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Short communication

Simultaneous detection of parainfluenza viruses 1 and 3 by real-time reverse transcription-polymerase chain reaction

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A B S T R A C T

Human parainfluenza virus (HPIV) types 1 and 3 are major viral pathogens responsible for upper and lower respiratory tract infections. The diagnosis of these two species is achieved generally by specific reverse transcription-polymerase chain (RT-PCR) reaction methods. In this study, a real-time RT-PCR was developed using a common pair of primers–probe (HPIV-1+3) for the simultaneous detection of both HPIV-1 and HPIV-3 genomes. Results obtained in a 10-fold dilution series assay demonstrate a high sensitivity of the assay with a lowest detection limit of approximately one plasmid copy for both HPIV-1 and HPIV-3. A comparison of HPIV-1 and HPIV-3 clinical sample detection between specific HPIV-1/HPIV-3 pairs of primers–probes and the HPIV-1+3 combination clearly shows that the latter is significantly more sensitive (gain of about five threshold cycles) than the former for HPIV-3 detection, while equivalent values are observed for HPIV-1. The HPIV-1+3 combination constitutes a more rapid, more sensitive, and less expensive alternative than classical or multiplex real-time RT-PCR assays usually used in clinical laboratories.

Human parainfluenza viruses (HPIV) types 1 and 3 are members of the *Respirovirus* genus belonging to the Paramyxoviridae family. HPIV-1 and HPIV-3 genomes are characterized by a non-segmented, negative-sense RNA of about 15,000 nucleotides encoding for six genes. HPIV-1 and HPIV-3 cause upper and lower respiratory tract infections in infants, young children, and immunocompromised individuals (Apalsch et al., 1995; Henrickson, 2003; Karron et al., 1994; Mufson et al., 1973; Singh-Naz et al., 1990; Welliver et al., 1982; Wendt and Hertz, 1995; Whimbey et al., 1993). At the upper respiratory tract level, HPIV-1 and HPIV-3 infections are associated with pharyngitis, otitis, conjunctivitis, and croup. HPIV-1 is considered to be the most frequent cause of croup. At the lower respiratory level, these viruses are known to cause tracheobronchitis, bronchiolitis (a characteristic feature of HPIV-3) and pneumonia (Monto, 1973; Vainionpaa and Hyypia, 1994). Studies suggest that HPIV may cause up to 40% of acute viral respiratory tract illness in children (Reed et al., 1997). In addition, HPIV-3 has been linked also to meningitis in both children (Arguedas et al., 1990; Arisoy et al., 1993) and adults (Vreede et al., 1992). Therefore, due to the clinical impact and the wide geographical distribution of HPIV-1 and HPIV-3, many studies have been conducted to identify a rapid and sensitive HPIV diagnostic tool.

Classical and multiplex reverse transcription-polymerase chain reactions (RT-PCR) (Aguilar et al., 2000; Bellau-Pujol et al., 2005; Coiras et al., 2004; Corne et al., 1999; Echevarria et al., 1998, 2000; Erdman et al., 2003; Fan and Henrickson, 1996), as well as real-time RT-PCR with specific primers and probes for each type of HPIV (Hu et al., 2005; Kuypers et al., 2006; Templeton et al., 2004), have been demonstrated to be much more sensitive and specific than both viral culture or immunofluorescence-based detection methods (Erdman et al., 2003; Syrmis et al., 2004; Templeton et al., 2004, 2005; van Kraaij et al., 2005).

Taking advantage of the genomic similarity between HPIV-1 and HPIV-3, this study describes the validation of a unique and highly sensitive real-time RT-PCR assay allowing the simultaneous detection of HPIV-1 and HPIV-3 genomes. This new combination (named HPIV-1+3 throughout the paper) represents the first set of common primers–probe designed to regroup HPIV detection. Previous specific HPIV-1 and HPIV-3 single or multiplex RT-PCR detection methods reported in the literature have aimed to target mostly the nucleocapsid (N) or the hemagglutinin–neuraminidase surface glycoprotein (HN). Based on an extensive alignment including 72 HPIV-1 and HPIV-3 complete and partial sequences available in Genbank, conserved regions between HPIV-1 and HPIV-3 were screened. Although no suitable regions were found within the N and HN coding sequences, this alignment highlighted a short con-

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Table 1
Primers and probe for HPIV-1+3 real-time RT-PCR assay. Nucleotide differences between PIV1-3 F1 and PIV1-3 F2 are shown in bold type.

