
Establishment of RT-PCR and Real Time RT-PCR Assays for Diagnosis of Nipah Virus Encephalitis

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ABSTRACT: Nipah virus (NiV) still remains a public health problem in Asia after its first outbreak in Malaysia during 1998-1999. NiV-associated encephalitis is known to cause a high mortality rate because of no specific treatment. Laboratory investigation for NiV infection, therefore, plays an important role on care, prevention and control. To strengthen laboratory capacity and capability on NiV diagnosis, we have established detection methods for NiV-associated encephalitis diagnosis which are Reverse transcription-polymerase chain reaction (RT-PCR) and Real time RT-PCR. The techniques aim to detect N gene of Nipah virus. Our study demonstrated that the limit of detection of RT-PCR and Real time RT-PCR assays was 10^4 copies/reaction and 10^2 copies/reaction, respectively. The assays were shown to be specific for NiV only. Determination of NiV in cerebrospinal fluid (CSF) from 30 patients with encephalitic symptoms found negative for N gene, suggesting that at present there is no Nipah infection in Thailand.

Key words: Nipah virus, RT-PCR, Real Time RT-PCR

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Introduction

Nipah Virus (NiV) has been recognized as a zoonotic paramyxovirus that is a causative agent of highly fatal and febrile human encephalitis.⁽¹⁾ It was firstly emerged in Malaysia during September 1998–April 1999. The virus was identified and further named after the village that the outbreak occurred.^(1, 2, 3) The natural reservoir of NiV is presumed to be fruit bats of the genus *Pteropus*. Pigs are the primary animal species affected by NiV infection of which direct contact or ingestion of objects or materials contaminated with urine, feces or saliva of infected fruit bats are major routes.^(4, 5, 6,) Moreover, the virus can spread among pigs by direct contact and by aerosol. Human and other animal species become infected by having close contact with sick pigs or objects contaminated with the virus.^(6, 7) Human illness can vary from no signs to death. Flu-like symptoms, such as fever, headache and muscle pain, vomiting and sore throat are initially seen, and may possibly follow by neurological signs such as disorientation, dizziness that indicate acute encephalitis. A case–fatality rate of 40% was reported during the outbreak in Malaysia.^(7, 8, 10) Subsequent outbreaks were widely reported in Southeast Asia such as Singapore, India and Bangladesh.⁽⁴⁾

Human-to-human transmission of NiV was also documented during the outbreak in Faridpur, Bangladesh. The transmission was occurred through close contact with people's secretions and excretions.⁽⁸⁻¹¹⁾ Additionally, transmission of the virus was also reported within a health-care setting, where 75% of cases occurred among hospital staff or visitors in Siliguri, India.⁽⁸⁾ Furthermore, recent cases of NiV in Bangladesh, have revealed person-to-person transmissions. It was thought that the outbreak initially started when children ate fruit contaminated by saliva and urine of fruit bats.⁽⁶⁾ Moreover, there were reports of positive detection of NiV antibodies among bats in Cambodia and Thailand^(12, 13), suggesting a possibility of human infection in Thailand and its neighboring countries.

It has been known that no effective treatment and vaccine are currently developed. Intensively supportive care with treatment of symptoms is the only approach to managing the infection in people.^(6,7) Laboratory investigation is, therefore, highly important for NiV detection in responses to public health concerns. Several technics can be used to diagnosis of NiV infection such as virus isolation, immunohistochemistry, electron microscopy, serum neutralization, ELISA, polymerase chain reaction (PCR) and sequencing. Each method has limitation and requirement on performance.

Nipah virus is classified as a hazard group 4 or Biosafety level 4 (BSL-4) pathogen, therefore, virus isolation and serum neutralization test⁽¹⁰⁾ require biosafety level 4 laboratory. In addition, there are no commercially viral antigens available for ELISA method. It is then suggested that samples preparation and RNA extraction can be performed in BSL-3 laboratory with the highest level of personnel protective equipment (PPE) according to biosafety and biosecurity rules and regulations.^(10, 20, 21) PCR and real time PCR methods can be conducted in a molecular laboratory. Owing to the fact that National Institute of Health (Thai NIH) of

Department of Medical Sciences plays major roles on detection of emerging and re-emerging pathogens affected Thailand public health and security concerns. Hence, laboratory preparedness for NiV infection is essential at Thai NIH, the national reference laboratory, according to International Health Regulation 2005 (IHR 2005). The present study was aimed to establish diagnostic methods for NiV encephalitis in humans.

