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Journal of Virological Methods 214 (2015) 15–24

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

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Article history:
Received 15 May 2014
Received in revised form 15 October 2014
Accepted 21 October 2014
Available online 4 December 2014

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.

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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,

5× reaction buffer, and 200 units of RevertAid M-MLV reverse transcriptase.

2.5. Multiplex RT-PCR for the identification of common respiratory viruses

The products of the reverse transcription reactions were subjected to multiplex PCR using the Seeplex RV15 ACE Detection kit (Seeplex RV15, Seegene Inc., Seoul, Korea). The primer mixes contained an internal control template and a primer pair used to validate the PCR. Three reactions (A set: human adenovirus, human coronavirus 229E/NL63, human parainfluenza virus 2, human parainfluenza virus 3, human parainfluenza virus 1; B set: human coronavirus OC43, human rhinovirus, human respiratory syncytial virus A, influenza A virus, human respiratory syncytial virus B; and C set: human bocavirus 1/2/3/4, influenza B virus, human metapneumovirus, human parainfluenza virus 4, human enterovirus) were set up for each sample. The PCR conditions were as follows: initial denaturation at 94 °C for 15 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 5 min. The multiplex PCR products were confirmed by visualization after electrophoresis on 2% agarose gels.

2.6. Molecular typing of HRV in clinical samples

Molecular typing of HRV-positive samples was performed by VP4/VP2 typing after nested PCR amplification (McIntyre et al., 2013b; Wisdom et al., 2009). The first-round PCR was performed using outer primer pair, 445 s (sense: 5′-CCG GCC CCT GAA TGY GGC TAA-3′) and 1104as (antisense: 5′-ACA TRT TYT SNC CAA ANA YDC CCA T-3′). One microliter of the primary product was then used for the nested PCR reaction. Nested PCR was performed using the inner primer pair, 533 s (sense: 5′-ACC RAC TAC TTT GGG TGT CCG TG-3′) and 1066as (antisense: 5′-TCW GGH ARY TTC CAM CAC CAN CC-3′). The conditions for both PCR reactions were as follows: 30 cycles at 94 °C (30 s), 50 °C (30 s), and 72 °C (90 s), followed by a final extension at 72 °C for 5 min. The final product was visualized on agarose gels and sequenced in both directions using primers 533 s and 1066as (Macrogen, Seoul, Korea). Molecular typing was undertaken by performing a nucleotide BLAST search on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was performed in a real-time thermal cycler (CFX96 Real-time system, Bio-Rad, CA, USA). The reaction mixture was made by adding a primer set to the real-time PCR premix kit (IQ SYBR® Green Supermix, Bio-Rad, CA, USA). The conditions for real-time PCR were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and 80 °C for 2 s. The fluorescence signal was read at 80 °C for each cycle. The reaction was followed by post-melting temperature analysis (from 65 to 95 °C in 0.5 °C increments).

2.8. Design of degenerate primers

2.8.1. Analysis of the VP4/VP2 amino acid sequence

The VP4/VP2 region is the most common target for HRV molecular typing. Therefore, the amino acid sequences in this region were first examined to identify a suitable PCR target. To amplify HRV-C specifically, the primers must target conserved species-specific sequences. The conservation of HRV-C-specific sequences must satisfy certain conditions. They must be conserved within HRV-C but show different levels of conservation in HRV-A or -B (sequences showing either no conservation or complete conservation among all three species will be non-specific). In addition, targeting conserved sequences in HRV-C alone will not guarantee specificity because other species may harbor the same sequences by chance. All rhinovirus subtype sequences were downloaded from the NCBI Genbank database (Supplementary Table). Next, multiple sequence alignments (MSA) were performed for each species using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to identify conserved amino acids within the VP4/VP2 region. The amino acid sequences of HRV-A, -B, -and -C were aligned and sequence logos generated using Weblogo 3 (http://weblogo.threeplusone.com). The amino terminal (N-term) region of VP2 was selected for a HRV-C-specific identification (Fig. 1a) based on the following observations: amino acid (aa) nine was Y, Y, and F/Y; aa 13 was I, V, and L; aa 14 was I, Q/E, and K; aa 13 was I, V, and L; aa 18 was R, L, and I; aa 25 was S, T, and T; aa 27 was D, E, and D; aa 31 was A, A, and T; aa 32 was V, Y, and Y; aa 34 was G/A, A/C, and A; aa 36 was G, A, and G; and aa 37 was V, E, and E in HRV-A, HRV-B, and HRV-C, respectively. The position of the selected region within the HRV capsid complex was then checked. The capsid structure of HRV14 (PID: 4HRV) was displayed in PyMol (ver. 1.3; http://www.pymol.org/). The target amino acids formed a secondary structure comprising extended beta-sheet and loop regions, and were located on the inner side of VP2 in close contact with VP3 (Fig. 1b).

