RESPONSE OF LABEO ROHITA TO DNA VACCINE AGAINST EDWARDSIELLA TARDA

Dissertation submitted in partial fulfilment of the requirements for the degree of

M. F. Sc. (AQUATIC ANIMAL HEALTH MANAGEMENT)

by

Ranjeeta Kumari, B. F. Sc.
(AAH-MA2-05)

CENTRAL INSTITUTE OF FISHERIES EDUCATION
(University under sec.3 of UGC Act)

Indian Council of Agricultural Research

Versova, Mumbai – 400 061

JUNE 2014
DEDICATED TO:

My parents & my beloved husband

......all praise is to almighty God!!!
DECLARATION

I hereby declare that the dissertation entitled “Response of Labeo rohita to DNA Vaccine against Edwardsiella tarda” is an authentic record of the work done by me and that no part there of has been presented for the award of any degree, diploma, associateship, fellowship or any other similar titles.

Ranjeeta Kumari

Date: 30th June, 2014

Place: CIFE, Mumbai

M. F. Sc. Student
AAHMA-02-05
Central Institute of Fisheries Education
ACKNOWLEDGEMENTS

Writing this most difficult but non-taxing chapter is a soulful acknowledgement of gratitude towards people who stood to extend their helping hands in various ways during my academic life and especially during this study. While few names are being mentioned many more are missing, but I hope those names shall be read between the lines, I owe deep seated gratitude to all these missing names.

It is exquisitely a jubilating occasion and unique opportunity to express my deepest sense of indebtedness and everlasting gratitude to my esteemed guide Dr. Megha K. Bedekar, Senior scientist, Aquatic Environment & Health Management Division, CIFE, Mumbai, for suggesting this research topic and for his indefatigable effort, constructive comments, scholarly research guidance, heart warming encouragement, love, affection and especially his friendly gesture at every step of my dissertation work.

I owe my obligations to my advisory committee member, Dr. G. Rathore, Principal Scientist and Dr. M. Makeş, Senior Scientist, Aquatic Environment and Health Management division, for his precious help and constant support throughout my research work.

My sincere thanks to my advisory committee members, Dr. K. V. Rajendran, Principal Scientist, Aquatic Environment & Health management division, Dr. Gayatri Tripathi, Senior Scientist Aquatic Environment and Health Management division, for their valuable suggestions, indefatigable efforts, constructive comments, scholarly research guidance, comments and constant encouragement during my research work.

I profess my heartfelt gratefulness to Dr. W. S. Lakra, Director, CIFE, Mumbai, for providing me all the facilities to complete my dissertation work.

I owe a deep sense of reverence to all my respected teachers, faculty members of Fish Pathology & Microbiology Dept. Dr. K. Pani Prasad, Principal Scientist, Dr R. P. Raman, Senior Scientist and Sc. Dr. Kundan Kumar for their amicable co-operation and help.

I offer my indebtedness to the library staff for their timely help in providing books and journals necessary for my research work. My special thanks to Nalini madam for her timely help and constant encouragement.
I set forth my heartfelt thanks to my beloved senior Praveena di, Saima di, Roshani di, Abhay sir, Gazanand sir, Anutoshi sir, Dipika di, Pushpa di, Rakhi di, Kavita di, Himanshu sir, Dhawal Sir, Parth sir, Sheetal di, Angel for their kind help and suggestions throughout my work.

I sprightly acknowledge to my junior Sajal, for his timely support, help and continuous inspiration.

And then there are those who were sharing my tears and smiles. For their love and affection, I have nothing to pay in return, except candid promise of warm remembrance for the life. This comes as a royal salute to you dear Kunal, Sarvendra, Pooja, Aadi, Anirbaan, Jackson, Chinmayee, Mira, Shweta, C. Yadii, Aman Divakar, Ramesh, Amit. My special thanks to Saima di and Roshani di who helped me a lot in last step of my research work and remarked forever gratitude for them.

I especially like to acknowledge to my dearest Rakhi didi and Praveena didi for their timely support, help, valuable suggestions, without whom my research might not be a successful one. For you no words in the dictionary can express my soul felt gratitude and can value your support, care, support that i got in every worst as well as joyful situations during my research period. This is really unforgettable in my life time.

I am thankful to my sweet beloved juniors Sampa, Pooja, Shushila, Nazneen, Biswajeet, Tasok for their kind encouragement and help. A special note to More, Balu and Thambe Bai for their help and cooperation.

Words cannot express my heartfelt love and gratitude to my beloved Papa, Mummy, Bhaiya, Bhabhi, my father-in-law, mother-in-law for all their love, support and blessings whose selfless, empathy are the constant source of inspiration and whose guidance always propel me toward the brighter horizon of happiness. I feel sorry for being absent and unavailable to my family in times of difficulty as well as joy for too long. Really, there are no words to convey my gratitude to my beloved husband Rajeev kumar for his love, care and encouragement.

Lastly, I bow my head and bend my knees before the Almighty God who is most benevolent, beneficent and whose blessings have solely contributed for my success and for giving me enormous strength to overcome the adversities in my life.

30th June

Ranjeeta Kumari
M.F.ScCIFE- MUMBAI
सारांश

एक व्यापक स्पेक्ट्रम सुरक्षात्मक प्रतिजन के रूप में E. tarda के एक पुन: संयोजक गर्ल्सराल्डेर्ड-3-फॉर्सेट डिहाइड्रोजेन (GAPDH) एवं, IFNγ एक सहायक के रूप में रोह में E. tarda के खिलाफ उल्लेखनीय संरक्षण पेश करने के लिए सिद्ध किया गया है। यहाँ, इस प्रतिजन से टीका किये गए रोह की सुरक्षा और प्रतिरक्षा प्रतिक्रिया का अध्ययन किया गया। इस टीके के निर्माण की प्रभावकारिता चुनौती सुरक्षा अध्ययन, ऐसे SOD के उत्पादन, GPx और iNOS उत्पादन और टीके के प्रतिजन के रूप में सेल्यूलर प्रतिरक्षा प्रेरक गतिविधियों द्वारा निर्धारित किया गया था। व्यक्तिगत अध्ययन में E. tarda के साथ चुनौतीपूर्ण रोह में दो जीव, प्रयोग में एक समूह के मछलियों के बचाव के लिए GAPDH टीके एवं अन्य समूह के मछलियों के बचाव के लिए GAPDH + IFN जीन और चुनौतीपूर्ण रोह में सेल्यूलर प्रतिरोध क्रियाओं के प्रभावोत्पादक गतिविधियों जैसे SOD के उत्पादन, GPx एवं iNOS का उत्पादन का भी निर्धारण किया गया। इंजेक्शन के माध्यम से इन दो जीवों के थल्जिम्ब द्वारा टीका किये गए मछलियों में संचयी मृत्यु दर कम पायी गयी जब E. tarda प्रतिजन के साथ चुनौती थी जबकि विशिष्ट एंटीबॉडी और प्रतिरक्षित सीरा के जीवाणुनाशक गतिविधियों का उत्पादन रोह के साथ SOD के उत्पादन के बाद 40 गुना प्रतिवर्तन संचयी मृत्यु दर कम पायी गयी। इसका परिणाम E. tarda के खिलाफ GAPDH टीका की तुलना में GAPDH + IFN एक एक प्रभावी टीका उम्मीदवार को सुधित करता है और यह जीवाणु के रोह के बचाव के प्रतिरक्षा तंत्र को अन्तर्गत भी प्रदान करता है।
ABSTRACT

As a broad spectrum protective antigen, a comparative study on DNA immunization with recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) construct of *E. tarda* and a bicistronic construct expression GAPDH plus IFNγ of *L. rohita* as adjuvant was undertaken. Here, the protection and immune responses of rohu vaccinated with this antigen were studied. The efficacy of DNA vaccines construct was determined by challenge protection study, the cellular immune-effector activities such as production of SOD, GPx and iNOS production and antibody response. Fish were immunized with plasmids via intra muscular injection exhibited a low cumulative mortality when challenged with *E. tarda* antigen, while statistically high levels of specific antibodies, iNOS gene expression, WBC count, SOD and GPX enzymes were observed in immunized group (p < 0.05), where expression of iNOS gene has been found to increase up to 40 fold change after challenging with bacteria in GAPDH+IFN group which is higher as compared to other groups and GAPDH vaccine. The experiment revealed that the highest protection and immune response was generated in the GAPDH+IFN group compared to the other two groups and this might be explained by the specific cellular immune response generated in this vaccinated group against the specific antigen.
### CONTENTS

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>PARTICULARS</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1-4</td>
</tr>
<tr>
<td>2</td>
<td>REVIEW OF LITERATURE</td>
<td>5-17</td>
</tr>
<tr>
<td>3</td>
<td>MATERIALS AND METHODS</td>
<td>18-35</td>
</tr>
<tr>
<td>3.1</td>
<td>Chemicals and Kits</td>
<td>18</td>
</tr>
<tr>
<td>3.2</td>
<td>Glass and plastic wares</td>
<td>18</td>
</tr>
<tr>
<td>3.3</td>
<td>Buffers and other reagents</td>
<td>18</td>
</tr>
<tr>
<td>3.4</td>
<td>Preparation of glasswares</td>
<td>18</td>
</tr>
<tr>
<td>3.5</td>
<td>Preparation of bicistronic construct of 2 genes</td>
<td>18</td>
</tr>
<tr>
<td>3.6</td>
<td>Fish</td>
<td>19</td>
</tr>
<tr>
<td>3.7</td>
<td>Experimental design</td>
<td>19</td>
</tr>
<tr>
<td>3.8</td>
<td>Collection of haematological parameters</td>
<td>21</td>
</tr>
<tr>
<td>3.8.1</td>
<td>Collection of blood samples</td>
<td>21</td>
</tr>
<tr>
<td>3.8.2</td>
<td>Serum protein</td>
<td>21</td>
</tr>
<tr>
<td>3.8.3</td>
<td>TLC (Total leukocyte count)</td>
<td>21</td>
</tr>
<tr>
<td>3.8.4</td>
<td>TEC (Total erythrocyte count)</td>
<td>22</td>
</tr>
<tr>
<td>3.9</td>
<td>Sample preparation for enzyme assay</td>
<td>22</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>3.9.1</td>
<td>Enzyme assay</td>
<td>22</td>
</tr>
<tr>
<td>3.9.1.1</td>
<td>Assay of Superoxide Dismutase (SOD, EC 1.15.1.1)</td>
<td>22</td>
</tr>
<tr>
<td>3.9.1.2</td>
<td>Assay of Glutathione peroxidase (GPx, EC 1.11.1.9)</td>
<td>23</td>
</tr>
<tr>
<td>3.10</td>
<td>Preparation of bacterial cultures</td>
<td>23</td>
</tr>
<tr>
<td>3.10.1</td>
<td>Preparation of whole cell of <em>E.tarda</em> for Challenge</td>
<td>23</td>
</tr>
<tr>
<td>3.10.1.1</td>
<td>Estimation of lethal dose 50 (LD&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>23</td>
</tr>
<tr>
<td>3.11</td>
<td>Plasmid isolation from vaccine groups</td>
<td>24</td>
</tr>
<tr>
<td>3.11.1</td>
<td>Quantification of plasmid</td>
<td>25</td>
</tr>
<tr>
<td>3.12</td>
<td>Confirmation of insert by restriction analysis of recombinant clones in plasmid</td>
<td>25</td>
</tr>
<tr>
<td>3.13</td>
<td>Immunization and challenge</td>
<td>25</td>
</tr>
<tr>
<td>3.14</td>
<td>Tissue collection and preservation</td>
<td>26</td>
</tr>
<tr>
<td>3.14.1</td>
<td>Histopathology</td>
<td>26</td>
</tr>
<tr>
<td>3.15</td>
<td>Gene expression study</td>
<td>26</td>
</tr>
<tr>
<td>3.15.1</td>
<td>Total RNA extraction of tissues</td>
<td>27</td>
</tr>
<tr>
<td>3.15.2</td>
<td>Nucleic acid purification</td>
<td>27</td>
</tr>
<tr>
<td>3.15.3</td>
<td>DNAse treatment</td>
<td>27</td>
</tr>
<tr>
<td>3.15.4</td>
<td>Complementary DNA (cDNA) synthesis</td>
<td>28</td>
</tr>
<tr>
<td>3.15.5</td>
<td>Polymerase chain reaction</td>
<td>28</td>
</tr>
<tr>
<td>3.15.6</td>
<td>Optimization of PCR conditions</td>
<td>28</td>
</tr>
<tr>
<td>3.15.7</td>
<td>Agarose gel electrophoresis</td>
<td>29</td>
</tr>
<tr>
<td>3.16</td>
<td>Real time PCR</td>
<td>29</td>
</tr>
<tr>
<td>3.16.1</td>
<td>Relative quantification of iNOS gene using real-time PCR</td>
<td>29</td>
</tr>
<tr>
<td>3.16.2</td>
<td>Statistical analysis</td>
<td>30</td>
</tr>
<tr>
<td>3.17</td>
<td>Development of Mab-based competitive ELISA for detection and quantification of antigen specific antibodies</td>
<td>32</td>
</tr>
<tr>
<td>3.17.1</td>
<td>ELISA</td>
<td>32</td>
</tr>
<tr>
<td>3.18</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to check functionality of Mab</td>
<td>33</td>
</tr>
<tr>
<td>3.19</td>
<td>Western blotting</td>
<td>35</td>
</tr>
<tr>
<td>3.20</td>
<td>Statistical analysis</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>RESULTS</td>
<td>36-53</td>
</tr>
<tr>
<td>4.1</td>
<td>Confirmation of bicistronic construct</td>
<td>36</td>
</tr>
<tr>
<td>4.2</td>
<td>Western blotting</td>
<td>36</td>
</tr>
<tr>
<td>4.3</td>
<td>Fish</td>
<td>36</td>
</tr>
<tr>
<td>4.4</td>
<td>Bacteriology</td>
<td>36</td>
</tr>
<tr>
<td>4.5</td>
<td>Total plate count</td>
<td>37</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>4.6</td>
<td>Challenge study</td>
<td>37</td>
</tr>
<tr>
<td>4.6.1</td>
<td>Estimation of Relative percentage survival (RPS)</td>
<td>37</td>
</tr>
<tr>
<td>4.7</td>
<td>Haematological Assay</td>
<td>39</td>
</tr>
<tr>
<td>4.7.1</td>
<td>Total leucocyte count (TLC)</td>
<td>39</td>
</tr>
<tr>
<td>4.7.2</td>
<td>Total erythrocyte count (TEC)</td>
<td>39</td>
</tr>
<tr>
<td>4.8</td>
<td>ENZYME STUDY</td>
<td>39</td>
</tr>
<tr>
<td>4.8.1</td>
<td>GPX activity</td>
<td>39</td>
</tr>
<tr>
<td>4.8.2</td>
<td>SOD activity</td>
<td>40</td>
</tr>
<tr>
<td>4.9</td>
<td>Clinical signs and pathological changes</td>
<td>40</td>
</tr>
<tr>
<td>4.10</td>
<td>HISTOPATHOLOGY</td>
<td>40</td>
</tr>
<tr>
<td>4.10.1</td>
<td>Kidney</td>
<td>40</td>
</tr>
<tr>
<td>4.10.2</td>
<td>Liver</td>
<td>40</td>
</tr>
<tr>
<td>4.11</td>
<td>Competitive ELISA</td>
<td>41</td>
</tr>
<tr>
<td>4.12</td>
<td>PCR amplification of iNOS gene</td>
<td>41</td>
</tr>
<tr>
<td>5.</td>
<td>DISCUSSION</td>
<td>55-62</td>
</tr>
<tr>
<td>6.</td>
<td>SUMMARY</td>
<td>63-64</td>
</tr>
<tr>
<td>7.</td>
<td>REFERENCES</td>
<td>65-82</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No</th>
<th>Particulars</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Experimental design</td>
<td>20</td>
</tr>
<tr>
<td>Table 2</td>
<td>Primers used in this study</td>
<td>31</td>
</tr>
<tr>
<td>Table 3</td>
<td>Composition of 12% resolving gel</td>
<td>34</td>
</tr>
<tr>
<td>Table 4</td>
<td>Composition of 5% stacking gel</td>
<td>34</td>
</tr>
<tr>
<td>Table 5</td>
<td>Estimation of Relative percentage survival (RPS)</td>
<td>38</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Particulars</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agarose gel electrophoresis (0.8%) showing RE analysis of pIRES vector confirming GAPDH and IFN gene.</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Western blot of 37 kDa of GAPDH protein</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Test fish, <em>Labeo rohita</em> (rohu) (avg. wt. 20±6.6g).</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Colonies of <em>E. tarda</em> on S.S. agar, streaked from kidney of <em>Labeo rohita</em></td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>Total WBC count after immunization with GAPDH and GAPDH+IFN constructs on various time points</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>WBC count of different groups immunized at 0 day and 21 day and given bacterial challenge at 35\textsuperscript{th} day</td>
<td>44</td>
</tr>
<tr>
<td>7</td>
<td>TEC from four studied groups which were vaccinated at 0\textsuperscript{th} and 21\textsuperscript{st} day and challenged with <em>E. tarda</em> at 35\textsuperscript{th} day.</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>GPx activity on 15 day after first immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>GPx activity on 35 day after second immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.</td>
<td>46</td>
</tr>
</tbody>
</table>
10 GPx activity on 37 days after 48 hrs post challenge in control (unimmunized) group, GAPDH group, GAPDH+IFN group, challenge control.

