principal vector of the dengue virus. A total of 16 *Ae. albopictus* larvae were recorded in pre-monsoon (2009), 1,221 in monsoon (2009), and 179 in post-monsoon seasons (2010) from 892 houses in Kathmandu and 734 in Lalitpur district. Positivity of *Ae. albopictus* larvae was recorded in discarded tires lying outdoors at all the habitats searched for mosquitoes. However, immature stage *Ae. albopictus* were also observed in metal drums and in metal containers in Lalitpur district. We confirmed the presence of the mosquito at Dhading and Tanahu for the first time during the epidemic in 2010.

DISCUSSION

In this paper, we reported the first isolation of dengue virus strains from patients in Nepal during the 2010 epidemic. All of the strains belong to DENV-1, and the analysis of the E-coding region of the genome provided insights in understanding the status of dengue in this country. It was during this epidemic that the affected areas expanded for the first time and included the highlands of Nepal. This phenomenon may be related to climate changes attributable to global warming. In fact, the information in this report raises concern that new areas may become susceptible to dengue outbreaks in the near future.

The eleven DENV-1 strains isolated in this study formed a monophyletic clade. This suggests that a DENV-1 population, possibly circulating in the Indian subcontinent, was introduced into the Nepali lowlands and subsequently spread to the highlands during the outbreak. Nepal shares a long open border with India, and Nepalese people visit India frequently for jobs and business opportunities. Movement of people between the two countries is facilitated by the fact that no visa is required. In the 2010 outbreak, it is likely that migrant workers infected with dengue virus in India returned to Nepal and subsequently transmitted the disease to the local community through the mosquito vector. Moreover, the DENV-1 outbreak in Nepal coincided with that in neighboring India [22] and Pakistan [23], indicating that the DENV-1 outbreak in Nepal was initially caused by virus strains from India. However, information from India is still limited.

Goncalvez et al. [24] previously divided DENV-1 into five genotypes. From the clustering pattern on the phylogenetic tree of the E region, all the eleven Nepalese strains belong to genotype V, a cosmopolitan genotype containing American, West African and Asian strains. In particular, the Nepalese strains are close to the Asian subcluster. Patil et al. [25] and Domingo et al. [26] reported that Indian isolates form phylogenetic clusters. However, the Nepalese strains were phylogenetically separate from these Indian clusters. By using the NS5-3'-UTR region, Nepalese strains form a subcluster with strains isolated from India in 2006–2007 (Fig. 4). As more data from India are obtained in the future, it will become easier to elucidate the ecology of DENV in the Trans-Himalayan countries. The increasing movement of people between Nepal and India may allow a more frequent introduction of the dengue viruses in Nepal. In addition, the population of cities in Nepal is increasing, particularly Kathmandu, a rapidly growing urban center with 4 million inhabitants at present. This city is also suffering from limited infrastructure, scarcity of water and poorly planned housing. Moreover, it reported that the average temperature of Kathmandu is continuously rising [27], thus potentially facilitating further successful vector mosquito infestations in the highland areas of Nepal.

Nepal may be at a higher risk of DF/DHF outbreaks in future post-monsoon seasons. For this reason, the government of Nepal has recently identified dengue as an important emerging disease, and sanitation, mosquito control and health education programs are being conducted accordingly.

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Development of simple and rapid assay to detect viral RNA of tick-borne encephalitis virus by reverse transcription-loop-mediated isothermal amplification

Daisuke Hayasaka*†, Kotaro Aoki† and Kouichi Morita

Abstract

Background: Tick-borne encephalitis virus (TBEV) is a causative agent of acute central nervous system disease in humans. It has three subtypes, far eastern (FE), Siberian (Sib) and European (Eu) subtypes, which are distributed over a wide area of Europe and Asia. The objective of this study was to develop a simple and rapid assay for the detection of TBEV RNA by using reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) method that can differentiate the three subtypes of TBEV and can be used for clinical diagnosis and epidemiological study.

Methods: Primers for TBEV-specific and subtype-specific RT-LAMP assay were designed to target the consensus sequence in NS1 of all subtypes and the consensus sequence in the E gene of each subtype, respectiveluy. *In vitro* transcribed RNA of Oshima strain that belongs to FE subtype was serially diluted and used to examine the sensitivity of the assay. Cross-reactivity of subtype-specific RT-LAMP assay was tested by using the RNA of Oshima and Sofjin (FE), IR-99 (Sib) and Hochosterwitz (Eu) strains. RNA extracted from the mixtures of TBEV and ticks, and of TBEV and human blood, and the mouse tissues infected with TBEV, were evaluated in the assay. Positive amplification was observed by real-time monitoring of turbidity and by visual detection of color change.

Results: The sensitivity of TBEV-specific RT-LAMP assay was 10^2 copies of target RNA per reaction volume. FE-specific RT-LAMP assay amplified viral genes of Oshima and Sofjin strains but not of IR-99 and Hochosterwitz strains, and of Japanese encephalitis virus. RT-LAMP assay for Sib and for Eu specifically amplified viral genes of IR-99 and Hochosterwitz strains, respectively. We also showed that tick or human blood extract did not inhibit the amplification of viral gene during the assay. Furthermore, we confirmed that the TBEV RT-LAMP could detect virus RNA from peripheral and central nervous system tissues of laboratory mice infected with TBEV.

Conclusion: TBEV RT-LAMP assay offers a sensitive, specific, rapid and easy-to-handle method for the detection of TBEV RNA in tick samples and this may be applied in the clinical samples collected from TBE-suspected patients.

Keywords: TBEV, RT-LAMP, Far-eastern subtype, Siberian subtype, European subtype

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Background

Tick-borne encephalitis virus (TBEV), which belongs to the genus *Flavivirus* in the family *Flaviviridae*, is a causative agent of acute central nervous system (CNS) disease in humans and animals [1,2]. Its genomic RNA consists of one open reading frame and encodes three structural proteins (C, M and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [3]. TBEV is transmitted by *Ixodes* tick species and rodents in nature, and infects humans through the bite of an infected tick [1,2,4]. It is distributed over a wide area of Europe and Asia, and is geographically and genetically divided into three subtypes, far eastern (FE), Siberian (Sib) and European (Eu) [5-7]. The vector of FE and Sib subtypes is *Ixodes persulcatus* and that of Eu subtype is *Ixodes ricinus* [7].

It has been suggested that the FE subtype is associated with more severe disease than the other subtypes, although the morbidity rate for the disease is different in Europe and Russia, perhaps at least partly due to a selective registration mostly of severe cases in these areas [3,7,8]. This raises concern on the risk of tick-borne encephalitis (TBE) in each country or endemic area, where different TBEV subtypes may be distributed. Therefore, surveillance on the prevalence of TBEV in ticks or serological monitoring of rodent is required to assess the risk of human infection caused by this virus in the endemic areas [9-11].

In human cases, TBE characteristically takes a biphasic course involving an acute febrile illness, and a period of apparent recovery, followed by a neurological syndrome [2,12]. The neurological symptoms include headache, meningitis, meningoencephalitis and meningoencephalomyelitis, the latter being observed in the most severe cases. When death happens, it is usually within 5 to 7 days from the onset of neurological signs [1]. However, such clinical features are not unique to TBE, thus, laboratory diagnosis is required to distinguish it from other neurological disorders [12,13].

For the surveillance on the prevalence of TBEV in ticks and other animals, or for clinical diagnosis, molecular techniques based on genomic detections such as reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) have been developed [10,11,14-19]. However, RT-PCR method requires specific equipment such as thermal cycler, electrophoresis tank and UV illuminator. rRT-PCR has some advantages over conventional RT-PCR and allows high magnitude of amplification, but it requires high-precision instruments for the amplification and a complicated method for the detection of amplified products.

The loop-mediated isothermal amplification (LAMP) assay is a rapid, accurate, and cost-effective diagnostic method to amplify the DNA under isothermal conditions at 60°C to 65°C [20,21]. The one-step RT-LAMP assay requires six primers: two inner primers and two outer primers that define the target region, and two loop primers for increasing the sensitivity of the assay. The final DNA products of this assay consist of multiple loops with several repeats of the target sequence, and can be detected by agarose-gel electrophoresis, real-time monitoring of turbidity or visualization of color change. This method has been used to detect a number of RNA viruses including some flaviviruses [22-26]. Its advantage of over RT-PCR and rRT-PCR is that it makes use of inexpensive and simple equipment such as a heating block or a water bath. Thus, the LAMP method has a potential for use as a simple tool for rapid laboratory confirmation of infectious disease even in a resource-limiting setting.

In this study, we report the development of one-step RT-LAMP assay specific to TBEV and to each of the three subtypes. This assay will be useful in the surveillance of the prevalence of TBEV in ticks and other animals in the field and in the diagnosis of TBEV infection in humans.

