Discussion
Plants and animals in marine environments are subject to disease, parasites, and tissue pathologies such as tumours. Fish in intensive aquaculture conditions - often characterized by high densities, poor water quality, and temperature extremes succumb to viral, bacterial and other infectious diseases, leading to high rates of mortality. The ecological impact of these diseases can negatively effect entire populations of commercially important fishes of a particular geographical area. Yet the availability of efficient methods of diagnosis and prevention through vaccination is seriously lacking. During the past couple of decades, fish farming (aquaculture) has become an increasing important food production industry and its significant role as a significant food source has been realized. Therefore, attempts are being made world over to apply biotechnology to isolate and identify causative pathogenic agents and to study the molecular basis of disease processes.

In humans, immunization has been used to induce target memory responses to antigens in order to prevent disease expression on secondary exposures to these antigens. Positive outcomes have been demonstrated in many human vaccination programs and various animal models have been developed to test such vaccines (Foxwell et al., 2001; Altboum et al., 2002). However, limited progress has been made in the field of aquaculture vaccination.

Vaccination, which has been pivotal to other animal industries such as poultry, is required to help control fish diseases, which become a special threat when a large number of fish are enclosed on a single site. The development of novel drugs and vaccines along with more efficient systems for their delivery to enhance immune protection of commercially important fish and to increase adaptability to fish farming in closed environments is underway. For the development of vaccines for application in aquaculture, where the investment for mass immunization programs is low, the vaccines need to be economical, stable, and effective.

Most of the vaccines against fish bacterial diseases that are in use commercially are based on formalin-killed pathogen or live attenuated vaccines for the control of certain diseases. Recently there has been overwhelming desire to adopt the recombinant route for developing the vaccines against fish pathogens. Some of the pathogens against which recombinant and DNA vaccines have been developed are viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus.

* Aeromonas hydrophila, is a primary pathogen, isolated from a wide-range of freshwater fish species worldwide (Eurell et al., 1978). Diseases caused by this bacterium are a major problem for the aquaculture industry, resulting in significant
economic losses. Current measures used to control Aeromonas infections are treatment with chemicals and antibiotics which are not always effective and also lead to the production of disease resistance in the bacteria along with the risk of environmental pollution. Hence, vaccination is the alternative to chemicals and antibiotics as it is cost effective and has been shown to be an efficacious approach to induce immunity against pathogens.

Vaccines based on outer membrane vesicles require special safety for preparation. The bacteria, that are used to prepare outer membrane vesicles, must be inactivated. Furthermore, the presence of lipooligosaccharide (LOS, endotoxin) in the vaccine can cause toxicity. From these points of view, vaccines based on purified outer membrane proteins (OMPs) isolated from an LPS-free environment in non-pathogenic bacteria, are preferred. Thus, proteins produced through the recombinant DNA technology route are advantageous over purified proteins.

The outer membrane channel protein (maltose inducible porin) of A. hydrophila is a potential vaccine candidate against Aeromonas infection as it meets to the requirement of an ideal vaccine candidate i.e. being non toxic and essential for the survival of the pathogen. Outer membrane proteins have been reported to be highly immunogenic by many investigators (Quinn et al., 1993; Negm and Pistol, 1999; Iovane et al., 1998; Galdiero et al., 1999). A 28 kDa outer membrane porin of A. salmonicida has been reported to confer protective immunity against bacterial infection (Lutwych et al., 1995). In India, little work has been carried out using the recombinant and DNA vaccination approach for the development of vaccines against any organism that infects fish. Hence the present study was undertaken to evaluate the potential of A. hydrophila maltose inducible porin as a recombinant and DNA vaccine candidate.

