Journal of Virological Methods 234 (2016) 156–159

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Rapid detection of infectious rotavirus group A using a molecular beacon assay

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Article history:
Received 5 February 2016
Received in revised form 18 April 2016
Accepted 27 April 2016
Available online 27 April 2016

Keywords:
Cell culture
Molecular beacon
Rotavirus

A B S T R A C T

Rapid, sensitive and specific methods are necessary to detect and quantify infectious viruses. Cultivating and detecting enteric viruses in cell culture are difficult, thus impairing the advancement of knowledge regarding virus-induced diarrhea. Rotavirus (RV) detection has been conducted by serological or molecular biology methods, which do not provide information regarding viral infectivity. Molecular beacons (MBs) have demonstrated efficacy for viral detection in cell culture. We propose a MB assay to detect human rotavirus group A (HuRVA) in cell culture. MA104 cells were mock-infected or infected with HuRVA strains (RotaTeq® vaccine and K8 strains), and a specific MB for the HuRVA VP6 gene was used for virus detection. Mock-infected cells showed basal fluorescence, while infected cells exhibited increased fluorescence emission. MB hybridization to the viral mRNA target of HuRVA was confirmed. Fluorescence increased according to the increase in the number of infectious viral particles per cell (MOI 0.5–MOI 1). This technique provides quick and efficient HuRVA detection in cell culture without a need for viral culture for several days or many times until cytopathic effects are visualized. This methodology could be applied in the selection of samples for developing RV vaccines.

1. Introduction

Rotaviruses (RVs) are members of the *Reoviridae* family, *Sedoreovirinae* subfamily and *Rotavirus* genus (ICTV, 2014). The non-enveloped virions are 100 nm in diameter, and a triple-layer capsid surrounds the genome, which is composed of 11 double-stranded RNA (dsRNA) segments. Each dsRNA encodes at least one structural protein (VP1-4 and VP6-7) or non-structural protein (NSP1-6), except for segment 11, which has overlapping open reading frames (ORFs) for NSP5 and NSP6 (Estes and Kapikian, 2007; Greenberg and Estes, 2009). According to the serological reactivity and genetic variability of VP6, at least eight different groups or species (RVA-RVH) have been identified (Matthijnssens et al., 2012).

RVs are the leading cause of life-threatening diarrheal diseases among young children under the age of five years, with approximately 800,000 deaths worldwide annually (Liu et al., 2012). RVs replicate in intestinal tract enterocytes, and viral shedding into the stool occurs with or without signs of gastroenteritis. Therefore, for children and asymptomatic adults, virus transmission is usually fecal-oral through direct contact with RV or with contaminated fomites, food, water, and environmental surfaces (Arnold et al., 2009).

In the RV replication cycle, upon virion entry in the cell, the outer capsid, which is formed by VP4 and VP7 proteins, is removed, and the virion-associated transcriptase is activated. The resulting mRNAs function as either messengers for translation into proteins or templates for replication of progeny genomes. The negative-strand is synthesized during RV infection by replicase (RNA-dependent RNA polymerase enzyme) (Estes and Kapikian, 2007).

The inability to easily cultivate and detect many enteric viruses in cell culture systems impedes the advancement of knowledge regarding virus-induced diarrhea. The isolation and cultivation of infectious human RVs (HuRVs) directly from fecal specimens is difficult and does not work for every sample. HuRVs vary in their capacity to grow in culture, with average titers of 1- to 3-logs less compared with animal strains of RV, which are easily cultivated in continuous cell lines and which produce high titers (10^7 to 10^8 plaque-forming units per milliliter [PFU/mL]) (Arnold et al., 2009). Sometimes, cultivating RVs from feces requires multiple passages in primary cells before growth in continuous cell lines because RVs are fastidious and require several weeks to produce observable cytopathogenic effects (CPEs) (Arnold et al., 2009; Otto et al., 2015).

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http://dx.doi.org/10.1016/j.jviromet.2016.04.017
0166-0934/

Electron microscopy has traditionally been used to detect RV from feces; however, this technique lacks sensitivity, is very labor-intensive and requires highly trained personnel (Sidoti et al., 2015). RV detection is also commonly conducted by serological methods (enzyme immunoassays) or molecular biology techniques (e.g., reverse transcription polymerase chain reaction [RT-PCR], quantitative reverse transcription PCR [RT-qPCR], nucleic acid sequence-based amplification [NASBA]) (Banga-Mingo et al., 2014; Barman et al., 2014; Mo et al., 2015). However, these techniques only allow antigen or genome detection without providing information regarding viral infectivity. Therefore, rapid, sensitive and specific detection methods are necessary to detect and quantify infectious RVs.

Molecular beacons (MBs) were first reported in 1996 (Tyagi and Kramer, 1996) and have been widely applied in various in vitro hybridization assays (Han et al., 2013; Monroy-Contreras and Vaca, 2011). MBs are single-stranded nucleic acid probes that possess a stem-loop structure and that are doubly labeled with a fluorophore and a quencher at the 5′ and 3′ ends, respectively. These probes are capable of generating a fluorescent signal in the presence of a target that forces a structural change, causing fluorescence via resonance energy transfer. In the absence of a target, the MB retains its stem-loop conformation, and the fluorescence is quenched by proximity with the quencher (Han et al., 2013; Tyagi and Kramer, 1996).