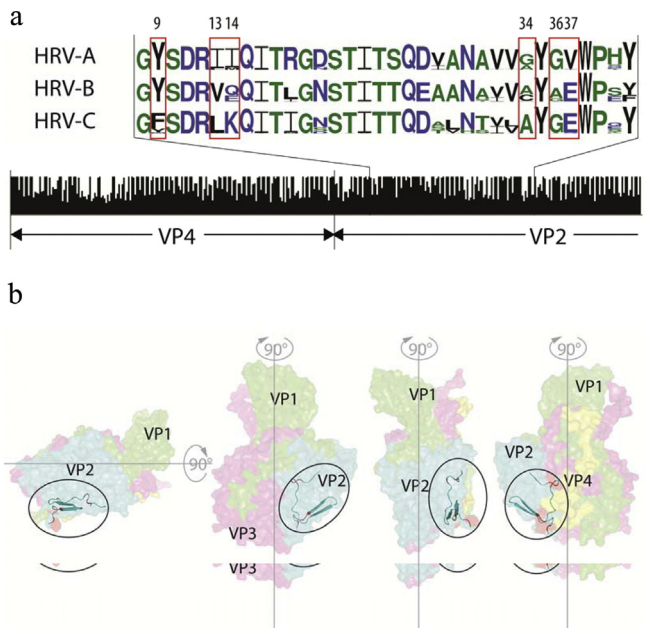

Fig. 1. Analysis of VP4/VP2 amino acids sequences. (a) Conserved amino acids (aa) sequences within the VP4/VP2 proteins of three HRV species. Top: the sequence logos of the aligned aa sequences within species A, B, and C. Numbers indicate aa positions from the VP2 N-terminus. The boxed aa represent the putative target sequence for HRV-C-specific primer design. Bottom: frequency of conservation among the aligned aa sequences for all HRVs. (b) Circles show the locations of putative target regions used for HRV-C-specific primer design. Crystal structure of the HRV-B14 capsid protein is shown.

2.8.2. Confirmation of target specificity for HRV-C-specific PCR

The VP2 region (aa 9–38) was selected as the target for the HRV-C-specific PCR primers. Phylogenetic analysis of all HRV subtypes was performed to check the species specificity of the target region. Phylogenetic trees were constructed from the tree file using forester (https://code.google.com/p/forester/wiki/forester). HRV-A, -B, and -C were grouped into complete clades (Fig. 2a). Therefore, amino acids within the target regions showed sufficient variation to enable discrimination of the HRV subtypes. Next, the genomic sequences encoding the target region were examined. The common clade containing HRV-B and HRV-C branched from HRV-A.

