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Original Research

Development and evaluation of a quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction assay for detecting Langya, Mojiang, Nipah, and Cedar viruses

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ABSTRACT

The emerging viruses within the genus *Henipavirus* in the family *Paramyxoviridae* pose a great threat to public biosafety. To develop a quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay is pivotal for the early warning of the potential of zoonotic infectious diseases. Specific primers and probes were designed for the relatively conserved regions based on whole genome sequences of Langya virus (LayV), Mojiang virus (MojV), Nipah virus (NiV), and Cedar virus (CedV), followed by the establishment of a quadruple real-time fluorescence-based qRT-PCR detection method. No cross-reactivity was observed with other viral nucleic acids. The optimal linear detection range for LayV, MojV, NiV, and CedV was 10^1-10^8 copies/μL, and the lower limit of detection was 10 copies/μL. Three different DNA concentrations of LayV, MojV, NiV, and CedV (10^4, 10^5, and 10^6 copies/μL) were tested 14 times, achieving good repeatability. The standard deviation of the cycle threshold values for each concentration was <0.5 and the coefficient of variation was <3 %. Furthermore, the amplification efficiency of quadruple real-time fluorescence-based qRT-PCR was >90 %, and the correlation coefficient was >0.99. The established quadruple real-time fluorescence-based qRT-PCR assay for the detection of LayV, MojV, NiV, and CedV exhibits good sensitivity, specificity, and repeatability. Therefore, it can be used to detect *Henipavirus* and other related clinical specimens.

1. Introduction

The recent human infections of the monkeypox virus and avian influenza viruses, etc., suggest that viruses can rapidly mutate and that human behavior often affects the course of the viral epidemic [1,2]. Globally, approximately 70 % of emerging infectious diseases are transmitted to humans through animal interactions because human populations are expanding into natural areas [3]. Therefore, it is vital to focus on these epidemics and prevent the spread of viruses. The viruses in the genus *Henipavirus* in the family Paramyxoviridae can cause zoonotic diseases. In the late 1990s, a zoonotic natural epidemic infection due to the Nipah virus (NiV) was discovered. This virus primarily attacks the nervous and respiratory systems of humans and pigs [4]. To date, NiV outbreaks have occurred in Malaysia, Singapore, India, Bangladesh, and other countries [5]. Pathogenic ecological studies have reported that fruit bats belonging to the genus *Pteropus* (flying foxes) are not only the natural hosts of NiV but also the main vector organisms that cause NiV infections in humans and pigs [6]. Bats are the natural hosts of NiV as well as asymptomatic carriers; however, they excrete the virus *via* saliva, urine, semen, and feces [7]. Humans contract the disease *via* NiV-infected pigs, which are the intermediate NiV hosts [8]. Virus spillover from bats to pigs occurs because of the consumption of fruits that are partially eaten or contaminated by NiV-infected bats [9]. Virus transmission from pigs to humans occurs *via* direct contact with the infected pigs, and human–human infection can occur *via* direct contact, aerosols, or fomites. Recently, NiV antibodies or nucleic acids were detected in fruit bat samples from Cambodia, Thailand, Vietnam, and India [10,11].

In 2022, a new virus outbreak causing Langya virus (LayV) infection was detected in patients with fever in the eastern provinces of

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HIGHLIGHTS

Scientific question

Nipah virus (NiV) disease is one of the eight most lethal pathogens as per the World Health Organization's priority pathogens list in the *Research and Development Blueprint* due to its high mortality rate, zoonosis, possible human-to-human transmission, and lack of specific drugs and vaccines. Recently, a new henipavirus outbreak causing Langya virus (LayV) infection was detected in patients with fever in the eastern provinces of Shandong and Henan in China. Therefore, rapid detection of viral nucleic acids is imperative for the early detection of infected cases.

Evidence before this study

Traditional serological detection assays are prone to cross-reactivity and exhibit low sensitivity and specificity. However, the detection method of real-time fluorescent fluorescence-based quantitative reverse transcription polymerase chain reaction quantitative reverse transcription polymerase chain reaction (qRT-PCR) has high sensitivity and specificity. According to the review of relevant literature, it was found that the previous studies did not establish the detection method of LayV or Mojiang virus (MojV) single and multiple real-time fluorescence-based qRT-PCR for NiV, LayV, MojV, and Cedar virus (CedV).

New findings

In this study, a quadruple real-time fluorescence-based qRT-PCR assay was developed to specifically amplify four target genes in samples containing multiple viral nucleic acids mixed in one tube and their respective single tubes. Molecular biology assays, particularly the quadruple real-time fluorescence-based qRT-PCR, exhibit the advantages of good repeatability, high sensitivity, short detection time, and strong specificity.

Significance of the study

Specific primers for virus detection were designed and combined to optimize the real-time fluorescence-based qRT-PCR method, which could simultaneously distinguish NiV, LayV, MojV, and CedV in one detection reaction, This method can provide reference information for rapidly screening suspected infected people and technical support for the differential diagnosis of related diseases. Furthermore, it is suitable for laboratories at the provincial, municipal, and county levels.

Shandong and Henan in China [12]. LayV, named after the Langya town in Shandong Province, is a unique phylogenetically zoonotic henipavirus [13]. The seroprevalence rates of this virus were 2 % and 5 % in goats and dogs, respectively, in serosurveys conducted on domestic animals; of the 25 wild small mammals examined, LayV RNA was primarily detected in shrews (27 %). However, human–human transmission remains unreported [14]. LayV infection is associated with febrile illness and pneumonia [13]. In China, LayV has been identified as a new member of the genus *Henipavirus*; phylogenetically, it is most closely related to the Mojiang virus (MojV) [14]. In June 2012, three miners in Mojiang Hani Autonomous County in China died of severe pneumonia [15]. While investigating bat, rat, and muskrat samples from the cave, a new rodent-borne paramyxovirus, MojV, was identified [16]. Wu et al. [17] reported that three of the nine anal swab samples collected from yellow-breasted rats were positive for MojV. This finding suggests that yellow-breasted rats are the natural host of this virus. Cedar virus (CedV) is another recognized species of *Henipavirus* that has been isolated from bat urine. This virus is commonly found in the flying fox community of bats in the cedar forests of Queensland, Australia [18]. CedV can be replicated in guinea pigs and ferrets without causing significant clinical diseases, and these two animals may be the natural hosts of CedV [18]; however, CedV is not associated with any zoonotic events. LayV, MojV, NiV, and CedV are all negative-stranded RNA viruses belonging to the genus *Henipavirus* of the family *Paramyxoviridae* [19].