Name	Sequence (5′–3′)	Label (5′, 3′)	Gene
PIV1-3 F1	ATCCAAGAGG**R**GGAATAGA		L
PIV1-3 F2	A**C**CCAAGAGG**G**GGTATAGA		L
PIV1-3 R	GTCTCCTTGAACCATTGC		L
PIV1+3 probe	TCTATAAGTGCAATMCATCTAGCAGCTGTT	FAM, TAMRA	L

served region within the viral polymerase encoding region (L) where primers and probe recognizing both HPIV-1 and HPIV-3 could be identified. Interestingly, this region overlaps two conserved motifs (named B and C) within the catalytic domain for L polymerisation (Poch et al., 1990). Nucleotide sequences of these primers and probe are shown in Table 1. Based on this finding, a unique reverse primer (PIV1-3 R) and probe (PIV1-3 probe) were able to be designed to detect all HPIV-1 and HPIV-3 genomes, whereas the use of two forward primers (PIV1-3 F1 and PIV1-3 F2) was required. However, PIV1-3 F1 and PIV1-3 F2 are similar in length, position and differ only at three positions in their sequences (Table 1). Primers and probe were screened by NCBI nucleotide BLAST to exclude any cross-reaction with human cellular sequences or other virus targets. Of note, an alignment was performed including all the above-mentioned HPIV-1 and HPIV-3 sequences as well as 20 HPIV-2 sequences (full-length and partial genomes) and the few HPIV-4 partial sequences available in Genbank. Given that these viruses belong to two different genera (HPIV-2 and HPIV-4 belong to the *Rubulavirus* genus), this alignment did not allow pointing of any common conserved regions either among the four HPIV species genomes, or between HPIV-2 and HPIV-4 species.

Primers were obtained from Invitrogen (Basel, Switzerland). The PIV1-3 probe (Applied Biosystems, PE Europe BV, Basel, Switzerland) was labelled at the 5′-end with the 6-carboxyfluorescein (FAM) and at the 3′-end with the 6-carboxytetramethylrhodamine (TAMRA) fluorescent quencher. In brief, after RNA extraction by the guanidinium–thiocyanate method (Roche Applied Science, Rotkreuz, Switzerland), the synthesis of cDNA was performed with random hexamers (Roche) at 42 °C using the Reverse Transcriptase Superscript II (Invitrogen) according to the manufacturer's instructions. cDNA was amplified using a TaqMan® 7500 (Applied Biosystems) thermocycler under the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, 55 cycles of 15 s at 95 °C and 1 min at 55 °C. After assessment of the optimal primers–probe concentrations, the reaction was performed in 20 μl containing 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.6 μM of PIV1-3 F1, 0.6 μM of PIV1-3 F2, 0.9 μM of PIV1-3 R, 0.25 μM of PIV1-3 probe and 5 μl of cDNA. Results were analysed using the SDS 1.4 programme (Applied Biosystems).

To assess the analytical sensitivity of detection, this new HPIV-1+3 combination was tested using a 10-fold dilution series of cloned PCR products obtained from HPIV-1 and HPIV-3 clinical samples (Fig. 1) amplified with 5′-ccataatccaagaggggaatag-3′ and 5′-gattgtctccttgtaccattgc-3′ and 5′-tcataacccaagaggggtatag-3′ and 5′-gattgcttccttgaaccattgc-3′ primers, respectively, before cloning into pCR2.1-TOPO plasmid (Invitrogen). As expected, two and zero mismatches were present between PIV1-3 probe and HPIV-1 and HPIV-3 clinical sample sequences, respectively. The experiment showed the conservation of a wide linearity that ranged between 1 and 10^6 copies of input together with a high detection level by the HPIV-1+3 combination for both HPIV types. HPIV-1 and HPIV-3 were both detected at a level of approximately one copy of cloned amplified product (Fig. 1). In addition, the sensitivity of the HPIV-1+3 combination was tested against the serial dilutions of HPIV-1 (ATCC VR-94) and HPIV-3 (ATCC VR-93) cultured and titrated stock. The lowest limits of HPIV-1 and HPIV-3 detections were of 0.01 and 0.1 of the 50% tissue culture infectious dose ($TCID_{50}$), respectively.

To determine the specificity of the HPIV-1+3 combination, the set of primers–probe was tested for cross-reactivity with a large panel of respiratory viruses with $TCID_{50}$ ranging between 10^4 and 10^8 including other paramyxoviruses. Several respiratory syncytial virus types A and B, coronaviruses OC43, E229, NL-63 and HKU1, influenza virus types A and B, metapneumoviruses, HPIV-2 and HPIV-4, and rhinovirus types A and B remained undetected by the assay after 55 amplification cycles, while all were detected efficiently by their specific primers–probe combination and cell culture.

