cross-reactivity between *A. caviae* IgY antibodies and *A. hydrophila* antigens, as well as between *A. hydrophila* antibodies and *A. caviae* antigens. The OD values from the indirect ELISA tests were significantly higher in the test wells compared

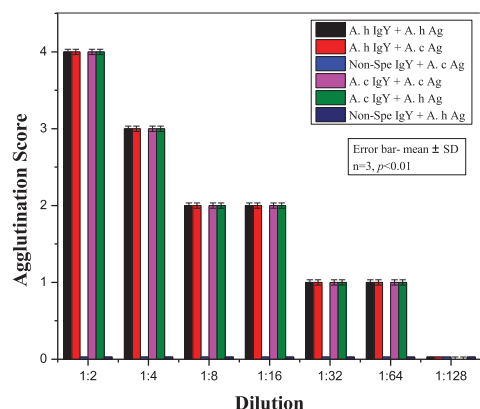


Fig. 1. Micro agglutination test. Here, A.h represents *A. hydrophila*, A.c represents *A. caviae* and Ag represents antigen. Statistical analysis using the Chi-square test revealed significant differences among groups.

to the control wells with nonspecific IgY antibodies, up to a dilution of 1:10000 (Fig. 2). This indicates a significant degree of cross-reactivity between *A. caviae* and *A. hydrophila*.

One-way ANOVA revealed a statistically significant difference among the ELISA OD values of the five experimental groups, $F(4,10) = 178.44$, $p = 2.99 \times 10^{-8}$. Tukey's post-hoc test further confirmed that all antigen-antibody combinations produced significantly higher OD values compared to the nonspecific IgY control ($p < 0.05$).

The growth inhibition study result of species specific IgY antibodies showed inhibition of *A. caviae* growth in the diameter of the 28 mm zone formed around the wells with IgY antibodies of *A. hydrophila* (Fig. 3). Similarly the growth of the *A. hydrophila* can effectively inhibit in the diameter of the 24 mm zone formed around the wells IgY antibodies of *A. caviae*. One-way ANOVA revealed significant differences among groups, $F(4,10) = 84.90$, $p = 1.12 \times 10^{-7}$, $n = 3$. Tukey's post-hoc test showed significant pairwise differences ($p < 0.05$).

4. Discussion

The primary objective of this study was to assess the potential of chicken egg yolk antibodies (IgY) generated against *A. caviae* as an alternative, sustainable approach for managing *Aeromonas* infections in aquaculture. Our findings confirm that IgY antibodies were successfully purified from immunized hens and demonstrated significant dose-dependent biological activity *in vitro*.

The results from the growth inhibition assay clearly demonstrated that *A. caviae* IgY inhibits the growth of the heterologous species, *A. hydrophila*, and *vice versa*. This inhibitory activity, combined with the successful agglutination observed in the tube agglutination test, confirms the successful generation of functionally active, cross-reactive antibodies. Importantly, these results are restricted to the laboratory environment. These promising *in vitro* findings indicate the possibility of therapeutic or prophylactic benefits, encouraging additional research to establish their effectiveness (Qin et al., 2018; El-Kafrawy et al., 2023). The observed activity strongly warrants further investigation, but *in vivo* validation is strictly required to establish protective potential in live fish or crustacean models.

The successful cross-reactivity between *A. caviae* IgY and *A. hydrophila* antigens, confirmed by both Indirect ELISA and agglutination, suggests the presence of shared antigenic determinants or

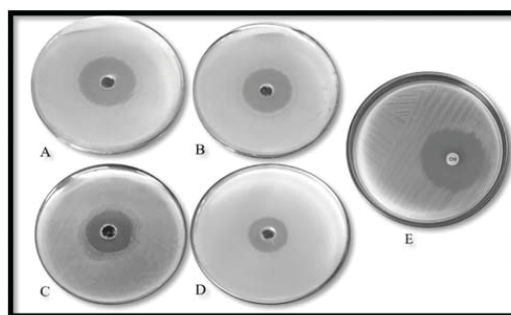


Fig. 3. Growth inhibition assay: A: *A. hydrophila* IgY antibodies inhibit the growth of *A. hydrophila* (zone 28 mm), B: *A. caviae* IgY antibodies inhibit the growth of *A. caviae* (zone 25 mm), C: *A. hydrophila* IgY antibodies inhibit the growth of *A. caviae* (zone 24 mm), D: *A. caviae* IgY antibodies inhibit the growth of *A. hydrophila* (zone 21 mm), E: control with antibiotic disc (zone 35 mm).

conserved epitopes on the surface of both pathogens. The successful cross-reactivity observed between *A. caviae* IgY and *A. hydrophila* antigens suggests the presence of shared, conserved surface epitopes. Indeed, reverse-vaccinology analyses have identified outer-membrane proteins such as PilQ and TolC that are highly conserved across *Aeromonas* species (Zhang et al., 2023), and cross-protection by IgY generated against *A. veronii* has been demonstrated *in vivo* against *A. hydrophila* (Chen et al., 2025). The existence of common O-antigens in *Aeromonas* spp. further supports this hypothesis (Cipriano, 2001).

Both *A. caviae* and *A. hydrophila* possess Lipopolysaccharide (LPS), which contains highly conserved core and lipid A regions. IgY may be targeting these structurally similar polysaccharide chains (O-antigens or common LPS core components) rather than species-specific epitopes (Turska-Szewczuk et al., 2014; Forn-Cuní, 2017).

