Development of a real-time RT-PCR assay for the detection of pan-human parechoviruses

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Research Article

Keywords: Human parechoviruses, VP1 region, real-time RT-PCR, Typing, Phylogenetics

Posted Date: July 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-736674/v1>

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Version of Record: A version of this preprint was published at Virology Journal on November 20th, 2021.
See the published version at <https://doi.org/10.1186/s12985-021-01689-8>.

Abstract

Background

Parechoviruses (PeV-As), which constitute a new genus within the family *Picornaviridae*, have been associated with numerous localized outbreaks of serious diseases, such as coryza, pneumonia, maculopapular exanthem, and conjunctivitis. However, only a few laboratories worldwide routinely conduct tests for the identification of this group of viruses; presently, to the best of our knowledge, no laboratory in China conducts routine test for PeV-A identification. Therefore, in this study, we aimed to develop and validate a real-time RT-PCR assay for the identification of PeV-As.

Methods

To designed and validate a real-time PCR primer-probe targeting the *5'-UTR* region of PeV-As, the *5'-UTR* sequences of PeV-As available in GenBank were aligned using the MUSCLE algorithm in MEGA v7.0. Thereafter, the highly conserved *5'-UTR* region was selected, and its primer-probe sequence was designed using Primer Premier v5.0. This primer-probe sequence was then evaluated for specificity, sensitivity, and repeatability, and for its validation, it was tested using fecal samples from 728 healthy children living in Beijing (China).

Results

The PeV-A real-time RT-PCR assay detected only the RNA-positive standards of PeV-A genotypes (1–8, 14, 17, and 18), whereas 72 serotypes of non-PeV-A EV viruses were undetected. In addition, the *VP1* region of these 11 PeV-A genotypes that tested positive were amplified using the primers designed in this study. Typing results indicated that eight, one, and two strains of the 11 were PeV-A1, PeV-A4, and PeV-A6, respectively. We also determined and presented the genetic characterization and phylogenetic analyses results corresponding to these 11 *VP1* region sequences. Furthermore, the real-time RT-PCR assay showed good repeatability, reproducibility, and sensitivity.

Conclusion

Our findings suggested that the real-time RT-PCR assay developed in this study can be applied for routine PeV-A identification. Additionally, we believe that our results will serve as a foundation for further studies on PeV-As and facilitate the expansion of the gene sequence information available in GenBank.

1. Background

Human parechoviruses (PeV-As), which are RNA viruses that are closely related to enteroviruses of the family *Picornaviridae*, were first isolated several decades ago (1). They are positive-strand RNA viruses

belonging to the genus *Parechovirus*, which is part of the expanding *Picornaviridae* family. Currently, 19 PeV-As are known to infect humans. Specifically, human parechoviruses type 1 and 2 (PeV-A1 and PeV-A2, respectively), which were first identified in 1956 during a summer outbreak of diarrhea in children, were classified as echoviruses type 22 and 23 in the genus *Enterovirus* (EV), respectively (2, 3). The roles played by these pathogens in human diseases were recently elucidated via molecular diagnostic techniques. In 1997, advances in molecular biology led to the discovery of significant differences between the genomic structures, encoded proteins, and biological properties of these pathogens and those of enteroviruses, resulting in them being renamed parechoviruses type 1 and 2, respectively (4). However, similar to enteroviruses, PeV-As, which primarily replicate in the gut, are typically transmitted via the fecal-oral route but can also be transmitted via the nasopharyngeal route (5). The PeV-A genome is approximately 7300 bp long and divided into four distinct regions, namely, the 5'-untranslated region (5'-UTR), an open reading frame region (ORF), the 3'-UTR region, and the poly(A) tail region (6, 7). Additionally, PeV-A capsids each comprise 60 copies of three viral proteins, VP0, VP1, and VP3 (8), that cleave post-translationally from a single polyprotein that forms the basic building block of the capsids. Moreover, in most small RNA viruses, VP0 is cleaved into fragments VP2 and VP4 following viral particle assembly (9). Nonetheless, unlike the VP0 of other small-RNA viruses, the VP0 of PeV-As remains intact, resulting in this capsid containing only three instead of four distinct polypeptides (8, 10). The slow, laborious, and insensitive cell culturing processes that are used to diagnose PeV-A infections have been largely replaced by nucleic acid amplification procedures such as reverse transcription polymerase chain reaction (RT-PCR), which can be used to target the conserved 5'-UTR region (11–15). Furthermore, in 1999, a molecular typing method based on aligning the RT-PCR-amplified sequences of the VP1 coding region with sequence data corresponding to all PeV-A genotypes (the nucleotide sequence consistency in the VP1 region of identical strains was greater than 75%) was introduced as a more effective technique for genotype determination (16, 17).

