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Promiscuous T cell epitopes boosts specific IgM immune response against a P0 peptide antigen from sea lice in different teleost species

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ABSTRACT

The development of vaccines employing conserved protein antigens, for instance ribosomal protein P0, has as disadvantage the high degree of identity between pathogen and host proteins due to possible induction of tolerance or auto antibodies in the host organism. To overcome this drawback, peptide-based vaccines have been designed with a proved high efficacy. The use of defined peptides as antigens has the problem that they are generally poor immunogenic unless coupled to a carrier protein. Several studies have established the potential for promiscuous T cell epitopes incorporated into chimeric peptides to enhance the immunogenicity in mammals. On the contrary, studies about the role of these epitopes on teleost immune system are scarce. Therefore, the main objective of our present study was to evaluate the potential of promiscuous T cell epitopes to boost specific IgM immune response in teleost fish against a peptide antigen. With this aim, we used a peptide of 35 amino acids from the ribosomal P0 protein of *Lepeophtheirus salmonis*, an important parasite in salmon aquaculture. We fused this peptide to the C-terminal of T cell epitopes from tetanus toxin and measles virus and produced the chimeric protein in *Escherichia coli*. Following vaccination, IgM antibody production was monitored in different immunization schemes in Tilapia, African catfish and Atlantic salmon. The results demonstrated for first time that the addition of T cell epitopes at the N-terminal of a target peptide increased IgM specific response in different teleost species, revealing the potential of this approach to develop peptide-based vaccines for aquaculture. The results are also of great importance in the context of vaccine development against sea lice using ribosomal protein P0 as antigen taking into account the key role of P0 in protein synthesis and other essential physiological processes.

1. Introduction

The development of a vaccine candidate based on conserved proteins between the pathogen and the host has as a drawback that the high degree of identity can result in the induction of tolerance or the generation of auto antibodies in the host organism. To overcome this disadvantage, peptide-based vaccines have been designed with a proved high efficacy; for example, a vaccine developed against ticks based on a 20 amino acid peptide of the acidic ribosomal protein P0 from *Rhipicephalus* sp. ticks [1,2]. The use of peptides as antigens has the problem that they are generally not very immunogenic unless coupled to a carrier protein. There are a number of contributing reasons, among which is that the peptide sequences used to present epitopes are generally short. Therefore, they contain insufficient information to fold into the correct shape necessary to mimic conformation-dependent epitopes and even if the peptide is recognized by a B cell, these cells must still receive help from a helper T cell recognizing a sequence within the same immunogen. Traditionally the site of T cell recognition has been provided by a carrier protein to which peptides are covalently coupled [3]. Several studies have established the potential for promiscuous T cell epitopes (TCE's) incorporated into chimeric peptides to enhance the immunogenicity of other epitopes

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within the chimeric peptide in mammalian immune systems [4–7].

Teleost fish has an adaptive immune system, as they have immunoglobulins, T cell antigen receptors, major histocompatibility complex class I and II molecules, spleen and thymus and many other features, which are similar to and in some cases differ from those of the mammalian immune system [8]. In order to overcome the problem associated with the poor immunogenicity of peptides, multiple antigen peptide system (MAPS) was used to bypass the need of a carrier protein for antibody production in rainbow trout *Oncorhynchus mykiss* [9]. A MAP is based upon the use of an inert lysine core with radial branches to which the peptides are attached. The number of peptides assembled on the core is usually 4 or 8 and results in a large macromolecule with a unique three-dimensional configuration. This conformation allows significant signal amplification, bypassing the need of a carrier protein for antibody production [10]. To test the effectiveness of MAPS, rainbow trout were immunized with two MAPS containing the decapeptide Gonadotropin Releasing Hormone (GnRH). One of these MAPS was heterologous and contained alternating sequences of GnRH and a measles virus T cell epitope. The results show that MAPS are a suitable delivery system in fish for the generation of anti-peptide antibodies but not a real improvement was seen with the addition of TCE's. In another previous work, promiscuous TCE's from measles virus fusion protein (MVF) (288–302) [11] and *Clostridium tetani* tetanus toxin (tt) P2 epitope (830–844) [12] were used to construct chimeric fusion proteins with OspA to determine if these mammalian TCE's could enhance the immunogenicity of recombinant OspA within the salmonid immune system. OspA is a 17 kDa putative outer surface protein from *Piscirickettsia salmonis*, the etiological agent of salmonid rickettsial septicaemia (SRS) and a devastating disease of farmed salmonid fish. The authors demonstrated that addition of these TCE's dramatically improved the efficacy of the OspA vaccine, reflected by a three-fold increase in vaccine efficacy. Nevertheless, the mechanisms of the increased efficacy were not measured at that time [13] and no further studies about the role of mammalian TCE's on teleost immune system or the potential of this strategy to develop peptide-based vaccines has been published.

The aim of the present study was to evaluate the potential of TCE's to boost specific IgM immune response in teleost fish against a peptide antigen. In this connection and based on the results obtained in ticks, we were interested also in the development of a vaccine against sea lice based on ribosomal P0 protein. Sea lice are crustacean ectoparasites affecting Atlantic salmon (*Salmo salar*) production worldwide, causing huge economic losses [14]. Thus, we used a peptide of 35 amino acids from the ribosomal P0 protein of *Lepeophtheirus salmonis* (the most important sea lice species affecting salmon aquaculture in northern hemisphere) as antigen. This peptide was chosen from a highly immunogenic region within the P0 protein, which also showed low sequence identity (45%) with *S. salar* P0 protein. We fused this peptide to the C-terminal of TCE's from tetanus toxin and measles virus positioned in tandem, similar to the strategy used by Kuzyk et al., 2001 [13] and produced the protein in *Escherichia coli*. Later, IgM antibody production was monitored after vaccination in Tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*) and Atlantic salmon. The results demonstrated for first time that addition of TCE's at the N-terminal of small peptide antigen increased systemic IgM specific response in different teleost species, revealing the potential of this approach to develop peptide-based vaccines for aquaculture.

