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INFLUENCE OF DIMERIZED LYSOZYME (KLP-602) ON THE IMMUNE RESPONSES INDUCED BY FUROGEN VACCINE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT

In the present study the possible stimulation of nonspecific and specific immune response after immunization induced by immersion Furogen (Aqua Health Ltd) vaccine were analysed in rainbow trout (*Oncorhynchus mykiss*). The modulation was attempted using a natural immunostimulant, dimerized lysozyme (KLP-602). Five groups of rainbow trout were given intraperitoneal injections of KLP-602 two days before vaccine, with vaccine, and two days after vaccine applicated by immersion. The results of cellular study showed that KLP-602 applicated before or with Furogen vaccine statistically significant ($P<0.05$) increased the total immunoglobulin secreting cells (ISC) and specific antibody secreting cells (ASC) levels, compared to the KLP-free (only vaccinated) group of fish. Analysis of the results shows no significant differences between group of fish where KLP-602 was applicated after vaccination and KLP-free (only vaccinated) group of fish. Analysis the results of humoral study shows that KLP-602 applicated before or with vaccine significantly increased ($P<0.05$) the total immunoglobulin levels and specific antibody levels in serum, compared to the KLP-free (only vaccinated) group of fish. Also results shows no significant differences between group of fish where KLP-602 was applicated after vaccination and KLP-free (only vaccinated) group of fish. The higher responses after the application of KLP-602 before or with vaccine were observed.

Key words: Lysozyme dimer, fish, Furogen vaccine, ASC, specific immune response

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INTRODUCTION

Application of vaccines has been an important method in prevention of fish against bacterial diseases. Some vaccines and immunization techniques when actually applied to hatchery conditions are not as effective as they should be. Therefore, research is concentrating on how to improve the potency and efficacy of the antigens and how to optimally activate the nonspecific defence mechanisms and specific cellular and humoral immune response [1, 2, 4, 8, 10, 13, 17, 18]. One of the most frequent uncertainties regarding the use of vaccines is effective protection over a long time. The use of adjuvants and immunostimulants in fish culture offers a wide range of attractive methods for inducing and modulating protection against diseases. Adjuvants and immunomodulators used to enhance the nonspecific defence mechanisms and a specific immune response has been divided into general group of function. Adjuvants are usually mixed and injected with an antigen preparation, which elevates specific immune response [1, 4, 19, 21]. The application of immunostimulants for the activation of the effectiveness of vaccines is a promising new development in aquaculture. Natural and synthetic immunostimulants activate the nonspecific cellular and humoral defence mechanisms and specific immune response if they are administered before, with, or after vaccines [1, 2, 9, 10, 11, 13, 14, 15].

Lysozyme is a monomeric enzyme with bacteriolytic properties and is ubiquitous in its distribution among living organisms. The functional activity of lysozyme is attacks structures containing muramic acid and has antibacterial and anti-inflammatory properties. Under natural condition, several substances achieve their full effectiveness only as dimers or polymers. Therefore, the question arose as to whether greater activity can be generated through dimerization. The ensuing experimental and clinical studies showed that lysozyme dimer (KLP-602) was less toxic than its monomer, and that it is highly active against viral and bacterial infections in humans and animals [6, 7]. Experimental studies on fish showed that dimerized lysozyme (KLP-602) activated the nonspecific cellular defence mechanisms and protection against infectious pancreatic necrosis virus (IPNV) and furunculosis in salmonids [15, 16, 17]

In this study, we determined the influence of dimerized lysozyme (KLP-602) on the non-specific and specific immune response induced by anti-*furunculosis* (Furogen) vaccine in rainbow trout (*Oncorhynchus mykiss*).

MATERIAL AND METHODS

Five hundred healthy rainbow trout (*Oncorhynchus mykiss*), weighing 70-80 g were used for the experimental study. The fish were held in five tanks of 500 L (100 fish per tank) in 12 ± 1°C spring water and fed twice daily with commercial pellets (Trouw France).

Anti-*furunculosis* immersion vaccine (Furogen, Aqua Health Ltd, Canada) was used for immunization of fish, according to the protocol presented by Aqua Health Ltd.

The dimerized lysozyme (KLP-602) from Nika Health Products (USA) was diluted in PBS to dose of 100 µg/kg of body weight for intraperitoneal injection. KLP-602 was applied by injection 2 days before, with, or 2 days after the vaccine administered by immersion. First control group (only vaccinated) was injected with PBS at similar protocol with KLP-602 and immunized by Furogen immersion vaccine at similar time. Second control group (non-vaccinated) was injected only with PBS.