Except for some studies on ribavirin and favipiravir possessing antiviral activity against NiV, no specific drugs or vaccines against henipaviruses have been approved for human use [20]. Furthermore, at present, no effective treatment regimens are available for LayV. However, unlike NiV and Hendra virus, there have been no deaths reported for LayV. Surveillance (both humans and animals) and limiting contact with infected animals are the only sensible public health interventions at present. The early identification of new virus outbreaks and the establishment of risk communication mechanisms are crucial for preventing evolution into pandemics [21,22].

The traditional detection methods include pathogen isolation and culture and immunological detection methods. However, these methods have cumbersome operation processes, low sensitivity, and a long detection time; therefore, they fail to meet the requirements of rapid diagnosis. Studies have reported that real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay exhibits high sensitivity and specificity and can detect the virus during the early disease stage; at present, it is the most common detection method used in various laboratories [23–25]. TaqMan fluorescent probes only bind to the target sequence and remain unaffected by nonspecific products. The fluorescence reporter groups of different wavelengths can label different probes; therefore, probes with different fluorescent labels can be used in the same reaction system to simultaneously detect multiple targets, thereby saving experimental costs and considerably shortening detection time [25]. The reaction principle, reaction reagents, and operation process of multiplex qRT-PCR are similar to those of traditional PCR; however, multiplex qRT-PCR can be used to simultaneously detect or identify various pathogenic microorganisms, genetic diseases, and cancer genes. Furthermore, compared with traditional PCR, multiplex qRT-PCR has the advantages of high efficiency, high throughput, simplicity, low cost, and strong systematics [23]. In the present study, we establish a quadruple real-time fluorescence-based qRT-PCR assay for detecting LayV, MojV, NiV, and CedV. This assay can rapidly and accurately differentiate the infection caused by the corresponding viruses, monitor the outbreaks caused by henipaviruses, and provide technological tools for related studies.

2. Materials and methods

2.1. Primer and probe design

The whole genome sequences of LayV, MojV, NiV, and CedV were retrieved and downloaded from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) database of the National Center for Biotechnology Information (NCBI), and analyzed and compared by the MEGA11 version 11.0.11 software ClustalW. Based on the principles of the primer and probe design for TaqMan PCR, the relatively conserved L and N regions were selected as the target gene fragments for amplification. Pick Primer and Primer Express 3.0 (Thermo Fisher Scientific, Waltham, MA, USA) in the NCBI were used to design the primers and probes. Sequences were obtained from GenBank. GenBank accession numbers used were YP009094088 for MojV-N, AFP87274 for CedV-N, UUV47242 for LayV-L, NP112028 for NiV-L. Basic Local Alignment Search Tool (BLAST) was used to verify the broad spectrum and speci-

ficity of these primers and probes. All primers and probes were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The probe for LayV was labeled with 5′6-FAM and 3′-BHQ1; the probe for MojV was labeled with 5′-ROX and 3′-BHQ2; the probe for NiV was labeled with 5′-VIC and 3′-BHQ1; and the probe for CedV was labeled with 5′-CY5 and 3′-BHQ2. All primers and probes were centrifuged at 12,000 × *g* for 2 min and diluted to a storage concentration of 100 μmol/L with sterile double distilled water according to the instructions. Finally, they were stored in a refrigerator at 4 °C for subsequent use. All fluorescent probes were stored in the dark.

2.2. Preparation of the positive control DNA templates for LayV, MojV, NiV, and CedV

Based on the L and N gene fragment sequences of LayV, MojV, NiV, and CedV, DNA fragments containing the fragment sequences for a quadruple real-time fluorescence-based qRT-PCR assay were synthesized, cloned into the pUC57 vector, transformed into *Escherichia coli*, purified using a plasmid DNA extraction kit (Takara Bio, Liaoning, China), and used as a positive control DNA template. The copy number was 3×10^{10} copies/μL. The positive control DNA was diluted to a concentration of 1×10^{10} copies/μL and stored at −80 °C.

2.3. A quadruple real-time fluorescence-based qRT-PCR assay

The One-Step PrimeScript RT-PCR Kit (Perfect Real Time; Takara Bio, Liaoning, China) was used according to the manufacturer's protocol. A reaction mixture of 25 μL and amplification conditions as per the protocol were used. The reaction mixture comprised the following components: 0.5 μL each of four 10 μmol/L upstream and downstream primers, 0.25 μL each of four 10 μmol/L probes, 4 μL of the positive control DNA template, 12.5 μL of 2 × One-Step RT-PCR Buffer III, 0.5 μL of TaKaRa Ex Taq HS (5 U/μL), and 0.5 μL of PrimeScript RT Enzyme Mix II. The remaining volume was supplemented with RNase-free distilled water. Amplification was performed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA). The amplification conditions were as follows: reverse transcription at 45 °C for 5 min; pre-denaturation at 95 °C for 10 s; and 40 amplification cycles at 95 °C for 5 s and 60 °C for 30 s. The fluorescence signal was collected at 60 °C during the 40 amplification cycles and annealing-extension stages.

2.4. Construction of the standard curve of the detection system and sensitivity analysis

The plasmids of the corresponding viruses were diluted in a 10-fold isocratic gradient with sterilized double-distilled water and amplified by multiplexed assays using 1×10^8 to 1×10^1 copies/μL as templates. Each concentration was detected in triplicate in one run. The standard positive control templates with different dilution levels were amplified using the quadruple real-time fluorescence-based qRT-PCR method under optimal reaction conditions. At the end of the reaction, the standard curve was plotted with the cycle threshold (Ct) value of the cycle number as the Y-axis and the logarithm of the initial copy number concentration of the positive control template as the X-axis.

2.5. Specificity analysis of the detection system

The positive nucleic acids of other *Paramyxoviridae* viruses, including human metapneumovirus (HMPV), respiratory syncytial virus (RSV), human parainfluenza virus type 3 (HPIV-3), and human parainfluenza virus type 4 (HPIV-4), were used as the examination samples, with negative and positive controls set simultaneously. These nucleic acid samples were amplified using the developed quadruple real-time fluorescence-based qRT-PCR assay, which was followed by specificity analysis.

2.6. Stability of the test system

The obtained LayV, MojV, NiV, and CedV standards were diluted 10-fold. The positive control DNA templates were diluted to 10^6, 10^5, and 10^4 copies/μL and then detected using the established quadruple real-time fluorescence-based qRT-PCR assay system and reaction conditions. Three parallel holes were set up for each concentration, and 14 parallel repeated tests were performed. The stability of the detection system was evaluated based on the mean, standard deviation (SD), and coefficient of variation (CV) of the Ct values obtained in the experiment. The CV values between and within the batches were calculated using the following formula: $CV = SD / mean$.