**CLONING OF THE lamB GENE OF A. HYDROPHILA**

The genomic DNA isolated from both the strains A. hydrophila was of high molecular weight, which is a prior requirement for successful PCR amplifications. Hence this DNA was used for further studies. The lamB gene is a conserved feature of the genus Aeromonas, hence gene specific primers were designed from the sequence information of the lamB gene of A. salmonicida (Dodsworth et al., 1993). When initial attempts to amplify the gene of interest did not result in any amplification, hot start PCR was performed. Hot start PCR is straightforward solution to difficulties encountered during the amplification of genes using genomic DNA as a template. Genomic DNA being large has greater degree of secondary structure. The initial heat
denaturation step in "Hot start PCR protocol" provides an enhanced opportunity for the high molecular weight genomic DNA to denature fully and interact with the reaction components. It also eliminates the nonspecific reactions (particularly production of primer dimers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermocycling (Chou et al., 1992). Vent DNA polymerase was chosen for the purpose of amplification as it has several advantages over Taq DNA polymerase. Vent DNA Polymerase is a high-fidelity thermophilic DNA polymerase with a 5 to 15 fold higher fidelity than that observed for Taq DNA Polymerase (Mattila et al., 1991; Eckert and Kunkel, 1991). This high fidelity derives in part from an integral 3' → 5' proofreading exonuclease activity in Vent DNA Polymerase (Mattila et al., 1991). In addition, it generates blunt-ended DNA fragments enabling one to directly clone the extension products in blunt ended vectors. Last but not the least, Vent DNA Polymerase is an extremely stable enzyme at both high and low temperatures.

No difference in the amplification profile was observed when two different concentrations of Mg^{++} were tried. The release of an insert of 1.2 kb instead of the expected ~1.4 kb fragment upon digestion with Xho I and Xba I was surprising. Sequence analysis of the cloned insert revealed the absence of the reverse primer used for amplification. It was evident that some sequences from both the vector and the insert were deleted in the resultant clone. Although the chosen host E. coli strain, DH5α was RecA−, it is possible, though highly unlikely, that somehow, the RecA function reverted to normalcy during subculturing. This could have allowed recombination between the Hind III site of the vector and the gene, resulting in the deletion of the reverse primer along with part of the insert. The experiment was repeated several times using different lots of host cells. However, the deletion of ~200 bp from the reverse primer end was observed in every attempt, as the released insert was approximately 1.2 kb while the PCR amplified product used for cloning was ~ 1.4 kb.

An alternative explanation for such persistent deletion of a fragment in the cloned gene may be a possible triplex formation during IPTG induction, which was used for blue-white selection during cloning. The formation of a triplex by the fold back model substitutes the role of Rec A protein and so the homologous recombination (RecA independent) can occur during induced transcription. The negative super helical strain generated by active transcription of the downstream gene induces poly(dG)-poly(dC) sequences to adopt a triple-helix structure in vivo and this structure brings
two remote sequences together to stimulate homologous recombination (Kohwi and Panchenko, 1993). It is possible that the region with the reverse primer and some part of the vector sequence has the propensity to form a triplex during IPTG induced transcription.

Therefore, in the next cloning attempt, the Hind III site was deleted from the vector by digestion with Xba I and Xho I which flank the Hind III site. Successful cloning was achieved and the transformants upon restriction analysis released an insert of the expected ~1.4 kb. The sequence information of the putative positive designated pAhlamB clone gave the expected results and the reverse primer was found to be present. The cloned gene was found to be of 1351 bases and an internal Hind III site was present at 151 bp from the reverse primer.

Genome database search showed significant similarities to other bacterial porins. The percentage identity of the AhlamB sequence with A. salmonicida lamB sequence was found to be 86%. Further analysis of the deduced amino acid sequence using CLUSTAL W program with the sequences of A. salmonicida, A. veronii, E. coli, S. typhimurium and V. parahaemolyticus revealed significant homology with the porins of these species. The percentage of identities/similarities shared with other LamB-like proteins of A. salmonicida, A. veronii, V. parahaemolyticus, S. typhimurium and E. coli were 81%, 64%, 61%, 38% and 35%, respectively. The rooted tree generated by Phylip's Drawgram shows that AhlamB is most closely related to A. salmonicida and V. parahaemolyticus rather than A. veronii. The closeness of A. hydrophila lamB to V. parahaemolyticus lamB supports the fact that members of the genus Aeromonas were previously placed in the family Vibrionaceae (Véron, 1965). E coli and S. typhimurium belonging to the same family (Enterobacteriaceae) are more closely related as compared to A. hydrophila.