MBs are widely used in biology, chemistry, medical science and biotechnology for biomolecular recognition (Tan et al., 2005). Some basic applications of MBs include pathogen and single nucleotide polymorphism (SNP) detection. These probes are also applied in disease diagnosis by the rapid and sensitive direct detection of genomic sequences (DNA or RNA) in living cells, by in situ hybridization, or by detection of nucleic acids during target sequence amplification (Han et al., 2013). Some infectious viruses, such as hepatitis A virus (Yeh et al., 2008), murine norovirus-1 (Ganguli et al., 2011), coxsackievirus (Wang et al., 2005), poliovirus (Cui et al., 2005; Sivaraman et al., 2013), bovine respiratory syncytial virus (Santangelo et al., 2006) and influenza A virus (Wang et al., 2008), have been detected by probing the endogenous nucleic acids with MBs. However, no studies have reported the detection of HuRVs by MBs.

In this study, we applied MB methodology for the rapid detection of HuRVA (human rotavirus group A) adapted to cell cultivation.

2. Materials and methods

2.1. Cell culture and viruses

African green monkey kidney epithelial cells (MA104) were grown in minimal essential medium (MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Nutricell, Campinas, SP) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Sigma-Aldrich, St. Louis, MO) in a humidified 5% CO_2 at 37 °C.

HuRVA strains (RotaTeq® vaccine and K8 strains) were propagated in MA104 cells. Briefly, HuRVA strains were inoculated into confluent cells for 2 h at 37 °C using serum-free MEM supplemented with 5 μg/mL porcine trypsin (Sigma-Aldrich, St. Louis, MO) to activate the virus. After the adsorption, the inoculum was removed, and serum-free MEM with 2 μg/mL porcine trypsin was added to the cells. The cells were observed daily until an approximately 90% CPE was detected. The viral suspension were stored at −80 °C and titrated based on the Reed-Muench method to determine the 50% tissue culture infective dose (TCID50) (Reed and Muench, 1938). Then, the relation 1 TCID50/mL = 0.7 PFU/mL was used to determine the virus titer in PFU/mL (Carter and Saunders, 2007).

2.2. MB synthesis

The probe was designed based on alignment of the VP6 genomic sequences of various HuRVA strains available in the GenBank database. The DNA folding form program (http://mfold.rna.albany.edu/?q=mfold/dna-folding-form) was used to predict the thermodynamic properties and the secondary structures of the MB. The 22-base pair MB sequence, 5′-[FAM]CGCG***A****TTAGTTCAGTCCAATTCATGCC***T**CGCG[DABCYL]-3′, was designed to be complementary to a coding region from the inner capsid protein VP6 gene from HuRVA and was synthesized by Sigma-Aldrich (St. Louis, MO). In the MB sequence, the stem is underlined, and the hybridization domain (loop) is in italics. MBs were resuspended in Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) to a final solution of 100 μM and stored at −20 °C.

The thermal denaturation profile was determined using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) by monitoring the fluorescence of a solution containing 200 nM of MB and a solution with 200 nM of MB and 400 nM of a synthetic oligonucleotide target, 5′-GGCATGAATTGGACTGAACTAA-3′. Both solutions were dissolved in 10 mM Tris-HCl (pH 8.0) containing 3.5 mM $MgCl_2$. The emitted fluorescence of MB and of MB plus target was monitored as the temperature was decreased from 90 °C to 10 °C at a rate of 1 °C/min.

2.3. MB assay

MA104 cells (1×10^4 cells/well) were cultured to approximately 90% confluence in an eight-well Lab-Tek II CC2 chamber slide system (Nalge Nunc, Rochester, NY) at 37 °C in 5% CO_2. The cells were mock-infected or infected with a multiplicity of infection (MOI) of 0.5 (1.25×10^5 PFU/mL) of RotaTeq® vaccine strain with or MOIs of 0.5 and 1 (2.5×10^5 PFU/mL) of K8 strain in a serum-free MEM supplemented with 5 μg/mL porcine trypsin for 2 h at 37 °C. Then, the virus inoculum was removed, the cells were washed three times with TBSS buffer (Tris-buffered saline solution; 0.05 M Tris-HCl, 0.01 M Tris, 0.28 M NaCl, 0.98 mM Na_2HPO_4, 10 mM KCl, pH 7.4), and serum-free MEM with 2 μg/mL porcine trypsin was added to the cells. After the cells were incubated for 24 h, the medium was aspirated, and the cells were washed three times with TBSS. Cell fixation, permeabilization and incubation were performed as described previously by Wang et al. (2005). Briefly, the cells were fixed with 2% (wt/vol) paraformaldehyde in TBSS buffer for 30 min at room temperature, washed three times with TBSS buffer, and incubated with 0.1% Triton X-100 in TBSS buffer for 5 min at 4 °C for permeabilization. After the cells were washed three times with TBSS buffer, mock-infected or infected cells were incubated with 6.4 μM MB for 1 h at room temperature in the dark. Finally, the slides were washed three times with TBSS buffer and observed under a fluorescence microscope (LEICA DM 2500—Wetzlar, Germany).

3. Results and discussion

3.1. MB design

The MB was designed based on an alignment of the VP6 genomic sequences of various HuRVA strains available in the GenBank database. Because the stem sequence is typically 5–7 base pairs long in each end and the loop sequence generally has 15–30 bases (Han et al., 2013), the MB was designed with 5 and 22 bases in the stem and loop, respectively. The enhanced selectivity of the MB results from the hairpin stem-loop structure in the nonhybridized state with more stable stems that are ensured by the increase in G-C content or by the stem length. However, increasing the stem