2.7. Application of the quadruple real-time fluorescence-based qRT-PCR assay

The established quadruple real-time fluorescence-based qRT-PCR assay system and optimal reaction conditions were used to test 24 bat anal swab samples collected in 2015, and 33 bat pharyngeal swab samples collected in 2019 in China. Positive nucleic acids from the wild-type Malaysian NiV strain were used to validate the established quadruple real-time fluorescence-based qRT-PCR assay. Positive DNA templates were also used as the positive control, and sterile water was used as the negative control.

3. Results

3.1. Specificity of the detection system

The specific primers and probes for LayV, MojV, NiV, and CedV were designed and synthesized with lengths of the amplified target fragments 151, 109, 100, and 161 bp, respectively (Table 1, Supplementary Table S1). The positive nucleic acids of HMPV, RSV, HPIV-3, and HPIV-4 and plasmid standards of LayV, MojV, NiV, and CedV were evaluated *via* two approaches: by mixing all samples into one tube (Fig. 1A) and their respective single tubes (Fig. 1B). The results showed that the established quadruple real-time fluorescence-based qRT-PCR assay specifically amplifies LayV, MojV, NiV, and CedV, with no positive amplification signals for the other *Paramyxoviridae* viruses, indicating a high specificity (Fig. 1, Supplementary Table S2).

Table 1
Sequence information on the primers and probes used in the real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay.

Virus	Primer probe name	Fluorescent labeling	Sequences (5′-3′)
LayV	L-F		TGATGTGCCAGACGAAGGAAT
	L-R		GTCACCTTGAACGACAGCAG
	L-P	5′6-FAM, 3′BHQ1	TCATCCGACATCCCATGGGAGGT
MojV	M-F		TCCTCTAAAGTGAGCCCAAAGG
	M-R		GTGCCTCAGATTTTCTTGCCTC
	M-P	5′ROX, 3′BHQ2	CCGACATGTCTGTCAGAGACCGT
NiV	N-F		CCAGTGATCCATACTCGGGC
	N-R		GGGTTCGGTGAGTTCCTCAG
	N-P	5′VIC, 3′BHQ1	TTGCCTGACTCACAAAGCATCACT
CedV	C-F		GGATCATGCCCAGAGTTCCT
	C-R		CTGCCAGCCTCTGTCTCAAT
	C-P	5′CY5, 3′BHQ2	TGACGAGCATCACATCCACCGT

Abbreviations: LayV, Langya virus; MojV, Mojiang virus; NiV, Nipah virus; CedV, Cedar virus.

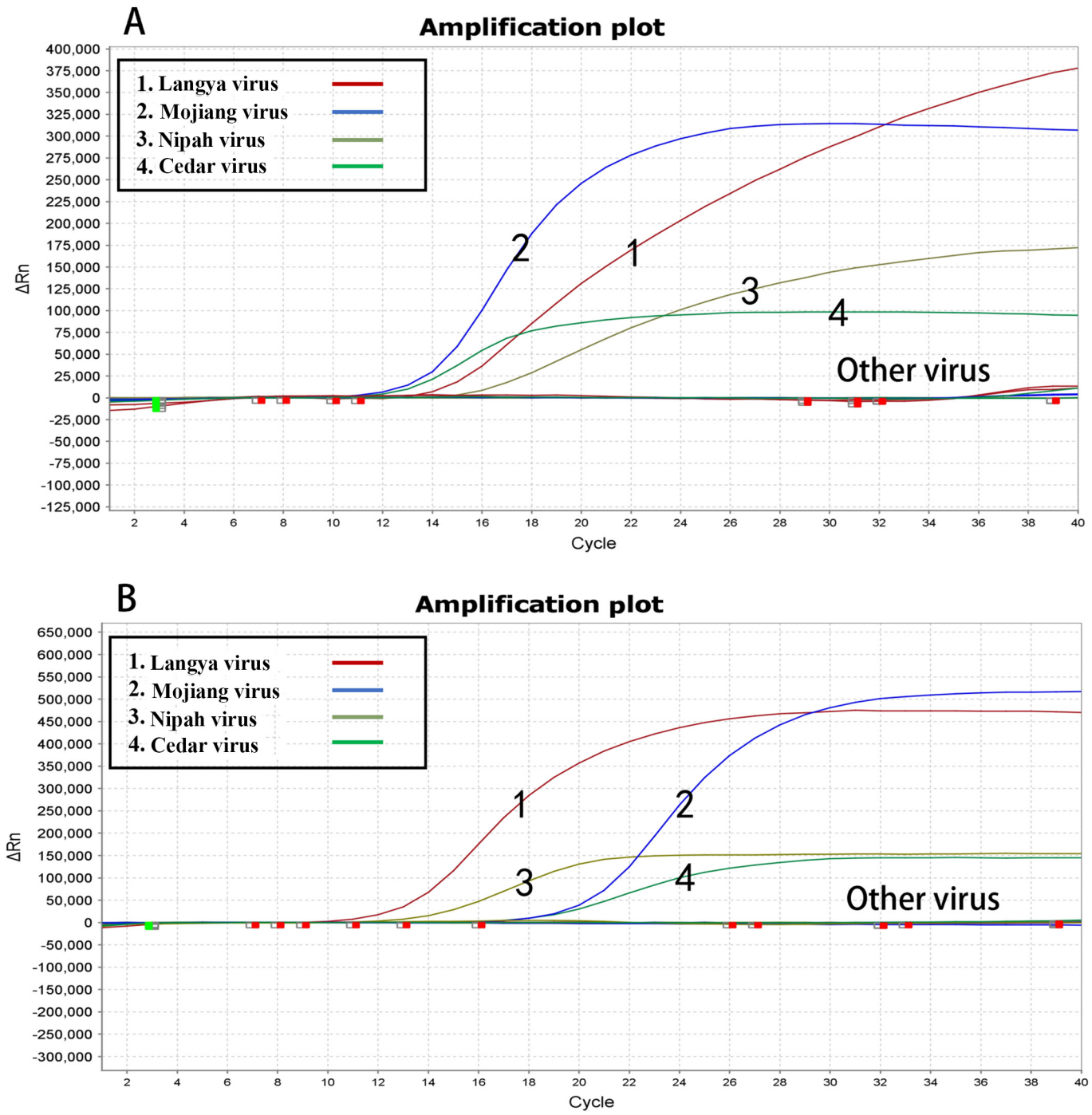

Fig. 1. Specific evaluation of the quadruple real-time fluorescence-based qRT-PCR assay. A) Multiple viral nucleic acid samples mixed in one tube. B) Each viral nucleic acid sample in a single tube. Abbreviation: qRT-PCR, quantitative reverse transcription polymerase chain reaction.