Materials and Methods

Preparation of standard Nipah RNA (N gene)

Plasmid DNA, containing Nipah N gene region, was purified using the Invitrogen Endo-free Plasmid Maxi Kit (Cat. no. 12362). One ug of template DNA was required for one reaction (20 ul) of transcription reaction (using MEGAScript kit; Ambion, Cat. no. 1334). One reaction mixture was consisted of 1 ug of DNA template, 2 ul of ATP, CTP, GTP, UTP, 10x buffer, enzyme mix and nuclease free water. An amplification cycle was 37 °C for 4 hrs, followed by 4 °C forever. DNase (1 ul) was added into a reaction tube after completion of amplification cycles and further incubated at 37 °C for 20 min. followed by 4 °C forever. Removal of unincorporated nucleotides and buffer from transcription products with the MEGA clear kit (Ambion, Cat. no. 1908) was done according to the manufacturer's recommendations.⁽¹⁴⁾ RNA transcripts were further used to establish RT-PCR and real time RT-PCR assays for NiV infection.

Estimation of RNA concentration and copy number

RNA concentration was measured by Nanodrop. The RNA preparation was considered as protein-free where the OD260/280 ratio was higher than 1.8. In this study, concentration of RNA is 1,356 ng/ul. Calculation of copy number was based on the formulas: Molecular Weight (MW) of 1 mole of RNA = number of base pairs × 340 Daltons/base pair = X g. (340 Dalton is the average MW of ribonucleotide monophosphate). The number of molecules of the template per gram was calculated using Avogadro's number (6.022×10^{23} copies or molecules/mole). Avogadro's number/MW = copies/g

Copies/g can be converted to copies/ag by dividing with 10^{18}

One ag contains approximately 1 copy of transcript.⁽¹⁴⁾

Determination of detection limit of NiV by RT-PCR assay

Serial (ten-fold) dilution of standard RNA stock (10^{12} copies) with nuclease free water was done to obtain working concentrations of 10^6 to 10^0 copies/ul. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the One Step RT-PCR kit (QIAGEN, Germany). Forward and reverse primers, provided by US-CDC, were NVNBF4 (5'-GGAGTTATCAATCTA-AGTTTAG-3') and NVNBR4 (5'-CATAGAGATGAGTGTAAGC-3'), respectively. RT-PCR was performed in a 50 ul reaction mixture containing 5 ul of RNA sample, 1x reaction buffer, 0.4 mM dNTP mix, 0.2 uM of primers, 0.2U RNase inhibitor and 2 ul of Enzyme Mix. Thermal cycling

conditions were 50 °C for 30 min., 95 °C for 15 min. and 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and 72 °C for 10 min. Reaction products of 203 bp were analyzed by 2% of agarose gel electrophoresis and ethidium bromide staining⁽¹⁵⁾.

Determination of detection limit of NiV by Real time RT-PCR assay

The real time RT-PCR was performed with the SuperScript[®] III Platinum[®] One-Step Quantitative RT-PCR System Kit (Invitrogen, USA). The real time RT-PCR reaction, performed for 25 ul, was tested in duplicate using

NVBNF2B (5'-CTGGTCTCTGCAGTTATCACCATCGA-3') and

NVBN593R (5'-ACGTACTIONAGCCCATCTTCTAGTTTCA-3') as forward and reverse primers, respectively. NVBN542P (5'-CAGCTCCCGACACTGCCGAGGAT-3') was used as a probe, labelling with a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), and a non-fluorescent quencher, black hole 1 (BHQ1), at the 5' end and the 3', respectively. One reaction contained 1x of 2x SS buffer, 0.4U RNase Inhibitor (Applied Biosystems, Foster City, CA), 0.9 uM of forward and reverse primers, 0.4 uM of probe, 0.05U/ul of ROX and 0.1U/ul of SS III/Taq mix. The assay was run on ABI 7500 FAST (Applied Biosystems, Foster City, CA). The thermal cycling conditions were: (a) 1 cycle of 48 °C for 30 min and 95 °C for 10 min and (b) 45 cycles of 95 °C for 15 sec and 60 °C 1 minute. To determine efficiency of the assay, the threshold was set by choosing the values closest to the idealized slope (-3.33) and correlation coefficient (r^2) was equal 1, while the non-template control (NTC) signal remains below the threshold setting (undetermined).

Results were interpreted as positive, equivocal and negative (undetermined) when Ct values were below 38, between 38 and 39, and equal or more than 40, respectively.⁽¹⁶⁾

Analytical specificity

To evaluate the analytical specificity of RT-PCR and real time RT-PCR assays, RNA samples derived from West Nile virus, Japanese Encephalitis virus and Dengue virus, obtained from Arbovirus section, Thai NIH, were used. Each of RNA transcripts was prepared and tested as described in aforementioned methods.