11 SOD activity on 15 day after first immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

12 SOD activity on 35 day after booster immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

13 SOD activity on 37 day at 48 hrs post challenge in control (unimmunized) group, GAPDH group, GAPDH+IFN group and in challenge control.

14 Accumulation of fluid in the abdomen and dropsy

15 Diseased fish showing protruded vent and haemorrhages all over the body

16 Diseased fishes showing reddening of fins.

17 Swollen abdomen of diseased fish

18 Haemorrhages all over the body of fish

19 Darkened body pigment of diseased fish

20 Healthy kidney.

21 Severely necrotized tubular cells along with marked extensive renal interstitial haemorrhage (Challenge group kidney)
22 Kidney tissue showing swelling in tubular cells characterized by the hypertrophy of cells (narrowing of tubular lumen) (GAPDH vaccinated group kidney)

23 Tissue section of kidney showing mild oedema (arrow) and renal tubules with sloughed basement membrane (GAPDH+IFN vaccinated group kidney)

24 Liver tissue showing oedema due to necrotized hepatocytes and atrophied acinar cells (GAPDH vaccinated group liver)

25 Liver tissue exhibiting hepatocytes with pyknotic nuclei, dilated and congested sinusoids (arrow) and oedema at places (GAPDH+IFN vaccinated group liver)

26 Healthy liver

27 Liver tissue displaying diffused necrotized hepatocytes, damaged hepatic cords along with oedema at places (Challenge control)

28 Percentage inhibition of antibody binding in different groups

29 Agarose gel electrophoresis showing RT-PCR based expression of iNOS in positive control, GAPDH and GAPDH+IFN vaccinated group.

30 Agarose gel electrophoresis showing RT-PCR based expression of β actin in kidney tissue in positive control, GAPDH and GAPDH+IFN vaccinated group.
Fold change in expression of iNOS gene in blood sample collected after each vaccination.

Fold change in expression of iNOS gene in different groups after challenging with bacteria.
## ACRONYM

<table>
<thead>
<tr>
<th>Abbr</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>@</td>
<td>At</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>&gt;</td>
<td>Greater than</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm³</td>
<td>Cubic micrometre</td>
</tr>
<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine-tetra Acetic acid</td>
</tr>
<tr>
<td>\g</td>
<td>Gram</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg/g</td>
<td>Milligram per gram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
</tbody>
</table>
ng : Nanogram

OD : Optical Density

PBS : Phosphate buffered saline

Pg : Picogram

Rpm : Revolution per minute

SE : Standard Error

SPSS : Statistical Package for Social Science

UV : Ultraviolet
1. INTRODUCTION

Among the fish producing countries, India stands second in terms of both, total fish production and aquaculture production globally. The freshwater aquaculture contributes about 85% of the total Indian aquaculture production in 2009-10 (Ayyappan et al., 2011). The Indian freshwater aquaculture is mainly carp based with Indian Major Carps (IMCs) viz catla (Catla catla), rohu (Labeo rohita) and mrigal (Cirrhinus mrigala) contributing more than 85.5% of the total freshwater fish production (Ayyappan et al., 2011). Among the IMCs, rohu is one of the most preferred species in the country and commands a higher price in the market. The species is also an excellent game fish owing to its easy acceptance of angler's bait. Andhra Pradesh, West Bengal, Assam and Odisha are the most important states for aquaculture production and rohu is the most preferred species in these states and also in other states of the country (FAO 2009). The intensive culture practices render increase in transmission of infectious diseases. Controlling disease problems in aquaculture industry is a major concern. Although introduction of vaccines has greatly reduced the traditional antibiotic mode of control, the limited knowledge on immune system of fish confines the development of new vaccines based on non-empirical strategies (Sommerset et al., 2005)

Bacterial pathogens are one of the major causes of aquaculture diseases (Castro et al., 2006). Bacteria are among the highly encountered causes of diseases in cultured warm water fish. Out of the most annihilating bacteria Edwardsiella tarda (E. tarda), act as a predominantly enteric pathogen of both freshwater and brackishwater fishes (Plumb, 1994). It is mainly responsible for causing a serious systemic bacterial disease called as edwardsiellosis, having a worldwide distribution and affecting a variety of fish taxa (Austin and Austin, 1999; Maiti et al., 2009). The bacterium act as a pathogen with wide range of hosts other than fish such as reptiles, birds, mammals including humans (Plumb 1999; Park and Oh, 2008). It is the aetiological agent of several pathologic symptoms of fresh water and marine fish, particularly causing entero hemorrhagic septicaemia (Benli and Yildiz, 2004). This disease had a disastrous effect on fish culture, occurred in Northern and Southern Europe in turbot (Castro et al., 2006). Disease signs may
include extensive skin lesions that progress into necrotic abscesses, distended abdomen, and swollen anus due to the accumulation of ascitic fluid, along with pigment loss, enlarged kidneys, and abscesses on the internal organs (Mohanty et al., 2007). Several potential virulence factors of *E. tarda* have been reported. *E. tarda* is a member of family Enterobacteriaceae. It is a Gram-negative, motile, rod shaped bacterium which can affect warm and cold water species of fish. It is cytochrome oxidase negative and can ferment glucose with production of acid and gas. Also it can produce both indole and hydrogen sulphide. In fact, the first isolation of the bacterium was in Japan, where Hoshina (1962) named the organism *Paracolobactrum anguillimortiferum*. The name *E. tarda*, which is now accepted world-wide, was proposed by Ewing et al., (1965). Based on phenotypic characteristics, *E. tarda* isolates were grouped into 3 different biogroups (Ewing et al., 1965, Grimont et al., 1980): (1) sucrose (suc)-, mannitol (manol)-, and -arabinose (ara)- negative, and hydrogen sulphide (H2S) positive ‘wild-type’ strains associated with humans and fish infections; (2) suc+, manol+, ara+, and H2S–Biogroup 1 strains isolated from diseased zoo animals (reptiles and birds); and (3) suc+, manol–, ara+, and H2S– Biogroup 2 strains, which have only been isolated from humans.

Vaccine efficacy of recombinant Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *E. tarda* against edwardsiellosis, and production of toxin and enzymes, such as hemolysins, (Hirono et al.,1997) Iron-regulated haemolysin gene from *E. tarda* and catalases, a major catalase (KatB) that is required for resistance to H2O2 and phagocyte-mediated killing in *E. tarda* have been found to play important roles in *E. tarda* pathogenesis (Srinivasan et al., 2003).

Use of chemicals and antibiotics to control diseases are common method. But these methods have some negative effects like accumulation in animal flesh, emergence of drug resistant strain and aquatic environment contamination etc. Medicated feed containing antibiotics is currently used to control Edwardsiella infection. This method is less effective. Genetic engineering (GE) strategies, such as production of transgenic fish, genetically modified (GM) plants as edible vaccines and GM feed (based on plants or micro-organisms), and DNA vaccines may offer
technical solution for some of the disease problems. Vaccines offer the most efficient way to control infectious pathogens. Vaccination is the most effective method for reducing suffering and economic losses due to the infectious diseases. But in some cases live vaccines with attenuated agent would be of concern. This is the interest behind the concept of DNA immunisation or third generation of vaccine. DNA vaccination is defined as the intentional transfer of genetic material to somatic cells for the purposes of influencing the immune system (Foss and Rogne et al., 2003).

Prevention of edwardsiellosis by vaccination is a great goal of research people, so that fish producers throughout the world can be stable. Vaccine is a biological preparation that improves immunity to a specific disease. Information on the virulence, antigenicity and antibiotic susceptibility is needed for prevention, rapid diagnosis, and therapy of E. tarda infection. Many virulence genes of E. tarda have been identified for clarification of the virulence mechanisms for production of hemolysins, intracellular replication in macrophage, and the ability to invade epithelial cells. In addition to the virulence genes, genes encoding antigenic proteins have been explored for development of a vaccine and immunodiagnostics. Various methods are employed in order to correct the bacterial disease.

The DNA based vaccination or genetic immunization is a very promising research field since long years ago. DNA vaccines represent a major advance in fight against infectious diseases. DNA vaccine has several practical and immunological advantages than traditional antigen vaccines. Unlike traditional types of vaccines (bacterin, subunit vaccine, and attenuated live vaccine) that are composed of pathogenic organisms or cellular components of the pathogen, DNA vaccines are genetic vaccines in which the genetic material, i.e., the coding sequence of the antigen, is carried on and expressed from a DNA plasmid. Following vaccination, the plasmid DNA is taken up by host cells, and the antigenic protein is expressed and synthesized by the transcription and translation machineries of the vaccinated animal (Tonheim et al., 2008). Studies in mammalian models have indicated that while bacterins and purified subunit vaccines induce mainly humoral immunity, DNA vaccines can induce both humoral and cellular immunities (Coban et al., 2008).
In case of *E. tarda* main virulence associated systems like type 3 and type 4 secretion systems, the LuxS/Al-2 quorum sensing system have been identified. Main vaccine candidates include sialidase NanA, Outer membrane Proteins (OMP), dermatotoxin, hemolysins, catalase, EseDs and GAPDH. Of these GAPDH in the outer membrane is reported to be highly antigenic. So it is considered to be a strong vaccine candidate to counter both Gram negative and Gram positive bacterial infections (Kawai *et al*., 2004). Many cytokine and immune-related genes have been identified and characterized in fish in recent years, which has helped in numerous studies on the expression of these genes during disease development and understanding molecular pathogenesis (Lindenstrom *et al*., 2004; Purcell *et al*., 2004; Coban *et al*., 2008). Cytokines like interleukin 1b (IL-1b) is one of the pivotal early response pro-inflammatory cytokines that enables organisms to respond to infectious insults, inducing an inflammatory cascade, along with other defensive responses. After this stimulation, acute phase response related genes, such as transferrin and ceruloplasmin, are induced. Other gene products such as lysozyme, protease inhibitors, and complement proteins may play a role in further restricting their multiplication or pathogenesis (Magnadottir *et al*., 2006).

Since immunogenicity generated by one immunogenic gene is not always enough to generate sufficient immune response, researchers are always in search of some additional genes which can be incorporated in the vaccine to enhance the immune response against vaccine. These genes are generally called as immune adjuvants. Interferon gamma (IFN-γ) is having a major role in adaptive cell mediated immune responses being produced by CD4+ T helper 1 (Th 1) lymphocytes and CD8+ cytotoxic T lymphocytes (CTL) in response to MHC-presented antigens (Biron and Sen, 2001). Based on many published reports IFN-γ appears to be the best immunoadjuvant especially against viruses and intracellular pathogens. In this background the objective of the present study is

- To study the responses generated in *Labeo rohita* against DNA vaccine construct containing GAPDH gene of *Edwardsiella tarda* and Interferon gamma of *Labeo rohita*. 

22
2. REVIEW OF LITERATURE

2.1. Edwardsiellosis in fish

*Edwardsiella tarda* is the causative agent of edwardsiellosis and also a common epizootic disease found in cultured (Meyer & Bullock 1973, Wakabayashi & Egusa 1973, and wild fish species (Francis-Floyd *et al.* 1993, Baya *et al.* 1997., Kusuda & Kawai 1998). *Edwardsiella tarda* causes systemic hemorrhagic septicemia in a variety of freshwater and marine fish species. Fishes infected by *E. tarda* develop extensive skin lesions and swelling occur, necrosis in internal organs, finally leading to mass-mortality outbreaks of edwardsiellosis. It has been reported in tropical and subtropical areas of Africa, America, Asia and Australia (Thune *et al.*; 1993). Edwardsiellosis has been described to occur in different geographical regions having an extensive host range (Kourany *et al.*, 1977; Coles *et al.*, 1978; Cook *et al.*, 1985; Janda *et al.*, 1993), and had resulted in severe economic losses in the aquaculture industries (Mohanty *et al.*, 2007).

