First isolation of viral haemorrhagic septicaemia (VHS) virus from turbot (*Scophthalmus maximus*) cultured in the Trabzon coastal area of the Black Sea in Turkey

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Abstract

Viral haemorrhagic septicaemia virus (VHSV) was isolated from cultured turbot fry and broodstock samples in the Trabzon coastal area of the Black Sea, Turkey in 2004. The virus was isolated in EPC and RTG-2 cell lines and identified by immunoperoxidase test, immunofluorescence test and enzyme linked immunosorbent assay. The results of applied immunological identification tests confirmed each other. This is the first report on the isolation of VHSV from cultured turbot in Turkey.

Introduction

Viral haemorrhagic septicaemia (VHS) is a serious infectious disease caused by a virus belonging to the Novirhabdovirus genus within the family *Rhabdoviridae*. Viral haemorrhagic septicaemia virus (VHSV) has been isolated from a number of wild marine species and at least 45 different freshwater and marine species have tested positive for the virus (Skall et al., 2005). Stone et al. (1997) suggested that all marine fish species are potential carriers of VHSV. VHSV has been isolated from freshwater and marine fish species in Europe, North America and East Asia (Dixon et al., 1997, Meyers et al., 1994, Mortensen et al., 1999, Takano et al., 2000). This infection is very important to many European countries because of the economical consequences for farmed salmonids. Many of the VHSV isolates from wild marine species have been shown by experimental bath challenge to infect turbot and cause high mortality (Castric & de Kinkelin, 1984, King et al., 2001). Natural infections with VHSV have also caused significant mortality in turbot in aquaculture (Schlotfeldt et al. 1991, Ross et al. 1994, Skall et al., 2005).

Anaemia, massive haemorrhaging and/or congestion and exophthalmia are among the signs of VHS. Necrotic changes are seen in the haematopoietic tissue. Gills, liver and kidney are also pale and contain necrotic areas (Wolf, 1988, Bruno & Poppe, 1996).

The diagnosis of VHS is based on the isolation of VHS virus in cell culture followed by identification by neutralisation, immunoperoxidase test (IP), immunofluorescence test (IFT), enzyme linked immunosorbent assay.

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(ELISA) or identification by reverse transcription polymerase chain reaction (RT-PCR) (Anonymus 2003, Anonymus 2001).

This report describes the first isolation of VHSV from cultured turbot in the Trabzon coastal area of the Black Sea in the North part of Turkey using standard screening methods.

Materials and methods

Fish samples
A total of 600 15-day old turbot fry (200 from each tank) and organ material from 15 broodstock fish sampled from three different tanks from the Central Fisheries Research Institute (CFRI) in the Trabzon coastal area of the Black Sea were transported on ice (4-8°C) to the Virology Laboratory of Bornova Veterinary Control and Research Institute.

All the samples were examined according to standard virological procedures (Anonymus 2001). Two hundred turbot fry from each tank were homogenized together to obtain three samples of turbot fry from three tanks for viral examination. Necropsy was performed in the laboratory using kidney, spleen, heart and brain tissues of five broodstock fish in each sample. Processed samples were incubated with rabbit antisera against both the Sp and Ab serotype of infectious pancreatic necrosis virus (IPNV) for 1 h at 15°C. Both anti-IPNV serum treated and non-treated aliquots of supernatants were used for viral examinations.

Virus isolation
Epithelioma papulosum cyprini (EPC) and rainbow trout gonad (RTG-2) cell lines were grown on 25cm² tissue culture flasks in cell culture medium (EMEM supplemented with 10% FCS, 1% antibiotic-antimycotic solution and 1% HEPES).

1/10 and 1/100 dilutions of the anti-IPNV serum treated and non-treated aliquots of supernatants of sample homogenates were inoculated into 25cm² tissue culture flasks with 24 h-old cultures of EPC and RTG-2 cell lines. Reference virus strains (VHSV-919, VHSV-961 were obtained from Dr. D. Fichtner and Dr. R. Riebe (FLI, Insel Riems, Germany) in 2004) were also inoculated at the same dilutions with samples following the same procedure. Inoculated cell culture flasks were incubated at 15°C for 1 h for adsorption following which cell culture medium was added to each cell culture flask. Cultures were examined for cytopathic effect (CPE) following a 10-day incubation at 15°C. Supernatants were then transferred to new cell cultures of the respective cell lines and incubated at 15°C for one week. Flasks were examined daily for CPE formation.