The blood and pronephros were separated from 10 fish of each group before and 7, 14, 21, 28, 35, 45 and 60 days after immunization. The serum was separated after centrifugation of blood. Single cell suspecions from pronephros were obtained by teasing the tissues in the medium through a nylon mesh, and cells were washed in heparinized Hank's balanced salt solution (HBSS) and isolated on a Gradisol L (density 1.077; Polfa) gradient. Counts of living cells from pronephros were made with trypan blue using a haemocytometer.

The ELISPOT assays for the quantification of total immunoglobulin secreting cells (ISC) and specific antibody secreting cells (ASC) were used, according to the protocol presented for rainbow trout by Siwicki and Dunier (12).

The total immunoglobulin (Ig) levels in serum was measured by spectrophotometric method (3) and specific antibody levels in serum was analyzed using the enzyme-linked immunosorbent assay (ELISA) test, according to the method presented by Cossarini-Dunier (5).

Statistical analyses were performed using Student's t-test. Differences on means (n=10 fish for each value) are considered statistically significant at $P<0.05$. The standard deviation (SD) was always within 5 % of the means.

RESULTS AND DISCUSSION

The influence of dimerized lysozyme (KLP-602) administered before, with, or after the vaccine on the immune response analyzed by enumeration of total immunoglobulin secreting cells (ISC) and specific antibody secreting cells (ASC) are summarised in Fig. 1 and Fig. 2. The results showed that KLP-602 applicated before or with Furogen vaccine statistically significant ($P<0.05$) increased the total ISC and specific ASC levels, compared to

the KLP-free (only vaccinated) group of fish. Analysis of the results shows no significant differences between group of fish where KLP-602 was applicated after vaccination and KLP-free (only vaccinated) group of fish. The number of total ISC and specific ASC increased rapidly and the highest, statistically significant ($P<0.05$) levels were observed between 14 and 60 days after immunization in fish from groups when KLP-602 was administered before and with vaccine, compared to only vaccinated (KLP-free) fish.

Fig 1. The influence of dimerized lysozyme (KLP-602) applied before, with or after vaccination with Furogen vaccine on the number of total immunoglobulin secreting cells (ISC), compared with these in vaccine only and non-vaccinated (control) rainbow trout (mean, SD≤5% of mean, n=10)

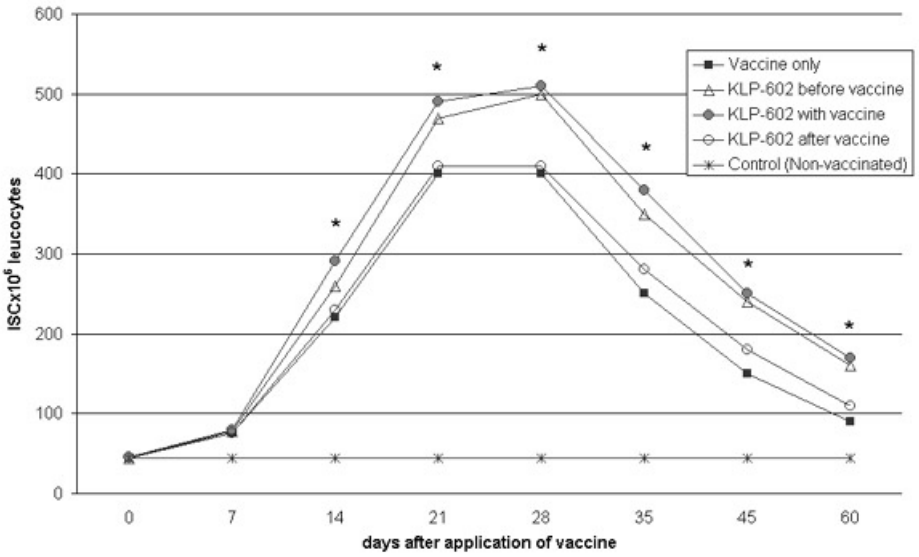
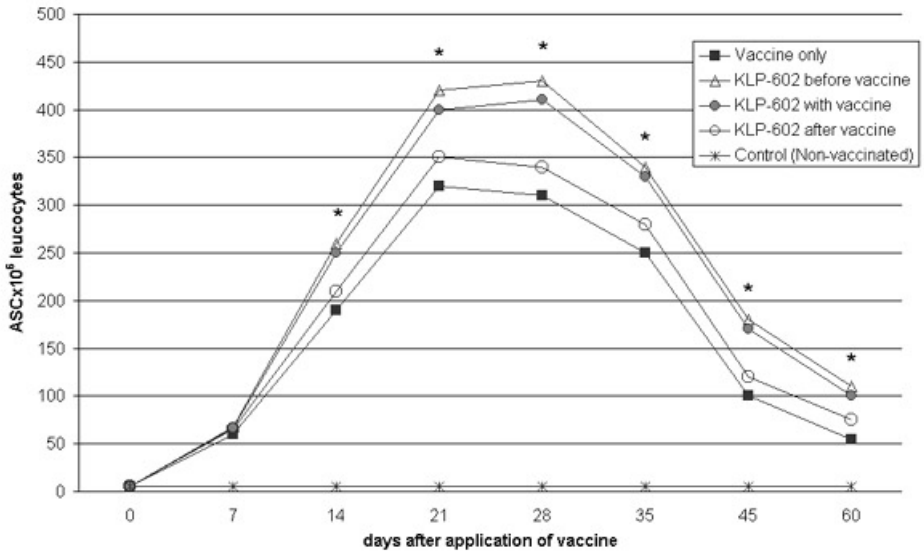