Investigation of NiV in clinical specimens

Thirty cerebrospinal fluids (CSF) were collected from patients with encephalitic symptoms and were suspected for Japanese Encephalitis (JE) infections. All were diagnosed as non-JEV infection and non-dengue virus infection by ELISA method at Arbovirus section of Thai NIH. RNA was extracted from CSF samples using a NucleoSpin RNA extraction kit (Macherey-Nalgal, Duren, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) and real time RT-PCR were performed according to the established methods. RNA transcripts at concentration of 10^5 copies/ul and 10^3 copies/ul were used as positive controls for RT-PCR and real time RT-PCR, respectively.

Results

Determination of detection limit of NiV by RT-PCR

The number of Nipah RNA copies obtained from plasmid DNA containing N gene, NiV RNA, ranging from 10^6 to 10^0 copies/ul, was diluted and used to determine detection limit of RT-PCR assay. The detection limit of the established assay was 10^4 copies per reaction, as demonstrated in Fig. 1.

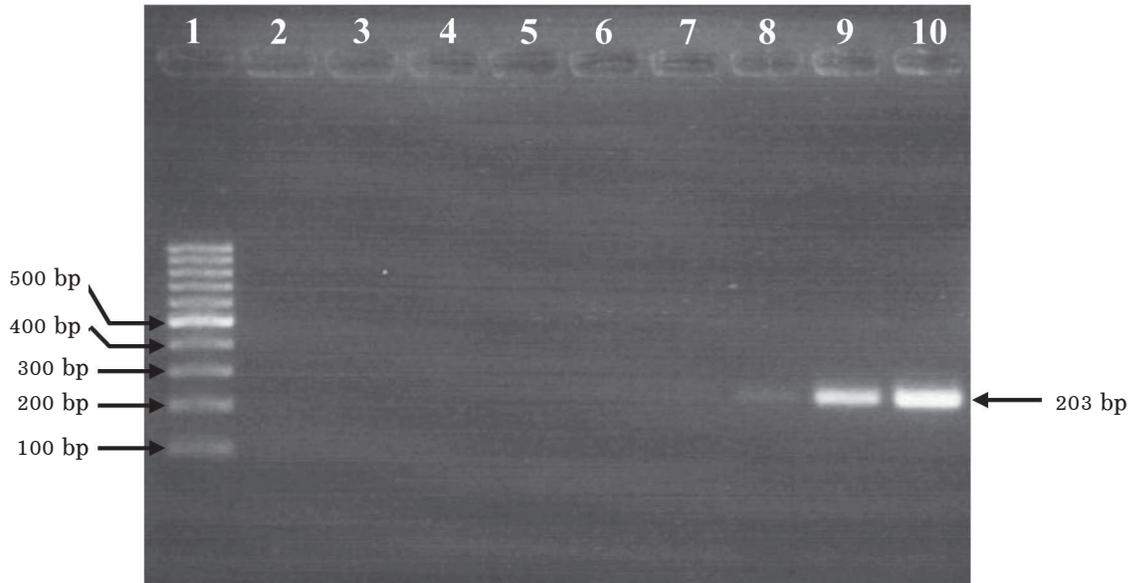


Fig. 1: Agarose gel electrophoresis of detection limit of NiV by RT-PCR assay was 10^4 copies per reaction. Lane 1 = 100 bp DNA marker, Lane 2 = Distilled water, Lane 3 = Negative control, Lane 4 = Standard RNA 10^0 copies, Lane 5 = Standard RNA 10^1 copies, Lane 6 = Standard RNA 10^2 copies, Lane 7 = Standard RNA 10^3 copies, Lane 8 = Standard RNA 10^4 copies, Lane 9 = Standard RNA 10^5 copies, Lane 10 = Standard RNA 10^6 copies.

Determination of detection limit of NiV by Real time RT-PCR

NiV RNA, ranging from 10^6 to 10^0 copies/ul, was used to determine detection limit of real time RT-PCR assay. Amplification plots of each RNA concentration were shown in Fig. 2. Our method also showed that the idealized slope was -3.362 and a correlation coefficient (r^2) was 0.999 (Fig. 3), indicating good efficiency of the established method.

Our study revealed that Ct values of 10 copies per reaction were 38.47 and 38.79, while those of 10^2 copies per reaction were 35.68 and 35.54. According to the interpretation criteria, the detection limit of real time RT-PCR assay of our performance was 10^2 copies per reaction.