Of the 19 parechovirus genotypes (PeV-A1–19) that are currently known to infect humans, genotypes 1 and 3 are most commonly associated with diseases (18, 19). It has also been observed that PeV-As, which commonly cause high persistent fevers in infants aged less than 5 months, are associated with sepsis-like conditions. Reportedly, the outbreak of a sepsis-like disease in children that occurred in Australia between 2013 and 2014 was caused by PeV-A3 (20). Additionally, in 2014, a study in Kansas and Missouri demonstrated that PeV-A3 can cause severe neurologic illness in neonates, and younger infants are more likely to require intensive care owing to this infection (21). It is also known that maculopapular exanthema, which is a common and distinctive rash involving the extremities, with palmar and plantar erythema is associated with PeV-A3 infection. In recent years, PeV-A-related disease outbreaks have also been reported in Japan and Croatia (22, 23). To effectively treat such PeV-A-associated diseases, the development of a sensitive and reproducible PeV-A identification method with good specificity is necessary. Therefore, in this study, we developed and validated a real-time RT-PCR assay for the identification of PeV-As using 728 clinical samples. Positive samples were amplified via nested RT-PCR, and the VP1 region was sequenced using the primers designed in this study. We also performed characterization and phylogenetic analysis on the VP1 sequence. The results of this study

provide a firm theoretical basis for further studies on PeV-As and help to expand the gene sequence information available in GenBank.

2. Methods

2.1 Real-time RT-PCR

2.1.1 Primer and probe design

The 5'-UTR sequences of PeV-As available in GenBank were aligned using the MUSCLE algorithm in MEGA v7.0 (24). Based on the results of this multiple sequence alignment, the highly conserved 5'-UTR region was selected, and the 5'-UTR primer-probe sequence (Table 1) was designed in accordance with the principles for designing TaqMan RT-PCR primer and probe sequences using Primer Premier v5.0 (Premier Biosoft, Palo Alto, CA, USA). In addition, the designed primer and probe sequences were screened via BLAST to detect the possibility of cross-reactivity with non-PeV-As.

Table 1
Forward (F) and reverse (R) primers and probe (P) for the detection of human parechovirus (PeV-A) and primers for amplification of the VP1 region of PeV-A

Name	Primer sequence (5'-3')	Nucleotide Position (nt)*	Reference
PeV-A-5'UTR-F	TGACGTGTGGGAACTTRTTGG	352–372	This study
PeV-A-5'UTR-R	CCTTCGTGGGCCYTACAAGTAGTG	538–561	This study
PeV-A-5'UTR-P	CYCTGGGGCCAAAAGCCAAGGTTT	433–456	This study
PeV-A-VP1-F1	TTYTCYACTTGGATGMGGAARA	2159–2180	This study
PeV-A-VP1-R1	ATRCTYGACATHARNCCWGC	3392–3411	This study
PeV-A-VP1-F2	CNTGGGGYTCVCARATGGAYYT	2340–2361	This study
PeV-A-VP1-R2	CCATARTGYTTRTARAAVCCYCTRT	3081–3105	This study
* Relative to the Harris PeV-A1 strain			

2.1.2 Preparation of positive standards

Only 11 PeV-A genotypes (1–8, 14, 17, and 18) with 5'-UTR region sequences were available in the GenBank database. Thus, the 5'-UTR regions of these strains, PeV-A1 (*Harris strain*), PeV-A2 (*Williamson strain*), PeV-A3 (*A308/99 strain*), PeV-A4 (*K251176-02 strain*), PeV-A5 (*CT86-6760 strain*), PeV-A6 (*NII561-2000 strain*), PeV-A7 (*PAK5045 strain*), PeV-A8 (*BR/217/2006 strain*), PeV-A14 (*V3C strain*), PeV-A17 (*M36/CI/2014 strain*), and PeV-A18 (*GhanaA36 886 strain*), were used as target sequences. After their synthesis, they were cloned into the p-GEM-T Easy Vector system (Promega, Madison, WI, USA).