Virus identification
Immunoperoxidase test (IP), immunofluorescence test (IFT) and enzyme linked immunosorbent assay (ELISA) were carried out using commercially (BIO-X Diagnostics, Belgium and TEST-LINE Clinical Diagnostics, Czech Republic) available kits for the identification of virus. The supernatants of CPE positive samples and reference virus strain positive controls were inoculated onto 24-well cell culture plates with 24 hour-old cultures of EPC and RTG-2 cell lines and incubated at 15°C for IP and IFT. When the CPE was clearly visible (within 48-72 h) the media in the wells were discarded and monolayers were fixed using acetone solution.
Results

Macroscopic observations
Exophthalmia displayed in both eyes, darkening of the body, pale coloration of gills, haemorrhaging of the head and the fin bases, pale and off-colour areas in liver and haemorrhages in the intestinal tract were observed macroscopically during the necropsy of broodstock samples (Figure 1).

Virus isolation
After ten days of incubation, CPE was clearly visible in both EPC and RTG-2 cultures

Immunoperoxidase test (IP) and Immunofluorescence test (IFT)
The tests were carried out according to manufacturers’ instructions of the kit (BIO-PEROX VHS, BIO-X Diagnostics, Belgium and BIO-fluo VHS-BioK006, BIO-X Diagnostics Belgium).

Enzyme linked immunosorbent assay (ELISA)
The supernatants of CPE positive samples were tested by ELISA using the VHSV Ag ELISA kit of TEST-LINE Clinical Diagnostics Ltd., Czech Republic, according to their recommendations.
inoculated with samples of turbot fry and broodstock. The supernatants of cell cultures were passaged to new cultures of respective cell lines. In second and third passages CPE was clearly visible within seven days. CPE was observed both in cultures inoculated with anti-IPNV treated and non-treated supernatants of sample homogenate (Figure 2).

**Virus identification**

For virus identification in cell culture three different test methods were used. VHS virus was detected in samples from both turbot fry and broodstock and identified using IP. As a result of the IP test, red plaque formations were observed in the infected cell layer with field isolates and also with reference virus strains (positive controls, data not shown). There was no red plaque formation in the negative control or cell control wells.

Granular positive staining was observed around the peripheral part of the cytoplasm of infected cells by IFT. Staining was never detected in any negative controls (Figure 3).

In ELISA, cell culture isolates of both turbot fry and broodstock were tested against both a positive and negative control antigen. The absorbance values of tested samples were compared with the mean absorbance values of positive and negative antigen wells. As a result of repeated tests the mean absorbance values of tested samples were calculated to be at least three or four times greater than the mean absorbance of negative controls. According to the kit instructions, these results were interpreted to be VHSV positive.

**Discussion**

This study represents the first isolation of VHSV from cultured turbots in Turkey. The standard methods for virus isolation in cell culture and subsequent immunological identification using tests recommended by the Office International des Epizooties (OIE) and European Commission Directive (Anonymous 2003, Anonymous 2001) were used for the isolation and identification of VHSV from three pooled samples of turbot fry and three pools of turbot broodstock from different tanks. Virus isolates obtained from samples considered to be VHSV positive in both cell lines were identified by IPT, IFA and ELISA. The results of the tests confirmed the findings in all cases. Turbot fry and broodstock
samples were sent to Virology Laboratory of Bornova Veterinary Control and Research Institute (NRL) because of observed levels of high cumulative mortality at the hatchery of CFRI. As shown in Figure 1, most of the external and internal clinical signs of VHS disease were observed in the broodstock samples during necropsy. The VHS outbreak occurred in spring 2004 in Turkey when water temperatures were fluctuating and the mortality rates reached 99% in 15-20 day-old turbot fry.

The broodstock turbot were collected by CFRI from the Black Sea. There is a filtration system at the marine water inlet to prevent bacterial contamination originating from sea water but this system was not able to exclude viruses. This factor may be considered as a possible source of infection.

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References


