Journal of Virological Methods xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells☆

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Article history:
Received 8 July 2013
Received in revised form 7 October 2013
Accepted 11 October 2013
Available online xxx

Keywords:
Infectious pancreatic necrosis virus
AGK cells
Mx
Protein shutdown

A B S T R A C T

Inactivated whole virus vaccines represent the majority of commercial preparations used to prevent infectious pancreatic necrosis (IPN) in salmonids today. The production of these vaccines requires high virus concentrations that are resource-demanding. In this study, we describe the cultivation of high yields of IPN virus in Asian Grouper strain K (AGK) cells. The mechanism by which this is achieved was investigated by comparison with a commonly used salmonid cell line (RTG-2 and CHSE-214 cells). The cells were counted before and sequentially after infection. Thereafter, protein shut down, virus yields and apoptosis were assessed. The effects of poly(I:C) pre-treatment and Mx expression on IPNV concentrations were examined and the results show that high virus yields were associated with high cell numbers per unit volume, delayed cell death and apoptosis in AGK cells while the opposite was observed in RTG-2 cells. Poly(I:C) treatment and Mx expression resulted in a dose-dependent inhibition of virus multiplication. The production capacity of AGK and CHSE-214 cells were compared and higher split ratio and shorter split interval of AGK cells documents dramatic differences in virus antigen production capacity. Collectively, the results suggest that high cell numbers and prolonged survival of AGK cells are responsible for the superior virus yields over RTG-2 and higher split ratio/shorter split interval makes AGK superior over CHSE cells.

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1. Introduction

Infectious pancreatic necrosis (IPN) is one of the most important diseases of farmed salmonids the world over. Originally known to affect fry at the point of start-feeding and in smolts shortly after sea water transfer, the disease now afflicts fish at all stages of production (Roberts and Pearson, 2005). IPN is caused by an un-enveloped double stranded RNA virus, the IPN virus (IPNV) that is a prototype of the genus Aquabirnavirus in the family *Birnaviridae* (Cohen et al., 1973; Duncan and Dobos, 1986). The virus genome consists of two segments, A and B, with the former encoding structural proteins VP2 and VP3, and non-structural proteins VP4 and VP5 (Dobos, 1976; Havarstein et al., 1990). Segment B encodes VP1, the RNA dependent RNA polymerase (Duncan et al., 1991).

Disease prevention and control of IPN include vaccination of parr during the fresh water stage with the purpose to protect them against disease during the first 2 months post sea transfer. Several vaccines are available, most of which are based on inactivated whole virus but also on subunit preparations (Sommerset et al., 2005) although their performance is equivocal. At experimental level, inactivated whole virus vaccines have shown better protection than recombinant subunit vaccines, the former holding promise as effective vaccines for the future (Munang'andu et al., 2012, 2013a). The efficacy of the vaccines relies on inclusion of high antigen content.

Several cell lines have been tested for their suitability in the propagation of IPNV (Lannan et al., 1984) and are indeed in use in several laboratories for example rainbow trout gonad 2 (RTG-2) and Chinook salmon embryo 214 (CHSE-214) cells. The yields of virus that these cell lines give differ between cell lines and even between virus isolates. In general, the Sp serotype yields lower quantities (10^6–10^7 pfu/ml) in RTG-2 cells compared to CHSE-214 (10^8 pfu/ml) (Song et al., 2005) or even up to 10^{10} $TCID_{50}$/ml (Skjesol et al., 2009). The difference in yields between RTG-2 and CHSE-214 cells has been attributed to the former's ability to elicit an interferon (IFN) response believed to be absent in the latter (Macdonald and Kennedy, 1979; Jensen et al., 2002). Other factors including apoptosis and necrosis as well as the rates at which they occur may also contribute to the virus yield although this is not well documented. As stated above, vaccine production, challenge

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0166-0934/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2013.10.020

Please cite this article in press as: Chen, L., et al., Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells. J. Virol. Methods (2013), http://dx.doi.org/10.1016/j.jviromet.2013.10.020

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models, immunological and biological assays demand the cultivation of virus in large quantities. We report the use of Asian Grouper strain K (AGK) cells that support the cultivation of high titres of IPNV (10^9–10^{10} $TCID_{50}$/ml) (Munang'andu et al., 2012). These cells are derived from the skin of a crossbreed between Orange-spotted grouper (*Epinephelus coioides*) and Malabar grouper (*Epinephelus malabaricus*). The cell line is not a mono-clone but is composed of different cell types. AGK cells grow at a higher temperature, can be split at high ratios and have a much shorter turn-around time making them a very valuable resource for easy production of IPNV in large quantities within a short period of time.

The purpose of the present study was to investigate and document the factors that permit the production of high titres and high volumes of IPNV using AGK cells. A good understanding of these factors will contribute to more efficient production of antigens that are required in the development of protective vaccines against IPNV.

2. Materials and methods

2.1. Cells

Rainbow trout gonad 2 cells (RTG-2; ATCC CCL-55) were maintained at 20 °C with L-15 medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS), 10% L-glutamine and 1 μl/ml of gentamicin. Asian Grouper strain K (AGK) cells (Munang'andu et al., 2012) were maintained at 28 °C with L-15 media of the same composition but supplemented with 7.5% FBS while Chinook salmon embryo cells (CHSE-214; ATCC CRL-1681) were maintained at 20 °C also with the same medium but containing 10% FBS.

