Fish & Shellfish Immunology 32 (2012) 1088–1094

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Protection and antibody response induced by intramuscular DNA vaccine encoding for viral haemorrhagic septicaemia virus (VHSV) G glycoprotein in turbot (*Scophthalmus maximus*)

P. Pereiro [a], A. Martinez-Lopez [b], A. Falco [b], S. Dios [a], A. Figueras [a], J.M. Coll [c], B. Novoa [a,*], A. Estepa [b,**]

[a] *Instituto de Investigaciones Marinas (IIM), CSIC, Vigo 36208, Spain*
[b] *Instituto de Biología Molecular y Celular (IBMC), Miguel Hernández University, 03202 Elche, Spain*
[c] *Dpto Biotecnología, INIA, 28040 Madrid, Spain*

ARTICLE INFO

Article history:
Received 17 January 2012
Received in revised form
1 March 2012
Accepted 5 March 2012
Available online 13 March 2012

Keywords:
DNA vaccine
VHSV
Turbot
Glycoprotein G
Rhabdovirus

ABSTRACT

Turbot (*Scophthalmus maximus*) is a high-value farmed marine flatfish with growing demand and production levels in Europe susceptible to turbot-specific viral haemorrhagic septicaemia virus (VHSV) strains. To evaluate the possibility of controlling the outbreaks of this infectious disease by means of DNA vaccination, the gpG of a VHSV isolated from farmed turbot ($VHSV_{860}$) was cloned into an expression plasmid containing the human cytomegalovirus (CMV) promoter (pMCV1.4-G_{860}). In our experimental conditions, DNA immunised turbots were more than 85% protected against $VHSV_{860}$ lethal challenge and showed both VHSV-gpG specific and neutralizing antibodies. To our knowledge this is the first report showing the efficacy of turbot genetic immunisation against a VHSV. Work is in progress to determine the contribution of innate and adaptive immunity to the protective response elicited by the immunization.

1. Introduction

Viruses are probably the most destructive pathogens in fish aquaculture and they are a serious concern since no specific chemotherapies are available. Among the 9 notifiable fish diseases (diseases with great social and economic and/or public health repercussion or with present or potential risk for the aquaculture industry) registered at the 2009 Aquatic Animal Health Code of the OIE (Office International des Epizooties, now the World Organization for Animal Health) (http://www.oie.int), 7 are caused by viruses. Furthermore, two of them (viral hemorrhagic septicaemia, VHS, and infectious hematopoietic necrosis, IHN) are caused by novirhabdoviruses hence showing their high risk to worldwide aquaculture.

To date, rhabdoviral disease prevention by means of vaccination is considered a very efficient strategy for the control and prevention of these diseases. For the salmonid fish novirhabdoviruses VHSV and IHNV, it has been demonstrated that DNA vaccines based on the glycoprotein G (gpG) gene are able to confer long-term protection against homologous challenges as well as short and unspecific protection against heterologous challenge [1–5]. In fact, a DNA vaccine to IHNV (Apex-IHN, Aqua Health Ltd, Canada) has been approved for commercialization in July 2005 by the Canadian Food Inspection Agency, Veterinary Biologist and Biotechnology Division [6,7]. In spite of this, DNA vaccines still face several challenges mainly concerning safety and delivery routes what constitutes an obstacle for their approval in many countries, as it occurs in European ones.

Among the fish novirhabdoviruses, VHSV is indeed one of the most important viral diseases of fish in European aquaculture [8,9]. In addition, VHSV is spreading to wild ecosystems as it is being isolated all over the world from an increasing number of free-living marine fish species [10–13]. To date, phylogenetic studies have grouped all these isolates into four genotypes (I–IV) [14,15]. Moreover, it has been also reported that susceptibility of the host species to VHSV infections depends on the genotype [16].

Due to intensive farming conditions, disease outbreaks caused by VHSV have become a severe problem for global turbot industries [14,17]. For instance, after the outbreak at the Gigha turbot farm (Scotland), caused by the UK-860/94 strain [17] 14 tonnes of fish were sacrificed (with subsequent relevant economic losses) [18]. Therefore, the need for the development of effective means of disease control is urgent. However, it is still unknown whether or not a DNA vaccine against VHSV based on its gpG gene can confer

* Corresponding author. Instituto de Investigaciones Marinas (IIM), CSIC, Vigo 36208, Spain.
** Corresponding author. Miguel Hernandez University, Instituto de Biologia Molecular y Celular (IBMC), Avenida de la Universidad s/n, 03202 Elche, Alicante, Spain. Tel.: +34 966 658 436.
E-mail address: aestepa@umh.es (A. Estepa).

1050-4648/$ – see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.fsi.2012.03.004

turbot protection against VHSV lethal challenge. In contrast, a vaccine for Japanese flounder (*Paralichthys olivaceus*) based on the use of VHSV strains isolated in their farm outbreaks [19,20] has been developed. Interestingly, a DNA vaccine to VHSV has been also used to confer short-term unspecific protection to turbot against nodavirus infection [21,22].

Since VHSV virulence in host species is genotype related [16], in this work a DNA vaccine encoding the gpG from a virulent VHSV strain isolated from a turbot outbreak (UK-860/94, genotype III) [14,17] was constructed, characterized and its protective role determined in juvenile turbots. The results showed that at least over the time scale and conditions analysed, DNA vaccination of turbot with the gpG protein of VHSV confers resistance and elicits specific antibodies with neutralising activity. To our knowledge this is the first published report describing the successful immunisation of turbot with a DNA vaccine against VHSV.

