ANTIGEN
Chapter II – ANTIGEN

2.1. INTRODUCTION

An antigen is a substance that is responsible for the production of specific antibody molecules when introduced into tissues of susceptible animals. A small site on antigen to which a complementary antibody may specifically bind is called an epitope. An immunoglobulin capable of specific combination with the antigen that causes its production in susceptible animals is called an antibody (Dhasarathan et al., 2010).

*A. hydrophila* is a primary pathogen (Esteve et al., 1993), secondary pathogen (Joice et al., 2002) and opportunistic pathogen (Dooley and Trust 1988 and Lio-po et al., 1996) of a variety of aquatic (fish) and terrestrial animals, including humans. It is a ubiquitous, free living, gram negative bacterium, mainly found in water and water related environments and causes a wide variety of symptoms (Hazen et al., 1978). The disease caused by *A. hydrophila* is called Motile Aeromonad Septicaemia (MAS) and this pathogen is associated with number of other diseases in fish, for example, epizootic ulcerative syndrome (EUS) as a secondary pathogen (Roberts, 1993; Pathiratne et al., 1994 and Lio-po et al., 1998).

Progress has been made towards the goal of active immunization against many of the major opportunistic pathogens. This approach is facilitated by the facts that compromised patients respond immunologically to active vaccination and the fact that...
65% of surgeries are elective, which would indicate that populations at risk could be identified for vaccination. The discovery and application of new antigens and techniques, particularly conjugative vaccine technology have created optimism. A vaccine based on fibronectin binding protein induces protective immunity against mastitis in cattle and it may also be used as vaccine in humans.

The present analysis focuses on the effect of 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 on the fresh water fish, *Catla Catla*.

2.2. MATERIALS AND METHODS

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.

2.2.1. Preparation of bacterial antigen

The isolated bacterial pathogen, *A. hydrophila* was subcultured in trypticase soy broth and used to prepare different types of antigens.
2.2.1. a. Preparation of whole cell bacterial antigen

The *A. hydrophila* culture was centrifuged at 3000rpm for 30 minutes, pellet is washed with PBS and then packed cells were resuspended to desired concentration (2.5 ×10⁵ cells / ml) in PBS after counting in haemocytometer.

2.2.1. b. Preparation of whole cell antigen with antiserum

The prepared whole cell bacterial antigen was injected (primary and secondary doses) into the laboratory acclimatized fishes. After primary and secondary dose immunization, antiserum was collected from immunized fishes and mixed with whole cell bacterial antigen in the ratio 1:1. The mixed antigen and antiserum (immune complex) used as antigen for further analysis.

2.2.1. c. Preparation of heat killed antigen

The cultured bacterial organism (approximately 2.5 ×10⁵ cells / ml) was heated at 60°C for 1 hr in a water bath. The above heat killed culture was centrifuged at 3000rpm for 15 minutes then pellets washed with PBS once and used for further analysis.

2.2.1. d. Preparation of heat killed bacterial antigen with antiserum

The prepared heat killed bacterial antigen was injected (primary and secondary doses) into the laboratory acclimatized fishes. After primary and secondary dose immunization, antiserum was collected from immunized fishes and mixed with heat killed whole cell bacterial antigen in the ratio of 1:1. The mixed antigen and antiserum (immune complex) used as antigen for further analysis.
2.2.1. e. Preparation of nucleotide antigen

Five ml of overnight culture (2.5 ×10^4 cells/ml) was taken in a centrifuge tube, and was spun at 10,000 rpm for 5 minutes. Pellet was washed with water followed by addition of 467µl of TE buffer to it. The mixture was mixed well by vortex and added 30µl of 10% SDS and inverted the tube for few times to mix well. Then it was incubated at 37°C for one hour. Equal volume of phenol – chloroform solution was added to this and spun at 10,000 rpm for 5 minutes. Aqueous layer was transferred carefully to another tube containing 1/10th volume of 3M sodium acetate and 0.6 volume of isopropanol and mixed with glass rod. The pooled DNA was resuspended in 100µl of TE buffer and used as PCR sample.

2.2.2. Polymerase chain reaction (PCR)

2.2.2. a. Pre – PCR

In pre – PCR, DNA was extracted from *A. hydrophila* culture following the method developed at Shri Me Disease Laboratory (SDDL) of Tamilnadu veterinary and animal science university, Chennai which is as follows: 200µl of *A. hydrophila* was added with 800µl of DNA Extraction buffer. Then the sample was incubated at room temperature for 30 minutes. The mix was then centrifuged at 5000 rpm for 5 minutes at 4°C. 500µl of supernatant was transferred to 1.5ml eppendorf tube and 500µl of ice cold ethanol was added to the supernatant and mixed well. The mixture was then centrifuged at 14000 rpm for 20 minutes at 4°C and the supernatant was discarded. The pellet formed was washed by centrifugation with 500µl of 95% ethanol at 10000 rpm for 10 minutes at 4°C and the supernatant was discarded. The pellet obtained was then washed with 500µl of 90% and 70% ethanol at 10000 rpm for 10 minutes respectively at
4°C and the supernatant was discarded. The obtained pellet was dried at 37°C. The pellet was resuspended in 25µl of distilled water and stored at -20°C and used as template for PCR amplification.