2. Materials and methods

2.1. Cloning and expression in Escherichia coli of TT-P0 antigen

The tt P2 (830–844 QYIKANSKFIGITEL; GenBank X04436) and MVF protein (288–302 LSEIKGVIVHRLEGV; GenBank M81903) TCE's were obtained by PCR from the plasmid 0807716_ttantigen-MVF_prote_pGA14 generated by GENEART containing the synthetic gene. The PCR primers used to amplify the TCE's were A and B (Table 1) and they contain *Nco* I-*Hind* III restriction sites. A 35 aa peptide between the amino acids 267 to 301 from *L. salmonis* P0 sequence (pP0) was amplified from P0 cDNA previously isolated in the laboratory and already cloned in pMOS-Blue (GE Healthcare) by using the primers C and D. These primers contain the restriction sites for *Hind* IIII and *Xho* I endonucleases (Table 1) to allow the fusion to the C-terminal of TCE's. The PCR fragments were sub-cloned into pGEM-T-easy (Promega), extracted by the corresponding endonuclease digestion and the fragments were inserted into the corresponding cloning sites of pET28a. The final vector was titled as pET28a-TT-P0.

For expression of the recombinant polypeptide, the pET28a-TT-P0 expression plasmid was transformed into *E. coli* BL21 (DE3) strain. Single clones of BL21 (DE3) transformed with pET28a-TT-P0 were grown overnight at 37 °C in Luria Bertani (LB) medium containing 50 μg/mL of kanamycin. Cultures were then diluted (1:20) in fresh LB medium and grown at 37 °C until the OD_{600} reached approximately 0.5. The expression of recombinant proteins was initiated by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma) to a final concentration of 1 mM and incubation continued another 6 h for induction of recombinant protein expression.

2.2. TT-P0 protein purification

After induction, the bacterial cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell pellet was resuspended in 300 mM NaCl, 10 mM Tris, pH 6 and the cells were disrupted in French Press (Ohtake, Japan) at 1200 kgf/cm^2. After bacterial cells disruption, the cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell pellet containing the protein was resuspended in 150 mL of Solubilization buffer (300 mM NaCl, 10 mM Tris, 10 mM Imidazole, 6 M urea, pH 8) and it was incubated for 2 h at room temperature with gentle agitation. Afterwards, the sample was centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatant was used for further purification steps. Affinity chromatography was performed under denaturing conditions employing IMAC Sepharose™ Fast Flow (GE Healthcare) according to the manufacturer's instructions. The clarified lysate with 10 mM Imidazole was loaded onto the previously equilibrated column with equilibration buffer (NaCl 300 mM, Tris-HCl 10 mM, Imidazole 10 mM, urea 1.5 M, pH 8) at a flow rate of 1 mL/min. Then, wash was performed with the same buffer but 40 mM Imidazole. Protein elution was done with 200 mM Imidazole. For refolding, the fraction purified by affinity chromatography was dialyzed against NaCl 150 mM, Tris-HCl 10 mM, pH 8 buffer. Each fraction was checked by 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis under reducing conditions. Protein concentration was determined with a BCA protein assay kit (Pierce) according to the manufacturer's instructions. The purity of recombinant protein was assayed by densitometry scanning of protein gels taking into account total protein concentration.

2.3. Protein gel electrophoresis and western blotting

Protein samples were loaded on 15% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blotting was performed using anti-His monoclonal antibody peroxidase conjugate (Sigma) at a dilution 1:2000, or a rabbit serum against P0.

The polyclonal sera against P0 was prepared in New Zealand White rabbits (6 weeks old) that were immunized subcutaneously with three doses (weeks 0, 3 and 7) containing 500 μg of synthetic 35 aa P0 peptide (CIGB peptide synthesis department) conjugated to KLH (Sigma) per dose in Freund's complete adjuvant (Sigma) at week 0 and Freund's incomplete adjuvant at weeks 3 and 7. Blood extraction was performed one week after the last immunization. After washing with PBS-Tween 0.01% once and with PBS twice, the membrane was incubated with a 1:100 dilution of the polyclonal serum against P0 for

Table 1
Primers used for cloning of promiscuous T cell epitopes and P0 into pET28a *Escherichia coli* expression vector. Endonuclease restriction sites are underlined.

Primers	Sequence 5′-3′	Direction	Application
A	CCATGGGACAATACATCAAGGCTAACTCC	Forward	Amplification of TCE's with *Nco* I site for insertion into *E. coli* expression vector
B	AAGCTTGGTACCAACACCCTCTAATCTG	Reverse	Amplification of TCE's with *Hin*d III site for insertion into *E. coli* expression vector
C	AAGCTTGAATATCTGGCTGATCCCA	Forward	Amplification of P0 with *Hin*d III site for insertion into *E. coli* expression vector
D	CTCGAGCTCAGGTTCATCCGCCTTAG	Reverse	Amplification of P0 with *Xho* I site for insertion into *E. coli* expression vector

2 h. After the washing steps, the membrane was incubated with gentle shaking for 1 h at room temperature with 1:5000 dilution of anti-rabbit polyclonal antibody-horseradish peroxidase (HRP) conjugate (Amersham Biosciences) as secondary antibody. Chromogenic detection was carried out using ECL detection system.

2.4. In-gel protein digestion and mass spectrometry analysis

The identity of the purified protein was confirmed by mass spectrometry analysis. The Coomassie blue-stained band was excised from SDS-PAGE gels and incubated at 37 °C with 50% acetonitrile in 1% ammonium bicarbonate until they became colorless. The gel slice was dried and rehydrated in 25 mM ammonium bicarbonate buffer containing sequencing grade trypsin at 12.5 ng/μL. The in-gel digestion was for 16 h at 37 °C. The resulting proteolytic peptides were passively eluted in 0.2% of formic acid solution, desalted by using a ZipTips reverse phase micro column and loaded into gold-coated borosilicate nanotips for mass spectrometry analysis.