Outer membrane proteins (OMPs) are essential for the survival and physiology of Gram-negative bacteria, serving critical functions such as nutrient uptake, environmental sensing, and host interaction (Lin et al., 2002; Nikaido, 2003). Many OMP families-including porins, adhesins, and other structural membrane proteins-are highly conserved across related bacterial species due to their fundamental roles in maintaining membrane integrity and metabolic exchange. In *Aeromonas* spp. specifically, several major OMPs have been shown to possess conserved epitopes capable of eliciting cross-reactive antibody responses (Beaz-Hidalgo and Figueras, 2013). Therefore, antibodies targeting these shared OMP antigens are likely responsible for the observed cross-reactivity and growth inhibition among closely related *Aeromonas* isolates.

In addition, the molecule's high sialic-acid content and extensive glycosylation contribute to its structural stability, resistance to degradation, and effective binding interactions at mucosal surfaces (Qiu et al., 2025a,b). These biochemical features support IgY's rapid local action at sites of infection, enabling it to neutralize pathogens or their toxins before they establish disease. Importantly, IgY achieves these effects without activating mammalian complement or binding Fc receptors, reducing the risk of inflammatory responses (Taylor et al., 2009; El-Kafrawy et al., 2023).

Future studies utilizing Western blot analysis or epitope mapping are needed to precisely identify and characterize these shared molecular targets. This study provides a solid foundation for utilizing cross-protective IgY in aquaculture. However, we acknowledge several critical limitations like the absence of *in vivo* challenge experiments prevents any definitive conclusion regarding the protective efficacy, absorption, half-life, or therapeutic dose required in an aquaculture setting.

In conclusion, this research successfully demonstrated the *in vitro*

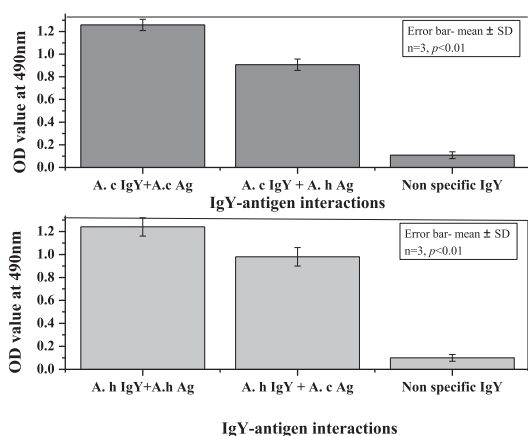


Fig. 2. The cross-reactivity of the IgY antibodies with *Aeromonas* antigens by indirect ELISA. (Where, A.c IgY + A.c Ag- *A. caviae* IgY + *A. caviae* Antigen, A.c IgY + A.h Ag- *A. caviae* IgY + *A. hydrophila* Antigen, A.h IgY + A.h Ag- *A. hydrophila* IgY + *A. hydrophila* Antigen and A.h IgY + A.c Ag- *A. hydrophila* IgY + *A. caviae* Antigen).

inhibitory activity and significant cross-reactivity of *A. caviae* IgY against *A. hydrophila*. This cross-protective capability highlights the promise of IgY technology as a vast, sustainable intervention against Motile *Aeromonas* Septicemia. However, progression to *in vivo* testing is the required next step to validate the practical utility of this approach.

CRedit authorship contribution statement

Thushara James: Writing – original draft, Methodology, Investigation, Conceptualization. **Denoj Sebastian:** Writing – review & editing, Supervision. **Rahiman K.M Mujeeb:** Writing – review & editing, Validation, Supervision, Formal analysis, Data curation. **MD Dinesh:** Writing – review & editing, Validation, Supervision, Data curation. **Rani S Suja:** Writing – review & editing, Validation, Supervision.

Ethical clearance

For this experiment, we followed the Prevention of Cruelty to Animals (PCA) Act 1960 and Breeding and Experimentation Rules 1998, and other amendments. All these experiments were reviewed and approved by IBSC and IAEC committees, CVAS, Mannuthy, Thrissur.

Institutional Biosafety Committee Approval No: IBSC-21–13
Institutional Animal Ethical Committee Approval No: IAEC/CVASMTY16/2021

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Importance, Production and Use of Egg Yolk Antibody (IgY) in Aquaculture

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ABSTRACT

Aquaculture industry has become the only rapidly developing farming sector worldwide; estimated to reach 62 per cent of the total global production by 2030. It is the controlled cultivation of aquatic flora and fauna and providing a protein food source for human consumption. The mass death of farmed fauna or flora owing to illnesses, climate change, and improper handling are among the major issues facing aquaculture production. For a decade, antibiotic treatment was the sole approach

to combat predicted microbial illnesses. Improper addition (overdosing), residual difficulties, microbe resistance, and the emergence of new types of microbial species are all adverse effects of antibiotic use in aquaculture. Vaccines, probiotics and prebiotics, among other technologies were developed to address these challenges. The importance of IgY technology, production and various levels of use in aquaculture system is highlighted in this chapter.

Introduction

During 2018, global aquaculture production reached a new high of 114.5 million tons in live weight; with a total farmgate sale value of USD 263.6 billion. The entire aquaculture production consisted of 82.1 million tons of aquatic animals, 32.4 million tons of aquatic algae and 26,000 tons of ornamental seashells and pearls. Inland aquaculture produces 51.3 million tonnes of aquatic animals in the year 2018, accounting for 62.5 per cent of global farmed food fish production (FAO, 2020). Fish and fish products are considered as the World's most traded foods, thus it is not surprise that 67 million tonnes of fish (live weight equivalent) were traded worldwide with an overall export value of USD 164 billion. This translates to over 38 per cent of all fish harvested or farmed on the planet. The total amount of fish produced is predicted to increase from 179 million tonnes in 2018 to 204 million tonnes by 2030 (FAO, 2020). Developing countries, mainly in Asia are by far predominant producers with China, Indonesia, India, Viet Nam and Peru being the key producers in 2018.

