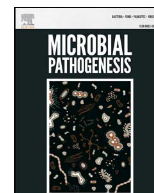
Microbial Pathogenesis 114 (2018) 68–74

Contents lists available at ScienceDirect

Microbial Pathogenesis

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



Development of indirect immunofluorescence assay for $TCID_{50}$ measurement of grass carp reovirus genotype II without cytopathic effect onto cells

Qing Wang[a,1], Hualiang Xie[a,b,1], Weiwei Zeng[a,*], Linchuan Wang[b], Chun Liu[a], Jiexing Wu[a], Yingying Wang[a], Yingying Li[a], Sven M. Bergmann[c,**]

[a] Key Laboratory of Fishery Drug Development of Ministry of Agriculture, Key Laboratory of Aquatic Animal Immune Technology of Guangdong Province, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, Guangdong, PR China

[b] College of Veterinary, South China Agricultural Universtiy, Guangzhou, PR China

[c] Institute of Infectology, Friedrich-Loffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald–Insel Riems, Germany

A R T I C L E I N F O

Keywords:
Immunofluorescence assay
$TCID_{50}$
qPCR
Grass carp reovirus
Genotype II
Cyenopharyngodon idellus

A B S T R A C T

Grass carp reovirus (GCRV) caused severe hemorrhagic disease with significant losses of fingerling and yearling grass carp, *Cyenopharyngodon idellus*, in southeast Asian. It was first isolated in 1983 in China, and clade analysis of the different GCRV isolates indicates there are at least three different genotypes I, II, and III. In recent years, GCRV genotype II has been determined as a dominant virus type which cause severe obvious clinical signs in fish but no cytopathic effect onto presently available cell culture. $TCID_{50}$ is one of standard method to quantity infectious virus particles. In the present study, an indirect immunofluorescence assay (IFA) was developed using antibody against a protein encoded by segment 10 of GCRV genotype II. Moreover, the specific assay to differentitate GCRV of different genotypes and a sensitive assay for determination of GCRV genotype II were developed respectively. The results showed the IFA only can recognize genotype II virus at the lowest initial concentration of 550 genomic copies/ml. Furthermore, comparison of results obtained from qPCR and the $TCID_{50}$ assay combined IFA was conducted. The results indicated that $TCID_{50}$ of GCRV isolates JX0901 and HZ08 differs with 2 log steps reduction in the numbers of viruses compared with the number of genome copies detected by qPCR. The immunofluorescence assay developed is sensitive, specific, and the $TCID_{50}$ combined with IFA will be a standardizable technique for the quantitation and detection of infectious GCRV in cell culture without cytolysis.

1. Introduction

Grass carp, *Ctenopharyngodon idellus*, is one of the main freshwater fish species farmed in China. The annual production of grass carp in China reached 5.90 million tons recorded in 2016, accounting for 20.95% of the harvest of all freshwater Chinese fisheries in 2016 [1]. It is also the most commonly cultivated fish in the world and is cultured extensively in more than 40 countries [2]. However, frequent outbreaks of hemorrhagic disease caused by grass carp reovirus (GCRV) has severely hampered the grass carp culture worldwide [3].

GCRV is a member of the Aquareovirus genus in the Reoviridae family, which was created by the International Committee on Taxonomy of Viruses in 1991 [4]. Based on RNA–RNA blot hybridization, serological tests, and/or sequence comparisons, those viruses are classified and grouped as Aquareovirus A to G (AQRV-A to AQRV-G) [5]. Due to GCRV (isolate GCRV 873) and Golden shiner reovirus sharing high-genome sequence identity, GCRV was moved from a tentative classification to AQRV-C [6].

