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http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

Development of an Inactivated Iridovirus Vaccine Against Turbot Viral Reddish Body Syndrome

FAN Tingjun*, HU Xiuzhong, WANG Liyan, GENG Xiaofen, JIANG Guojian, YANG Xiuxia, and YU Miaomiao

Department of Marine Biology, *College of Marine Life Sciences*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract Turbot (*Scophthalmus maximus* L.) reddish body iridovirus (TRBIV) was propagated in turbot fin cells (TF cells) and inactivated as the TRBIV vaccine with its protection efficiency evaluated in this study. TF cells were cultured in 10% bovine calf serum (BCS)-containing MEM medium (pH7.0) at 22℃, in which TRBIV propagated to a titer as high as $10^{5.6}$ $TCID_{50}$ mL^{-1}. The TRBIV was inactivated with 0.1% formalin and formulated with 0.5% aluminum hydroxide. The inactivated vaccine caused neither cytopathogenic effect (CPE) on TF cells nor pathogenic effect on turbots. After being administered with the vaccine twice *via* muscle injection, the turbot developed high-tittered TRBIV neutralizing antibodies in a dose-dependent manner. The vaccine protected the turbot from dying with an immunoprotection rate of 83.3% as was determined *via* subcutaneous vaccination in the laboratory and 90.5% *via* bath vaccination in turbot farms, respectively. The inactivated vaccine was very immunogenic, efficiently preventing turbot from death. It holds the potential of being applied in aquaculture.

Key words turbot reddish body iridovirus; turbot fin cell; inactivated vaccine; *Scophthalmus maximus*

1 Introduction

Turbot is an appreciated flatfish for its firm white flesh and subtle and refined flavor. It is not only a rich source of protein and vitamins B3 and B12, but also the source of minerals including selenium, magnesium and phosphorous. After being imported from Europe, it is cultured in quantity in China (Lei, 2000). One serious problem for turbot farming is infectious diseases, especially those by diverse viruses (*e.g.*, turbot viral reddish body syndrome, TVRBS; lymphocystis). These diseases dramatically influenced the turbot farming in Europe and China. The methods for controlling these diseases are needed urgently at present.

DNA-containing turbot reddish body iridovirus (TRBIV) causes TVRBS. It is a member of the iridovirus family and 125 nm in diameter and its genome sequence has been determined (Shi *et al*., 2010). Turbot is highly sensitive to TRBIV at all its developmental stages. Once being infected, turbot quickly develops abnormal behavior and their ventral surface and fins become reddish. Over 20% of the infected turbots may die from this disease, causing a dramatic economic loss (Fan *et al*., 2006; Shi *et al*., 2004). At present, the only reliable way for preventing TRBIV infection is vaccination. Vaccines against viruses infecting animals including freshwater fish and humans have been developed using *in vitro* cultured cells. However, the commercial vaccine against TRBIV is not available for turbot. It becomes possible to develop a vaccine against TRBIV at present as a turbot fin (TF) cell line and a spotted halibut kidney cell line have been established (Fan *et al*., 2007; Wang *et al*., 2010). In this study, a safe and effective vaccine against TRBIV was developed in TF cell line.

* Corresponding author. Tel.: 0086-532-2031637
E-mail: tjfan@ouc.edu.cn

2 Materials and Methods

2.1 Turbot

Both TVRBS and morbidity-free healthy turbot (*Scophthalmus maximus* L.), about 20 cm in body length, were obtained from Hesheng turbot farm (Laizhou, Shandong, China) and maintained at 22℃ in aerated seawater.

2.2 Cell Preparation

TF cell line at passage 127 (Fan *et al*., 2007) was used for virus propagation. Cells were cultured at 22℃ in 10% bovine calf serum (BCS) (Thermo Fisher Scientific Inc., Waltham, MA, USA)-containing minimal essential medium (MEM, Invitrogen, Carlsbad, CA, USA) (pH 7.0).