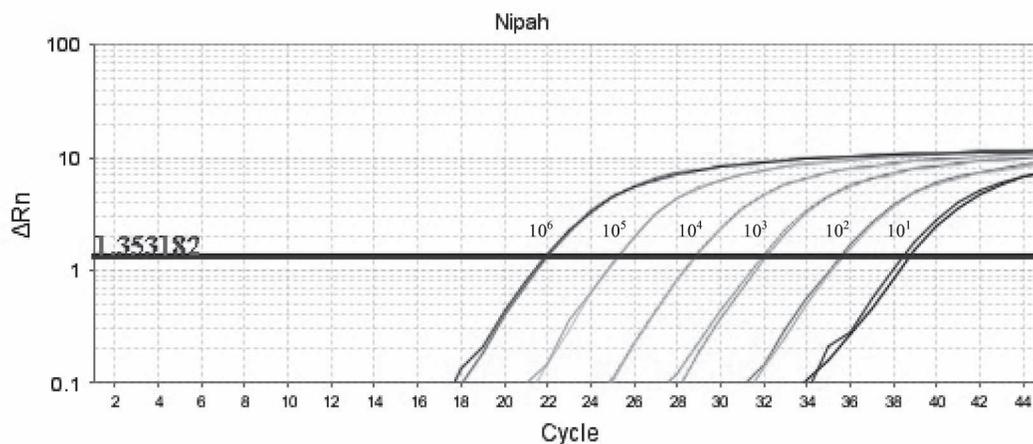


Fig. 2: Amplification plots of detection limit of Real time RT-PCR assay. Amplification plots were realized on 10-fold dilutions of NiV RNA standard. Tests were performed in duplicate from 10^0 to 10^6 copies/reaction

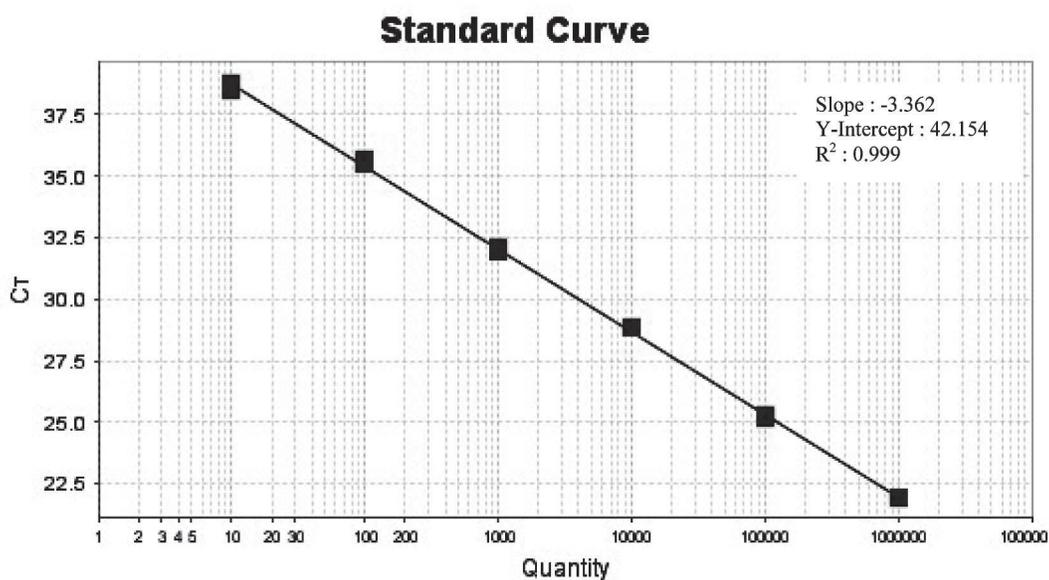


Fig. 3: Standard curve obtained from 10 fold dilutions of NiV RNA. Standard Ct values calculated from results obtained in Fig. 2.

Analytical specificity

The specificity of our established assays was verified using a series of 10-fold dilutions of RNA of West Nile, Japanese Encephalitis and Dengue viruses. Each RNA was diluted in a 10-fold dilution and PCR assays were performed, accordingly. Our results indicated that all samples were negative for Nipah virus by RT-PCR (Fig. 4) and real time RT-PCR (Fig. 5).

