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Molecular and biological characterization of the human-bovine rotavirus-based reassortant rotavirus

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ABSTRACT

Rotaviruses (RV) are the leading cause of acute infantile gastroenteritis, associated with elevated mortality in low-income countries. Morbidity and mortality, length and rates of hospitalization due to RV gastroenteritis are dropping. Improving the quality of newborns life is an ongoing challenge for health-care providers. In this study, homemade reassortant human-bovine rotavirus was developed and biological activity and molecular characterization of candidate vaccine were evaluated for the vaccine stability. Virus titration and purification of reassortant rotavirus strains were evaluated by plaque assays, electropherotyping. The genetic stability after first, third and sixth passage was by sequencing.

Due to WHO recommendation, developingment of national capacity for vaccine production in appropriate quantities and at affordable prices is the cornerstone of developing global vaccination policies. Such studies are critical to producing national vaccines and modeling herd protection.

1. Introduction

Rotavirus (RV) is a non-enveloped double-stranded RNA virus, belongs to the Reoviridae family. Rotavirus is an important cause of acute, often severe viral gastroenteritis in infants and young children worldwide, especially in low income countries. The rotavirus genome consists of 11 segment of dsRNA [14], with three concentric layers of proteins that surrounds the viral genome in the infectious mature viral particle. The outer protein layer is composed of the VP4 and VP7 proteins, which independently elicits neutralizing antibodies and induces protective immunity [3].

The identification of protective vaccine is vital for the vaccine development. This demands for systematic research to find appropriate vaccine candidates for testing. The high morbidity and mortality of RV infections have persuaded the development of RV vaccines for reducing, length and rates of hospitalization. In developing countries > 85% of circulating rotavirus strains are G1 to G4, and G9 together with P1A[8] or P1B [4] serotypes [18].

The RV dsRNAs can be separated by polyacrylamide gel electrophoresis (PAGE). This showed a typical dsRNA characteristic profiles, demonstrating four definite size classes of segments, according to their molecular weight [5,17]. Differences in the mobility of individual RNA segments in PAGE allow a genetic characterization of RV strains. Due to the segmented nature of the rotavirus genome, gene re-assortments between bovine and human-infecting viruses have been generated during co-culture with high MOI or in immune suppressed individuals. Reassortment is a developmental approach for all segmented viral genomes such as RVs. In reassortment strategies, segment(s) of bovine and human-infecting viruses have been replaced by each other's which cause disappearing of normal segment pattern on PAGE. In bovine-human reassortants, two distinct rotavirus strains exchange gene segments and create diverse progeny. Due to the segmented composition of the rotavirus genome, co-infection between two rotavirus strains has been exploited to naturally derive reassortant vaccine strains with desired specific combinations of genome segments derived from each of the parental rotavirus strains (M [4]. Regarding the high frequency of rotavirus infection in Iran, continuous surveillance is needed to provide information about the occurrence of new rotavirus strains. This surveillance program would be considered in production of efficient vaccines. This assists the policy makers to decide on rotavirus vaccine development [2].

In this study, we try to create reassortant virus between RF (G6P[1]) and RV4 (G1P[8]) rotaviruses through co-culturing of two related strains. In reassorted viruses human rotavirus RV4 as a parental virus

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was nominated and only VP7 gene of interest was replaced in the backbone of RF rotaviruses. However, in addition to development of reassorted virus, the molecular characteristics of the reassortant strain was shown by genomic stability. The aim of this study was full characterization of the molecular and biological properties of the resultant reassortant virus, assigning and stability of parental origin for VP7 gene segment 9, sequencing of the target gene and comparing with human and bovine counterpart RV strains. Biological activity and molecular characterization were evaluated in cell culture by endpoint dilution assay, and PCR-based methods respectively. The potency, antigenicity, and physical/biochemical heterogeneity would be introduced in the near future in vivo.

2. Material and methods

2.1. Cells and viruses

African green monkey kidney MA104 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Bovine Rotavirus RF rearranged (re) was prepared in our lab. Bovine rotavirus RF (G6P[1])with normal pattern was kindly supplied by Dr. Pothier, France. Human rotavirus RV4 (G1P[8]) was purchased from European Collection of Cell Cultures (ECACC). All viruses were propagated in MA104 cells at a multiplicity of infection (M.O.I.) of 0.1. Plaque assay for virus titration and purification was performed for virus selection and isolation.