Fig 2. The influence of dimerized lysozyme (KLP-602) applied before, with or after vaccination with Furogen vaccine on the number of specific antibody secreting cells (ASC), compared with these in vaccine only and non-vaccinated (control) rainbow trout (mean, SD≤5% of mean, n=10)



The effect of dimerized lysozyme (KLP-602) applicated before, with, or after the vaccine on the total immunoglobulin levels in serum were presented in Fig. 3, and on the specific antibody levels in serum analysed by ELISA test are showed in Fig. 4. Analysis of these results shows that KLP-602 applicated before or with vaccine significantly increased ($P<0.05$) the total immunoglobulin levels and specific antibody levels in serum, compared to the KLP-free (only vaccinated) group of fish. Also results shows no significant differences between

group of fish where KLP-602 was applicated after vaccination and KLP-free (only vaccinated) group of fish. The growth of total immunoglobulin levels in serum between 14 and 28 days after immunization in each experimental group of fish were observed, but statistically significant ($P<0.05$) higher levels in fish applicated KLP-602 before and with vaccine were observed, compared to KLP-free group of fish. The similar pattern was observed in specific antibody levels in serum. The specific antibody levels increased rapidly in serum between 14 and 35 days after immunization in each experimental group, but the significant ($P<0.05$) higher levels in fish administered KLP-602 before or with vaccine were observed, compared to KLP-free group of fish. To the end of the experimental study (day 60), the higher total immunoglobulin and specific antibody levels in serum in fish administered KLP-602 before and with vaccine were observed, compared to the only vaccinated group of fish and group when KLP-602 was applicated after vaccine.

Fig 3. The influence of dimerized lysozyme (KLP-602) applied before, with or after vaccination with Furogen vaccine on the number of total immunoglobulin levels in serum, compared with these in vaccine only and non-vaccinated (control) rainbow trout (mean, SD≤5% of mean, n=10)