3.2. Standard curve, sensitivity and stability of the detection system

The standard curve equations of the viruses using the detection system were as follows:

For LayV,

$$y = -3.3350x + 37.3507 \quad (1)$$

$R^2 = 0.9999$

For MojV,

$$y = -3.4462x + 38.8185 \quad (2)$$

$R^2 = 1.0000$

For NiV,

$$y = -3.3650x + 40.9995 \quad (3)$$

$R^2 = 0.9999$

For CedV,

$$y = -3.4666x + 37.8589 \quad (4)$$

$R^2 = 0.9998$

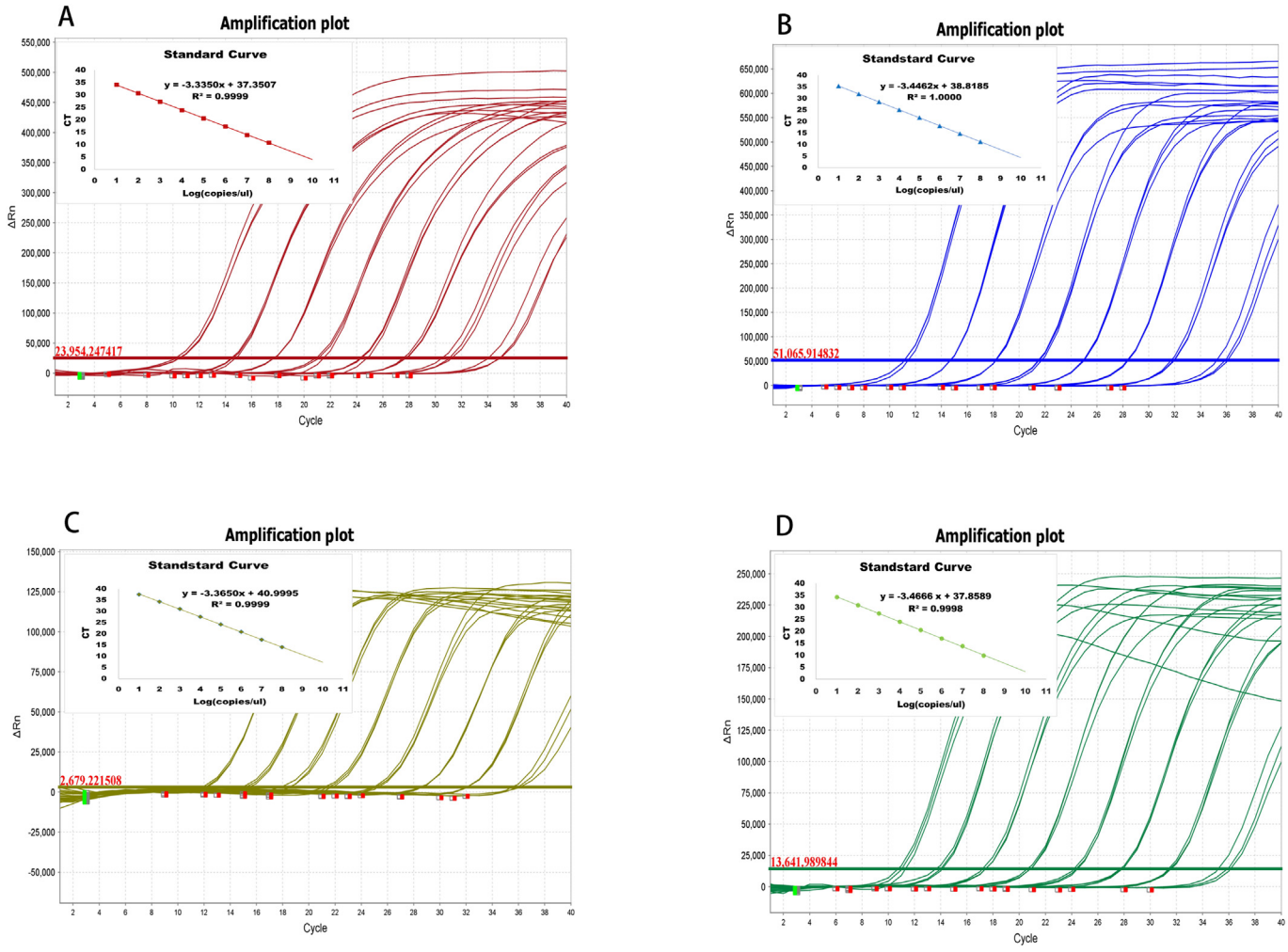

Fig. 2. Sensitivity analysis and the standard curves of the established quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay for the four viruses. A) Langya virus, the standard curve was $y = -3.3350x + 37.3507$, $R^2 = 0.9999$. B) Mojiang virus, the standard curve was $y = -3.4462x + 38.8185$, $R^2 = 1.0000$. C) Nipah virus, the standard curve was $y = -3.3650x + 40.9995$, $R^2 = 0.9999$. D) Cedar virus, the standard curve was $y = -3.4666x + 37.8589$, $R^2 = 0.9998$.

Table 2
Stability analysis of the established quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay for the four viruses.

Nucleic acid concentration	LayV (copies/μL)			MojV (copies/μL)			NiV (copies/μL)			CedV (copies/μL)		
	10^6	10^5	10^4	10^6	10^5	10^4	10^6	10^5	10^4	10^6	10^5	10^4
Ct (mean)	17.57	20.86	24.23	22.16	25.24	28.74	19.10	22.49	25.46	17.14	20.68	24.11
SD	0.29	0.23	0.21	0.49	0.47	0.40	0.28	0.36	0.45	0.19	0.34	0.36
CV (%)	1.67	1.10	0.86	2.23	1.87	1.39	1.47	1.60	1.77	1.10	1.63	1.50
Detection rate (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Abbreviations: Ct, cycle threshold; SD, standard deviation; CV, coefficient of variation; LayV, Langya virus; MojV, Mojiang virus; NiV, Nipah virus; CedV, Cedar virus.

The PCR efficiency of each virus was as follows: LayV, 99.46 %; MojV, 95.06 %; NiV, 98.23 %; and CedV, 94.30 %; with all curves exhibiting a good linear relationship (Supplementary Table S3). Furthermore, the lower limit of detection of the established method was 10^1 copies/μL, and the linear range of the standard curve was 1.0×10^8 - 1.0×10^1 copies/μL, and no amplification of the negative controls was detected (Fig. 2).

