Volume 1 (2026)
https://doi.org/10.22452/jtoh.vol1.2
JOURNAL OF TROPICAL One Health
SHORT COMMUNICATION

Discovery of a Japanese Encephalitis Virus Nakayama Substrain Highlights the Dual Lessons of Molecular Characterization and Laboratory Biosafety

Jefree Johari, Shih Keng Loong, Juraina Abd-Jamil, Chee-Sieng Khor, Boon-Teong Teoh, Vunjia Tiong, Kim Kee Tan, Sazaly AbuBakar*

Tropical Infectious Diseases Research & Education Centre, Higher Institution Centre of Excellence, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author: sazaly@um.edu.my

ARTICLE HISTORY

Received: 11 Sep 2025
Revised: 12 Feb 2026
Accepted: 14 Apr 2026
Published: 30 Jun 2026

ABSTRACT

Japanese encephalitis virus (JEV) remains an important cause of viral encephalitis in Asia, with high mortality and neurological sequelae. During a routine inventory at a Malaysian research institution, an unregistered vial labeled "JEV" was recovered from an unsecured −80 °C freezer. The sample, designated MY8662, was investigated under biosafety level 3 conditions. Inoculation of Vero cells produced cytopathic effects, and real-time RT-PCR confirmed JEV identity. Full-genome sequencing revealed 99.68% nucleotide and 99.88% amino acid similarity to the Nakayama vaccine strain, with 35 nucleotide substitutions resulting in four amino acid changes, including novel substitutions in the envelope and NS4A proteins. Phylogenetic analysis clustered MY8662 closely with Nakayama, suggesting a substrain relationship. Replication kinetics in Vero cells showed exponential viral RNA increase with a doubling time of 2.6 hours, comparable to other JEV strains. The incident also revealed biosafety concerns associated with obscure inventories, prompting corrective measures including access-controlled biorepositories and barcode-based inventory systems. This case illustrates the dual significance of forgotten laboratory materials, providing insights into JEV genomic diversity while underscoring the importance of sustainable biosafety and biosecurity practices. Strengthened institutional oversight and risk-based management are essential to prevent accidental exposures or loss of infectious agents.

Key words: Malaysia, arbovirus, Flaviviridae, zoonosis, biosafety, biosecurity

Introduction

Japanese encephalitis (JE) is the most important viral encephalitis in Asia, with an estimated 67,000 cases annually and significant morbidity and mortality (Campbell et al., 2011). The Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, circulates between birds and Culex mosquitoes, with pigs serving as amplifying hosts. Although humans are dead-end hosts, outbreaks continue to occur across Southeast Asia, including Malaysia (Khor et al., 2020). The Nakayama strain, isolated in 1935, remains a prototype genotype III (GIII) JEV strain and the basis of inactivated vaccines. JEV comprises five genotypes with up to 19% nucleotide divergence; Nakayama's position as a GIII prototype makes it a useful benchmark for molecular comparison (Mansfield et al., 2025). Understanding substrain diversity has implications for virulence and immunity, but equally important is the safe handling of pathogens. Global inconsistencies in dengue virus (DENV) biosafety classification highlight the risks of poor harmonization and the need for transparent, evidence-based risk assessment when managing flaviviruses (Le & Blacksell, 2025). The World Health Organization emphasizes sustainable biosafety practices based on local risk assessments (WHO, 2020). Historical lapses, such as the discovery of forgotten smallpox vials at the US NIH in 2014, highlight the risks posed by inadequate inventory control (CDC, 2014). Here, we describe the recovery and molecular characterization of an undocumented JEV specimen in Malaysia and discuss both its genetic features and biosafety implications.

Materials and methods

In 2009, during a routine inventory reconciliation, an undocumented vial labeled 'JEV' was discovered in an