Aquaculture is a vital part of the economy in both developed and developing countries. It provides food, employment, and income at various levels of business. However, the loss of crops due to climate change, disasters, diseases or other factors which causes financial burden to the persons or company involved directly or indirectly. Disease problems constitute the largest single cause of economic losses in aquaculture sector (Meyer, 1991; El-son *et al.*, 2021). Concurrent with the rapid growth and intensification of aquaculture industry increased use of water bodies, pollution, globalization, and transboundary movement of aquatic fauna, the list of new bacterial pathogens isolated from fish has been steadily increasing (Harvel *et al.*, 1999; Pekala, 2018). Bacterial disease in aquaculture is the major constrain of the cultivation of aquatic species and cause significant economic impact on production globally (Lu *et al.*, 2021; Condon *et al.*, 2022). The disease development is a complicated process, dependent not only on bacterial species being capable of causing health disorders, but also on the immune status of fish, environmental conditions as well as the virulence of the disease agent.

Antibiotic Treatments and its Drawbacks in Aquaculture

Antibiotic usage in aquaculture provides a potentially broader environmental exposure pathway for drug dispersion through water, which has substantial ecosystem health implications as compared to antimicrobial use in terrestrial food

animal production (Cabello, 2006; Lulijwa *et al.*, 2020; Holmstrom *et al.*, 2003; Boxall *et al.*, 2004). In addition to disease regulation, the aquatic environmental microbiome's function in nutrient cycling, sustaining biodiversity, carbon sequestration, and freshwater availability remain important research inquiries (Kummerer, 2009). Antimicrobial residues in the aquatic environment change the environmental microbiome and accordingly ecosystem regulatory, provisioning and supporting capabilities (Sarmah *et al.*, 2006; Larsson *et al.*, 2018).

The use of antimicrobials may also serve as reservoirs for antimicrobial resistance genes that provide key routes for human and animal exposure to antimicrobial resistant bacteria (Cabello, 2013; Lulijwa *et al.*, 2020; Larsson, 2018; Heuer, 2009; Aedo *et al.*, 2014; Miller, 2018). Although the mechanisms and directionality of transference can be challenging to establish, shared antimicrobial resistance genes encoded on mobile genetic elements in pathogens isolated from humans, terrestrial and aquatic animals as well as the environment point toward pathways for movement of resistance genes across compartments (Cabello, 2016; Holmstrom *et al.*, 2003; Larsson, 2018; Heuer, 2009; Aedo *et al.*, 2014). Such pathways are illustrated by a putative aquaculture origin for the emergence and dissemination of select plasmid-mediated colistin resistance genes (Shen *et al.*, 2019; Cabello *et al.*, 2017). Increasing use of antimicrobials in humans and food producing animals is driving antimicrobial resistance (Laxminarayan *et al.*, 2013), which is amongst this era's defining global health challenges. Treatment failure rates are increasing as the incidence of antibiotic resistant infections in animal agriculture rises, jeopardising sustainable food animal production and animal welfare.

Egg Yolk Antibodies (EYA) and Mode of Action

Due to the emerging drug resistance in various microorganisms, traditional antibiotic treatment are becoming less effective; thus modern research has mainly focused on developing proficient alternative approaches. Instead of antibiotics for prophylactic and immunotherapeutic use prebiotics, probiotics, various immune stimulating agents and vaccines are the leading alternatives in aquaculture industry. Vaccines get wide acceptance for the fact that there is no risk of drug resistance development in vaccinated animals and protection of minority unvaccinated animals through herd immunity (Gudding, 2014). Out of these valuable therapies, recent studies proved that the application of chicken egg yolk antibodies (EYA) will act as promising alternative mechanism for diagnosis and treatments against various pathogenic organisms and infectious diseases in both humans and animals (Xu *et al.*, 2011; Mohammad *et al.*, 2016; Hu *et al.*, 2019; Paul *et al.*, 2019; Lu *et al.*, 2020; Lee *et al.*, 2021; Shen *et al.*, 2021; Wei *et al.*, 2021). The chicken egg yolk antibodies are very cheaper and easy to produce in large amounts; whereas other polyclonal and monoclonal antibodies are expensive and difficult to produce. This chapter presents an overview on egg yolk antibodies, their salient features, advantages and the potential therapeutic applications of IgY for the prevention and treatment of infectious diseases in aquaculture.

More than 100 years ago, Klemperer (1893) was first demonstrated the avian maternal antibodies are transferred from serum to egg yolk in order to protect the developing embryo from potential pathogens. However, there was no scientific application for this knowledge during that period. When animal welfare became a matter of serious ethical concern for the scientific community, the concepts of Klemperer began to receive more interest. In the year 1996, the workshop of European Center for the Validation of Alternative Methods (ECVAM) also suggested the use of IgY rather than IgG, since then the production and use of IgY technology has become considered an internationally accepted practice (Schade and Hlinak, 1996). Currently, the benefits of IgY technology and its universal application is extensive at large-scale in the field of research, diagnosis, medicine and immunotherapy.

The three classes of immunoglobulins in birds are IgA, IgM and IgY. Immunoglobulin IgY are considered as the human immunoglobulin G (IgG) homolog due to the supposed similarity between the two (Leslie *et al.*, 1969). However, in comparison with IgG, the IgY has a higher molecular weight of 180 kDa than IgG (150 kDa) and also contains a large heavy chain of 65 to 68 kDa (Warr *et al.*, 1995). The major advantage of this higher chain stiffness is that it may be associated with more IgY resistance to proteolytic degradation as well as fragmentation (Spillner *et al.*, 2012). Furthermore, IgY is more hydrophobic than the IgG and has an isoelectric point between 5.7 and 7.6. In chicken antibodies, often IgG and IgY are interchanged because they are functionally equivalent to each other and also the antibodies in hens serum is IgG and once when it is maternally transferred from hens serum to egg yolk it is called as IgY, which is an egg yolk antibody in high concentrations (Narat, 2003). Antibodies are transferred from hen to chick via the latent stage of the egg and play an important role in immunological function for the relatively immuno-incompetent chick to resist various infectious diseases. Serum IgY is selectively transferred from the hen's circulatory system across the oolemma into the maturing oocyte in the ovarian follicle (Rose *et al.*, 1981).