2.3 Virus Propagation

Cells were cultured to logarithm phase and infected with

TRBIV isolated from viscus of turbot with typical TVRBS (Fan *et al*., 2006). After homogenization and centrifugation, the virus-containing supernatant was filtrated through a 0.45 μm filter and used for inoculation. The inoculated cells were motionlessly placed for 2 h first and then cultured at 22℃ in 10% BCS-containing MEM (pH 7.0) for 4 d until typical cytopathogenic effect (CPE) formed. The culture was frozen and thawed with viruses harvested by centrifugation at 9000 *g* for 90 min. The viruses in the supernatant were tittered with the hemagglutination method (Yin and Liu, 1997).

2.4 Formalin Inactivation of Viruses

Formalin (37% formaldehyde, Sigma-Aldrich, St. Louis, MO, USA) was diluted with 0.8% saline (pH 7.0) to 1% and filtered through a 0.22 μm membrane. The viruses were inactivated with nine volumes of 1% formalin at 37℃ for 48 h. The stock vaccine was stored at 4℃.

2.5 Vaccine Formulation

Two point five liters of aluminum hydroxide gel (4%, Sigma-Aldrich) was mixed with 16.5 L of 0.8% sterilized saline and 1.0 L of stock vaccine. The aliquots of mixture in 10 L plastic buckets were stored at 4℃ and diluted 20 folds before use.

2.6 Security Evaluation

Ten healthy turbot individuals (TVRBS-free and about 20 cm in body length) were inoculated with 0.5 mL vaccine *via* muscle injection and the other ten with 0.5 mL 0.8% sterile saline as the control. The fish were fed and monitored daily.

TF cells at passage 127 were cultured to logarithm phase and then harvested and mixed with 1.5 mL stock vaccine, vaccine and 0.8% sterile saline (control), respectively before they were motionlessly placed for 2 h. Cells were harvested and mixed with 5 mL of 10% BCS-containing MEM and incubated at 22℃ with their morphology monitored daily.

2.7 Turbot Immunogenicity Assay

Ten turbots (about 20 cm in body length) were injected with 0.1 mL of vaccine and the other 10 with 0.1 mL of 0.8% sterile saline in each position, 3 positions being selected in each turbot. Fifteen days after injection, blood was collected from tail fin vein with a 2.5-mL syringe. The blood cells were separated from serum by centrifugation at 200 *g* for 10 min. Then they were washed 3 times with 0.8% saline and centrifugation at 200 *g* for 10 min each time. Finally they were resuspended in 0.8% saline to a final concentration of 1.0%. The hemagglutination-inhibition (HI) assay was conducted in a 96-well microtiter plate (V-bottom) (Rose Scientific Ltd., Edmonton, Alberta, Canada) with the method described by Clarke and Casals (1958). The vaccine (25 μL each well) was mixed with the gradient dilution (1:2 to $1:2^7$) of serum (25 μL each well) and incubated at 22℃ for 30 min. Blood cell suspension (50 μL of 1.0% dilution) was then added into each well, mixed thoroughly and incubated at 22℃ for 60 min. Wells without immune serum were used as TRBIV controls. Three independent measurements were performed and the mean of HI assay was evaluated.

2.8 Immunoprotection Assays on Laboratory Scale

Sixty healthy turbot individuals (TVRBS-free and about 20 cm in body length) were divided randomly into 6 groups with 10 individuals in each. Three groups were inoculated with 0.5 mL vaccine *via* subcutaneous (sc) route (0.1 mL each spot, 5 spots each fish), while the other 3 groups were inoculated with 0.5 mL 0.8% sterile saline. After fed for 15 d, all turbot individuals were challenged with 0.3 mL of vaccine *via* muscle injection and monitored daily. Three independent measurements were performed and the mean of animal survivals was calculated. The immunoprotection rate of the vaccine was calculated according to the formula from Su *et al.* (2008): (death rate without vaccine inoculation-death rate with vaccine)/death rate without vaccine

2.9 Immunoprotection Assaying in Fish Farm

Protection assay was conducted in Hesheng turbot fish farm (Laizhou, Shandong, P. R. China) with 20400 turbot individuals (about 20 cm in body length). These fish were found to be infected by TRBIV with a death rate of about 15% (data not shown). The turbots were divided randomly into 6 groups, 3400 individuals in each. Three groups were dipped in vaccine at 22℃ for 30 min by 2 successive bath immunizations at an interval of 7 d, while the other 3 groups were dipped in sea water under the same condition. All of the turbot were fed routinely and their death rate was monitored. Three independent assays were performed with immunoprotection rate calculated as described above.