Thereafter, the plasmids containing these target sequences were cloned by TsingKe Biological Technology Limited (Beijing, China). Additionally, the plasmids were linearized using restriction endonuclease *Sac* I and DNA purification was performed using AMPure XP (Beckman Coulter, CA, USA). The purified linear plasmids were transcribed *in vitro* using a RiboMAX™ Large Scale RNA Production System-T7 kit (Promega, Hilden, Germany) and thereafter purified using the RNeasy Mini Kit (Promega, Hilden, Germany). To calculate the copy numbers, the purified RNA was then spectrophotometrically quantified using the Qubit™ RNA BR Assay Kit (Invitrogen, Eugene, OR, USA).

2.1.3 Reagents, reaction systems, and real-time RT-PCR settings

As a template, 3- μL samples of the nucleic acids were used (the total reaction volume was 25 μL) in a One Step PrimeScript™ RT-PCR Kit (Perfect Real Time, TaKaRa, Dalian, China). The concentrations of each primer and probe in the reaction mixtures were 0.8 and 0.4 μM , respectively. The reaction settings were as follows: the system was maintained at 42°C for 10 min and 95°C for 1 min. This was followed by two 40 cycles of amplification at 95°C for 10 s and 55°C for 40 s. All the real-time RT-PCR experiments were performed using QuantStudio 5 instruments (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

2.1.4 Specificity testing

To assess the specificity of the PeV-A assay, 72 known non-PeV-A strains, including 16 serotypes of EV-A (CVA2–A8, CVA10, CVA12, CVA14, CVA16, EV-A71, EV-A76, EV-A89, EV-A90, and EV-A120), 45 serotypes of EV-B (CVB1–B6, CVA9, E1–7, E9, E11–21, E24–27, E29–33, EV-B74–B75, EV-B79–B81, EV-B83, EV-B85, EV-B93, EV-B97, and EV-B106), and 11 serotypes of EV-C (PV-1, PV-3, CVA1, CVA11, CVA13, CVA17, CVA20–A21, CVA24, EV-C96, and EV-C99) were tested together using 11 types of PeV-A (1–8, 14, 17, and 18) RNA-positive standards. Enterovirus-specific real-time RT-PCR assays showed that all the non-PeV-A strains were positive. Thereafter, they were stored in our laboratory. Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.

2.1.5 Sensitivity and stability testing

Globally, the most popular PeV-A types are PeV-A1 and PeV-A3. Therefore, the sensitivity and stability of the probes was evaluated based on these two PeV-As. The limit of detection (LOD) of the real-time RT-PCR assay was evaluated via a serial 10-fold dilution of PeV-A1 (2.1×10^8 – 2.1×10^1 copies/ μL) and PeV-A3 (1.9×10^8 – 1.9×10^1 copies/ μL). The results were interpreted as 'positive' for threshold cycle (Ct) values ≤ 35 and 'negative' for Ct > 35 . The obtained PeV-A1 and PeV-A3 RNA standards were diluted 10-fold. Thereafter, five different concentrations (2.1×10^7 – 2.1×10^3 and 1.9×10^7 – 1.9×10^3 copies/ μL for PeV-A1 and PeV-A3, respectively) were selected as templates based on three parallel replicate experiments. The standard deviation (SD) and coefficient of variation (CV) of the Ct values calculated for each dilution based on the triplicates were used to evaluate the reproducibility of the real-time RT-PCR assay. In this regard, the approximate proportion of the Ct value that could be explained by the copy number was

determined based on the coefficient of determination (R^2), and SD served as an indicator of the variability of the Ct value; the larger this value, the greater the variability of the Ct value. CV = standard deviation/mean was used to compare the degree of variability between different gradients; the larger the CV value, the greater the relative variability.

