

Original Article

Asian Pacific Journal of Tropical Medicine

apjtm.org

doi: 10.4103/apjtm.apjtm_957_23

Advancing public health preparedness: Establishment of Nipah virus molecular diagnostic test at the National Reference Laboratory, Sri Lanka

Hewa Babarandage Chathurika Harshani, Denagama Vitharanage Rishan Geeth Ruwan, Udage Kankanamge Isuru Udara Samaraweera, Dedunu C U Dias Weligamage, Janaki I Abeynayake✉

Medical Research Institute, Colombo-08, Sri Lanka

ABSTRACT

Objective: To establish Nipah virus diagnostic capabilities at the National Reference Laboratory in Sri Lanka using the NIV Pune real-time PCR kit.

Methods: Strict safety precautions were adhered during testing due to the high pathogenicity of the Nipah virus, with all diagnostics conducted in a BSL2+ laboratory at the Medical Research Institute in Sri Lanka. RNA extraction was performed using the QIAamp Viral RNA Mini kit. The NIV Pune in-house real-time PCR kit was employed, following established primer/probe sequences and controls. The assay was validated using the Rotor-Gene Q Series Real-time PCR platform.

Results: The validation run of the Nipah virus real-time PCR test demonstrated robust performance, with positive controls consistently detecting Nipah RNA at a Ct value of 21.50±0.01. Negative controls confirmed assay specificity with an external negative control which was also used as an extraction control and showed no interference. The internal control exhibited stable behavior, enhancing confidence in PCR results. The qPCR analysis graph illustrated the successful detection of internal and positive controls, validating the reliability of the assay.

Conclusions: Establishing Nipah virus diagnostic capabilities in Sri Lanka signifies a proactive and collaborative response to the persistent global health threat.

KEYWORDS: Nipah virus; Real-time PCR; Validation; Molecular diagnostics

1. Introduction

Surveillance of emerging infectious disease remains a critical though challenging task[1,2]. The Nipah virus has become one of the major global health concerns related to infectious diseases, requiring careful attention and comprehensive understanding[3]. The Nipah virus belongs to the Paramyxoviridae family and was initially discovered in 1999, during an outbreak that affected both humans and pigs in Malaysia. Subsequent outbreaks were reported in Singapore, followed by a period of dormancy in Malaysia. However, sporadic outbreaks occurred in local areas of Bangladesh and Eastern India from 2001 onwards. A recent outbreak in Kerala, South India, in late August 2023, resulted in 6 infections and 2 deaths, with 700 potential contacts quarantined and tested[4]. WHO

Significance

Previous knowledge highlights the critical need for reliable diagnostic tools for Nipah virus, a significant global health threat. This study adds to this by successfully establishing Nipah virus diagnostic capabilities in Sri Lanka using the NIV Pune real-time PCR kit, demonstrating robust performance and assay specificity. This enhances the nation's preparedness and response to potential Nipah virus outbreaks.

✉To whom correspondence may be addressed. E-mail: janakiiabeynayake@yahoo.com

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2024 *Asian Pacific Journal of Tropical Medicine* Produced by Wolters Kluwer-Medknow.

How to cite this article: Harshani HBC, Ruwan DVRG, Samaraweera UKIU, Weligamage DCUD, Abeynayake JI. Advancing public health preparedness: Establishment of Nipah virus molecular diagnostic test at the National Reference Laboratory, Sri Lanka. Asian Pac J Trop Med 2024; 17(8): 369-374.

Article history: Received 15 November 2023 Revision 14 March 2024
Accepted 21 March 2024 Available online 30 August 2024

has classified Nipah disease as one of the top emerging pathogens due to its significant public health threat[5].

Nipah virus is known to be zoonotic, originated in bats and spread to humans. Nipah virus infections have been associated with severe respiratory and neurological symptoms, with mortality rates of 40% to 75% of human cases[5,6]. The virus has been responsible for sporadic outbreaks in various regions of Southeast Asia, including Bangladesh and India, drawing attention due to its potential for rapid transmission and the lack of specific antiviral treatments[7]. The dynamic nature of viral outbreaks emphasizes the vital importance of early detection and prompt response in reducing the impact of infectious diseases.