2.2. Systematic Classification

The systematic classification of *E. tarda* is as follows (Brenner *et al.*, 2005)

Super kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus:*Edwardsiella*

Species: *tarda*
2.3. Morphology

The bacteria are short, rod shaped, measuring 0.5-0.8 × 0.8-1.5 μm arranged singly. They are Gram negative, motile, non-spore forming and non- capsulating. Physiologically they are aerobic and facultatively anaerobic in nature (Wang et al, 1993).

2.4. Biochemical characters

Many workers have studied biochemical characteristics of *E. tarda*. Positive reactions have been reported for the production of hydrogen sulphide on TSI, indole, MR/VP (+/-), catalase, lysine decarboxylase and ornithine decarboxylase. The organism is also found to produce acid and gas from glucose. Negative reaction has been noted for urease, gelatin, oxidase, phenylalanine and arginine (Ewing et al, 1965; Wang et al, 1993). Ewing *et al.* (1965) had noted negative reactions for lactose, sucrose, mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, arabinose, raffinose, rhamnose, xylose, starch and cellobiose. However it was later found that the organisms could utilize cellobiose, sucrose and arabinose (Wang *et al*, 1993).

2.4.1. Cultural characters

The bacterium grows slowly on nutrient agar requiring 18-24 h of incubation at 25°C to form typical small, circular, transparent and slightly raised colonies (Choi, 1991). A selective medium *Edwardsiella ictaluri* medium (EIM) was formulated for the isolation of *Edwardsiella* species, which inhibits the growth of most Gram-negative bacterial organisms. The bacteria that grew on EIM were easily differentiated from *Edwardsiella* spp. based on colony morphology (Shotts and Waltman, 1990). Cysteine, Methionine, Nicotinic acid, Phenylalanine, Threonine, Valine, Aspartic acid, Glutamic acid and Isoleucine formed the essential components of the media required for the growth of the organisms (Hoshina, 1962; D’Empaire, 1969). *E. tarda* specific media was designed taking into account the typical biochemical characteristics of *E. tarda* by decarboxylation of lysine,
formation of hydrogen sulphide from thiosulphate and the lack of mannitol or sucrose fermentation. Moreover, the inclusion of colistin in the medium would severely restrict the variety of enteric bacteria, allowing a better isolation of *E. tarda* (Muyembe et al, 1973). ET medium was evaluated in parallel with the commercial Salmonella–Shigella agar (SS), which is usually employed for the selective isolation of enteric bacilli. The results obtained showed that ET-1 is distinctly selective for the isolation of *E. tarda*, allowing its recovery from mixed cultures and natural samples.

### 2.4.2. Epizootiology

The precise source of *E. tarda* infection is unknown. Nevertheless, it has been speculated that the snakes or faecal contamination from humans, or other animals may have been involved in the initial outbreak. To date, the disease has been reported from various fish species, including Channel catfish (Meyer and Bullock, 1973), Japanese eel (Miyazaki and Egusa, 1976), mullet (Kusuda et al, 1976), tilapia (Kubota et al, 1981), chinook salmon (Amandi et al, 1982), flounder (Nakatsugawa, 1983), common carp (Sae-Oui et al, 1984), striped bass (Herman and Bullock, 1986), turbot (Nougayrede et al, 1994), Asian catfish (*Clarias batrachus*) (Sahoo et al, 1998), koi (Sahoo et al, 2000), climbing perch (*Anabas testudineus*) (Sahoo et al, 2000), tilapia (Clavijo et al, 2002), Indian major carps (Swain and Nayak, 2003), European eel (Alcaide et al, 2006), and Senegalese sole (Castro et al, 2012). Environmental parameters namely water temperature and quantity of organic matter in the water influences the severity of disease outbreak. In particular it is noteworthy that most of the disease outbreaks occur at high water temperature that is 30°C. There is some evidence that *E. tarda* exists in water within the vicinity of fish farms (Minagawa et al, 1983; Park et al, 1983). However it is unclear whether *E. tarda* should be regarded as primary or opportunistic pathogen of fish. Indeed it may comprise part of normal microflora of fish species (Wyatt et al, 1979). *E. tarda* has long been considered as an unusual human pathogen. It is primarily associated with gastroenteritis, wound infections such as cellulitis or gas gangrene and generalized infections in humans with impaired immune systems (Thune et al, 1993; Plumb, 1999; Srinivasa Rao et al, 2001; Nucci et al, 2002).
2.4.3. Pathogenecity

Pathogenicity of *E. tarda* has been described in catfish by Meyer and Bullock (1973) and eels by Miyazaki and Egusa (1976a, 1976 b); Liu and Tsai (1987) and Kuo (1977). Lee et al. (1990) reported that healthy snakeheads (*Channa argus*) when injected with *E. tarda* produced ascites and haemorrhagic ulcers within 5 days. In another incident of outbreak of *E. tarda* infection in cultured bastard halibut, the diseased fish showed abdominal inflammation, accumulation of hemorrhagic ascitic fluid and greyish white spot on liver. Kodama et al. (1987) reported that in an infectious disease caused by *E. tarda* in young Japanese flounder (*Paralicthys olivaceous*), deep ulcerative lesions and loss of skin was evident. Hemorrhage and loss of fins, protrusion of rectum and swelling of the spleen were also observed. Experimentally infected rohu with *E. tarda* were characterized by erratic swimming behavior, anorexia and listless-ness. The infected fishes showed small, rounded deep brick coloured erythematous lesions at the posterior end of the body along with shrunken eyes, emaciation and lethargy (Sahoo and Sahoo, 1997).

2.5. Diagnosis

Presumptive diagnosis of *E. tarda* is based on clinical signs and on isolation and serological identification of the causative agents. But clinical signs vary depending on different fish host and severity of disease (Bullock et al, 1985). Mohanty and Sahoo (2007) have mentioned that the symptoms or gross signs are not of much help in arriving at a diagnosis as many of these symptoms and signs match those of infection with other bacteria.

The gross features of *Edwardsiella* septicaemia are cutaneous lesions extending down into the musculature (Roberts, 2012). In mild infections, the only manifestation of the disease is small cutaneous lesions (3–5 mm in diameter) located on the posteriollateral parts of the body. As the disease progresses, abscesses develop within the muscles of the flanks or caudal peduncle (Mohanty and Sahoo, 2007). Loss of pigmentation over the lesions is common (Sahoo et al,
Internally the most common gross lesion consists of light-colored nodules on the kidneys, spleen, or liver (Bullock et al., 1985).

Histologically the lesion is characterized by focal necrosis, extending from muscle, haemopoietic tissue and liver parenchyma to perforate the abdominal walls (Miyazaki and Egusa, 1976). *E. tarda* infection causes hypertrophy of the liver cells and enlargement of their nuclei (Miwa and Mano, 2000). According to Mohanty and Sahoo (2007) nephritis is found most often followed by suppurative hepatitis on histopathological examination. Roberts (2012) has mentioned that there is usually a cellular infiltrate beneath the fibrinous layer in case of fibrinous peritonitis.

Hematological parameters have been regarded equally significant indicators of fish health (Chen et al. 2004, Martins et al. 2004). Studies demonstrated that the bacterial infection may be the cause of reduction in the number of erythrocytes in the blood and in the hematocrit percentage (McNulty et al. 2003, Benli & Yildiz, 2004, Shoemaker et al. 2006b). Analyses of blood parameters of *E. tarda*-infected tilapia fish expressed reduction in haematocrit values, haemoglobin, erythrocytes, total plasma proteins, Mg2+, K+, Na+, Cl−, and increment in the total leukocyte count and level of plasma glucose (Benli and Yildiz 2004). Caruso et al (2002) observed that there was a considerable increase in the plasma lysozyme level in *E. tarda*-challenged sheatfish (*Silurus glanis*). A positive correlation between superoxide production during respiratory burst activity and survival against challenge by *E. tarda* was noticed in *coho salmon* (Balfry et al., 1994) and rohu (*Labeo rohita*) (Mohanty et al., 2007), which could help as an immunological marker for immunity. Serum samples can be diagnosed using dot, indirect or competitive ELISA methods and antigens can be detected by agglutination tests (Swain et al., 2001, Swain and Nayak et al., 2003).

Rogers (1981) developed a fluorescent antibody test and enzyme immunoassay that can identify *E. tarda* both in culture and in infected tissues. Horiuchi et al. (1980) also demonstrated that an indirect fluorescent antibody test in which tissue impressions are used was effective in detecting and diagnosing *E. tarda* in Japanese eels (*Anguilla japonica*). PCR-based diagnosis of *E. tarda*
infection in blood samples of oyster toadfish was reported by Horenstein et al. (2004). They had also tried more sensitive real-time PCR methods.

2.6. Virulence factors

*E. tarda* survive in their host by utilizing several important substances and abilities that serve as virulence factors in the host. A study carried out using green fluorescent protein (GFP) showed that both avirulent and virulent *E. tarda* were able to adhere to, invade, and replicate in the carp epithelial papilloma (EPC) cell line using host microfilaments and protein tyrosine kinase (Ling et al., 2000). Histopathological and infection kinetics studies using GFP revealed that the gill, gastrointestinal tract, and body surface are the sites of entry of the virulent strain (Ling et al., 2001). Type III secretion system (T3SS) and type VI secretion system (T6SS) play important roles in adherence, penetration, survival, and replication of *E. tarda* in epithelial cells and phagocytes. The T6SS of *E. tarda* comprises 16 genes, and 13 of the encoded proteins are involved in the secretion of *E. tarda* virulence protein (EvpP) (Zheng et al., 2007). In *E. tarda*, T3SS proteins include the *E. tarda* secretion system apparatus (EsaB and EsaN), effectors (EseB, EseC and EseD), chaperones (EscA, EscB and EscC), and regulators (EsrA, EsrB and EsrC) (Tan et al., 2005; Zheng et al., 2007; Wang et al., 2010). Proteomic studies have revealed that EseB, EseC and EseD are the major ECP, and mutations of these genes in *E. tarda* reduces virulence compared to parental *E. tarda* (Tan et al., 2002). It has been reported that *E. tarda* produces two kinds of hemolysin; one is a cell associated, iron-regulated hemolysin, encoded by *ethA* and *ethB*, that is secreted as an extracellular protein (ECP) under iron-regulated conditions (Janda et al., 1993), and the other is an extracellular hole-forming hemolysin distinct from EthA and EthB that is not regulated by iron (Chen et al., 1996; Hirono et al., 1997; Strauss et al., 1997). A recent functional study demonstrated that EthA is critical for invasion *in vivo* and *in vitro*, and is regulated by the two-component system EsrA-EsrB and nucleid protein Hha (Wang et al., 2010). Other enzymes, including catalase, chondroitinase, dermatotoxin, protease, and collagenase, are also important for the pathogenesis of *E. tarda* (Ullah et al., 1983; Srinivasa et al., 2003; Wang et al., 2009; Jiao et al., 2010).
2.7. Immune mechanism

Various surveys have indicated that *E. tarda* is able to live and replicate in phagocytes, leading to systemic infections (Ainsworth *et al*., 1990; Srinivasa *et al*., 2001; Pirarat *et al*., 2007; Ishibe *et al*., 2008; Ishibe *et al*., 2009). Virulent *E. tarda* opsonized with the serum of blue Gourami can replicate within phagocytes and fails to induce an oxidative burst, perhaps offering a mechanism for avoiding phagocyte-mediated killing (Srinivasa *et al*., 2001). A subsequent survey revealed that the construction of the catalase (KatB) gene of *E. tarda* is responsible for the resistance to H$_2$O$_2$ and phagocyte-mediated killing (Srinivasa *et al*., 2003). Likewise, a comparison of the response of peritoneal macrophages from olive flounder to high- and low-virulence *E. tarda* proved that merely the highly virulent strain is able to resist reactive oxygen species generated by macrophages, and survive and replicate within macrophages (Ishibe *et al*., 2008). In a subsequent study it was demonstrating that virulent *E. tarda* elicits a significantly greater induction of nitric oxide and tumor necrosis factor TNF-α production by macrophages, actions that may account for the pathogenicity of *E. tarda* infection (Ishibe *et al*., 2009). A study of *E. tarda* septicemia revealed that *E. tarda* induces systemic immunosuppression through lymphocyte apoptosis, which suppresses systemic immune responses during the initial stage of septicemia (Pirarat *et al*., 2007). The host also seems to acquire with the immune mechanisms for avoiding or resisting the propagation of *E. tarda*. On examining the pathogenic capacity of motile and non-motile *E. tarda* strains toward olive flounder, red sea bream, and yellow tail, it showed that all strains were virulent in the olive flounder and yellow tail, whereas only atypical strains showed mortality in the red sea bream (Matsuyama *et al*., 2005). These findings suggest that immune mechanisms involved in recognition of and resistances against *E. tarda* vary among hosts. In zebra fish (*Danio rerio*), experimental infection with *E. tarda* resulted in an acute elevation of the expression of inflammatory cytokines, interleukin-1β (IL-1β) and TNF-α (Pressley *et al*., 2005). Indian major carp challenged with *E. tarda* exhibited a significant induction of
immune responses and expression of several immune related genes, including IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), complement component C3, β-microglobulin, CXCa, and C-type and G-type lysozyme (Mohanty et al., 2010). Aoki and his colleagues surveyed over a thousand genes in olive flounder infected with *E. tarda* using microarray analyses, identifying 36 genes that were differentially expressed between susceptible and resistant olive flounder groups (Matsuyama et al., 2007; Yasuike et al., 2010; Aoki et al., 2011). Notably, 3 days post challenge, MHC class I antigen processing- and presenting-related factors were highly expressed in resistant groups, but susceptible groups showed high expression of factors involved in innate immune responses (Yasuike et al., 2010).

Understanding the virulence factors of *E. tarda* may inform the evolution of protection strategies against edwardsiellosis in fish. Recent advance in analytical methods, such as genomics and proteomics, has revealed important virulence factors, including T3SS, T6SS, and two component systems. Verjan et al. demonstrated seven antigenic proteins, which were detected as lipoproteins, periplasmic proteins, exported, and secreted proteins (Verjan et al., 2005). Additional proteomic studies on outer membrane proteins (OMP), ECP, and outer membrane vesicles (OMV) may also lead to the development of efficient protection strategies against edwardsiellosis (Tan et al., 2005; Tan et al., 2002; Sakai et al., 2009; Srinivasa et al., 2004; Park et al., 2011; Lee et al., 2009; Kumar et al., 2007; Vinogradov et al., 2005; Tang et al., 2010; Kumar et al., 2010). In addition, recognizing the entire genome sequence of *E. tarda* would enhance our understanding of the relationship between *E. tarda* and the host, and further the development of new prophylactic and therapeutic strategies for managing edwardsiellosis in fish (Wang et al., 2009).

