CLINICAL STUDY

Establishment of reverse transcription polymerase chain reaction assay for simultaneous detection of human parainfluenza virus types 1 to 4 in children with influenza like illnesses

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ABSTRACT

OBJECTIVES: The aim of this study was to develop an in-house multiplex reverse transcription polymerase chain reaction (mRT-PCR), which can recognize HPIV1-4 in clinical samples.
BACKGROUND: Human parainfluenza virus (HPIV) is one of the major causes of viral respiratory infections and can affect people at any age, especially infants and young children.
METHODS: Four sets of specific primers targeting conserved areas of hemagglutinin–neuraminidase (HN) genes of HPIV1-4, were designed and tested with type-related plasmid controls. Specificity and sensitivity of mPCR were tested. One-step mRT-PCR was set up using a viral panel containing 10 respiratory viruses, including HPIVs. One hundred nasopharyngeal samples of respiratory infection patients were tested using the set One-step mRT-PCR.
RESULTS: The specificity of set mPCR for HPIV1-4 using plasmid positive controls was proved and reaction sensitivity was measured. The specificity of set mRT-PCR was confirmed and 4 and 5 out of 100 clinical samples were HPIV1 and HPIV2 positive, respectively.
CONCLUSION: The developed one-step mRT-PCR in this study is an effective and specific assay for clinical diagnosis of HPIV1 to 4 *(Tab. 1, Fig. 6, Ref. 28)*. Text in PDF *www.elis.sk*.
KEY WORDS: human parainfluenza virus, human, reverse transcriptase PCR, respiratory tract infections.

Introduction

Respiratory tract infections in infants and children 5 years old or younger are a major cause of morbidity and mortality. Viruses are considered the main reason for these infections. Human Parainfluenza viruses can infect persons at any age, but are the second-leading cause of respiratory illness hospitalizations in young children (1–3). HPIVs are enveloped, single stranded, non-segmented negative sense RNA viruses that belong to Paramyxoviridae family and have four types based on genetic and antigenic characteristics and clinical presentation and differ in seasonality worldwide. HPIV types 1 and 3 belong to Respirovirus genus and types 2, and 4 belong to Rubulavirus genus (4–6).

The HPIVs can cause a wide spectrum of respiratory diseases in children, including rhinorrhea, cough, bronchiolitis, croup, and pneumonia. The majority of cases are mild upper respiratory tract infections that can be asymptomatic, but in the immunocompromised host, HPIV infection can cause lower respiratory tract infections, become persistent, or the patient may develop secondary bacterial infection, which all have worse outcomes and can lead to death (7–10). Even though efforts have been made, currently no licensed specific antiviral therapy or vaccine is available against the HPIVs (11–14).

In past studies, several methods have been used to detect these viruses in patients with upper and lower respiratory tract diseases, which differ in expenses, sensitivity, specificity, ease of use and turnaround time. These methods include viral isolation, direct viral antigen detection, serological techniques, immunofluorescence or enzyme immunoassay and nucleic acid amplification methods like reverse transcription polymerase chain reaction (RT-PCR). Studies comparing these different methods seem to suggest that RT-PCR, considering reliability, specificity, speed, reproducibility of results and other aspects, is one of the best choices for this purpose at present and can be widely used in health care systems (5, 15, 16).

In this study, we developed a one-step multiplex RT-PCR using type-specific primer pairs targeting the conserved regions of the HN gene of HPIV1-4 to detect any of four HPIV types in 100 inpatient respiratory samples with influenza-like illnesses' symptoms.

The purpose of the present study, considering the importance of accurate diagnosis in health care system's goal of lowering medical costs and preventing the unnecessary use of antibiotics, was to

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develop a one-step multiplex RT-PCR that is able to detect types 1-4 of HPIV in any one clinical respiratory sample.

Materials and methods

Primer design

The nucleotide sequences of all HN genes were obtained from the National Center for Biotechnology Information (NCBI) and aligned to find the most conserved regions. Using AlleleID software, four type-specific primer pairs were designed for a conserved region of the each HPIV HN gene. Thermodynamic characteristics and ability of the primers to function properly in a multiplex RT-PCR were analyzed using Oligo6 and Oligo analyzer software. The designed primers were synthesized by Tag Copenhagen (Denmark). The Primers sequences are displayed in Table 1.