2.2 Clinical samples for validation

2.2.1 Sample material

To further validate this method, experiments were conducted using stool from healthy children in Beijing. This study involved no human experimentation. Within the June–November 2019 period, a total of 728 stool samples were collected from 549 healthy children living in Beijing, China. All the 549 children, who participated in the study, were aged below 5 years. Additionally, the children's parents agreed to the use of their stool samples for research purposes, and the study was approved by the Second Ethics Review Committee of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. All the experimental protocols were approved by the National Institute for Viral Disease Prevention and Control, and all the methods were performed in accordance with the approved guidelines. Of these 549 children, 284 (51.73%) were boys, and 265 (48.29%) were girls. The stool samples were collected from children living in five districts in Beijing (Haidian, $n = 179$; Daxing, $n = 183$; Dongcheng, $n = 117$; Huairou, $n = 122$; and Fengtai, $n = 127$). These samples were sent to the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (CDC) for evaluation.

2.2.2 Stool sample treatment and nucleic acid extraction

All the fecal samples were intended for nucleic acid extraction; therefore, they required special handling. The stool samples (approximately 2 g each) were dissolved in 10 mL of phosphate-buffered saline containing antibiotics and thereafter treated with 1 g of glass beads and 1 mL of chloroform, followed by vigorous shaking for 20 min using a mechanical shaker and centrifugation for 20 min at $1500 \times g$ in a refrigerated centrifuge. Then, the supernatant was stored at -80°C until further analysis. RNA was extracted from the stool suspension using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).

2.2.3 Comparison of different real-time RT-PCR assays for PeV-A detection

A TaKaRa One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) was used to detect viral RNA via real-time RT-PCR using the probe/primer sequences of PeV-A designed in this study and the probe/primer sequences of PeV-A reported in a previous study (AN345, AN344, and AN257) (25). The reaction settings corresponding to the probe/primer sequences designed in this study were as follows: the system was maintained at 42°C for 10 min and at 95°C for 1 min, followed by two 40 cycles of amplification at 95°C for 10 s and at 55°C for 40 s. The reaction settings corresponding to primer/probe sequences, AN345, AN344, and AN257, for PeV-A detection were set as previously reported (25).

2.2.4 *VP1* region amplification and sequencing and molecular typing

The *VP1* region of PeV-As was amplified via nested PCR using the Primescript One Step RT-PCR Kit Ver.2 (TaKaRa). The first reaction system (25 µL) contained 3 µL of the original sample template RNA, 1 µL of PrimeScript 1 Step Enzyme Mix, 12.5 µL of 2× 1 Step Buffer (Dye Plus), 7.5 µL of RNase Free dH₂O, 0.5 µL of forward primer-F1, and 0.5 µL of reverse primer-R1 (all the final concentrations were 20 µM). The reaction settings were as follows: the system was maintained at 50°C for 30 min and at 94°C for 2 min, followed by four 40 cycles of amplification at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 20 s, and finally 72°C for 7 min. In the second reaction system, the template was the product of the previous reaction, and the primers were F2/R2 (Table 1). The reaction settings were as follows: the system was maintained at 94°C for 2 min, followed by four 40 cycles of amplification at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 20 s, and finally 72°C for 7 min. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), resulting in each strand being sequenced at least once. The EV Genotyping Tool and BLAST were used to identify the parechovirus type, based on the obtained partial *VP1* sequences (26).

2.2.5 Phylogenetic analysis

The *VP1* sequences of the PeV-A strains were aligned with PeV-A prototype strains using the MUSCLE algorithm implemented via MEGA v7.0(24). A nucleotide identity matrix and an amino acid identity matrix were generated using BioEdit v7.0.9.0 (27). Eleven strains as well as the nucleotide and amino acid sequences that showed more than 90% similarity to those of the *VP1* region, based on BLAST, were downloaded to construct a maximum likelihood (ML) tree. The ML trees constructed using the GTR + G model, as suggested by ModelGenerator0.85, were then implemented in MEGA with 1000 bootstrap replicates. In addition, to verify the best topology, ML trees were constructed using RAXML version8.2.12 (28).

3. Results

3.1 Real-time RT-PCR

3.1.1 Specificity testing

In this study, performing the real-time RT-PCR assay for the identification of PeV-As led to the detection of only the RNA-positive standards of PeV-A genotypes (1–8, 14, 17, and 18), while 72 serotypes of non-PeV-A EV viruses were undetected (Fig. 1).