Results

Sensitivity of TBEV-specific RT-LAMP assay

The sensitivity of TBEV-specific RT-LAMP assay that targets the consensus sequence in NS1 gene of all TBEV subtypes was examined by using 10-fold serial dilutions of *in vitro* transcribed RNA of the FE-subtype Oshima strain (Figure 1A). The detection limit was 10^2 copies of target RNA per reaction volume. All positive amplifications were achieved in less than 50 min. Agarose gel electrophoresis of the RT-LAMP products showed the typical ladder-like pattern of bands (Figure 1B). The amplified products were confirmed to have the expected nucleotide sequences (data not shown). Change of color to cloudy yellow was observed directly by the naked eye (Figure 1C) and fluorescent detection was observed under UV irradiation (Figure 1D) in samples with at least 10^2 copies. On the other hand, the detection limit of the TaqMan rRT-PCR assay was 10^3 copies of target RNA per reaction volume. Thus, our TBEV-specific RT-LAMP assay was more sensitive for the transcribed RNA of the Oshima strain than the rRT-PCR assay with TBEV-specific primers reported previously [14].

TBEV-subtype specific RT-LAMP assay

TBEV-specific RT-LAMP assay could clearly detect viral gene amplifications for the representative strain (s) of all subtypes of TBEV used in this study such as Oshima and Sofjin (FE), IR-99 (Sib) and Hochosterwitz (Eu), but not for JaOArS982 strain of Japanese encephalitis virus (JEV) (Figure 2A). All the positive amplifications were achieved in less than 50 min.

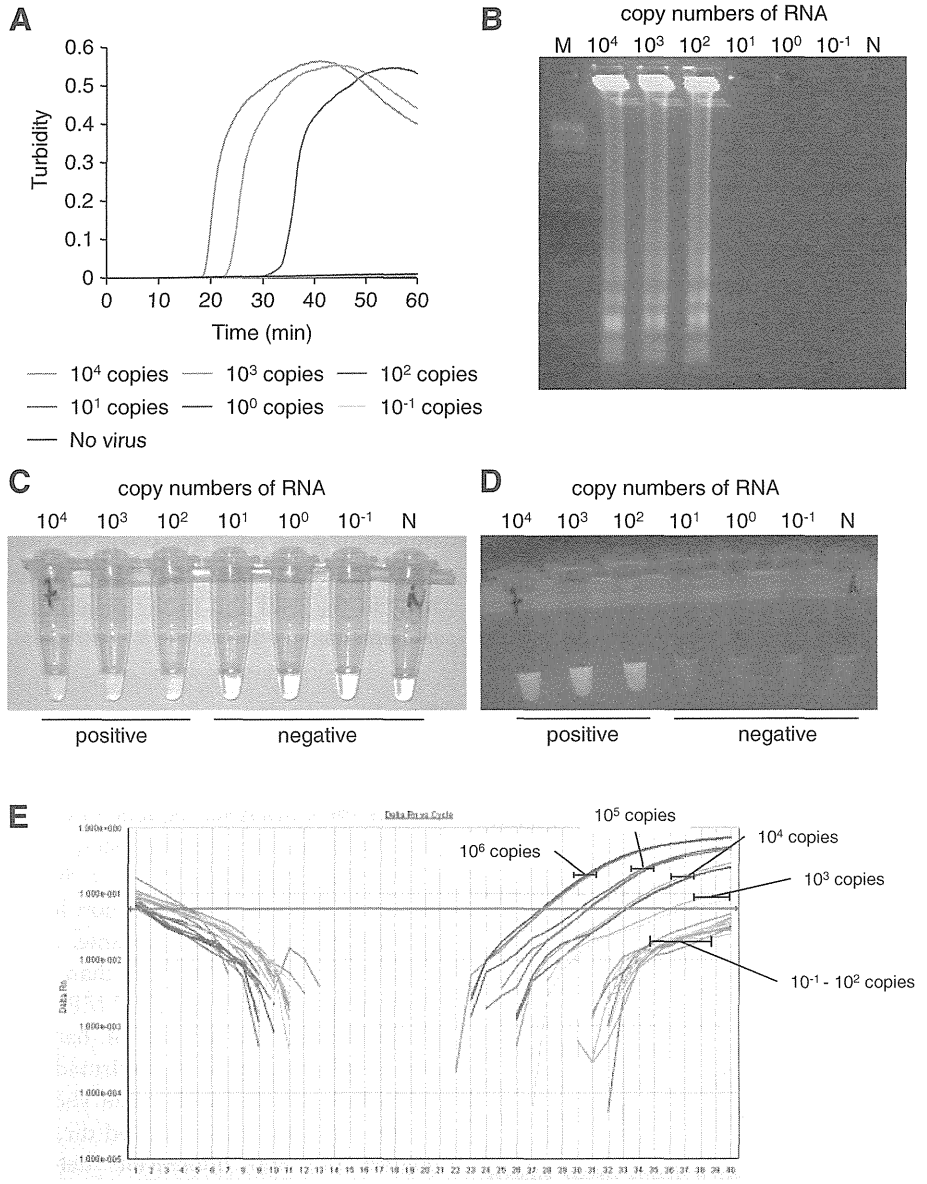

Figure 1 Gene amplification and detection of the TBEV by TBEV-specific RT-LAMP assay. (**A**) Real-time kinetics of the RT-LAMP amplification was monitored by real-time turbidimeter. *In vitro* transcribed RNA of TBEV Oshima strain was serially diluted to make 10^{-1} to 10^{4} copies. The reaction was repeated three times and a representative result is shown. (**B**) Agarose gel electrophoresis profile of the RT-LAMP products. M indicates 100-bp DNA marker (Sigma). N indicates a sample containing no viral RNA. (**C** and **D**) Visual detection of the RT-LAMP products amplified in a reaction tube. Positive amplification is indicated by change of color to cloudy yellow (**C**) or by fluorescent green under UV irradiation (**D**). (**E**) Real-time kinetics of TaqMan rRT-PCR with TBEV-specific primer/probe set. *In vitro* transcribed RNA of Oshima was serially diluted to make 10^{-1} to 10^{6} copies. The reaction was repeated three times and a representative result is shown.

On the other hand, FE-specific RT-LAMP assay could amplify viral genes only of Oshima and Sofjin strains but not of IR-99 and Hochosterwitz strains, and JEV (Figure 2B). Sib- and Eu-specific RT-LAMP assay could amplify viral genes only of IR-99 and Hochosterwitz strains, respectively, but not of the other subtype strains and JEV (Figures 2C and 2D). Each subtype-specific RT-LAMP assay exhibited either similar or shorter reaction time for positive amplification when compared with TBEV-specific RT-LAMP assay done in the same RNA samples (Figure 2). These observations indicate that the sensitivity of each subtypes-specific RT-LAMP assay was either similar or higher compared with TBEV-specific RT-LAMP assay.

rRT-PCR assay using TBEV-specific primers could clearly detect viral gene amplifications for all subtypes of

