Fan *et al. Virology Journal* (2025) 22:61
https://doi.org/10.1186/s12985-025-02671-4

Virology Journal

METHODOLOGY Open Access

A *Taq*Man probe-based multiplex real-time quantitative pcr for simultaneous detection of kobuvirus, parechovirus B, rosavirus B, and hunnivirus carried by murine rodents and shrews

Shunchang Fan[1†], Minyi Zhang[1†], Yucheng Li[1], Jingli Tian[1], Juxian Xian[1] and Qing Chen[1*]

Abstract

Background Picornaviruses, common infectious agents in humans and various animal species, pose significant health threats. Conventional monoplex PCR is widely employed in laboratory diagnostics but is relatively time-intensive and laborious.

Results In this study, we developed a multiplex *Taq*Man probe-based real-time quantitative PCR (qPCR) assay for the rapid and simultaneous detection of kobuvirus, parechovirus B, rosavirus B and hunnivirus in murine rodent and shrew samples. The approach demonstrated high sensitivity and specificity, with detection limits of 1×10^2 copies/μL for kobuvirus, parechovirus B, and rosavirus B, and 50 copies/μL for hunnivirus. Evaluation using 149 clinical samples showed strong concordance with conventional PCR methods.

Conclusions This work developed an effective multiplex qPCR method for the simultaneous detection of emerging picornaviruses particularly in rodents, including kobuvirus, parechovirus B, rosavirus B, and hunnivirus. Our findings contribute valuable insights into the monitoring and prevention of zoonotic diseases associated with these pathogens.

Keywords Multiplex qPCR, Picornavirus, Rodent, Shrew, Detection

†Shunchang Fan and Minyi Zhang contributed to the work equally and should be regarded as co-first author.

*Correspondence:
Qing Chen
qingchen@smu.edu.cn
[1]Department of Epidemiology, School of Public Health, Southern Medical University, Guangzhou 510515, China

Background

Picornaviruses, classified within the family *Picornaviridae*, represent a diverse group of non-enveloped viruses characterized by positive-sense single-stranded RNA genomes. As of March 2024, the family *Picornaviridae* encompasses 159 species organized into 68 genera, many of which exhibit significant zoonotic potential and are capable of spreading widely among humans and various animal species, thereby resulting in critical health impacts [1].

Rodents are recognized vectors for more than 60 human infectious diseases, transmitting pathogens via their urine, feces, or through arthropod ectoparasites [2]. Likewise, shrews are predominantly ground-dwelling, yet knowledge of the pathogens they harbor remains limited [3, 4]. Currently, an increasing number of picornaviruses have been detected in these animals. Monitoring the molecular epidemiological characteristics of picornaviruses in these small mammals could help mitigate potential risks to humans.

Kobuvirus, one of the picornaviruses of interest, was initially identified in the stool of patients with gastroenteritis and has been proposed as a potential causative agent of diarrhea [5, 6]. Subsequently, it has been detected in various mammalian species. Previous studies reported that kobuvirus was detected in 50% and 55.5% of murine rodent fecal samples from the USA and Hungary, respectively [7, 8]. Parechovirus B (Ljungan virus), initially isolated from voles, is linked to conditions such as myocarditis, insulin-dependent diabetes mellitus, and Guillain-Barré syndrome [9]. Fevola C. et al. detected parechovirus B in tissue from various rodents and shrews across 12 European countries, identifying bank voles as the primary host (overall prevalence: 15.2%) [10]. Additionally, rosavirus B and hunnivirus are recently identified picornaviruses, first discovered in rodents within the past decade [11]. Although there is no report of human infection with rosavirus B and hunnivirus, considering the evidence of cross-species transmission [12], the possibility of these novel picornaviruses infecting humans cannot be ruled out. In our previous research in southern China, we found that the overall prevalence of kobuvirus in several murine rodents was 23.0%, with hunnivirus at 17.6% and rosavirus reaching 32.5%, with frequent coinfections of these picornaviruses [11–13]. These findings support that picornaviruses are prevalent in murine rodents and shrews. Despite these insights, the role of rodent-derived picornaviruses in the evolution, transmission dynamics, and biological impact of the family *Picornaviridae* remains poorly understood, which underscores the urgent need for comprehensive epidemiological surveillance and molecular studies to elucidate their geographic distribution, genetic diversity, and clinical relevance.

The surveillance of viruses carried by animals requires rapid, sensitive, and specific methods. The current standard for detecting picornaviruses predominantly relies on monoplex PCR, a technique that employs a single set of primers to target one virus at a time [11, 13, 14]. While monoplex PCR is straightforward and offers high specificity, it is time-consuming, cost-inefficient, and lacking the capability for quantitative detection of multiple target viruses. Real-time Quantitative PCR (qPCR), first introduced in 1993, has emerged as a more sensitive and quantitative alternative for monitoring DNA amplification reactions [15, 16]. Given the high incidence of co-infections among picornaviruses within a single host, the development of a rapid and accurate diagnostic tool is essential for elucidating the epidemiological characteristics of these potential diarrheal pathogens. This study aims to develop a multiplex qPCR assay that will enable effective detection, identification, and differentiation of kobuvirus, parechovirus B, rosavirus B and hunnivirus, thus addressing a critical gap in our understanding of rodent-derived picornaviruses.