Plasmid control

In order to prepare a control template for PCR, complete HN gene of each human parainfluenza virus type (1 to 4) were synthesized and inserted into pBSK plasmid (pBluescript SK (+), BIO-MAIK- Canada). Plasmids (lyophilized powder) were prepared as instructed by the manufacturer, and were introduced into E.coli TOP10 F′ using thermal shock method. After 18 hours, the plasmids were extracted using YTA Plasmid DNA Extraction Mini Kit, and the concentration was determined by spectrophotometry at 260nm and stored at –20 °C for further use.

To evaluate primers accuracy and functionality, preliminary singleplex PCR assays were done using primers and related control template. Total volume of each reaction was 25 μl, containing 0.4 μM of each primer and 1 ng template. The thermal profile consisted of an initial step at 95 °C for 10 minutes, 35 cycles of 15 seconds at 95 °C, 20 sec at 55 °C and 40 sec at 72 °C, followed by the final extension at 72 °C for 10 min. PCR products were evaluated using gel electrophoresis.

PCR sensitivity

In order to determine the test sensitivity, plasmids were linearized by ScaI enzyme (Jena Bioscience) as recommended by BioEdit and Snap Gene Viewer software. Serial dilutions (10^{-1} to 10^{-10} ng/mL) of linearized plasmids were entered as the template in a PCR test, and the minimum concentration, which resulted in a visible band in gel electrophoresis, was labeled as the detection limit of the test. The gene copy number can be calculated using the formula below.

number of copies(molecules) = (amount of amplicon(ng)* 6.022×10^{23}) / (length of dsDNA amplicon * 1×10^9 * 650)

PCR specificity

To ensure the specificity of each primer, and to rule out possible cross reaction, each primer pair along with 3 other unrelated HPIV plasmids were entered in a PCR reaction.

Multiplex PCR

Multiplex PCR was accomplished to amplify HPIV1, HPIV2, HPIV3, and HPIV4 target sequence using specific primers in a single PCR reaction. The reaction was performed in a total volume of 25 μl, contained 0.4 μM of each HPIV1-4 specific primers and 1ng of HPIV1-4 template plasmid.

RT-PCR specificity

In order to evaluate the specificity of designed primers for detection of viral RNA in clinical samples, a viral panel (Vircell – AMPLIRUN TOTAL RESPIRATORY VIRAL PANEL CONTROL (SWAB)) containing 10 respiratory viruses was used as the positive control in a multiplex RT-PCR test. The panel included inactivated adenovirus type 4, coronavirus, influenza A H1N1, A H3 and B viruses, HPIV types1, 2, and 3, and respiratory syncytial virus (subtypes A and B). The lyophilized panel was prepared as instructed by the company and the viral ribonucleic acids were extracted using High Pure Viral RNA Kit (ROCH– Switzerland).

Multiplex RT-PCR was done using designed primers and YTA (Yekta Tajhiz Azma) one step RT-PCR smart mix kit. The reaction was carried out in 25 μl of reaction mixture consisting of 12.5 μl one-step Multiplex master mix, 2 μl of primer mix (0.4 μM of HPIV1-4 primers), and 10.5 μl of template. Thermal profile of the RT-PCR consisted of an initial cDNA step of 30 min at 50 °C followed by 10 min at 95 °C, 35 cycles of 15 s at 95 °C, 20 s at 55 °C, 40 s at 72 °C and a final extension at 72 °C for 10 min. Each test contained negative and internal controls.

Application of multiplex RT-PCR to detect HPIVs in clinical samples

In this study, 100 nasopharyngeal swab samples from patients (≤ 5 years of age) admitted to hospital for Influenza-like illness symptoms were chosen. Samples were collected from March 2013 to March 2016 (from provinces Semnan, Zanjan and Markazi in Iran) by hospital´s medical staff and transferred in viral transport media at 4 °C to The Influenza and other Respiratory Viruses De-

Tab. 1. HPIV1-4 primers sequences and characteristics.