The term IgY is originally coined to refer to the larger molecular weight IgY found in egg yolk, but it is now accepted that IgY is the major antibody in both the blood and yolk in concentration around 10-20mg/ml (Warr *et al.*, 1995). It makes up about 75 per cent of the total immunoglobulin pool and the serum concentrations of IgY, IgA, and IgM have been reported to be 5.0, 1.25, and 0.61mg/ml, respectively (Leslie *et al.*, 1973). The concentrations of IgA (~0.7 mg/ml) and IgM (~0.15mg/ml) in egg white are relatively low; while that of IgY (8-25 mg/ml) in egg yolk is much high (Rose *et al.*, 1974). A typical egg of an immunized hen contains about 100mg of IgY and a single immunized hen lays approximately 325 eggs per year, resulting in a total production yield of up to 40g of IgY per hen per year (Pauly *et al.*, 2009).

IgY can be purified via a variety of nontoxic methods including water dilution, low pH-induced precipitation, as well as polyethylene glycol, dextran sulfate and xanthan gum-induced precipitations (Akita *et al.*, 1993) or NaCl extraction (Kubickova *et al.*, 2014). Therefore, in terms of environmental impact, IgY can

be efficiently collected and processed on an industrial scale without the usage of harsh chemicals and the production of toxic by-products (Yokoyama *et al.*, 1993). Huge quantities of IgY antibodies can be harvested from the eggs laid by a single immunized hen, and the overall yield is comparable to the amount that can be collected from large mammals such as goats and cows and 18-times higher than what is collected from rabbits (Karlsson *et al.*, 2004).

IgY may provide a low cost (only a few cents to produce an oral or nasal dose), safe and fast approach (produced in 5-8 weeks, including the time required for immunogen preparation) for rapid prophylaxis and therapeutic development against a variety of pathogens. In particular, passive immunoprophylaxis by surface treatment with IgY (e.g. via nasal spray, lozenges *etc.*) may be an effective method of capturing pathogens before they enter the body of both humans and animals. This approach may be especially attractive in limited resource countries where vaccination is unavailable, where new viral variants may evade immunity produced by vaccines or previous infection, or where immediate immunity is required (Lee *et al.*, 2021).

IgY used as prophylaxis can also be tailored to be highly specific and is not dependent on the host immune response. This latter feature may enable its clinical application for protecting a wide range of especially vulnerable patients, including the elderly, immune-compromised and young children (Rahman *et al.*, 2013). In addition, in animals raised for conventional antibody production, IgG is the main serum antibody collected. In contrast, IgY antibodies can be easily harvested and used in diagnostic assays, with lower background and cross-reactivity compared with current standards using IgG in animals raised for conventional antibody production.

The exact mechanisms through which IgY counteracts pathogen activity have not been determined. However, several mechanisms have been proposed by Xu *et al.* (2011), Abbas *et al.* (2019) and Paul *et al.* (2019) including agglutination of bacteria (Tsubokura *et al.*, 1997; Thu *et al.*, 2017), inhibition of adhesion (Jin *et al.*, 1998; Lee *et al.*, 2002; Xu *et al.*, 2011), opsonization followed by phagocytosis (Nie *et al.*, 2004; Zhen *et al.*, 2008) and toxin neutralization (Wang *et al.*, 2011; Thu *et al.*, 2017). Among these, inhibition of adhesion is considered the primary mechanism by which specific IgY functions. Briefly, IgY antibodies generated against intestinal disease causing organisms may reduce the incidence of disease by preventing the attachment of pathogens to the intestine such as blocking the mucosal receptor, interfering with binding to mucins, or neutralizing the colonization factor (Jin *et al.*, 1998).

The IgY Technology Based Production and Purification of IgY Antibodies

Currently, there are several methods of isolation and purification of functionally active chicken IgY from egg yolk described, even for large-scale purification. Nevertheless, purity and yield vary greatly from method to method and require

intense optimization for each experiment (Carlander, 2002). The choice of a specific method depends on the yield and purity desired, final application of the IgY as well as material cost, technology, labor skills and scale of extraction. First step is the chicken immunization followed by the yolk IgY extraction, the antibody characterization and finally IgY applications in various kinds of assays (Figure 15.1, Alvaro *et al.*, 2017). Chickens are a suitable model to produce IgY by immunization with bacterial or viral antigens (killed form). To improve the specific IgY response it is demanded the use of adjuvants like Freund's adjuvants for immunization procedures, which is the most frequently used adjuvant. Intramuscularly way is a common way for chicken immunization to produce specific IgY. Alternatively, subcutaneous way provides satisfactory amounts of specific antibody (Behn *et al.*, 2001).

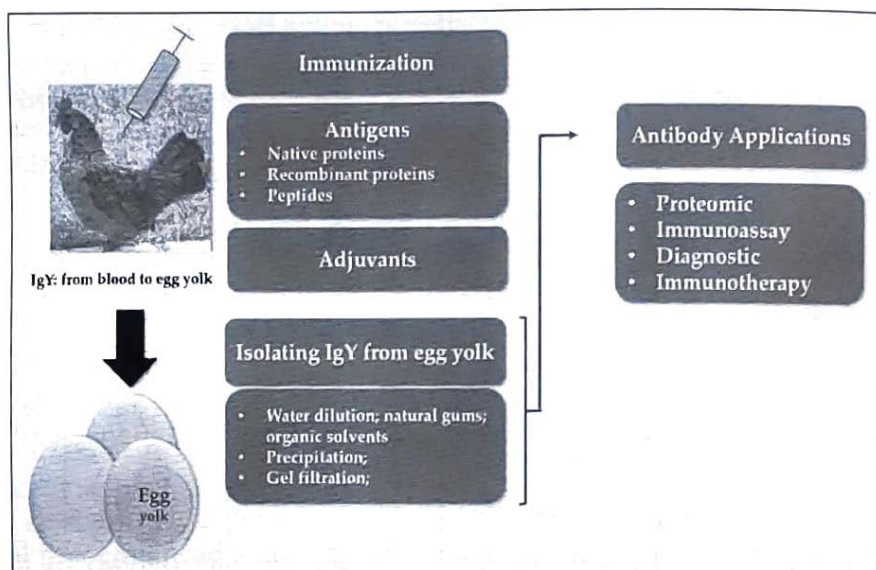


Figure 15.1: General Layout for Producing Specific IgY Antibody.