Methods

Design of primers and probes

For kobuvirus, rosavirus B, and hunnivirus, the primer and probe sequences were designed to target highly conserved regions of the viral genome with the coding sequences of the RNA-dependent RNA polymerase (RdRp) gene. These regions were selected based on multiple sequence alignments of available genomic sequences from representative strains of kobuvirus, rosavirus B, and hunnivirus deposited in GenBank. While, for parechovirus B, 5'UTR is the most common region for detection. MEGA11 software was utilized for multiple sequence alignments, and Oligo 7 software was employed for the ultimate determination of primer-probe sets. The specific sequences for each target virus were provided in Table 1. To facilitate multiplex detection, four fluorochromes with distinct wavelengths, including FAM, CY5, Texas Red, and VIC, were selected for the modification of probes targeting kobuvirus, parechovirus B, rosavirus B, and hunnivirus, respectively. Quenching was achieved using BHQ1, BHQ2, and BHQ3. The primers and probes were synthesized by BGI Genomics Co.,Ltd. (Shenzhen, China).

Construction of plasmid standards

Apart from the 5' UTR region of parechovirus B, which was obtained via gene synthesis, viral RNA from positive samples for the other three picornaviruses was extracted using the MiniBEST Viral RNA/DNA Extraction Kit (Takara Bio Inc.). Following extraction, reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science), with random

Table 1 Primers and probes for the multiplex real-time quantitative PCR assay

Target virus	Primer/Probe Name	Sequence 5′-3′	Length (bp)
Kobuvirus	q-Kobu-F	TGTCATCTACGCYACWGAACCC	197 bp
	q-Kobu-R	CCAWCACWGGGTGGATGTAGA	
	Probe-Kobu-F	FAM-TGGACGCTTGTCATCAGGAACAAACCAG-BHQ1	
Parechovirus B	q-PeVB-F	GTGGCTTTCACCTCTRGACAGT	129 bp
	q-PeVB-R	GTAATCCGYTGGCACTCAAGTAGC	
	Probe-PeVB-F	CY5-CATTCCACACCCGCTCCACGGTAGA-BHQ3	
Rosavirus B	q-Rosa-F	ACGCYCTTGCCGAYGTCCTMA	165 bp
	q-Rosa-R	TCAAYGARTTGAACACRCTAGTCC	
	Probe-Rosa-R	Texas Red-CCYGACGGCATGGCRCCATCRAG-BHQ2	
Hunnivirus	q-Hunni-F	AGAGCAARATGTGGACCTGTG	149 bp
	q-Hunni-R	TCCGTTTGCCAAAGCAGCTC	
	Probe-Hunni-F	VIC -CTTTGGGACTGACAACCTGGACCCGAT-BHQ1	

hexamers serving as the primers. Amplification of target genome fragments was conducted using the primers listed in Supplemental Table S1. Subsequently, viral genome fragments from amplification or gene synthesis were cloned into the pMD18-T vector (Takara Bio Inc.). The ligation reaction comprised 2 μL of viral genome fragment, 1 μL of the pMD18-T clone vector, 2 μL nuclease-free water, and 5 μL of Solution I, with ligation performed at 16 °C for 2 to 3 h.

Subsequently, the recombinant plasmid was transformed into DH5α competent cells (Takara Bio Inc.). Transformants were cultured on solid Luria-Bertani (LB) medium containing ampicillin (Amp) and incubated overnight at 37 °C. Selected monoclonal colonies were inoculated into LB liquid medium with Amp and shaken at 150 rpm for 5 h. Conventional PCR was performed using pMD18-T vector-specific primers (Supplemental Table S1) to amplify the bacterial culture, followed by verification through sequencing. The amplification product length for positive bacterial cultures was expected to equal the combined lengths of the empty vector amplification fragment (155 bp) and the target viral nucleotide fragment. Bacterial transformants containing the correct plasmids were cultured in LB medium supplemented with Amp and incubated overnight at 37 °C. Plasmid extraction was performed using the TIANprep Mini Plasmid Kit (TIANGEN Biotech (beijing) Co.,Ltd.).

The plasmids were linearized using restriction enzymes prior to quantification to ensure accurate quantification of plasmid copy numbers. SnapGene software was employed to confirm the absence of restriction enzyme sites in the target fragment. Quantification of linearized standard plasmids was conducted using OD260 measurements (Thermo Scientific NanoDrop One), and expressed as a copy number. The standard plasmid copy number was calculated using the following formula: y (copies/μL) = (6.02×10^{23}) × concentration (ng/μL) × 10^{-9} DNA) / (DNA length × 660). The final concentration of the plasmids was adjusted to 10^7 copies/μL and then ten-fold serially diluted to 10^0 copies/μL before storage at -80 °C in aliquots.

Reaction conditions for monoplex real-time quantitative PCR assay

To evaluate the amplification performance of each primer-probe set, monoplex qPCR was conducted for each virus. Ten-fold diluted plasmids were used as templates to establish standard curves, from which the R^2 (correlation coefficient) values and standard equations were calculated. The reaction system of monoplex qPCR comprised 0.2 μM of each primer, 0.1 μM of each *Taq*-Man probe, 1 μL of template, 10 μL of 2 × Conc. Premix Ex Taq (Takara Bio Inc.), with nuclease-free water added to reach a final volume of 20 μL. Amplification was carried out on a Roche LightCycler® 96 Instrument (Roche Life Science) with the following program: 95 °C for 30 s; followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s; and a final cooling step at 37 °C for 300 s. Fluorescence signals were automatically collected at the end of each cycle.

Optimization of reaction system for multiplex real-time quantitative PCR assay

The four sets of primers and probes validated through monoplex qPCR were then utilized in the development of multiplex qPCR approach. System optimization involved adjusting the concentration and ratio of primers and probes as previously reported [17]. During the optimization process, the concentration of primers was varied from 0.1 μM to 0.3 μM, and the concentration of probes was ranged from 0.1 μM to 0.4 μM. With 10^5 copies/μL standard plasmids as the template, the fluorescence intensity and Cq values were compared to identify the best combination. The same instrument and qPCR program used for monoplex qPCR were employed.