The emergence of Nipah virus infection in Kerala, India, underscores the importance of understanding its epidemiology and the potential threat it poses to neighboring countries like Sri Lanka. Studies have demonstrated the epidemiology, transmission dynamics, and control measures of Nipah virus from various affected regions including Malaysia, Singapore, Bangladesh, and India[1,6,8]. Sri Lanka is close to the affected areas in location and has close interactions in travel and trade with these areas. As a result, vigilance and preparedness are essential to prevent and manage potential outbreaks in Sri Lanka.

Setting up a Nipah virus diagnostic test at the National Reference Laboratory at Medical Research Institute in Sri Lanka is a crucial endeavor in recognition of this urgency. Precise identification of Nipah virus typically involves serological, molecular, or virological examinations, such as western blotting, enzyme-linked immunosorbent assay, plaque assay, immunofluorescence staining, PCR-based genome detection, quantitative PCR, and virus isolation[1,9,10]. The importance of molecular techniques in detecting viral RNA was highlighted by Wong *et al*.[11] in the context of real-time PCR for the diagnosis of Nipah virus. These techniques provide fast and accurate results that are essential for early intervention and containment measures. In addition to securing high sensitivity and specificity in detecting Nipah virus cases, the real-time PCR methodology expedites the process of making decisions regarding public health responses[12]. This development in diagnostic technology is essential to strengthening the National Reference Laboratory's capacity to effectively monitor and manage the Nipah virus cases in Sri Lanka. The primary goal of the project is to strengthen Sri Lanka's public health system by establishing advanced Nipah virus diagnostic tool at National Reference Laboratory at Medical Research Institute. By accelerating the identification of Nipah virus cases, this proactive action hopes to enable more timely and focused public health responses.

2. Materials and methods

Strict safety precautions were taken during the testing due to high level of pathogenicity of the Nipah virus[10]. All diagnostic operations were carried out at the Biosafety 2^+ ($BSL2^+$) laboratory at Medical Research Institute in Sri Lanka, assuring the highest levels of protection from containment.

RNA extraction was done using QIAamp Viral RNA Mini kit (REF: 52906, LOT: 166024854, Qiagen, Germany) following manufactures' instructions. This kit, renowned for its dependability and efficiency, makes it easier to extract precisely Nipah virus RNA while preserving the integrity of genetic material for further steps.

In the diagnostic protocol, the in-house real-time PCR kit sourced from the National Institute of Virology (NIV) in Pune was employed, utilizing the original primer/probe sequences as detailed by Guillaume *et al*.[10]. Accordingly, the forward primer (Ni-NP1209) sequence was 5' GCAAGAGAGTAATGTTCAGGCTAGAG 3', and the reverse primer (Ni-NP1314) sequence was 5' CTGTTCTATAGGTTCTTCCCCTTCAT 3'. The fluorescent probe (Ni-NP1248Fam) sequence was 5' TGCAGGAGGTGTGCTCATTGGAGG 3'. This study utilized internal controls, as well as positive and negative controls provided by the in-house real-time PCR kit (NIV, Pune). An external negative control was employed, derived from a sample received at Medical Research Institute for measles PCR testing, collected in accordance with guidelines provided by Ministry of Health, Sri Lanka. In this study, five samples (CSF=3, Swab=2) from three Nipah suspected patients, which had been received by the Medical Research Institute for viral studies, were collected. These specimens, also transported in viral transport medium, had been meticulously collected in accordance with the guidelines set forth. These samples were tested in a separate run by using established PCR protocol.

In the present study, the Rotor Gene Q Series Real-time PCR platform (SN: R0619121, GTIN: 04053228010838, Qiagen, Germany) was employed for the execution and validation of the assay. It is noteworthy that the utilization of this kit underwent thorough validation within the study, ensuring its precision, sensitivity, and specificity in the detection of Nipah viral RNA.