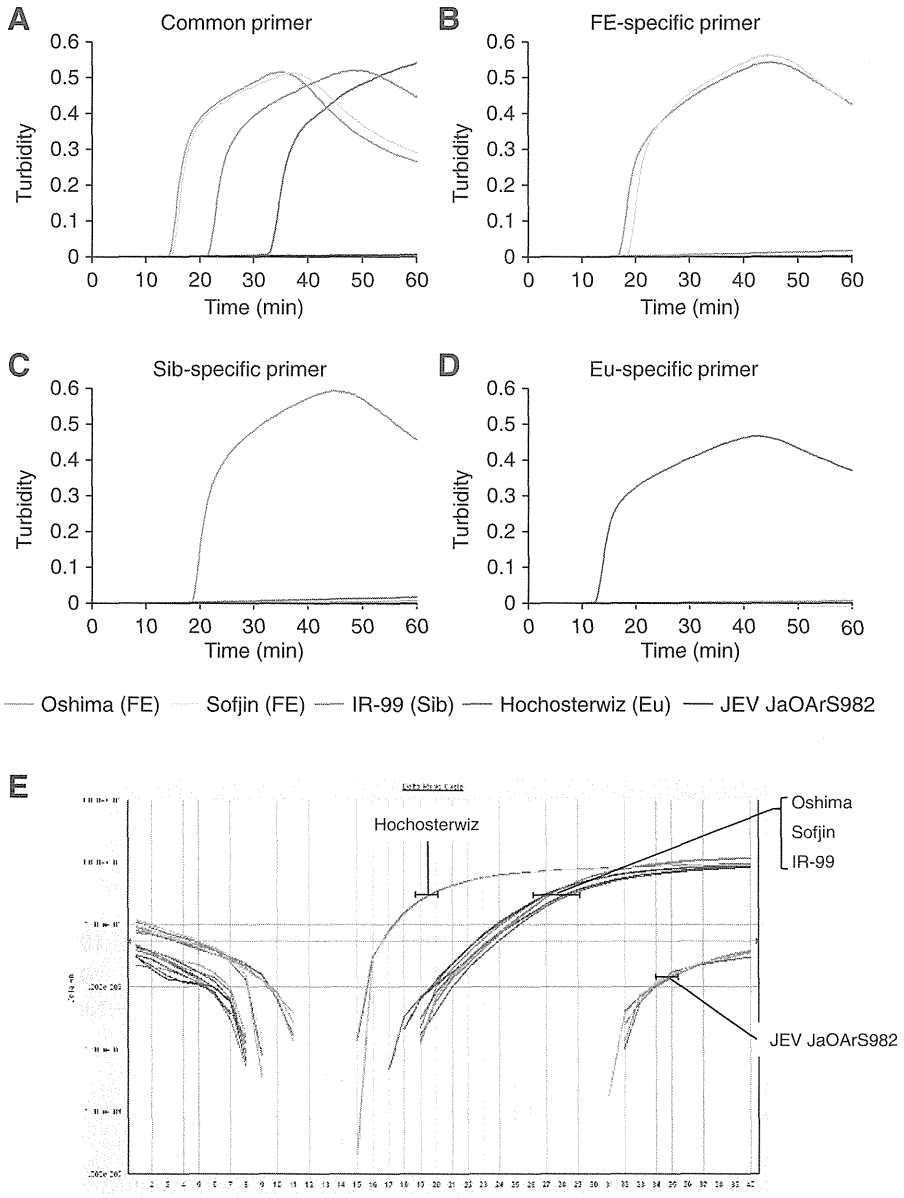

Figure 2 Gene amplification and detection of different TBEV subtypes by subtype-specific RT-LAMP assay. Real-time kinetics was monitored by the turbidity of RT-LAMP products after using 10 ng of RNA samples extracted from supernatants of cultured cells infected with the Oshima (FE), Sofjin (FE), IR-99 (Sib) and Hochostervitz (Eu) strains and JEV JaOArS982 strain. Each viral gene was amplified by (**A**) TBEV-specific RT-LAMP assay, (**B**) TBEV FE subtype-specific RT-LAMP assay, (**C**) TBEV Sib subtype-specific RT-LAMP assay, or (**D**) TBEV EU subtype-specific RT-LAMP assay. Each reaction was repeated three times and a representative result is shown for each sample. (**E**) Real-time kinetics of TaqMan rRT-PCR with TBEV-specific primer/probe set using 10 ng of RNA samples extracted from supernatants of cultured cells infected with the Oshima (FE), Sofjin (FE), IR-99 (Sib) and Hochostervitz (Eu) strains and JEV JaOArS982 strain. The reaction was repeated three times and a representative result is shown.

TBEV with the Hochosterwitz showing early amplification than the other strains (Figure 2E). These results indicate that our RT-LAMP assay can specifically amplify the target RNA of all TBEV subtypes as the rRT-PCR assay established previously [14].

Evaluation of TBEV-specific RT-LAMP assay for amplification of gene from TBEV mixed with tick homogenates

Next, we examined the possibility of using the TBEV-specific RT-LAMP assay for epidemiological study. To

confirm the detection of TBEV gene in tick samples, we mixed TBEV Oshima strain prepared at different dilutions (10^{-1} to 10^{6} pfu) with pooled tick homogenates and extracted RNA from these mixtures. TBEV-specific RT-LAMP assay could amplify specific TBEV gene in the RNA extracted from tick samples with more than 10^{2} pfu of TBEV/10 mg of ticks (Figure 3A). Change of color to cloudy yellow was visualized in positive samples, but not in control sample containing no virus (Figure 3B). These results suggest that tick extract did not inhibit the amplification of viral gene in the assay.

Evaluation of TBEV-specific RT-LAMP assay for amplification of gene from TBEV mixed with human blood

To show the possibility of using the TBEV-specific RT-LAMP assay for clinical diagnosis, we tried to detect the viral RNA of TBEV Oshima strain mixed in human blood samples. TBEV-specific RT-LAMP assay could amplify TBEV gene in the RNA samples extracted from human blood samples containing more than 10^{1} pfu of TBEV/200 μl of blood (Figure 4A). Color changed to cloudy yellow in positive samples, but not in control sample containing no virus (Figure 4B). These results suggest that blood extract did not inhibit the amplification of viral gene during the assay.

Evaluation of TBEV-specific RT-LAMP assay for detection of TBEV gene in TBEV-infected mouse tissues

To show the possibility of applying TBEV-specific RT-LAMP assay for clinical samples, i.e. biopsies samples, we further attempted to detect the viral RNA in the tissues of laboratory mice infected with TBEV. As we previously showed, infectious viruses could be detected in the peripheral tissues 1 to 7 days post-infection (pi) and in the central nervous system (CNS) 5 days pi [27]. Thus, we collected blood, plasma, liver, spleen and brain samples of mice infected with the Oshima strain of TBEV on the 5th and 9th days pi. TBEV-specific RT-LAMP assay could amplify TBEV RNA in the peripheral tissues (blood, plasma, liver and spleen) of all mice and in the brain of one mouse at 5 days pi (Figure 5A and 5B). On the 9th days pi, all mice were positive for TBEV RNA in their blood and brain. Color change was not visualized in samples from mock-infected mice at 9 days pi (Figure 5A and 5B). These results closely reflected the time course of virus replication *in vivo* as reported previously [27].

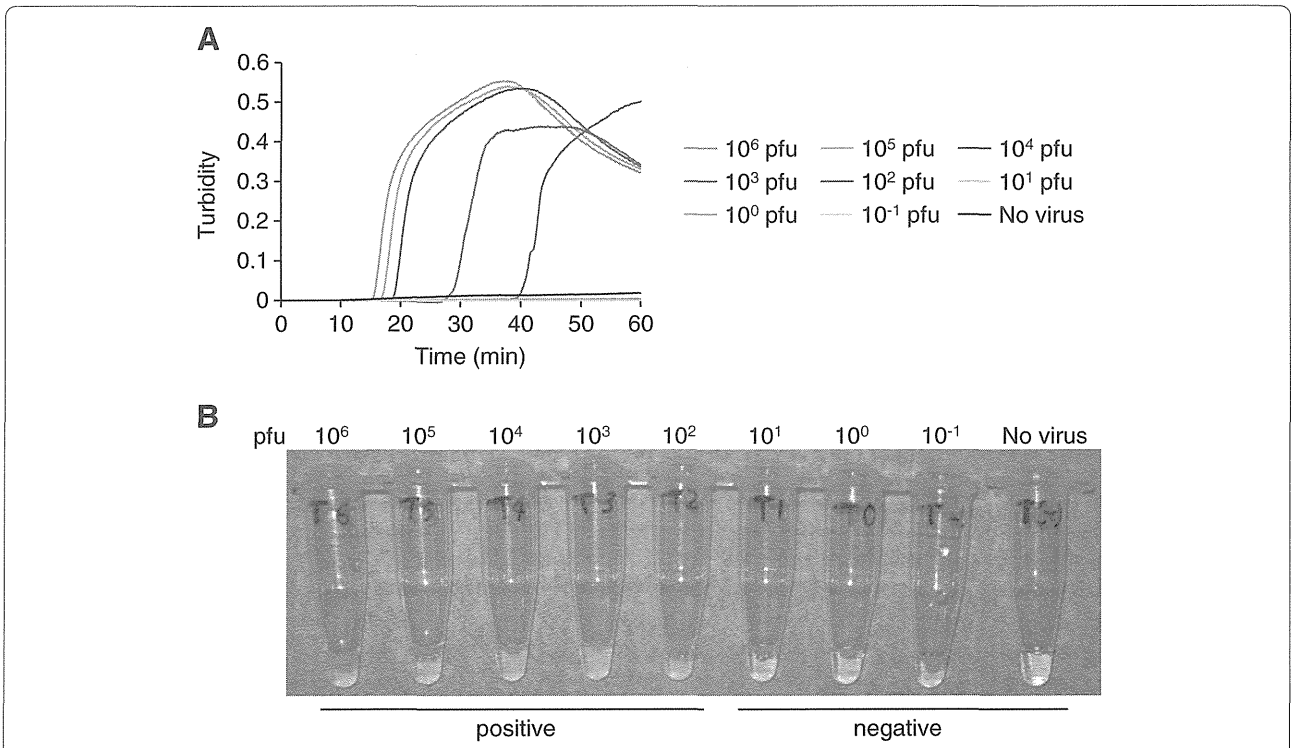

Figure 3 Detection of TBEV gene from a mixture of TBEV and tick homogenates by TBEV RT-LAMP assay. (**A**) Real-time kinetics monitored by the turbidity of RT-LAMP products after using 10 ng of RNA extracted from each mixture of TBEV and pooled tick homogenates. (**B**) Visual detection of color change to cloudy yellow in the RT-LAMP products. The reaction was repeated three times and a representative result is shown. RNA was extracted from 10 mg of ticks containing TBEV Oshima strain of different concentrations between 10^{-1} to 10^{6} pfu. Approximately 10 μg of RNA was recovered from one sample.