Sensitivity of multiplex real-time quantitative PCR assay

The sensitivity of the multiplex qPCR assay was evaluated using ten-fold serially diluted standard plasmids, ranging from 10^0 to 10^7 copies/μL. A positive Cq value cut-off of 35 was chosen based on previous studies [16, 18, 19]. The lowest concentrations detectable by multiplex qPCR, as indicated by the standard curves for each picornavirus, were considered rough limits of detection. In this study, the rough limits of detection for 4 picornaviruses were all 10^2 copies/μL. To further evaluate sensitivity, duplicate detections were performed using the standard plasmids based on the following concentrations: 100 copies/μL, 50 copies/μL, 20 copies/μL, and 10 copies/μL. The reliable limit of detection was defined as the concentration at which all replicates gave positive results.

Specificity and repeatability of multiplex real-time quantitative PCR assay

The specificity of the multiplex qPCR assay was evaluated to exclude potential false positives from other viruses. With exception of target viruses, cardiovirus, enterovirus G, and bocavirus were included as control viruses to assess the specificity of the multiplex assay. For control viruses, we employed the same nucleic acid extraction and reverse transcription methods as described before to obtain cDNA libraries, and confirmed by conventional PCR and sequencing. Higher detection limit of conventional PCR can ensure that the concentration of the control virus is sufficient for detection. In repeatability testing, we conducted three replicates in one experiment for each concentration, which were regarded as intra-group replicates. Additionally, to assess the inter-group reproducibility, we repeated the experiment three times, with a one-week interval between each repetition. The repeatability of the multiplex qPCR assay was evaluated by calculating the coefficient of variation (CV) for each target virus. The CV was defined as the ratio of the standard deviation to the mean Cq value, expressed as a percentage.

Detection of clinical samples using multiplex real-time quantitative PCR assay

One hundred and forty-nine fecal samples of murine rodents and shrews collected from four areas of southern China: Shanwei in Guangdong Province, Yiyang in Hunan Province, Xiamen in Fujian Province, and Malipo in Yunnan Province. The procedures for sampling and sample processing were reviewed and approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University, China. All mammals were captured alive and maintained in full adherence to the requirements outlined in the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China. Viral nucleic acid was extracted for each sample through a MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa, Japan). The viral nucleic acid was subsequently reverse transcribed into cDNA via a Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). The cDNA from all stool specimens were utilized to validate the multiplex qPCR method. The 20 μL reaction system comprised 10 μL 2 × Conc. Premix Ex Taq (Takara Bio Inc.), 6 μl nuclease-free water, 0.2 μM of each forward/reverse primer, 0.1 μM of each probe, and 1 μL of cDNA template. Furthermore, all samples were subjected to conventional monoplex PCR methods and sequencing for additional confirmation.

Results

Monoplex real-time quantitative PCR detection of a single virus

To facilitate the development of a multiplex qPCR assay, monoplex qPCR approaches were initially established for each target virus. The sequences of the primers and probes designed in this study are presented in Table 1. Probes for kobuvirus, parechovirus B, rosavirus B, and hunnivirus were labeled with FAM, VIC, Texas Red, and Cy5, separately. The resulting standard curves from each monoplex qPCR demonstrated excellent correlation coefficients and amplification efficacy (Supplemental Figure S1). These findings confirm the suitability of the four sets of primers and probes for the subsequent establishment of the multiplex qPCR methodology.

Reaction system optimization for multiplex real-time quantitative PCR assay

To determine the optimal working concentrations of the four sets of primers and probes, multiplex qPCR was performed with varying concentrations under consistent reaction conditions. By comparing fluorescence intensity and Cq values (Supplemental Table S2 and Figure S2), we determined that the optimal concentration for probes and primers was 0.2 μM and 0.1 μM, respectively. The final reaction volume of 20 μL consisted of 10 μL 2 × Conc. Premix Ex Taq (Takara Bio Inc.), 6 μl nuclease-free water, 0.2 μM of each forward/reverse primer, 0.1 μM of each probe, and 1μL of template. Fluorescence signals were collected using the Roche LightCycler® 96 Instrument (Roche Life Science).

Standard curves and sensitivity of multiplex real-time quantitative PCR assay

Standard curves were constructed based on the Cq values obtained from ten-fold serial dilutions of the four constructed plasmid standards. The specific amplification curves and standard curves are shown in Fig. 1. The results indicated that the multiplex qPCR effectively amplified target viral genome sequences, with high correlation values observed for kobuvirus ($R^2 = 0.993$),