Virus	Primer	Sequence	Product Size	Position on HN$_c$ gene
HPIV$_a$1 (Accession number:AF457102.1)	forward primer	5´-AGG AAT TGG CTC AGA TAT GC-3´	149 bp	451-470
	reverse primer	5´-ACT TGG TCC AGG TAA TAA TGA G-3´		578-599
HPIV2 (Accession number:X57559.1)	forward primer	5´-GGA TAA TAC AAC AAT CTG CTG-3´	337 bp	786-806
	reverse primer	5´-ATG AGA CCA CCA TAT ACA GG-3´		1103-1122
HPIV3 (Accession number:AB012132.1)	forward primer	5´-TAA TAT GAC AGA TGA YAC AAT GC-3´	484 bp	1005-1024
	reverse primer	5´-YGG GTA TGG AGG TCT TGA AC-3´		1466-1485
HPIV4 (Accession number:AB543336.1)	forward primer	5´-TCA AAC CCA TTG GTG TTA TAC-3´	558 bp	546-566
	reverse primer	5´-CTC CCT GAG AAG TAA GTT ATT G-3´		1082-1103

a – human parainfluenza virus, b – base pair, c – hemagglutinin neuraminidase
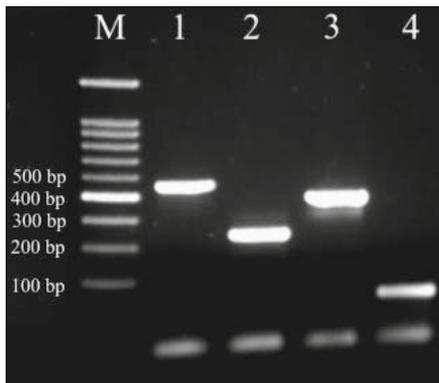
Fig. 1. Results of singleplex PCR reactions with designed primers and their related plasmid template. Lane M, Marker; Lane 1, HPIV-4(Positive control(PC)); Lane 2, HPIV-2(PC); Lane 3, HPIV-3(PC); Lane 4, HPIV-1(PC).
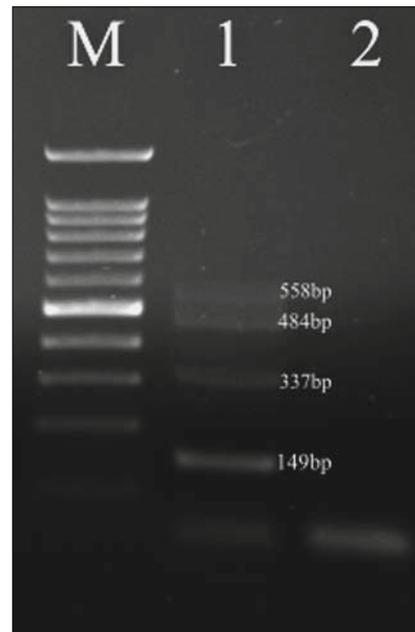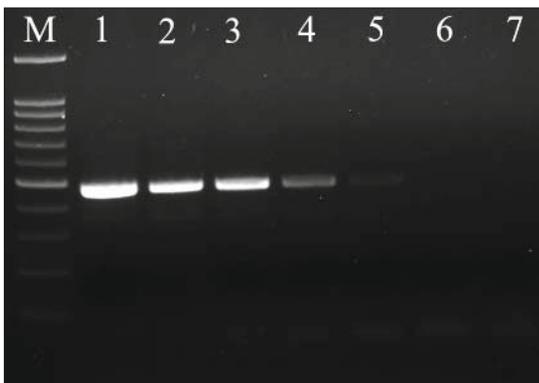

Fig. 2. Results of singleplex PCR reactions on different concentrations of HPIV-3 positive control with related primer pair. Lane M, Marker; Lane 1 to Lane 7, 10^-1 ng/microl to 10^-6 of linearized plasmid template, respectively.

Fig. 3. Result of multiplex PCR on all 4 types of HPIVs plasmids and all four types of primer pairs. Lane M, Marker; Lane 1, HPIV-1, 2, 3 and 4 positive controls; Lane 2, negative control.
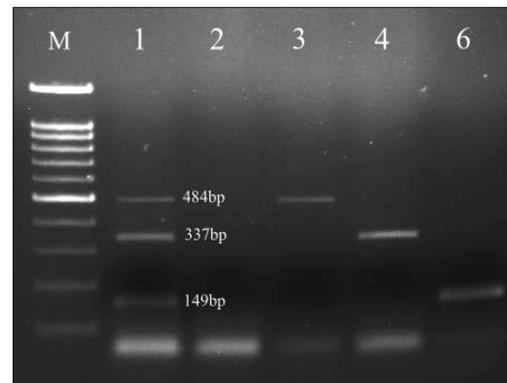

