Journal of Virological Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Degenerate PCR primer design for the specific identification of rhinovirus C

Young Ran Nam[a], Uk Lee[a], Han Seok Choi[a], Kyong Jin Lee[a], Nari Kim[a], Yong Ju Jang[b], Chul Hyun Joo[a,c,*]

[a] Department of Microbiology, University of Ulsan College of Medicine, Seoul, Republic of Korea
[b] Department of Otolaryngology, Asan Medical Center, Seoul, Republic of Korea
[c] Cell Dysfunction Research Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

Article history:
Received 15 May 2014
Received in revised form 15 October 2014
Accepted 21 October 2014
Available online xxx

Keywords:
Rhinovirus C
Degenerate PCR primer
RT-PCR

A B S T R A C T

Human rhinovirus (HRV)-A and -B is a common cause of upper respiratory tract infections. Recently, a third species, HRV-C, was categorized based on molecular typing studies. The results showed that the HRV-C genome had diverged from that of HRV-A and -B. Despite its late identification, increasing evidence suggests that HRV-C causes more severe pathogenic infections than HRV-A or -B; however, a large amount of epidemiological data is required to confirm this association in different clinical settings. Consequently, a simple and rapid method for identifying HRV-C is required to expedite such epidemiological studies. Here, two degenerate primer sets (HEV and HRVC) were designed based on bioinformatic analyses. The HEV set targeting the fifth IRES domain sequence within the 5′-UTR, which is highly conserved among enteroviruses, was designed to detect all enteroviruses, whereas the HRVC set, which targeted the VP2 coding region, was designed to detect HRV-C alone. Both primer sets were tested against a panel of standard enteroviruses and clinical lavage samples. HEV detected all enteroviruses tested whereas HRVC was specific for HRV-C. Although the primer design strategy was confirmed with a limited number of samples, extensive tests are required to be applied in clinical settings.

1. Introduction

1.1. Rhinovirus

Human rhinovirus (HRV) is the main cause of the common cold. Although the infection is generally self-limiting, it can lead to life-threatening conditions, including pneumonia (Jartti et al., 2004) and bronchiolitis (Lemanske et al., 2005). The virus can also aggravate asthma in both adults (Ferreira et al., 2002; Zambrano et al., 2003; Miller et al., 2007) and children (Hayden, 2004; Takeyama et al., 2012). Rhinoviruses belong to the genus *Enterovirus* within the family, *Picornaviridae*, and are classified into two types based on the serotype: human rhinovirus A (HRV-A) or human rhinovirus B (HRV-B). Approximately 150 different serotypes have been identified to date.

1.2. Rhinovirus C

The high sensitivity of reverse transcription polymerase chain reaction (RT-PCR) has led to a marked increase in the number of viruses identified as the cause of respiratory infections (Erdman et al., 2003; Jennings et al., 2004; Weinberg et al., 2004). Along with advances in automatic nucleotide sequencing technology, RT-PCR speeds up the identification of unculturable viruses. Consequently, molecular typing has become an essential tool for viral taxonomical studies. Since the introduction of molecular typing, increasing evidence suggests that some unculturable rhinoviruses have genomic sequences that are divergent from those of HRV-A or HRV-B. Recently, HRV-C was identified based on molecular typing studies (Ledford et al., 2004; Lau et al., 2007; Lee et al., 2007; McIntyre et al., 2013a). The International Committee on Taxonomy of

Abbreviations: HRV, human rhinovirus; HRV-A, human rhinovirus A; HRV-B, human rhinovirus B; HRV-C, human rhinovirus C; RT-PCR, reverse transcription polymerase chain reaction; HEV, human enteroviruses; ORF, open reading frame; UTR, untranslated regions; QRT-PCR, quantitative real-time PCR; MSA, multiple sequence alignments; N-term, amino terminal; aa, amino acid.

* Corresponding author at: Department of Microbiology, University of Ulsan College of Medicine, Asanbyeongwon-gil 86, Songpa-gu, Seoul 138-736, Republic of Korea. Tel.: +82 2 3010 4269.
E-mail address: chjoo@amc.seoul.kr (C.H. Joo).

http://dx.doi.org/10.1016/j.jviromet.2014.10.021
0166-0934/

Viruses had since revised the classification of HRV based on molecular typing results (Adams et al., 2013). Currently, 51 subtypes of HRV-C have been classified based on their genomic sequences.

1.3. Mutations in the rhinovirus genome

HRV are non-enveloped positive single-stranded RNA viruses that are classified as human enteroviruses (HEV). The HEV genome contains a single open reading frame (ORF) that encodes a polyprotein. Host and viral proteases cleave the polyprotein into four capsid structural proteins (VP1, VP2, VP3, and VP4) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). The ORF is sandwiched between the 5′ and 3′ untranslated regions (UTR), which are critical for translation and replication of the viral genome. HEV evolution is driven by the selection of quasispecies under certain environmental pressures. Quasispecies represent a pool of genetically diverse progeny that originate from mutations driven by two factors: rapid virus replication and an error-prone viral RNA polymerase. Although the error-prone viral RNA polymerase generates multiple random mutations, not all viral sub-genomic regions are affected equally. The ORF can tolerate silent or non-critical amino acid mutations; however, the UTRs are greatly affected by mutations because a single nucleotide change can destroy the tertiary structure and function of the RNA. As a consequence, capsid structural proteins are highly variable, the non-structural viral proteins are less variable, and the UTRs are the least variable.