The mature porin possesses some characteristics that are typical of other bacterial porins, including a theoretical acidic pi of 4.6 and high glycine content (~12%). Two cysteine residues are present which is also a characteristic feature of the highly related LamB porins of A. veronii, E. coli, S. typhimurium, V. parahaemolyticus. The two cysteines form a disulphide bridge in loop 1 (Cowan 1993, Schirmer et al., 1995).

**EXPRESSION AND PURIFICATION OF MALTOSE-INDUCIBLE PORIN**

In order to use porin as a potential recombinant vaccine candidate, it is vital to express the recombinant protein in vivo. Prokaryotic expression systems are best used to express large quantities of proteins for studies of protein structure and for
antibody production (Kleid et al., 1981; Amann et al., 1985; Pillai et al., 1996).

To improve the yield and easy purification, different fusion systems have been employed which mainly include fusion with staphylococcus Protein A (Nilsson et al., 1985), maltose binding protein malE (di Guan et al., 1988) and thioredoxin (La Vallie et al., 1993). The expression of proteins in E. coli offers a number of advantages as the methods are relatively inexpensive, proteins can be over-expressed with high yields, and expression can be easily regulated. Immobilized metal ion chromatography has opened new prospects for efficient purification of proteins with engineered poly-histidine tags (Gaberc-Porekar and Menart, 2001). Poly Histidine tags form high affinity complexes with immobilized divalent metal ions (such as Ni^{2+}) even in the presence of high concentrations of chaotropic agents (e.g., urea or guanidium hydrochloride), thereby allowing isolation of the tagged protein from a crude cellular extract. Histidine tags at the N- or C- terminus have been routinely used for expression and easy purification of various proteins (Janknecht and Nordheim, 1992; Schon and Schumann, 1994; Mukhija et al., 1995; Ito et al., 2003). The His-tagged protein is bound to Ni^{2+} - nitrilotriacetic acid resin and eluted with imidazole, which competes with the histidine residues. Due to its small size, His-tag is poorly immunogenic and is not required to be cleaved from the fusion protein after purification (Mukhija et al., 1995).

The mature porin had to be expressed as a recombinant protein, therefore the signal sequence was not required. PCR amplified mature lamB gene (lacking the signal sequence) was initially cloned in the prokaryotic expression vector pQE 30, which has a 6xhis-tag for easy purification using Ni-NTA affinity chromatography. Several attempts to express the LamB protein using this expression system did not give positive results. The expression of membrane proteins poses unique challenges to scientists working in this field (reviewed by Tate and Grisshammer, 1996; Loll, 2003). Often, overexpression of these proteins is deleterious to the membrane and is consequently toxic to the host organism (Miroux and Walker, 1996). Also, proper folding of proteins within the membrane is rate limiting for any expression system. Lastly, many membrane proteins undergo post-translational modifications that are not faithfully reproduced in a heterologous expression system (Sarramegna et al., 2003). In the present study, in the absence of the signal sequence in the cloned gene, it is possible that the expressed rPorin does not fold properly and gets degraded in the cytoplasm. It is also likely that the expression is hindered due to a frameshift or a point mutation that could have been introduced during PCR amplification and cloning. This was ruled out by DNA sequencing which revealed no
frameshift or substitution mutation. It has been reported that some cDNA that were transcribed successfully and the RNAs encoding them could be translated in vitro, failed to express in vivo (Alone, 2001). Therefore, due to the unpredictable nature of heterologous expression, there is a need to test multiple vectors and bacterial hosts if initial heterologous expression efforts are unsuccessful (Baneyx, 1999; Hanning and Makrides, 1998). By testing multiple expression systems to express a number of proteins, it has been established that some proteins that fail to express in one vector can be successfully expressed by altering the expression vector or changing the host (Sijwal et al., 2001).