3. Results

The findings from Nipah virus real-time PCR test validation run provide a thorough assessment of the assay's performance. The assay's capacity to detect the virus was validated by the positive controls (PC1 and PC2), which detected Nipah RNA with a constant Ct value of 21.50±0.01 (mean±SD). The final interpretation of "Negative" for the absence of Nipah RNA and the lack of detection in the negative controls (NC1 and NC2) verified the specificity of the test. The robustness of the assay was further supported by the results

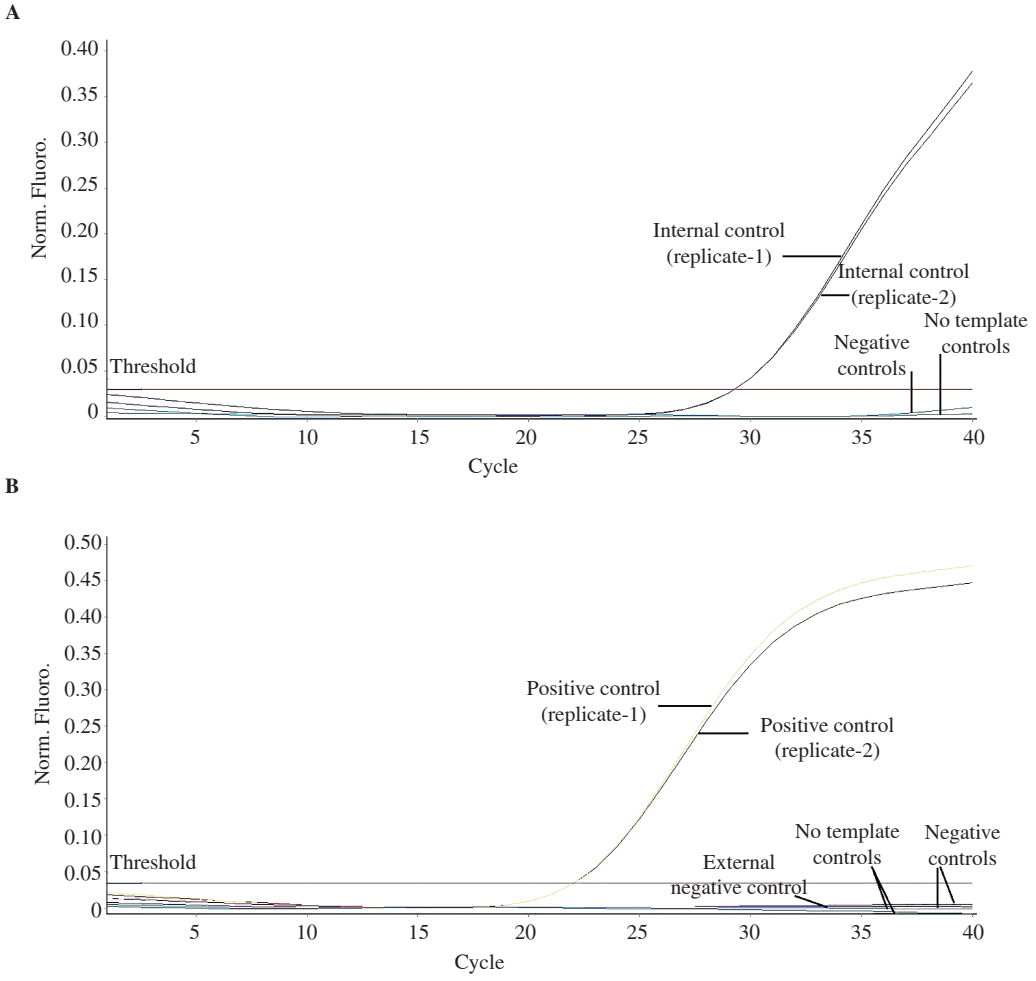

Figure 1. The graph illustrates the real-time polymerase chain reaction (qPCR) analysis of Nipah virus. The X-axis denotes PCR cycles, while the Y-axis represents fluorescence signals. (A) depicts the successful detection of the Internal Controls (IC) with specific samples labeled as Internal Control-1, Internal Control-2, Negative Control, and No Template Control. (B) showcases the successful detection of the positive controls with samples labeled as Positive Control-1, Positive Control-2, Negative Controls, External Negative Control, and No Template Controls.