The standard deviation of the Ct values of the parallel replicate experiments for each sample was 0.19 - 0.49, and the CV was 0.86 % - 2.23 %. These results indicate that the detection system exhibits good stability (Table 2).

3.3. Application of the quadruple real-time fluorescence-based qRT-PCR assay

The established quadruple real-time fluorescence-based qRT-PCR assay was used to detect nucleic acids from samples of the Malaysian NiV strain. All results were positive, indicating that the established

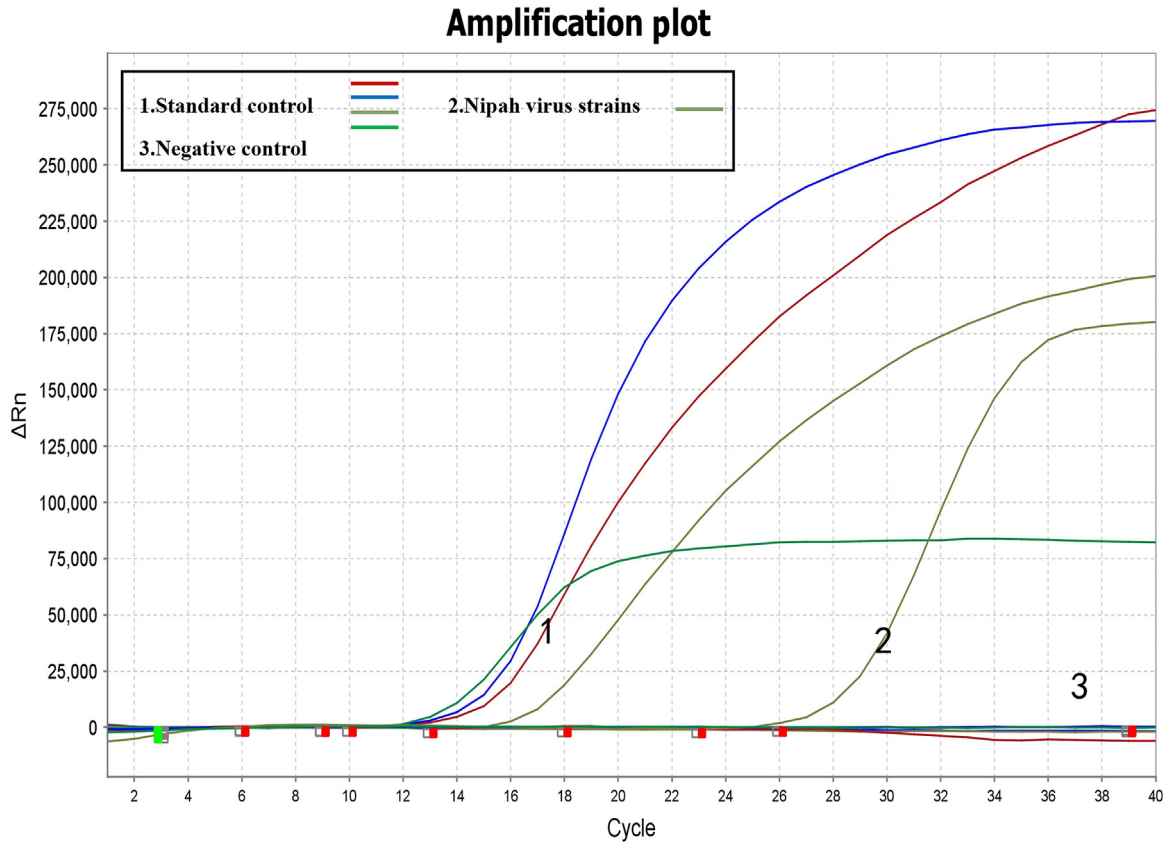

Fig. 3. Quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay to detect Nipah virus strains.

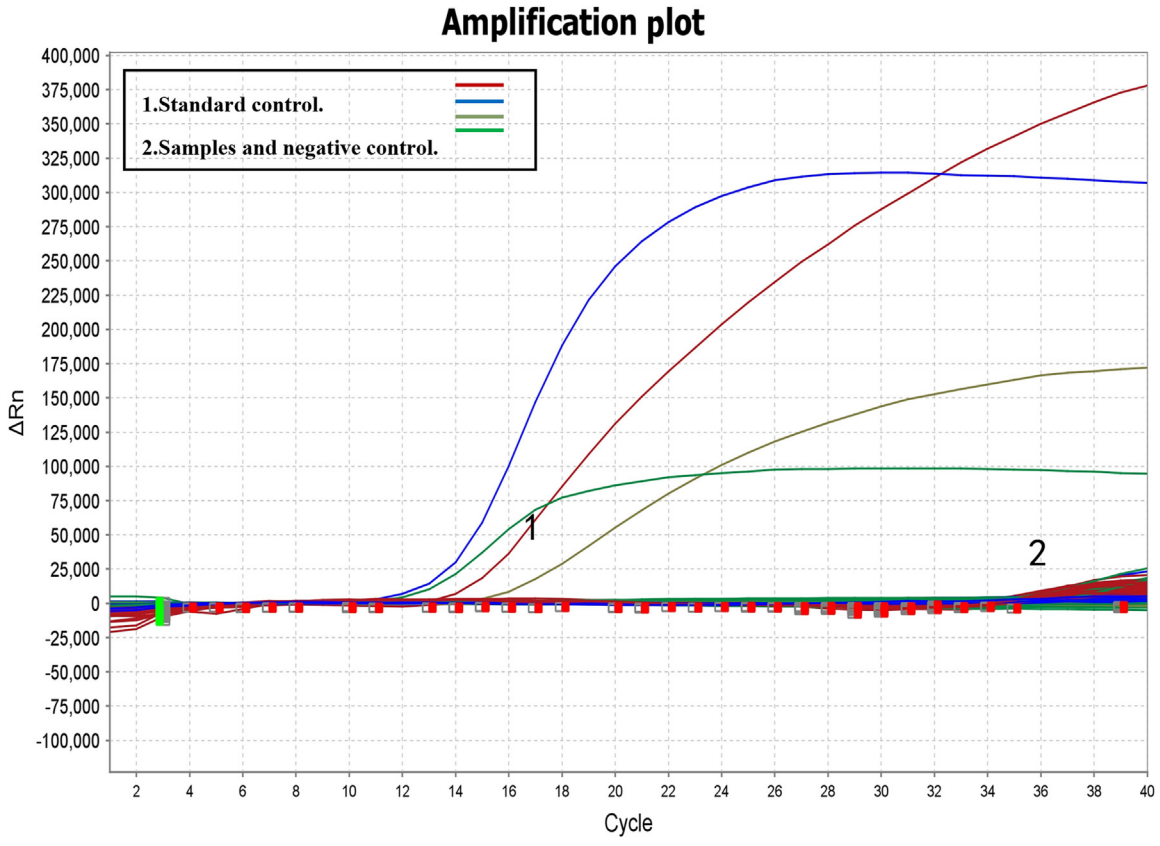

Fig. 4. Quadruple real-time fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay for sample detection.

detection method can effectively detect the abovementioned two NiV strains (Fig. 3). Using the quadrupole real-time fluorescence-based qRT-PCR assay established in this study, nucleic acids were detected in 24 bat anal swab samples from Xiaozhu and Yuteng Villages, Huaiji County, Zhaoqing City, Guangdong Province, and 33 bat pharyngeal swab samples from Dongyuan County, Heyuan City, Guangdong Province, and the results were negative (Fig. 4).