Low-energy ESI-MS and MS/MS spectra were acquired using a QTOF-2™ mass spectrometer from Waters (Manchester, UK). The capillary and cone voltages were set to 1200 and 35 V, respectively. The multiply-charged signals of highest intensity corresponding to tryptic peptides were further analyzed by ESI-MS/MS using appropriate collision energies to obtain either partial or complete amino acid sequences.

2.5. Fish immunization experiments

Fish immunization experiments are summarized in Table 2.

2.5.1. Animals

Tilapias (*Oreochromis niloticus*) were obtained from the Aquaculture Research Station at the Center for the Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. African catfish (*Clarias gariepinus*) were provided by the Center for Aquaculture of Mampostón (CPAM). Atlantic salmon (*Salmo salar*) were from the Aquaculture Research Station (Tromsø, Norway).

All animal experiments were previously approved by the Ethics Committee of the CIGB, Havana, Cuba (Tilapia and African catfish) or by 'FDU' (http://www.mattilsynet.no/fdu/) to be in accordance with the animal welfare act as required by Norwegian law (Atlantic salmon). Prior to vaccination and sampling, fish were anaesthetized with benzocaine at recommended doses.

2.5.2. Adjuvants

All antigens, unless specified, were formulated in Montanide ISA 50 V2 adjuvant (Seppic, France) at a ratio 50/50. Formulations were made in a Politron (Ultra-Turrax T25, IKA WERKE, Germany).

2.5.3. Experiments in tilapia

2.5.3.1. Experiment 1. Animals were maintained in a circular 500 L tanks supplied with recirculating fresh water at 26 ± 2 °C and 12:12 light-dark cycle. Fish were fed with pelleted feed (CENPALAB, Cuba). Ten tilapias per group (65 ± 5 g) were immunized by intraperitoneal (i.p.) injection on days 0 and 15. Three experimental groups were settled. One group of Tilapia was injected with the TT-P0 antigen at the dose of 1 μg per g of body weight (1 μg/gbw). The second group received the same quantity of 35 aa synthetic P0 (CIGB synthesis Department, Cuba) and the third group (Control) received equal volume of buffer (NaCl 150 mM, Tris-HCl 10 mM, pH 8). Blood was collected from the caudal vein of all fish on days 0 and 21 days after re-immunization and serum was prepared for antibody detection.

2.5.3.2. Experiment 2. A second experiment was developed to evaluate the need of the oil adjuvant to obtain the antibody response. A similar immunization scheme than in previous experiment was developed but the experimental groups were: buffer in Montanide ISA 50 V2, TT-P0 purified protein without adjuvant and TT-P0 adjuvated in Montanide ISA 50 V2. Blood was collected from the caudal vein of all fish on days 0 and 15, 21 and 28 days after re-immunization.

2.5.3.3. Experiment 3. A third similar immunization scheme was performed to compare the antibody titers against P0 induced by TT-

Table 2
Experimental design for fish immunization experiments.

Specie	Experimental Design Groups	Fish number per group	Immunization time in days (degree days***)	Blood Extraction** time after re-immunization in days (degree days***)
Oreochromis niloticus (Tilapia)	G1: Placebo G2: Synthetic P0 G3:TT-P0	10	0 and 15 (390)	21 (546)
	G1: Placebo G2: TT-P0 G3: TT-P0- M-ISA50*	10	0 and 15 (390)	15 (390), 21 (546) and 28 (728)
	G1: Placebo G2: TT-P0-M-ISA50* G3: P0-my32-M- ISA50*	10	0 and 15 (390)	7 (182), 15 (390) and 21 (546)
Clarias gariepinus (African catfish)	G1: Placebo G2: TT-P0	12	0 and 15 (390)	15 (390)
Salmo salar (Atalntic salmon)	G1: Placebo G2: TT-P0	120	0 and 35 (350)	34 (340)

*M-ISA50: adjuvant MONTANIDE™ ISA50 (Seppic, France).
**In all the experiments, blood was collected also at day 0 (pre-immune serum).
***Degree days = Temperature °C x days (For tilapia and catfish: water temperature = 26 °C; for salmon water temperature = 10 °C).

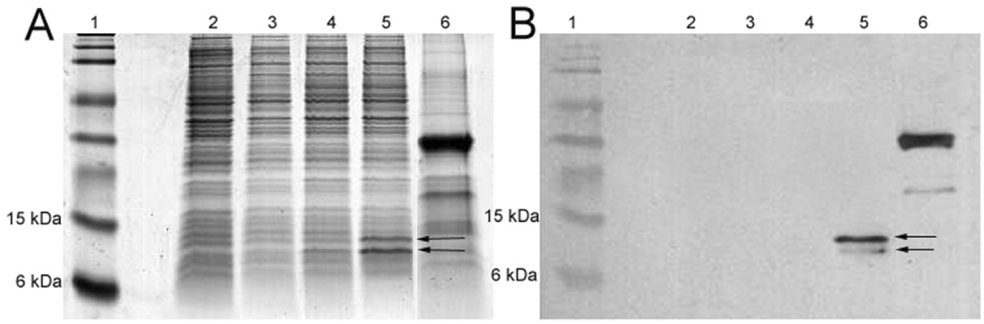


Fig. 1. Production in *E. coli* of TT-P0 protein. (A) SDS-PAGE 15% (B) Western blotting using anti-His monoclonal antibody. Lane 1: Molecular weight marker Prestained SDS-PAGE Standards Broad Range (BioRad, EE. UU), Lanes 2–5: BL21 (DE3)-TT-P0 cell extracts 0, 2, 4 and 6 h post-induction with IPTG. Lane 6: P0-my32 protein previously obtained in the laboratory. Arrows indicate TT-P0 protein.

P0 protein and P0 fused to a carrier protein. One group of tilapias was i. p. injected with the TT-P0 antigen at 1 μg/gbw, the second group received the same quantity of another P0 based antigen (P0-my32) and the third group (Control) received equal volume of buffer. Blood was collected from the caudal vein of all fish on days 0 and 7, 15 and 21 days after re-immunization.