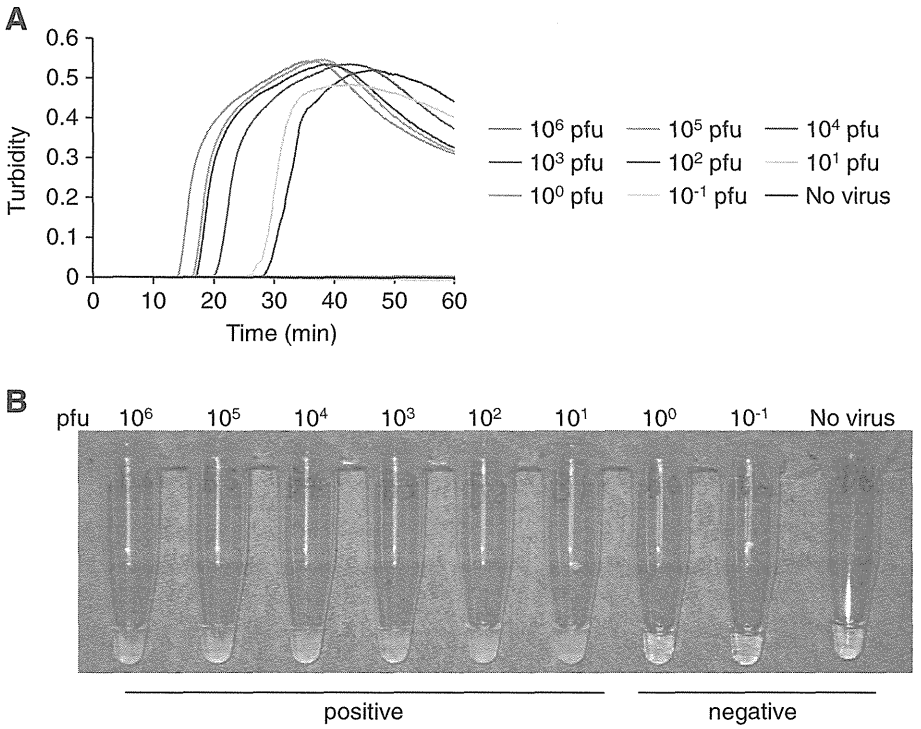

Figure 4 Detection of TBEV gene from a mixture of TBEV and human blood by TBEV RT-LAMP assay. (**A**) Real-time kinetics monitored by the turbidity of RT-LAMP products after using 10 ng of RNA extracted from each mixture of TBEV and human blood sample. (**B**) Visual detection of color change to cloudy yellow in the RT-LAMP products. The reaction was repeated three times and a representative result is shown. RNA was extracted from 200 μl of blood containing TBEV Oshima strain of different concentrations between 10^{-1} to 10^6 pfu. Approximately 2 μg of RNA was recovered from one sample.

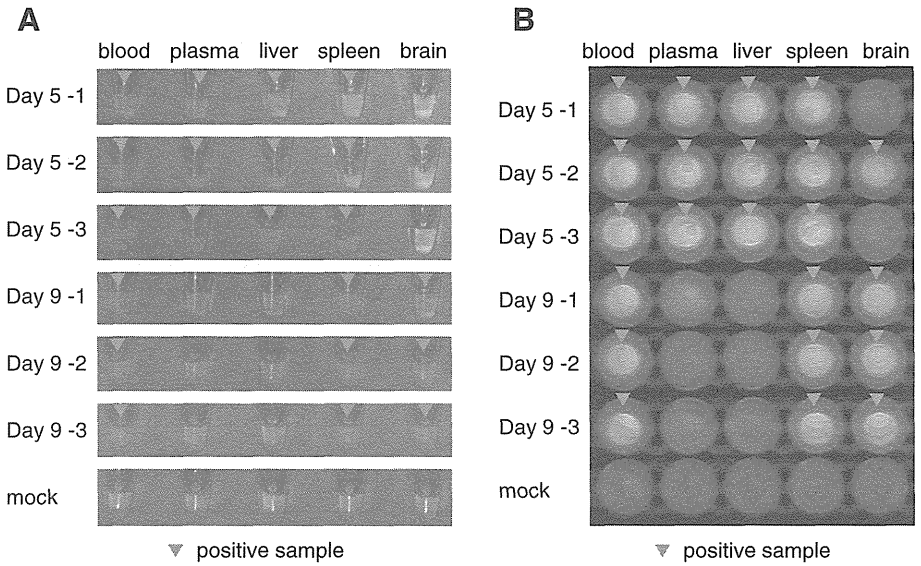

Figure 5 Detection of TBEV gene from mouse tissue samples by TBEV RT-LAMP assay. Visual detection of RT-LAMP products using 10 ng of RNA samples extracted from blood, plasma, liver, spleen and brain samples collected from laboratory mice on the 5th and 9th day following peripheral infections with TBEV Oshima strain. Three mice in each group and one mouse for mock infected mice were used. Positive amplification was indicated by the change of the color to cloudy yellow (**A**) or fluorescent green under UV irradiation (**B**). The reaction was repeated three times for each sample and a representative result is shown.

Discussion

This is the first report of an RT-LAMP assay for detection of TBEV gene and for differentiating all the three subtypes (FE, Sib and Eu) of TBEV from each other. We also raised the possibility that this assay will be useful for the detection of viral RNA from tick and clinical samples.

Over the last decades, TBE endemic areas and human cases have increased [7,28], although the disease severity caused by different subtype of TBEV may vary [3,7,8]. Co-circulation of the different subtypes exists in some countries, e.g. Latvia [29]. Therefore, investigation of the distribution of different subtypes in the field is important for epidemiological studies. In Europe, prevalence of TBEV in ticks is determined by identifying viral RNA through RT-PCR or rRT-PCR [10,11,30]. However, the first report on the use of real-time rRT-PCR [15] could not differentiate between subtypes. To differentiate TBEV subtype, some techniques have been developed, e.g. multiplex RT-PCR and pyrosequencing technique combined with rRT-PCR method [14,17]. However, PCR-based method has its own disadvantages due to the high cost of equipment and the requirement for highly skilled personnel. In this study, we showed that the TBEV subtype-specific RT-LAMP assay could differentiate subtypes with high sensitivity. The positive amplification could be confirmed by simple and direct visualization without the use of any expensive equipment. Furthermore, we also showed that tick extract did not inhibit the quality of the assay. Therefore, this assay might be suitable for the epidemiological study of TBEV and in field work.

For current laboratory diagnosis of TBE, serological assays are usually used [31,32]. In particular, IgM capture ELISA has been widely used for rapid diagnosis [33], because IgM antibody is detected in serum or CSF in the early phase of disease. However, IgM antibody appears as late as about two weeks after infection, and can persist for up to 10 months after vaccination or natural infection of a person [28]. Viral RNA is detectable before appearance of IgM antibody. Therefore, PCR-based TBEV diagnosis is an important alternative to serological methods in the early phase of the disease [15,19].

Owing to the simple and rapid performance of TBEV RT-LAMP assay, it has advantages over IgM capture ELISA or PCR-based TBEV diagnosis. Here, we showed that TBEV RT-LAMP assay could detect virus RNA from peripheral and CNS tissues following TBEV infection and was not inhibited by blood. Therefore, the assay we developed in this study will be useful for timely diagnosis and rapid laboratory confirmation of TBE suspected case-patients in TBE endemic countries especially in areas with resource-limiting condition.

Conclusion

In conclusion, the TBEV RT-LAMP assay offers a sensitive, specific, rapid and easy-to-handle method for the detection of TBEV RNA in clinical samples obtained from patients during the first phase of their illness or in tick samples. We hope that this new technique will contribute to practical molecular diagnostic tool as well as in epidemiological surveillance.

Methods

Design of RT-LAMP assay primers for TBEV

A set of six primers comprising two outer primers (forward outer primer F3 and backward outer primer B3), two inner primers (forward inner primer FIP and backward inner primer LB), and two loop primers (forward loop primer FP and backward loop primer BP), were designed for the LAMP assay by using a software PrimerExplorer (version 4; Eiken Chemical Co., Ltd. Tokyo, Japan). The TBEV-specific primers were designed to be specific for NS1 gene based on the consensus sequence identified in 47 strains of TBEV. On the other hand, the TBEV subtype-specific primers were designed to be specific to E gene based on each consensus sequence identified in 63, 96 and 182 strains for FE, Sib and Eu subtypes, respectively. All primers were synthesized by Genenet Co., Japan. The details for each primer and the location in the genomic sequences are shown in the Table 1.

Cell and virus

Stocks of the Oshima (FE) [34], Sofjin (FE), IR-99 (Sib) [5] and Hochosterwitz (Eu) strains of TBEV, and JaOArS982 strain of JEV were prepared in baby hamster kidney (BHK) cells [5,34]. Hochosterwitz strain was kindly provided by F.X. Heinz (Medical University of Vienna), and the supernatant of infected BHK cells was kindly provided by K. Yoshii, (Hokkaido University). Virus titers were determined by plaque forming assays done in BHK cells and were expressed as pfu/ml [27,35]. The BHK cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co.) containing 10% fetal calf serum (FCS). All experiments using live TBEV were performed in a biosafety level 3 laboratory of the Institute of Tropical Medicine, Nagasaki University according to standard BSL3 guidelines.

Mice

B6 mice were purchased from Japan SLC Corporation. Five week old female mice were subcutaneously inoculated with 10^3 pfu of the Oshima strain of TBEV diluted in EMEM containing 2% FCS. Mock infected mice were inoculated with the supernatant medium of uninfected BHK cells. At 5 and 9 days pi, blood, liver, spleen and brain samples were collected after euthanasia. Blood samples were centrifuged to obtain plasma. The animal experimental protocols were approved by the Animal Care and Use Committee of the Nagasaki University (approval number: 100723–1/1008050873).