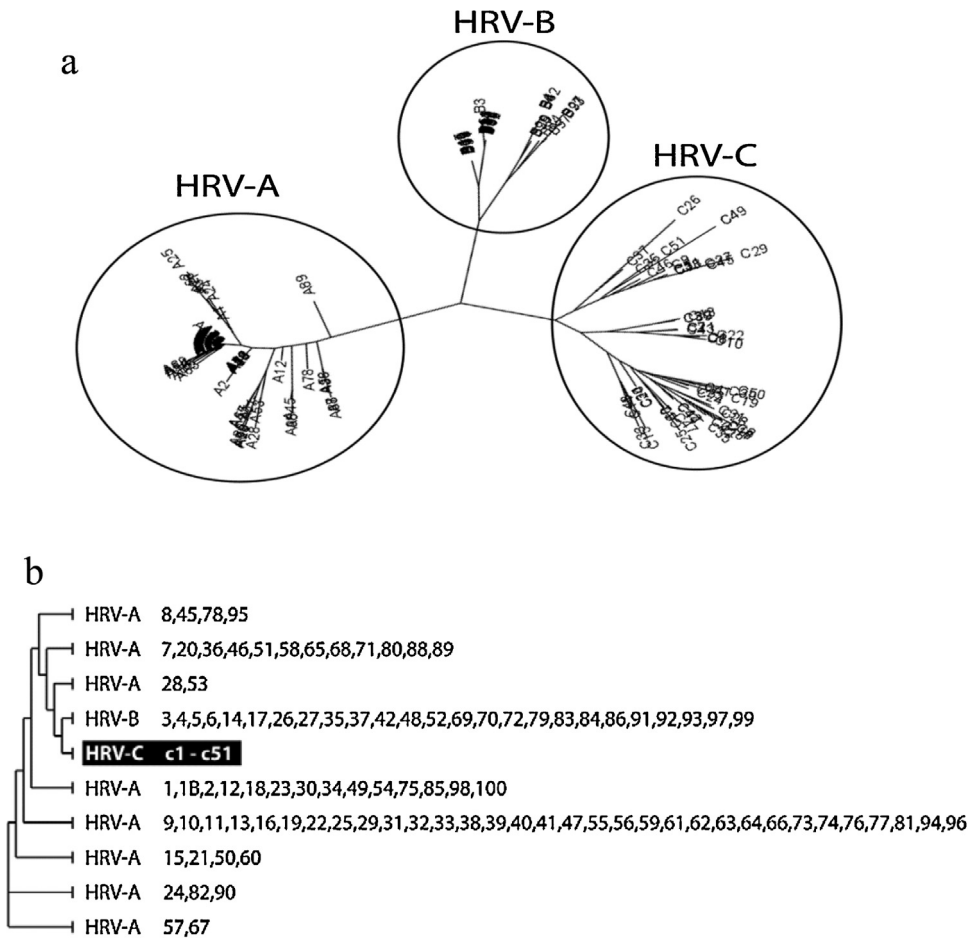

Fig. 2. Confirmation of HRV-C target specificity. Phylogenetic analysis was performed using amino acid (a) and nucleotide (b) sequences. Unrooted phylogenetic tree shows the targeted amino acid sequences among all HRV species. Square cladogram shows the HRV nucleotide sequences encoding the target region.

The HRV-C clade branched from HRV-B (Fig. 2b). These results confirmed that the genomic sequence encoding the target region within VP2 was an ideal target for HRV-C-specific amplification.

2.8.3. Design of the degenerate HEV and HRVC primer sets

Two primer sets were designed: HEV was designed to target sequences common among all human enteroviruses, whereas HRVC was designed to target a specific sequence within HRV-C. HEV targeted the fifth IRES domain within the 5′-UTR, whereas HRVC targeted aa 8–37 within the VP2 region (Fig. 3a). MSAs were performed to identify conserved nucleotides encoding the target region. The corresponding coding sequences within HRV-A, -B, -and -C were then aligned and conservation logos generated to design the HRVC primers. The MSA results were compared and the following nucleotides selected as specific targets for the HRVC sense primer: the first and second codons for the 10th Ser, the third codon for the 12th Arg, the first codon for the 13th Leu, and the first and second codons for the 14th Lys. The following nucleotides were selected for the antisense primer: the second codon for the 34th Ala, the second codon for the 37th Glu, and the second codon for the 41st Tyr. Importantly, the "AA" at the 3′ end of the sense primer and the "GC" at the 3′ end of the antisense primer are critical for HRV-C-specific amplification (Fig. 3b). The HEV set was designed to supplement the HRVC set and targeted nucleotides 469–585 within the 5′-UTR. This primer set detects all human enteroviruses because it targets mutation-intolerant positions located within the fifth domain of the IRES. The sites targeted by this primer set are conserved throughout all HEVs, except for a single degenerate code (Fig. 3c).

3. Results

3.1. Characteristics of the HEV and HRVC primers

The HEV and HRVC primer sets were tested using a series of 10-fold dilutions (10^8–10^2 copies/μl) of the HRV-C51 standard plasmid. The efficiency, *R* square value, and melting temperature peak of the HEV primer set were 129.8%, 0.997, and 85 °C, respectively. The values for HRVC were 114.8%, 0.989, and 80.4 °C, respectively (Fig. 4a). Both primer sets amplified their target sequences over a wide range of plasmid concentrations. However, the Ct values for HRVC were about 10 cycles higher than those for HEV at the same concentrations, reflecting the higher degeneracy of the HRVC set. The primer concentration in a conventional PCR reaction has little effect on the reaction; however, the primer concentration is critical when using a highly degenerate primer. Therefore, the HRV-C51 standard was amplified at primer concentrations ranging from 20 to 500 pmoles. The Ct values for the HEV set were similar at different concentrations. However, the Ct values for the HRVC set decreased as the primer concentration increased (Fig. 4b). An approximately 25-fold higher concentration of the HRVC primers was required to obtain an amplification level similar to that obtained with the HEV primers.

