EXTRACTION AND PURIFICATION PATHOGENIC Escherichia coli FOR HUMORAL IMMUNE RESPONSE

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Abstract:

In the present investigation, the affected gold fish was obtained from the fish rearing farms and microorganisms were isolated from the organs such as muscle, gill, liver and intestine. The highest microbial load ($6.3 \pm 0.4 \times 10^7$ cfu g^{-1}) was observed in muscle tissue of the infected *C. auratus*. The lowest microbial load ($4.3 \pm 0.7 \times 10^4$ cfu g^{-1}) was found in intestine of the fish *C. auratus*. The percentage distributions of mycotic and bacterial isolates are observed**.** The biochemical characterization of the pathogenic isolates was performed and the microorganisms were identified up to the generic level. Based on the results of lethal concentration and minimum inhibitory concentration test to the isolates, *E.coli* was found to be highly antigenic to the fish *Catla catla and hence E.coli strain was selected for preparation of antigen.* From the *E.coli* strain five different types of antigens such as heat killed antigen, whole cell antigen, heat killed antigen with antiserum, whole cell antigen with antiserum and nucleotide antigens were prepared and injected in to the experimental fish (*Catla catla*)groups for the study of immunomodulation. Analysis of immunogenicity of antigens against the fish *Catla catla* was estimated. The *E.coli* produced β hemolytic pattern on the blood agar plate. B Lymphocytes counts using rosette forming assay revealed significant decrement in pathogens exposed fishes than in control. In conclusion, the results from the present investigation suggest that it is impendingly achievable to develop a commercial vaccine against the *E.coli* using immune complexes which will overcome the issues of the heterogenicity of the bacterium. It is also possible to improve the vaccine by adding additional antigens to other diseases to this formulation. The aquaculture desperately needs such a formulation to manage and overcome the distressing disease problem caused by the microorganisms.

Keywords: *Catla catla, Humoral immune response, E.coli and pathogens.*

1. Introduction

Bacterial diseases cause almost more suffering and death than any other infectious agents. It remains a major health problem of global concern [1]. The previous work suggest that about 2 billion people are infected with bacterial infectious organisms, of which 8-10 million develop active disease with 2 million deaths annually [2]. For diagnostic purposes purified protein derivatives are used to detect immune reaction to bacterial antigens [3]. The evolution of antimicrobial drug resistance is a major impediment to infectious disease control. Disease prevention methods such as improved hygiene, antibiotics and vaccine developments have proven successful in controlling many diseases, but the effectiveness of vaccines depends on the standing and evolving variability of serotypes in a given population of bacterial pathogens [4]. Therefore, there is a need to identify and evaluate bacterial antigens for the specific diagnosis of bacterial disease and to develop effective and safer vaccines to replace allopathic drugs. There are many diseases known today for which no efficacious vaccines exist and the use of low efficacy vaccines may actually reduce profitability [5].

Fish diseases caused by *Aeromonads* is considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced production and low quality of aquatic organisms [6]. Most of their earlier research on the fish immunology has focused on the comparative aspect of the immune system with fish and other species. Nevertheless, recent research has focused on understanding how the fish immune system responds to foreign agents or how innate resistance can be improved to produce stock of fish with superior resistance [7].

Non specific defense mechanisms such as the skin and scales and lytic enzymes of the mucus and serum and cellular mechanisms like monocytes, macrophages, neutrophils and cytotoxic cells are present in fish [8]. Isolation of drug resistant microorganisms from food is an increasing public health problem. Being easily spread among the population, they can cause an epidemic outbreak, especially if sanitary conditions are not optimum [9]. Several defined antigens of bacterial organisms have become available either through the purification of natural

antigens using biochemical techniques or by producing large quantities of recombinant antigens using molecular biology procedures [10]. Some of these antigens have been shown to be useful as new vaccine candidates against bacterial disease [11].

A new technique for the prevention of fish diseases is rapidly emerging as a result of research into the development of fish vaccines. Fish immunology has a more recent history than human and veterinary immunology but the techniques used are similar. However, methods of administering vaccines to fish differ and are dependent upon species, pathogen, temperature, and environment [10]. Vaccination is still the best way for prevention of bacterial diseases. Undesirable side effects, supplementary vaccines prepared from whole bacteria, are avoided by the use of antigenic bacterial sub units. Bacterial sub units are mainly poor immunogens. Antigens bound in an immune complex (IC) with specific antibodies effects an improved immune response rather than antigen alone. Hence in the present study attempts have been made to assess antigenicity of pathogen and compare immune response of fish, *Catla catla* against antigens and immune complex.