2.5.4. Experiment in African catfish

Animals were maintained in a circular 500 L tanks supplied with recirculating fresh water at 26 ± 2 °C and 12:12 light-dark cycle. Fish were fed with pelleted feed (CENPALAB, Cuba). Twelve African catfish (65 ± 5 g) per group were immunized by i. p. injection on days 1 and 15. One group of catfish was injected with the antigen TT-P0 (1 μg/gbw). Control fish were immunized with buffer. Blood was collected from the caudal vein of all fish at days 0 and 15 days after re-immunization (day 30 from the beginning of the experiment).

2.5.5. Experiment in Atlantic salmon

Atlantic salmon (40 ± 5 g) were kept in a circular 500 L tanks supplied with recirculating fresh water for 2 weeks at an ambient temperature of approximately 10 °C with 24 h illumination (summer stimuli) for acclimation. Fish were fed with a commercial pellet diet (Nutra Olympic, Skretting). One hundred and twenty fish were placed in each tank per group and two experimental groups were settled. Control group received PBS and the other group received 1 μg/gbw of TT-P0, both by i. p. injection. Fifteen days after first vaccination, the fish were transferred to the sea water. The fish were kept under the following conditions during the experiment: Temperature: 10 °C; Light: 24 h; Oxygen level: ~80–90%; Salinity: 34–35 ppt. After 20 days in sea water (35 days from first vaccination), a booster was given. Blood samples were taken at day 0 and 69 (34 days after booster) from 30 fish per group.

2.6. Serum IgM levels

P0-specific IgM antibodies in the serum of vaccinated fish were determined by indirect ELISA. High binding microtiter plates (Nunc, Denmark) were coated for 16 h at 4 °C with 10 μg/mL of 35 aa synthetic P0. After three washes with PBS-Tween 0.05%, blocking was performed with 5% skimmed milk in PBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 120 mM NaCl, pH 7.4) for 2 h at room temperature (RT). Afterwards, two-fold serial dilutions starting from undiluted serum were applied and incubated for 16 h at 4 °C. Bound antigen-specific antibodies were incubated for 2 h at 25 °C with anti-tilapia IgM or anti-catfish IgM or anti-rainbow trout/Atlantic salmon IgM monoclonal antibody (ADL Aquatic Diagnostics, UK), depending on the specie evaluated, and they were detected by sequential incubation for 1 h at 25 °C with anti-mouse IgG conjugated with peroxidase (Sigma) according with the instructions of the manufacturer.

After washing, the chromogen TMB in substrate buffer was added and incubated for 10 min or until color development. After stop the reaction, color intensity was measured at 450 nm with a Varioskan Flash microplate reader. Antibody titers were defined as the dilution of serum giving twice the OD value of the pre-immune serum for each animal. Cut off value was defined for each experiment and it was set at twice the lowest dilution of the negative control serum (pre-immune serum). An internal positive control was included in each assay.

2.7. Western blotting for P0 recognition by immunized fish sera

Recombinant TT-P0 purified protein was loaded on 15% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blotting was performed as described above but using as primary antibody serum from immunized tilapia at a dilution 1:100 and anti-tilapia IgM as secondary antibody.

2.8. Statistical analysis

All the statistical analysis was done in Graphpad Prism version 6.0. In the case of comparison of two groups, Unpaired *t*-test or Mann-Whitney test was performed were done depending on the normal distribution and data variance. In case of three experimental groups, One-way ANOVA or Kruskall-Wallis test was done followed by Tukey or Dunn's Multiple Comparison post-test in each case. The normal distribution of data was analyzed with D'Agostino Pearson's test and the variance homogeneity with Bartlett's test.

3. Results

3.1. Production of recombinant TT-P0 protein

Analysis by SDS-PAGE and western blotting with the anti-His showed two bands between 6 and 15 kDa in the lanes corresponding to the cell extracts of the strains of *E. coli* BL21 (DE3) transformed with the genetic construction pET28-TT-P0 at 6 h post-induction with IPTG (Fig. 1). This agrees with the expected molecular weight for the TT-P0 protein of 8.3 kDa, according to the prediction based on the amino acid sequence deduced from the nucleotide sequence. The result of the western blotting also confirmed the presence of the tail of six His (Fig. 1B). After cell disruption most of the protein was obtained forming inclusion bodies (data not shown).

The purification of the TT-P0 protein, after its solubilization in urea, was carried out by affinity chromatography to metal chelates. For the optimization of this process, different imidazole molarities were tested. The wash step of the TT-P0 protein was carried out at a concentration of 40 mM of imidazole where important contaminants are eliminated without significant losses of the proteins of interest. Elution at 200 mM concentration of Imidazole resulted in TT-P0 protein with purity higher than 90% (Fig. 2). Purified protein was recognized in western blotting by the anti-His monoclonal antibody (Fig. 2B) and a polyclonal antibody against P0 generated in rabbits (Fig. 2C). These antibodies reacted with the two bands between 6 and 15 KDa and also with other bands

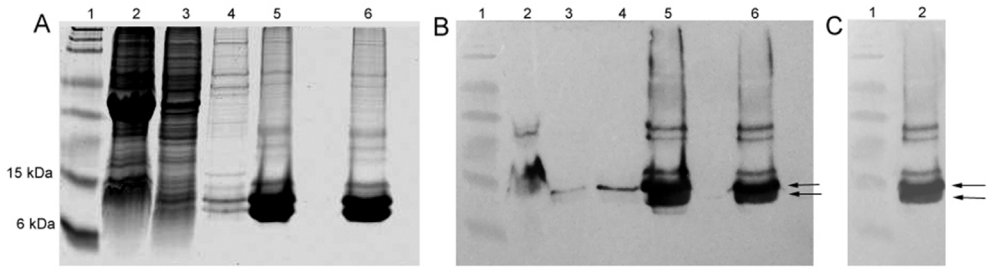
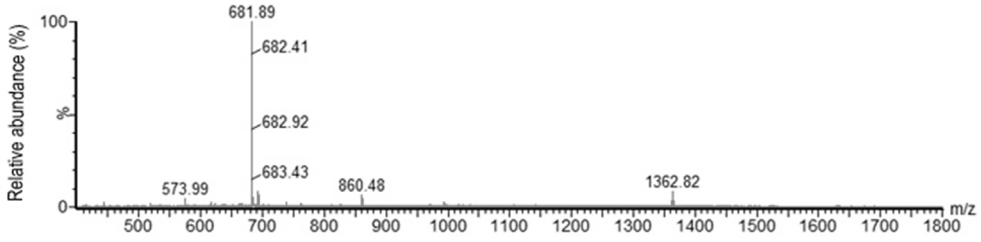


