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Molecular detection of human parechovirus in under-Five-Year-Old Children with gastroenteritis

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A R T I C L E  I N F O

Article history:
Received 18 August 2016
Received in revised form 26 September 2016
Accepted 3 October 2016

Keywords:
Parechovirus
Real time-PCR
Gastroenteritis
RNA extraction
coi-infection

A B S T R A C T

Background: Currently, RT-PCR is used widely and considered to be a convenient, useful, and powerful method for molecular diagnosis, to detect pathogens from clinical specimens.

Objectives: In this work we describe the development of an in-house Real-time Taqman PCR assay for quantification of HPeV in stool specimens.

Study designs: A total of 137 fecal specimens previously screened for rotavirus and adenovirus were tested for HPeV virus.

Results: A total of 11 out of 137 (8%) episodes of acute gastroenteritis were associated with HPeV genomic detection with median viral load 14678 ± 28927 genomes/mg fecal specimens. There was no significant difference in the detection rate between male and female (54.5% (6/11) vs. 45.5% (5/11). Among the 11 HPeV-positive cases, 2 were also positive for other viral pathogens, including rotavirus (n = 2).

Conclusion: In conclusion, the development of a laboratory designed Real Time PCR TaqMan assay for quantitative detection of HPeV and the optimization and standardization of this assay using stool of children with acute gastroenteritis are described.

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1. Background

Gastroenteritis is a common disease in children, characterized by diarrhea, vomiting, abdominal pain, and fever. Especially in low-income countries, gastroenteritis in children is related to substantial mortality and morbidity. Pediatric gastroenteritis remains the second most common cause of child mortality worldwide [1]. Diarrheal disease can be caused by a wide spectrum of parasitic, bacterial, and viral pathogens. In the past decade, there has been a great shift from conventional methods to identify viral pathogens to the utilization of polymerase chain reaction (PCR) assays, which have proven their advantages over previously used techniques, such as rapid antigen detection and viral culture [2]. The major advantages of molecular diagnostic methods are lower detection limits and, therefore, higher analytical sensitivity for common viruses such as rotavirus (RV) and adenovirus (AdV), and their ability to detect in cultivable viruses such as norovirus (NoV), sapovirus (SaV), and astrovirus (AstV) [3–6] or more cultivable viruses such as Human Parechoviruses (HPeVs) [7]. Additionally, real-time PCR has the competence to determine the viral load in a semi-quantitative way, expressed as the amount of amplification cycles (cycle threshold (Ct) - value) necessary to detect the pathogen.

Human parechoviruses belong to the family of Picornaviridae, a highly diverse group of small, non-enveloped, single-stranded RNA viruses of positive polarity, many of which cause disease in humans [7]. The genera within the family include important pathogens such as rhinoviruses, enteroviruses, polioviruses and hepatoviruses. On the basis of the phylogenetic analyses of the VP1 (Viral Protein capsid-coding region) encoding region, 16 types have been described. Type 1 (HPeV1) and type 2 (HPeV2) were discovered in children with diarrhea in the United States in 1956, initially designated as echovirus types 22 and 23. In pediatrics, HPeV1 and HPeV2 cause mild gastrointestinal or respiratory illness; more serious diseases have been occasionally reported, including myocarditis, encephalitis, pneumonia, flaccid paralysis, meningitis, fatal neonatal infection and Reye syndrome [7]. HPeV3 not only causes mild gastrointestinal and respiratory tract illness, but also severe illnesses such as sepsis and conditions involving the central nervous system (CNS) [7], whereas HPeV4–8 seem to cause dis-
eases similar to those associated with HPeV1 and HPeV2 infections [7]. HPeV9–16 seem to be rare in human diseases. Little is known about this viral agent causing acute gastroenteritis in Italy, except for one study of HPeV detection in acute gastroenteritis in infants and children less than 5 years old [8]. Thus, the role and effect of HPeV infection in acute gastroenteritis is unclear.

2. Objectives

This paper presents the novel realtime RT-PCR assay for the detection of HPeVs for clinical fecal samples collected from hospitalised children with acute gastroenteritis in Piedmont (northern Italy) from December 2014 to April 2015.

3. Study designs

3.1. Virus

Human Parechovirus type 1 ATCC VR-52 (WWR International Milano Italy) were used as control.