Table 1. Results interpretation table of Nipah virus by NIV Pune Rt-PCR kit.

Target	Nipah (FAM)	RNase P (HEX)	Remark	Action
Positive	[+] Ct value⩽38	[+] Ct value⩽35	Nipah RNA detected by real time rt-PCR	Report the results. Test is valid.
Negative	[-] Ct value>38	[+] Ct value⩽35	Nipah RNA not detected by real time rt-PCR	Report the results. Test is valid.
Inconclusive	[-] Ct value⩽38	[-] Ct value>35	Specimen inconclusive for the presence of Nipah RNA real time rt-PCR.	Repeat the extraction. If unable to resolve inconclusive result for specimens, request collection of additional specimens from the patient. Report the inconclusive results.

Ct: Cycle threshold; FAM and HEX are reporter dyes.

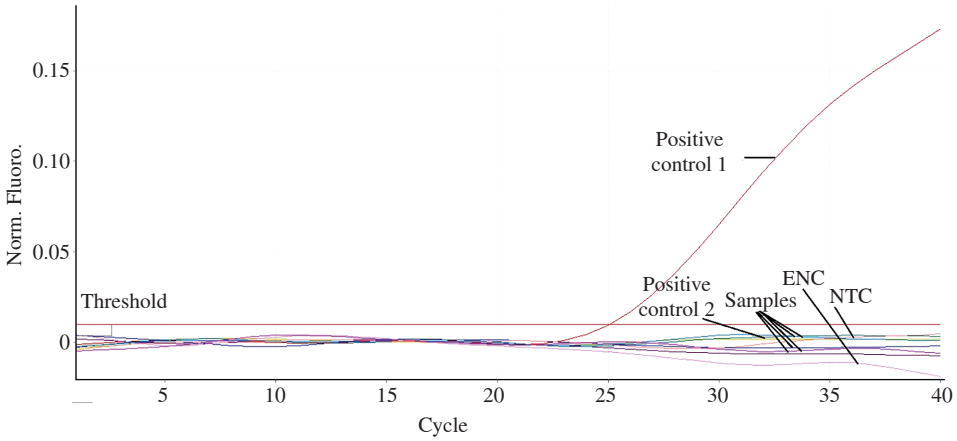

Figure 2. Real-time polymerase chain reaction (qPCR) analysis of Nipah virus during the second run of the study. The X-axis represents PCR cycles, while the Y-axis denotes fluorescence signals. Positive control 1 (neat) exhibits a positive curve, indicating the presence of Nipah virus, whereas positive control 2 (diluted) shows negative results. All five tested samples display negative results for the PCR assay. Additionally, both the no template control (NTC) and the external negative control (ENC) demonstrate negative results, confirming the validity of the PCR assay.

of the external negative control, which was derived from a measles run and showed no interference from the extraction procedure with either the RNaseP gene or Nipah targets. The absence of Nipah RNA in the template controls (NTC1 and NTC2) confirmed the assay's specificity (Figure 1).

The second run of the assay was also conducted to analyze the Nipah virus target (FAM) cycle threshold (Ct) values and internal quality control (IQC) Ct values. Notably, positive control PC1 (Neat) exhibited a Ct value of 26, indicating a positive result, while PC2 (Diluted) showed a negative result. Among the clinical samples, Ct values (IQC) ranged from 23 to 36 with all samples, including cerebrospinal fluid (CSF) and swab specimens, interpreted as negative (Figure 2).

In Figure 1A, the amplification plot of the internal control (IC) mirrors that of the target gene, exhibiting a consistent baseline and characteristic exponential phase. This consistent behavior serves as a critical indicator of the overall robustness of the PCR process. The stability of the IC is crucial, especially when interpreting the amplification plot of the target gene. If challenges are observed in the target gene amplification, yet the IC demonstrates anticipated performance, it implies that the issue is likely confined to the target gene itself and not from general PCR inhibition or technical constraints. This underscores the value of the IC as a reliable internal reference, providing confidence in the accuracy and specificity of the observed PCR results.