The immunized Chickens lay daily source of eggs was collected and surfaces sterilized using water or any sterilizing agents. The collected eggs marked properly and stored at suitable temperature for purification. The yolk antibody extraction begins by separating yolk and egg white. After removal of the vitellin membrane, the yolk is put into a measuring cylinder. The next steps in IgY extraction are devoted to removing the lipid content and obtaining a water soluble phase (WSP) antibodies enriched (Lee *et al.*, 2014). The consequent purification procedures vary widely in type and combination and from laboratory to laboratory. The degree application; for example, for oral administration, the whole yolk can be used, and on the other hand, for a reagent, isolated IgY from the mixture of yolk molecules is necessary (Behn *et al.*, 2001).

Removal of lipid content of egg yolk is a very important step in the extraction of IgY. Several natural gums, as carrageenan and xanthan gum, were found to be effective for removal of yolk lipoprotein as a precipitate, resulting in a water soluble fraction with very low lipid concentration (Hatta *et al.*, 1990). Optionally, organic solvents (chloroform) have been used as a mean to remove yolk lipid content at the initial step of IgY isolation. The water dilution method is a widespread procedure for the yolk lipid removal, which provides IgY enriched water soluble fraction (Behn, 2001; Ren *et al.*, 2016).

Several methods can be used for the extraction of IgY from egg yolk and commercial extraction kits are also available (Van Regenmortel, 1993). After the extraction step, three types of separation can be applied to eliminate the contaminants: precipitation with ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ (Ko and Ahn, 2007), or sodium sulfate (Wooley and Landon, 1995), or caprylic acid-ammonium sulfate (McLaren *et al.*, 1994), chromatography (Hansen, 1998) and filtration (Hernandez-Campos *et al.*, 2010). Salt based yolk antibody precipitation is promising to be a more efficient and useful purification method for the large scale IgY from egg yolk preparation (Ko and Ahn, 2007). Then IgY precipitated by using $(\text{NH}_4)_2\text{SO}_4$ and it could be removed by dialysis. Additionally, by following precipitation procedures, the IgY antibodies may be further purified by DEAE cellulose ion-exchange column chromatography (Selvan *et al.*, 2012) or ionic-liquid based aqueous biphasic systems (Taha *et al.*, 2015). The purity could be confirmed by SDS-polyacrylamide gel electrophoresis (Carroll and Stollar, 1983).

Stability and Safety of Chicken Antibodies (IgY)

Even though it is a protein molecule, IgY is resistant to heat and pH, being stable between 30° and 70 °C and active between pH 3.5 and 11. However, the affinity of IgY to its antigen decreases with increasing temperature. One of the most useful characteristics of IgY is its stability during the processing steps and under physiological conditions after administration (Kovacs-Nolan and Mine, 2012). IgY were found to be stable during storage at room temperature or at 4°C for 6 months without losing their activity (Nilsson *et al.*, 2012). Furthermore, IgY antibodies can be stored for 5–10 years at 4°C without significant loss of their activity; they can even retain their activity after storage for 6 months at room temperature or for 1 month at 37°C (Larsson *et al.*, 1997). Even if IgY is a protein molecule, it is resistant to temperature and pH. IgY is being stable between upto 70°C and active between pH 3.5 to 11 (Pereira *et al.*, 2019). According to Shimizu *et al.* (1992) and Hatta *et al.* (1993), IgY is stable at temperature ranging between 60°C and 70°C. IgYs are preferably stored at -20°C for long-term storage; storage at -70°C can cause up to 50 per cent loss of their activity (Staak *et al.*, 2001). The stability of IgY is not affected by freeze-drying, although repeated freeze-thaw cycles might compromise its activity (Shimizu *et al.*, 1988). One report of Fu *et al.* (2006) showed that the thermal stability of IgYs was not affected by lyophilization at 90°C for 15 minutes.

The activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3 (Shimizu *et al.*, 1988). IgY is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. Hatta *et al.* (1993) demonstrated that almost all of the IgY activity was lost following digestion with pepsin, but 39 per cent and 41 per cent of the activity remained even after 8h of incubation with trypsin or chymotrypsin respectively. The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive. However, proteins may suffer from a loss of activity as a result of conformational changes, aggregation or adsorption (Skrabanja *et al.*, 1994). However, Shimizu *et al.* (1988) reported freezing and freeze-drying did not affect the activity of IgY unless repeated several times. Fu *et al.* (2006) examined the thermal stability of IgY at various temperatures ranging between 25 and 90°C for 15min treatment, before and after freeze-drying process. Based on their observation, freeze-dried IgY had shown a good thermal stability with no significant reduction in reactive activity except at 90°C, but this was also observed for the non-freeze-dried IgY.