Table 1 Primer sets designed for RT-LAMP assay detection of TBEV in this study

Primer	Sequence	Genome position*	Length (bp)
TBEV-F3	ACCATAAATGCCGACTGTGA	3319–3338	20
TBEV-B3	TGCCACCACCATTGAGC	3500–3516	17
TBEV-FIP	CGGCAGCACCACTCTGGAATTCGGGGGCTTCTGTGAGGA	3345–3361	39
		3385–3404	
TBEV-BIP	TGCACACTACCTCCAGTGACGTGAATTCATGAACAGGACGTATTTCC	3409–3430	47
		3462–3480	
TBEV-LF	ACCTTGCCACTCTCTGTGG	3365–3383	19
TBEV-LB	ACGGGGACAGACTGTTGGTATGC	3436–3485	23
FE-F3	TTGACCTTGCTCAGACTGTC	1547–1566	20
FE-B3	CGTCCATTTTCACAGCGTGT	1713–1732	20
FE-FIP	TGAACCAGTCCCGGTGGACCTTGGAGCTTGACAAGACCTC	1569–1589	40
		1615–1633	
FE-BIP	GCCCTGCCGTGGAAACATGAGGAGCTCCAAACTCAACCA	1642–1663	39
		1694–1710	
FE-LF	CAGGCCGTCGGTAGGTGTTC	1591–1610	20
FE-LB	GGGAGCACAAAACTGGAACA	1662–1681	20
Sib-F3	CCACTCTGGCTGAAGAACAT	1211–1230	20
Sib-B3	ATTGGCGGCAACGTAGTC	1417–1434	18
Sib-FIP	GCCCTTTCCAAACAGTCCGCAAGCACGGTGTGCAAGAGAG	1240–1255	40
		1285–1305	
Sib-BIP	TGAGGCAAAGAAGAAGGCCACTCGTGTGTGGCTCAACCTTA	1335–1356	41
		1395–1413	
Sib-LF	CCCAGCCTCGATCACTCTGGT	1256–1276	21
Sib-LB	GGACATGTGTATGACGCCAACAAG	1357–1380	24
EU-F3	CGCAAAACTGGAATAACGCA	1667–1686	20
EU-B3	CCACTTCGCAGGTCACATG	1831–1849	19
EU-FIP	ACTCCAGTCTGGTCTCCGAGGACTGGTTGAATTTGGGGCTC	1691–1711	41
		1741–1760	
EU-BIP	GTTACTGAAGGCTCTCGCTGGGGCCACTCTTCAGGTGGTACT	1761–1783	42
		1811–1829	
EU-LF	ACACGTCCATCTTGACAGCGT	1715–1735	21
EU-LB	GTTCCTGTGGCACACATTGA	1783–1802	20

*genome position is based on the sequence of Neudoerf strain (accession no. TEU27495).

In vitro transcription and quantification

To obtain a quantitative RNA standard with which to examine the detection limit of RT-LAMP assay, a plasmid containing full length sequence of the Oshima strain of TBEV was used for RNA transcription [36]. The plasmid was digested with Not I and Sma I (Takara) for 1 hour and the fragment was purified with QIAquick Gel Extraction Kit (Qiagen). The number of DNA copies/μl was calculated based on a previous report [37]. The DNA fragment was then transcribed *in vitro* with SP6 transcriptase and digested with DNAse by using mMESSAGE mMACHINE (Ambion) according to the manufacturer's protocol. The RNA was purified with an RNeasy minikit (Qiagen). The number of RNA copies/μl was determined by using the following formula: concentration of RNA (g/μl)/[(transcript length in nucleotides × 340)] × 6.022×10^{23}, where the RNA length is 4966 base. The RNA concentration was measured with a NanoDrop ND-1000 apparatus (Thermo Scientific). The target RNA copy number was calculated and serial dilutions ranging from 10^{-1} to 10^{4} RNA copies were used to determine the range of quantification.

RNA extraction

A 250 μl volume of culture supernatant from infected BHK cells was mixed with 750 μl of Isogen-LS (Nippon Gene) and genomic viral RNA was extracted according

to the manufacturer's protocol. Ten-fold serial dilutions (10^{-1} to 10^{6} pfu) of the Oshima strain of TBEV were prepared. Each diluted sample was added to 10 mg of pooled ticks (*Ixodes persulcatus*, laboratory breeding ticks was kindly provided by S. Konnai, Hokkaido University) and homogenized in 1 ml of Isogen (Nippon Gene). A 200 μl volume of healthy human blood was mixed with 50 μl of the Oshima strain of TBEV prepared at different concentrations (10^{-1} to 10^{6} pfu) and with 750 μl of Isogen-LS. RNA was extracted according to the manufacturer's protocol. Total RNAs of mouse blood and plasma samples were extracted using Isogen-LS (Nippon Gene). Total RNAs of mouse livers, spleens and brains were extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was dissolved in DPEC-treated water and stored at −80°C until use. The experiment using human blood was performed with the approval of ethics committee of the Institute of Tropical Medicine, Nagasaki University (approval number: 121226102).

RT-LAMP

RT-LAMP was performed in a final reaction volume of 25 μl using a Loopamp RNA amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) with 5 pmol of the primers F3 and B3, 20 pmol of the primers LF and LB, and 40 pmol of the primers FIP and BIP. One microliter of the extracted RNA was used as template per reaction. The RT-LAMP reaction mixtures were incubated at 62.5°C for 60 min and inactivated at 80°C for 5 min.

Analysis of RT-LAMP product

A real-time turbidity caused by the accumulation of magnesium polyphosphate was monitored spectrophotometrically at 650 nm with LA-320C Loopamp real-time turbidimeter (Teramecs, Japan). The results were analyzed by the LA-320C software package (Teramecs). The product was electrophoresed on an agarose gel in Tris-acetate-EDTA (TAE) buffer followed by staining with ethidium bromide. Amplified products were sequenced to show that they matched the expected nucleotide sequences. Positive amplification was shown by the specific ladder-like pattern on a UV transilluminator at 320 nm. For visualization of the positive reaction, fluorescent detection reagent (FD; Eiken Chemical Co., Ltd. Tokyo, Japan) was added to the reaction mixture and a change of color was recognized directly (transparent to cloudy yellow color) or under UV irradiation (fluorescent green).

rRT-PCR

One step rRT-PCR was done with One Step PrimeScript® RT-PCR Kit (TAKARA BIO INC.) according to the manufacturer's instructions. TBEV-specific primers and probe that detect all TBEV subtypes described in the previous paper [14] were synthesized by Genenet Co., Japan. Hydrolysis minor groove binder probe (ABI PRISM® TaqMan® MGB probe) was obtained from Applied Biosystems. The rRT-PCR assay was performed with an Applied Biosystems 7500 rPCR system. One microliter of the extracted RNA was used as the template in each reaction mixture. A sample with the growth curve crossing the threshold line within 40 cycles was considered positive.

Competing interests

The authors declared they have no competing interests.

Authors' contributions

DH designed the experiment, performed live virus infection, extracted RNA, did RT-LAMP assay, and wrote the manuscript. KA designed the primers for RT-LAMP assay, extracted RNA, and carried out RT-LAMP assay and rRT-PCR. KM participated in discussion of the results and helped in drafting the manuscript. All authors read and approved the final manuscript.

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CASE REPORT Open Access

An approach for differentiating echovirus 30 and Japanese encephalitis virus infections in acute meningitis/encephalitis: a retrospective study of 103 cases in Vietnam

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Abstract

Background: In recent decades, Echovirus 30 (E30) and Japanese encephalitis virus (JEV) have been reported to be the common causative agents of acute meningitis among patients in South East Asia. An E30 outbreak in Vietnam in 2001–2002 gained our interest because the initial clinical diagnosis of infected patients was due to JEV infection. There are few clinical insights regarding E30 cases, and there are no reports comparing E30 and JEV acute meningitis/encephalitis cases based on clinical symptoms and case histories. We therefore aimed to identify reliable clinical methods to differentiate E30 and JEV acute meningitis/encephalitis.

Methods: A retrospective, cross-sectional study was conducted to compare E30 and JEV acute meningitis/encephalitis cases. We collected and analyzed the clinical records of 43 E30 confirmed cases (E30 group) and 60 JEV confirmed cases (JEV group). Clinical data were compared between the E30 and the JEV groups. Differences of clinical parameters were analyzed by certain statistical tests.

Results: Fever, headache, and vomiting were the most common symptoms in both the E30 and the JEV groups. Combined symptoms of headache and vomiting and the triad of symptoms of fever, headache, and vomiting were observed in more patients in the E30 group (E30 vs. JEV: 19% vs. 0%, $p < 0.001$; 74% vs. 27%, $p < 0.001$, respectively). On the other hand, strong neurological symptoms such as seizure (5% vs. 73%, $p < 0.001$) and altered consciousness (12% vs. 97%, $p < 0.001$) were manifested primarily in the JEV group. CSF leukocytosis was observed predominantly in the E30 group (80 vs. 18 cells/μL, $p = 0.003$), whereas decreasing CSF sugar level was observed predominantly in the JEV group (58.7 vs. 46.9 mg/dL, $p < 0.001$).

Conclusion: Fever, headache, vomiting, absence of neurological symptoms (seizure, altered consciousness), and presence of CSF leukocytosis are important parameters to consider in differentiating E30 from JEV cases during early infection. Then, proper measures can be adopted immediately to prevent the spread of the disease in the affected areas.