3.1.2 Sensitivity testing

To determine the sensitivity of the PeV-A real-time RT-PCR assay, serial dilution of RNA transcripts derived from the clones of the 5'-UTR region of the 11 PeV-A genotypes (1–8, 14, 17, and 18) was performed, and the corresponding Ct values were recorded (Fig. 2). Thereafter, standard curves were constructed using the Log value of the RNA copies as the x-axis and the Ct value as the y-axis. The coefficient of determination (R^2) values were 0.9987 and 0.9982, respectively, and the LODs were both 10^2 copies/ μ L.

3.1.3 Repeatability testing

To determine the repeatability of the real-time RT-PCR assay, five RNA-positive standards with different PeV-A1 and PeV-A3 concentrations (2.1×10^7 – 2.1×10^3 copies/ μ L and 1.9×10^7 – 1.9×10^3 copies/ μ L, respectively) were tested using three parallel replicates. The SD values corresponding to the different dilutions were all less than 0.5, while the CV values were all less than 2% (Table 2), indicating good repeatability.

Table 2
Intra-assay reproducibility of the PeV-A real-time RT-PCR

Copy no. (copies/ μ L)	Ct values ^a								CV ^b	
	Mean		SD		Max		Min		PeV-A1	PeV-A3
	PeV-A1	PeV-A3	PeV-A1	PeV-A3	PeV-A1	PeV-A3	PeV-A1	PeV-A3		
10^7	14.46	15.99	0.49	0.09	14.51	16.05	14.18	15.89	0.011	0.005
10^6	18.86	19.13	0.07	0.20	18.90	19.32	18.77	18.93	0.003	0.010
10^5	22.00	22.98	0.14	0.21	22.10	23.22	21.84	22.84	0.006	0.009
10^4	26.02	26.81	0.16	0.18	26.20	27.02	25.88	26.68	0.006	0.007
10^3	29.68	30.19	0.04	0.24	29.73	30.45	29.64	29.98	0.001	0.008
^a Maximum (Max) and minimum (Min) values are within 3 SD from the mean Ct.										
^b Coefficient of variation.										

3.2 Validation of clinical specimens

3.2.1 Virus detection and genotyping

Using the probe-primer designed in this study, 11 samples tested positive for PeV-A, eight of which were positive with the probe-primers, AN345, AN344, and AN 257. The prevalence of PeV-A infection did not differ significantly between boys and girls ($P > 0.1$). Additionally, the partial VP1 region (696–702 bp) of the 11 strains was successfully determined via RT-PCR, and three types of PeV-As, including PeV-A1 ($n =$

8), PeV-A4 (n = 1), and PeV-A6 (n = 2), were identified using the Online Enterovirus Genotyping Tool (Table 3).

Table 3
Comparison of two methods used to test 728 fecal samples

Virus type	Probe-primer	No. positive ^a	Genotype (No.) ^b	Reference
PeV-A	PeV-A-5'UTR-F/R/P	11	PeV-A1 (n = 8), PeV-A4 (n = 1), PeV-A6 (n = 2)	This study (25)
	AN344, AN345, AN257	8	PeV-A1 (n = 7), PeV-A6 (n = 1)	
^a Results of real-time RT-PCR test.				
^b Result of molecular typing detected via RT-PCR.				

3.2.2 Genomic characterization of *VP1* region

The nucleotide sequences of the 11 strains isolated from the stool samples were compared pairwise with those corresponding to the PeV-A1 (*Harris strain*), PeV-A4 (*K251176-02 strain*), and PeV-A6 (*NIJ561-2000 strain*) prototype strains, as well as other PeV-A prototype strains. Similarities between the nucleotide sequences of the *VP1* region and the amino acid sequences of eight PeV-A1 strains and the PeV-A1 prototype strain were 73.9–75.4% and 87.9–89.2%, respectively. Additionally, the similarities between the nucleotide sequences of the *VP1* region and amino acid sequences of the PeV-A4 strain and PeV-A4 prototype strain were 82.6% and 95.6%, respectively, and those between the nucleotide sequences of the *VP1* region and amino acid sequences of the two PeV-A6 strains and the PeV-A6 prototype strain were 94.7–95.6% and 96.9%, respectively.