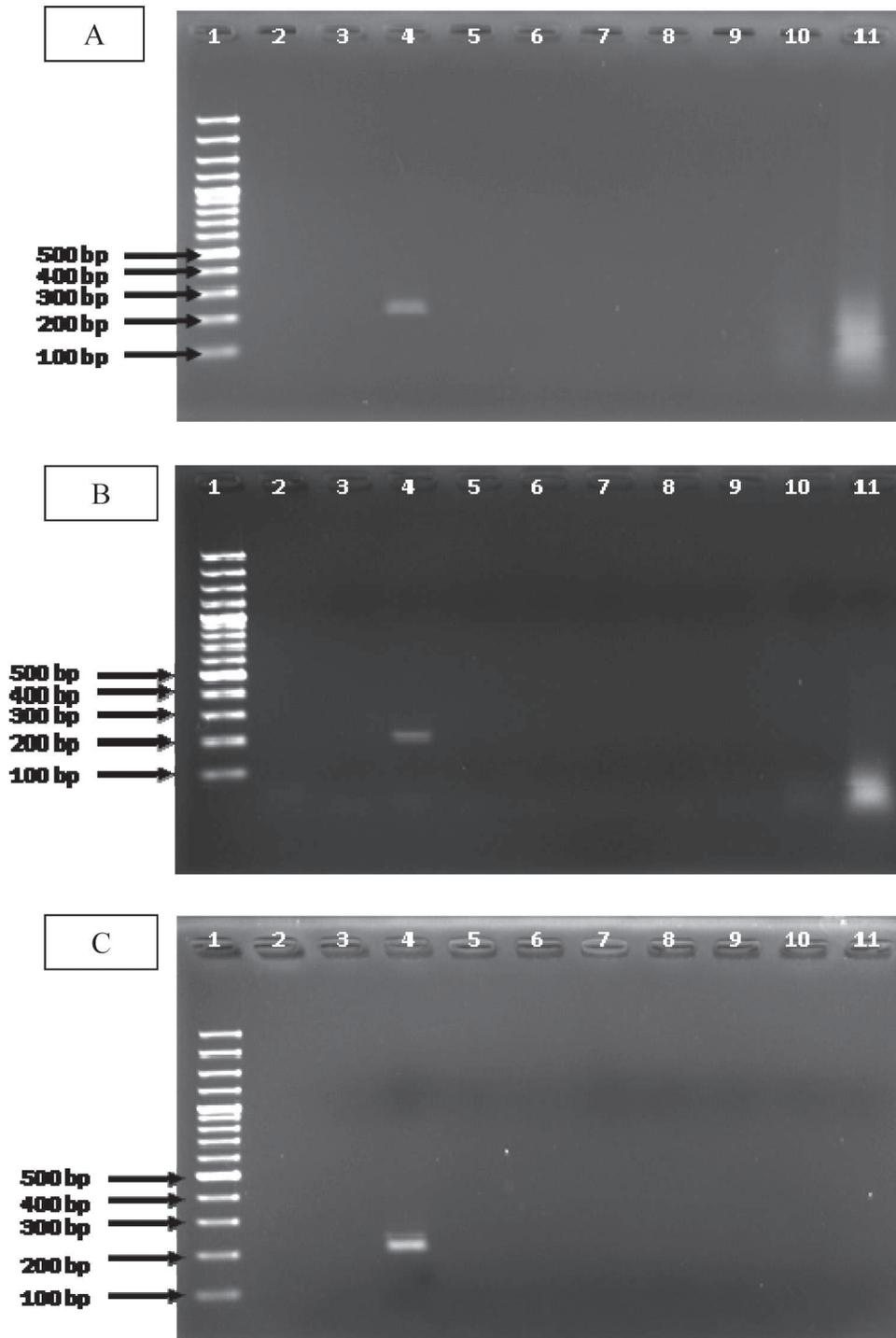
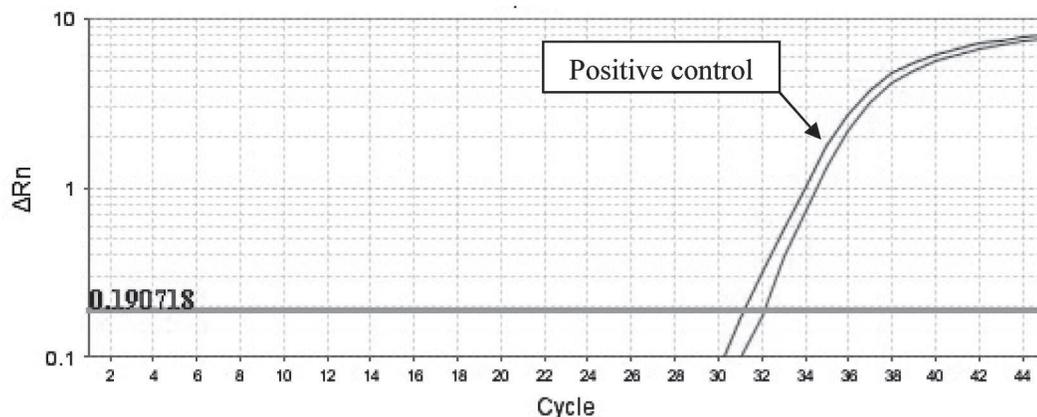
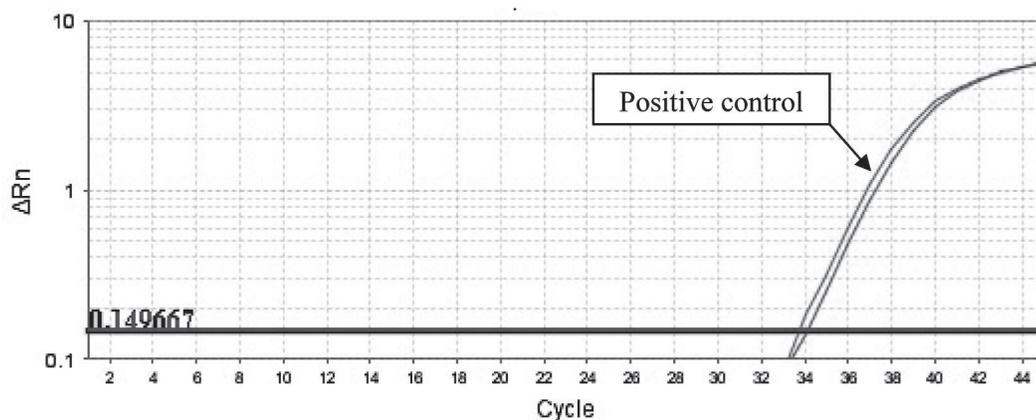