Keywords: Echovirus 30, Japanese encephalitis virus, Acute meningitis/encephalitis

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Background

E30 is a positive-strand RNA virus that belongs to the family *Picornaviridae*, genus *Enterovirus* [1]. It is transmitted to humans by the oral-fecal route [2]. E30 is responsible for many sporadic outbreaks of aseptic meningitis in many countries because of its easier transmission [1-9]. Japanese encephalitis virus (JEV) is a mosquito-borne virus that belongs to family *Flaviviridae*, genus *Flavivirus* [10]. It can cause meningitis or encephalitis in humans. JEV causes approximately 30,000-50,000 meningitis/encephalitis cases annually in Asia, and it is one of the leading causes of the meningitis/encephalitis cases worldwide [10,11]. In recent decades, many E30 outbreaks have occurred in JEV-endemic Asian countries around Vietnam [3,5,12-14]. According to several case reports on meningitis in these countries, an epidemic season of E30 overlapped with that of JEV [5,12,15,16]. Hence, certainty on clinical diagnosis cannot be guaranteed, especially during the early course of illness.

The general symptoms of *Enterovirus* meningitis are headache, nausea, and vomiting. Common cold symptoms are also observed [1,5]. In some instances, severe illness characterized by paralysis and encephalitis leads to death [9,13]. These symptoms varying from mild to severe manifestations are quite similar to those due to JEV infection [11,17,18]. Thus, a correct identification of the causative agent is difficult to determine based on the clinical symptoms.

Several reports showed that JEV is one of the leading causes of acute meningitis/encephalitis in Vietnam [15,16,18]. However, the number of JEV-confirmed cases was not high enough, and some of the patients were found to actually be infected by enteroviruses instead [18,19]. In this report, we consider only those patients whose admitting diagnosis was acute meningitis/encephalitis, and whose infection was confirmed to be due to E30 or JEV by laboratory procedures. Our study focused on the clinical information of these patients, and we found that specific clinical symptoms and laboratory findings could give the clinicians/epidemiologists a more reliable method for differentiating E30 and JEV cases as early as possible.

Materials and methods

Ethical statements

This study was approved by the Institutional Review Board of the National Institute of Hygiene and Epidemiology (NIHE), Vietnam (No: 01 IRB, November 7, 2005, No: 33 IRB, December 15, 2011).

Specimen collection

Cerebrospinal fluid (CSF) specimens were collected only from patients who were clinically diagnosed to have acute meningitis/encephalitis at the time of admission and whose clinical records were available. The patients were from the National Hospital of Pediatrics (NHP) in Hanoi, Northern Vietnam and from Bac Giang General Hospital (BGGH) in Bac Giang, Northern Vietnam. The period of collection at the NHP was from 2001–2002, when an E30 outbreak occurred in Hanoi. The period of collection at BGGH was from 1999 to 2008. NIHE collects clinical specimens from BGGH annually because it is located in a JEV endemic area [16].

Laboratory investigation

CSF specimens were sent to the NIHE for laboratory diagnosis. The E30 cases were identified by the neutralization test (NT) using anti-E30 serum [20]. Several samples that were unidentified by the NT were subjected to the virus isolation and gene amplification method as described below. The JEV cases were confirmed by IgM Capture ELISA [16].

Virus isolation, RT-PCR, and sequencing

Eight unidentified samples were subjected to virus isolation. Each CSF specimen was inoculated in human rhabdomyosarcoma cells (RD cells). The cells were incubated at 37°C with 5% CO_2 until the cytopathic effect (CPE) was observed under a microscope [21,22]. Then, the infected culture fluids (ICFs) were collected and kept at −80°C prior to use. The viral RNA was isolated from the ICFs by the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions [23]. To amplify the complete VP1 gene of E30, RNA templates were subjected to reverse transcription and polymerase chain reaction (RT-PCR) using the forward primer 5′-GCRTGCAATGAYTTCTCWGT-3′ and the reverse primer 5′-GCICCIGAYTGITGICCRAA-3′ [24]. The amplicons were sequenced using the ABI PRISM 3100-*Avant* Genetic Analyzer [25].

Phylogenetic analysis

Phylogenetic analysis of selected strains of human E30 from different geographical origins was performed based on the VP1 gene sequences (Figure 1). Alignment of these sequences was performed by Clustal W version 2.0 [26], and a neighbor-joining tree [27] was generated using MEGA 5.0 software [28]. The prototype strain Farina of Echovirus 21 (GenBank accession number: AY302547) was used as the out-group. The reliability of the phylogenetic tree was determined by a bootstrap resampling test with 1,000 replicates.

Clinical data and statistical analysis

The clinical data from E30-confirmed cases (E30 group) and JEV-confirmed cases (JEV group) were included in the statistical analysis. The statistical analysis was conducted using R version 2.15 software [29]. The difference between

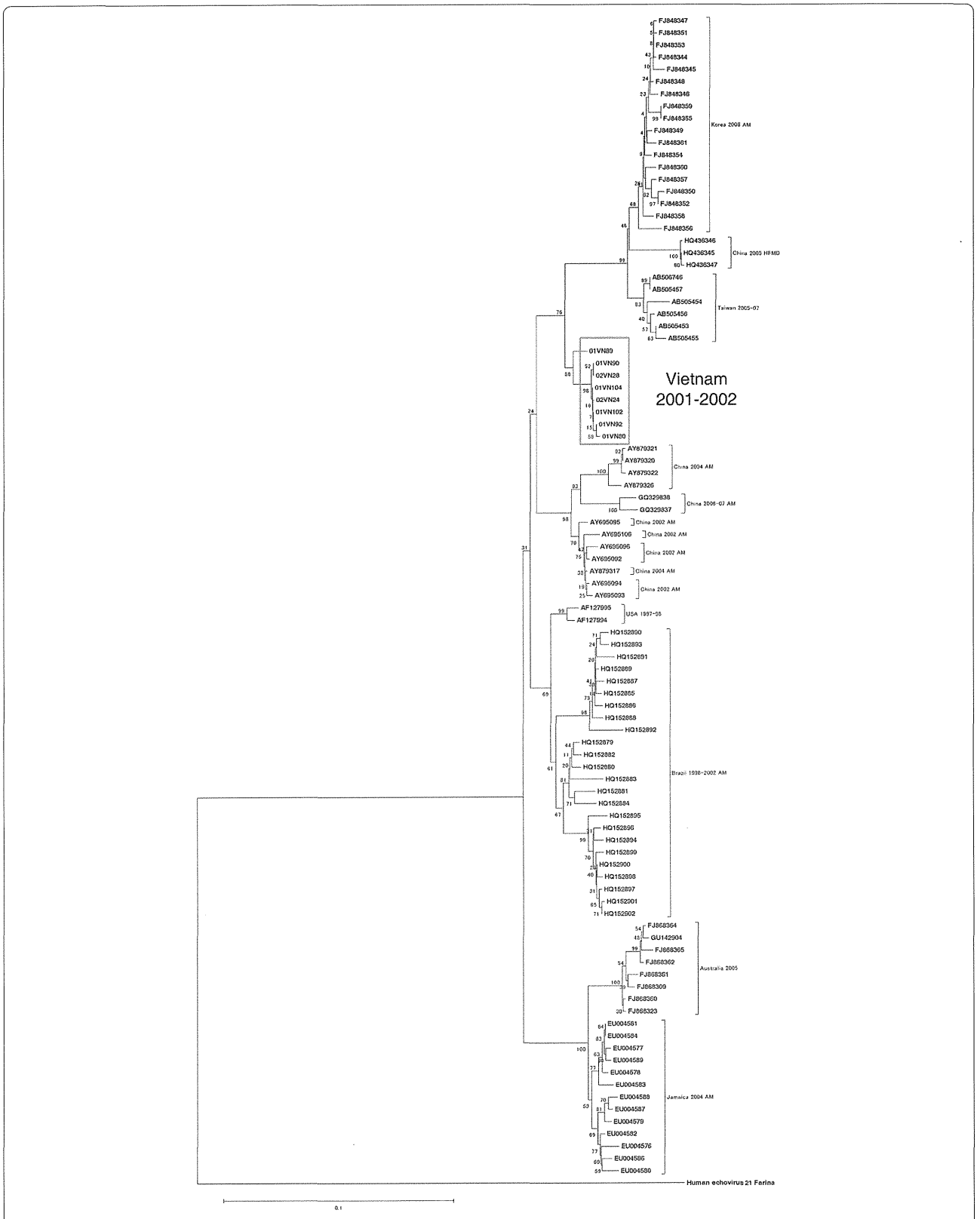

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Phylogenetic analysis of the selected E30 strains. A phylogenetic analysis of VP1 gene sequences from the selected human E30 strains from different geographical origins was conducted. The neighbor-joining tree was generated using MEGA 5.0 software, and the prototype strain Farina of echovirus 21 was used as the outgroup. The eight Vietnamese isolates are highlighted in the red colored box. The sequences reported here have been deposited in GenBank with the accession numbers of KC999616 to KC999623.

the E30 and the JEV groups was tested by Chi-squared tests and Fisher Exact tests. The distribution of values between these groups was compared by the F test, and the difference of values between them was assessed by Student's t-tests or Welch tests. A P value of less than 0.05 was accepted as significant.

Results

Laboratory confirmation of E30 and JEV infection

Eighty-eight patients from NHP were confirmed to have E30 infection based on NT (80 patients) and virus isolation (8 patients) (Figure 2). One hundred thirty-four patients at BGGH were confirmed to have JEV infection by IgM Capture ELISA (Figure 2).