Most of the safety studies are conducted in humans and revealed IgYs are safer than IgGs because they do not bind to human Fc receptors or fix mammalian complement components. Hence they do not trigger potentially dangerous immune responses (Carlander *et al.*, 1999). The researchers found that the levels of pro-inflammatory cytokines tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were very low in cell cultures treated with IgY compared with the high levels of TNF- α and GM-CSF in cells treated with lipo-polysaccharides, indicating that IgYs do not cause inflammatory responses in lung cells and can thus be safely used for prevention of airway infections (Pasteur *et al.*, 2010). Additionally, oral IgY antibodies have been applied to treat rotavirus infections in humans and to treat pulmonary *Pseudomonas aeruginosa* infections, and no negative side effects of IgY treatment have been observed in up to 10 years of use (Nilsson *et al.*, 2007). The safety of intranasal IgY delivery has been documented for the treatment of influenza virus (Nguyen *et al.*, 2010) and acute and chronic pharyngitis for human (Xie *et al.*, 2004).

Advantages of Chicken Antibodies (IgY)

The chicken immune system has been studied for many years, and these studies have contributed substantially to the understanding the fundamental concepts of immunology and the development of different immunoglobulin classes (Carlander *et al.*, 1999). In a report of Sharma (1997), the use of avian antibodies offers many advantages over mammalian antibodies. Nowadays small quantities of avian antibodies are only used because of the limitation of awareness and handling techniques while using chickens. Traditionally, commercially available polyclonal antibodies have been produced in animals such as mice, rats, rabbits, sheep, goats, and horses, and are generally obtained from sera after immunization (Schade *et*

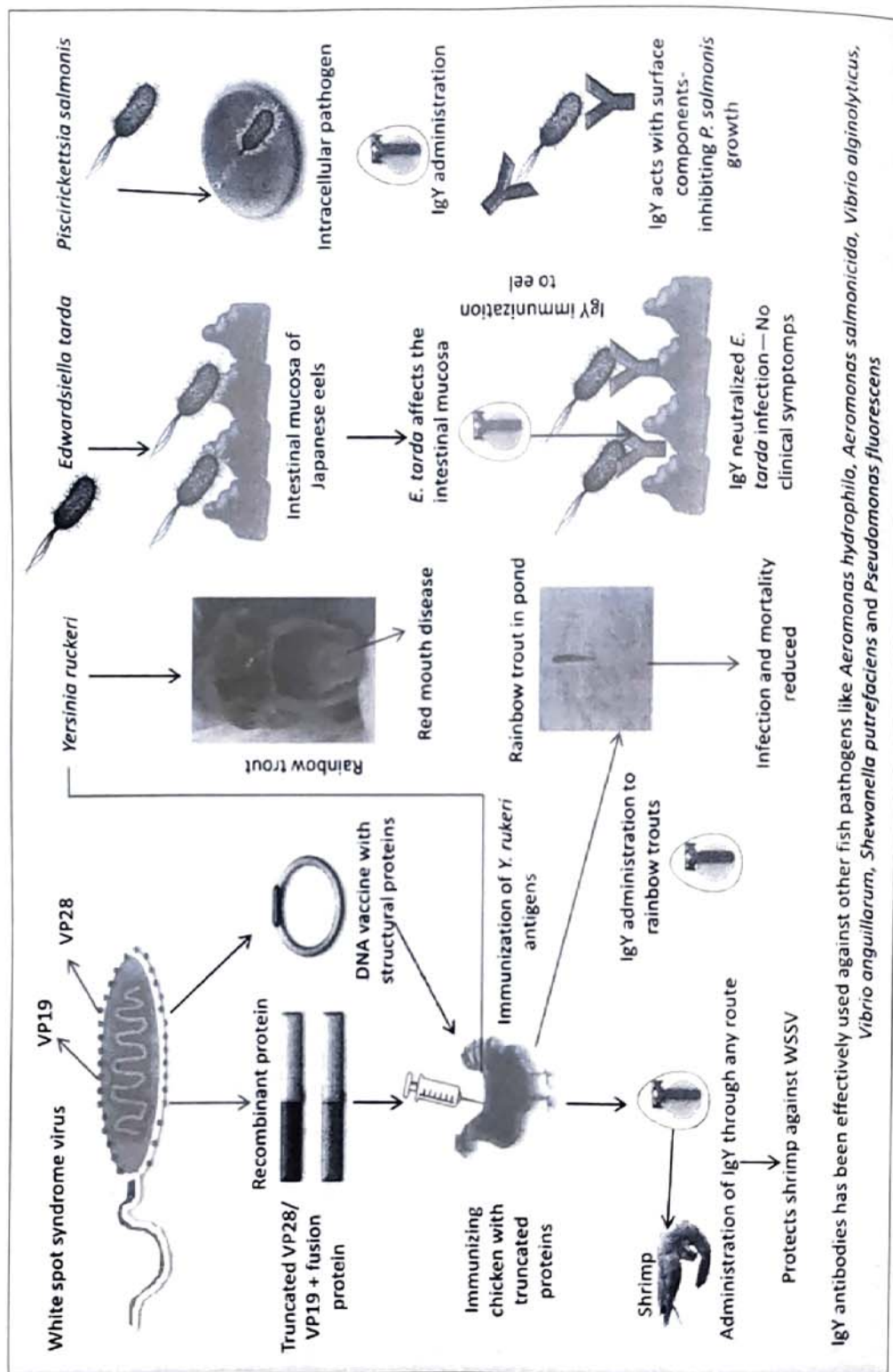
al., 1996). However, these antibodies cannot be prepared on an industrial scale because of the difficulty in obtaining large quantities of blood and concerns about animal welfare (Li *et al.*, 2017). The use of hybridoma technology has been used for the preparation of monoclonal antibodies; however it is still far from the successful commercialization of therapeutic monoclonal antibodies due to the expensive cost.