Fig. 4: RT-PCR detection of NiV infection using RNA samples of West Nile (A), Japanese Encephalitis (B) and Dengue virus (C) and electrophoresed on 2% agarose gel. Lane 1 = 100 bp DNA marker, Lane 2 = Distilled water, Lane 3 = Negative control, Lane 4 = Positive control, Lane 5 to Lane 11 = Sample concentration from 10^{-6} to undiluted, respectively. All samples shown negative results.

A) West Nile Virus



B) Japanese Encephalitis Virus



C) Dengue Virus

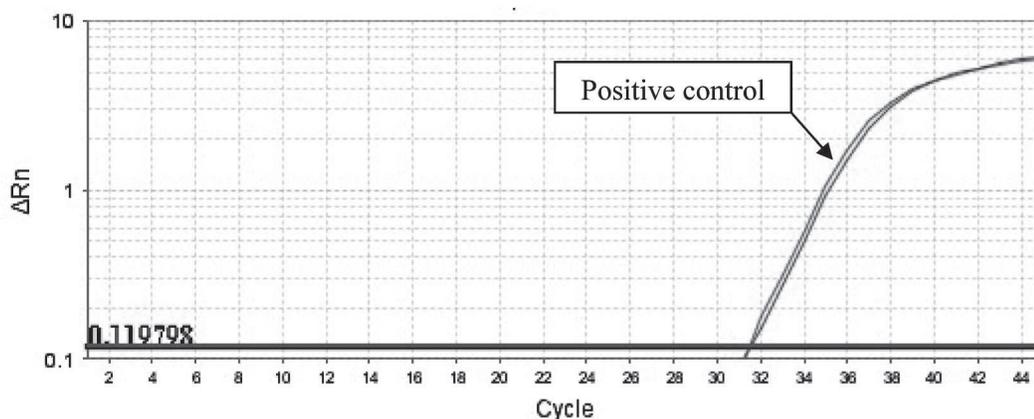


Fig. 5: Real time RT-PCR detection of NiV using RNA samples of West Nile virus (A), Japanese Encephalitis virus (B) and Dengue virus (C). Each sample was tested at concentration of 10^{-6} to undiluted.

Testing on encephalitis samples

Our established assays were further performed to investigate for NiV in CSF specimens of patients suspected of JEV infection. The specimens were previously determined for JEV and dengue virus using ELISA methods. We received the specimens after diagnosis as non-JEV and non-dengue virus infections. Our findings showed that all samples were negative for Nipah virus as determined by RT-PCR (Fig. 6). The real time RT-PCR was additionally tested in duplicated, with NiV RNA concentration of 10^3 copies/ul as a positive control. All samples were also shown for Nipah virus (Fig. 7)