### 2.8. Need for fish vaccine

Among the troubles that the fish and shellfish industries have to address, infectious pathogens are the most important (Meyer et al., 1991; Aasjord et al., 1994; Mialhe et al., 1995). Eruptions can cause severe losses to fish farmers and in many instances, there are no preventive or therapeutic measures available. Chemicals and antibiotics can be utilized to control bacterial and parasitic diseases, but these products frequently cause unwanted side effects such as accumulation in
the flesh of animals, development of drug resistant strains, and pollution of the aquatic environment (Munn et al., 1994; Press et al., 1995). Therefore, most research efforts are concentrating on prevention rather than treatment with chemicals, which should only be considered when vaccines are unavailable for specific pathogens or when they give way to prevent an eruption.

Vaccines have long demonstrated their efficacy in herd protection, merely in the aquaculture industry, they are in a comparatively early stage of growth. Most of the commercially available vaccines protect fish against bacterial diseases, and are normally made of inactivated bacteria which can be used either by immersion or by injection with an oil adjuvant (Newman et al., 1993; Aasjord et al., 1994; Schnick et al., 1997). Immunization by antigen encoding plasmid DNA exhibits very strong and long-lasting humoral and cellular immune response. This access also offers economic, environmental and safety advantages, which are especially attractive for the aquaculture industry (Heppell et al., 2000; Kwang et al., 2000).

2.9. DNA vaccines

DNA vaccines are plasmids that encode antigenic proteins derived from pathogenic microorganisms. The plasmid is inserted into the host animal, where the exogenous antigen is grown, thus inducing host immune reaction. Compared to traditional types of vaccine, DNA vaccine has the vantage of being cheap, secure, unchanging, and stimulating both humoral and cellular immunities (Heppell et al., 2000; Tonheim et al., 2008). In the past decade, DNA vaccine has been extensively examined for the prevention of aquaculture diseases caused by viral factors, especially infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and hirame rhabdovirus (Leong et al., 1994; Lorenzen et al., 1998; Corbeil et al., 1999; Takano et al., 2004; Garver et al., 2005). The first vaccines against infectious bacterial diseases in farmed fish were developed in the 1970s and used on a commercial scale in the early 1980s. Evolution of DNA vaccines only started experimentally in the early 1990s and there is only limited proof about their effectiveness and adverse effects in fish. Moved over the operating mechanism of DNA vaccine and its success with viral pathogens, it is potential that this kind of vaccine would be effective against E. tarda, which is
recognized to be an intracellular pathogen and can survive in host phagocytes (S.H. Ling et al., 2000; Srinivasa et al., 2001). Two *E. tarda* antigens, Eta6 and FliC, which are homologues to an ecotin precursor and the FliC flagellin, respectively, were detected by in vivo-induced antigen technology from a pathogenic *E. tarda* strain isolated from diseased fish. When applied as a subunit vaccine, purified recombinant Eta6 was moderately protected against lethal challenge of *E. tarda* in a Japanese flounder model, whereas purified recombinant FliC showed no apparent immunoprotectivity. Likewise, DNA vaccines based on eta6 and Flic in the form of plasmids pEta6 and pFliC induced, respectively, moderate and marginal protection against *E. tarda* infection. To improve the vaccine efficacy of eta6, a chimeric DNA vaccine, pCE6, was built, which encodes Eta6 fused in-frame to FliC. pCE6 was found to cause a significantly higher degree of protection than pEta6. Also, another chimeric DNA vaccine, pCE18, which expresses FliC fused to a previously identified *E. tarda* antigen Et18, exhibited significantly stronger protective immunity than the DNA vaccine based on et18 alone. Fish immunized with pEta6 and pCE6 produced specific serum antibodies and elicited significantly enhanced expression of the genes encoding elements that are involved in both innate and adaptive immune reactions. Furthermore, the induction magnitudes of most of these factors were significantly higher in pCE6-immunised fish than in pEta6-immunised fish. Several DNA vaccines were developed experimentally against *E. tarda* infection to induce protective immunity. Sia 10, the antigen from *Streptococcus iniae* administered in the form of DNA vaccine produces protective immunity. Sun et al. (2012) constructed TX5RMS10 which is a combination of Sia10 antigen and TX5RM as a carrier.

### 2.9.1. GAPDH as protective antigen

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that takes part in the propagation of bacterial energy, which is indispensable for maturation in the absence of neoglucogenic substrates. The GAPDH is considered a classical cytosolic glycolytic protein. GAPDH is widely present in Gram-positive bacteria (Iddar et al., 2005) and is a common protein of Gram-negative bacteria (Villamon et al., 2003). Coalition of the GAPDH amino acid sequences of various Gram-negative bacteria (*Escherichia coli, Salmonella*...
enterica, Shigella flexneri, and Vibrio cholerae) showed that the sequences are highly preserved and the similarities between these bacteria are ≥80% (Liu et al., 2005).

A conserved 37 kDa outer membrane protein (OMP) of E. tarda was found to effectively protect Japanese flounder (Paralichthys olivaceus) against different serotypes of E. tarda, and N-terminal amino acid sequence analysis indicated that the 37 kDa OMP is homologous to GAPDH, a cytoplasmic enzyme common in organisms (Kawai et al., 2004). Later, an immunization experiment of Japanese flounder with rGAPDH showed that rGAPDH effectively protected Japanese flounder from experimental E. tarda infection (Liu et al., 2005). Of late, it was found (Li et al., 2012) that localizations of GAPDHs from six pathogenic bacteria including E.tarda, E. ictaluri, Aeromonas hydrophila, Vibrio anguillarum, V. alginolyticus and V. harveyi were similar to many other causative agents like group A streptococci, Streptococcus pneumoniae (Bergmann et al., 2004) and Bacillus anthracis (Matta et al., 2010). In addition, rGAPDHs gave remarkable cross immunity in the zebrafish model and could be created as a broad spectrum vaccine candidate against multiple microbial infections in aquaculture (Li et al., 2012). The recombinant Streptococcus agalactiae GAPDH was found to be a stimulatory protein that induced the proliferation and differentiation of B cells in mice (Madureira et al., 2007).

2.9.2. Adjuvants in DNA vaccines

Vaccination is the most adequate method to control infectious diseases that threaten the aquaculture industry worldwide. Unfortunately, vaccines are generally not able to aid protection on their own; especially those vaccines based on recombinant antigens or inactivated pathogens. Thus, the role of adjuvants or immunostimulants is often necessary to enhance the vaccine efficacy. To maximize the efficacy of vaccines, especially those containing poorly antigenic components or highly purified antigens, adjuvants are usually added. Adjuvants enhances response to vaccines and/or balance/shift the Th1/Th2 immune response. They can cut the amount of antigen to be injected or the numbers of doses administered, and they may promote prolonged immunologic memory. To increase immunogenicity of a
given DNA vaccine one may consider optimization by including co-injected adjuvant, either being traditional aluminum salts, polysaccharides (e.g. zymosan, glucans, Chitosan), different liposomes, synthetic polymers and TLR agonists. Moreover, plasmid encoded cytokine adjuvants may also be one approach to increase the immunogenicity. IL-2, IFN-γ, IL-12, GM-CSF and IL-15 have been proven to regulate immune responses when co-encoded by the DNA vaccine (Saade et al., 2012). One may also apply the concept of immune modulating effects chemokines, transcription factors and/or co-stimulatory factors assembled into the plasmid vectors. Using fish models, merely one report on the use of cytokine adjuvants encoded by the same plasmid as the expression plasmid has been issued. Caipang et al., 2009, used Japanese flounder interferon regulatory factor-1 (IRF-1) cloned into a plasmid DNA vector containing the major capsid protein gene of sea bream iridovirus. The antibody levels of fish injected with this vaccine were not significantly higher with the control plasmid without IRF-1 gene (Caipang et al., 2009).

Mixing adjuvant with DNA vaccines enhances their strength, enhancing both helper and cytotoxic T cell responses. This is observed with both established and experimental immune potentiators. To stimulate immune response adjuvant-active molecules can combine with the plasmid of interest. They are made exclusively by cells of the immune system and are more important in immune response (Schroder et al., 2004). To increase vaccine potency and efficacy of poor performing DNA vaccines one should explore strategies such as inclusion of molecular adjuvants perhaps in combination with targeting carrier systems such as nano- and microparticles. Although transfection levels are much depressed, studies have demonstrated a persistence of transgene expression at the injection site that might coincide with a time when the fish would normally be ready for slaughter (Tonheim et al., 2008). The demonstration that mycobacterial DNA had adjuvant activity, preceded to the discovery that the adjuvant activity correlated with a higher content of CpG motifs present in bacterial nucleic acids. DNA containing CpG motifs is one of the most potent cellular adjuvants (Weiner et al., 1997).

2.9.3. Construction of bicistronic vector
Unlike most eukaryotic mRNA in which ribosomes scan from the 5' end until the initiation codon is reached, ribosomes are able to begin translation at internal ribosome entry sites (IRES) in messenger RNA of the picornaviruses (Jackson et al., 1990; Jang et al., 1988), such as encephalomyocarditis virus (ECMV). These IRES elements can be transferred from their viral setting and linked to unrelated factors to produce polycistronic RNAs. pIRES is a mammalian expression vector that allows to express two genes of interest at high levels by cloning them into multiple cloning sites (MCS) A and B. These MCSs are located on either side of the internal ribosome entry site (IRES) from the Encephalomyocarditis virus (ECMV), which allows translation of two consecutive open reading frames from the same messenger RNA. pIRES utilizes a partially disabled IRES sequence which will have the affect of a reduced rate of translation initiation at the second, downstream cloned gene relative to that of the first. The MCSs and IRES sequence are downstream of the immediate early promoter of cytomegalovirus. The intervening sequence between immediate early PCMV and the MCS is an intron that is efficiently spliced out following transcription. SV40 polyadenylation signals downstream of the MCS direct proper processing of the 3' end of the mRNA from your gene of interest. Bacteriophage T7 and T3 promoters are located upstream and downstream of MCS A and B, respectively. pIRES uses the neomycin resistance gene (Neor) to permit selection of transformed cells. The SV40 origin also allows for replication in mammalian cells expressing the SV40 T antigen. The vector backbone also contains the b-lactamase gene for ampicillin resistance and a ColE1 origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production. Kadam et al. (2009) developed a bicistronic DNA vaccine construct co-expressing immunogenic protein of mycobacterium and interferon gamma in mice models, to enhance immunogenic effect of DNA vaccines. The use of a bicistronic expression cassette encoding both the genes of interest and the selection marker minimizes the formation of clones expressing the resistance marker, but not the gene of interest (Rees et al., 1996). So in many cases, eliminate the need to isolate, analyze, and propagate single clones.
3. MATERIAL AND METHODS

The present work was carried out in the laboratory of Aquatic Animal Health Management division of the Central Institute of Fisheries Education, Mumbai, India.

3.1. Chemicals and Kits

All the chemicals and kits used in the study were of molecular biology grade.

3.2. Glass and plastic wares

All the glass wares used for this study were from Borosil India. Microfuge tubes, PCR tubes, pipette tips, spreaders and sterilised petriplates were from Tarsons and Axygen, USA. Cell culture flasks and plates were from Nunc, Denmark.

3.3. Buffers and other reagents

The recipes of various buffers and reagents used for the study are described in appendix.

3.4. Preparation of glasswares

All the glasswares were soaked overnight in mild and soft detergent solution. Next day, they were washed thoroughly in tap water and finally with triple glass distilled water. After air drying, they were wrapped in brown paper and sterilised in hot air oven at 160 °C for 90 minutes.

3.5. Preparation of bicistronic construct of 2 genes

The construct of cloned genes of GAPDH and GAPDH+IFN genes were available in the AAHM lab. It was just revived from the master plate to LB ampicillin plates for fresh growth of culture. Revival of constructs were done every 15 days to maintain its viability for further use.
3.6. Fish

Test fish, *Labeo rohita* (rohu) of an average weight 20±6.6g (mean ± SD, N = 30±15) were obtained from local farm at Mumbai,Maharastra. Approximately 45 fish in each group and also for control were reared in 500 L tanks with well-aerated flowing water at 25°C. Fishes were fed with 0.5 mm commercial dry pellets corresponding to 2-3% of the fish body weight per day for the entire experiment.

3.7. Experimental design

There were 4 groups in this experiment viz, positive challenge control, negative control, GAPDH plasmid vaccinated group, GAPDH + IFN plasmid vaccinated group. The latter 2 groups were first immunized at 0 day and a booster dose was also given at 21st day. At 35th day of experiment all the 3 groups i.e., challenge control, GAPDH and GAPDH+IFN were challenged with antigen i.e. *E. tarda* (ET-1). For checking the response of vaccine, blood, liver, kidney tissue samples were collocated at 0 day (1st vaccination) 21st day (booster), 35th day (pre challenge) at 2nd 4th and 6th day (post challenge) from all the groups and subjected to various tests. Further to check response of vaccine construct serum and blood samples were collected at each vaccination day (0 and 21st) and 24, 48, 96 h post vaccination for WBC count and iNOS gene expression.
Table 1.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>NEGATIVE CONTROL</th>
<th>GROUP A</th>
<th>GROUP B</th>
<th>POSITIVE CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO. OF FISH</td>
<td>30±15</td>
<td>30±15</td>
<td>30±15</td>
<td>30±15</td>
</tr>
<tr>
<td>TREATMENT</td>
<td>None</td>
<td>I/m Vaccination with construct containing GAPDH gene of <em>E. tarda</em></td>
<td>I/m Vaccination with construct containing <em>GAPDH</em> gene and IFN γ</td>
<td></td>
</tr>
<tr>
<td>BOOSTER</td>
<td></td>
<td>AT 21&lt;sup&gt;ST&lt;/sup&gt; DAY</td>
<td>AT 21&lt;sup&gt;ST&lt;/sup&gt; DAY</td>
<td></td>
</tr>
<tr>
<td>CHALLENGE</td>
<td>None</td>
<td>AT 35&lt;sup&gt;TH&lt;/sup&gt; DAY</td>
<td>AT 35&lt;sup&gt;TH&lt;/sup&gt; DAY</td>
<td>AT 35&lt;sup&gt;TH&lt;/sup&gt; DAY</td>
</tr>
<tr>
<td>MORTALITY</td>
<td>Mortality and symptom (survival ratio)</td>
<td>Mortality and symptom (survival ratio)</td>
<td>Mortality and symptom (survival ratio)</td>
<td></td>
</tr>
</tbody>
</table>
3.8. Collection of haematological parameters

3.8.1. Collection of blood samples

Blood samples were collected from the caudal vein in aseptic condition by puncturing the caudal peduncle with tuberculin syringe (1 ml). With fish less than six inches in length, puncture of caudal peduncle proved most feasible (Hesser, 1960). The blood samples collected in small glass vials were treated with 20 μl of 2.7% EDTA to prevent coagulation. Aliquots of pooled blood samples of 2 to 3 fishes were used for different estimations. The different haematological analyses were carried out as per described methods.