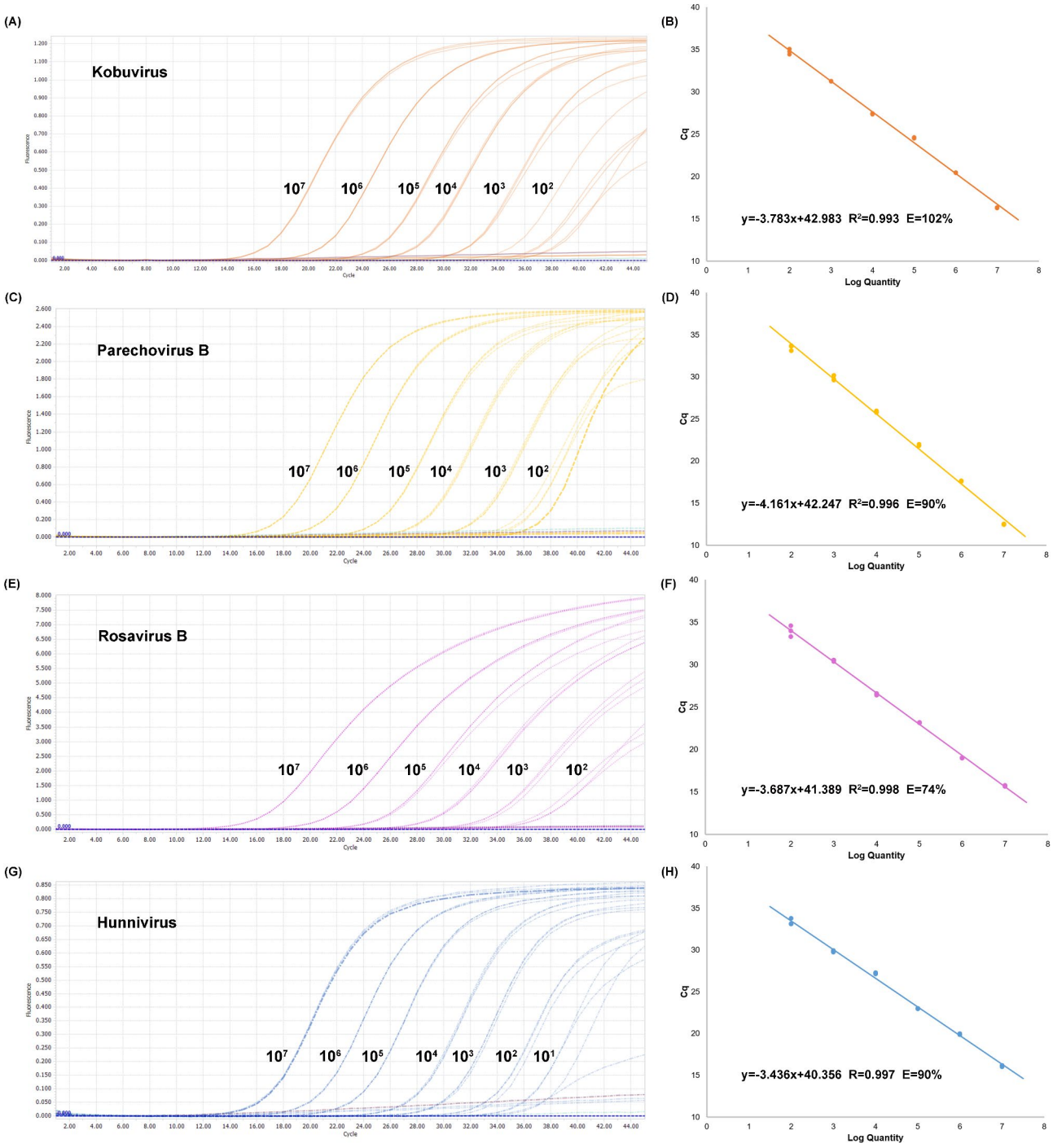

Fig. 1 Amplification curves and standard curve of multiplex real-time quantitative PCR. (**A**) and (**B**) are amplification curves and standard curve of kobuvirus, respectively; (**C**) and (**D**) are amplification curves and standard curve of parechovirus B, respectively; (**E**) and (**F**) are amplification curves and standard curve of rosavirus B, respectively; (**G**) and (**H**) are amplification curves and standard curve of hunnivirus, respectively

parechovirus B ($R^2 = 0.996$), rosavirus B ($R^2 = 0.998$), and hunnivirus ($R^2 = 0.997$). This illustrates excellent linear amplification across a range of 1×10^2 to 1×10^7 copies/μL per reaction.

Based on the standard curves, the limit of detection for multiplex qPCR was preliminarily established at 100 copies/μL. To further evaluate sensitivity, duplicate detections were performed on the standard plasmids of the following concentrations: 100 copies/μL, 50 copies/μL, 20 copies/μL, and 10 copies/μL. The results of this sensitivity assessment are summarized in Supplemental Table S3. The limit of detection was determined to be 100

Table 2 Specificity of the multiplex real-time quantitative PCR assay

Template *	Multiplex qPCR in This Study (Cq Value)			
	Kobuvirus	Parechovirus B	Rosavirus B	Hunnivirus
Kobuvirus	24.14	(–)	(–)	(–)
Parechovirus B	(–)	23.06	(–)	(–)
Rosavirus B	(–)	(–)	21.06	(–)
Hunnivirus	(–)	(–)	(–)	27.31
Enterovirus G	(–)	(–)	(–)	(–)
Bocavirus	(–)	(–)	(–)	(–)
Cardiovirus	(–)	(–)	(–)	(–)

*The templates for kobuvirus, parechovirus B, and rosavirus B were all 10^5 cpoies/μL plasmids. The template for hunnivirus was 10^4 cpoies/μL plasmid. Regarding enterovirus G, bocavirus, and cardiovirus, positive cDNAs were considered as templates.

(–): Negative detection.