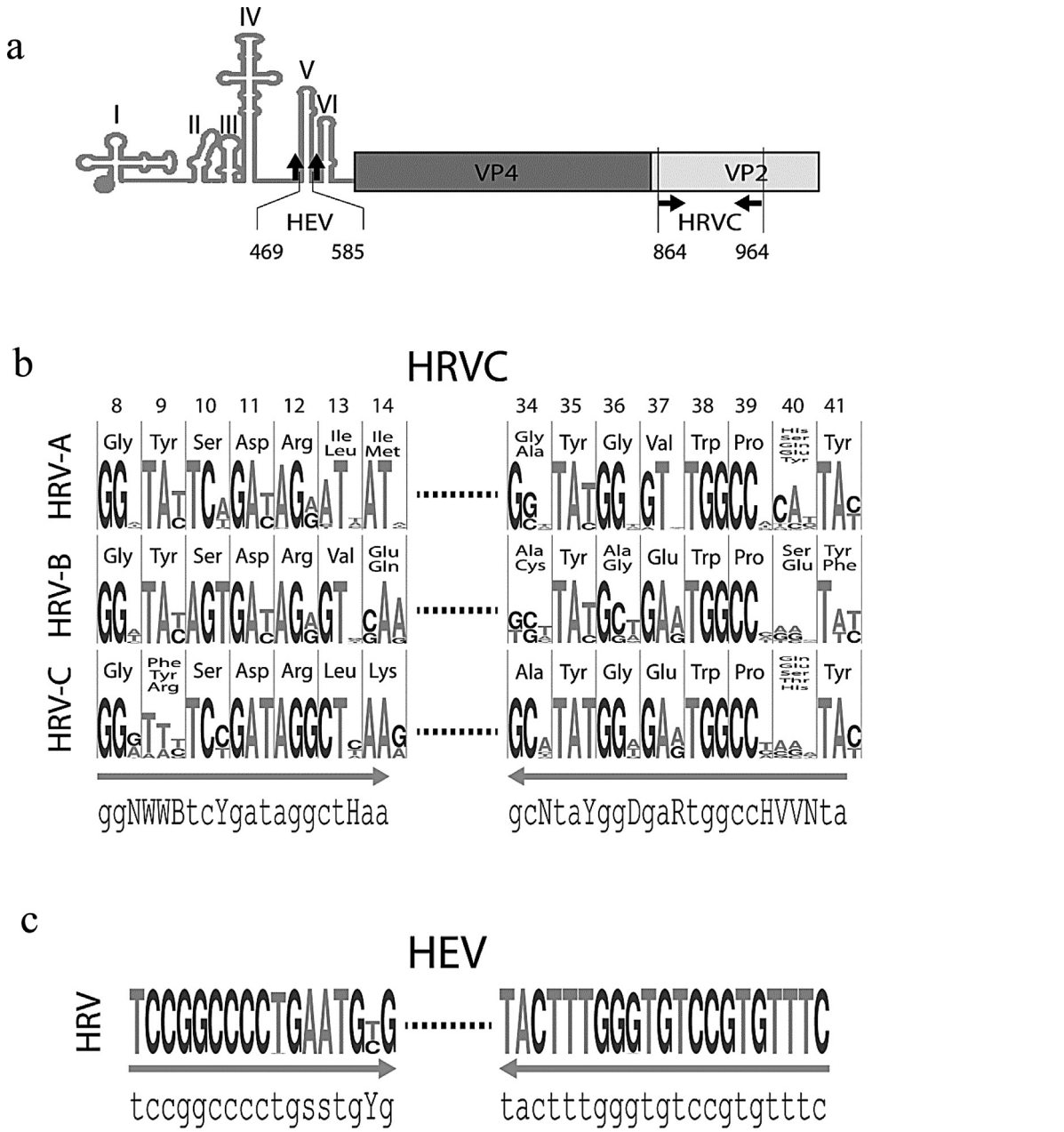

Fig. 3. Design of the HEV and HRVC primers. (a) HEV and HRVC primer target region in the HRV genome. HEV detects all human enteroviruses and HRVC detects rhinovirus C. Arrows indicate the position and direction of each primer. The HEV target sequence is located in the fifth IRES domain of the 5′-UTR. The HRVC target is located within the viral protein 2 (VP2) coding region. (b) HRVC degenerate primers. Sequence logos show the target nucleotide sequences in the VP2 target region. Amino acids are numbered from the VP2 N-terminus. Arrows indicate the position and direction of the degenerate primers. Bottom: degenerate HRVC primer sequences. Mixed nucleotides codes; N(A+T+C+G), W(A+T), B(G+T+C), Y(C+T), H(A+T+C), D(G+A+T), R(A+G), and V(G+A+C). (c) HEV degenerate primers. The sequence logo shows the conserved nucleotide sequences common to all HRV. Bottom: degenerate HEV primer sequences.