Fig. 4. Result of multiplex and monoplex RT-PCR on viral panel nucleic acid. Lane M, Marker; Lane 1, result of multiplex RT-PCR, showing HPIV1, 2 and 3 fragments; Lane 2, Negative control; Lane 3-5, results of monoplex RT-PCR on viral panel using HPIV3, HPIV2 and HPIV1 specific primers, respectively.

partment, Pasteur Institute of Iran, Tehran and were prepared and stored at −70 °C.

The samples were chosen from influenza negative specimens (tested previously following WHO protocol).

Viral RNA was extracted from patient samples and multiplex RT-PCR was performed on each sample using designed primers to detect HPIVs 1-4 in a single tube.

Results

Plasmid control

Singleplex PCR reactions, using designed primers, and plasmids (containing HPIV types 1-4 HN gene) were performed. Each PCR reaction produced a product with desired band size on gel electrophoresis. No other band other than predicted product was observed (Fig. 1).

PCR specificity and sensitivity

The results of PCR reactions, each containing one primer pair and 3 unrelated templates, showed no band in gel electrophoresis, which proved that the designed primers were indeed type-specific.

To obtain the minimum concentration of plasmid template, PCR reactions with different concentrations of each linearized plasmid and type-specific primers were performed. The least concentration, in which a visible band in gel electrophoresis could be seen, was considered as the limit of detection. For instance, the result for HPIV-3 is shown in Figure 2. The limit of detection was 20, 4.4×10^3, 5.4×10^3 and 5.3×10^3 plasmid copies for type 1, type 2, types 3 and type 4, respectively (Fig. 2).

Multiplex PCR

In a single-tube PCR reaction, containing all 4 primer pairs with their respective plasmid templates, the set up multiplex-PCR ability in simultaneous recognition of HPIV1-4 was confirmed
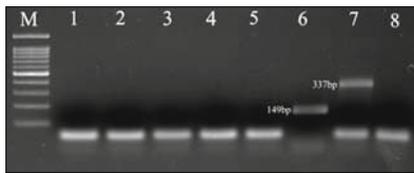
Fig. 5. Results of multiplex RT-PCR on clinical samples. Lane M, Marker; Lane 1 to 5, HPIV1-4 negative; Lane 6, HPIV-1 positive; Lane 7, HPIV-2 positive; Lane 8 HPIV1-4 negative.
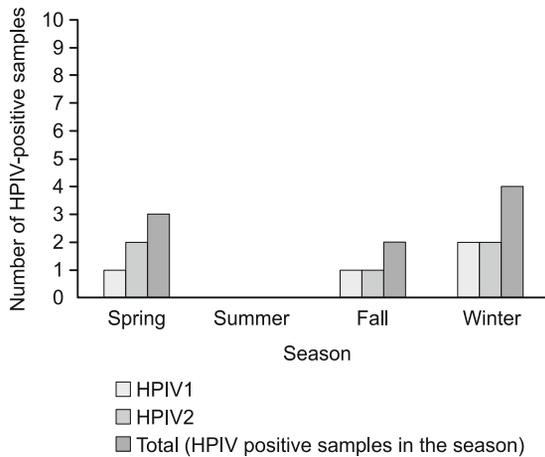

Fig. 6. All HPIVs detected in children younger than 5, during 2013-2016.

by observing 4 separate bands with expected sizes in gel electrophoresis (Fig. 3).

RT-PCR specificity

A series of monoplex and multiplex RT-PCR assays were performed on a viral panel, containing 10 respiratory viruses including HPIV1-3. In a multiplex RT-PCR assay, three different fragments related to HPIV1-3 (149, 337 and 484 bp, respectively) were observed. In monoplex tests, one type-specific primer pair was used in each reaction and desired bands were observed. The result confirmed that in-house RT-PCR could detect HPIVs in the presence of various viral RNA (Fig. 4).

Detection of HPIVs in clinical samples

Multiplex RT-PCR was performed on 100 inpatient samples. Nine out of 100 tested samples (9%) were positive for the nucleic acid of one type of HPIVs, four HPIV1 and five HPIV2 (Fig. 5). Seasonal distribution of these HPIV-positive samples is shown in Figure 6.