When the attempts to express A. hydrophila porin in pQE30 failed, attempts were made to express the protein in an alternate prokaryotic expression system, pRSET-A, as successful expression of a Nesseria outer membrane porin (PorB) in this expression system has been achieved (Wright et al., 2002). The pRSET vectors are pUC-derived expression vectors designed for high level protein expression and purification from cloned genes in E. coli. High levels of expression of DNA sequences cloned into the pRSET vectors are made possible by the presence of the T7 promoter. In addition, DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. The fusion tags are 6xHis, Xpress Epitope, and EK cleavage site. The T7 expression system relies upon an enzyme called T7 RNA polymerase. While T7 RNA Polymerase is not endogenous to bacteria, some strains of E coli [like BL21(DE3) and BL21(DE3) pLysS] have been engineered to carry the gene encoding for this RNA Polymerase in a piece of DNA called the DE3 bacteriophage lambda lysogen.

Successful and efficient expression of the recombinant porin (rPorin) showing a band at ~49 kDa was achieved using pRSET expression system. The increase in the size of the expressed rPorin is due to the presence of a stretch of 37 amino acids preceding the cloned mature porin gene comprising of polyhistidine (6xHis tag) region, T7 gene 10 leader sequence, Xpress epitope and enterokinase cleavage site. It is possible that this stretch of amino acids residues before the protein of interest, prevents its degradation in the cytoplasm in addition to already known functions. Presence of the T7 gene 10 leader sequence has not been reported to affect the antibody response and bactericidal activity of the Neisseria meningitides class I porin as a fusion protein (Ward et al., 1996).

Since the recombinant proteins are desired to be expressed at maximum possible levels, optimization of the inducer concentration was carried out. The time of
maximum expression after induction was also determined. In order to purify rPorin, it was desirable to know where in the cell rPorin is targeted. Localization of target protein expression revealed that rPorin was being expressed in the form of inclusion bodies. Overexpression of genetically engineered proteins in bacteria often results in the accumulation of the protein product in inactive insoluble deposits inside the cell, called inclusion bodies. Porin is an outer membrane protein and targeting of the overexpressed protein to cytoplasm tends to form inclusion bodies as the folding environment in the cytoplasm is different from that of outer membrane (Makrides, 1996). The recombinant protein expressed as inclusion bodies did not undergo degradation in cytoplasm as the inclusion bodies are refractile to intracellular proteases. It is highly likely that the aggregated protein in inclusion bodies is packed together in a conformation that resists degradation (Shen, 1984; Kopito, 2000).

Recombinant mature porin expressed in the form of inclusion bodies was solubilized and purified in the presence of 8 M urea, using the denaturing-purification protocol. In order to study the immunogenicity of a protein, it is important to have the protein free of denaturants. Therefore, it was essential to purify the recombinant protein in the absence of urea. The protein eluted in imidazole-urea buffer was subjected to dialysis against 1× PBS with step wise reduction in the concentration of urea. This resulted in the precipitation of the protein. The aggregation of proteins before they are completely refolded leads to precipitation and this may have been the case when the purified samples were dialyzed. To prevent the aggregation of partially refolded protein, an alternate approach was used. The 8 M urea-solubilized proteins bound to the Ni\(^{2+}\)-NTA resin were gradually exposed to lower urea concentrations in the buffer. This prevented the refolding-intermediates from aggregating as one end of the polypeptide was immobilized on the Ni\(^{2+}\)-NTA resin. After complete removal of urea, the rPorin was eluted in a soluble form in phosphate buffer saline.