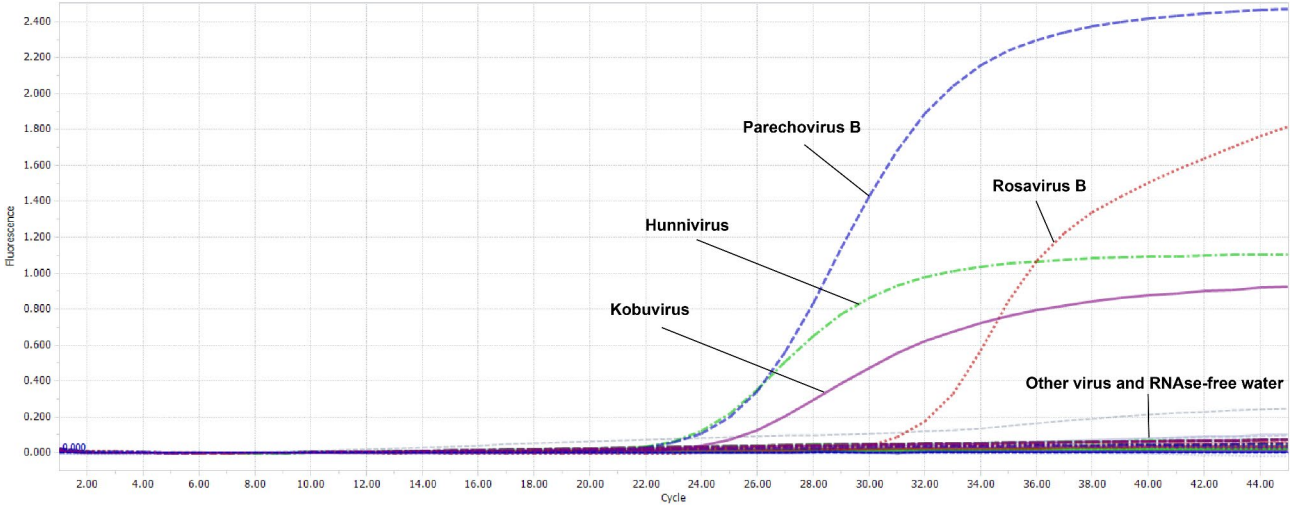

Fig. 2 Analytical specificity of the multiplex real-time quantitative PCR. Negative samples included enterovirus G, bocavirus, cardiovirus, and the negative control

copies/μL for kobuvirus, parechovirus B and rosavirus B, while a lower limit of 50 copies/μL was established for hunnivirus.

Specificity and repeatability of multiplex real-time quantitative PCR assay

The specificity of the four primer pairs and probes was rigorously tested and is presented in Table 2; Fig. 2. Each primer pair successfully amplified only the corresponding target viral genomes, with no observed cross-amplification or non-specific amplification.

To assess the variability of the multiplex qPCR assay, each concentration of the standard plasmid underwent repeated testing for both intra-group and inter-group analysis. The percentage of CV of Cq values ranged from 0.01 to 1.77% for intra-group analysis, and from 0.11 to 2.80% for inter-group analysis (Table 3), indicating high repeatability and precision of the assay.

Detection of clinical samples using multiplex real-time quantitative PCR assay

The established multiplex qPCR assay was used to analyze 149 fecal samples collected from murine rodents and shrews, including *Rattus norvegicus*, *Rattus tanezumi*, *Rattus losea*, *Mus caroli*, and *Suncus murinus*, across various regions.The information of the species was presented in Supplemental Table S4. The results revealed detection rates of 20.1% (30/149) for kobuvirus, 33.6% (50/149) for rosavirus B, and 12.1% (18/149) for hunnivirus, while none of the samples tested positive for parechovirus B. Co-infection with picornaviruses was identified in 21 fecal samples, resulting in a co-infection rate of 14.1%. Among the 21 samples with co-infections, 15 were positive for two viruses, and 6 were positive for three viruses. Moreover, all samples underwent validation through conventional monoplex PCR and sequencing, with results fully corroborating those obtained from the multiplex qPCR assay.

Table 3 Intra- and inter-assay coefficients of variation of multiplex real-time quantitative PCR

Target virus		intra-group analysis			inter-group analysis		
		Cq (means)	SD	CV(%)	Cq (means)	SD	CV(%)
Kobuvirus	S7	16.68	0.08	0.48	16.42	0.23	1.41
	S6	20.25	0.05	0.25	20.24	0.22	1.06
	S5	24.65	0.08	0.32	24.22	0.68	2.80
	S4	27.26	0.03	0.11	26.97	0.62	2.30
	S3	30.76	0.09	0.29	30.86	0.34	1.09
	S2	34.39	0.61	1.77	34.74	0.37	1.07
Parechovirus B	S7	15.81	0.01	0.06	15.70	0.11	0.70
	S6	19.23	0.04	0.21	19.08	0.13	0.69
	S5	23.11	0.01	0.04	23.03	0.19	0.81
	S4	26.67	0.07	0.26	26.43	0.27	1.04
	S3	30.95	0.05	0.16	30.82	0.29	0.95
	S2	34.31	0.13	0.38	34.39	0.48	1.40
Rosavirus B	S7	12.48	0.05	0.40	12.75	0.28	2.16
	S6	17.6	0.03	0.17	17.79	0.30	1.71
	S5	21.9	0.08	0.37	21.92	0.54	2.44
	S4	25.84	0.10	0.39	25.86	0.43	1.64
	S3	29.84	0.29	0.97	29.89	0.35	1.18
	S2	33.47	0.32	0.96	33.73	0.29	0.87
Hunnivirus	S7	16.18	0.16	0.99	16.21	0.12	0.75
	S6	19.59	0.02	0.10	19.78	0.17	0.85
	S5	22.72	0.04	0.18	22.73	0.25	1.08
	S4	26.38	0.08	0.30	26.46	0.71	2.68
	S3	29.64	0.11	0.37	29.84	0.21	0.70
	S2	32.99	0.07	0.21	33.08	0.24	0.73

SD: Standard error

CV: The coefficient of variation

Discussion

In recent years, the emergence of zoonotic diseases caused by novel viruses has attracted widespread attention. Among these, picornaviruses are notable as some of the most common infectious pathogens threatening human health. Due to genetic mutations and recombination events, picornaviruses exhibit a high degree of genetic variability and can infect a wide range of hosts, from lower vertebrates to mammals, leading to diseases in both humans and animals [20]. Murine rodents and shrews, which possess fast life cycles and occupy diverse geographic ranges, are reservoirs for a lot of zoonotic diseases caused by various pathogens [21–26]. However, knowledge regarding the picornaviruses carried by murine rodents and shrews remain comparatively limited. Currently, there are ten virus genera within the family *Picornaviridae* have been identified in murine rodents and shrews [1], Among these picornaviruses, kobuvirus, rosavirus, and hunnivirus present the high prevalence and common co-infection in these small mammals [11–13]. Moreover, parechovirus B infection is associated with a variety of diseases, such as myocarditis, insulin-dependent diabetes mellitus, and Guillain-Barré syndrome [9]. Thus, in order to improve the efficiency of laboratory diagnostics, this study aimed to establish a multiplex qPCR assay capable of simultaneously and quantitatively detecting kobuvirus, parechovirus B, rosavirus B, and hunnivirus within a single reaction tube.

