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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 reaction (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.

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erved 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 HPN-2 sequences (full-length and partial genomes) and the few HPN-4 partial sequences available in Genbank. Given that these viruses belong to different genera (HPIV-2 and HPIV-4 belong to the Rhabdovirus 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-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 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′-ccataactcaaggggggaat-3′ and 5′-gcattccttgagacctgc-3′ and 5′-ctataaccaaggggtattag-3′ and 5′-gcattccttgagacctgc-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 (TCID₅₀), 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 TCID₅₀ ranging between 10⁴ and 10⁶ 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 (Carbino et al., 2006; Regamey et al., 2008) were reanalysed in parallel experiments. The threshold (C₇) 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 C₇ on average) compared to the specific HPIV-3 set of primers–probe (mean of C₇ 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

Table 1

| Name       | Sequence (5′-3′) | Label (5′, 3′) | Gene     |
|------------|-----------------|---------------|----------|
| PIV1-3 F1  | ATCCAAAGGCRGGAATAGA | L             |          |
| PIV1-3 F2  | ACACCAAGGCGGATAGA | L             |          |
| PIV1-3 R   | GCTCTCCTGACCATGC | L             |          |
| PIV1+3 probe | TCTTAAAGTCCATCTCATGCAGCTGTT FAM, TAMRA | L             |          |

Fig. 1. Analytical sensitivity of the HPIV-1+3 real-time PCR assay. (A) Tenfold dilution of HPIV-1+3 cloned PCR product (5×10⁻⁹ to 5×10⁻¹ copies per reaction). Copy numbers are plotted versus the threshold cycle (C₇). (B) Tenfold dilution of HPIV-3 cloned PCR product (5×10⁻⁹ to 5×10⁻¹ copies per reaction). Copy numbers are plotted versus the C₇. 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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