Within our PCR experiment (Figure 1B), we incorporated dual replicates of the positive control, a deliberate measure to enhance the reproducibility and dependability of our assay. Under identical experimental conditions, both replicates of the positive control demonstrated successful amplification of the target sequence. This observed consistency serves as a robust indicator, instilling confidence in the precision and accuracy of our experimental results. Moreover, the inclusion of these replicates functions as a stringent quality control measure, effectively detecting and addressing any potential technical issues that might emerge during the course of the experiment.

The interpretation of the results was followed by instructions provided by the ICMR, Pune Kit (Table 1). This offers precise instructions for reporting results that are positive, negative, or inconclusive depending on specific Ct threshold values. When the Ct threshold value for the Nipah target is $\leqslant$38 and the RNaseP threshold value is $\leqslant$35, it is interpreted as positive, signifying the successful detection of Nipah RNA. When the RNaseP threshold value is $\leqslant$35 and the Ct threshold value for the Nipah target is >38, it is interpreted as negative, confirming the lack of Nipah RNA. It is suggested to repeat the extraction in the event of inconclusive results, which indicate ambiguity regarding the existence of Nipah RNA. If the results remain inconclusive, it is recommended to further collect specimen from the patient (Table 1).

4. Discussion

This study highlights the proactive laboratory measures undertaken in Sri Lanka in response to the global threat posed by the Nipah virus outbreak. Since its discovery in 1999, the Nipah virus has sporadically affected various regions, notably Bangladesh and India[13]. The recent outbreak in Kerala, India, underscores the virus's enduring presence, emphasizing the importance of cross-border preparedness measures. One significant initiative involves

establishing Nipah virus diagnostic capabilities at the National Reference Laboratory in Sri Lanka using the NIV Pune real-time PCR kit, thus enhancing the nation's preparedness in public health[14].

Molecular diagnostic techniques, particularly PCR and genome sequencing, play a crucial role in Nipah virus detection[15]. Nucleic acid amplification tests like PCR are preferred due to their high sensitivity, capable of detecting as few as 20 viral genomes. However, their infrastructure requirements may pose challenges, and their sensitivity may decrease with viral genome mutations or variations in probe-design regions with new strains. To address this, probes are designed for highly conserved sequences found in all known strain sequences of the targeted pathogen[16].

In the early stages of illness, laboratory diagnosis typically involves real-time PCR analysis of samples obtained from throat and nasal swabs, cerebrospinal fluid, urine, and blood. As the disease progresses and during recovery, antibody testing using enzyme-linked immunosorbent assay methods is commonly performed[17]. The adoption of real-time PCR is crucial to meet the urgent need for accurate diagnosis, particularly in settings lacking established Nipah virus detection methods, such as the Medical Research Institute Sri Lanka. Real-time PCR offers high specificity and sensitivity, enabling rapid and reliable diagnosis within hours[18].

Several PCR assays have been developed for Nipah virus detection, including both in-house assays and commercial kits like the NIV Pune PCR kit. For instance, Tong *et al*.[19] described a nested reverse transcriptase PCR (RT-PCR) targeting a conserved region of the L gene, while Wacharapluesadee *et al*.[20] designed a duplex RT-PCR for RNA detection from bat urine, targeting a conserved region of the *N* gene. However, the present study employed real time reverse transcriptase PCR which can be used to get both qualitative and quantitative results.