Although viral isolation and culture remain important tools for confirming the presence of infectious viruses, their application can be limited by sensitivity and requiring high technical expertise [27]. Rapid detection technologies, such as transmission electron microscopy (TEM), necessitate advanced equipment and specialized laboratory facilities [27]. Immunological assays including enzyme-linked immunosorbent assays (ELISA) and colloidal gold immunochromatographic assays (GICA), are commonly utilized in laboratory diagnostics but typically require well-defined serological profiles [28]. Given these constraints, PCR and qPCR have become the preferred method for rapid detection in clinical settings, with multiplex PCR recognized as a more efficient diagnostic approach [29]. In this study, we successfully developed a two-step multiplex qPCR that enables the simultaneous detection of four picornaviruses within a single reaction. RNA is more susceptible to degradation during the extraction and preservation processes. Thus, constructing cDNA libraries for these picornaviruses enables long-term storage. In addition, using a portion of the cDNA in the second qPCR step facilitated the optimization of

reaction conditions for each target, while also minimizing the consumption of the original clinical samples.

Real-time fluorescence PCR techniques, including *Taq*Man probe-based and dye-based quantitative PCR, are instrumental in this context. SYBR Green dye is more general but less specific than that of fluorescent probe, and it is not suitable for simultaneous detection of multiple viruses. Conversely, *Taq*Man probe-based qPCR provides high specificity and a reduced risk of sample contamination. However, designing four sets of primers and probes that function together posed significant challenges. Through bioinformatics analysis, we designed primers and *Taq*Man probes targeting highly conserved regions of the genome sequences of kobuvirus, parechovirus B, rosavirus B, and hunnivirus, ensuring their melting temperature were closely aligned to maximize amplification efficiency. After comprehensive optimization, we selected the current primer-probe sets as they provided the best balance of efficiency, specificity, and multiplex compatibility. Moreover, the utilization of four distinct fluorophores (FAM, Texas Red, CY5, and VIC) allowed for the effective differentiation of the four picornaviruses in our multiplex qPCR.

The *Taq*Man probe-based multiplex qPCR assay established in this work demonstrated excellent linear amplification across a range of 1×10^2 and 1×10^7 copies/μL. This level of sensitivity surpasses that of conventional monoplex RT-PCR, which typically ranges from 1×10^4 to 1×10^5 copies/μL [17, 30, 31]. For instance, Zhao et al. developed a multiplex RT-PCR for detecting classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis virus (TGEV), with similar detection limits not falling below 1×10^3 copies/μL [31]. Our multiplex qPCR assay achieved a limit of detection as low as 1×10^2 copies/μL for each picornavirus, comparable to other multiplex qPCR assays for different viral pathogens [17, 32, 33]. This enhanced sensitivity facilitates more robust surveillance of these picornaviruses in murine rodent and shrew populations. Nevertheless, increased sensitivity may also elevate the risk of false positive results, underscoring the necessity for stringent adherence to laboratory protocols to mitigate cross-contamination and aerosol transmission.

The design of probe sequences from the most conserved region of the target viral genome sequences contributes to the higher specificity of *Taq*Man probe-based qPCR compared to SYBR Green dye methods. Our results confirmed that each set of primers and probe in the multiplex qPCR specifically detected only the target virus, with no cross-reactivity observed. The fluorescence intensity and amplification efficiency for kobuvirus, parechovirus B, and hunnivirus were not adversely affected in the multiplex assay. However, the efficiency for rosavirus B was slightly reduced, which could be due to primer and probe competition in the multiplex system, as suggested in previous studies [32].

Detection of clinical samples using the multiplex qPCR revealed a notable incidence of co-infection among picornaviruses in murine rodents and shrews. Such viral co-infections are prerequisites for genetic recombination, with the simultaneous infection of individuals by different strains potentially leading to the emergence of more virulent and transmissible viral strains [34, 35]. Consequently, the co-infections of picornaviruses in murine rodent and shrew populations raise public health concerns regarding potential outbreaks of zoonotic diseases, emphasizing the critical need for enhanced viral surveillance in these small mammal animals. To the best of our knowledge, this is the first multiplex qPCR assay developed for the simultaneous detection of kobuvirus, parechovirus B, rosavirus B, and hunnivirus. The method presented herein enables efficient detection of co-infections with these picornaviruses and significantly reduces both labor and material costs.

Conclusions

In conclusion, this work developed a *Taq*Man probe-based multiplex qPCR method with high sensitivity and repeatability for the simultaneous detection of emerging picornaviruses particularly in murine rodents and shrews, including kobuvirus, parechovirus B, rosavirus B, and hunnivirus. Our findings contribute valuable insights into the monitoring and prevention of zoonotic diseases associated with these pathogens.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02671-4 .