3.8.2. Serum protein

Serum protein was estimated by Biuret method (Reinhold, 1953) using Innoline™ Total Protein Plus kit. Proteins present in the serum binds with copper ions in an alkaline medium of the biuret reagent and produce a purple coloured complex, whose absorbance is proportional to the protein concentration. 3 test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. 1ml of biuret reagent and 2ml of distilled water were added in all the test tubes. 0.05 ml of protein standard was taken in the test tube labelled as standard and 0.05 ml of serum was added in to the test tube labelled as test. It was then mixed well and incubated at 37°C for 10 minutes. The absorbance of Standard (S) and Test (T) were measured against blank (B) in a spectrophotometer at 630 nm. Test samples were diluted 1/5 DW The calculation was done as follows:

\[
\text{Total proteins in gm}\% = \frac{\text{Absorbance of Test (T) \times n}}{\text{Absorbance of Standard (S)}}
\]

Where, \( n = \) Concentration of Standard

3.8.3. TLC (Total Leukocyte count)

Blood 20 μl was mixed with 3980 μl of WBC diluting fluid in a clean volume. The mixture was shaken well to suspend the cells uniformly in the solution. Care was taken that there were no air bubbles trapped. The no. of cells counted in 4
squares under high power (40X) magnification of light microscope. The no. of WBC per mm$^3$ of the blood sample was calculated using the following formulae: -

No. of WBC/mm$^3 = N \times \text{dilution/area counted} \times \text{depth}

= N \times 20/4 \times 0.1

= N \times 50

Where, N denotes the total no. of WBC counted in 4 squares of haemocytometer.

3.8.4. TEC (Total erythrocyte count)

Blood 20 µl was mixed with 3980 µl of RBC diluting fluid in a clean vial. The mixture was shaken well to suspend the cells uniformly in the solution. A small drop of this mixture was charged to Nenbauer’s counting chamber of haemocytometer. Care was taken that there were no air bubbles trapped. The no. of RBC were counted in 5 groups of squares. All the cells lying inside the 5 small squares are counted under high power (40X) magnification of light microscope. The no. of WBC per mm$^3$ of the blood sample is calculated using the following formulae: -

No. of RBC/mm$^3 = N \times \text{dilution/area counted} \times \text{depth}

= N \times 200/0.2 \times 0.1

= N \times 10000

Where, N is the total no. of RBC counted in 5 square of haemocytometer.

3.9. Sample preparation for enzyme assay

Blood serum was collected at the end of selected test period was stored at -20°C and was used for enzyme assay with or without dilution as and when required.

3.9.1. Enzyme assay

3.9.1.1. Assay of Superoxide Dismutase (SOD, EC 1.15.1.1)

Assay of Superoxide Dismutase was done using Abcam® Kit (ab65354, Superoxide Dismutase Activity Assay Kit). The method uses WST-1 (Water Soluble Tetrazolium) that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. This inhibition activity of SOD was determined by colorimetry in 96 well microplate. The
absorbance was recorded by Biotek® microplate reader at 450nm. The results were expressed in U/ml or as inhibition %. (1 unit (U) is the amount of enzyme that catalyses the reaction of 1 μmol of substrate per minute.)

3.9.1.2. Assay of Glutathione peroxidase (GPx, EC 1.11.1.9)

Assay of Glutathione peroxidise was carried out using Abcam® kit (ab102530, Glutathione Peroxidase Assay Kit). In this method of assay, GPx reduces Cumene Hydroperoxide while oxidizing glutathione (GSH) to oxidized glutathione (GSSG). The generated GSSG is reduced to GSH with consumption of NADPH by Glutathione Reductase (GR). The decrease of NADPH is proportional to GPx activity which was read in a 96 well microplate using Biotek® microplate reader at 340nm. The change in the optical density was read at interval of 5min. Nadph curve was generated using standard NADPH solution included in the kit and GPx activity was calculated using the given formula and expressed in mU/ml.

3.10. Preparation of bacterial cultures

The bacterium *E. tarda* used in the study was obtained from laboratory of Aquatic Animal Health Management, CIFE. Pure culture of the bacterium was inoculated in Brain Heart Infusion Broth (BHI broth) (Himedia, India). The broth was incubated for 18-24 hours at 37°C. One loop of bacteria from the incubated broth was streaked on Nutrient Agar and Salmonella Shigella Agar (SS agar) media. On SS Agar bacterial colonies appeared as transparent with black centres.

3.10.1. Preparation of whole cell of *E.tarda* for challenge studies

Black dot colony of *E.tarda* grown on Salmonella shigella agar was scrapped using a sterile glass rod and added to 5 ml of sterile nutrient broth. After confirmation of cfu/ml by the method of Swain et al (2003), desired amount of bacterial broth were washed 3 times in phosphate buffer saline (PBS), pH 7.4 by centrifugation at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in PBS.

3.10.2. Estimation of lethal dose 50 (LD$_{50}$ )
Ten sterile test tubes were arranged in test tube stand and to all the tubes 9 ml of sterile PBS was added. To the first tube 1 ml broth culture of *E. tarda* and was added and the contents were mixed thoroughly. 1 ml of first tube was transferred to the next tube. This procedure was repeated up to the last tubes. 0.1 ml of sample from each of the dilution was transferred to the sterile petriplates containing nutrient agar and incubated at 25 °C for 16-18 hrs. The LD50 value was calculated by the method described by Reed and Muench (1938). The no. of bacteria was calculated as follows.

\[ \text{Cfu/ml} = \text{No. of colonies} \times \text{Reciprocal of dilution factor} \times 10 \]

### 3.11. Plasmid isolation from vaccine groups

Plasmid designated pGPD and pGPD/IFN contained GAPDH gene and GPDH+IFN gene respectively. The construct was available in the AAHM laboratory. Plasmid was isolated from the master plate of construct of GAPDH and GAPDH+IFN gene separately. For this the construct plate was first revived into new LB agar plate containing 50 µg ampicillin followed by plasmid extraction in bulk.

Plasmid extraction was done by using maxi kit SurePrep® Plasmid Maxi Kit(Anion). First of all culture was grown overnight for 12-16 hrs and then harvested by centrifuging at 6000×g for 15 min at 4°C. The bacterial pellet was then re-suspended in 10 ml buffer P1. Then 10 ml of P2 buffer was added, it was mixed thoroughly by vigorously inverting 4-6 times, and then incubated at r.t.(15-25°C) for 5 min. After that 10 ml of P3 buffer which was prechilled, was mixed thoroughly by vigorously by inverting 4-6 times. It was then incubated on ice for 20 min. It was then centrifuged at 10000×g for 30 min at 4°C. The supernatant was then re-centrifuged at 10000 for 20 min at 4°C. Then it was equilibrated by a QIAGEN-tip 500 by applying 10 ml buffer QBT, and the column was allowed to be emptied by gravity flow. Then the supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. Then the QIAGEN-tip was washed with 2×30 ml buffer QC. The buffer QC was allowed to move through the QIAGEN-tip by gravity flow. The DNA was eluted with 15ml buffer QF into a clean 50ml vessel. For constructs larger then 45kb, prewarming the elution buffer to 65°C may help to increase the yield. The DNA was precipitated by adding 10.5ml (0.7 volumes) at r.t.
isopropanol was added to the eluted DNA and mixed. It was centrifuged at 10000×g for 30 min at 4°C. Then carefully the supernatant was decanted. After that the DNA pellet was washed with 5 ml 70% ethanol at r.t. and centrifuged at 10000 for 20 min. Then decant the supernatant carefully. The pellet was then air dried for 5-10 min and DNA was redissolved in a 1 ml NFW.

3.11.1. Quantification of plasmid

Plasmids were quantified using a Nanodrop (Thermo Scientific), NFW (1 µl) was used to set the blank, which was used to elution of plasmid. Reading for plasmid samples were then taken, nucleic acid concentration was obtained in terms of ng/µl along with their corresponding 260-280 absorbance ratios which indicates purity of the nucleic acids.

3.12. Confirmation of insert by restriction analysis of recombinant clones in plasmid

To confirm the presence of GAPDH and IFNγ gene in plasmid vector double enzyme digestion reaction was carried out. Plasmid DNA (1µL), 10X FD buffer (2 µL), Xho I (1µL), Mlu I (1µL) and nuclease free water (15µL) were mixed thoroughly in ice to make a 20 µL reaction volume. The mixture was incubated at 37°C for 5 minutes and 80°C for 10 minutes. The digestion reaction was done in 0.5 mL tubes. The digested samples were analysed on 1.2% agarose gel in 0.5X TAE buffer. Gel was examined on UV transilluminator and photographed by gel documentation system. The positive clone was designated as pTZ/GPD. Similarly, for IFNγ gene identification similar process is proceeded except the enzymes SALI (1µL), and Not I (1µL) were used for digestion reaction. The positive clone was designated as pIRES / GPD and pIRES / GPD+IFN.

3.13. Immunization and challenge

An appropriate amount of plasmid was dissolved in distilled water, and then stirred at room temperature for 2 h to obtain a stable emulsion. The rohu were divided randomly into 3 groups (30 fish per set), and designated groups GAPDH, GAPDH/IFN and negative control. Fish in group I were vaccinated with GAPDH plasmid (10 µg for each fish) (Zhang et al., 2012) by i/m injection, while the
fish in group II were injected GAPDH+IFN plasmid in the same amount. Samples were stored at −80 °C until RNA extraction.

In addition, blood was collected from the vaccinated fish (3 fish on each occasion) at suitable post vaccination time points mentioned earlier. The blood clotted at room temperature for 1–2 h and then stored at 4°C overnight. Serum was collected after centrifugation at 1000 × g for 10 min, divided into several aliquots and stored at −20°C until use.

In the challenge experiment, fish of each group (20 fish per group) immunized for 35 days were intramuscularly challenged with 100 µl of ET-1 that had been cultured in a NB medium to mid-logarithmic phase, washed and resuspended in PBS to 1.0 × 10^6 CFU ml^−1. The fish were monitored for mortality for a period of two weeks, and the cumulative mortality was calculated according to the following formula: = ( total no fish dead / total number of fish ) × 100, (Amend, 1981). All fish were anaesthetized as described previously when the fish were vaccinated, sampled and challenged, and all the experiments involved with fish were carried out.

3.14. Tissue collection and preservation

The sampling was done every alternate day of post infection of all 4 groups i.e. challenge control, negative control, GAPDH and GAPDH+IFN vaccinated groups. The organs (kidney, liver) were collected by using sterile scissors and forceps. The specimen were preserved 10 % formalin solution for histopathology, and in RNA later or immediately kept at -80°C for further use.

3.14.1. Histopathology

Histopathology was performed according to the method of Pirarat et al. 2006. Briefly, kidney and liver tissues of the fish that had survived bacterial challenge were removed and fixed in 10% neutral buffered formalin. The fixed tissues were processed, and the sections were stained with haematoxylin and eosin. After staining, the sections were observed under microscope.

3.15. Gene expression study
The expression of inducible nitric oxide synthase (iNOS) gene was studied as indicator of interferon response in all groups in blood or tissue (kidney and liver). Before going for real-time PCR primer efficiency was checked by conventional reverse transcription PCR.

3.15.1. Total RNA extraction of tissues

Total RNA was isolated from Blood, Kidney and liver tissues following Sambrook et al. (2001) with some modifications. Briefly, 100 mg of tissue was taken directly in a 2 ml homogenizing tube and homogenized in 1 ml of Trizol reagent (Invitrogen, USA) using a tissue homogenizer (Micro smash MS-100) for 40-60 sec at room temperature. After incubating the homogenate for 5 min at r.t for lysis, it was centrifuged for 5 min and aqueous phase was transferred to a fresh tube. To the lysate, 200 µl chloroform (0.2 vol/ml Trizol reagent) was added, mixed by vigorous vortexing and incubated at r.t for 10 min. The samples were centrifuged at 12000 rpm for 15 min at 4°C and the upper aqueous phase was transferred to a fresh 1.5 ml tube. RNA was precipitated using 500 µl of isopropanol (0.5 vol/ml Trizol reagent), for 10 min at r.t. following centrifugation at 12000 rpm for 15 min at 4°C. The RNA pellet was washed with chilled 70% ethanol (1 vol/ml Trizol reagent), air dried and re-suspended in 35 µl of nuclease free water. RNA was stored at -20°C for short term use (within a week) and -80°C for long term storage.

Concentration of RNA was measured using a Nanodrop (Thermo Scientific). Total RNA integrity was checked by agarose gel electrophoresis.

3.15.2. Nucleic acid purification

Total RNA isolated using Trizol reagent may contain significant amounts of genomic DNA and protein. For that we employed a new protocol called double purification method.

3.15.3. DNAse treatment

The total RNA was treated with DNAse I ‘RnaSE free’ (MBI fermentas, USA), briefly, 2500 ng of total RNA, 2.5 µl of buffer (10X), 2.5 µl DNAse was taken in 0.5 ml microcentrifuge tube and the volume was made up to 17.5 µl with NFW.
The reaction mixture was incubated at 37 °C for 30 min then 2.5 µl of 25 Mm EDTA was added and the reaction was terminated by a 10min exposure to 65°C. RNA was stored at -20°C for short term use (within a week) and -80°C for long term storage. Concentration of RNA was measured using a Nanodrop (Thermo Scientific).

3.15.4. Complementary DNA (cDNA) synthesis

The mRNA from purified total RNA was reverse transcribed to first strand cDNA using an oligo dt primer (MBI Fermentas, USA), following manufactures instruction. Briefly, 1000 ng of total RNA and 1 µl oligo dT (0.5 µl) was taken in 0.5 ml microfuge tube and total reaction mixture volume was made to 13.25 ul with NFW. The tube was incubated at 65°C for 5min, chilled on ice to remove secondary structures and briefly centrifuged. Then 4 µl 5X RT buffer, 0.25 µl RNase inhibitor (40 U/ µl), 2 µl dNTP mix (10mM) and 1µl RevertAid M-MuLV Reverse Transcriptase (200U/ µl) were added. The tube was mixed gently, centrifuged briefly and incubated at 42°C for 1hr. the reaction was terminated at 70°C for 10 min.