Fig. 2. Purification of TT-P0 protein by metal affinity chromatography. (A) SDS-PAGE 15% (B) western blotting using anti-His monoclonal antibody. Lane 1: Molecular weight marker Pre-stained SDS-PAGE Standards Broad Range (BioRad, EE. UU), lane 2: TT-P0 solubilized with 8 M urea, lane 3: Not-bound fraction, lane 4: Wash at 40 mM Imidazole, lane 5: Elution at 150 mM Imidazole, lane 6: Purified TT-P0 after buffer exchange (C) western blotting using *anti*-P0 polyclonal antibody. Lane 1: Molecular weight marker Pre-stained SDS-PAGE Standards Broad Range (BioRad, EE. UU), lane 2: Purified TT-P0 after buffer exchange. Arrows indicate TT-P0 protein.

A



B

Protein	Sequence	Calculated mass (Da)	Experimental mass (Da)
TT-P0	LEYLADPSK	1034.53	1034.56
	FASVAAA…	1231.66	1231.70
	FIGITELLSEIK	1361.78	1361.78
	LEGVGTKLEYLADPSK	1718.91	1718.96
	FIGITELLSEIKGV…	2023.18	2023.23

C

MGQYIKANSKFIGITELLSEIKGVIVHRLEGVGTKLEYLADPSKFASVAAAPAAGATKAAAAAPAKADE

PELEHHHHHH

Fig. 3. Mass spectrometry identification of TT-P0 protein. (A) ESI-MS/MS spectrum of the TT-P0 tryptic digestion (B) Peptides sequences obtained (C) Underlined the sequence region obtained.

with higher molecular weight that could be protein aggregates. For this reason we decided to corroborate the sequence of the bands at expected size by mass spectrometry.

The mass spectrum of tryptic digestion of the most intense band showed few signals (Fig. 3A), typical of a low molecular weight protein and multiple tryptic sites. The most intense signals were fragmented and the MS/MS spectra were analyzed manually. The sequences obtained (60% coverage from full sequence) confirmed the identity of TT-P0 chimeric protein (Fig. 3B and C).

3.2. Promiscuous T cell epitopes boosts the humoral immune response to P0 peptide in tilapia

In order to evaluate the immune-potentiating action of the TCE's on the pP0 peptide, an immunization scheme was performed on tilapia. Sera from tilapia were evaluated by indirect ELISA at 21 days after re-immunization. Chimeric TT-P0 protein adjuvanted in Montanide ISA 50 V2 induced a specific antibody response against pP0 after intraperitoneal immunization ($p < 0.001$) whereas the synthetic peptide in the same adjuvant or placebo (buffer in Montanide) does not produce any IgM response (Fig. 4A). In this group, the 67% of the fish had specific IgM titers. The specificity of the response was confirmed by *western blotting*. The results showed that sera from TT-P0 immunized fish are able to recognize the antigen. On the contrary, sera from synthetic pP0 immunized fish didn't give any signal (Fig. 4B).

In order to compare the response induced by immunization of TT-P0 with or without the oil adjuvant, we conducted another experiment in tilapia. In this experiment, only the group immunized with TT-P0 adjuvanted in Montanide ISA 50 V2 had significant higher IgM titers ($p < 0.01$) as compared to buffer-adjuvanted injected group and 80% of immunized fish developed an antibody response. Significant antibody titers were developed in all sampling points assayed after re-immunization. In TT-P0 injected group without adjuvant, only 43% of fish had titers against pP0 (Fig. 5A).

To compare with another carrier, a third experiment in tilapia was performed. IgM response to TT-P0 immunization was compared to IgM response against P0-my32. As result, the specific IgM titers against P0 were significantly higher in sera from TT-P0 immunized fish as compared to buffer injected ($p < 0.01$) and 70% of immunized fish developed the response after 7, 15 and 21 days after booster whereas only