3.2. Target sequence selection

Primers and probes specific to HPeV were designed corresponding to nucleotide positions 518–616 (positions follow Human parechovirus 1 strain BJ-37358, Genbank: Kj659491.1). This region shows high conservation among different strains of HPeV and cover all the 8 human pathogen types. The forward and reverse primer sequences were 5’- CACATGTGAAGCCACGGA -3’ and 5’-GGCCCCAGATCAGATCCA –3’, respectively. The probe sequence was 5’- CAGTGGCTCTTGATGCCTGACTTCT – 3’ labeled with JOE at the 5’ end and a BHQ2 at the 3’ end. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The primers for GAPDH were previously published [9].

3.3. Fecal specimens

A total of 137 fecal specimens previously screened for rotavirus and adenovirus were tested for HPeV virus. The samples were collected from under-five-year-old children with acute gastroenteritis in pediatric Hospital Regina Margherita of Turin in Italy from December 2014 to April 2015. The study was approved by our institutional Ethics Committee on 24/11/2014 prot. Number 116918.

3.4. Nucleic acid extraction

A total of 50 μg of fecal specimens (previously diluted 1/10 in 0.9% NaCl buffer saline solution) DNA were extracted with RNAzol (QIAGEN, Valencia, CA) as previously report and on the automated extractor Maxwell (Promega, Madison, WI) using simply RNA Blood Kit protocol without modification. Samples were eluted in 50 μL by both methods.

3.5. Reverse transcription (RT)

For reverse transcription, 5 μL of the stored, extracted RNA was added to a reagent mixture consisting of 5x First Strand Buffer (Applied Biosystems, Carlsbad, CA, USA), 10 mM dNTPs (Sigma, St. Louis, Missouri, USA), MuLV (50 U/μL) (Applied Biosystems, Carlsbad, CA, USA), random primer (50 μM) (hexa-deoxyribonucleotide mixture) (Promega), RNase Inhibitor (20 U/μl) (Promega), and distilled water. The total volume of reaction mixture was 50 μL. RT reaction was carried out at 42 °C for 15’, followed by 99 °C for 5’ and then 5’ °C for 5’. The optimization of the PCR reaction was performed by determining the optimal amount of primers and probe, as well as the cycling condition. The optimized HPeV PCR reaction contained 5 μL of extracted DNA, 200 nM of HPeV primer forward, 900 nM of HPeV primer reverse, 200 nM of the HPeV FAM probe, 1 x PCR Master Mix (Applied Biosystems), in a total of 20 μL reaction. The PCR amplification was carried out for 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, 1 min at 60 °C on the 7500 ABI real time instrument (Applied Biosystems).

3.6. Real time PCR

The accuracy or intra- and inter-assay variability (CV) was evaluated using different concentrations of standard plasmids (ranging from 10^4 to 10^2 copies/reaction) spiked in stool matrix within a single run (n = 10) or different run experiments (n = 10). The efficiency value, defined as 10^(-1/slope) and ranging usually from 1.7 to 2.2, was evaluated.

3.10. Inhibition studies

Extraction inhibition and PCR inhibition were assessed by spiking stool matrix not containing HPeV with known concentrations of the pGastro2 control prior to and after DNA extraction. Plasmid concentrations used were within 1 log of the limit of sensitivity of the assay.

3.11. Calibration curves

The pGastro2 was serially diluted to construct the calibration curve. The dynamic range, which is defined as the range of dilutions in which a linear regression curve can be constructed, was evaluated using 10-fold dilutions (from 10^10 to 10^6) of pGastro2 copies/μL. The limit of detection is defined the lowest concentration of target quantified. The sensitivity of the assay was evaluated by using serial dilution (from 10^3 to 10^5) of pGastro2. Negative control, a white control, a positive control were included in each batch of test samples for checking up carry-over contamination. The panel was tested in triplicate for 5 days to determine the intra- and inter-assay variability.

3.12. Specificity

The analytical specificity of the RT-PCR assay was determined by testing coxsackievirus types B1 (ATCC VR-28), B2 (ATCC VR-29) and B3 (ATCC VR-30), enterovirus type 68 (ATCC VR-561), 69 (ATCC VR-785), 70 (ATCC VR-836) and 71 (ATCC VR-1432), human respiratory syncytial virus (RSV-A [ATCC VR-26], human parainfluenza
viruses (PIV 1 [ATCC VR-94], PIV 2 [ATCC VR-92], PIV 3 [ATCC VR-93]), influenza viruses (influenza A virus H1N1 [ATCC VR-95], H3N2 [ATCC VR-547], influenza B virus [ATCC VR-101]), human adenovirus 3 (ATCC VR-3), 5 (ATCC VR-5) and 7 (ATCC VR-7), herpes simplex virus 1 (ATCC VR-260), human cytomegalovirus (ATCC VR-538), Epstein-Barr virus (ATCC VR-602), human coronavirus types 229E (ATCC VR-740) and OC43 (ATCC VR-1558), Legionella pneumophila (ATCC 33152), Bacillus cereus (ATCC 14579), Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922), haemophilus influenzae (ATCC 49766), staphylococcus aureus (ATCC 25923) and lactobacillus plantarum (ATCC 8014).