According to Mazzola *et al*.[15], various real-time RT-PCR assays have been developed by different institutions for the detection of Nipah virus (NiV) and Hendra virus (HeV). These assays target specific genes such as the *P*, *M*, and *N* genes of NiV/HeV. The CSIRO/AAHL in collaboration with the University of Cambridge and the Zoological Society in the UK developed a real-time RT-PCR assay targeting the *P*, *M*, and *N* genes. This assay provides a comprehensive approach for detecting both NiV and HeV[21]. Another approach is the multiplex bead-based real-time RT-PCR assay developed by CSIRO/AAHL in Australia. This assay targets the *N* and *P* genes of NiV and HeV simultaneously, allowing for efficient detection of both viruses in a single reaction[19]. The CDCs in the USA and Kenya have developed a multiplex array card real-time RT-PCR assay with a 26-multiplex panel, including the NiV *N* gene. This assay offers the capability to simultaneously detect multiple pathogens, including NiV, providing a comprehensive surveillance tool[22]. Similarly, the Kenya Medical Research Institute in collaboration with the CDCs in the USA and Kenya developed a multiplex array card real-time RT-PCR assay with a 21-multiplex panel, including the NiV *N* gene. This assay also allows for the simultaneous detection of multiple pathogens, including NiV, aiding in rapid and efficient diagnosis[23]. In contrast, the NIV Pune real time PCR kit, used in the present study, focuses on amplifying a conserved region of the *N* gene similar to the assay developed by Guillaume *et al*.[10].

Compared to other in-house assays, the NIV Pune PCR kit offers standardized and validated protocols for Nipah virus detection. It employs TaqMan Real-Time PCR, ensuring specificity for Nipah virus without amplification of other paramyxoviruses in One-step real time reverse transcriptase PCR.

The choice between in-house assays and commercial kits often depends on factors such as laboratory resources, expertise, and specific research or diagnostic requirements. While in-house assays may offer flexible, customized, commercial kits like the NIV Pune PCR kit provide standardized protocols and validated performance, ensuring accuracy and reliability in Nipah virus detection.

As a limitation of this study, we only used swab samples and CSF samples from the patients for the validation, therefore, further studies are needed for the other types of samples. Additionally, as there was no Nipah-positive animal/human samples available for validation, a positive control provided with the NIV Pune kit was utilized. This limitation is recognized and will be rectified in future research efforts.

The establishment of Nipah virus diagnostic capabilities in Sri Lanka brings about several crucial benefits. Rapid identification and confirmation of Nipah virus cases empower timely public health responses, enabling authorities to implement effective containment measures. This proactive approach not only safeguards the health of the population but also contributes to regional and global efforts in preventing the spread of Nipah virus.

In conclusion, the establishment of Nipah virus diagnostic testing at the National Reference Laboratory in Sri Lanka signifies a proactive response to a persistent global health threat. By adopting advanced molecular diagnostic tools, Sri Lanka not only strengthens its own defenses but also to the collective global effort against emerging infectious diseases. This initiative serves as an evidence to the nation's commitment to public health and its role in the broader context of global health security.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgments

We acknowledge the staff of the Department of Virology, Medical Research Institute, Sri Lanka.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

Conceptualization: HBCH, JIA; data curation: HBCH, JIA; formal analysis: HBCH, JIA; investigation: DVRGR, UKIUS; writing original draft: HBCH, JIA; writing-review & editing: JIA, HBCH, DCUDW.

References

[1] Abeynayake JI, Chathuranga GP, Fernando MAY, Sahoo MK. Surveillance of emerging SARS-CoV-2 variants by nanopore technology-based genome sequencing. *Asian Pac J Trop Med* 2023; **16**(7): 313-320.

[2] Biswas S, Khuntia HK, Bal M, Pati S, Ranjit M. Emerging and re-emerging diseases: A narrative review. *One Health Bull* 2023; **3**(1): 20.

[3] Chua KB, Goh KJ, Wong KT, Kamarulzaman A, Tan PSK, Ksiazek TG, et al. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *Lancet* 1999; **354**(9186): 1257-1259.

[4] Palihawadana P. *Weekly epidemiological report; Nipah virus infection.* [Online]. Available from: https://www.epid.gov.lk/storage/post/pdfs/Vol%2039%20NO%2008%20English.pdf. [Accessed on 28 November 2023].

[5] World Health Organization. *Nipah virus outbreaks. 2020.* [Online]. Available from: https://www.who.int/news-room/fact-sheets/detail/nipah-virus. [Accessed on 25 November 2023].

[6] Luby SP, Gurley ES, Hossain MJ. Transmission of human infection with Nipah virus. *Clin Infect Dis* 2009; **49**(11): 1743-1748.