2. Materials and methods

2.1. Cell cultures and virus

The fish cell line EPC (Epithelioma papulosum cyprinid) [23] purchased from the American Type Culture Collection (ATCC number CRL-2872) was used in this work. Recently, the ATCC has revealed that the EPC cell line, originally deposited as a carp (*Cyprinus carpio*) cell line, is in fact a fathead minnow (*Pimephales promelas*) cell line. EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (FCS) (Sigma, Che. Co, St. Louis, Ms, USA), 1 mM Piruvate (Gibco, Invitrogen corporation, UK), 2 mM Glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone.

Viral haemorrhagic septicaemia virus UK-860/94 ($VHSV_{860}$) isolated from farmed turbot in England [14,17] was propagated in EPC cells at 14 °C as previously described for other VHSV isolates [24]. Supernatants from $VHSV_{860}$-infected EPC cell monolayers were clarified by centrifugation at 4000 × g during 30 min and kept in aliquots at −70 °C. Viruses from clarified supernatants were concentrated by ultracentrifugation at 100,000 × *g* for 45 min [16]. The virus stock was titrated in 96-well plates according to Reed and Muënch [25].

2.2. Plasmids

The expression vector pMCV1.4 (Ready-Vector, Madrid, Spain) (2863 bp) [26] containing the CMV regulatory sequences was used. The regulatory sequences of pMCV1.4 vector promoter included an enhancer/promoter sequence (740 bp from the human cytomegalovirus, CMV, major immediate-early gene) and an intron sequence (~200 bp of a synthetic chimeric intron).

To obtain the pMCV1.4-G_{860} construct, the gpG cDNA sequence from $VHSV_{860}$ (Gene bank accession number AY546628) was synthesised by BioS&T (Montreal, Canada) and then cloned into the vector pMCV1.4 digested with the restriction enzymes KpnI and XbaI following standard procedures. Finally, the sequence of the gpG gene in the construct pMCV1.4-G_{860} was confirmed by DNA sequencing.

2.3. Cell transfection assays

Cell transfection assays were performed as previously described [27,28]. Briefly, EPC cell monolayers grown in culture flasks of 75 cm^2 were detached using trypsine (Sigma), washed, resuspended in culture medium supplemented with 10% of FCS and dispensed into 24-well plates at a concentration of 2.5×10^5 cells per well in a final volume of 250 μl. The following day, the pMCV1.4-G_{860} or the pMCV 1.4 (0.5 μg) complexed with 0.7 μl of FuGene 6 (Roche, Barcelona, Spain) were incubated for 15 min in 50 μl of RPMI-1640 containing 2 mM Cl_2Ca and then added to each well in 200 μl of culture medium with 10% of FCS. As an additional control, EPC cells were transfected with FuGene 6 without DNA following the same procedure. The plates were further incubated at 20 °C for 2 days. As an additional control, the cells were also transfected with the previously characterised pMCV1.4-$G_{07.71}$ [29] encoding the cDNA sequence of the gpG of VHSV strain 07.71 (genotype I) isolated from rainbow trout *Oncorhynchus mykiss* [30].

2.4. Turbots

Juvenile turbot of approximately 2.5 g each, obtained from a VHSV-free commercial farm (Insuiña S.L., Mougás, Galicia, Spain), were maintained in 500 L fibreglass tanks at the IIM (CSIC) facilities at 18 °C with a re-circulating saline-water system and fed daily with a commercial diet (LARVIVA-BioMar, France). Prior conducting experiments, fish were acclimatised to laboratory conditions for 2 weeks and then it was confirmed that their sera were negative for the presence of antibodies against VHSV as below described.

2.5. DNA immunisation protocol

DNA immunisation of turbot was carried out following procedures for fish genetic immunisation previously described [31]. Briefly, turbots were anaesthetised by immersion in 50 μg/ml buffered tricaine methanesulfonate (MS-222; Sigma) prior to handling and then divided into 6 tanks (52 fish/tank). Turbots from two tanks were then intramuscularly injected (i.m.) with one of the following: 50 μl of PBS (non-immunised or control fish), 50 μl of PBS containing 2 μg of pMCV1.4 or pMCV1.4-G_{860}. The replicate tanks were placed alternatively to minimize the influence of tank position. Three days after immunisation (Fig. 1), 3 fish from each group (a total of 6 fish/treatment) were sacrificed by overexposure to MS-222 and muscle (site of injection) was removed and processed for real time quantitative PCR (RT-qPCR) as indicated below. Moreover, at day 30 post-immunisation (Fig. 1) blood from the caudal vein was extracted (3 fish per group or 6 fish per treatment) in order to study antibody production. An additional untreated group composed by 23 fish was also added in order to control possible mortalities caused by maintenance conditions.

2.6. Analysis of VHSV-G protein expression in transfected cells and DNA immunised-fish muscle

The expression of gpG_{860} in EPC transfected cells as well as in turbot muscle tissue was analysed at transcriptional levels by RT-qPCR. Cell RNA extraction and cDNA synthesis were performed as previously described [29]. RT-qPCR was carried out using the gpG_{860} gene primers and probe designed to detect specifically the

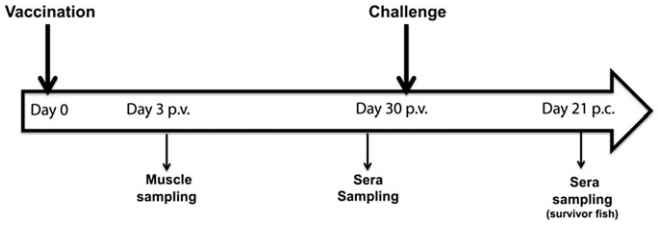

Fig. 1. Schematic overview of vaccination/challenge protocol and sampling procedure. Vaccination/challenge protocol carried out for determining the vaccine effectiveness. Moreover, muscle and sera samples were collected at the indicated time points in order to analyse the immune response. p.v., post-vaccination; p.c., post-challenge.