2. Materials and Methods

The infected fish samples *Mugil Sp* was collected in pre sterilized container from the fishing area in Pondi, Tamilnadu, India. The collected fish sample was transported to the laboratory in an icebox for further analysis. From the infected fish, *Mugil Sp* pathogenic strains (*Escherichia coli*) were isolated and prepared the whole cell bacterial antigens, heat killed antigen, whole cell antigen with antiserum, heat killed antigen with antiserum and nucleotide antigen. LC_{50} values of all bacterial pathogens in *Catla catla* were calculated. From the LC_{50} value sublethal concentration of antigens were given to the test fish, *Catla catla* for further study.

2.1.Humoral immune response estimation: The humoral immune response of fish, *Catla catla* administered with antigens was analyzed by screening of antibody, B-cell erythrocyte assay and plaque forming cell assay (PFC).

Analysis of cell mediated immune response: The cell mediated immune response of fish, *Catla catla* administered with antigens were analysed by T-cell erythrocyte rosette assay, Delayed type hypersensitivity (DTH) and lymphocyte migration inhibition test.

Immunopropylaxis analysis: From the tested bacterial antigens, highly virulent *antigen* was used to study immunopropylatic activity in fish, *Catla catla*.

2.2. Enumeration of bacterial organisms

From the infected fish samples muscles, gills, liver and intestine were dissected and used for pathogen isolation. The pathogenic strains were isolated with help of sterile swab on dissected samples and spread over the nutrient agar plates. The plates were incubated at 37ºC for 24 - 48 hrs. After incubation total heterotropic bacterial population (THBP) was enumerated and recorded. The morphologically different microbial strains were identified in bacterial plates. The colonies were isolated and purified by re-streak method. The isolated colonies were streaked on nutrient agar slants, incubated overnight at 37º C. The following tests were performed for identification of selected colonies isolated from the fish samples. A loopful of culture was inoculated and cultured for standarfd biochemical characterization.

2.3. Antigen preparation

Thoroughly checked fishes which record good sexual and physical health were only chosen for the present study. A total of 36 fishes grouped into 6 cages (6 fishes each) were treated with different types of antigens such as whole cell bacterial antigen, whole cell bacterial antigen with antiserum, heat killed antigen, heat killed bacterial antigen with antiserum and nucleotide antigen and one set left as control. Pathogens were found to adversely affect the fish growth, feed consumption and reproductivity.

2.4. **Analysis of immunogenicity of antigens**

The prepared bacterial antigen administered to the fish, *Catla catla.* After administration of antigen the changes of fish and immunogenicity of antigens were screened. Due to administration of antigen the growth of the body weight was determines followed by consumption food value and reproductivity also screened by standard methods. Character change of fish movement in habitat was observed.

2.5. Humoral immune response

In the present study humoral immune response was analyzed by antibody titration. B cells e rosette assay and plaque forming cell assay techniques are carried out. The test fish, *Catla catla*

divided into 6 groups (each groups contain 6 animals) and treated with different types of antigen (One group served as control) prepared from *E.coli* .

2.6. Screening of antibody: From the normal and antigen treated fishes five ml of blood sample was collected and serum separated for antibody screening. Quantization of serum antibodies was carried out by antibody titre plate technique containing respective antigens. 25µl of physiological saline was added into all wells of microtitre plate then 25µl of antiserum was added into the first well of microtitre plate, the antiserum was seriously diluted in the well of the first row till the 11^{th} well of the microtitre plate leaving the 12^{th} well as positive control. Then 25µl of 1% test antigen in saline were added to all the wells of the microtitre plate. The plate was hand shaken for the effective mixing of reagents and incubated for an hour 37˚C. The highest dilution of serum samples which shows detectable agglutination was recorded and expressed in $\log_2 2$ titre of the serum. E-roseette assay used for detection of B cell in this study.

3. Results and Discussion

The isolation of microorganism is based on the infected fish species, its disease status, clinical signs and biochemical diagnosis. The infected gold fish from which the bacterial strains were identified**.** The results of the quantitative estimation of microbial count in muscle, gill, liver and intestine of fish are given in the **Table .1.**

Table 1. Enumeration of THBP in *C.auratus* fish samples (Muscle, gill, liver and intestine).

S. No	Sample	Colony forming unit g-1 (CFU g-1)
1	Muscle	$6.3 \pm 0.4x10^7$
2	Gill	$5.7 \pm 0.6x10^6$
3	Liver	$7.2 \pm 0.9x10^5$
4	Intestine	$4.3 \pm 0.7x10^4$

(Values are mean ± SD)

The highest microbial load ($6.3 \pm 0.4x10^7$) cfu g^{-1} was observed in muscle tissue of the fish sample and the lowest microbial load ($4.3 \pm 0.7x10^4$) cfu g^{-1} was found in intestine of the infected fish.