[7] Chattu VK, Kumar R, Kumary S, Kajal F, David JK. Nipah virus epidemic in southern India and emphasizing "One Health" approach to ensure global health security. *J Family Med Prim Care* 2018; **7**(2): 275-283.

[8] Garbuglia AR, Lapa D, Pauciullo S, Raoul H, Pannetier D. Nipah virus: An overview of the current status of diagnostics and their role in preparedness in endemic countries. *Viruses* 2023; **15**(10): 2062.

[9] Gurley ES, Montgomery JM, Hossain MJ, Bell M, Azad AK, Islam MR, et al. Person-to-person transmission of Nipah virus in a Bangladeshi community. *Emerg Infect Dis* 2007; **13**(7): 1031-1037.

[10] Guillaume V, Lefeuvre A, Faure C, Marianneau P, Buckland R, Lam SK, et al. Specific detection of Nipah virus using real-time RT-PCR (TaqMan). *J Virol Methods* 2004; **120**(2): 229-237.

[11] Crameri G, Wang LF, Morrissy C, White J, Eaton BT. A rapid immune plaque assay for the detection of Hendra and Nipah viruses and anti-virus antibodies. *J Virol Methods* 2002; **99**(1-2): 41-51.

[12] Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; **348**(20): 1967-1976.

[13] Wong KT, Shieh WJ, Kumar S, Norain K, Abdullah W, Guarner J, et al. Nipah virus pathology working group. Nipah virus infection: Pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am J Pathol* 2002; **161**(6): 2153-2167.

[14] Mazzola LT, Kelly-Cirino C. Diagnostics for Nipah virus: A zoonotic pathogen endemic to Southeast Asia. *BMJ Glob Health* 2019; doi: https://doi.org/10.1136/bmjgh-2018-001118.

[15] Garbuglia AR, Lapa D, Pauciullo S, Raoul H, Pannetier D. Nipah virus: An overview of the current status of diagnostics and their role in preparedness in endemic countries. *Viruses* 2023; **15**(10): 2062. doi: https://doi.org/10.3390/v15102062.

[16] Chong HT, Kunjapan SR, Thayaparan T, Tong J, Petharunam V, Jusoh MR, et al. Nipah encephalitis outbreak in Malaysia, clinical features in patients from Seremban. *Can J Neurol Sci* 2002; **29**(1): 83-87.

[17] Bruno L, Nappo MA, Ferrari L, Di Lecce R, Guarnieri C, Cantoni AM, et al. Nipah virus disease: Epidemiological, clinical, diagnostic and legislative aspects of this unpredictable emerging zoonosis. *Animals (Basel)* 2022; **13**(1): 159.

[18] Centers for Disease Control and Prevention (CDC). *Nipah virus (NiV).* [Online]. Available from: https://www.cdc.gov/vhf/nipah/index.html. [Accessed on 12 June 2023].

[19] Tong S, Chern SW, Li Y, Pallansch MA, Anderson LJ. Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. *J Clin Microbiol* 2008; **46**(8): 2652-2658.

[20] Wacharapluesadee S, Lumlertdacha B, Boongird K, Wanghongsa S, Chanhome L, Rollin P, et al. Bat Nipah virus, Thailand. *Emerg Infect Dis* 2005; **11**(12): 1949-1951.

[21] Feldman KS, Foord A, Heine HG, Smith IL, Boyd V, Marsh GA, et al. Design and evaluation of consensus PCR assays for henipaviruses. *J Virol Methods* 2009; **161**(1): 52-57.

[22] Liu J, Ochieng C, Wiersma S, Ströher U, Towner JS, Whitmer S, et al. Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including Ebola virus. *J Clin Microbiol* 2016; **54**(1): 49-58.

[23] Onyango CO, Loparev V, Lidechi S, Bhullar V, Schmid DS, Radford K, et al. Evaluation of a TaqMan array card for detection of central nervous system infections. *J Clin Microbiol* 2017; **55**(7): 2035-2044.

Publisher's note

The Publisher of the *Journal* remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Edited by Pan Y, Lei Y, Zhang Q