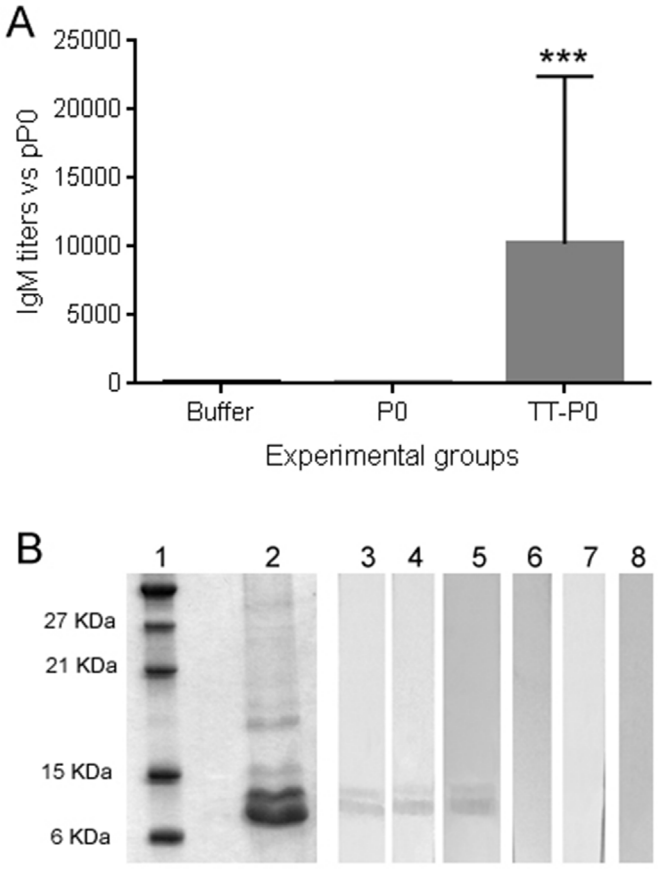


Fig. 4. (A) IgM response in tilapia (*Oreochromis niloticus*) immunized with TT-P0 antigen in comparison with synthetic P0. Tilapia (n = 10) were injected twice intraperitoneally (days 0 and 15) with the antigens formulated in Montanide ISA 50 V2. Three experimental groups were settled: Buffer, injected with synthetic peptide (P0) and injected with recombinant TT-P0 protein. Blood was collected 21 days after re-immunization. Data represents the mean +SD. The statistical analysis of data was performed using a Kruskall-Wallis followed by Dunn's multiple comparison test. *** indicates $p < 0.001$ **(B)** Specific recognition of TT-P0 by sera from immunized tilapia. Lanes 1 and 2: SDS-PAGE 15%, lane 1: Molecular weight marker (BioRad), Lane 2: purified TT-P0. Lanes 3–8: *western blotting* using as primary antibody the sera from injected fish as primary antibody and anti-tilapia IgM as secondary antibody. Lanes 3–5 immunized with TT-P0. Lanes 6–8 immunized with synthetic pP0.

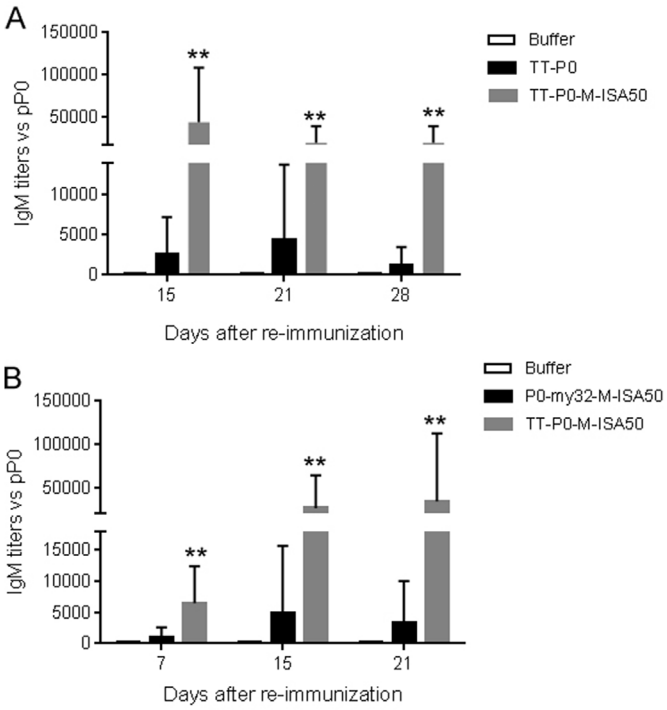


Fig. 5. (A) Comparison of antibody response in tilapia (*Oreochromis niloticus*) immunized with TT-P0 antigen in Montanide or without oil adjuvant. Three experimental groups were settled: Buffer, injected with recombinant TT-P0 protein adjuvanted in Montanide ISA50 V2, injected with recombinant TT-P0 protein without adjuvant. **(B)** IgM antibody response in tilapia (*Oreochromis niloticus*) immunized with TT-P0 antigen in comparison with P0-my32. Three experimental groups were settled: injected with buffer, injected with recombinant TT-P0 protein and a third group injected with recombinant P0-my32. Antigens were formulated in Montanide ISA 50 V2. In each experiment, tilapias (n = 10) were injected twice intraperitoneally (days 0 and 15). Data represent the mean +SD. The statistical analysis of data was performed using a Kruskall-Wallis followed by Dunn's multiple comparison test. ** indicates $p < 0.01$.

20% had IgM titers in P0-my32 vaccinated fish (Fig. 5B).

3.3. Promiscuous T cell epitopes enhances IgM response in African catfish and Atlantic salmon

We also test the potential of TT-P0 antigen to induce antigen-specific IgM response in other teleost species such as African catfish and Atlantic salmon. Two groups of catfish were immunized with TT-P0 and buffer (negative control). Fifteen days after booster, the IgM antibody response against P0 was measured by ELISA. Results show that P0-specific total IgM response was developed in 100% of TT-P0 injected animals compared to no response in buffer injected group ($p < 0.001$) (Fig. 6A) and in all cases antibody titers were equal or higher than 4000. In Atlantic salmon, TT-P0 was also able to induce specific IgM response 34 days after booster (Fig. 6B), being the antibody titers significantly higher compared to negative control group ($p < 0.001$).

4. Discussion

The prevention of diseases is essential for the development of sustainable aquaculture worldwide. In this context, vaccination is the most effective method for combating diseases and currently there are some commercially available vaccines for use in fish. Modern advances in vaccines and vaccinology offer valuable opportunities to discover new vaccine candidates to combat fish pathogens such as parasitic agents, for which vaccines are still lacking [15,16].

The present results clearly show that the fusion of TCE's to the N-terminal of P0 peptide and production of chimeric protein in *E. coli* constitute a suitable delivery system in fish for the generation of *anti*-P0 antibodies, suggesting the use of this cost-effective strategy to develop vaccines against conserved antigens in fish. Increased antibody titers had been found in human and murine studies using tt P2 and MVF epitopes [4,6]. The tt P2 and MVF epitopes have been established as strong T helper cell epitopes that exhibit universal antigenicity in mammals. This universality of the TCE's was confirmed in the present study by the induction of specific IgM titers against pP0 after intraperitoneal injection in three different teleost species: Tilapia, African catfish and Atlantic salmon. Previously, its action in terms of increased vaccine efficacy was also demonstrated in salmon coho [13]. These TCE's are MHC class II restricted and are capable to bind MHC class II molecules from a wide variety of haplotypes [11,12]. Farmed fish are outbred and presumably exhibit heterologous MHC haplotypes. Therefore, the expansion of protection across MHC haplotypes is highly beneficial to fish vaccinology.