2.10 Statistical Analysis

Data were expressed as mean ± standard deviation (n=3) with significance tested using one way analysis of variance. Values with $P<0.01$ were considered significant.

3 Results

3.1 Propagation of TRBIVs in TF Cells

Once inoculated with TRBIV, TF cells formed typical CPE on day 3 (Fig.1b). TRBIV propagated well in TF cells. The virus titer reached $10^{5.6}$ $TCID_{50}$ mL^{-1}. Many virions with typical morphology scattered in cytoplasm as was observed on day 4 (Fig.1c, d).

3.2 Security of Inactivated Vaccine

The vaccine was safe to turbot. Both the injected with vaccine and the injected with saline water survived well without TVRBS pathogenic changes appearing in 15 d after injection and TVRBS pathogenic changes were found in dissection. TF cells inoculated with either vaccine or sa-

line water were normal in fibroblast morphology without CPE found in 5 d after inoculation (Fig.2). The vaccine was unable to induce CPE in TF cells and it was considered safe.

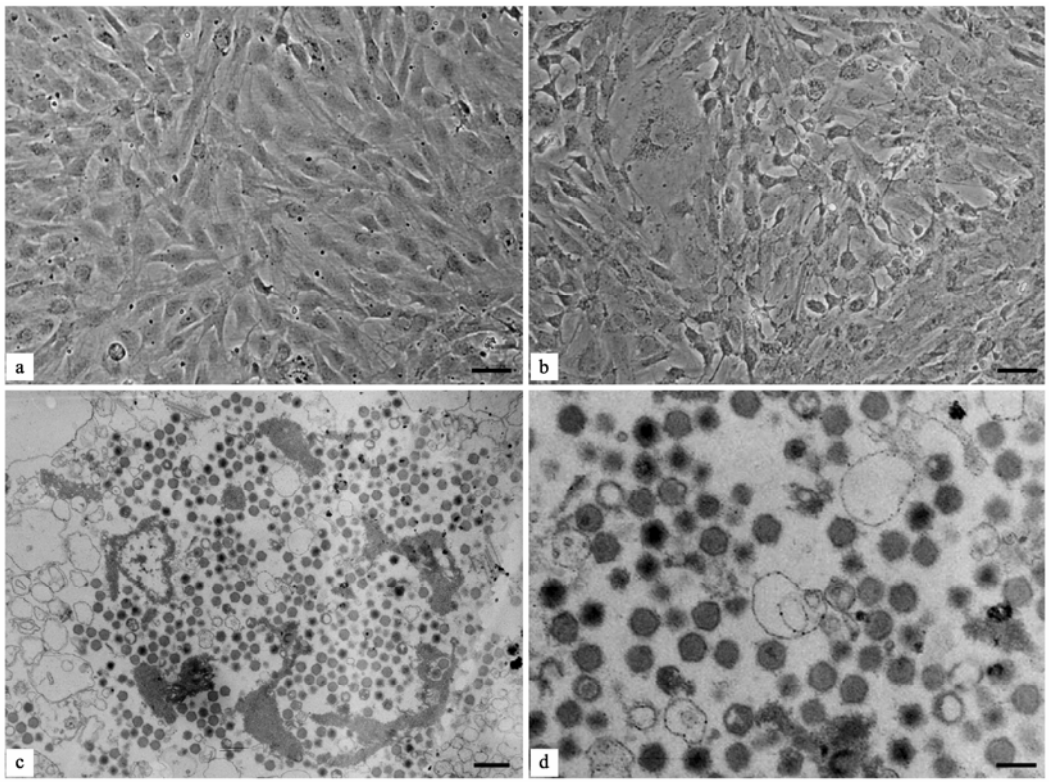

Fig.1 Morphology and ultrastructure of TF cells inoculated with TRBIVs. a, Morphology of TF cells without inoculation of TRBIVs, showing the fibroblast-like morphology of TF cells *in vitro*. Scale bar: 40 μm; b, Morphology of TF cells inoculated with TRBIVs, showing the shrinked morphology of TF cells and the CPE they formed. Scale bar: 40 μm; c, Ultrastucture of a TF cell inoculated with TRBIVs, showing the propagated TRBIV virions in the cytoplasm. Scale bar: 500 nm; d, Ultrastucture of TRBIV virions in a TF cell, showing the morphology of the TRBIV virions. Scale bar: 200 nm.