3.15.5. Polymerase chain reaction

DNA amplification by PCR was carried out using the standard protocol. The amplification reaction was carried out in a 25 µl final volume in 0.2 ml PCR tubes in a heated lid Thermocycler (ABI, USA). Briefly, reaction mix was prepared by adding 50 ng DNA, 10 pmol of each forward and reverse primer, 200 µM each dNTPs, 0.75 U Taq polymerase, 1.5 mm MgCl₂, 1x assay buffer and volume was made up to 25 ul with sterile DMW. The reaction mix was constituted on PCR cooler (Eppendorf). The contents were mixed by tapping and briefly spin before placing in the Thermocycler. The PCR program was optimized as below.

3.15.6. Optimization of PCR conditions

The annealing temperature for the primers combinations were optimized by using gradient and touchdown PCR. The general PCR program was carried out for the sequence consisted of an initial denaturation at 94°C for 5 min followed by 30 cycles of 95°C for 40 sec, annealing temperature for 40 sec and 72°C for 1 minute. The final extension was done for 10 min at 72 °C. The MgCl₂ concentration in the
reaction mixture was 1.5 mm. Cycling conditions were same for both beta actin gene and iNOS genes except annealing temperature. The PCR products were run on 1% gels for further analysis.

3.15.7. Agarose gel electrophoresis

The PCR products were run in 1% Agarose gels using 0.5x TAE buffer (for reagent compositions please see Appendix II). Ethidium bromide (0.5ug/ml) was added to molten Agarose just before pouring. Generuler 50bp plus ladders from Fermentas were used to determine the size of the DNA fragments. DNA samples were mixed with 6x loading dye and loaded onto the gel. Electrophoresis was carried out in a horizontal submersible unit in 0.5x TAE buffer at 80 V. Gels were photographed using MegaC apt gel documentation system and the molecular weight was determined using GeneTools software version 2.0.

3.16. Real time PCR

3.16.1. Relative quantification of iNOS gene using real-time PCR.

Real time PCR amplifications was carried out in ABI 7500 Real Time PCR detection system (Applied Biosystems). The 25 µl reaction mix volume contained 12.5 µl of Maxima SYBER Green qPCR Master Mix (MBI Fermentas, USA), 0.5µl of (0.3µM) each gene-specific primers and 2µl (50ng) of cDNA. The default thermal profile was used for PCR amplification and it consisted of initial denaturation of 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extention at 60°C for 1min. Melting curve analysis of the amplification products were performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. For each sample two well replicates of each 10µl were used.

The threshold cycle (Ct) was calculated for each RT-PCR. The amount of RNA transcripts of iNOS were normalized to βactin gene and comparison of control Vs treated groups. ΔΔ ct method was used to analyse the data (Livak and Schmittgen, 2001).

\[
\text{Fold change} = 2^{(\Delta \text{ct Test} - \Delta \text{ct control})}
\]
3.16.2. Statistical analysis

The differences in fold change was calculated by between treatments were tested for statistical significance using one-way ANOVA followed by Duncan’s new multiple range test using the statistical package, (SPSS Inc., USA). P value below 0.05 was considered statistically significant. The results were expressed as the mean ± S.D.
### TABLE. 2: PRIMERS USED IN THIS STUDY

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENC E</th>
<th>PRIMER SEQUENCE</th>
<th>ANNEALING TEMP.</th>
<th>AMPLICON SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-actin</td>
<td>Forward</td>
<td>CCATGTTGCACACTTGAT GGA</td>
<td>56° C</td>
<td>56 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCACTGCTGCAAAAG AAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INOS</td>
<td>Forward</td>
<td>AAGGAAAGCCTCCTAATC GTTGT CCATTCCTGGACAGTCT CCAT</td>
<td>57° C</td>
<td>62 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.17. Development of Mab-based competitive ELISA for detection and quantification of antigen specific antibodies

ELISA for detection of antigen specific antibodies was standardized using *E. tarda* GAPDH specific 3G12 MAbs developed in our lab. MAbs were used for competitive binding with *E. tarda* against serum collected from various experimental groups.

3.17.1. ELISA

The competitive ELISA was performed using 96 well polystyrene ELISA plates (Nunc, Denmark). Serum from all four groups ie negative control, challenge control, GAPDH and GAPDH +IFN groups were collected at day 0 (pre vaccination), day 21st (2nd vaccination), 35th day (pre challenge), 37th day (post challenge), 39th (post challenge). Hundred μl *E. tarda* cells having 1 × 10⁵ CFU were incubated with sera samples @ 1: 200 dilution in blocking buffer. Hundred μl of this mix was coated in respective wells. The plate was washed thrice with 200 μl PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) and blocked with 200 μl of blocking buffer for 1 hour at 37ºC. The wells were further washed with PBS-T thrice. Culture supernatant containing MAbs@ 1:200 (Anti-*E.*tarda Ig) were added to each well at 50 μl per well. Eight wells were coated with MAbs only and not antigen antiserum combination. Readings of these wells were taken as reference for studying the competition between MAbs and serum. The plates were incubated for 1 h at 37 ºC and further washed with PBS-T thrice. Further, 50 μl of goat anti-mouse alkaline phosphatase was added to each well at a dilution of 1:2000 and incubated for 30 min at 37ºC. The plates were thoroughly washed with PBS-T five times and substrate (100 mg/ml of O-phenylenediamine tetra hydrochloride and 40 μl of H₂O₂ (30% v/v) in 5 ml of citrate-phosphate buffer, pH 5) was added to each well at the rate of 100 μl per well. The plates were incubated for 5-10 minutes at 37 ºC in dark chamber. After that the reaction was stopped with stop solution (3M H₂SO₄) upon addition of 50 μl to each well and finally the optical density (OD) was recorded at 492 nm in microplate reader (Biorad, USA).

The result were expressed as percentage inhibition, derived by following formula:
100- (mean OD$_{492}$ of test serum X 100)/(mean OD$_{492}$ of MAbs)

3.18. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to check functionality of Mab.

SDS-PAGE was carried out to confirm the immunoreactivity of MAbs against *E. tarda* culture, following the method of Sambrook *et al.* (2001). Discontinuous SDS-PAGE of 12% resolving (Table 3) and 5% stacking (Table 4) gels was casted.

SDS-PAGE apparatus was assembled and the bottom was sealed with 2% agarose. All the ingredients of resolving gel given in the table above were added in the same order and mixed gently. The solution was poured into the gel module leaving sufficient space for stacking gel. Precaution was taken to avoid trapping of any air bubbles in the gel. The gel was left without disturbing for 10-20 minutes for polymerisation. Stacking gel was prepared and layered above the separating gel. The comb was fitted into the separating gel to form the sample wells and kept undisturbed for 15 minutes at room temperature for the gel to polymerise. During this period, the sample preparation was done. About 10 μL of sample ie 10$^5$ *E. tarda* cells were lysed and boiled with 2X sample loading buffer. After the polymerisation of the stacking gel, the comb was removed carefully and the wells were washed with distilled water. The samples and protein marker were loaded in the wells. The upper and lower chambers of the electrophoresis apparatus were filled with 1X tris glycine buffer.

The unit was connected to the electrophoresis power pack with anode at the bottom and was run at 76 V till tracking dye reached the upper margin of resolving gel. Then 96 V was applied till the protein bands reached to the bottom of the gel. Power supply was turned off after the complete run and the unit was disassembled. The gel was carefully separated from the glass plate and transferred to Coomassie blue staining solution for overnight. The gel was destained with repeated changes of destaining solution until the background of the gel was clear. The gel was photographed in gel documentation system.
### Table 3: Composition of 12% resolving gel

<table>
<thead>
<tr>
<th>Gel volume</th>
<th>10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.3</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>4.0</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
</tr>
</tbody>
</table>

### Table 4: Composition of 5% stacking gel

<table>
<thead>
<tr>
<th>Gel volume</th>
<th>5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.4</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>0.83</td>
</tr>
<tr>
<td>1.0 M Tris (pH 6.8)</td>
<td>0.63</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05</td>
</tr>
</tbody>
</table>
3.19. Western blotting

Western blotting was done to check the specificity of Anti-mouse IgG antibody to the GAPDH. Protein bands in the unstained gel were transferred into the nitrocellulose membrane (Whatman, Germany). A set of two extra thick blot papers (Bio Rad) and a nitrocellulose paper was cut to the size of the gel. The nitrocellulose paper was activated by soaking in the distilled water for 5 minutes. The gel, extra thick papers and activated nitrocellulose paper was soaked in the transfer buffer for 15 minutes. The gel was transferred to the semi-dry blotting apparatus (Bio-Rad, USA) in the following order: Extra thick blot paper → nitrocellulose membrane → gel → extra thick blot paper. After the last thick paper was placed, a glass rod was rolled over it to release any trapped air bubble. The apparatus was closed and connected to the power supply unit and electrophoresed at 10 V for 25 minutes.

After electrophoresis, the nitrocellulose membrane was removed and dried using filter paper. For further process amresco western blot kit was used (Amresco, USA). The membrane was blocked with blocking buffer for 30 minutes at room temperature with gentle agitation. After the blocking buffer discarded specific MAbs (3G12)(GAPDH supernatant) was added as 1:200 dilution and incubated for 2 h at r. t. with gentle agitation. The membrane was washed twice with 20 mL of wash buffer for 5 minutes at r. t with gentle agitation. Anti-mice secondary antibody solution was added at 1:2000 concentration and incubated for 30 minutes at r. t with gentle agitation. The membrane was washed twice. The bounded secondary-HRP conjugated antibody was detected by incubating the membrane in BCIP/NBT (Sigma, USA) substrate solution at room temperature until the violet colour developed. The membrane then washed with distilled water for 5 minutes. The membrane was air dried and stored in dark.

3.20. Statistical analysis

All the data were statistically analysed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan’s multiple range test was used to determine the significant differences between the means. Comparisons were made at the 5% probability level.
4. RESULTS

In present study, two genes namely GAPDH from *E. tarda* and IFN-γ from *L. rohita* which were first amplified and cloned in a bicistronic vector pIRES in different frames. Expression of both the genes was checked in plasmid by RE digestion before using it.

4.1. Confirmation of bicistronic construct

Before starting the work first we confirmed the positive clone by restriction analysis for releasing of GAPDH fragment from the plasmid by double digestion with *Mlu* I and *Xho* I enzymes and IFN-γ gene by double digestion with *Sal* I and *Not* I enzymes. Release of 996 bp GAPDH fragment and 665 bp IFNγ fragment was seen. After this the positive construct was confirmed and was designated as pIRES/GPD/IFNγ (Fig 1). The construct containing only GAPDH gene in frame A was designated as pIRES/GPD.

4.2. Western blotting

Western blot was carried out to check the immune reactivity of MAb (3G12) against GAPDH protein of *E. tarda* Specific reactions were detected with the MAb. The MAb designated as 3G12 showed a clear specific reaction with the GAPDH protein and inactivated *E. tarda* lysate at molecular weight of 37 kDa (Fig. 2).

4.3. Fish

Test fish, *Labeo rohita* (rohu) of an average weight 20±6.6g were taken (Fig.3).

4.4. Bacteriology

The colonies of the bacteria isolated from diseased fishes were identified as that of *E. tarda* on the basis of colony morphology, staining characteristics and biochemical properties. The colonies appeared transparent with black centre after overnight incubation at 37°C in Salmonella shigella (SS) agar medium (Fig.4). In
other solid media like nutrient agar plates and BHI agar plates, they appeared as smooth, circular (0.5 - 1 mm), whitish translucent about 0.2 mm raised from base, and on staining with Grams stain appeared gram-negative small rods to coccobacilli. The length was about 2 - 4.5 times more than the width and was non-sporing and non-capsulated bacteria.

**4.5. Total plate count**

Based on the results of this preliminary experiment of LD₅₀, the immunized fish in the each groups were challenged by a dose of 1.0 × 10⁶ colony-forming units (CFU) / fish with intraperitoneal injection. Mortality was recorded twice a day for 2 wk.

**4.6. Challenge study**

**4.6.1. Estimation of Relative percentage survival (RPS)**

Relative percentage of survival = \( \{1 - \left( \frac{\text{Percentage mortality in vaccinated group}}{\text{Percentage mortality in control group}} \right) \right\} \times 100 \)

From the second day after infection, fish in the challenged control group started to die, and the survival rate decreased sharply until the fourth day after infection to a final survival rate of 40%. The final survival rate of the immunized group was 66.66% in GAPDH+IFN group whereas 51.11% in GAPDH group. Fish immunized with GAPDH+IFN maintained a markedly higher survival rate than the unimmunized fish (Table: 5); 3 groups were significantly different (p < 0.005).
Table 5:

<table>
<thead>
<tr>
<th>DAYS POST CHALLENGE</th>
<th>% survival in GPADH+IFN group</th>
<th>% survival in GAPDH vaccinated group</th>
<th>% survival in challenge control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; day</td>
<td>95.55</td>
<td>93.33</td>
<td>88.88</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>86.66</td>
<td>84.44</td>
<td>51.11</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>77.77</td>
<td>73.33</td>
<td>46.66</td>
</tr>
<tr>
<td>8&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>66.66</td>
<td>55.55</td>
<td>44.44</td>
</tr>
<tr>
<td>10&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>66.66</td>
<td>51.11</td>
<td>40</td>
</tr>
</tbody>
</table>
4.7. Haematological Assay

4.7.1. Total leucocyte count (TLC)

Total WBC count was estimated at 0 hr, 24 hr, 48 hr, 96 hr post immunization of first dose of vaccine and similarly done after booster dose of vaccine treatment just to study the change in WBC count after plasmid injection in the fish. So, it was found that there is significant increase in WBC count at 48 hrs after first immunization and booster immunization. The mean WBC count given in Fig.5.

After challenging with bacteria challenge control group and immunized group showed significantly (P < 0.05) higher WBC count in comparison to negative control group. There was an increase in WBC counts upto 48 hrs of post challenge with bacteria *E. tarda*. The mean WBC count are shown in Fig.6.

4.7.2. Total erythrocyte count (TEC)

Challenge control and immunized group showed significantly (P < 0.05) lower RBC counts in comparison to negative control group (Fig.7). At 37th day there is reduction in RBC count after bacterial challenge. Mean value of RBC counts of control and treatment group are shown in Fig.7.

4.8. ENZYME STUDY

4.8.1 GPx activity

The present result significantly showed that GPx activity in vaccinated group is enhanced as compared to control group, even after challenged by *E. tarda* bacteria, GAPDH and GAPDH+IFN vaccinated group showed enhanced expression. Therefore, it is found that vaccinated group show significantly (p < 0.05) higher GPx activity in comparison to positive and negative control group. Maximum
GPx activity was observed after 48 hrs of challenge (37 day). The mean values of GPx activity of all control and immunized groups are shown in fig. 8, 9, 10.