2.2.2. b. PCR

PCR amplification of aerolysin and hemolysin genes were performed in a total volume of 25µl with 2X PCR Master Mix, 1µl of extracted template DNA, 1µl of each forward and reverse primers specifics for aerolysin genes. The PCR mixture was subjected to PCR amplification of aerolysin genes with a denaturation at 95°C for 5 minutes and 30 cycles with denaturation at 95°C for 15 seconds, annealing at 66°C for 30 seconds, extension temperature at 72°C for 30 seconds and a final extension at 72°C for 7 minutes for amplification of aerolysin gene. The PCR mixture was subjected to PCR amplification of hemolysin gene with 30 cycles with denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes.

2.2.2. c. Post - PCR

PCR amplified products of aerolysin and hemolysin genes were separated by agarose gel electrophoresis and visualized using a gel documentation unit. 1.5% agarose gel was prepared by dissolving 0.6g of agarose in 40 ml of TBE buffer (1X). The mixture was heated in a microwave at full power for 2 minutes and swirled at intervals to dissolve the agarose to create wells. The gel was allowed to solidify for 30 minutes. The comb was then removed, the gel was placed in the gel tank and covered with TBE buffer (1X). PCR product (8 µl) and loading dye (4 µl) were mixed and loaded on the wells. The tank
was connected to the electrophoresis power pack and the PCR products were separated by running a gel at 110V and 150mA for 1 hour. The products were observed in a gel documentation unit under UV illumination. The amplified DNA content was used as nucleotide antigen.

2.2.3. 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 determined followed by consumption food value and reproductivity also screened by standard methods. Character change of fish movement in habitat was observed.

2.2.4. Hemolytic activity

Hemolysis of pathogenic antigen was analysed using standard method. The hemolytic plates were enriched with 5% whole sheep blood. *A. hydrophila* culture was streaked on to the medium; the plates were incubated at 30°C and were checked for the type α or β hemolytic activity after 24 hours.

2.3. Results and Discussion

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 *A. hydrophila* challenge.
Table 2.1: Analysis of immunogenicity of antigens against the fish *Catla catla*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Character</th>
<th><em>A. hydrophila</em> whole cell bacterial antigen</th>
<th><em>A. hydrophila</em> whole cell bacterial antigen with antiserum</th>
<th><em>A. hydrophila</em> heat killed antigen</th>
<th><em>A. hydrophila</em> heat killed antigen with antiserum</th>
<th><em>A. hydrophila</em> Nucleotide antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Character change</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Induce NSI</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Induce HI</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4.</td>
<td>Induce CMI</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>5.</td>
<td>Vaccination</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+= Moderate change observation,  ++ = Positive observation
++++= Highly positive observation
Virulence of *Aeromonas* sp was multifunctional and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesions, agglutinations and various hydrolytic enzymes (Janda *et al*., 1996). Virulence factors are present in two forms, cell associated structure and extracellular products.

*Aeromonas* strains are piliated, a detailed analysis of such structure was not undertaken untill recently. However, Carrello *et al*., (1988) observed two distinct morphological types of fimbriae on *Aeromonas* strains under transmission electron microscope, one is designated a “straight” pilus and other one is more curvilinear and termed “ flexible”. Kay and Trust (1991), identified an additional layer external to the cell wall in auto aggregating strains of *A.salmonocida* by transmission electron microscope that were pathogenic. The spontaneous loss of this layer from virulent *A.salmonocida* strains resulted in significant increase in 50% lethal doses (LD$_{50}$) of these strains.

The hemolysin produced by some *Aeromonas* sp is termed “Aerolysin”. And it possesses both hemolytic and enterptoxic activity. This hemolytic enterotoxin (aerolysin), has been shown to share significant homology with the cytotoxic enterotoxin (Act). It has two cytotoxic toxins (Alt and Ast) as reviewed by Xu *et al*., (2001). Early investigations of *Aeromonas* toxins were responsible for recognition of “aerolysin” in several species of *Aeromonads*. The toxins reported by various investigations have shown fundamental differences in properties, making comparisons difficult (Buckley and Howard 1999 and Chakrabarthy *et al*., 1984).
The *catla catla* fish samples used in this study are shown in **Plate 2.1**. The hemolytic activity in the blood agar plate confirmed that the isolate was hemolytic in nature as shown in **Plate 2.2**. PCR amplification of the DNA extracted from the isolate, using the Aerolysin primers of Kingkombe *et al.*, (1999) and hemolysin primers of Sen and Rodgers, (2004) resulted in the expected product size of 232 base pairs and 597 base pairs respectively as shown in **Plate 2.2**.

Many other factors also contribute to the virulence of *A.hydrophila*, such as α-amylase, protease, DNAase and heamagglutination ability. Few of the virulence factors were tested using the cell – free culture supernatant after overnight cultivation of the isolates in Brain Heart Infusion Broth (BHI). Antibiotics are the major agents for controlling *A.hydrophila* (Fang *et al.*, 2004). Several workers described effective antibiotics such as sulfonamide, chloramphenical, neomycin, sulfamethoxazole, trimethoprim, streptomycin, naladixic acid, oxolinic acid, neomycin and sarafloxacin rifampicin oxytetracycline, cephamycins and moxalactam ciprofloxacin amoxicillin and enrofloxacin (Ilhan *et al.*, 2006; Ansary *et al.*, 1992; Tafalla *et al.*, 1999; Landre *et al.*, 2000 and Ko *et al.*, 2003). *A. hydrophila* is also found to be sensitive to other chemotherapeutants such as amino acid derived hydroximates (Walter *et al.*, 1999) and hydrogen peroxide (H₂O₂) (Mitchell and Plumb, 1980),
Plate. 2.1. *Catla catla* fish used in the challenge study.

Plate.2.2. PCR Amplification of the Aerolysin and Hemolysin Gene Associated with Toxin Production