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Shunchang Fan, Minyi Zhang and Qing Chen conceived and designed the study. Shunchang Fan, Yucheng Li, Jingli Tian, and Juxian Xian performed the experiments. Shunchang Fan undertook the data analyses and wrote the original draft of the manuscript. Minyi Zhang and Qing Chen contributed to the manuscript review and editing. All the authors have read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (no. 82273697) and the Guangdong Basic and Applied Basic Research Foundation (no. 2023A1515110994).

Data availability
The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The research protocol was approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University, China, and met the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China. Not applicable for consent to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 16 December 2024 / Accepted: 15 February 2025
Published online: 06 March 2025

References

1. Picornaviruses H. [Available from: https://www.picornaviridae.com/. Accessed 10, 2024.
2. Meerburg BG, Singleton GR, Kijlstra A. Rodent-borne diseases and their risks for public health. Crit Rev Microbiol. 2009;35(3):221–. https://doi.org/10.1080/10408410902989837. 70.
3. Xiong YQ, Mo Y, Chen MJ, Cai W, He WQ, Chen Q. Detection and phylogenetic analysis of torque teno virus (TTV) carried by murine rodents and house shrews in China. Virology. 2018;516:189–95. https://doi.org/10.1016/j.virol.2018.01.017
4. Sasaki M, Orba Y, Ueno K, Ishii A, Moonga L, Hang'ombe BM, Mweene AS, Ito K, Sawa H. Metagenomic analysis of the shrew enteric virome reveals novel viruses related to human stool-associated viruses. J Gen Virol. 2015;96(Pt 2):440–. https://doi.org/10.1099/vir.0.071209-0. 52.
5. Yamashita T, Ito M, Tsuzuki H, Sakae K. Identification of Aichi virus infection by measurement of immunoglobulin responses in an enzyme-linked immunosorbent assay. J Clin Microbiol. 2001;39(11):4178–80. https://doi.org/10.1128/jcm.39.11.4178-4180.2001
6. Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, Isomura S. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. J Infect Dis. 1991;164(5):954–7. https://doi.org/10.1093/infdis/164.5.954
7. Boros Á, Orlovácz K, Pankovics P, Szekeres S, Földvári G, Fahsbender E, Delwart E, Reuter G. Diverse picornaviruses are prevalent among free-living and laboratory rats (Rattus norvegicus) in Hungary and can cause disseminated infections. Infect Genet Evol. 2019;75:103988. https://doi.org/10.1016/j.meegid.2019.103988
8. Firth C, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, Ng J, Garcia J, Bhuva NP, Lee B, Che X, Quan PL, Lipkin WI. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio. 2014;5(5):e01933–14. https://doi.org/10.1128/mBio.01933-14
9. Niklasson B, Hörnfeldt B, Lundman B. Could myocarditis, insulin-dependent diabetes mellitus, and Guillain-Barré syndrome be caused by one or more infectious agents carried by rodents? Emerg Infect Dis. 1998;4(2):187–93. https://doi.org/10.3201/eid0402.980206
10. Fevola C, Rossi C, Rosso F, Girardi M, Rosà R, Manica M, Delucchi L, Rocchini D, Garzon-Lopez CX, Arnoldi D, Bianchi A, Buzan E, Charbonnel N, Collini M, Ďureje L, Ecke F, Ferrari N, Fischer S, Gillingham EL, Hörnfeldt B, Kazimírová M, Konečný A, Maas M, Magnusson M, Miller A, Niemimaa J, Nordström Å, Obiegala A, Olsson G, Pedrini P, Piálek J, Reusken CB, Rizzolli F, Romeo C, Silaghi C, Sironen T, Stanko M, Tagliapietra V, Ulrich RG, Vapalahti O, Voutilainen L, Wauters L, Rizzoli A, Vaheri A, Jääskeläinen AJ, Henttonen H, Hauffe HC. Geographical distribution of Ljungan Virus in Small mammals in Europe. Vector Borne Zoonotic Dis. 2020;20(9):692–702. https://doi.org/10.1089/vbz.2019.2542
11. Zhang M, Li Q, Wu F, Ou Z, Li Y, You F, Chen Q, Epidemiology. Genetic characterization, and evolution of Hunnivirus carried by Rattus norvegicus and Rattus tanezumi: the First Epidemiological evidence from Southern China. Pathogens. 2021;10(6). https://doi.org/10.3390/pathogens10060661
12. Zhang M, Fan S, Liang M, Wu R, Tian J, Xian J, Zhou X, Chen Q. A panoramic view of the molecular epidemiology, evolution, and cross-species transmission of rosaviruses. Vet Res. 2024;55(1). https://doi.org/10.1186/s13567-024-01399-3
13. Zhang M, You F, Wu F, He H, Li Q, Chen Q. Epidemiology and genetic characteristics of murine kobuvirus from faecal samples of Rattus losea, Rattus tanezumi and Rattus norvegicus in southern China. J Gen Virol. 2021;102(9). https://doi.org/10.1099/jgv.0.001646
14. Lim ES, Cao S, Holtz LR, Antonio M, Stine OC, Wang D. Discovery of Rosavirus 2, a novel variant of a rodent-associated picornavirus, in children from the Gambia. Virology. 2014;454–455:25–33. https://doi.org/10.1016/j.virol.2014.01.018