2.2. Assay of viral infectivity

MA104 cells were infected as previously described [19]. The viral infectivity of rotaviruses was quantitated by infecting cells with serial 10-fold dilutions of viruses, and the infectious titer was determined. Two days after infection, cells were monitored for cytopathic effects, and CCID50 values were calculated according to the Karber method [10].

2.3. Plaque assay

The plaque assay as a biological assay was used for the quantification of RV. This assay is based on the CPE, which was caused by active and viral replicability of RV in cultured cells and introduced to plaque-forming units per milliliter of virus (PFU/mL). Neutral red was used as a vital stain for visualization of the plaques. In this case the living cells uptake the neutral red, whereas the lysed infected cells are transparent and form the plaque. After inoculation the agar containing 1:1 mixture of 1.6% (w/v) agar and serum-free 2 × DMEM was overlaid. Once the agar was solidified, the plates were transferred to a 37°C-incubator until plaques are visible. Mixed infection for generation of reassortant rotavirus strains after preparing the suitable condition and optimization of plaque and electropherotyping assays, two target viruses were twice plaque purified. Six-well plate culture of MA104 cells were co-infected with parental strains RF and RV4 viruses at an MOI of 1. The virus seed during co-infection was isolated and amplified from confidential plaques and serial passages were down for generation of viral stocks in MA104 cells. In some circumstances mixed virus populations were treated with specific antisera for removing unwanted parental viruses. The lysate was serially diluted and plated on MA104 cells in a six-well plate for plaque formation and one more purifying step [7,8]. Each plaque was propagated in one well of six-well plate until exhibition of cytopathic effect.

2.4. Characterization of generated reassortant rotaviruses during co-infection

Rotaviruses replicate and assemble within cytoplasmic inclusions in the infected cells, parallel to the native parental rotaviruses.

Table 1
Primer Names and Sequences information, and nucleotide position of primer's in the RF strains.

Primer Name	Primer Sequence 5′ 3′	Position (rotavirus RF strain)
NSP1F	GCTTTTTTTATGAAAAGTCTTGTGG	2-56 nt
NSP1R	GTTCACATTTTATGCTGCCTAG	1545-1566 nt
NSP2F	CGTCTCAGTCGCCGTTCG	12-29 nt
NSP2R	GGTCACATAAGCGCTTTCAATT	1038-1059 nt
NSP3F	ATTTAATGCTTTTCAGTGGTTGATGC	4-29 nt
NSP3R	GGTCACATAACGCCCCTATAGCC	1056-1078 nt
VP7Fcon	GAGAATTTCCGTCTGGCTARC	13-33 nt
VP7Rcon	TGCCACCATTTTTTCCARTTY	909-929 nt

R; A or G.
Y; C or T.

The infected cells subjected for RNA extraction following freezing and thawing three times.

2.5. RNA extraction

Viral genomic RNA of reassortant virus was extracted from plaque purified infected cell supernatant using RNAX-Plus solution (Cinnagen, Iran) according to the manufacturer's instructions. The purified RNA was then re-suspended in electrophoresis sample buffer prior to analysis on the gels.

2.6. RT-PCR analysis

Full segments amplification of important rotavirus genome segments were performed by RT-PCR for detection of normal or reassort segments. The list of primer sequences used for RT-PCR analysis in this study was shown in Table 1. For confirmation of probable rotaviruses with abnormal pattern, the universal primers to VP7 was applied and followed by sequencing.

2.7. Electropherotyping of viral genomes

The RNA electropherotypes of parental and the reassortant rotavirus strains were analyzed by PAGE and silver staining based on Laemmli discontinuous buffer system [12]. Cell lysates were mixed with reducing buffer (2% SDS, 20% glycerol, 5 M urea, 4% 2-mercaptoethanol, and 0.1% bromophenol blue), heated at 68 °C for 10 min, and subjected to electrophoresis. Polyacrylamide gels (12%) were used for viral RNA analysis using 20 mA for 14 h. The resulting migration patterns of the genome segments were visualized by staining the gel with silver nitrate (BioRad, Horizontal Electrophoresis Systems).

2.8. The stability assay

In order to characterize reassortant rotavirus strain stability, six times serial passage was done in cell culture and followed by RNA extraction, cDNA synthesis, and partial sequencing. Genetic stability of reassortant virus after first, third and sixth passage was confirmed by sequencing.

3. Results

3.1. Mixed infection

MA104 cells were coinfected at an optimized condition between the RF X RV4 viruses at an MOI of 1. About one hundred plaques separately were picked up after 18–24 h and analyzed in order to identify the reassortant viruses. Two reassortant viruses have been observed. The genetic structure of the viruses has been extensively studied.