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Parenteral immunization of PLA/PLGA nanoparticle encapsulating outer membrane protein (Omp) from *Aeromonas hydrophila*: Evaluation of immunostimulatory action in *Labeo rohita* (rohu)

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Advanced vaccine research approaches needs to explore on biodegradable nanoparticles (NPs) based vaccine carrier that can serve as antigen delivery systems as well as immuno-stimulatory action to induce both innate and adaptive immune response in fish. Immunogenicity of PLA and PLGA NPs encapsulating outer membrane protein (Omp) antigen of *Aeromonas hydrophila* were evaluated through intra-peritoneal injection in fish, *Labeo rohita*. Antigen loaded PLA-Omp (223.5 ± 13.19 nm) and PLGA-Omp (166.4 ± 21.23 nm) NPs were prepared using double emulsion method by efficiently encapsulating the antigen reaching the encapsulation efficiency 44 ± 4.58% and 59.33 ± 5.13% respectively. Our formulated PLA Omp and PLGA-Omp NPs were in nanometer range (<500 nm) and could be successfully endocyted in the body. Despite low antigen loading in PLA-Omp, it showed considerably slower antigen release in vitro than PLGA-Omp NPs. Other physical properties like zetapotential values and poly dispersity index (PDI) confirmed the stability as well as monodisperse nature of the formulated nanoparticles. The spherical and isolated nature of PLA-Omp and PLGA-Omp NPs were revealed by SEM analysis. Upon immunization of all antigenic formulations (PLA-Omp NP, PLGA-Omp NP, FIA-Omp, PLA NP, PLGA NP, PBS as control), significant higher bacterial agglutination titre and haemolytic activity were observed in case of PLA-Omp and PLGA-Omp immunized groups than rest groups at both 21 days and 42 days. The specific antibody response was significantly increased and persisted up to 42 days of post immunization by PLA-Omp, PLGA-Omp, FIA-Omp. PLA-Omp NPs showed better immune response (higher bacterial agglutination titre, haemolytic activity, specific antibody titre, higher percent survival upon *A. hydrophila* challenge) than PLGA-Omp in *L. rohita* confirming its better efficacy. Comparable antibody response of PLA-Omp and PLGA-Omp with FIA-Omp treated groups suggested that PLA and PLGA could be replacement for Freund's adjuvant (for stimulating antibody response) to overcome many side effects offering long lasting immunity. Our encouraging results suggest that PLA/PLGA nanoparticles based delivery system could be a novel antigen carrier for parenteral immunization in fish.

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1. Introduction

Today's leading research on vaccine approaches mostly focuses on the development of naturally acquiring immunity by inoculation of non-pathogenic but immunogenic components of the pathogenic variants. The issues of vaccine safety are growing in alarming rate owing to their weak immunogenicity, poor immunization procedures and failure to acquire booster doses to potentiate prime doses. These are some of the strong reasons which have paved the way towards research for development of new generation therapeutic vaccine. Adjuvants are used for development of new generation vaccine formulations to serve in multiple ways; to enhance immunogenicity, provide antigen-dose sparing, to accelerate a robust immune response, reduce the need for booster immunizations, increase the duration of protection, or improve efficacy in immunocompromised individuals in human and other live stocks. Hence, there is an urgent need for improved and effective, with or without adjuvant based novel vaccine formulations with minimal compositions of purified proteins, peptides etc. for particular disease antigen in question. Because of the chemical

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nature and mode of action, the safety and efficacy of adjuvants in vaccine formulations vary to a greater extent. There are reports which describes about the adverse effects and hyper activation of immune system, neurotoxicity and other detrimental effects when adjuvants are used for prolonged period. Therefore, a relatively new system which is stable with antigen combination and can provide comparable immunity to that of adjuvants eventually can be treated as complete replacement for the existing adjuvants in use should take the lead for development of advanced vaccine formulations. The strategy here is to develop such a system.

Currently, controlled delivery systems consisting of biodegradable microspheres/nanosphere act as a promising vaccine carrier because it can potentially deliver the antigens to the desired locations at predetermined rates and durations to generate an optimal immune response. The strategies that have been tested include use of polymeric carriers such as chitosan, poly-(D, L-lactide-co-glycolide) (PLGA), and poly (lactic acid) (PLA) as adjuvant or delivery carrier [1]. Indeed, polymeric micro- and nanoparticles have been used to develop vaccines against many infectious diseases [2,3]. In fact, the immune response elicited by polymeric particles might be influenced by number of factors, such as inner structure of particulate carriers, use of adjuvant, surface charge, particle size, and surface hydrophobicity. These parameters can also affect differential uptake by macrophages and dendritic cells, which in turn play an important role in generating the right kind of immune response [4]. PLGA/PLA polymers have the advantage of being well characterized and commercially available for drug delivery systems [5]. Among the biodegradable microparticles, PLGA microparticles have been proved as a vaccine carrier in fish [6].