3.2.3 Phylogenetic analysis of the *VP1* region

A phylogenetic tree was established based on the nucleotide sequences of the *VP1* coding regions of the 11 PeV-A strains identified and all the PeV-A prototype strains in the GenBank database (Fig. 3(a)). This phylogenetic tree indicated that eight strains clustered with the PeV-A1 prototype strain, while one strain clustered with the PeV-A4 prototype strain, and two strains clustered with the PeV-A6 prototype strain, confirming the molecular typing results.

The phylogenetic tree based on the nucleotide sequences downloaded from BLAST and eight *VP1* region sequences showed that three of the PeV-A1 strains clustered with strains isolated from Belarus, while the remaining five PeV-A1 strains clustered with strains isolated from Hong Kong (Fig. 3(b)). Moreover, the phylogenetic tree based on the PeV-A4 strains showed that the PeV-A4 strain clustered with strains

isolated from Hong Kong (Fig. 3(c)), while the two PeV-A6 strains clustered with strains isolated from Hong Kong (Fig. 3(d)).

4. Discussion

Parechoviruses constitutes a new genus within the *Picornaviridae* family, and PeV-A1, which is considered to be a very common pathogen, is prevalent worldwide (29). PeV-A3 has caused several global outbreaks of severe diseases in children (20, 22, 23). However, to the best of our knowledge, no laboratory in China conducts routine tests for the identification of these viruses. Even though parechoviruses and enteroviruses are similar in terms of genomic structure (1), enterovirus testing methods do not detect parechoviruses. Thus, molecular diagnostic methods, such as real-time RT-PCR, are becoming increasingly important in disease diagnosis owing to their rapid sensitivity. Furthermore, sensitive nucleotide detection methodologies contribute to a better understanding of the epidemiology and natural history of diseases. Therefore, in this study, we designed and validated a real-time RT-PCR primer-probe targeting the 5'-UTR region of PeV-A. PeV-A-positive standards were tested together with 72 serotypes of known non-PeV-A strains (enteroviruses), and the results showed no cross-reactions and specifically detected only PeV-As. The LOD of this real-time PCR assay was 10^2 copies/ μ L, indicating that the assay system has good sensitivity. Additionally, the *CV* values corresponding to this assay were all less than 1.5%, demonstrating that it shows good reproducibility. Given that the 5'-UTR regions of different types of PeV-As are highly conserved, it is possible that this assay has potential for application in detecting new or emerging PeV-As.

When 728 stool samples from healthy children in Beijing, China, were tested using this established real-time PCR method as well as a previous method(25), 11 and eight samples tested positive, respectively. Nested PCR amplification of the *VP1* region was performed on the 11 positive real-time PCR samples, and their *VP1* sequences were successfully obtained using the primer sequences designed in this study. Additionally, EV Genotyping Tool, BLAST, and ML tree results, based on the *VP1* region, showed that of the 11 strains isolated from the stool samples, eight were PeV-A1, one was PeV-A4, and two were PeV-A6. Comparisons also revealed that the nucleic acid-based similarity between the eight PeV-A1 strains and the prototype strain was not very high (73.9–75.4%), and the combination of the results of nucleic acid similarity analysis with the ML tree based on the PeV-A prototype strains indicated that PeV-A1 exhibits a large evolutionary trend in China. In addition, the ML tree, which was established via BLAST analysis, showed that three of the PeV-A1 strains clustered with strains isolated from Belarus, while the remaining five PeV-A1 strains, as well as the PeV-A4 and PeV-A6 strains, clustered with strains isolated from Hong Kong. Thus, a majority of the isolates were endemic. Reportedly, parechoviruses are associated with a wide range of gastrointestinal, respiratory, and central nervous system infections (30). Although parechovirus infections cannot be treated directly, a rapid and sensitive detection method for their identification may reduce hospitalization and shorten the duration of antibiotic treatment. Therefore, we believe that our study lays the foundation for further research on PeV-As and helps expand the gene sequence information available in GenBank.