3.13. Sequence analysis

The PCR products generated from the PCR were purified and subjected to sequencing to confirm the amplicons of target sequences.

4. Results

4.1. Viral nucleic acid extraction

The performance of the two systems was assessed and compared on 137 fecal specimens undergoing routine testing in under-Five-Year-Old Children with gastroenteritis. For this purpose we compared the results of the GAPDH amplification and demonstrated that the two systems was equivalent (Fig. 1).

Consistent results of HPeV copies were obtained when 50 μg of samples were extracted and eluted in 50 μL by both manual (RNAzol protocol) and automated (Maxwell) methods (Fig. 2).

4.2. Limit of detection

The limit of detection of the assay, defined by the lowest concentration of viral target that was detected with 95% probability, was 10 copies/reaction when pGastro2 was loaded directly into the PCR reaction and 12.5 copies/mg when positive HPeV viral genome was spiked into matrix (fecal specimens).

4.3. Linearity

The amplification efficiency, defined by the standard curve slope, was 3.46. The consistency of replicates was measured by the correlation coefficient (R²), which indicates the linearity of the Ct values plotted in the standard curves. The R² index was 0.9982.

4.4. Accuracy

Accuracy can be calculated by the following equation: \(10^{(-1 \cdot \text{slope})}\). The accuracy was expressed as the coefficient of variation (CV) in the log10 values of the concentration. CV value of the Ct and is reported in Table 2.

4.5. Specificity

None of tested microorganism resulted positive, thus indicating that this method is specific for HPeV.

4.6. Quantification of HPeV in fecal specimens

A total of 137 (from 70 males and 67 females) fecal specimens previously screened for rotavirus and adenovirus were collected from infants and children with acute gastroenteritis in pediatric Hospital Regina Margherita of Turin in Italy from December 2014 to April 2015. The mean age of the tested cases were 21.6 months. All fecal specimens were tested for the presence of HPeV with specific primers and probe. A total of 11 out of 137 (8%) episodes of acute gastroenteritis were associated with HPeV genomic detection with median viral load 14678 ± 28927 genomes/mg fecal specimen, calculated with formula above:

\[
\text{genomes/mgFeces} = \frac{\left( \text{genomes/reaction} \div 50 \right) \times \text{RNAconcentration}}{\text{mgFeces} \div 10}
\]

There was no significant difference in the detection rate between male and female (54.5% [6/11] vs. 45.5% [5/11]). Among the 11 HPeV-positive cases, 2 were also positive for other viral pathogens, including rotavirus (n = 2). The results shown in Table 1 revealed that there was no difference in term of symptoms (diarrhea, vomiting and temperature) between HPeV positive and

### Table 1
Clinical features of parechovirus infected children.

| Symptoms/Features | PARECHOV positive | PARECHOV negative | p-Value | OR (95% CI) |
|-------------------|-------------------|-------------------|---------|-------------|
| Diarrhea + vomiting | 8/11 (72.7%) | 95/126 (75.4%) | 1 | 0.8702 |
| Vomiting | 2/11 (18.2%) | 13/126 (10.3%) | 0.3444 | 1.932 |
| N' ep/day: 1–5 | 5/11 (45.5%) | 72/126 (57.1%) | 0.5339 | 0.6250 |
| N' ep/day: 5–10 | 2/11 (18.2%) | 29/126 (23%) | 1 | 0.7433 |
| N' ep/day: >10 | 3/11 (27.3%) | 7/126 (5.6%) | 0.0342 | 6.375 |
| Diarrhea | 1/11 (9.1%) | 18/126 (14.3%) | 1 | 0.6000 |
| N' ep/day: 1–5 | 4/11 (36.4%) | 74/126 (58.7%) | 0.2063 | 0.4015 |
| N' ep/day: 5–10 | 3/11 (27.3%) | 34/126 (27%) | 1 | 1.015 |
| N' ep/day: >10 | 2/11 (18.2%) | 5/126 (4%) | 0.0988 | 5.378 |
| Fever (>38 °C) | 5/11 (45.5%) | 80/126 (63.5%) | 0.3320 | 0.4792 |
| Age <24 m | 7/11 (63.6%) | 81/126 (64.3%) | 1 | 0.9722 |
| Coinfections (Rota-Adeno-Cultured Bacteria) | 2/11 (18.2%) | 41/126 (41.5%) | 0.5019 | 0.4607 |
| Coinfections: Rotavirus | 2/11 (18.2%) | 36/126 (28.6%) | 0.7272 | 0.5556 |
| Coinfections: Adenovirus | None | None | None | None |
| Coinfections: Cultured Bacteria | None | None | None | 3/126 (2.4%) |
negative clinical fecal specimens except for vomiting in which 3/11 (27.3%) HPeV positive vs 7/126 (5.6%) HPeV negative have had more than 10 episode per day (Table 1).