The growing bacterial infections in various aquaculture systems are gaining much more concerns now days because of increased disease modalities affecting food market, human health and ultimately economic value including the added cost of controlling these diseases, recovery and wellbeing. *Aeromonas hydrophila* is a pathogenic gram negative bacterium associated with lower vertebrates like fish, amphibians [7]. Aeromoniasis, an important fish disease is caused by this bacterium [8]. *A. hydrophila* infections in fishes have been reported from time to time in many asian countries including China, Philippines, Thailand, and India [9]. Fish are free-living organisms mainly dependent on their innate immune system for survival. Non-specific immunity act as primary defence mechanism in fish and it also plays a key role in development of acquired immune response [10]. Like mammals, humoural immunity in fish involves the secretion of specific immunoglobulin directed to neutralize antigens and to activate complement cascade in response to pathogens [11].

The Outer Membrane (OM) of *A. hydrophila* is a complex structure which consists of mainly lipopolysaccharide (LPS), phospholipids and a group of outer membrane proteins (Omps). The OM protein of pathogenic gram-negative bacteria is mainly responsible for establishing initial adherence, modulate host pathogen interaction, overall survival of the organism and propagation of virulence factor [12]. It has also protective antigenicity, because OM components are easily recognized as foreign substances by immunological defence systems of the hosts. Omps are reported to be conserved among different serovars. Some of them serve as adhesins and play an important role in virulence [13]. Omps are located at host–bacterial interface in *A. hydrophila* and can be targeted for drug therapy [13].

Omps are highly immunogenic due to their exposed epitopes on the cell surface. There are safety concerns with use of oil adjuvant (Freund's incomplete adjuvant; FIA) in multivalent vaccine formulations for fish due to generation of adverse side effects (e.g., adhesions). Therefore, alternative molecules or certain combinations of them (targeting specific cellular responses) as adjuvants are desirable in order to enhance overall immunogenicity and efficacy without reducing protection levels [14]. There are only few reports available on PLA/PLGA NP based vaccine formulations with adjuvant like effects and sustained target specific release to generate an optimal immune response for which more elaborate and conclusive studies are needed to define the protective roles of PLA/PLGA nanoparticulate system. In the present study, PLA/PLGA NPs encapsulating Omp antigen from *A. hydrophila* are compared with adjuvant formulations to develop a better antigenic carrier model in fish. The efficacy is also compared between PLA/PLGA encapsulated Omp NPs immunized groups and a correlation is established evaluating the results of innate and adaptive immune response studies in *Labeo rohita*.

2. Materials and methods

2.1. Materials

The polymer; polylactic acid (PLA; molecular weight: 85,000–160,000 Da), poly (D, L-lactic-co-glycolic) acid (PLGA 50: 50; molecular weight: 40,000–75,000 Da), N-Lauroylsarcosine sodium salt, trehalose dihydrate and poly vinyl alcohol (PVA) were procured from Sigma Aldrich, USA. Chloroform was purchased from Merck India Pvt Ltd. Ultrapure water from Milli-Q water system (Millipore, USA) was used throughout the study.

2.2. Preparation of OM proteins

In order to isolate the proteins of the OM of *A. hydrophila*, the respective strain was grown to late exponential phase. Cells were harvested from I L culture (4500 × g, 15 min, 4 °C), washed twice in 10 mM HEPES, pH 7.5, and resuspended in 10 mM HEPES, pH 7.5, with one Sigma FAST™ protease inhibitor cocktail tablet (EDTA free) per 20 gm cell mass. Cells were disrupted by ultrasonication for 30 min at 10 W an interval of 30 s. Unbroken cells were removed by centrifugation (4000 × g, 15 min). The supernatant containing the OM proteins was transferred into a new tube and centrifuged again (13,000 × g, 30 min, 4 °C). The pellet was resuspended in 0.8 ml 10 mM HEPES, pH 7.5, plus 1% N-Lauroylsarcosine sodium salt and incubated for 30 min. After centrifugation (13,000 × g, 30 min, 4 °C), the pellet was washed once with 1 ml of 10 mM HEPES, pH 7.5, and resuspended in 50 µl of 10 mM HEPES, pH 7.5. The protein concentration was determined by Pierce™ BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Scientific). Purified OM proteins were lyophilized and stored at −80 °C for further use.

2.3. Preparation of biodegradable and biocompatible based NPs

NPs containing different antigens were formulated using a double emulsion-solvent evaporation technique with little modification [15]. In this method, aqueous solution of antigen dissolved in 500 µl of citrate buffer (pH = 8) was emulsified with 100 mg of PLGA/PLA in chloroform solution (3 ml) to get a primary emulsion. Emulsification was carried out by ultrasonicator (Sartorius LABSONIC® M) at amplitude of 5 for 20 s in an ice-cooled bath. The primary emulsion (w/o) was further emulsified in an aqueous PVA solution (10 ml, 2% w/v) in a drop-wise manner and then emulsified with high speed homogenizer for 2 min at 10000 rpm. The w/o/w double emulsion was added to 80 ml double distilled water and was stirred in 500 rpm for 2 h at room temperature to allow the organic solvent evaporation. The NPs were recovered by centrifuge at 37,500 g for 45 min and washed thrice with sterile deionized water. Then the particles were lyophilized using 2.5% trehalose dihydrate as cryoprotectant.

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