3.2. Testing the primer sets against standard lab strains

The designed primer sets were tested against 17 standard enteroviruses. The HEV primer set detected all enteroviruses, with a melting temperature peak ranging from 83 °C to 86.2 °C. The difference in melting temperature indicates that the GC content of the PCR amplicons generated by these primers was different. Notably, the HRVC set only amplified RVC51, with a melting temperature of 80.4 °C (Fig. 5a). Next, the primer target sequences were analyzed. The sequences within the genomic regions of the tested viruses targeted by the HEV set were identical (Fig. 5b). Therefore, the regions targeted by the HEV set were suitable for detecting HEV in clinical samples with high sensitivity. However, as expected the HRVC target site in different viruses harbored multiple mismatches. In particular, the sense primer in the HRVC set was specific for the conserved 3′ "AA" di-nucleotides in HRV-C51, and the antisense primer in the HRVC set was specific for the conserved "GC" di-nucleotides in HEV-B6, HEV-E9, HEV-E11, and HRV-C51. As a result, only the HRV-C51 sequence was amplified by the HRVC primer set.

3.3. Testing the primer sets on clinical nasal lavage samples

Next, the primer sets were tested against nasal lavage samples collected from patients with chronic rhinosinusitis. These samples were suitable for evaluating the primers due to the high incidence of

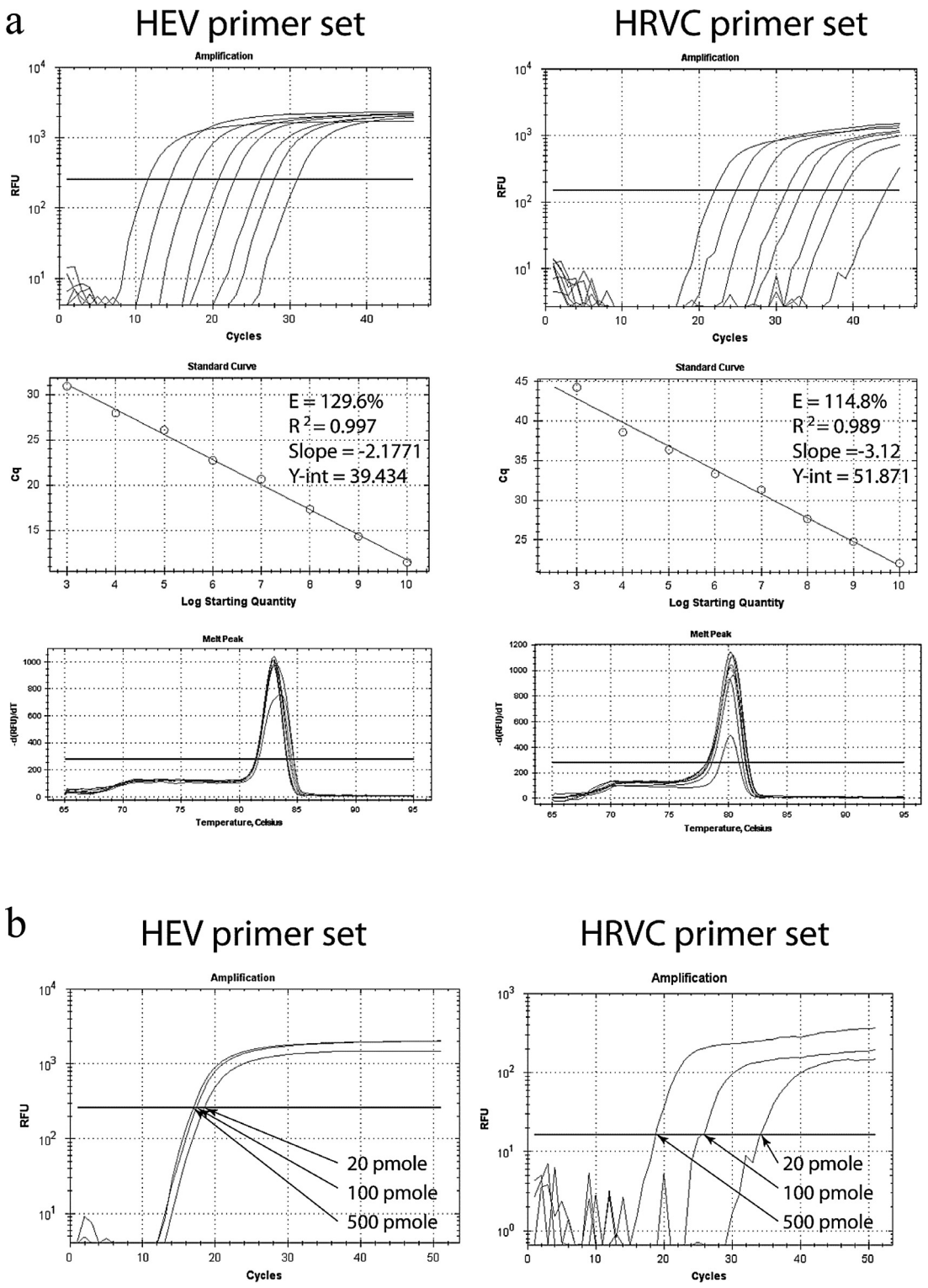

Fig. 4. PCR characteristics of the HEV and HRVC primers. (a) Characteristics of the HEV and HRVC primer sets. Top panel: amplification curves generated by serial dilutions of the standard HRV-C51 plasmid (10^8–10^2 copies/μl). Middle panel: linear regression analysis of the standard curve. Bottom panel: melting curve analyses. (b) Amplification curves generated using increasing primer concentrations (20, 100, and 500 pmoles).