The GCRV genome consists of 11 linear segments (S1–S11) of double-stranded RNA (dsRNA) with a total size of approximately 24 kb. Wang et al. first reported the existence of at least three GCRV genotypes in China [7]. Among different genotypes, the viral protein 6 (VP6) similarity was less than 20% among three groups with representative isolates: GCRV 873 (group I), HZ08 (group II), and 104 (group III) [7]. In the recent years, more than 40 GCRV isolates, including GCRV 873, GCRV 875, GCRV 991, GCRV 097, GCRV 109, GCRV JX-0901, GCRV

* Corresponding author. Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, Liwan District, 510380, China.
** Corresponding author. Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Institute of Infectology, Südufer 10, 17493 Greifswald-Insel Riems, Germany.
E-mail addresses: zww8810303@163.com (W. Zeng), sven.bergmann@fli.bund.de (S.M. Bergmann).
[1] Qing Wang and Hualiang Xie contributed equally to this work.

https://doi.org/10.1016/j.micpath.2017.11.042
Received 11 November 2017; Received in revised form 22 November 2017; Accepted 23 November 2017
Available online 24 November 2017
0882-4010/ © 2017 Published by Elsevier Ltd.

HZ08, GCRV GD108, GCRV JX01, GCRV JX02, GCRV HuNan1307, HeNan988, GCRV 918, have been identified and sequenced partially or completely [8–10]. More than 80% of isolates belong to genotype II, indicating that grass carp hemorrhagic disease predominantly caused by genotype II in China [11].

Interestingly, GCRV from different genotypes represent diverse biological properties. Aquareoviruses can be replicated in fish cell cultures and usually produce large syncytia as a characteristic CPE [12]. JX-0901 (genotype I) could induce large syncytia in different cell lines, however, HZ08 (genotype II) and HGDRV (genotype III) did not. After inoculation of isolate HZ08, there was no obvious CPE observed in Grass carp swimming bladder (GSB) cells [13]. So the reproductive ability of different viruses in vitro and in vivo mostly were determined by qRT-PCR, not onto cells by titration using the $TCID_{50}$ assay.

DNA-amplifing techniques, such as quantitative PCR (qPCR), are the most sensitive and rapid methods for the detection and quantification of pathogens in vitro and in vivo samples [14,15]. However, PCR based techniques target genome copies from both active replicated and inactivated pathogens. In some cases, it may be necessary to analyze the infectivity of the viral particles. Cell culture techniques for detecting and quantification of viruses include usually cytopathic-effect-related assays, such as $TCID_{50}$ assay [16]. However, some viruses, such as genotype II GCRV, cannot produce obviously a cytolytic CPE onto cell lines, which needs the help of other techniques to determine the concentration of infectious virus particle by the $TCID_{50}$ assay.

In the present study, IFA has been developed, and the capacity of the $TCID_{50}$ assay combined with IFA for virus quatification by titration of genotype II GCRV in cell culture was determined. A relation between isolate values quantified by the $TCID_{50}$ assay and by qPCR was obtained from the analysis of two GCRV isolates incubated for two weeks. The IFAs developed thereafter is sensitive, specific, and the $TCID_{50}$ assay combined with IFAs will be a standardizable technique for the quantitation and detection of infectious GCRV without cytosis in cell culture.

2. Materials and methods

2.1. Cell lines

The proboscis snout into fibers (PSF) cell line derived from grass carp, was used for GCRV replication [10]. PSF cells were replicated using M199 medium (Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS; HyClone™, GE Healthcare Life Sciences, Logan, UT, USA) at 28 °C incubation temperature. The concentration of FBS in the cell culture medium was reduced to 5% for virus propagation.

2.2. Virus proliferation

GCRV JX0901 (genotype I), GZ1208 (genotype I), HuNan1307 (genotype II), and HZ08 (genotype II) were isolated and stored in −80 °C before use in our laboratory, and 104 (also named HGDRV, genotype III) was generously provided by the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. For virus infection, 2×10^6 PSF cells were inoculated into a 25 cm^2 flask (Corning, USA) and incubated for 24 h at 28 °C to 85% confluence. After removing the medium, 1 mL of virus suspension with an initial concentration corresponding to 5.5×10^3 copies/ml (HZ08), 5.1×10^3 copies/ml (JX0901) and 4.7×10^3 copies/ml (104) were inoculated onto the cell culture respectively. After adsorbtion of virus suspension for 1 h, the cell culture flasks were then emptied and filled up with 5 mL of maintenance medium containing 5% FBS including control flasks without virus inoculation. The cells were incubated at 28 °C for up to 14 days post infection (dpi).