1.4. Selection of appropriate PCR target regions

Molecular typing is currently the only method of identifying HRV-C, and successful PCR amplification is a prerequisite for molecular typing. The selection of an appropriate PCR target for the typing procedure is complicated because although successful amplification requires that the primer must target sequences that are highly conserved among subtypes, molecular typing requires sufficient variation within the amplicon to enable discrimination between different subtypes. Previous studies used different sub-genomic regions as PCR targets for molecular typing; for example the 5′-UTR (Mori and Clewley, 1994), VP4/VP2 (Savolainen et al., 2002), VP1 (Ledford et al., 2004), 3D polymerase (Savolainen et al., 2004), and a partial 2A sequence (Laine et al., 2005).

1.5. The need for a simple method of identifying HRV-C

Despite delayed identification due to culture difficulties, studies suggest that HRV-C may cause more severe respiratory diseases than HRV-A or -B (McErlean et al., 2007; Miller et al., 2009; Bizzintino et al., 2011; Denlinger et al., 2011). HRV-C also shows seasonal patterns that are different from those of HRV-A or HRV-B, suggesting a different pathophysiology (Savolainen-Kopra et al., 2009; Kaida et al., 2011; Miller et al., 2011). However, this association is inconclusive because there is insufficient epidemiological data. Also, a previous report suggests that the identification of HRV-C in patients affected by lower respiratory illnesses during the winter months would require a change of treatment modality (Linder et al., 2013). Therefore, a simple, efficient, and rapid method of identifying HRV-C is required to identify HRV-C in various clinical settings; such a method would complement the rather laborious and time consuming method of molecular typing. Therefore, the aim of this study was to design degenerate primer sets that enable the specific identification of HRV-C by RT-PCR.

2. Materials and methods

2.1. Cells and virus

Human coxsackievirus A9 (ATCC# VR-1014), B1 (ATCC# VR-1032), B3 (ATCC# VR-30), B5 (ATCC# VR-185), B6 (ATCC# VR-155), human echovirus 6 (E6, ATCC# VR-1044), E7 (ATCC# VR-1047), E9 (ATCC# VR-1050), E11 (ATCC# VR-1052), E25 (ATCC# VR-1066), E30 (ATCC# VR-1072), human enterovirus 71 (EV71, ATCC# VR-1432), human rhinovirus 21 (HRV-A21, ATCC# VR-1131), 16 (HRV-A16, ATCC# VR-283), 1B (HRV-1B, ATCC# VR-1645), and 14 (HRV-B14, ATCC# VR-284) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Vero cells (ATCC, Manassas, VA) were used for virus culture. Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (50 mg/ml). The cells were cultured at 37 °C in a 5% CO_2 incubator. All viruses were propagated and stored as described previously (Ahn et al., 2003).

2.2. Clinical nasal lavage samples

Nasal lavage samples were collected from patients admitted to Asan Medical Center (Seoul, Korea) for endoscopic surgery from summer to winter, 2011. In the operating room, lavage samples were collected immediately after the induction of general anesthesia. Briefly, a balloon catheter was placed posterior to the nasal cavity and choana, and the nostrils were aerosolized with sterile saline solution (0.9% NaCl) at room temperature using a needle-free syringe. This procedure was repeated until 10 ml of lavage fluid was recovered. The samples were frozen immediately and stored at −70 °C until use. The study plan was approved by the Institutional Review Board of the Asan Medical Center. All participants provided informed consent.

2.3. Standard cloning procedure and generation of standard curves

A standard plasmid containing the 5′-UTR and VP4/VP2 sequences of HRV-C51 was cloned. RT-PCR was then performed using the following primer pair: sense, 5′-CCC GCT AGC ACT ACT TTG GGT GTC CGT GT-3′; antisense, 5′-CCC AAG CTT GGT AAT TTC CAC CAC CAN CC-3′. The amplified product was digested with *Nhe*I and *Hind*III and inserted into the pCMBB vector as described previously (Joo et al., 2010). The Qiagen Miniperp kit (Qiagen, Hilden, Germany) was used to extract plasmid DNA from the overnight culture in LB broth (containing 100 μg/ml of ampicillin). The success of the cloning was checked by sequencing the plasmids. The plasmid DNA concentration was measured using a spectrophotometer (NanoDrop 1000; NanoDrop Technologies, Wilmington, DE). The standard plasmid stock was diluted to 5×10^5 plasmids/μl and 10-fold serial dilutions were performed down to 5×10^0 plasmids/μl. Two microliters of each dilution were then used for in real-time RT-PCR (10–10^6 plasmids/reaction). The amplification efficiency was calculated from the slope of the best-fit linear regression of the standard curve.

2.4. Extraction of viral RNA and reverse transcription

Viral RNA was extracted from 140 μl of virus stock or nasal lavage sample using the QIAamp Viral RNA Mini Kit (Qiagen). Reverse transcription was then performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). The final reaction mixture (20 μl) contained 5 μM random hexamers, 1 mM of each dNTP, 20 units of RiboLock RNase Inhibitor,

Please cite this article in press as: Nam, Y.R., et al., Degenerate PCR primer design for the specific identification of rhinovirus C. J. Virol. Methods (2014), http://dx.doi.org/10.1016/j.jviromet.2014.10.021