common respiratory viral infections. Common respiratory viruses were detected using a commercial multiplex PCR kit (Table 1). Common respiratory viruses were detected in 15 out of 23 samples (L1–15). Co-infection by more than two viruses was confirmed in nine samples (L2, L4, L6–7, and L11–15). Notably, HRV (A/B/C) was detected in 10 samples (L1–10). To identify HRV species, VP4/VP2 molecular typing was performed using HRV-positive samples. One sample was untypeable due to PCR failure. In all, nine HRVs were typed: HRV-A in six samples, HRV-B in one sample, and HRV-C in two samples. The HEV and HRVC primer sets were then tested.

a

	EV		RVC	
	Copies/mL	MT	Copies/mL	MT
HEV-B1	5.5E+06	85.0	0.0E+00	0
HEV-B3	2.8E+07	85.6	0.0E+00	0
HEV-B5	2.3E+07	86.0	0.0E+00	0
HEV-B6	7.0E+08	86.2	0.0E+00	0
HEV-E6	9.1E+07	86.0	0.0E+00	0
HEV-E7	1.2E+08	84.8	0.0E+00	0
HEV-E9	1.1E+08	85.6	0.0E+00	0
HEV-E11	1.6E+07	84.4	0.0E+00	0
HEV-E25	2.7E+07	84.6	0.0E+00	0
HEV-E30	6.6E+07	85.2	0.0E+00	0
HEV-CA9	2.1E+08	86.0	0.0E+00	0
HEV-EV71	8.3E+06	85.2	0.0E+00	0
HRV-1B	1.7E+06	84.2	0.0E+00	0
HRV-A16	3.4E+06	83.6	0.0E+00	0
HRV-A21	2.4E+07	83.8	0.0E+00	0
HRV-B14	6.8E+07	83.8	0.0E+00	0
HRV-C51	1.0E+09	83.0	2.3E+08	80.4

b

HEV primer set

```
HEV-B1     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-B3     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-B5     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-B6     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-E6     tccggcccctgaatgcggataa.........tactttgggtgtccgtgtttc
HEV-E7     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-E9     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-E11    tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-E25    tccggcccctgaatgcggataa.........tactttgggtgtccgtgtttc
HEV-E30    tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-CA9    tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HEV-EV71   tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HRV-1B     tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HRV-A16    tccggcccctgaatgtggctaa.........tactttgggtgtccgtgtttc
HRV-A21    tccggcccctgaatgtggctaa.........tactttgggtgtccgtgtttc
HRV-B14    tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
HRV-C51    tccggcccctgaatgcggctaa.........tactttgggtgtccgtgtttc
           tccggcccctgaatgYg               tactttgggtgtccgtgtttc
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HRVC primer set