2.3. Antibodies

To confirm the optimal antibody as primary reagent for IFA, three virus specific polyclonal antisera were investigated in present study, including anti-GCRV II segment 6, segment 8 and segment 10, respectively. All the polyclonal antisera were prepared from rabbit and kept at Pearl River Fisheries Research Institute. IFAs were performed as described insection 2.4. After a final wash with 0.05% PBST three times, the cell nuclei were stained with 4′,6′-diamidino-2′-phenylindole (DAPI) (Beyotime Biotechnology, China) for 10 min at room temperature and were analyzed using confocal laser scanning microscopy (Carl Zeiss, Germany).

2.4. Indirect immunofluorescence assay (IFA)

An IFA based on that described previously by Wang et al. was modified for the present study [17]. PSF cells (2.5×10^5 cells mL^{-1}) were incubated in 24-well plates (Lab Tek NUNC) and infected with GCRV isolates JX0901 (genotype I), GZ1208 (genotype I), HuNan1307 (genotype II), HZ08 (genotype II), and 104 (genotype III), respectively. Infected cells were incubated with GCRV JX0901 (genotype I), GZ1208 (genotype I) and 104 (genotype III) for 4 days, or with HuNan1307 (genotype II) and HZ08 (genotype II) for 7 days at 28 °C. After this period, the maintenance medium was removed, and the virus-infected cells were fixed with ice-cold absolute methanol (−20 °C) for 10 min, followed by rinsing with PBS. The fixed cells were allowed to dry on air for at least 10 min. Additionally, 0.5% v/v triton X-100 (Sigma, Germany) diluted in PBS was applied to each well for 10 min. After washing three times with sterile PBS, cells were overlaid with PBS containing 5% w/v bovine serum albumin (BSA) and incubated for 30 min at 37 °C. The polyclonal antibody rabbit antiserum anti-GCRV S10 was used as the first antibody with a 1:100 dilution in PBS. After 1 h incubation at 37 °C, the cell monolayers were rehydrated by rinsing three times with PBS. Coverslips from each well were stained using 100 μL goat anti-rabbit immunoglobulin G (IgG) conjugated with fluorescein isothiocyanate (FITC; Sigma, Missouri, USA) and incubated for a further 1 h at 37 °C. FITC-stained cells were then rinsed with PBS, and immediately the examined under a NIKON fluorescence microscope (Tokyo, Japan). The IFAs were checked during 14 days in order to determine the optimal day of examiniation.

2.5. Tissue culture infectious dose ($TCID_{50}$/mL)

PSF cells were grown in 96-well plates (Lab Tek NUNC) until they reached 85% confluence. GCRV isolate HZ08 suspensions with an initial concentration of 5.5×10^6 copies/ml was diluted in serial 10-fold dilutions to 10^{-8}. Cell culture medium was removed and 50 μL of each virus dilution was added (10 wells per dilution) to the cell monolayer and then incubated at 28 °C for 1 h. After incubation, the inoculated viral suspensions were removed and 100 μL of maintenance medium was added to each well. Plates were checked daily once for 7 days. Given that the viruses of GCRV genotype II used not produce any cytolysis onto cell lines, the wells with and without CPE were investigated by IFA. The IFAs were carried out according to the method described above. The virus titres were expressed as the 50% tissue culture infective dose (TCID50) according to the method of Reed and Muench [18].

2.6. Quantitative PCR (qPCR)

Total RNA was extracted from infected and non-infected PSF cells using TRIzol Reagent (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) methods for each genotype were established by Wang et al., in 2016 [13]. The extracted RNA was then reverse transcribed using the First Strand cDNA Synthesis Kit (Takara, Japan). cDNA was used as a template for qPCR, which was performed using Premix Ex TaqTM (Perfect Real Time; Takara, Japan) to assay samples with specific primers (Table 1). The results were subsequently

Download English Version:

https://daneshyari.com/en/article/8749852

Download Persian Version:

https://daneshyari.com/article/8749852

Daneshyari.com