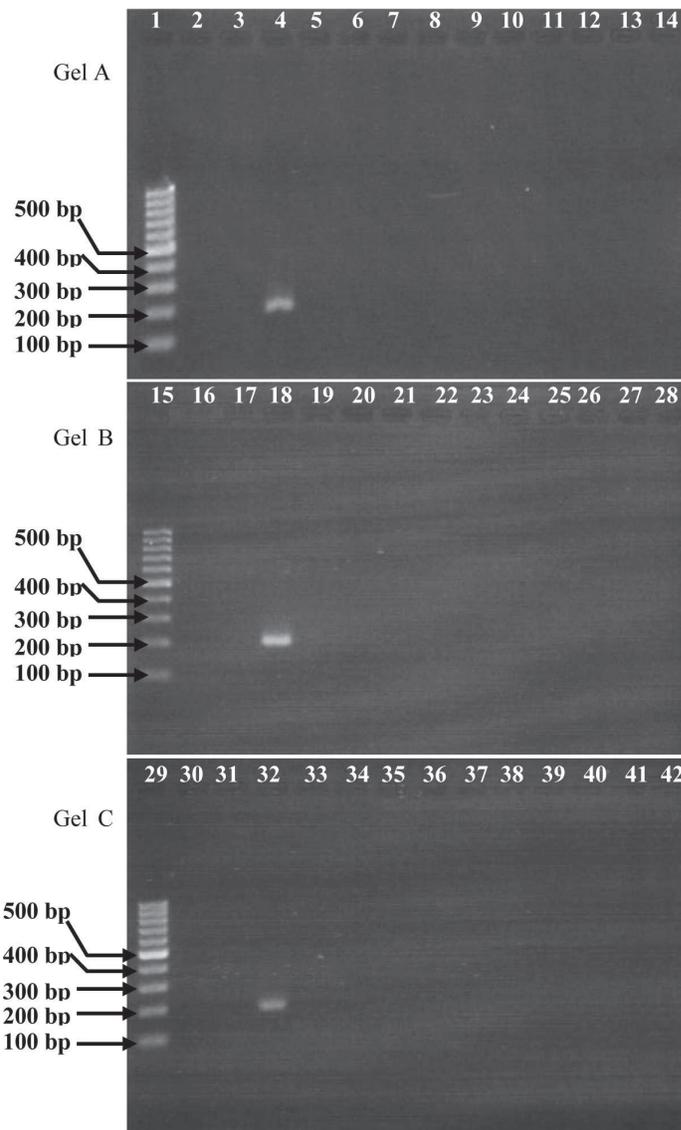


Fig. 6: RT-PCR detection of NiV infection in CSF collected from encephalitic patients with encephalitic symptoms. Lane 1, 15, 29 = 100 bp DNA marker, Lane 2, 16, 30 = Distilled water, Lane 3, 17, 31 = Negative control, Lane 4, 18, 32 = Positive control, Lane 5 to Lane 14 = Sample No. 1 to 10 (Gel A), Lane 19 to Lane 28 = Sample No. 11 to 20 (Gel B), Lane 33 to Lane 42 = Sample No. 21 to 30 (Gel C).

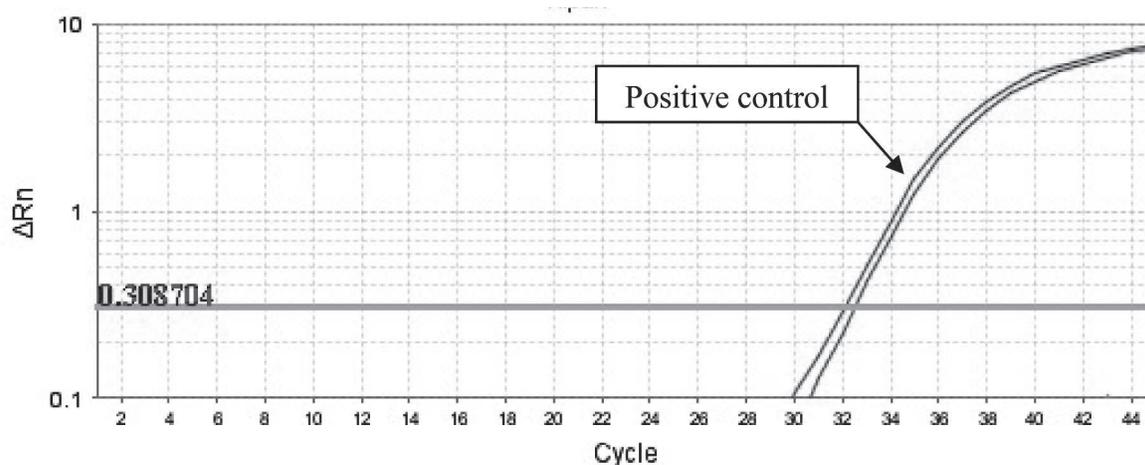


Fig. 7: Real time RT-PCR detection of NiV infection in CSF collected from patients with Encephalitic symptoms.