15. Higuchi R, Fockler C, Dollinger G, Watson R, Kinetic. PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnol (N Y). 1993;11(9):1026–30. https://doi.org/10.1038/nbt0993-1026
16. Wang R, Zhang W, Ye R, Pan Z, Li G, Su S. One-step multiplex TaqMan probe-based method for real-time PCR detection of four canine diarrhea viruses. Mol Cell Probes. 2020;53:101618. https://doi.org/10.1016/j.mcp.2020.101618
17. Pan Z, Lu J, Wang N, He WT, Zhang L, Zhao W, Su S. Development of a TaqMan-probe-based multiplex real-time PCR for the simultaneous detection of emerging and reemerging swine coronaviruses. Virulence. 2020;11(1):707–18. https://doi.org/10.1080/21505594.2020.1771980
18. Zhang L, Jiang Z, Zhou Z, Sun J, Yan S, Gao W, Shao Y, Bai Y, Wu Y, Yan Z, Sheng S, Lai A, Su S. A TaqMan Probe-based Multiplex Real-Time PCR for simultaneous detection of Porcine Epidemic Diarrhea Virus subtypes G1 and G2, and Porcine Rotavirus groups A and C. Viruses. 2022;14(8). https://doi.org/10.3390/v14081819
19. Zou J, Yu J, Mu Y, Xie X, Wang R, Wu H, Liu X, Xu F, Wang J, Wang Y. Development of a TaqMan-based multiplex real-time PCR for simultaneous detection of four feline diarrhea-associated viruses. Front Vet Sci. 2022;9:1005759. https://doi.org/10.3389/fvets.2022.1005759
20. Tapparel C, Siegrist F, Petty TJ, Kaiser L. Picornavirus and enterovirus diversity with associated human diseases. Infect Genet Evol. 2013;14:282– 93. https://doi.org/10.1016/j.meegid.2012.10.016.
21. Keesing F, Ostfeld RS. Emerging patterns in rodent-borne zoonotic diseases. Science. 2024;385(6715):1305–10. https://doi.org/10.1126/science.adq7993
22. Rabiee MH, Mahmoudi A, Siahsarvie R, Kryštufek B, Mostafavi E. Rodent-borne diseases and their public health importance in Iran. PLoS Negl Trop Dis. 2018;12(4):e0006256. https://doi.org/10.1371/journal.pntd.0006256
23. Han BA, Schmidt JP, Bowden SE, Drake JM. Rodent reservoirs of future zoonotic diseases. Proc Natl Acad Sci U S A. 2015;112(22):7039–44. https://doi.org/10.1073/pnas.1501598112
24. Dürrwald R, Kolodziejek J, Weissenböck H, Nowotny N. The bicolored white-toothed shrew Crocidura leucodon (HERMANN 1780) is an indigenous host of mammalian Borna Disease virus. PLoS ONE. 2014;9(4):e93659. https://doi.org/10.1371/journal.pone.0093659
25. Witkowski PT, Klempa B, Ithete NL, Auste B, Mfune JK, Hoveka J, Matthee S, Preiser W, Kruger DH. Hantaviruses in Africa. Virus Res. 2014;187:34–42. https://doi.org/10.1016/j.virusres.2013.12.039
26. Yanagihara R, Gu SH, Arai S, Kang HJ, Song JW. Hantaviruses: rediscovery and new beginnings. Virus Res. 2014;187:6–14. https://doi.org/10.1016/j.virusres.2013.12.038
27. Yu Z, Wu H, Huang Q, Zhong Z. Simultaneous detection of Marburg virus and Ebola virus with TaqMan-based multiplex real-time PCR method. J Clin Lab Anal. 2021;35(6):e23786. https://doi.org/10.1002/jcla.23786
28. Li X, Yin Y, Pang L, Xu S, Lu F, Xu D, Shen T. Colloidal gold immunochromatographic assay (GICA) is an effective screening method for identifying detectable anti-SARS-CoV-2 neutralizing antibodies. Int J Infect Dis. 2021;108:483–6. https://doi.org/10.1016/j.ijid.2021.05.080
29. Shi X, Liu X, Wang Q, Das A, Ma G, Xu L, Sun Q, Peddireddi L, Jia W, Liu Y, Anderson G, Bai J, Shi J. A multiplex real-time PCR panel assay for simultaneous detection and differentiation of 12 common swine viruses. J Virol Methods. 2016;236:258–65. https://doi.org/10.1016/j.jviromet.2016.08.005
30. Zhao PD, Bai J, Jiang P, Tang TS, Li Y, Tan C, Shi X. Development of a multiplex TaqMan probe-based real-time PCR for discrimination of variant and classical porcine epidemic diarrhea virus. J Virol Methods. 2014;206:150–5. https://doi.org/10.1016/j.jviromet.2014.06.006
31. Zhao Y, Liu F, Li Q, Wu M, Lei L, Pan Z. A multiplex RT-PCR assay for rapid and simultaneous detection of four RNA viruses in swine. J Virol Methods. 2019;269:38–42. https://doi.org/10.1016/j.jviromet.2019.04.001
32. Yu J, Zou J, Liu X, Pan Y, Mu Y, Li S, Wang J, Xu F, Wang Y. TaqMan-probe-based multiplex real-time RT-qPCR for simultaneous detection of GoAstV, GPV, and GoCV. Poult Sci. 2023;102(2):102396. https://doi.org/10.1016/j.psj.2022.102396

33. Huang X, Chen J, Yao G, Guo Q, Wang J, Liu G. A TaqMan-probe-based multiplex real-time RT-qPCR for simultaneous detection of porcine enteric coronaviruses. Appl Microbiol Biotechnol. 2019;103(12):4943–52. https://doi.org/10.1007/s00253-019-09835-7
34. Simon-Loriere E, Holmes EC. Why do RNA viruses recombine? Nat Rev Microbiol. 2011;9(8):617–. https://doi.org/10.1038/nrmicro2614. 26.
35. Su S, Bi Y, Wong G, Gray GC, Gao GF, Li S. Epidemiology, evolution, and recent outbreaks of Avian Influenza Virus in China. J Virol. 2015;89(17):8671–6. https://doi.org/10.1128/jvi.01034-15

Publisher's note