Maltose inducible porin has not been expressed as a recombinant protein. Despite the difficulties in expression of membrane proteins, several reports of have been put forth on expression of outer membrane porins from a number of bacteria (Aguero et al., 1987; Coulton et al., 1992; Koehler et al., 1992; Ward et al., 1996; Fenno et al., 1996; Fang et al., 2000; Rahman et al., 2000; Wright et al., 2002). However, only few reports are available on the immunogenicity of recombinant porins. Most immunogenic studies have been conducted with Nesserial porins (Ward et al., 1996; Wright et al., 2002). To the best of my knowledge, the present investigation is the first attempt to express a porin from Aeromonas. Though antigenicity of native porins

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of Aeromonas has been evaluated (Dodsworth et al., 1993; Lutwych et al., 1995), the present study is the first attempt to evaluate the recombinant maltose inducible porin as a potential vaccine candidate.

**IMMUNOGENICITY OF RECOMBINANT PORIN**

The eluted protein (rPorin) was purified to homogeneity, as evident by a single band. The purified rPorin was used for immunogenic studies in mice. Antisera raised in mice against rPorin cross reacted with the total outer membrane protein fraction isolated from A. hydrophila, in addition to its cross reactivity with the purified rPorin. There was no cross reactivity with the BSA or the protein molecular weight rainbow marker, suggesting that the antisera raised against the rPorin is highly specific.

Analysis of anti-rPorin antisera cross reactivity by ELISA showed that end point titres were detected up to a dilution of 1:1.6x10^5 after the second immunization. No significant difference was observed in the end point titres after the third and fourth immunizations. These results indicate that the rPorin is highly immunogenic, as it is able to generate such high end point titers just after two immunizations. Also, two different doses used did not exhibit any difference in the endpoint titres, indicating that a high immunogenicity is generated by immunization with small amounts of rPorin.

The antigen-specific antibody-subtyping of anti-rPorin antisera indicated a predominant IgG1 response. IgG1 titers were approximately twice as high when compared to IgG2a and IgG2b titers. It has been reported that clones of Th1 and Th2 cells specifically induce antigen specific B cells to secrete IgG2a and IgG1, respectively. A predominance of IgG1 indicates a dominant Th2 response. The secretion of IgG1 is induced by IL-4, while IFN-γ enhances the secretion of IgG2a (Stevens et al., 1988). The appearance of IgG2a and IgG2b at later time indicates the initiation of the Th1 response, in context to protectively. Antibodies elicited with the denatured PorA have been reported to be mainly of isotype IgG1, while the antibodies elicited with folded PorA showed a more equal distribution of isotypes IgG1, IgG2a and IgG2b (Jansen et al., 2000). It has been demonstrated that bactericidal antisera contain significant titers of the complement-binding IgG2a and IgG2b isotypes (Steeghs et al., 1999; Hoogerhout et al., 1995). The consensus view on immunity against bacterial invasion is that a Th2 response is beneficial since it promotes the production of toxin-neutralizing antibodies. Simultaneously, higher levels of IgG2a and IgG2b isotypes indicate a parallel Th1 response. The appearance of the IgG2a and IgG2b isotypes also has a role in the activation of the complement (Ey et al.,...
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1980; Germann et al., 1995) which might perform differently in animal models of bacterial infections. Variations in the isotype differences that are reported by different investigators, could be partly due to the type of microbes used and the target antigen applied (Michaelson et al., 2004). Further, antibody isotypes also depend on the adjuvant used and the confirmation of the antigen. Wright et al., (2002) have reported that the immunization of mice with rPorB adsorbed to Al(OH)₃ induced antibodies that were predominantly of the IgG₁ subclass. Immunization with porin incorporated into liposomes and micelles induced IgG₂a antibodies, whereas the addition of monophosphoryl lipid A (MPLA) substantially increased this subclass and also induced an IgG₂b response.

A mixed immune response has also been observed in the present immunization study indicating that activation of both the arms of the immune system. This may reflect that when an animal responds to a foreign antigen, there occurs a coordination of both types of responses and a polarized Th2 response is not sufficient for protective immunity to diseases (Ryan et al., 1998; Kamboj et al., 2001).