```
HEV-B1     tgtggttatagtgacagggttcga...tggtatggtgtgtggccagaatatt
HEV-B3     tgcggatacagtgacagggtgaga...ggctatggagtatggccagattatc
HEV-B5     tgtggttacagtgatagggttagg...tggatatggagtgtggcccacctact
HEV-B6     tgtgggtatagtgatagagttaga...cgcatatggggtgtggcccgattacc
HEV-E6     tgcggctacagcgaccgtgtgcga...aggtatggagtgtggcccgattatt
HEV-E7     tgtggttacagtgatagagtcaga...tggctacggcaggtggccagagtacc
HEV-E9     tgcggattcagtgatagagttagg...ggcgtatggtagatggcctacatatc
HEV-E11    tgtgggttcagtgatcgtgtacgc...cgcatatggtcgctggcccgaatacc
HEV-E25    tgtggatacagcgatcgtgttcga...tggtatggtgtctggccagagtatt
HEV-E30    tgtggttacagtgatcgagtgcgc...ggggtatggagtgtggcccacttact
HEV-CA9    tgcgggtacagcgatcgggttagg...ggggtatggtagatggcccacttacc
HEV-EV71   tgtggatacagtgatcgagtggcg...tggttatggtgagtggccttcctact
HRV-1B     tgtggctattcagataggattata...tggatatggggtttggccacactact
HRV-A16    tgtggatactctgatagaataatc...tggtatggggtatggccacactacc
HRV-A21    tgcggttattcggatagaatcata...tggttatggagtttggccacattact
HRV-B14    tgtggttatagtgatagagtacaa...gtgttatgctgaatggccagagtacc
HRV-C51    tgtggattctctgataggctaaag...ggcgtatggagagtggccacagtatc
           ggNWWBtcYgataggctHaa      gcNtaYggDgaRtggccHVVNta
```

Fig. 5. Real-time RT-PCR using standard HEV strains. (a) Real-time RT-PCR results using the HEV and HRVC primer sets. The number of copies/ml = amount of viral RNA in 1 ml of sample. MT: peak melting temperature calculated from post-PCR melting temperature analysis. (b) Degree of matching between the primers and the tested viral sequences. Nucleotides that match from the 3′ end of the corresponding degenerate primer are shown in bold. The boxes show the degenerate primer sequences. Mixed nucleotides codes: N(A+T+C+G), W(A+T), B(G+T+C), Y(C+T), H(A+T+C), D(G+A+T), R(A+G), and V(G+A+C).

HEV amplified all HRV-positive samples (L1–10) with a titer from 4.3×10^2 to 2.2×10^6, whereas it did not amplify HRV-negative samples (L11–23). Notably, the samples positive for other viruses (L11–15; RSV A/B, Flu A, PIV 1/3, and coronavirus) were not amplified by HEV. The HRVC primer set only amplified L6 and L8, which contained HRV-C23 and HRV-C51, respectively.

4. Discussion

4.1. Sequence conservation within the enterovirus genome

The genetic diversity of enteroviruses derives from the low fidelity of the viral RNA polymerase. The location of random

Table 1
Testing HEV and HRVC in clinical lavage samples.

ID	Sampling date	Multiplex PCR detection	Molecular typing	HEV		HRVC	
				Titer	MT	Titer	MT
L1	14-Jul-11	HRV	HRV-A22	5.4E+02	83.2	0	ND
L2	11-Aug-11	Adv, CV, PIV, Flu A, RSV A, HRV, MPV	HRV-A13	1.9E+03	85.8	0	ND
L3	28-Sep-11	HRV	HRV-A13	1.6E+03	85.8	0	ND
L4	11-Oct-11	PIV 3, HRV	HRV-A66	4.3E+02	86.2	0	ND
L5	12-Oct-11	HRV	HRV-B72	1.9E+04	86.4	0	ND
L6	13-Oct-11	CV, HRV	HRV-C23	8.2E+03	85	5.0E+04	79.6
L7	28-Oct-11	PIV 1,3, HRV	HRV-A13	2.4E+03	85.8	0	ND
L8	23-Nov-11	HRV	HRV-C51	2.2E+06	83.2	2.5E+06	79.4
L9	1-Dec-11	HRV	untypeable	5.0E+03	84.6	0	ND
L10	29-Dec-11	HRV	HRV-A63	8.8E+03	86.2	0	ND
L11	4-Aug-11	RSV A, Flu A		0	ND	0	ND
L12	11-Aug-11	PIV 1,3, RSV A		0	ND	0	ND
L13	18-Aug-11	CV, PIV 1		0	ND	0	ND
L14	8-Sep-11	RSV B, Flu A		0	ND	0	ND
L15	7-Oct-11	CV		0	ND	0	ND
L16	27-Jul-11	(−)		0	ND	0	ND
L17	4-Aug-11	(−)		0	ND	0	ND
L18	9-Aug-11	(−)		0	ND	0	ND
L19	10-Aug-11	(−)		0	ND	0	ND
L20	8-Sep-11	(−)		0	ND	0	ND
L21	6-Oct-11	(−)		0	ND	0	ND
L22	7-Oct-11	(−)		0	ND	0	ND
L23	9-Dec-11	(−)		0	ND	0	ND

ID: sample ID. Date: sampling date. Multiplex PCR detection: the results of common respiratory viruses detection (HRV, human rhinovirus; Adv, human adenovirus; CV, human coronavirus; PIV, human parainfluenza virus; Flu A, influenza A virus; RSV, human respiratory syncytial virus A; MPV, human metapneumovirus). Molecular typing: HRV type determined by VP4/VP2 sequencing. HEV: real-time RT-PCR results obtained using the HEV primer set. HRVC: real-time RT-PCR results obtained using the HRVC primer set. Titer: copy number of viral RNA in 1 ml of sample. MT: peak melting temperature calculated from post-PCR melting temperature analysis. (−): negative. ND: non-determined.

mutations is critical for the production of replication-compatible progeny. The 5′-UTR is highly conserved among enteroviruses because the functional folding structure is easily disrupted by a single mutation. Therefore, the HEV primer set was designed to target the fifth IRES domain within the 5′-UTR and detected all enteroviruses tested. This region is frequently selected for the PCR detection of enteroviruses (McLeish et al., 2012). Mutations have much less effect on the capsid-encoding sequences because mutations can be either silent or occur in the non-structurally critical loop regions of the capsid proteins. Thus, the HRVC primer set was designed to target the N-terminus of the VP2 protein and specifically amplified HRV-C (Fig. 3a).