4. Discussion

Henipavirus is the only genus in the family *Paramyxoviridae* that can cause highly pathogenic zoonotic diseases. To date, bats appear to be the primary natural host of heniparviruses. The flying fox species reside in various regions, ranging from Madagascar in the west, across the Indian subcontinent to Southeast Asia and Australia, and to Oceania in the east [26]. However, studies have reported that henipaviruses are also found in various other bat species [10,27,28]. Furthermore, these viruses have a very wide range of natural hosts, including multiple other mammals such as shrews, pigs, goats, cows, cats, dogs, and horses. This consideringly increases the risk of human transmission because these animals are frequently in close contact with humans [29]. The virus is transmitted through the oral cavity, genitourinary system, feces, and delivery fluid excretion among bats during grooming, mating, and fighting [30]. Henipaviruses can be transmitted among bats or to other species during spillover events. The transmission mechanism is *via* the body fluids of organisms and ingestion of contaminated food [31]. NiV is commonly transmitted by ingesting contaminated raw sap [32]. When palm sap is harvested, the infected bats may lick the sap [33]. When domestic animals consume these palm sap licked by infected bats or fruits contaminated with NiV-containing feces, urine, or saliva, the infected domestic animals can excrete the virus and transmit it to humans who are in close contact with them. This can increase the number of infected cases [33]. Close physical contact and / or contact with respiratory secretions are crucial routes of the human–human transmission of NiV. Several NiV outbreaks have occurred with which human–human transmission has occurred. Studies have reported that patients who cough are more likely to transmit NiV; furthermore, healthcare workers who share rooms and food or come in contact with NiV-infected patients are at extremely high risk, particularly when the healthcare worker touches the patient's saliva, thereby emphasizing that close contact is warranted for human–human transmission [32]. Another study has reported that the cadaver of a deceased patient with NiV infection can transmit the virus to those in close contact with the cadaver [34].

LayV is an RNA virus with a relatively high degree of variability. It has a relatively high error rate of genomic RNA replication mediated by an error-prone RNA-dependent RNA polymerase. Fortunately, LayV has directly not caused a public health concern to date [35]. Furthermore, it can undergo genetic evolution and rapid antigenic mutation in infected hosts. No targeted treatment or protective vaccine is available for this virus; however, doctors are trying to use different antiviral drugs to treat the symptoms. Therefore, establishing surveillance methods to achieve the timely, accurate, and comprehensive management of virus exposure risks and preparing for the early warning, prediction, disposal, and post-event recovery stages of emergencies are vital. Using the surveillance methods used during the 2018 NiV outbreak, such as culture isolation, serological methods, and molecular biology assays, to detect viral shedding and isolate the virus from possible animal hosts can help reduce the potential threat of LayV to humans [36]. Virus isolation and immunohistochemical assays of NiV must be conducted in biosafety level 4 laboratories, whereas the detection of inactivated clinical samples *via* real-time fluorescence-based qRT-PCR can only be conducted in biosafety level 2 laboratories [37]. Molecular biology assays, particularly real-time fluorescence-based qRT-PCR, exhibit advantages such as good repeatability, high sensitivity, short detection time, and strong specificity. They are promising tools for rapidly screening and identifying multiple pathogens simultaneously [38,39], which is suitable for laboratories at the provincial, municipal, and county levels.

LayV, MojV, NiV, and CedV are all negative-stranded RNA viruses belonging to the genus *Hennipavirus* of the family *Paramyxoviridae*. Traditional serological detection assays are prone to cross-reactivity and exhibit low sensitivity and specificity. Therefore, molecular biology-based diagnostic methods play an important role in the early diagnosis of viral infections. The detection and differentiation of these four viruses are important for rapidly identifying pathogens. According to the principles of real-time fluorescence-based qRT-PCR, the specific primers for detecting the four viruses were designed and combined to optimize the method, which could distinguish the four viruses in a single assay, saving the amount of reagents and samples used and shortening the pathogen detection time. In this study, the quadruple real-time fluorescence-based qRT-PCR assay was established to specifically amplify four target genes in a sample containing multiple viral nucleic acids mixed in one tube and their respective single tubes. The repeatability of the assay was determined using high, medium, and low concentrations of the nucleic acids of the four viruses, and the standard deviations of Ct values were <0.5, and the CV was <3 % in 14 independent quadruple real-time fluorescence-based qRT-PCR assays [37,40]. This indicated good specificity and repeatability. However, one limitation of this assay is the absence of specimens and strains of LaV, MojV, and CedV; hence, additional confirmation is required in the future.

Our established quadruple real-time fluorescence-based qRT-PCR assay has good specificity, sensitivity, and stability. This method simplifies the experimental steps, reduces the amount of samples required, and saves experimental costs. Furthermore, it provides reference information for rapidly investigating suspected infected people and technical support for the differential diagnosis of related diseases as well as valuable time for controlling the epidemic.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Wenjun He: Investigation, Formal analysis, Writing – original draft. **Tian Ma:** Conceptualization, Investigation. **Yalan Wang:** Conceptualization, Investigation. **Weifang Han:** Conceptualization, Investigation. **Jun Liu:** Conceptualization, Writing – review & editing. **Wenwen Lei:** Conceptualization, Supervision, Resources, Writing – review & editing. **Le Zhang:** Conceptualization, Writing – review & editing. **Guizhen Wu:** Conceptualization, Supervision, Resources, Writing – review & editing.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2024.02.002.

References

[1] W.J. Liu, J. Xu, The arms race in the war between virus and host: implications for anti-infection immunity, Infect. Dis. Immun. 2 (3) (2022) 129–131, https://doi.org/10.1097/id9.0000000000000062.