A high rate of proliferation of T-cells isolated from rPorin-sensitized mice stimulated rPorin in vitro, is indicative of the fact that rPorin contains T-cell stimulatory epitopes. Purified porin monomers and trimers have been reported to elicit T-cell proliferative responses, as measured by in vitro [³H]-thymidine incorporation (Tufano et al., 1984; Matsui and Arai, 1989).

It has been reported that protective immunity to enteric pathogens is partially dependent on the activation of cellular defense mechanisms by T cells. Intracellular bacteria and parasites stimulate MHC-II-restricted CD4⁺ T-helper cells, which consist of distinct subsets, designated Th1 and Th2 cells, based on their cytokine secretion profiles and functional characteristics (Mosmann and Coffman, 1989). Th1 cells secrete IL-2, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), TNF-β, and granulocyte/macrophage colony-stimulating factor and are associated with cell-mediated immunity and an Ig class switch to IgG2a. Th2 cells secrete IL-4, IL-5, and IL-10 and promote humoral immunity by activation of B cells and an Ig class switch to IgG1 and IgE. Th1 cells promote recovery and resistance to intracellular pathogens by the activation of antimicrobial effector functions of macrophages through their secreted cytokines. But Th1 cells also exert a protective role through direct cytotoxic activity against infected macrophages. A preliminary analysis of the cytokine profile of the culture supernatants 48 h after stimulation of T-cells of rPorin-sensitized mice indicated a mixed response in vitro as both IL-4 and IFN-γ could be detected.
POTENTIAL OF lamB AS A DNA VACCINE CANDIDATE

The existing approaches of vaccination have not always been an overwhelming success. Therefore, new methods for vaccine development are nonetheless of great interest. Scientific progress is founded - more frequently than imagined - on methodological innovation. Thus, when an occasional revolution in vaccine methodology comes along, it is always a welcome thing. New methods lead to new experimental approaches which give rise to new concepts. One of such methodological leaps would have occurred with the advent of DNA-mediated immunization, now colloquially known as DNA vaccines, a spin-off from the field of gene therapy. As the administration of such antigens posed a problem, attempts are continuously being made to simplify their administration. Scientists in this rapidly growing field of DNA vaccines are experimenting with a variety of needle free methods including nasal sprays, subcutaneous patches, and edible vaccines to deliver the genetic material. This certainly is an encouraging step, especially for the application of such a tool to aquaculture as it is more difficult and cumbersome to inject each fish individually. Genetic immunization has shown, in a number of animal models, to be an efficacious approach for inducing protective immunity to infectious diseases. In fish, DNA has been reported to be as effective as proteins at inducing an antibody response (Kanellos et al., 1999) and several reports have been put forth related to successful DNA immunization in fishes ((Winton, 1997; Anderson et al., 1996; Wells et al., 1997; Tighe et al., 1998; ; Kanellos et al., 1999a, Kanellos 1999b) A DNA vaccine has also been tested against viral hemorrhagic septicemia virus (VHSV) in trout (Lorenzen et al., 1998). Combined DNA immunization with the glycoprotein gene of VHSV and IHNV has been reported to induce double-specific protective immunity and non-specific response in rainbow trout (Boudinot et al., 1998). However, rarely is a report available on DNA immunization against Aeromonas infection. Only recently, two major outer membrane protein encoding genes of A. veronii have been evaluated as DNA vaccine candidates in spotted sand bass (Vazquez-Juarez et al., 2005) As infections of Aeromonas sp. are common among the Indian freshwater fishes, the present proposal is directed to apply the recombinant and DNA vaccine approach against this infection. The gene candidate selected is maltose-inducible porin which has been shown to elicit protective response in rainbow trout.