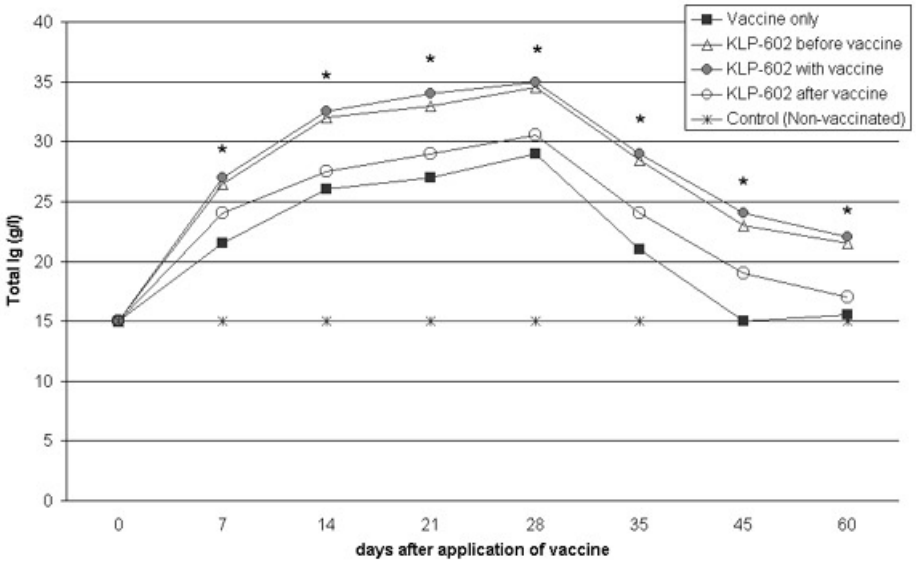
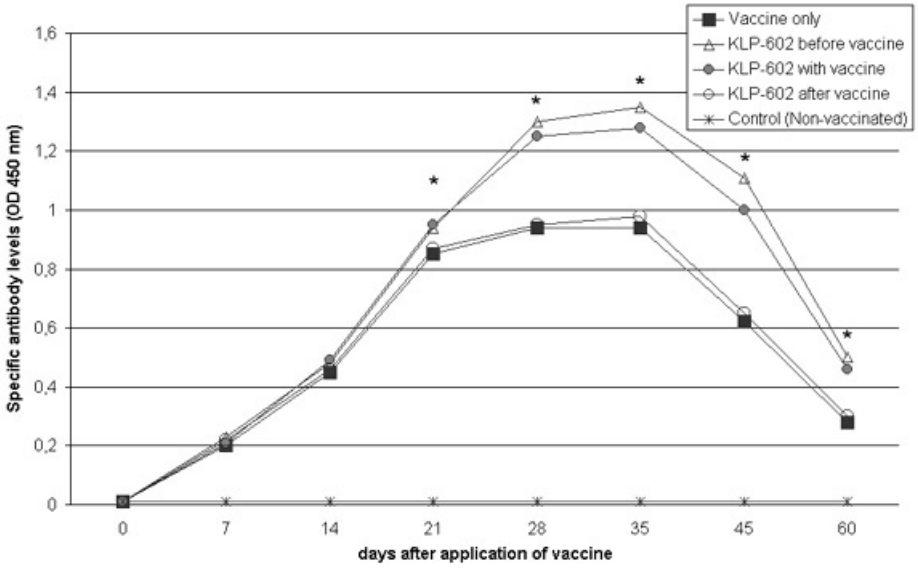


Fig 4. The influence of dimerized lysozyme (KLP-602) applied before, with or after vaccination with Furogen vaccine on the number of specific antibody levels in serum, compared with these in vaccine only and non-vaccinated (control) rainbow trout (mean, SD≤5% of mean, n=10)



In this experimental study, we observed the adjuvantic effect of dimerized lysozyme (KLP-602), with greater effects being observed before or at the time of vaccination. KLP-602 applicated by intraperitoneal injection before or at the time of immunization by immersion vaccine increased the cellular and humoral immune response in rainbow trout. Complete Freund's adjuvant (CFA) was one of the first immunostimulants used in human and animal to elevate the specific immune response, and it has also successfully been used in conjunction with the injection of bacterins [1, 10, 19]. Other immunostimulants and biological response modifiers that have been used in fish research including synthetic levamisole [11], bacterial lipopolisaccharides and glucan [2, 13, 17, 21]. Vaccines have been adsorbed to inert particles, such as bentonite and latex beads to carry the immunogens in attempts to maximise *in vivo* uptake for bath immunization. Each substance presents special problems in timing and methods of administration by injection, immersion, orally or flushes treatments, and dosage adjustments for size and fish species. In a previous work [15, 16] the authors observed the immunostimulatory effects of dimerized lysozyme (KLP-602) on the nonspecific cellular and humoral defence mechanisms and specific immune response analysed by antibody secreting cells (ASC) at doses between 10 and 100 µl. The experimental study presented by Studnicka et al. (20) suggest that KLP-602 have immunomodulatory influence on the cell-mediated immunity and specific immune response after suppression induced by xenobiotics.

In our study, we found that lysozyme dimer (KLP-602) activated the nonspecific and specific immune response after immunization by immersion with anti-*furunculosis* vaccine in rainbow trout. These results suggest that it is possible to activate the specific immune response by natural immunostimulant. Dimerized lysozyme (KLP-602) may also be added to viral vaccines to help elevate the specific response, thus acting as adjuvants for such changes as increased mobilisation of antigen uptake and activation of the antibody secreting cells in fish.

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