[2] C. Quan, Q. Wang, J. Zhang, M. Zhao, Q. Dai, T. Huang, Z. Zhang, S. Mao, Y. Nie, J. Liu, et al., Avian influenza a viruses among occupationally exposed populations, China, 2014–2016, Emerg. Infect. Dis. 25 (12) (2019) 2215–2225, https://doi.org/10.3201/eid2512.190261.

[3] C.K. Thakur, J.B. Adhikari, N. Gupta, P. Ghimire, M. Dhimal, Is the emergence of the zoonotic Langya virus amidst COVID-19 and monkeypox a cause for concern?, Future Virol 18 (1) (2023) 5–7, https://doi.org/10.2217/fvl-2022-0175.

[4] B.H. Harcourt, A. Tamin, T.G. Ksiazek, P.E. Rollin, L.J. Anderson, W.J. Bellini, P.A. Rota, Molecular characterization of Nipah virus, a newly emergent paramyxovirus, Virology 271 (2) (2000) 334–349, https://doi.org/10.1006/viro.2000.0340.

[5] M. Rahman, A. Chakraborty, Nipah virus outbreaks in Bangladesh: a deadly infectious disease, WHO South East Asia, J. Public Health 1 (2) (2012) 208–212, https://doi.org/10.4103/2224-3151.206933.

[6] K.B. Chua, C.L. Koh, P.S. Hooi, K.F. Wee, J.H. Khong, B.H. Chua, Y.P. Chan, M.E. Lim, S.K. Lam, Isolation of Nipah virus from Malaysian Island flying-foxes, Microbes Infect. 4 (2) (2002) 145–151, https://doi.org/10.1016/s1286-4579(01)01522-2.

[7] V.K. Chattu, R. Kumar, S. Kumary, F. Kajal, J.K. David, Nipah virus epidemic in southern India and emphasizing “One Health” approach to ensure global health security, J. Family Med. Prim. Care 7 (2) (2018) 275–283, https://doi.org/10.4103/jfmpc.jfmpc_137_18.

[8] L.M. Looi, K.B. Chua, Lessons from the Nipah virus outbreak in Malaysia, Malays. J. Pathol. 29 (2) (2007) 63–67.

[9] S.P. Luby, The pandemic potential of Nipah virus, Antiviral Res. 100 (1) (2013) 38–43, https://doi.org/10.1016/j.antiviral.2013.07.011.

[10] F. Hasebe, N.T. Thuy, S. Inoue, F. Yu, Y. Kaku, S. Watanabe, H. Akashi, D.T. Dat, T. Q. Mai le, K. Morita, Serologic evidence of nipah virus infection in bats, Vietnam, Emerg. Infect. Dis. 18(3) (2012) 536-537, https://doi.org/10.3201/eid1803.111121.

[11] P.D. Yadav, C.G. Raut, A.M. Shete, A.C. Mishra, J.S. Towner, S.T. Nichol, D.T. Mourya, Detection of Nipah virus RNA in fruit bat (Pteropus giganteus) from India, Am. J. Trop. Med. Hyg. 87 (3) (2012) 576–578, https://doi.org/10.4269/ajtmh.2012.11-0416.

[12] S. Chakraborty, D. Chandran, R.K. Mohapatra, M.A. Islam, M. Alagawany, M. Bhattacharya, C. Chakraborty, K. Dhama, Langya virus, a newly identified henipavirus in China - zoonotic pathogen causing febrile illness in humans, and its health concerns: current knowledge and counteracting strategies - correspondence, Int. J. Surg. 105 (2022) 106882, https://doi.org/10.1016/j.ijsu.2022.106882.

[13] X.A. Zhang, H. Li, F.C. Jiang, F. Zhu, Y.F. Zhang, J.J. Chen, C.W. Tan, D.E. Anderson, H. Fan, L.Y. Dong, et al., A zoonotic henipavirus in febrile patients in China, N. Engl. J. Med. 387 (5) (2022) 470–472, https://doi.org/10.1056/NEJMc2202705.

[14] O.P. Choudhary, Priyanka, M.L. Fahrni, A.A. Metwally, A.A. Saied, Spillover zoonotic 'Langya virus': is it a matter of concern? Vet. Q. 42(1) (2022) 172-174, https://doi.org/10.1080/01652176.2022.2117874.

[15] M.C. Rahalkar, R.A. Bahulikar, Lethal pneumonia cases in Mojiang miners (2012) and the mineshaft could provide important clues to the origin of SARS-CoV-2, Front. Public Health 8 (2020) 581569, https://doi.org/10.3389/fpubh.2020.581569.

[16] I. Rissanen, A.A. Ahmed, K. Azarm, S. Beaty, P. Hong, S. Nambulli, W.P. Duprex, B. Lee, T.A. Bowden, Idiosyncratic Mojiang virus attachment glycoprotein directs a host-cell entry pathway distinct from genetically related henipaviruses, Nat. Commun. 8 (2017) 16060, https://doi.org/10.1038/ncomms16060.

[17] Z. Wu, L. Yang, F. Yang, X. Ren, J. Jiang, J. Dong, L. Sun, Y. Zhu, H. Zhou, Q. Jin, Novel Henipa-like virus, Mojiang Paramyxovirus, in rats, China, 2012, Emerg. Infect. Dis. 20 (6) (2014) 1064–1066, https://doi.org/10.3201/eid2006.131022.

[18] G.A. Marsh, C. de Jong, J.A. Barr, M. Tachedjian, C. Smith, D. Middleton, M. Yu, S. Todd, A.J. Foord, V. Haring, et al., Cedar virus: a novel Henipavirus isolated from Australian bats, PLoS Pathog. 8 (8) (2012) e1002836, https://doi.org/10.1371/journal.ppat.1002836.

[19] J. Quarleri, V. Galvan, M.V. Delpino, Henipaviruses: an expanding global public health concern?, Geroscience 44 (5) (2022) 2447–2459, https://doi.org/10.1007/s11357-022-00670-9.

[20] S. Taseen, M. Abbas, F. Nasir, S. Wania Amjad, M.S. Asghar, Tip of the iceberg: emergence of Langya virus in the postpandemic world, J. Med. Virol. 95 (1) (2023) e28173, https://doi.org/10.1002/jmv.28173.

[21] M.T. Rahman, M.A. Sobur, M.S. Islam, S. Ievy, M.J. Hossain, M.E. El Zowalaty, A.T. Rahman, H.M. Ashour, Zoonotic diseases: etiology, impact, and control, Microorganisms 8 (9) (2020) 1405, https://doi.org/10.3390/microorganisms8091405.