A significant response was obtained only when the recombinant TT-P0 was injected in “water in oil” formulation with Montanide ISA 50

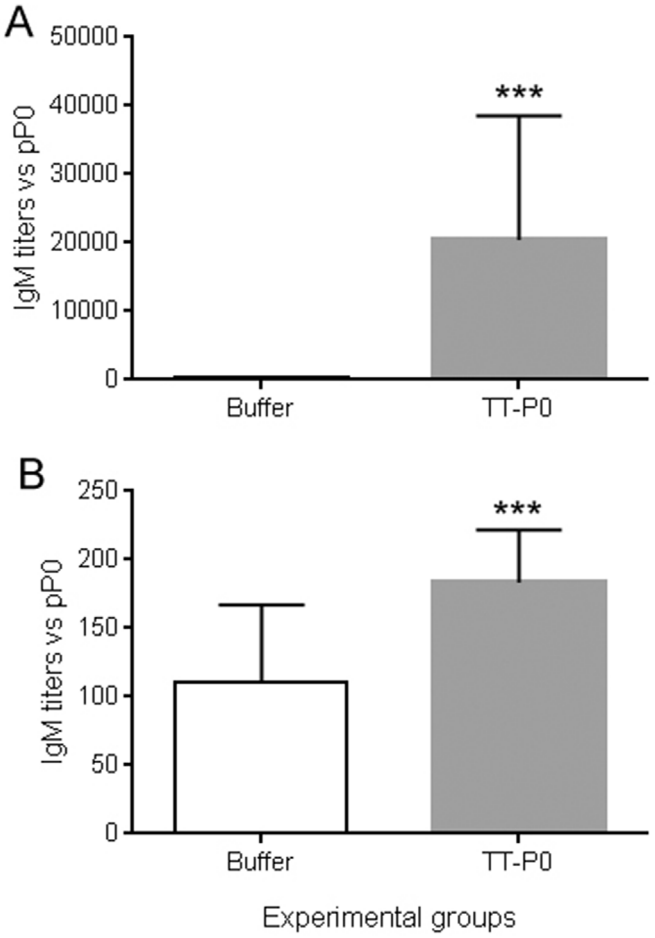


Fig. 6. (A) IgM antibody response in African catfish (*Clarias gariepinus*) immunized with TT-P0 antigen or buffer. Catfish (n = 12) were injected twice intraperitoneally (days 0 and 15). Data shows P0-specific IgM antibody response 15 days after booster. The statistical analysis of data was performed using a Mann-Whitney test. *** indicates $p < 0.001$. (B) IgM Antibody response in Atlantic salmon (*Salmo salar*) immunized with TT-P0 antigen. Salmon (n = 120) were injected twice intraperitoneally (days 0 and 35). The antigen was formulated in Montanide ISA 50 V2. Data shows P0-specific antibody response 34 days after booster of 30 animals per group represented as mean +SD. The statistical analysis of data was performed using a Mann-Whitney test. *** indicates $p < 0.001$.

V2. The need for an oil adjuvant had been demonstrated before in fish [9,17,18]. For example, MAPS administered in saline solution elicited no response in rainbow trout [9].

In this work, a 35 aa peptide of *L. salmonis* ribosomal protein P0 was chosen due to its less amino acid identity with respect to its hosts *S. salar* (45%). The L. *salmonis* pP0 amino acid identity was 48% comparing to the same region of P0 in *O. niloticus* (GenBank: FJ389919.1). As the P0 sequence in *C. gariepinus* was not reported, we made the comparison with P0 of another *Siluriformes*, *Ictalurus punctatus* and the amino acid identity was 41%, (GenBank: AAK95123.1). Thus, the selected peptide has also low identity with the same P0 region in the other teleost used in the study and thereof it's immunogenic also in these species. This sequence is part of a linear B cell epitope and presents a high degree of accessibility that suggests it is exposed on the surface of the protein, forming part of a natural epitope thereof. This agrees with that reported by Rodríguez-Mallon and colleagues, who used a 20-amino acid peptide as a vaccine candidate against another ectoparasite, the *R. sanguineus* tick with 90% efficacy [1]. P0 is essential for the assembly of the 60S ribosomal subunit and its absence leads to inactive ribosomes, absence of protein synthesis and cell death. P0 also acts as a regulatory element helping to adjust the metabolism of organisms to different environmental conditions. This protein is localized in the cell cytoplasm but it has been also reported in association with membrane proteins on the cell surface [19,20] and in tick saliva [21,22]. Despite it is localized in other compartments than the cytoplasm, several results challenge the paradigm that intracellular proteins are not capable of inducing a protective response against ectoparasite infestations [23]. Host antibodies may interact with arthropod intracellular proteins through a process that has not been fully characterized but results suggests that antibodies may be specifically transported across the midgut barrier into the hemolymph, and then enter into cells to interact with these intracellular proteins [23,24]. Nevertheless, other possibilities should be considered to explain the effect of the vaccination with intracellular antigens such as the effect of a host cell-mediated immune response and antibody responses that are cross-reactive with other proteins [24].

The chimeric protein TT-P0 was designed focused on locating the designed epitopes T, measles virus and tetanus toxin, fused in tandem towards the N-terminal of the peptide of 35 amino acids pP0, because it has been reported that tandem TCE's improves the immunogenicity of chimeric proteins, with respect to the fusion of a single T cell epitope [4,13,25].