2.2. Experimental design

RTG-2 and AGK cells were used to grow IPNV as a basis for understanding the mechanisms underlying differences in yields. CHSE-214 cells were used to quantify virus yields from both cell lines.

2.3. Assessment of IPNV yields in AGK and RTG-2 cells

A recombinant IPNV strain (rNVI-15PT) with amino acids Proline (P), Threonine (T) and Alanine (A) in positions 217, 221 and 247 in the VP2 protein, respectively (Song et al., 2005), was used to infect confluent cells. The cells were infected in triplicates by first adsorbing the virus for 2 h on a rocking board, then washing with PBS before replacing fresh maintenance media and incubating them at 15 °C. Virus samples from both intact cells and culture supernatants were harvested in parallel, on daily basis until full CPE. The virus yield in these samples was assessed by titration in CHSE-214 cells.

To assess the effect of virus to cell infection ratio on virus yields, the cells were infected with either MOI = 0.1 or MOI = 10. Virus yields were assessed as described above. In addition, quantitative RT-PCR was also used to assess virus yields of AGK cells from day 1 to 5.

2.4. Metabolic labelling of newly synthesized proteins

AGK and RTG-2 cells infected with IPNV (MOI = 0.1 and 10) were incubated for the required duration (up to 5 days maximum). To harvest the cells, they were first washed with PBS and then incubated for 1 h in Dulbecco's modified Eagle's medium without Methionine and supplemented with 0.1% FBS and 20 Ci [^{35}S]Methionine/ml. After incubation, the cells were washed three times with PBS and then lysed by using CelLytic M reagent (Sigma). The protein was separated by SDS-PAGE and blotted onto a poly(vinylidene) fluoride (PVDF) membrane. The membrane was then kept in a Phosphor cassette over-night prior to scanning using a Typhoon imager (GE Healthcare).

2.5. Transfection of poly(I:C) in AGK and RTG-2 cells followed by IPNV infection

To examine antiviral effects, AGK and RTG-2 cells were grown in 6-well culture plates until near confluence. Thereafter they were transfected with 3 μg of poly(inosinic:polycytidylic) acid (poly(I:C)) and 9 μl of FuGENE® HD Transfection Reagent (Roche) per well according to the manufacturer's instructions. 24 h later, the cells were infected with IPNV at MOI of 0.1. At 48, 72 and 96 h following infection, the viral proteins were assessed by Western blot.

2.6. Assessment of Mx expression following IPNV infection in AGK cells

AGK cells were infected with IPNV (MOI of 0.1 and 10). Sampling was done daily for 6 days. Total RNA was isolated by using the RNeasy Plus mini kit (Qiagen), and the concentration of RNA was determined by spectrophotometry (Nanodrop ND1000). For each sample, 500 ng of total RNA was subjected to cDNA synthesis using Transcriptor first-strand cDNA kit (Roche) in a total volume of 20 μl as described above. Mx expression was determined by quantitative real time PCR (qPCR) and this was performed by using Light-Cycler 480 SYBR green I Master mix on a LightCycler 480 thermocycler (Roche). 2 μl of cDNA was used as a template in a final volume of 20 μl. The mixtures were first incubated at 95 °C for 10 min, followed by 40 amplification cycles (10 s at 95 °C, 20 s at 60 °C, and 8 s at 72 °C).

The sequences of primers (Mx-574 Fwd and Mx-730 Rev) used to assess the expression of Mx are given in Table 1. The specificity of the PCR products from each primer pair was confirmed by melting-curve analysis and subsequent agarose gel electrophoresis. The $2^{-\Delta\Delta CT}$ method was used to calculate the amount of gene products as described previously (Nolan et al., 2006; Schmittgen and Livak, 2008). $2^{-\Delta\Delta CT}$ is the relative mRNA expression representing the fold induction over the control group. All quantifications were normalized to Cathepsin D. This gene has been demonstrated not to be induced in IPNV-persistently infected CHSE-214 cells (Marjara et al., 2010). In the present study, Cathepsin D expression was tested in AGK cells and found not to be induced by infection with IPNV.

2.7. Over-expression of Mx in AGK cells

The full Mx gene sequence (GenBank Accession No. KF148054) was amplified by PCR using the primers pcDNA3.1c-Mx-F and pcDNA3.1c-Mx-R (Table 1) containing BamHI/EcoRI restriction sites. A truncated form of the gene was used as a negative control and was produced using primers pcDNA3.1c-MxN-F and pcDNA3.1c-MxN-R. PCR was done as described above. 3 μg of PCR products and pcDNA3.1c vector were digested by using restriction enzyme BamHI and EcoRI (Promega) for 3 h and were directionally ligated using a ratio of 1:3. The plasmids were sequenced to confirm orientation and position of the gene as well as that of the His-tag.

Transfection of AGK cells was done in 6-well plates using 3 μg of plasmids containing the Mx gene and 9 μl of FuGENE® HD Transfection Reagent (Roche) per well according to the manufacturer's instructions. After 48 h, the cells were infected with IPNV (MOI of 0.1). 24 h post infection, the Mx and viral protein expression were detected by IFAT.

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