Recently, the chicken has attracted considerable attention as an alternative source of antibodies. IgY is deposited in the egg yolk in large quantities (Janson *et al.*, 1995), and it can be easily purified from the yolk by simple precipitation techniques, making chickens an ideal source for specific polyclonal antibodies (Gassmann *et al.*, 1990). From the point of view of animal welfare and bioethics, the production of antibodies using a chicken followed by their purification from eggs is more acceptable than preparation of mammalian antisera from blood. Moreover, immunization of the chicken is usually well-tolerated without the formation of abscesses and/or serious health problems, as is common for rabbits. The amount of injected mammalian antigen is often much lower than it is necessary for immunization of rabbits to assure an adequate immune response. Since the antibody is purified from egg yolks, not blood, the stress of the experimental animal is reduced to only the injection of the immunization doses. Animals do not need to be sacrificed to obtain blood for antisera production. Hens are able to produce antibodies with high titers against conserved mammalian proteins.

Hens therefore provide a more hygienic, cost efficient, convenient, and plentiful source of antibodies as compared to the traditional method of obtaining antibodies from mammalian serum (Gassmann *et al.*, 1990; Carlander *et al.*, 2000). Nakai *et al.* (1994) estimated that the productivity of antibodies in hens is nearly 18 times greater than that by rabbits based on the weight of antibody produced per head. Because of the high yolk IgY concentrations, over 100 mg of IgY can be obtained from one egg (Akita and Nakai, 1992). A laying hen produces approximately 20 eggs per month; therefore over 2 g of IgY per month may be obtained from a single chicken (Carlander *et al.*, 1999). In the egg, IgY is stable for months, and once purified it may be stored for years in the cold (Larsson *et al.*, 1993). As the industrial scale automated collection and separation of eggs is currently carried out, the large-scale production of specific IgY for immunotherapeutic purposes is feasible (Cotterill and McBee, 1995).

Applications of IgY Antibodies in Fishes and Aquatic Animals

IgY has been extensively used in health researches, as both therapeutic and diagnostic tool in human and veterinary filed as anti-bacterial, anti-viral, anti-fungal, anti-parasitic, anti-tumour, anti-obesity, anti-allergic and anti-venom activities (Pereira *et al.*, 2019). For the last three decades, IgY based disease diagnosis, prophylactic and disease treatments in aquaculture (Figure 15.2) have been reported and in recent years the development in this field is very promising (Kovacs-Nolan and Mine, 2012; Mohamed *et al.*, 2016; Kumaran *et al.*, 2018; Yi *et al.*, 2018; Eto *et al.*, 2019; Gao *et al.*, 2019; Nakamura *et al.*, 2019; Pereira *et al.*, 2019; Liu *et al.*, 2021; Zhang *et al.*, 2021).

Figure 15.2: Use of IgY in Aquaculture (Source: Mohammad *et al.*, 2016).

Application of IgY against Bacterial Diseases

Application of IgY in Crustaceans, Molluscs and Echinoderm

IgY antibodies have been developed against bacterial pathogens such as *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. splendidus*, *Enterobacter cloacae* etc.). Passive immunity could possibly protect the crustaceans, molluscs and echinoderms from several diseases including the diseases caused by stress, as they lack an adaptive immune system (Gao *et al.*, 2016a; b; Hu *et al.*, 2019). Study conducted by Li *et al.* (2006) showed anti-*A. hydrophila* IgY protect polyploid gibel carps when challenged with formalin inactivated *A. hydrophila*. The study by Qin *et al.* (2018) showed anti-*A. hydrophila* IgY antibodies significantly increased immunity and maintained the structural integrity after vaccination. Fernandes *et al.* (2019) from their study with anti *A. hydrophila* IgY showed exhibition of specificity and affinity for membrane and cytoplasmic *A. hydrophila* proteins, which help to detect the intra and extra cellular bacterial colonization in the host tissues. Thus, the findings claimed a new perspective on the use of IgY for the diagnosis and analysis of *A. hydrophila* pathogenesis in Nile tilapia (Fernandes *et al.*, 2019). *Aeromonas salmonicida* is a causative agent of ulcers on fish skin. When anti- *A. salmonicida* IgY added in powder form to the water environment of ornamental Koi carp (*Cyprinus carpio koi*), showed good prophylactic activity against the infection and protection from skin ulcer (Gan *et al.*, 2015).

Vibrio spp. are major causes of mortality in shrimp which is lacking adaptive immunity. The study of Gao *et al.* (2016a) and Kumaran *et al.* (2018) showed the protection against *V. harveyi* and *V. parahaemolyticus* on *Litopenaeus vannamei* and *F. indicus*. Toxin-producing *V. parahaemolyticus* caused acute hepatopancreatic necrosis disease (AHPND) to shrimp and anti-PirA-IgY added feed found an effective prophylactic method against this infection. Similar study by Keetanon *et al.* (2021) in the shrimp post larvae 12 (PL 12) of *L. vannamei* (Pacific white shrimp) infected with *V. parahaemolyticus* furnished reduction in mortality rate (25-40 per cent) in contrast to the control group (60 per cent) with IgY-feed proved the effects of IgY on growth, immune responses and pathogenic tolerance.

Experiment conducted with specific and nonspecific anti-*V. alginolyticus* IgY, enhancement of resistance against vibriosis was found in specific IgY feeding experiments and this study also reported the increased survival rate of 90 per cent when fed with specific IgY compared with 10–30 per cent survival rate with nonspecific IgY feed (Wu *et al.*, 2011). In IgY against *V. splendidus* (causing skin ulceration syndrome) was produced and injected intra peritoneal via. 80 per cent of survival rate was occurred in sea cucumber (*Apostichopus japonicus*) and burden of bacteria in IgY treated sea cucumber was also less in the respiratory tract and intestine when compared with untreated control (Li *et al.*, 2016).