Virus isolation, sequence comparison, and phylogenetic analysis

E30 was isolated from eight CSF specimens. The VP1 gene in these isolates was sequenced. The designated strain name of each isolate and the corresponding GenBank accession number (enclosed in parentheses below) were as follows: 01VN80 (KC999616), 01VN89 (KC999617), 01VN90 (KC999618), 01VN92 (KC999619), 01VN102 (KC999620), 01VN104 (KC999621), 02VN24 (KC999622), and 02VN28 (KC999623).

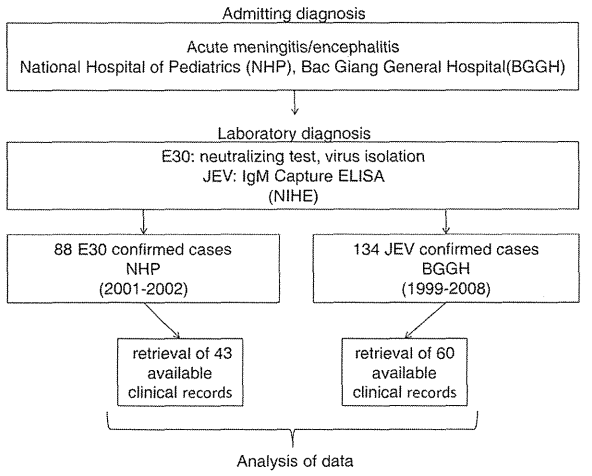

Figure 2 Flow-chart of the work and some of the results in this study. CSF specimens were collected from patients who were clinically diagnosed with acute meningitis/encephalitis at the time of admission to the National Hospital of Pediatrics (NHP) during 2001–2001 and Bac Giang General Hospital (BGGH) during 1999–2008. CSF specimens were sent to NIHE for laboratory diagnosis. A total of 88 patients from NHP were confirmed to be infected with E30 based on NT (80 patients) and virus isolation (8 patients) but only 43 patients had available clinical records. From BGGH, a total of 134 patients were confirmed to be infected with JEV based on the IgM Capture ELISA but only 60 had clinical records.

We constructed the NJ tree of these eight Vietnamese strains together with the 88 E30 strains from GenBank based on the VP1 gene sequence. Based on this tree, our identified strains were relatively close to the Asian strains that caused aseptic meningitis (Figure 1).

Epidemiological features

Most patients were under 15 years old in both the E30 and the JEV groups. The median age was 7 in the former group and 6 in the latter group. In the E30 group, there were 30 male patients (70%) and 13 female patients (30%), with a male-to-female ratio of 2.3:1. In the JEV group, there were 22 male patients (37%) and 38 female patients (63%), with a male-to-female ratio of 1:1.7. The percentage of JEV-vaccinated patients in the E30 group was significantly higher than that in the JEV group (E30 vs. JEV: 56% vs. 12%, $p < 0.001$) (Table 1). Fatal (12%) and sequelae (10%) cases were observed in the JEV group only (Table 1). Both E30 and JEV cases occurred more frequently during the summer season from May to July (Figure 3).

Clinical features during admission

The clinical features during admission of the E30 and the JEV patients are shown in Table 2. The vital sign parameters (body temperature, pulse rate, and respiratory rate) on admission day showed no significant difference between the two groups of patients. A longer hospitalization period was observed in the JEV group than in the E30 group (E30 vs. JEV: 7 [range: 3–23] vs. 9 [1–37] days, $p = 0.003$). More patients in the E30 group complained of headache (95% vs. 50%, $p < 0.001$), vomiting (98% vs. 33%, $p < 0.001$), abdominal symptoms (19% vs. 5%, $p = 0.048$), and neck or back pain (26% vs. 2%, $p < 0.001$). On the other hand, more patients in the JEV group had a higher maximum body temperature (38.0 [37.0-41.0] vs. 38.8 [37.0-40.0] °C, $p = 0.010$), altered consciousness (12% vs. 97%, $p < 0.001$), and seizures (5% vs. 73%, $p < 0.001$). The periods of altered consciousness (1 [1-3] vs. 3 [1-17] days, $p = 0.002$), seizures (1 [1] vs. 2 [1-7] days, $p < 0.001$), Babinski reflex (1 [1-3] vs. 2 [1-7] days, $p = 0.001$), and focal neurological signs (1 [1] vs. 2.5 [1-13] days, $p = 0.037$) lasted longer in the JEV group. Both groups had a similar percentage of patients with signs of meningeal irritations, whereas the duration period was longer in the JEV

Table 1 Basic patient information and outcome

		Median age [range] or number of patients (%)		
		E30 patients n = 43	JEV patients n = 60	p-value
Age		7 [0.4-14]	6 [0.8-21]	0.916
Sex	Male	30 (70)	22 (37)	
	Female	13 (30)	38 (63)	
Family/neighbor history[1]		1 (2)	1 (2)	0.628
JEV vaccination		24 (56)	7 (12)	<0.001*
Death		0 (0)	7 (12)	0.040*
Sequelae		0 (0)	6 (10)	0.039*

[1]family or neighbor showed similar symptoms as the patient at that time, *significant difference ($p < 0.05$).

group (1 [1-6] vs. 3 [1-14] days, $p = 0.002$). More patients in the JEV group experienced Babinski reflex (42% vs. 62%, $p = 0.047$), and with a longer duration (1 [1-3] vs. 2 [1-7] days, $p = 0.001$). Hemiplegia was observed only in the JEV group (25%, $p < 0.001$).

We also focused on the symptoms observed simultaneously. The combined symptoms of headache and vomiting and the triad of symptoms of fever, headache, and vomiting were observed in more patients in the E30 group (19% vs. 0%, $p < 0.001$ and 74% vs. 27%, $p < 0.001$, respectively). On the other hand, occurrence of the combined symptoms of fever and headache was observed only in the JEV group (18%, $p = 0.002$).

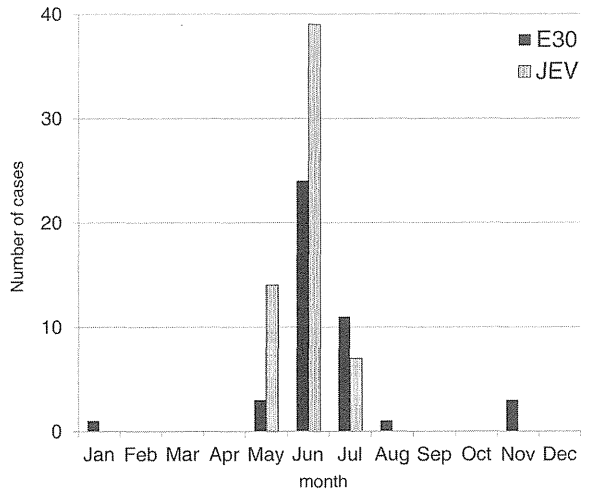

Figure 3 Monthly distribution and frequency of E30 and JEV cases. There were 43 confirmed E30 cases from NHP during 2001–2002 and 60 JEV confirmed cases from BGGH during 1999–2008. The E30 cases and the JEV cases were totaled separately according to the month and regardless of the year of occurrence. The total number of cases per month from January to December was plotted in the graph. The peak for both cases was from May to July.

Laboratory findings

The results of the CSF and blood examination are shown in Table 3. CSF leucocytes were significantly increased in the E30 group (80 [1–750] vs. 18 [5–350] cells/μL, $p = 0.003$). An increased number of both CSF neutrophils and CSF lymphocytes was observed in the E30 group (neutrophils: 26 [2–381] vs. 10 [3–140] cells/μL, $p = 0.033$, lymphocytes: 56 [3–676] vs. 12 [4–210] cells/μL, $p = 0.043$). The CSF sugar levels were significantly decreased in the JEV group (58.7 [27–90] vs. 46.9 [25–74] mg/dL, $p < 0.001$). Other indicators of CSF and blood components showed no significant difference between the E30 and the JEV groups.

Treatment features

The laboratory diagnosis required at least one week to complete, thus medication was given even before the results of the diagnosis became available. The medication histories of the E30 and the JEV groups are shown in Table 4. A history of the use of steroids showed no significant difference between the two groups (E30 vs. JEV: 98% vs. 97%). The duration of steroids use also showed no difference in both groups (3 [2-4] vs. 3 [2-15] days). More patients were prescribed mannitol (47% vs. 95%, $p < 0.001$) and antibiotics (35% vs. 95%, $p < 0.001$) in the JEV group. The duration of mannitol use was longer in the JEV group (1 [1-3] vs. 3 [1-7] days, $p < 0.001$). A history of the use of diazepam showed no significant difference between the two groups. The duration of diazepam use was longer in the E30 group (6 [2-14] vs. 2 [1-11] days, $p < 0.001$). More patients in the JEV group were prescribed barbiturates (33% vs. 63%, $p = 0.002$), chlorpromazine (5% vs. 23%, $p = 0.012$), fentanyl (0% vs. 33%, $p < 0.001$), and sodium valproate (0% vs. 33%, $p < 0.001$).

Discussion

E30 and JEV are the causative agents of acute meningitis/encephalitis in Asian countries. In the present report, Vietnamese patients with an admitting diagnosis of acute meningitis/encephalitis were confirmed to have either E30 or JEV infection by a laboratory diagnosis. The E30 CSF samples analyzed in the present study were collected during the outbreak in 2001–2002. Eight E30 strains were isolated from this outbreak, and the phylogenetic analysis showed that these are relatively close to the strains isolated during E30 outbreaks from other Asian countries (Figure 1).