When developing a new real-time RT-PCR assay, it is important to investigate its ability to detect clinical samples. Therefore, 20 HPIV-3 and 13 HPIV-1 clinical samples detected in bronchoalveolar lavage fluids or nasopharyngeal specimens between 1996 and 2007 using previously published methods (Garbino et al., 2006; Regamey et al., 2008) were reanalysed in parallel experiments. The threshold (C_T) values obtained with HPIV-1+3 were compared to those obtained by using two different specific combinations for HPIV-1 or HPIV-3 which were largely inspired from Corne et al. (1999) and Karron et al. (1994), respectively. All HPIV-1 and HPIV-3 clinical samples were detected by both methods. In concordance with results obtained in the analytical 10-fold dilution series experiment, the HPIV-1+3 exhibits a sensitivity similar to the specific HPIV-1 combination, whereas it is strongly increased (approximately 5 C_T on average) compared to the specific HPIV-3 set of primers–probe (mean of C_T values comparison between specific HPIV-1 and HPIV-3 primers–probe versus HPIV-1+3 combination: 38.45 ± 5.67 versus 37.33 ± 4.44 for HPIV-1 clinical samples and 33.22 ± 6.01 versus 28.44 ± 5.59 for HPIV-3 clinical samples). Since the stop/restart mechanism at each gene junction is not optimal, sequential HPIV-1 and HPIV-3 transcription leads to a higher transcription of the most proximal genes of the 3′-end of the genome with a further transcription decrease of the more proximal genes of the genome 5′-end. Thus, it can be expected that the HPIV-1+3 combination (designed in L) would be less sensitive than both HPIV-1

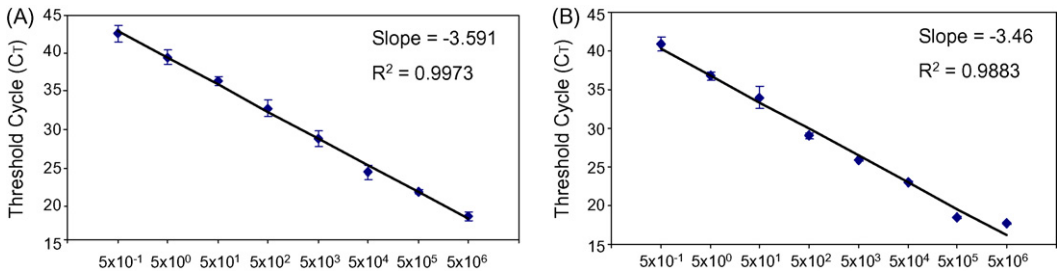

Fig. 1. Analytical sensitivity of the HPIV-1+3 real-time PCR assay. (A) Tenfold dilution of HPIV-1-cloned PCR product (5×10^6 to 5×10^{-1} copies per reaction). Copy numbers are plotted versus the threshold cycle (C_T). (B) Tenfold dilution of HPIV-3-cloned PCR product (5×10^6 to 5×10^{-1} copies per reaction). Copy numbers are plotted versus the C_T. Each dot represents the average of four independent experiments. As expected, the dilution with 5×10^{-1} cDNA copies was not detected statistically in all experiments and the values presented take into account only positive assays. Error bars indicate standard deviations.

and HPIV-3 specific combinations (both designed in HN). However, as mentioned above, these two specific sets of primers–probes were not optimal for real-time PCR assays as they resulted from slot-blot and RT-PCR–enzyme immunoassay (RT-PCR–EIA)-adapted methods, respectively. By using HPIV-1+3, the detection of HPIV-1 and HPIV-3 genomes are not only simplified, but also improved (at least for HPIV-3) as evidenced across temporally diverse clinical isolates.

The development of new broad-specificity real-time RT-PCR assays capable of detecting more than one specific virus without multiplexing is a strategy that needs to be considered in order to limit the complexity of the validation process, as well as to reduce the cost and the complexity of the assay. Thanks to a careful alignment of all available sequences, a relatively conserved target on the polymerase gene that could be used for HPIV-1 and HPIV-3 genome detection was identified. The resulting HPIV-1+3 combination can detect efficiently circulating HPIV-1 and HPIV-3 and, at least for HPIV-3, with a higher sensitivity than the former specific real-time RT-PCR assay. HPIV-1+3 could be extremely useful for the large screening of HPIV-1 and/or HPIV-3 in one assay, thus reducing costs and avoiding the need for a total number of analyses required by a factor of two.

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