For the expression of TT-P0 protein *in E. coli*, the pET28a vector was selected due to bacteriophage T7 promoter robustness that ensures high levels of expression of the genes under its control. In addition, it contains a coding sequence for a tail of histidine that can be fused to both the N-terminus and the C-terminus of the target protein and it is essential for further purification by affinity chromatography to metal chelates. The immune-identification by western blotting showed two bands at the expected size. Similar results were obtained by us [26], using pET28a as expression vector for the recombinant tilapia IFN-γ production. In this work both bands were identify as TT-P0 by mass spectrometry. Further deeper characterization by this technique could explain the difference in size between these two bands, for example if the lower band is a degradation product.

Three different Ig isotypes have been recognized in teleost fishes (IgM, IgD, and IgT/Z) [27]. IgM constitutes the most abundant Ig class in fish serum and this isotype plays most important role in the adaptive immune response at the systemic level but also has a role in mucosal immune responses [28]. IgD has a wide distribution among vertebrates; however, its function is still not well defined [28]. The most recently discovered Ig isotype is IgT/IgZ. This isotype can play a crucial role in the specific immune response against infectious agents in the intestinal, epithelial and gill mucosal surfaces [29,30]. Until now, commercial antibodies to detect teleost IgT are not available and for that reason we focus our studies on IgM response. The first immunological evaluation carried out in this work was the demonstration of the immune-potentiating effect of the T epitopes in the IgM type humoral immune response specific against the pP0 peptide in tilapia. The titers of antibodies obtained against the TT-P0 protein adjuvanted in Montanide ISA 50 V2 were statistically superior to those obtained for animals immunized only with the synthetic pP0 peptide formulated in the same adjuvant. The synthetic pP0 was not able to induce a specific IgM humoral immune response in tilapia. This is in consistent with the main disadvantage of peptide vaccines, which is the low immunogenicity. In the case of pP0, it contains only a linear B epitope, so it should not be recognized by T lymphocytes that cooperate in the induction of a specific humoral response and high affinity. Additionally, the group immunized with TT-P0 chimera showed the largest number of fish responders with higher titers. Concurrently, these results suggest that the incorporation of the TCE's in the chimeric construction provide an immunostimulatory effect in other T cells and in humoral epitopes within the peptide. The immunoidentification of the chimeric protein TT-P0 by western blotting using the sera of tilapia immunized with the formulations of the protein TT-P0 and synthetic pP0 in Montanide ISA 50 V2 confirmed the specific response against this antigen.

We studied the IgM response only after re-immunization due to previous demonstration that single immunization in tilapia didn't give statistically significant antibody titers compared to buffer injected animals. In the mentioned study, we only got significant different IgM titers compared to control group when we applied a re-immunization [18,31]. Other studies had been demonstrated the necessity of re-immunization to maintain antibody response and long-term protection, for example, in salmonids [32]. In the cited study high levels of specific IgM antibodies were observed after injectable vaccination, reaching a maximum concentration at 600–800 degree-days. The authors also demonstrated the necessity of oral re-immunizations to maintain protective antibody levels. These results showed that several oral immunizations are essential in the field to uphold a high level of specific anti-pathogens antibodies and, therefore, the protective status during the whole productive cycle. Taking into account the above mentioned results, the duration of salmon productive cycle, the fact that sea louse infected salmon in sea water and first vaccination occur in freshwater, we think that it is not possible to overcome the necessity of booster immunization. Then, for vaccine application in field conditions it will be mandatory to develop a feasible way to perform re-immunizations.

Previously, a chimeric protein based on pP0 fused to the N-terminal of the my32 protein of *L. salmonis* (P0-my32) was obtained by us. The chimeric protein P0-my32, adjuvanted in Montanide was able to induce a specific IgM type response against pP0 in tilapia [18]. This prototype provided 28–35 relative percent protection in *S. salar* vaccinated groups at 44 days post infection with the parasite under different vaccination-boost strategies in an immunization-challenge experiment in controlled laboratory conditions (unpublished results). Based on these results, we decided to compare the humoral immune response of this protein and the TT-P0 chimera adjuvanted in Montanide ISA 50 V2. In this experiment, higher titers against pP0 were obtained in animals immunized with TT-P0 and Montanide ISA 50 V2 compared to animals immunized with P0-my32, in addition to which a greater number of fish with titers higher than 1:1000 responded. This result suggests that T epitopes confer greater antigenicity to the pP0 peptide in terms of IgM response and it could be a potential candidate for sea lice vaccine development.

As observed in the experiments, the humoral response induced in the fish, especially tilapia and catfish, is very heterogeneous. This may be due to the fact that these species constitutes open genetic lines, since the animals are obtained by the uncontrolled crossing between unrelated individuals; unlike syngenic and pure lines, which constitute closed populations composed of genetically identical individuals.

In conclusion, we showed for first time that non-responsiveness to P0 peptide in teleost fish can be overcome by adding foreign T cell epitopes. The response is characterized by increased IgM titers and more fish responding to vaccination as compared to synthetic peptide or another chimeric protein P0-my32. TCE's, MVF and tt P2, which are highly immunogenic in human and murine models, were shown to retain their immunostimulatory properties not only in the context of the salmonid model but also in perciformes and siluriformes teleost species. The results are also important in the context of peptide-vaccine development. In other studies in teleost, chemical conjugation to carrier proteins such as hemocyanin from *Megathura cranulata* (KLH) had been used to increase the immunogenicity of peptides-based vaccines. This approach could have some disadvantages such as: the need of carrier purification from its natural source, reproducibility of chemical conjugation procedures and the fact that some fish could have natural antibodies to KLH, probably due to its presence in the marine environment. The potential lytic activity to KLH of natural antibodies might have decreased the serum half-life of the peptide, reducing its interaction with the fish immune system although this potential lytic action was probably negligible according to results obtained in sea bass [33]. The chimeric peptide herein can be obtained in controlled laboratory conditions by *E. coli* fermentation methodology adding another value to the approach used. Next steps are the validation of this vaccine candidate in a vaccination-challenge experiment in *S. salar* and deeper studies about the activation of immune response at mucosal level. Further characterizations of the same approach using other candidate peptides will broad the application range of this strategy for peptide-vaccine development to fight against important diseases in farmed species.

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