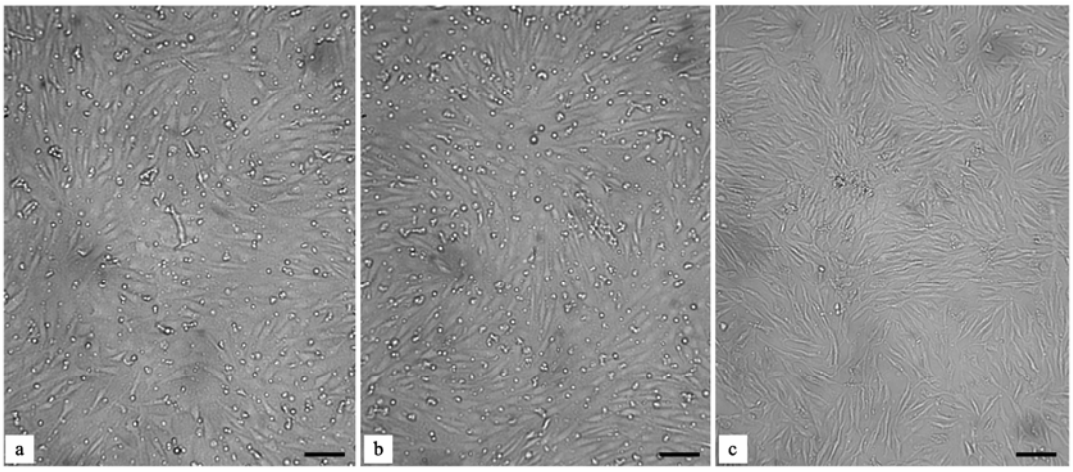

Fig.2 TF cells in the security test of TRBIV vaccine. a, TF cells inoculated with 1.5 mL of 0.8% sterile saline 7 d later; b, TF cells inoculated with 1.5 mL of 20× diluted TRBIV vaccine 7 d later; c, TF cells inoculated with 1.5 mL of undiluted TRBIV vaccine 7 d later. Scale bar: 20 μm.

3.3 Immunogenicity

HI titer of the serum of turbot individuals vaccinated 1:16 after challenged subcutaneously with 0.1 mL vaccine for 15 d, implying that the vaccine was obviously immunogenic, stimulating fish to produce antibody against TRBIV (Fig.3).

3.4 Immunoprotection Efficiency

The death rate of vaccinated fish was much lower than that of the control. The immunoprotection rate of the vaccine developed reached as high as 83.3%. Testing in farm showed that the immunoprotection rate of the vaccine developed reached 90.5% (Fig.4).

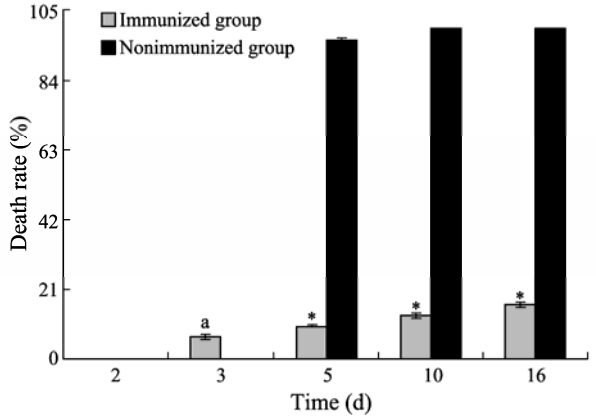

Fig.3 Immunoprotection assays of inactivated TRBIV vaccine with turbots in laboratory scale. Values are the average of three independent measurements in triplicate ± S.D. [a] The dead turbots had no features of TVRBS. [*]$P<0.01$.