The predominant strains *E.coli* was administered to the healthy normal juvenile *Catla catla* for determination of LC_{50}.The *Catla catla* fish used in the study. From the mortality rates the LC_{50} value was calculated and recorded in **Tables 2**. The LC_{50} value for the *Escherichia coli* was 3.16 x 10^6 CFU/ml.

Table 2. Analysis of LC_{50} value of *Escherichia coli* in *Catla catla*

Conc. of *E.coli* (cfu/ml)	**Initial Number**	**Dead**	**Survival**	**Dead ratio (%)**	**Survival Ratio (%)**	**Mortality**	**Cumulative mortality (%)**
10^8	10	10	0	30	0	30/30	100
10^7	10	8	2	20	2	20/22	90.90
10^6	10	5	5	12	7	12/19	63.16
10^5	10	5	5	7	12	7/19	36.84
10^4	10	2	8	2	20	2/22	9.90

In the present study the mean bacterial load was found to be high in muscle load ($6.3 \pm 0.4x10^7$) cfu g^{-1} followed by gills load ($5.7 \pm 0.6x10^6$) cfu/ml, liver ($7.2 \pm 0.9x10^5$) cfu/ml and intestine ($4.3 \pm 0.7x10^4$cfu/ml). Similarly Al-Harbi and Uddin [12] stated higher bacterial load in gills followed by intestine of hybrid tilapia. In the present study, 19 bacteria and one fungus were isolated from infected *Mugil Sp* . These results are supported by Katoch *et al.*, [13] who has reported 25 bacterial and fungal species in fresh water carp at Himachal Pradesh, India.

In the present study, *E.coli* was dominant in the bacterial isolates found in infected fish samples. The findings of the study are supported by Thampuran *et al.*, [14] who have reported the dominance of *E.coli* in the EUS affected *C. striatus*. Motile aeromonads have been associated with the surface of lesion in EUS fishes [15]. In the present investigation Gram negative (75%) bacteria comprised major part among the isolated microorganisms.

3.2. Antigen Analysis

In this study pathogen decreases the body weight compared to normal fishes. Some notable changes were also noted in activity, growth, feed consumption and reproduction. It is concluded, that the pathogenic organism acts as biotic stress to the host animals. The fish groups treated with antigen were found to be more susceptible to *E.coli* challenge.

Table 3. Analysis of immunogenicity of antigens against the fish *Catla catla*

S. No	**Character**	***E.coli* whole cell bacterial antigen**	***E.coli* whole cell bacterial antigen with antiserum**	***E.coli* heat killed antigen**	***E.coli* heat killed antigen with antiserum**	***E.COLI Nucleotide antigen***
1.	Character change	+	+	+	+	+
2.	Induce NSI	++	+	+	+	+
3.	Induce HI	++	++	++	++	++
4.	Induce CMI	+	+	+++	+++	++
5.	Vaccination	++	++	++	++	+++

+= Moderate change observatrion, ++ = Positive observation

+++= Highly positive observation

Virulence of *E.coli* was multifunctional and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesions, agglutinations and various hydrolytic enzymes [16]. Virulence factors are present in two forms, cell associated structure and extracellular products.

3.2.**Immune response**

Humoral immunity is mainly mediated by serum antibodies which are the protein secreted by B cell compartment of immune responses. The reaction between antigen and antibody involve several steps like recognition, stimulation and neutralization. It has been analyzed by different method of antigen antibody reactions which are presented here.

To optimize the serum dilution with saline, control serum was loaded over a range of dilution from 1:1250 to 1:320 and this appeared to be the highest antibody titre **(Table 4).** An estimation of antibody levels in the serum after an antigenic challenge will expose the functioning of humoral immune systems. In immunodeficient animal, antibody production was affected and hence humoral response against a disease causing antigen was less. But the immune complex treated animals enhance the production of antibody. In the present study, pathogen and heat killed pathogen treated fishes showed an antibody suppressive effect. The suppression of antibody reflects on the reduction of humoral immune response and this state subjects to easy infection. Immunomodulation of whole pathogen with antiserum treated fishes showed moderate change in antibody production. Cukrowsha *et al.,* [17] stated that the damage to splenic Ig secreting cell reduce antibody production in fishes. A similar observation was made in the present study too.

Table 4. Character analysis of *Catla catla* exposed to antigens

S. No	Character	Heat killed bacterial antigen	Whole cell bacterial antigen	Heat killed bacterial antigen with antiserum	Whole cell bacterial antigen with antiserum	Nucleotide antigen
1	Weight of the animal (gm)	20	20	25	28	20
2	Movement of the animal	Resting	Abnormal	Restless	Abnormal	Abnormal
3	Adulation days	24	27	32	24	9

The immune complex of the samples tested were immune enhancer for antibody production. This was expected in animals were obviously exposed immune complex it will resist many intestinal pathogens. Earlier studies reported cross reaction of *Escherichia coli* with antibody of many other pathogens such as *Citrobacter* sp., *Brucella* sp. and *Salmonella* sp. [18].