Discussion

Respiratory infections are one of the major health challenges around the world. These infections can cause a wide range of problems from minor symptoms to fatal diseases (17, 18). They are usually more serious and life-threatening in pediatric, geriatric population and patients with cardiac, respiratory, renal and immune deficiency diseases. According to WHO, these infections lead to death of 4 million children annually (19). The causative agents of 80 % of these infections are viruses. One of the important viruses that cause respiratory infections is HPIV, which is the second most common cause of these infections in children under 5 years old, after respiratory syncytial virus. Bronchiolitis, croup and pneumonia caused by HPIV has costed the USA health care system about 259 million dollars each year from 1991 to 2010 (1).

HPIVs can be transmitted through direct and indirect contact with infected discharges and respiratory droplets. Different factors such as: humidity, air flow rate, etc. have been proposed to be involved in transmission of the disease and seasonal pattern of its outbreaks (20), which shows the importance of health care planning in disease prevention in areas with a higher risk of outbreaks and antibiotic management, in case of outbreak (21).

Data about the efficacy of antiviral treatments against HPIVs are limited and contradictory. Ribavirin is proposed to be effective in transplant patients in one study, while negative results were seen in other studies. Although it is used to treat severe HPIV infections in transplant patients, no strong data exists on its benefit (11). On the other hand, efforts to develop a vaccine for these infections have not been successful (2). So, early and accurate diagnosis of the viral infection should still be the top priority of the health care system in order to prevent secondary bacterial infection, which increases morbidity and mortality and unnecessary use of antibiotics (18).

Radiographic methods are inefficient in diagnosing viral infections, as they cannot differentiate between different viruses, and even are unable to diagnose viral infections from bacterial ones. Considering the shortcomings of clinical methods, laboratory tests are needed. Different laboratory assays such as serological methods (including immunofluorescence and IgG measurement), viral culture, and molecular methods have been used. Every method has its advantages and disadvantages, but molecular methods like RT-PCR and real-time RT-PCR have been shown to have high efficiency and precision, are more rapid in virus detection -even in low viral loads-, and are able to determine the viral types and subtypes. Comparing to the other methods, nucleic acid based techniques cost less when used in large scales (16, 21–24).

It should be emphasized that the presence of viral RNA in patient sample alone can not prove that the present virus is the cause of the infection. Therefore, samples from patients. who present respiratory infection symptoms, should be evaluated for the presence of all common respiratory viruses, which suggests the use of multiplex methods. By using multiplex RT-PCR, fast, affordable, precise and definitive diagnosis can be achieved (10).

Real-time RT-PCR has been suggested by some studies to be more sensitive than RT-PCR, but conventional RT-PCR costs less and is more available for large-scale use as a regular test for diagnosing viral respiratory infections (18).

In consideration of all above-mentioned factors, a multiplex RT-PCR assay was chosen to be set up as a diagnostic test for recognizing all 4 types of HPIV in a single-tube reaction in nasopharyngeal samples.

Genes for HN, phosphoprotein, fusion protein, and nucleoprotein have been used as target genes for primer design in order to simulta-

neously identify HPIVs with RT-PCR (3, 25–27). Among all 4 types of HPIV, HN gene has the most genetic and antigenic differences (6), which makes it a very suitable choice in primer designing and was used as the target gene for the primers in present study. In addition, in a multiplex reaction, melting temperature of each primer, PCR product size, the final concentration of each primer, and other components of PCR should be considered to ensure yielding all desired products in the reaction (28). Taking all steps necessary to eliminate inhibitors and impurities in samples and avoiding contamination (especially in presence of plasmids) in the PCR process is crucial.

Although the set in-house multiplex RT-PCR was able to detect the nucleic acid of HPIV types in viral panel and clinical samples, the number of samples was not enough to draw a conclusion about the epidemiology and seasonal pattern of HPIVs. However, the most positive samples in our study were collected during winter (more than 44 percent of total positive samples).

In conclusion, the present study shows that the designed multiplex RT-PCR could detect HPIVs nucleic acid in clinical samples, which makes it an option for rapid and precise detection of HPIVs with a low cost in a larger scale and as a standard assay for diagnosis in health care protocols.

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