[22] R. Sah, A. Mohanty, S. Chakraborty, K. Dhama, Langya virus: a newly identified zoonotic henipavirus, J. Med. Virol. 94 (12) (2022) 5621–5622, https://doi.org/10.1002/jmv.28095.

[23] M.J. Mun, J.Y. Bae, J.H. Kim, S.B. Kim, I. Lee, J.I. Kim, M.S. Park, M.S. Park, Y.S. Nam, One-step multiplex real-time RT-PCR for detection and typing of dengue virus, Mol. Cell. Probes 43 (2019) 86–91, https://doi.org/10.1016/j.mcp.2018.10.001.

[24] G.F. Trindade, S.M.B. de Lima, C. Britto, A.G. Fernandes-Monteiro, Detection of yellow fever virus by quantitative real-time PCR (qPCR), Methods Mol. Biol. 2065 (2020) 65–77, https://doi.org/10.1007/978-1-4939-9833-3_6.

[25] S.M. Wang, U.H. Ali, S.D. Sekaran, R. Thayan, Detection and quantification of chikungunya virus by real-time RT-PCR assay, Methods Mol. Biol. 1426 (2016) 105–117, https://doi.org/10.1007/978-1-4939-3618-2_10.

[26] B.A. Clayton, L.F. Wang, G.A. Marsh, Henipaviruses: an updated review focusing on the pteropid reservoir and features of transmission, Zoonoses Public Health 60 (1) (2013) 69–83, https://doi.org/10.1111/j.1863-2378.2012.01501.x.

[27] J.F. Drexler, V.M. Corman, M.A. Müller, G.D. Maganga, P. Vallo, T. Binger, F. Gloza-Rausch, V.M. Cottontail, A. Rasche, S. Yordanov, et al., Bats host major mammalian paramyxoviruses, Nat. Commun. 3 (2012) 796, https://doi.org/10.1038/ncomms1796.

[28] A.J. Peel, D.R. Sargan, K.S. Baker, D.T.S. Hayman, J.A. Barr, G. Crameri, R. Suu-Ire, C.C. Broder, T. Lembo, L.F. Wang, et al., Continent-wide panmixia of an African fruit bat facilitates transmission of potentially zoonotic viruses, Nat. Commun. 4 (2013) 2770, https://doi.org/10.1038/ncomms3770.

[29] P. Lawrence, B. Escudero-Pérez, Henipavirus immune evasion and pathogenesis mechanisms: lessons learnt from natural infection and animal models, Viruses 14 (5) (2022) 936, https://doi.org/10.3390/v14050936.

[30] K. Halpin, A.D. Hyatt, R. Fogarty, D. Middleton, J. Bingham, J.H. Epstein, S.A. Rahman, T. Hughes, C. Smith, H.E. Field, et al., Pteropid bats are confirmed as the reservoir hosts of henipaviruses: a comprehensive experimental study of virus transmission, Am. J. Trop. Med. Hyg. 85 (5) (2011) 946–951, https://doi.org/10.4269/ajtmh.2011.10-0567.

[31] J.H. Epstein, S.J. Anthony, A. Islam, A.M. Kilpatrick, S. Ali Khan, M.D. Balkey, N. Ross, I. Smith, C. Zambrana-Torrelio, Y. Tao, et al., Nipah virus dynamics in bats and implications for spillover to humans, Proc. Natl. Acad. Sci. U. S. A. 117 (46) (2020) 29190–29201, https://doi.org/10.1073/pnas.2000429117.

[32] N. Nahar, R.C. Paul, R. Sultana, E.S. Gurley, F. Garcia, J. Abedin, S.A. Sumon, K.C. Banik, M. Asaduzzaman, N.A. Rimi, et al., Raw sap consumption habits and its association with knowledge of Nipah virus in two endemic districts in Bangladesh, PLoS One 10 (11) (2015) e0142292, https://doi.org/10.1371/journal.pone.0142292.

[33] M.S. Khan, J. Hossain, E.S. Gurley, N. Nahar, R. Sultana, S.P. Luby, Use of infrared camera to understand bats' access to date palm sap: implications for preventing Nipah virus transmission, Ecohealth 7 (4) (2010) 517–525, https://doi.org/10.1007/s10393-010-0366-2.

[34] A. Chakraborty, H.M. Sazzad, M.J. Hossain, M.S. Islam, S. Parveen, M. Husain, S.S. Banu, G. Podder, S. Afroj, P.E. Rollin, et al., Evolving epidemiology of Nipah virus infection in Bangladesh: evidence from outbreaks during 2010–2011, Epidemiol. Infect. 144 (2) (2016) 371–380, https://doi.org/10.1017/s0950268815001314.

[35] T.F. Kautz, N.L. Forrester, RNA virus fidelity mutants: a useful tool for evolutionary biology or a complex challenge?, Viruses 10 (11) (2018) 600, https://doi.org/10.3390/v10110600.

[36] R.K. Plowright, D.J. Becker, D.E. Crowley, A.D. Washburne, T. Huang, P.O. Nameer, E.S. Gurley, B.A. Han, Prioritizing surveillance of Nipah virus in India, PLoS Negl. Trop. Dis. 13 (6) (2019) e0007393, https://doi.org/10.1371/journal.pntd.0007393.

[37] K.S. Jensen, R. Adams, R.S. Bennett, J. Bernbaum, P.B. Jahrling, M.R. Holbrook, Development of a novel real-time polymerase chain reaction assay for the quantitative detection of Nipah virus replicative viral RNA, PLoS One 13 (6) (2018) e0199534, https://doi.org/10.1371/journal.pone.0199534.

[38] B. Gröndahl, W. Puppe, A. Hoppe, I. Kühne, J.A. Weigl, H.J. Schmitt, Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study, J. Clin. Microbiol. 37 (1) (1999) 1–7, https://doi.org/10.1128/jcm.37.1.1-7.1999.

[39] A.W. Paton, J.C. Paton, A.J. Lawrence, P.N. Goldwater, R.J. Harris, Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification, J. Clin. Microbiol. 30 (4) (1992) 901–904, https://doi.org/10.1128/jcm.30.4.901-904.1992.

[40] W. Wu, J. Wang, N. Yu, J. Yan, Z. Zhuo, M. Chen, X. Su, M. Fang, S. He, S. Zhang, et al., Development of multiplex real-time reverse-transcriptase polymerase chain reaction assay for simultaneous detection of Zika, dengue, yellow fever, and chikungunya viruses in a single tube, J. Med. Virol. 90 (11) (2018) 1681–1686, https://doi.org/10.1002/jmv.25253.