B Lymphocytes counts using rosette forming assay revealed significant decrement in pathogens exposed fishes than in control (Table 5). Of the two pathogens decrement in B lymphocyte it was much pronounced in *Escherichia coli* in the first and *Staphylococcus aureus* pathogens had more or less similar impact on B cell estimation. The present study, clearly confirms the decrement in B cell number in fish exposed to whole and heat killed pathogens. In this study conclude, impact of whole cell and heat killed pathogenic molecules on the synthesis, proliferation and activation of lymphocytes. Sujatha *et al.,* [3], reported that differentiation of B cell counts is affected by pathogens.

Table 5. Enumeration of B cells using rosette forming assay in fish *Catla catla* exposed to different sublethal concentrations of *E.coli* antigens

S.No	***E.coli* Test antigens**	**Number of B cells Rosette formed in 100 lymphocyte observed**		
		I week	**II week**	**III week**
1.	Control fishes	18	18	18
2.	Heat killed bacterial antigen	8	6	9
3.	Whole cell bacterial antigen	8	7	9
4.	Heat killed bacterial antigen with antiserum	9	8	11
5.	Whole cell bacterial antigen with antiserum	6	6	8
6.	Nucleotide antigen	8	6	9

Fishes exposed to pathogenic strains ($1/10^{th}$ sublethal concentration) for 3 weeks showed reduction in PFC. Effect or pathogenic antigens in direct spleenic plaque forming cells (1g M producing cells) showed a reduction in secondary plaque forming cell in the first 3 weeks and a time and dose dependent decrease in primary and secondary PFC response (Table 6)**.** The Peak antibody formation occurred in the first week of primary response and in the third week the secondary response in control as well as in treated animals was seen indicating that there was no delay in antibody formation. Moreover, the present study demonstrates that all pathogenic

antigen exposure resulted in a suppression of primary IgM type plaque forming cell in response to antigens in fishes.

Table 6. Plaque Forming Assay of fish *Catla catla* at different Time intervals

S.No	*E.coli* Test antigens	Distribution of PFC/10^6 Spleen cells		
		I week	II week	III week
1.	Control fishes	36.3	36.4	37.1
2.	Heat killed bacterial antigen	10.2	11.4	12.8
3.	Whole cell bacterial antigen	11.2	12.1	15.8
4.	Heat killed bacterial antigen with antiserum	10.7	11.6	12.9
5.	Whole cell bacterial antigen with antiserum	11.1	12.3	12.7
6.	Nucleotide antigen	10.3	11.7	12.8

A remarkable observation enhancement in B cell production is due to immune complex of antigens was noted in the present study. The enhancement of this type of immune responses confirms the potential of immune complexes to be used as vaccines.

E.coli is a well recognized pathogen particularly of freshwater fish with an almost worldwide occurrence. However, the organism is not only associated with fish diseases, but has been implicated as possible cause of human gastroenteritis leading to diarrhea *E.coli* In immunocompromised individuals, the organism may cause wound infections leading to septicaemia. To prevent diseases caused by this organism, various measures have been adopted, of which the use of antibiotics is common one. To a lesser extent, vaccines have been considered, although the process of immunization and vaccine development is costly and

relatively slow. The emergence of antimicrobial resistance among bacteria is makes them among the most difficult bacteria to treat in aquaculture situations. As the efficacy of antibiotic therapy rapidly wanes, attention must be focused upon new approaches to controlling infection. Vaccines are one of the few protective measures that can potentially save money and improve output of farming operations. The inductions of immune cells against the optimal concentration of *E.coli* were confirmed by comparing the several immunological assays in the normal fish. Immunomodulation to immune system was accessed directly by quantifying immunological factors that govern cell mediated and humoral immune response. All these tests indicate efficacy of immune complexes on immunomodulators. All immunoglobulin are antibodies and are produced in response to antigenic challenge. The synthesis of different types of immunoglobulin in normal fish and how far the heat killed pathogen and whole cell pathogen had impaired the immunoglobulin synthetic pathway were assessed. It can modulate the humoral mediated immunity.

In conclusion, the results from the present investigation suggest that it is impendingly achievable to develop a commercial vaccine against the *E.coli* using immune complexes which will overcome the issues of the heterogenicity of the bacterium. It is also possible to improve the vaccine by adding additional antigens to other diseases to this formulation. The aquaculture desperately needs such a formulation to manage and overcome the distressing disease problem caused by the microorganisms.

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