4.7. Sequence analysis

All the samples tested resulted HPeV type 1.

5. Discussion

Currently, RT-PCR is used widely and considered to be a convenient, useful, and powerful method for molecular diagnosis, to detect pathogens from clinical specimens. With the development of real time PCR assays, many of the limitations related to performing PCR amplification in a clinical laboratory have been overcome.

In this work we describe the development of an in-house Real-time Taqman PCR assay for quantification of HPeV in stool specimens and the assessment of a RNA extraction methods. The performance of the two RNA extraction systems, RNAzol and automated simply RNA Blood Kit (Maxwell) were compared. The difference resulted was not statistically significant. RNAzol produce a more concentrated RNA (p < 0.0001) but the amplification of GAPDH mRNA revealed equal results (p = 0.2647). All the samples resulted HPeV positive for both RNA extraction methods with a concordance of 100%.

The advantage of Real-Time PCR assay in comparison to conventional PCR, include a reduced turnaround time, minimizing the potential for carry-over contamination and the ability to scrutinize closely the assay performance, thus representing a suitable tool for routine diagnosis in virology. The development of a “in house” Real Time-RT-PCR assay, available for diagnostic routine, requires an analogous process applied to marked and approved commercial kit for diagnostic use [10,11]. This is realized by the standardization and optimization of amplification protocol. The sensitivity of our assay was found to be of 10 copies/reaction with a linearity range from $10^{10}$ to $10^2$ copies/reaction. The accuracy obtained in spiked test was good, with very low CV (Table 2).

Epidemiological studies of HPeV are required to further our understanding of disease causation and geographic distribution. We tested stool specimens collected from children less than 5 years old for HPeV between December 2014 to April 2015 in Piedmont, Italy. The percentage of HPeV-positive specimens (8%) was lower than that found in other studies in China (55% in Shanghai [12], 25.3% in Lanzhou [13] and 13.4%) but higher than Italian study (6% [8] or 1% [14]). The discrepancy might be due to the geographical location, the specific year of sample collection, or the use of different PCR methods. Several studies have evaluated the clinical impact of mixed infections believed to be the cause of severe diarrhea in children under 5, and they have found that the frequencies of mixed infections fluctuate between 5% and 34% [14–18] in agreement with our data (31.4%). It is difficult to compare such findings as dual infections are often misdiagnosed or not investigated at all in routine laboratory work. For example in our Institution only Rotavirus, Adenovirus and Cultivable bacteria are included in routine tests. Despite the evidence that HPeV play a significant role in various severe pediatric infectious diseases, diagnostic assays are not available for routine clinical practice in every center and therefore their involvement in clinical illnesses is substantially underestimated.

In conclusion, the development of a laboratory designed Real Time PCR TaqMan assay for quantitative detection of HPeV and the optimization and standardization of this assay using stool of children with acute gastroenteritis are described. There is a high degree of variability in analytical performance of quantitative PCR-based assays amongst clinical laboratories. Quantitative monitoring of HPeV loads for individual patients should be performed by the same assay and by the same laboratory. In addition, the interpretation
should be based on viral load trends instead of single determinations. The clinical utility of quantitative molecular assays, such as HPeV load, will be markedly improved. This method should solve an emerging clinical problem in the era of increased use of molecular diagnostic tools. Furthermore, more research is needed to clarify the specific characteristics of this clinically relevant group of viruses and develop appropriate treatment strategies.

Funding
This study was funded by Institutional grant University of Turin (ex 60% 2015).

Competing interests
None.

Ethical approval
The study was approved by our institutional Ethics Committee on 24/11/2014 prot. Number 116918.

Acknowledgments
The authors thank the members of the Department of Public Health and Pediatrics and gratefully acknowledge the Director AM. Cuffini.

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