Vaccination with outer membrane proteins has been shown to be effective against A. salmonicida and A. hydrophila (Hirst and Ellis, 1994; Lutwyche et al., 1995; Bricknell et al., 1999; Fang et al., 2000; Rahman et al., 2000). However, the need for isolation
and purification of the antigens prior to vaccination makes these subunit vaccines costly and the process cumbersome. DNA vaccines offer a more economic alternative to the conventional vaccination especially for fish. Therefore, in the present study, DNA immunization using lamB gene under the control of CMV promoter was also evaluated for its immunogenic potential, to be employed as an alternative vaccination approach.

Mammalian expression vectors have earlier been used for the immunization of fish. Sequences of transcriptional control in the standard vectors (promoter, enhancer, polyadenylation signal, etc.) seem to work efficiently in fish (Heppel et al., 2000). Of the various promoters assessed for their ability to drive expression foreign genes in fish tissue, the immediate early promoter of the cytomegalovirus (CMV) gives best results. It is the most widely used promoter in DNA vaccines reported till date, and its potency has been demonstrated in fish vaccination trials (Anderson et al., 1996; Hepppel et al., 1998; Lorenzen et al., 1999). For the purpose of studying the potential of A. hydrophila lamB gene as a recombinant plasmid based DNA vaccine candidate, the region encoding the mature porin was cloned into the mammalian expression vector pEGFP-N1. Since a termination codon was present in the cloned lamB gene, GFP expression was not achieved.

The recombinant plasmid (rCMV-lamB) was administered at a dose of 100 µg to the mice. A single booster of the same dose was given one week post immunization. Although it has been argued that boosters may not be needed to in case of DNA vaccines, primed-boosted vaccinations may be necessary to enhance the antigenecity of bacterial antigens such as outer membrane protein lamB (Skinner et al., 2003; Vordermeier et al., 2003). The antibody end point titres generated against the anti-rPorin-DNA antiserum of mice was detectable up to a dilution of 1:80. This could be considered a weaker immune response, when compared to the rPorin vaccination. The low antibody titers in genetic immunization have been associated with low levels of antigen expression in the host tissue. Several studies have found that codon usage optimization has an enhancing effect on the expression levels and the immunogenicity of DNA candidate vaccines (Stratford et al., 2000; Narum et al., 2001; Cid-Arregui et al., 2003). It is also possible that the lack of secretions of the antigen may have influenced the magnitude of the humoral response.

After intramuscular injection, plasmid DNA enters the myocytes by an unknown mechanism. Once the antigen is expressed, it is released from the myocytes either as a secreted protein or due to cell damage and/or death and it is then internalized.
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by the antigen presenting cells (Donnelly et al., 2000). Although a considerable humoral response can be obtained against outer membrane protein antigens that lack secretion signals (Zhang et al., 1999; Penttila et al., 2001), it has been recently reported that the antibody response is dramatically increased when antigens are expressed as extracellular proteins from plasmid expression vectors carrying the appropriate signal sequence (Smooker et al., 1999; Svanholm et al., 1999; Price et al., 2001). In the present study, no signal sequence was present in the plasmid based DNA vector to express lamB as an extracellular protein. It would be of interest to investigate the effect of signal sequence on immunogenicity of recombinant lamB DNA.

It has been reported that DNA priming followed by a protein booster results in remarkably high titres and enhanced neutralizing capacity of antigen-specific antibodies (Coban et al., 2004). Another group of mice that were immunized and boosted once with 100 µg of rPorin-DNA were given a single booster with 15 µg of rPorin. There was a remarkable increase in the antibody response with end point titres detected upto a dilution of 1: 800.

Thus, in this study we have cloned the lamB gene of Aeromonas hydrophila and expressed it with N-terminal His-tag in E. coli. The rPorin was found to be highly specific and immunogenic. The potential of lamB gene was also evaluated as a DNA vaccine candidate. This is the first report on the evaluation of recombinant porin and lamB based DNA vaccine of A. hydrophila for its immunogenic potential. Further studies will help determine if the immunogenic response attained by these immunizations, is protective or not. Extending these studies i.e. rPorin and lamB DNA vaccine immunization in fish will be of immense interest as Aeromonas remains the most common pathogens of fish.