Enterobacter cloacae caused mass mortality of *M. rosenbergii* zoeal stages in hatcheries during 2017 in China. Specific egg yolk powder treatment could achieve

the survival rate of 67 per cent after *E. cloacae* infection compared to 20 per cent survival in non-specific egg yolk powder and 17 per cent survival in no egg yolk powder treatment. The damage to hepatopancreas was significantly lower in *M. rosenbergii* treated with specific egg yolk powder than those treated with non-specific egg yolk powder provided a strategy to control the disease by using specific IgY (Gao *et al.*, 2019).

Application of IgY in Fishes

The passive immunization in fish is a good alternative to using antibiotics/chemotherapy, and it is always likely to be successful if the infection occurs through gastro-intestinal tract. Enhancement of phagocytic activity of macrophages for *V. anguillarum* in the presence of specific IgY and decreased levels of tumor necrosis factor-dinna (PaTNF-dinna), interleukin-1dippa (PaIL-1dippa), transforming growth factor-dippa (PaTGF-dippa) and leukocyte cell-derived chemotaxin-2 (PaLECT2) are reported in ayu fih, *Plecoglossus altivelis* against *V. anguillarum* after passive immunization by oral intubation with pathogen-specific IgY (Li *et al.*, 2014). Arasteh *et al.* (2004) reported the protection against *V. anguillarum* by using IgY antibodies through feeding, intraperitoneal injection or oral intubation.

V. harveyi has become a common cause of skin ulceration in Japanese pufferfish in recent years (*Takifugu rubripes*). The first publication on using IgY to manage bacterial illness in pufferfish found that prophylactic and therapeutic effects were achieved through oral administration and immersion of IgY and found improved macrophage phagocytosis of *V. harveyi* (Zhang *et al.*, 2021). The specific IgY incorporated feeding trial in carp (*Cyprinus carpio*) exhibited significant bacteriolytic effects on the non-O1 *V. cholerae* pathogen and control the disease (Gao *et al.*, 2017).

Loma salmonae is a microsporidian parasite infecting Pacific salmon and a polyclonal IgY preparation is found to be useful of detecting this parasite in Pacific salmon (Young *et al.*, 2007). Oliver *et al.* (2015) developed IgY against intracellular bacteria *Piscirickettsia salmonis* affecting salmonids causing the disease Piscirickettsiosis. After treating the cell line with specific IgY for 1 h, it was found that the cell line remained uninfected for up to 10 days, when infected with bacteria. Gutierrez *et al.* (1993) reported the neutralizing efficacy of IgY antibodies through oral route against *Edwardsiella tarda* infection and proposed that when Edwardsiellosis/paracolo disease is anticipated, daily administration of anti-*E. tarda* IgY antibodies could be a prophylactic measure. The anti-*E. tarda* IgY antibody test confirmed the prevention of paracolo disease by anti-*E. tarda* IgY antibody (Hatta *et al.*, 2008). Enhancement of passive immunity via feeding microencapsulated specific IgY against Edwardsiellosis, an extremely harmful disease can be caused by *Edwardsiella tarda* suggested the use of this IgY as a valuable preventative in turbot against *E. tarda* 2CDM001 infections (Xu *et al.*, 2020). Experiment conducted by Lee *et al.* (2000) in rainbow trout (*Oncorhynchus*

mykiss) fed with anti-*Yersinia ruckeri* IgY antibodies showed lower mortalities after 8 days and found lower number of infection in organs and intestine caused *Y. ruckeri* (pathogen of enteric redmouth disease). Pseudotuberculosis is another fish disease caused by *Photobacterium damsela* subsp. piscicida (Phdp) and Eto *et al.* (2019) from their study reported that anti-*Photobacterium damsela* IgY inhibited the dissemination of *Photobacterium damsela* that could be helpful for the treatment and prophylaxis of this disease.

Application of IgY against Viral Diseases

White spot syndrome virus (WSSV) infection is a major cause of Shrimp mortality in hatcheries and farms. The study of Kim *et al.* (2004) reported the survival rate of 50 per cent at day 15 post-challenge with WSSV against shrimp treated with 0.01 mg/10 µl of IgY (produced against the WSSV protein) and suggested these IgY as a potential immunotherapeutant to prevent WSSV infection in *P. chinensis*. By using specific IgY from the hens that had been immunized with inactivated WSSV to neutralize WSSV, the shrimp showed high survival rate (73.3 per cent) contrary to those hens that were immunized with DNA vaccine (33.3 per cent) (Lu *et al.*, 2008). Decreased mortality rate of 20 per cent was observed in crayfish treated with IgY antibodies from inactivated WSSV when compared with 80 per cent mortality with DNA vaccine (Lu *et al.*, 2009) suggested the protection of crayfish (*Procambarus clarkii*) against the WSSV. The results of Kumaran *et al.* (2010) suggested the use of IgY is an excellent biological source of medicine and prophylactic agent against WSSV infection in shrimps.

Carp are highly infected by Cyprinid herpesvirus 3 (CyHV-3), causes fatal disease with high mortality rate and it was reported that anti-CyHV-3 serum IgY from eggs could prevent the infection of carp brain cell. Liu *et al.* (2021) from their study suggested the effectiveness of groupers from red-spotted grouper nervous necrosis virus (highly infectious and lethal, mainly infects marine fish larvae) and found 70 per cent protection against this virus infection when fed with specific egg yolk powder.

Conclusion

IgY technology has been extensively used in health researches, as both therapeutic and diagnostic tool in human and veterinary field. However, the development of IgY technology is in a developing stage and there are scope for various diagnostic and therapeutic IgY antibodies in fisheries and aquaculture sector. This technology can be used in aquaculture or preventing and treating bacterial, viral and parasitic infection diagnosis, tumor diagnosis, hematological tests, various enzyme detection assays, identification of substances *etc.* Besides, IgY represents significant therapeutic resource instead of antibiotic application and also resource for controlling emerging viral infections for which there is no treatment.

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