The HEV primer set is supplementary to the HRVC set and is more sensitive due to its low degeneracy. Four possible combinations of results can be obtained when both primer sets are used together to test clinical samples: HEV-positive/HRVC-positive, HEV-positive/HRVC-negative, HEV-negative/HRVC-positive, and HEV-negative/HRVC-negative. The first combination provides strong evidence of HRV-C infection (see Table 1: L6 and L8). HEV-negative/HRVC-positive indicates a false-positive result and HEV-positive/HRVC-negative indicates an infection by other enteroviruses such as HRV-A or HRV-B (see Table 1: L1–5, L7, and L9–10). The last combination indicates no HEV infection.

4.2. Considerations when designing degenerate primers for PCR based on sequence analysis

Several factors were considered when designing the degenerate primers: 3′-end specificity, melting temperature, minimum degeneracy, and short amplicon size. The specificity of the primer is dependent on the 3′-end sequence, and the final specificity of the PCR is determined by the combined specificity of two primers. The primer must also have a suitable melting temperature. High degeneracy reduces the sensitivity of the PCR. A short amplicon reduces the chance of non-specific binding of degenerate primers during the annealing step. Specificity and degeneracy are the most critical factors. The reverse-codon method is conventionally used for degenerate primer design; however, we found that this strategy led to an unnecessary increase in degeneracy. For example, the first and second codons for the 10th Ser were T and C in HRV-A and HRV-C, but A and G in HRV-B (Fig. 3b). Therefore, our MSA-based design strategy is preferable to the reverse-codon-based method because it avoids unnecessary degeneracy. The HRVC primer set (designed using the MSA approach) had 1.5×10^6-fold degeneracy (288 of sense and 5184 for antisense primers); however, the set would have 2.7×10^9-fold degeneracy (82,944 for sense and 32,768 for antisense primers) if designed using the reverse-codon method.

Degenerate PCR primers can be used to detect a wide range of target sequences at the expense of sensitivity because the concentration of the matching primer is inversely correlated with degeneracy. However the low sensitivity can be overcome partially by increasing the concentration of the degenerate primer. Theoretically, if the degeneracy increases by 10-fold, the matching primer concentration decreases by 10-fold. However, this inverse correlation is not linear because primer binding can tolerate some mismatches. There is no accepted method of calculating the acceptable degree of mismatch at the desired melting temperature. Therefore, experimental “tuning” of the concentration is required before using degenerate PCR primers in a specific clinical setting.

4.3. Fluorescent signal reading temperature

The Taqman probe-based method is generally more sensitive than SYBR green II-based methods when used for real-time PCR; however, it is extremely hard to design degenerate primers and probe sets due to the exponential increase in degeneracy. In addition, the average melting temperature of the probe might be about 10 °C higher than that of the primers. Thus, probe-based methods are not currently applicable to the detection of specific virus subgroups by degenerative primers. The disadvantage of SYBR green II-based methods is the false-positive signal generated by primer dimer formation or non-specific reaction. This “noise” signal can

be minimized by reading the fluorescent signal at a temperature higher than the melting temperature of the non-specific amplicon. In our case, the signal was read at 80 °C because the melting temperature of the specific target amplicon was about 80 °C.

4.4. HRVC considerations

In this study, the design of the HRVC primer set was based on bioinformatics analyses using the sequences of standard HRV strains. Therefore, more extensive testing in different clinical settings is required in addition to parallel molecular typing, although the HRVC set was tested against a limited number of standard strains and clinical lavage samples. In particular, it is possible that the HRVC set will not amplify non-classified HRV-C strains because its design was based on 51 standard HRV-C sequences registered in GenBank. Therefore, constant "tuning" of the HRVC primer set using newly identified HRVC sequences will be required.

5. Conclusions

The present study reports the MSA-based degenerative primer design method. Two primer sets, HEV and HRVC, were designed to detect HRV-C by RT-PCR. The HEV primer set targeted the fifth loop within the IRES, which is highly conserved among enteroviruses. The HRVC primer set specifically targeted the VP2 coding region within HRV-C, which was identified by MSA and phylogenetic analyses. Both primer sets were tested against standard laboratory enterovirus strains and clinical nasal lavage samples. Although more extensive tests are required to be applied in clinical settings, the HEV primer set amplified all enteroviruses whereas the HRVC primer set was specific for HRV-C in a limited number of samples.

Acknowledgement

This study was supported by a grant of the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A110893).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.10.021.

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