4.8.2 SOD activity

Immunized group show significantly (p < 0.05) higher SOD activity after challenge in comparison to challenged group and control group. Maximum SOD activity was observed after 48 hrs. The mean values of SOD activity of all control and immunized groups and challenged group after giving first vaccine dose at 15 day, booster dose at 21st day and challenging with E. tarda bacteria at 37th day are shown in fig. 11, 12, 13 respectively.

4.9. Clinical signs and pathological changes

The diseased rohu showed accumulation of fluid in the abdomen and dropsy (Fig.14). Severely, affected fish showed signs of blood oozing out from vent (Fig.15), reddening of fins (Fig.16), swollen abdomen (Fig.17), haemorrhages all over the body (Fig.18), pigmented entire body (Fig.19).

4.10. Histopathology

4.10.1 Kidney

Severely necrotized tubular cells along with marked extensive renal interstitial haemorrhage was found in kidney tissue after 48 hrs of challenge with bacteria in Challenged group (Fig.21). Whereas mild oedema and renal tubules with sloughed basement membrane found in GAPDH+IFN vaccinated group (Fig.23) and swelling in tubular cells characterized by the hypertrophy of cells (narrowing of tubular lumen) found in GAPDH vaccinated group (Fig.22). These 3 groups histopathological signs are compared with healthy signs of control group (Fig.20).

4.10.2. Liver

Liver tissue displayed diffusely necrotized hepatocytes, damaged hepatic cords, along with oedema at places after 48 hrs of post challenge in positive control group (Fig.27) compared with normal liver of control (Fig.26). Liver tissue showed oedema due to necrotized hepatocytes and atrophied acinar cells in GAPDH vaccinated group (Fig.24) where as GAPDH+IFN vaccinated group showed liver
tissue exhibiting hepatocytes with pyknotic nuclei, dilated and congested sinusoids and oedema at places (Fig.25).

4.11. Competitive ELISA

Antibody responses were noted in all groups after primary and secondary immunizations, and 48 hrs and 96 hrs post challenge. Percentage inhibition activity calculated for each group showing significantly higher inhibition in GAPDH+IFN group as compared GAPDH group as well as challenge control group.

4.12. PCR amplification of iNOS gene

PCR amplification of 62bp of INOS gene was obtained at 57°C annealing temperature with forward and reverse set of primers at the rate of 25 pmol and 100 ng/μL DNA template concentration. The template DNA from E. tarda using the particular primers on repeated experiments produced highly reproducible single band consistently. The amplified DNA band was 62 bp in size on 1.2% agarose. Gene expression was studied at various intervals after first vaccination and booster vaccination at 0 hr, 24 hr, 48 hr, 96 hr respectively. There was significant difference of expression of iNOS among two vaccines in which GAPDH+IFN vaccinated group showed higher expression compared to other groups.(Fig.29).

The expression of iNOS gene was checked 2 days, 4 days, 6 days post challenge also in all the four groups (Fig.30). The expression here also of GAPDH+IFN vaccinated group showed high expression at 4th day but after that it reduced by attaining normalcy.
Fig. 1: Agarose gel electrophoresis (0.8%) showing RE analysis of pIRES vector confirming GAPDH and IFN gene
Lane M: 1kb plus molecular weight marker (100bp)
Lane 1 – Linearised pIRES vector
Lane 2 – release of GAPDH gene (996 bp)
Lane 3 – release of IFN gene (665 bp)

Fig. 2: Western blot of 37 kDa of GAPDH protein
Lane M: Prestained protein molecular weight marker
Lane 1,2,3: E. tarda cell lysate showing GAPDH protein reacted with Mab (3G12)
Fig. 3: Test fish, *Labeo rohita* (rohu) (avg. wt. 20±6.6g)

Fig. 4: Colonies of *E. tarda* on S.S. agar, streaked from kidney of *Labeo rohita*
Fig.5: Total WBC count after immunization with GAPDH and GAPDH+IFN constructs on various time points

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>241.7</td>
<td>239.0</td>
<td>214.0</td>
<td>245.0</td>
</tr>
<tr>
<td>GAPDH</td>
<td>238.0</td>
<td>245.0</td>
<td>270.0</td>
<td>289.0</td>
</tr>
<tr>
<td>GAPDH+IFN</td>
<td>240.0</td>
<td>259.0</td>
<td>289.0</td>
<td>289.0</td>
</tr>
</tbody>
</table>

First vaccination

<table>
<thead>
<tr>
<th></th>
<th>0h</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>245.0</td>
<td>234.0</td>
<td>239.0</td>
<td>237.0</td>
</tr>
<tr>
<td>GAPDH</td>
<td>249.0</td>
<td>269.0</td>
<td>290.0</td>
<td>246.0</td>
</tr>
<tr>
<td>GAPDH+IFN</td>
<td>257.0</td>
<td>290.0</td>
<td>320.0</td>
<td>290.0</td>
</tr>
</tbody>
</table>

Second vaccination

Fig.6: WBC count of different groups immunized at 0 day and 21 day and given bacterial challenge at 35th day

<table>
<thead>
<tr>
<th></th>
<th>1st vaccination (0 day)</th>
<th>2nd vaccination (21 day)</th>
<th>challenge (35th day)</th>
<th>2nd day post challenge (37 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>240</td>
<td>200</td>
<td>214</td>
<td>201</td>
</tr>
<tr>
<td>GAPDH</td>
<td>238</td>
<td>157</td>
<td>235</td>
<td>410</td>
</tr>
<tr>
<td>GAPDH+IFN</td>
<td>240</td>
<td>187</td>
<td>285</td>
<td>459</td>
</tr>
<tr>
<td>Challenge control</td>
<td>239</td>
<td>190</td>
<td>240</td>
<td>416</td>
</tr>
</tbody>
</table>

Total WBC post vaccination
Fig. 7: TEC from four studied groups which were vaccinated at 0<sup>th</sup> and 21<sup>st</sup> day and challenged with <i>E. tarda</i> at 35<sup>th</sup> day.
Fig. 8: GPx activity on 15 day after first immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

Fig. 9: GPx activity on 21 day after second immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

Fig. 10: GPx activity on 37 days after 48 hrs post challenge in control (unimmunized) group, GAPDH group, GAPDH+IFN group, challenge control.
Fig.11: SOD activity on 15 day after first immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

Fig.12: SOD activity on 21 day after booster immunization in control (unimmunized) group, GAPDH group, GAPDH+IFN group.

Fig.13: SOD activity on 37 day at 48 hrs post challenge in control (unimmunized) group, GAPDH group, GAPDH+IFN group and in challenge control.
Fig. 14: Accumulation of fluid in the abdomen and dropsy

Fig. 15: Diseased fish showing protruded vent and haemorrhages all over the body

Fig. 16: Diseased fishes showing reddening of fins.

Fig. 17: Swollen abdomen of diseased fish

Fig. 18: Haemorrhages all over the body of fish

Fig. 19: Darkened body pigment of diseased fish
Fig. 20: Healthy kidney

Fig. 21: Severely necrotized tubular cells (arrow head) along with marked extensive renal interstitial haemorrhage (arrow) (Challenged group kidney)

Fig. 22: Kidney tissue showing swelling in tubular cells characterized by the hypertrophy of cells (narrowing of tubular lumen)(arrow). (GAPDH vaccinated group kidney)

Fig. 23: Tissue section of kidney showing mild oedema(arrow) and renal tubules with sloughed basement membrane (arrow head) (GAPDH+IFN vaccinated group kidney)
Fig. 24: Liver tissue showing oedema (arrow) due to necrotized hepatocytes and atrophied acinar cells (arrow head) (GAPDH vaccinated group liver).

Fig. 25: Liver tissue exhibiting hepatocytes with pyknotic nuclei, dilated and congested sinusoids (arrow) and oedema at places (arrow head) (GAPDH+IFN vaccinated group liver).

Fig. 26: Healthy liver

Fig. 27: Liver tissue displaying diffused necrotized hepatocytes, damaged hepatic cords, along with oedema at places (arrow) (Challenge control).
Fig. 28: Percentage inhibition of antibody binding in different groups
Lane M: 50 bp molecular weight marker

Lane 1 - 16: expression of iNOS gene in kidney tissue on various days

**Fig.29:** Agarose gel electrophoresis showing RT-PCR based expression of iNOS in positive control, GAPDH and GAPDH+IFN vaccinated group.

Lane M: 50 bp molecular weight marker

Lane 1, 2, 3: Negative control of control, GAPDH and GAPDH+IFN vaccinated groups respectively.

**Fig.30:** Agarose gel electrophoresis showing RT-PCR based expression of β actin in kidney tissue in positive control, GAPDH and GAPDH+IFN vaccinated group.
Fig.31: Fold change in expression of iNOS gene in blood sample collected after each vaccination.
Fig.32: Fold change in expression of iNOS gene in different groups after challenging with bacteria.
5. DISCUSSION

Aquaculture is the fastest growing sector of agriculture in most part of the world. Aquaculture growth has been accompanied by economic loss due to fish diseases (Hanneson, 2003). Out of various infectious bacterial diseases, Edwardsielllosis caused by Edwardsiella tarda (E. tarda) is a dreadful problem. Unavailability of effective means of control and prevention especially the lack of effective vaccines cause heavy economic losses in aquaculture industries worldwide due to E. tarda infection. Vaccination is the most effective method for reducing suffering and economic losses due to the infectious diseases. But in some cases live vaccines with attenuated agent would be of concern. This is the interest behind the concept of DNA immunisation or third generation of vaccine. Improvisation of DNA vaccine with addition of some immunoadjuvants may deal with the questions raised over efficiency of DNA vaccines.

In this study, we analyzed the immunogenic potential of two DNA vaccine construct one containing GAPDH gene of E. tarda and another containing GAPDH+IFN of rohu. We found that GAPDH+IFN gene showed high immune-induction as compared to GAPDH, it also showed higher protective efficiency. Hence, it appears that under the experimental conditions, DNA construct expressing GAPDH and IFNγ induced protections. Although it is possible that these results were coincidental and could be different if conditions associated with the vaccination procedure, such as vaccine dose and delivery route, vary, they nevertheless suggest that in some cases, DNA vaccine is not superior to other types of vaccine as far as protective efficacy is concerned.

Two construct used in this study for immunization were previously cloned and eukaryotically expressed in our laboratory. Plasmids were designated as pIRES-GAPDH and pIRES-GAPDH/IFN, later one expressed two genes as a bicistronic construct. pIRES is a bicistronic vector that contains internal ribosomal entry site IRES, that allows expression of two genes independently under one promotor. We used this strategy to co-express IFNγ of rohu as immunoadjuvant.
along with GAPDH gene. We found that bicistronic construct can be effectively used as vaccine agent.

Bicistronic vectors have been used to design DNA vaccine against HIV infection, which contained gp120 and GM-CSF gene (Barouch et al., 2002), bicistronic DNA vaccine containing apical membrane antigen 1 and merozoite surface protein 4/5 can prime humoral and cellular immune responses and partially protect mice against virulent plasmodium chabaudi adami DS malaria (Rainczuk et al., 2004), and a bicistronic woodchuck hepatitis virus core and gamma interferon DNA vaccine can protect from hepatitis (Wang et al., 2007). Kadam et al., 2009, have reported that coexpression of IFNγ with a 16.8 kDa gene of Mycobacterium avium paratuberculosis can enhance immunogenicity of DNA vaccine using the same protein.

First of all we confirmed our clones for GAPDH and IFN insert by restriction digestion analysis and monoclonal antibody for its reactivity with E tarda by western blot. As these two things is main foundation of our rest of the studies. Our study was mainly based on two approaches 1) to study the immune response generated against both constructs after each vaccination for which we collected blood every alternate day after vaccine and booster day. 2) to study the challenge protection study, for this after 35th day of first vaccination we challenged two groups with E tarda culture and observed the symptoms, mortality and some parameters like stress enzymes, iNOS expression and blood picture.

5.1. Haematology

5.1.1. Erythrocytes

The present study revealed that in the case of bacterial challenge the total RBC count in blood significantly decreased initially by the 2nd post challenge day and at the 4th day. The reduction in RBC count after challenge with E tarda can be attributed to the ability of bacteria to haemolyse the erythrocytes as have been observed and explained by Oliver et al., 1981; Kumar et al., 2006 in their studies related to a toxigenic profile of Aeromonas hydrophila and A. sobria from the fish and challenged studies of rohu with A. hydrophila respectively. Hematological parameters have been considered as important indicators of fish health (Chen et al.,
2004, Martins et al., 2004). Studies demonstrated that the reduction in the number of erythrocytes in the blood and in the hematocrit percentage may be signs of bacterial infection (McNulty et al., 2003, Benli & Yildiz 2004, Shoemaker et al. 2006).

5.1.2. Total leukocyte count (TLC or WBC)

WBC count is an indicator of infection or nonspecific immune response. Cell proliferation is governed by IFN induction. In vaccine groups we observed that after 24 h of first vaccination there was no significant increase in WBC count but at 48 h the significant rise in WBC count was observed in GAPDH and GAPDH +IFN group, which reached to almost normal at 96 h post vaccination. The rise in WBC count may be attributed to trigger of immune mechanism. At second immunization the rise in WBC in both the groups was significant at 24 h and then remained there till 48 h and then reduced to normal at 96 h post boostering. The rise in WBC count at 24 h post booster indicate that the cells were already sensitized to vaccine so secondary response took less time as compared to first vaccination. The WBC count was higher in GAPDH + IFN group; this explains the role of IFN in cell proliferation.

When comparison was made with challenge control group the WBC count was observed significantly higher in challenged group and both vaccinated group compare to control group at 48 h post challenge. The WBC count of challenge and vaccine group were in the same range. Similar finding was also reported by Kumar et al., 2006 in an experiment associated with challenged study of rohu with A. hydrophila. This might have been due to increased production of WBC to counter against the bacteria. The fish groups showed a sharp increase in the leucocyte proliferation by head kidney after 2 days of infection. The significant augmentation of the proliferative responses indicated clonal expansion of rohu leucocytes after E. tarda infection. Such proliferative responses have also been detected in rohu inoculated with A. hydrophila (Das et al., 2009). Similar proliferative responses were also reported in other fish species (Marsden and Secombes, 1997; Marsden et al., 1995).
IFNγ indirectly induces cell proliferation by activating macrophages and increasing antigen presentation which induces IL2 receptors on T cell surface, thereby inducing cell proliferation. The results were in consensus as found by other workers who used cytokines as immunoadjuvant in bicistronic DNA vaccine. Chow et al. (Chow et al., 1997) have reported increased cell proliferation in group that received hepatitis B virus surface protein and IL2 as bicistronic DNA vaccine. Barouch et al. (Barouch et al., 2002) found twofold augmentation of cell proliferation in bicistronic group which coexpressed gp120 gene of HIV and GMCSF than in monocistronic gp120 immunized group. Kadam et al., also found that bicistronic vector expressing a 16.8 kDa protein of MAP along with IFNγ gene induce higher proliferative response than the protein alone.