The E30 acute meningitis/encephalitis outbreak in 2001–2002 gained our interest because nearly all of the patients were clinically diagnosed to have Japanese encephalitis at the beginning of their hospitalization. The reason

Table 2 Clinical features during admission

Clinical feature(s)	Median [range] or, number of patients with the feature (%)		p-value
	E30 patients	JEV patients	
	n = 43	n = 60	
Body temperature (°C)[1]	38.0 [36.2-41.0]	38.5 [36.5-40.0]	0.096
Pulse rate (beats per min.)[1]	100 [80–120] {34}[2]	100 [70–145] {58}[2]	0.092
Respiratory rate (breaths per min.)[1]	26 [20–45] {13}[2]	26 [20–50] {31}[2]	0.691
Length of stay in the hospital (day)	7 [3-23]	9 [1–37]	0.003*
Highest body temperature (°C)	38.0 [37.0-41.0]	38.8 [37.0-40.0]	0.010*
Period of fever[3] (day)	1 [1-14]	2 [1-7]	0.259
Headache	41 (95)	30 (50)	< 0.001*
Period of headache (day)	2 [1-7] {41}	2.5 [1-12] {30}	0.006*
Vomiting	42 (98)	20 (33)	< 0.001*
Period of vomiting (day)	1 [1-5] {42}	1 [1,2] {20}	0.526
Altered consciousness	5 (12)	58 (97)	< 0.001*
Period of altered consciousness (day)	1 [1-3] {5}	3 [1-17] {58}	0.002*
Seizure	2 (5)	44 (73)	< 0.001*
Period of seizure (day)	1 [1] {2}	2 [1-7] {44}	< 0.001*
Sign of meningeal irritation	30 (70)	41 (68)	0.877
Period of meningeal irritation (day)	1 [1-6] {30}	3 [1-14] {41}	0.002*
Babinski reflex	18 (42)	37 (62)	0.047*
Period of Babinski reflex (day)	1 [1-3] {18}	2 [1-7] {37}	0.001*
Focal neurological sign	2 (5)	10 (17)	0.070
Period of focal neurological sign (day)	1 [1] {2}	2.5 [1-13] {10}	0.037*
Dyspnea	2 (5)	10 (17)	0.070
Hemiplegia	0 (0)	15 (25)	< 0.001*
Fatigue	14 (33)	22 (37)	0.666
Abdominal symptom	8 (19)	3 (5)	0.048*
Neck or back pain	11 (26)	1 (2)	< 0.001*
Joint pain	1 (2)	0 (0)	0.235
Skin rash	0 (0)	1 (2)	0.395
Fever[3] + Headache	0 (0)	11 (18)	0.002*
Headache + Vomiting	8 (19)	0 (0)	< 0.001*
Vomiting + Fever[3]	4 (9)	2 (3)	0.232
Fever[3] + Headache + Vomiting	32 (74)	16 (27)	< 0.001*

[1]taken on the day of admission, [2]number inside curly brackets refer to the number of patients with the record, [3]fever defined as body temperature of over 38°C, *significant difference ($p < 0.05$).

for this initial diagnosis could be the lack of clear guidelines for differentiating E30 from JEV infections based on the clinical symptoms and case histories. Our study is the first report to show that certain clinical features could differentiate E30 from JEV cases.

We tried to compare the symptoms and clinical features between the two groups before admission, according to the information in the clinical record (unpublished data). However, there was no sufficient difference noted between the E30 and the JEV cases compared to those during admission (Table 2). Then, we focused our attention on the data during the admission period. Here, we noted that the most common symptoms were fever, headache, and vomiting for both the E30 and the JEV groups (Table 2). More patients in the E30 group had the combined symptoms of headache and vomiting and the triad of symptoms (fever, headache, and vomiting) (Table 2). Thus, it is concluded that these combined symptoms could indicate a high probability of E30 infection. Findings on magnetic resonance imaging (MRI) of patients in the E30 cases revealed no significant abnormalities in the brain [7]. It seems that the mild neurological symptoms observed in

Table 3 CSF and blood examination

	Median value (range), [number of patients]		p-value
	E30 patients n = 43	JEV patients n = 60	
CSF protein (mg/dL)	36.3 (14–144) [39]	44.5 (6–100) [47]	0.093
CSF sugar (mg/dL)	58.7 (27–90) [38]	46.9 (25–74) [47]	< 0.001*
CSF leucocyte (cells/μL)	80 (1–750) [41]	18 (5–350) [45]	0.003*
CSF neutrophil (cells/μL)	26 (2–381) [23]	10 (3–140) [35]	0.033*
CSF lymphocyte (cells/μL)	56 (3–676) [23]	12 (4–210) [35]	0.043*
Blood leucocyte (10E3 cells/μL)	10.4 (5.8-34) [19]	12.0 (3.0-30) [59]	0.711
Blood neutrophil (10E3 cells/μL)	8.2 (3.9-25) [18]	8.0 (1.1-26) [57]	0.368
Blood lymphocyte (10E3 cells/μL)	2.1 (1.3-9.6) [18]	3.2 (1.3-12) [57]	0.189

*significant difference (p < 0.05).

the E30 group are not from direct neuronal damage, but from increased intracranial pressure. Therefore, vomiting, which is one of the important signs of increased intracranial pressure, was often observed in the E30 group compared to the JEV group in this study.

More patients had altered consciousness (97%), seizure (73%), and hemiplegia (25%) in the JEV group (Table 2). These symptoms are strong signs of neural pathogenicity. It was reported that the JEV patients showed abnormalities in thalamus, basal ganglia, and cerebral cortex based on the results of computed tomography (CT) and MRI [30-32]. However, it is difficult to conduct these expensive examinations in a resource limiting setting in the rural areas of Asian countries. Neuropathological findings in fatal JEV cases showed that the virus caused broad inflammation in the central nervous system [33]. JEV infects neurons and causes direct damage [10,33] that consequently leads to many severe neurological symptoms. Thus, in areas where E30 and JEV outbreaks are overlapping, severe neurological symptoms could provide strong evidence of JEV infection.

The most critical laboratory finding in this report was the CSF leukocytosis in the E30 group (Table 3). An increase in CSF neutrophils was observed, and this was also reported in another study [34]. An increase in CSF lymphocytes was observed in the present study. Therefore, these results seem to suggest that CSF leukocytosis relates to the E30 infection.

Based on the medication history, more patients were prescribed barbiturates, chlorpromazine, fentanyl, and sodium valproate in the JEV group (Table 4). This seems to correlate with the longer period of neurological symptoms such as altered consciousness and seizures in the JEV

Table 4 Treatment features

	Number of patients (%), median day [range]		p-value
	E30 patients n = 43	JEV patients n = 60+	
Steroids	42 (98)	58 (97)	0.769
Period of treatment	3 [2-4]	3 [2-15]	0.204
Mannitol	20 (47)	57 (95)	< 0.001*
Period of treatment	1 [1-3]	3 [1-7]	< 0.001*
Antibiotics	15 (35)	57 (95)	< 0.001*
Period of treatment	8 [1-15]	8 [1-17]	0.688
Diazepam	23 (53)	32 (53)	0.988
Period of treatment	6 [2-14]	2 [1-11]	< 0.001*
Barbiturate	14 (33)	38 (63)	0.002*
Period of treatment	3.5 [1-14]	2.5 [1-11]	0.130
Chlorpromazine	2 (5)	14 (23)	0.012*
Fentanyl	0 (0)	20 (33)	< 0.001*
Sodium Valproate	0 (0)	20 (33)	< 0.001*

*significant difference (p < 0.05).

group. We would like to indicate that medication strategies have been established in each hospital, however some minor differences in these strategies exist between hospitals.

Patients in the E30 group had a higher male-to-female ratio of 2.3:1. A higher ratio was also shown in previous reports [34,35]. Boys usually prefer to play outside of their house compared with girls. This behavior could result in a more frequent exposure of boys to the pathogen.

Conclusion

Clinical information demonstrated that the combination of headache and vomiting and the triad of symptoms of fever, headache, and vomiting could indicate a high probability of E30 infection. A laboratory parameter of CSF leukocytosis also suggests E30 infection. On the other hand, strong neurological symptoms, such as altered consciousness, seizure, and hemiplegia, could indicate a high probability of JEV infection. These findings will help improve the clinical diagnosis of patients with E30 or JEV meningitis/encephalitis. Then, proper measures can be adopted for the clinical management of the patients at the early phase of admission before laboratory diagnosis becomes available.

Abbreviations

E30: Echovirus 30; JEV: Japanese encephalitis virus; CSF: Cerebrospinal fluid; NIHE: National Institute of Hygiene and Epidemiology; NT: Neutralization test; RD cells: Rhabdomyosarcoma cells; ICF: Infected Culture Fluid; CPE: Cytopathic effect; RT-PCR: Reverse transcription and polymerase chain reaction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DTH, NTT, HMD, and PTN contributed to the collection of the specimens and clinical diagnosis. NTP and FH contributed to the laboratory diagnosis. YT, LU, KO, and TN performed the genome analysis and cell culture. YT, DTT, PTN, and FH collected clinical information and analyzed these data. YT and KM designed the study. YT and LXL drafted the manuscript. All of the authors read and approved the final version of the manuscript.

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