Discussion

It has been noted that recent emerging infection in humans are often transmitted from infected animals, including Nipah virus (NiV). Detection of those zoonotic pathogens that cause diseases in human is considered as importance for reference laboratories of the country, according to International Health Regulations 2005 (IHR 2005).⁽²²⁾

RT-PCR and real time RT-PCR assays have been developed to diagnosis several kinds of viruses such as dengue, hepatitis C, human papilloma and influenza viruses. In this regards, we studied to established methods that are useful for NiV determination under current and acceptable laboratory facilities of NiV detection i.e., BSL-2 and BSL-3 laboratories, within Thai NIH. Our study demonstrated that the limit of detection of RT-PCR and real time RT-PCR assays was shown as low as 10^4 copies/reaction and 10^2 copies/reaction, respectively, which were in paralleled with the report of Guillaume V et al.⁽²¹⁾

We examined specificity of our assays and found that our established assays are definitely specific for NiV. Furthermore, NiV-associated encephalitis symptoms are similar to JEV-associated one and causative viral agents are also found in CSF.⁽¹⁹⁾ We, then, tested 30 CSF samples of encephalitic patients that were reported as negative for JEV and dengue virus infection. Using our established methods, we did not found any positive NiV-infected CSF. Our finding is supported current information that no Nipah virus infection is reported although anti-NiV antibodies were found in fruit bats in Thailand and Combodia.⁽¹²⁾

Conclusion

In this study, we reported the development of RT-PCR and real time RT-PCR assays for Nipah virus. The limit of detection of RT-PCR and real rime RT-PCR assays was 10^4 copies/reaction and 10^2 copies/reaction respectively. RT-PCR and real time RT-PCR are essential tools

for diagnosis of NiV infections in laboratory facilities of Thailand. Our study is also well-associated with global agenda on health security.

Acknowledgements

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พัฒนาวิธีตรวจวินิจฉัยโรคสมองอักเสบนิปาห์ โดยเทคนิค RT-PCR และ Real Time RT-PCR

อัจฉริยา ลูกบัว อธิวัฒน์ ปริมลศิริคุณาวุฒิ พัชชา อินคำสืบ ศิริมา ปัทมดิลก
และปฐม สวรรค์ปัญญาเลิศ

สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ ถนนติวานนท์ นนทบุรี 11000

บทคัดย่อ ไวรัสนิปาห์เป็นสาเหตุของการระบาดของโรคไข้สมองอักเสบนิปาห์ และเป็นโรคติดต่อจากสัตว์สู่คนที่ยังคงเป็นปัญหาสาธารณสุขในภูมิภาคเอเชีย หลังจากที่มีรายงานการระบาดครั้งแรกในประเทศมาเลเซีย ในปี พ.ศ. 2541-2542 ไวรัสนิปาห์จัดเป็นเชื้อไวรัสที่มีอัตราการป่วยตายสูง ปัจจุบันยังไม่มียาที่รักษาการติดเชื้อไวรัสนิปาห์โดยตรง อีกทั้งยังไม่มีวัคซีนในการป้องกันโรค ดังนั้นการตรวจวินิจฉัยการติดเชื้อไวรัสนิปาห์ในห้องปฏิบัติการจึงมีความสำคัญในการป้องกันและควบคุมโรค และเพื่อเป็นการเพิ่มประสิทธิภาพและศักยภาพห้องปฏิบัติการในการตรวจวินิจฉัยไวรัสนิปาห์ สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ ได้พัฒนาวิธีตรวจหาเชื้อไวรัสนิปาห์ทางห้องปฏิบัติการโดยเทคนิค RT-PCR และ Real Time RT-PCR เพื่อตรวจหาสารพันธุกรรมไวรัสนิปาห์โดยเลือก N gene เป็นยีนเป้าหมาย ผลการศึกษาพบว่าวิธี RT-PCR และ Real Time RT-PCR สามารถตรวจจับสารพันธุกรรมไวรัสนิปาห์ได้ที่ปริมาณน้อยที่สุดที่ 10^4 copies/reaction และ 10^2 copies/reaction ตามลำดับ เมื่อนำวิธีที่พัฒนาขึ้นมาทดสอบตัวอย่างน้ำไขสันหลังของผู้สงสัยป่วยโรคไข้สมองอักเสบ จำนวน 30 ตัวอย่าง พบว่าให้ผลเป็นลบทุกตัวอย่างทั้งสองวิธี จากผลการศึกษาแสดงให้เห็นว่าวิธีที่พัฒนาขึ้นมีความจำเพาะต่อไวรัสนิปาห์เท่านั้น และอาจกล่าวได้ว่ายังไม่มีกรณีการติดเชื้อไวรัสนิปาห์ในประเทศไทย