5.2. Expression of iNOS gene

We found that expressions of the genes coding for INOS were significantly elevated in GAPDH and GAPDH+IFN vaccinated fish. The induction level of the INOS gene was high in GAPDH+IFN vaccinated fish and was comparable to that in GAPDH vaccinated fish. These observations suggest that IFNγ probably exerts a positive effect on the expression of INOS gene.

In another set of comparison we analysed iNOS expression in blood cells of vaccinated groups, immediately after vaccination. Here again we observed significantly high expression of iNOS in GAPDH+ IFN group compared to GAPDH group. Again we observed effect of sensitization in terms of early iNOS expression at 24 h post booster, compared to 48 h after first vaccination in both the groups.

With respect to the effect of DNA vaccine on the transcription profile of the immune system, although relevant data are scarce for bacterial DNA vaccines, numerous studies in this respect have been carried out with viral DNA vaccines, most of which analyzed the effect of DNA vaccines at various stages prior to viral challenges, and the results have provided valuable insights to the operating mechanisms of DNA vaccines (Kurath et al., 2007; Kurobe et al., 2005; Purcell et al., 2006; Yasuike et al., 2007; Byon et al., 2005; Byon et al., 2006; Takano et al., 2004). In our study, since we were more interested in immune reactions that are likely to be
responsible for protection, we examined the expression of iNOS genes immediately following bacterial challenge in kidney tissue.

5.3. Humoral Immune response

A competitive ELISA test was performed to compare the antibody status. Since it was a competitive ELISA the results were inferred in terms of “percent inhibition”. Mabs developed against rGAPDH competed for binding to *E. tarda* with serum collected from different groups. The serum which contained less no. of antibodies bound to *E. tarda* antigen more MAbs were bound to *E. tarda* and higher optical density was recorded. High percentage inhibition was considered as representation of high antibody concentration in sample.

In present study we observed almost same level of antibody status of all the groups at day 0. There was insignificant increase in antibody concentration at 21 day of first vaccination however at 35 day there was significant rise in antibody concentration in both immunized groups. This indicated that optimum time taken for antibody response generation after DNA immunization is 35 days. After 48 h of challenge the antibody response in GAPDH+IFN was significantly high as compared to GAPDH and challenge control, which reduced at 96 h post challenge. The sudden rise in antibody in this group suggest role played by IFNγ in humoral antibody generation. This observation is in contrast to the that of Kadam et al, 2009, where antibody titre was not effected by IFNγ injection. Here we explain that IFNγ indirectly increase the antibody response. This result were also in contrast to the groups who find that codelivery of IFN-gamma or IL-4 encoding EG95 protein of *Echinococcus granulosus*, the causative agent of hydatid appeared to reduce the ability of the DNA vaccine to prime an IgG antibody response demonstrated the efficacy of the codelivery of cytokines to modulate immune responses generated in a DNA prime-protein boost strategy (Scheerlinck *et al.*, 2001).

5.4. Enzyme study

Enzymatic studies can be used as an important tool in the case of pathological conditions in animals; however this has not been used properly in the case of fishes. Under several stressful condition, different enzymes in the
plasma/serum may show variations. It has been observed that variation in GPx activity and SOD activity indicate exposure to cellular stress and adverse cellular effects (Dash et al., 2006). Glutathione peroxidases (GPxs) are key enzymes in the antioxidant defence system of living organisms, and protect organisms against oxidative stresses. Glutathione peroxidase enzymes are involved in the elimination of ROS produced in physiological and pathological processes. The present result significantly showed that GPx activity in vaccinated group is enhanced as compared to control group, even after challenge by *E. tarda* bacteria, GAPDH and GAPDH+IFN vaccinated group showed enhanced expression. Excessive ROS production has been found to oxidize and damage cell membrane, proteins, and nucleic acids (Hensley et al., 2000). In marine animals, the oxidative stress can be counteracted by non-enzymatic antioxidant and enzymatic antioxidant systems (Fernández et al., 2010). The superoxide production significantly (p < 0.05) decreased in the *E. tarda* challenged rohu to that in the positive control fish immediately after challenge till 48 hrs post-challenge (p.c.) after 96 hrs, The superoxide production in infected fish returned back near to control fish value. The elevated levels of the SOD in vaccinated fish response to *E. tarda* infection highlights their important functions in eliminating toxic reactive oxygen species (ROS) and protecting organisms from bacterial invasion in *L. rohita*.

Superoxide dismutases are enzymes that catalyze the dismutation of superoxide (O$_2^-$) into oxygen and hydrogen peroxide Thus, they are an important antioxidant defence in nearly all cells exposed to oxygen. SODs are ubiquitous metalloenzymes that can scavenge superoxides in response to various stresses. Immediately upon infection of Japanese flounder head-kidney macrophages, *E. tarda* also upregulated the gene expression of an iron-cofactored superoxide dismutase (FeSOD), while FeSOD mutants have increased sensitivity to H$_2$O$_2$; are more susceptible to macrophage-mediated killing; and exhibit reduced cellular invasion and bacterial dissemination (Cheng et al., 2010). The survivors on 96 hrs post-infection showed almost similar level to that of control fish, thus proving that these fish are coming back to normalcy.
5.5. Challenge protection assay

After 48 h of infection high mortality was observed in Challenge control group but in immunized group survival ratio was very high. This explained the effective response of immunization. The survival of the fish in immunized group may be attributed to many unexplained pathways involved along with humoral and cellular response.

5.6. Histopathology

On histopathological examination, classical signs of chronological development of *E. tarda* infection were observe in challenge control group. Suppurative interstitial nephritis is found most often followed by suppurative hepatitis. *E. tarda* infection causes hypertrophy of the liver cells and enlargement of their nuclei (Miwa and Mano 2000). Bacteria-laden phagocytes are found in the sinusoids of the anterior, liver and spleen. Liquefaction and gaseous necrosis are seen in the kidney, liver, spleen and body musculature leading to ulcer formation (Sahoo *et al.*, 2000; Jin *et al.*, 2000). Increased melanomacrophage reaction is frequently observed in the kidney. The gills show hyperplastic changes. Due to massive haemolysis of the red blood cells, the spleen accumulates haemosiderin pigments along with the presence of hyperaemia and necrotic changes (Herman and Bullock 1986; Darwish *et al.*, 2000). The authors have witnessed major damage to the primary ovarian follicles along with oophoritis in Asian catfish due to *E. tarda* infection (Sahoo *et al.*, 1998). In vaccine group also some histopathological lesions were observed but they were very less compare to challenge group. It explains that there was a mild infective stage in immunized fish which suppressed because of immune response generated by immune groups.

5.7. GAPDH

In the present study the 37 kDa GAPDH gene was used and expressed. On SDS-PAGE analysis of the recombinant GAPDH, the size of the protein was 37 kDa as expected. The GAPDH protein is the major OMP of *E. tarda*. It is highly immunogenic and offers protection to the fish when injected into them (Kawai *et al.*, 2000).
Hence the recombinant GAPDH produced in the present study has the potential as a vaccine in fishes.

5.8. IFN

IFN-γ is the key cytokine in Th1 type of immune response required for intracellular pathogens (Flynn and Chan, 2001). IFN-γ gene of fishes has similar functions in the innate and adaptive immune system as in mammals. The mature fish IFN-γs contain 156 to 167 amino acids and signal peptides of 23 to 24 amino acids. It can act as an ideal immunoadjuvant for the vaccine preparations. The IFN-γ gene of *L. rohita* can alter transcription in up to 30 genes producing a variety of physiological and cellular responses. Also it can induce the expression of intrinsic defence factors. IFN-γ has been identified and cloned from Fugu (Zou *et al.*, 2004), rainbow trout (Zou *et al.*, 2005), channel catfish (Milovanovic *et al.*, 2006), zebra fish (Igawa *et al.*, 2006), Atlantic salmon (Das *et al.*, 2007), common carp (Stolte *et al.*, 2008), gold fish (Grayfer and Belosevi., 2009), grass carp (Chen *et al.*, 2010). The protection offered to a host from whole pathogen by a single protein is always uncertain. So we tested the hypothesis that inclusion of IFN-γ gene in vaccine construct will enhance the immune response. We observed that inclusion of IFNγ in vaccine construct synergized the immune response generated by GAPDH.

Together these results indicate that GAPDH could generate immune response in rohu this response was increased by addition of IFNγ. It is true that this is a preliminary study and many factors like environment, dose of vaccine, load challenge, temperature of water, half life of vaccine ect needed to be addressed before declaring the vaccine construct as effective for immunization. Still our concept of adding IFN as immunoadjuvant is tested for the first time in fisheries science, and can give a new direction to vaccine research in aquaculture.
6. **SUMMARY**

*Edwardsiella tarda* is a Gram negative bacterium of the family *Enterobacteriaceae* and a serious pathogen of cultured and wild, freshwater as well as marine fishes. It is the causative agent of the systemic disease edwardsiellosis which is responsible for causing high morbidity and mortality in a wide range of freshwater and marine fishes. Vaccines provide an effective and eco-friendly substitute to antibiotics and chemicals. DNA vaccines as when compared to traditional vaccines have several practical and immunological advantages that make them very attractive for the fishery sector. In this situation exploring new potential vaccine candidate is a necessity to guard our culture practices.

Hence, in the present study, biscardstonic DNA vaccine construct was checked for its potency against *E. tarda* in one of the major carp *L. rohita*. Study revealed that GAPDH+IFN vaccine construct elicited better immune response compared to GAPDH construct alone. Ther immunogenicity is estimated by performing several experiments with relative survival rate of the immunized group was 66.66% in GAPDH+IFN group whereas 51.11% in GAPDH which can be efficient using against *E. tarda* infected fish.

Total leucocyte count was significantly higher in immunized group and the probable reason was induction of cell proliferation by IFNγ and antigenic gene GAPDH.

The enzymatic study revealed that GPx and SOD activity was enhanced in immunized group as compared to control groups. GPx and SOD activity proved that vaccine mimics antigenic property and put the host under stress, as seen in both vaccinated group.

Histopathological studies were conducted to correlate the findings of other two parameters i.e. haematology and enzyme study. It was clearly found that some pathological signs were reduced in vaccinated group when challenged with pathogenic bacteria *E. tarda* in compared to unimmunized challenged group where
severe haemorrhages, oedema and fully necrositized tubular cells were observed. But the signs were somewhat reduced in both vaccinated group.

Real time PCR diagnosis also revealed that immune related genes such as iNOS upregulation on getting bacterial infection. The production of nitric oxide (NO) was selected as a criterion of metabolic activity, which has significance for functional (immunological) activation of mononuclear phagocytes. The production of inducible NO is a stringently controlled process and there is a delicate balance between various microbial stimuli, host derived cytokines and other important factors in microenvironment vital for its regulation. Serum anti- E. tarda antibodies measured by ELISA was useful in evaluating the immunogenicity of vaccine construct. However, ELISA data in our study also indicated that the immune response of vaccinated group correlated well with protection efficacy.

The present study can be used as basic experimental model to compare the efficacy of vaccines with and without adjuvant. These preliminary observations need further confirmation for application in in-vivo like natural hosts for the development of an effective bicistronic DNA vaccine against Edwardsiellosis infection in rohu.
7. REFERENCES


Srinivasa, Rao, P.S., Lim, T.M. and Leung, K.Y., 2001. Opsonized virulent Edwardsiella tarda strains are able to adhere to and survive and


### APPENDIX – I

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxy Ribonucleic Acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy Ribonucleic Acid</td>
</tr>
<tr>
<td>gm</td>
<td>Gram</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Beratani</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimole</td>
</tr>
<tr>
<td>M-MuLV-RT</td>
<td>Moloney Murine Leukemia Virus Reverse Trans</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reac</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Dichlorobenzidine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
</tbody>
</table>
APPENDIX – II

- **Ampicillin solution**: 100 mg of ampicillin dissolved in 1 mL of 60% alcohol

- **Coomassie brilliant blue**:
  
  Coomassie brilliant blue R 250 : 0.10 gm  
  Methanol : 50 mL  
  Acetic Acid : 10 mL  
  Water : 40 mL

- **Ethidium bromide** (10 mg/mL/): 10 mg Ethidium bromide was dissolved in 1 mL TDW and stored at 4°C.

- **TAE (50X)**: Tris base-242g, glacial acetic acid-57.1 mL, 0.5 M EDTA-100 mL made upto 1000 mL and autoclaved.

- **TE buffer**: 10 mM Tris-HCl (pH -8), 1 mM EDTA (pH-8), autoclaved.

- **6X DNA loading dye**: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water.

- **70% alcohol**
  
  Absolute alcohol : 70 mL  
  Water : 30 mL

- **Stock Acrylamide Solution**
  
  Acrylamide 30% : 30.00 gm  
  Bisacrylamide : 0.80 gm  
  Water : 100 mL

- **Separating Gel Buffer**
  
  1.875 M Tris-HCl : 22.70 gm  
  Water : 100 mL 77
Polymerising agent

- Ammonium persulphate 5% : 0.5 gm/10 mL (prepare fresh before)
- TEMED : Fresh from refrigerator

Electrode buffer (pH 8.3)

- 0.05 M Tris : 12.00 gm
- 0.192 M Glycine : 28.80 gm
- 0.1% SDS : 2.00 gm
- Water : 2 L

Sample Buffer

- 0.125 M Tris-HCl Buffer pH 6.8 : 22.00 mL
- SDS : 0.50 gm
- Glycerol : 5.00 mL
- Mercaptoethanol : 2.5 mL
- Bromophenol blue : 0.25 mL
  (0.05% W/V solution in water)

Destainer

- Methanol : 50 mL
- Acetic acid : 10 mL
- Water : 40 mL

Transfer buffer

- Tris base : 1.16 gm
- Glycine : 0.585 gm
- Methanol : 40 mL
- SDS : 0.0375% 78
**Phosphate Buffer Saline Solution (pH 7.4)**

Sodium Chloride : 8.00 gm  
Disodium Hydrogen Phosphate : 1.44 gm  
Potassium Dihydrogen Phosphate: 0.24 gm  
Potassium Chloride : 0.20 gm  
Distilled water : 1 L  
Autoclave before use