This study has some limitations. First, even though the primer-probe used in this study was designed based on all the 5'-UTR region sequences in the current GenBank database, which show good specificity, sensitivity, and stability, during the long evolution of the viral genome, mutations, deletions, or gene recombination resulted in off-target primer-probe sequences and false negative results. Second, except for PeV-A1, 4, and 6 genotypes, we were not able to detect other PeV-A genotypes using this method. Finally, we only performed specificity studies on the enterovirus species A, B, and C and did not validate the accuracy of our system using other species. Therefore, we think that the real-time RT-PCR method for the detection of pan-PeV-As can be improved in future.

5. Conclusions

In this study, we developed and validated a real-time RT-PCR assay for the identification of PeV-As using 728 clinical samples. This real-time RT-PCR assay showed good reproducibility and specificity, and given that the 5'-UTR regions of different types of PeV-As are highly conserved, this assay method possibly has potential for application in the detection of new or emerging PeV-As. In addition, we amplified the *VP1* region of the 11 detected PeV-A genotypes using the primers designed in this study. Typing results indicated that eight, one, and two strains were PeV-A1, PeV-A4, and PeV-A6, respectively. We also presented the results of the genetic characterization and phylogenetic analyses of *VP1* region of these 11 genotype sequences. Therefore, we believe that our study provides a firm foundation for further studies on PeV-As and helps expand the gene sequence information available in GenBank.

Abbreviations

Enterovirus, EV; 5'-untranslated region, 5'-UTR; open reading frame, ORF; 3'-untranslated region, 3'-UTR; limit of detection, LOD; threshold cycle, Ct; standard deviation, SD; coefficient of variation, CV; coefficient of determination, R^2 ; Chinese Center for Disease Control and Prevention, CDC.

Declarations

Ethics approval and content to participate:

Written informed consent for the use of their clinical samples was obtained from all individuals or their guardians included in the study. The study was approved by the Second Ethics Review Committee of the National Institute for Viral Diseases Control and Prevention (IVDC), Chinese Center for Diseases Control and Prevention, all experimental protocols were approved by IVDC and the methods were carried out in accordance with the approved guidelines.

Consent for publication:

Not applicable

Availability of data and materials: *VP1* genome nucleotide sequences corresponding to the 11 PeV-A strains determined in this study have been deposited in the GenBank nucleotide sequence database under accession numbers MW448141–MW448151.

Competing interests: The authors declare that they have no competing interests.

Funding: This study was supported by the National Key Technology R&D Program of China [Project Numbers 2018ZX10711001, ZDZX-2018ZX10101002-001-003, 2017ZX10104001, and 2018ZX10101002-004-006]. We also acknowledge the funding received from the National key research and development project (Project No. 2021YFC0863000), and Beijing Natural Science Foundation (Project No. L192014). The funding bodies were not involved in the design of the study, clinical sample collection, data analysis, or interpretation. They were also not involved in the writing of the manuscript or in the decision to publish the results.

Author Contributions: **HL** conceived and performed the experiments, analyzed the data, drafted the manuscript, and prepared all the figures. **YZ** contributed to the conception and design of the experiments, supervised of projects, and polished the manuscript. **JX** conducted some of the experiments and analyzed the data. **KZ** Conducted some of the experiment. **ZH, YS, DW, TJ, DY, SZ, and WX** analyzed the data. All the authors read and approved the final manuscript.

Acknowledgments: We thank the local staff at Beijing Center for Disease Control and Prevention for collecting stool specimens for assay validation.

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Figures

Figure 1

Evaluation of the specificity of real-time RT-PCR assay for PeV-A detection

Figure 2

Dynamic range and linearity of PeV-A real-time assay. Threshold cycle (Ct) values (y-axis) were plotted against 10-fold dilutions of PeV-A1 and PeV-A3. R², regression coefficient.

Figure 3

Phylogenetic tree based on the sequences of the VP1 region of PeV-As. Maximum likelihood trees were constructed using the GTR + G model and implemented in MEGA7.0 with 1000 bootstrap replicates. The circles and squares represent the 11 detected PeV-A strains and the PeV-A prototype, respectively. The scale bars indicate the genetic distance. All panels have the same proportions. (a) Eleven PeV-A strains and PeV-A prototype strains, (b) Eight PeV-A1 strains and PeV-A1 sequences downloaded from BLAST

server, (c) One PeV-A4 strain and PeV-A4 sequences downloaded from BLAST server, (d) Two PeV-A6 strains and PeV-A6 sequences downloaded from BLAST server.