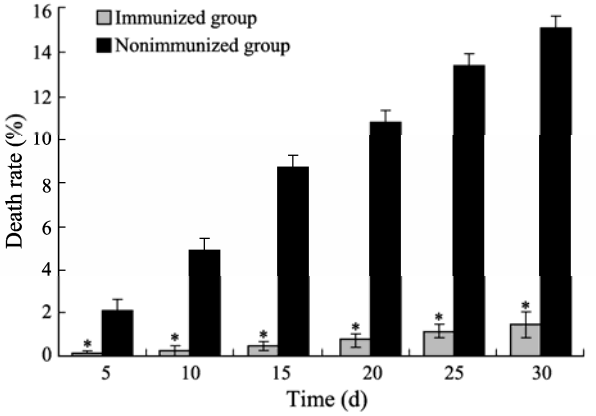

Fig.4 Immunoprotection assays of inactivated TRBIV vaccine with turbots in a fish farm. Values are the average of three independent measurements in triplicate± S.D. [*] $P<0.01$.

4 Discussion

The infective disease TVRBS is very serious in both fry and adult fish, which has caused dramatic economic loss in turbot farming in China. The disease occurred not only in turbot but also in other marine fishes (Grisez and Tan, 2005). At the end of 2006, loss related to this disease and medicament misusage in China was estimated to be 100 to 120 million dollars. Intensive effort has been made to reduce the loss in the last 4 years; however, turbot disease has not been efficiently controlled.

Over the last decade, prophylactic immunization (vaccination) has become a powerful tool for disease control in aquaculture (Evelyn, 2002; Gudding *et al*., 1999). Vaccines against infectious grass carp reovirus, hematopoietic necrosis virus, pancreatic necrosis virus and red sea bream iridovirus have been successfully developed for grass carp, rainbow trout, carp, catfish, red sea bream and Atlantic salmon (Dixon, 1997; Evelyn, 2002; Kurita and Inui, 1997; Nakajima *et al*., 1996). To our knowledge, the vaccine developed in this study is the first for turbot.

Most available fish vaccines against viral diseases are traditional inactivated ones (Sommerset *et al*., 2005). Instead of purified viruses, the medium supernatant of TRBIV-infected TF cells was directly used for vaccine preparation. TF cell line was derived from turbot fin, which is highly permissive to TRBIV isolates (Fan *et al*., 2006). The doubling time of TF cells was about 62.4 h when cultured in L-15 medium (20% BCS) at 22℃ (Fan *et al*., 2007) and typical CPE appeared in the TRBIV-infected cells cultured in L-15 (10% BCS) in 3 d (Fan *et al*., 2006). The short doubling time and the high efficiency of TRBIV proliferation of TF cells make the inactivated TRBIV vaccine feasible with a low price. The titer of TRBIV propagated in this cell line was as high as $10^{5.6}$ $TCID_{50}$ mL^{-1}.

Fish viruses were less susceptible to formalin at low concentrations (Arimoto *et al*., 1996; Frerichs *et al*., 2000); however, they were sensitive to formalin at higher concentrations (≥0.5%) (Anderson *et al*., 2008; Yamashita *et al*., 2005; Yamashita *et al*., 2009). In this study, we found that TRBIV can be completely inactivated by 0.1% formalin within 48 h at 37℃, faster than at 24℃ or 30℃ (Kai and Chi, 2008; Yamashita *et al*., 2005). Increase of temperature was harmless for TRBIV antigenicity (Anderson *et al*., 2008; Kai and Chi, 2008). The vaccine obtained in this study was safe both to turbot individuals and TF cells, and could be used safely in vaccination of farming turbots.

Various adjuvants were frequently utilized in fish viral vaccine formulation in order to enhance the vaccine's immunostimulating effects (Dixon, 1997; Midtlyng *et al*., 1996; Nakajima *et al*., 1997; Rønsholdt and McLean, 1999). We used 0.5% of aluminum hydroxide as an adjuvant in our TRBIV vaccine preparation. The vaccine developed showed obvious immunogenicity as the immunoprotection rate reached 83.3% on laboratory scale and 90.5% on farm scale, respectively, indicating the vaccine is promising for practical use. The vaccine developed in this study is similar to those for rainbow trout, catfish, red sea bream, Atlantic salmon and grouper (Dixon, 1997; Evelyn, 2002; Kai and Chi, 2008; Midtlyng *et al*., 1996; Nakajima *et al*., 1997). It can be applied to protect turbots from infection efficiently.

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