https://doi.org/10.22452/jtoh.vol1no1.2

unsecured -80 °C freezer. The vial was presumed to have been deposited prior to 2007, as laboratory biosafety protocols had been strengthened following a 2007 incident involving a *Bacillus* species initially suspected to be *Bacillus anthracis*, which required investigation under biosafety level 3 conditions (Loong et al., 2017). Despite these earlier corrective measures, the presence of this forgotten JEV vial highlighted persistent gaps in historical inventory control. The sample was immediately transferred to a biosafety level 3 facility for characterization. A biorisk assessment was conducted, emphasizing that biological safety levels should be set based on procedure-specific risks rather than nominal risk groups (Artika & Ma'roef, 2017). This assessment prioritized controls for aerosol-generating procedures, mandated sealed centrifuge rotors and certified Class II biosafety cabinets for manipulations, and refreshed sharps safety training. Vero cells were inoculated with the specimen and observed for cytopathic effects. Viral RNA was extracted and subjected to SYBR Green I-based real-time RT-PCR targeting the NS3 region (sense-direction: 5'-AGA GCG GGG AAA AAG GTC AT-3' and antisense-direction: 5'-CTT CAC GCT CTT CCT ACA GT-3', producing a 162-bp fragment) to confirm JEV identity. To obtain the complete genome, eleven pairs of overlapping primers were used, and bidirectional sequencing was performed (Supplementary Table S1). Sequences were assembled and aligned with 23 reference JEV strains available in GenBank. Phylogenetic analysis was performed using the maximum likelihood method with 1,000 bootstrap replications. Replication kinetics were determined by sampling infected culture supernatants at eight-hour intervals, quantifying viral RNA copy numbers using RT-PCR against a synthetic RNA standard. Given recommendations to integrate whole genome sequencing into JEV preparedness frameworks, sequencing was used to verify lineage and assess divergence from reference Nakayama sequences (Mansfield et al., 2025). A biorisk assessment of the unregistered vial prompted corrective actions, including enhanced access control, barcode-based inventories, and reconciliation of historical stocks.

Results and discussion

Inoculated Vero cells exhibited cytopathic effects by day four, including cell rounding and detachment, and real-time RT-PCR confirmed JEV identity. The isolate was designated MY8662. Genome sequencing revealed 10,977 nucleotides comprising a 95-nucleotide 5'-UTR, a 10,296-nucleotide open reading frame, and a 586-nucleotide 3'-UTR (Table 1). Pairwise comparison showed 99.68% nucleotide and 99.88% amino acid identity with the Nakayama strain. Thirty-five nucleotide differences produced four amino acid substitutions: prM-Lys16Asn, E-Glu83Lys, E-Ile176Thr, and NS4A-Phe260Val (Table 1). Phylogenetic analysis grouped MY8662 with Nakayama in a distinct branch with strong bootstrap support, suggesting a substrain relationship (Figure 1). Here we use 'substrain' to denote a variant of the prototype Nakayama strain with >99.5% nucleotide identity but consistent, reproducible sequence differences. Replication kinetics were assessed to determine whether the four amino acid substitutions in MY8662 affected viral growth efficiency. RNA copies rose exponentially with a doubling time of 2.6 hours (Supplementary Figure S1), comparable to other JEV strains (Yamaguchi et al., 2011), suggesting that these substitutions do not confer a replication advantage or disadvantage in Vero cells.

Table 1. Comparison of the complete nucleotide and deduced amino acid sequences between MY8662 and Nakayama strain.

Genome segment	Size		Nucleotide substitution		Amino acid substitution	
	Nucleotide	Amino acid	No. of substitution	% substitution	No. of substitution	% substitution
5' UTR	95	0	3	3.16	0	0.00
Capsid	381	127	1	0.26	0	0.00
Membrane	501	167	3	0.60	1	0.60
Envelope	1500	500	8	0.53	2	0.40
NS1	1245	415	5	0.40	0	0.00
NS2A	492	164	0	0.00	0	0.00
NS2B	393	131	1	0.76	0	0.00
NS3	1857	619	2	0.11	0	0.00
NS4A	801	267	3	1.12	1	0.37
NS4B	411	137	1	0.73	0	0.00
NS5	2715	905	3	0.11	0	0.00
3' UTR	586	0	5	0.85	0	0.00
Complete	10977	3432	35	0.32	4	0.12

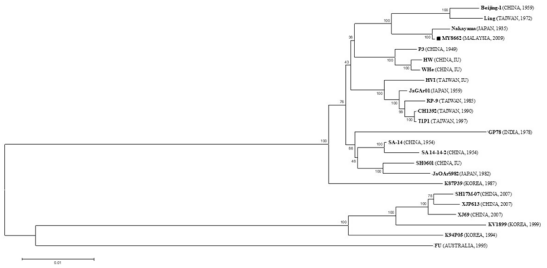

Figure 1. Phylogenetic tree based on complete nucleotide sequences of MY8662 (indicated by ■) and other selected 23 other JEV strains. The scale bar represents the number of nucleotide substitutions per site. Bootstrap confidence limits are shown at each node. The strain name is followed by country and year of isolation. IU – information unknown.

The amino acid substitutions warrant consideration. The prM-Lys16Asn change occurs at a conserved glycosylation site important for virion maturation (Bause, 1983; Kim et al., 2008). The E-Glu83Lys substitution may affect immune recognition, while E-Ile176Thr lies at a position associated with neurovirulence (Arroyo et al., 2001; Singha et al.,

2013). The NS4A-Phe260Val substitution is conservative and likely minimal in effect. These findings support MY8662 as a Nakayama substrain, but functional studies are required to clarify phenotypic significance. Beyond molecular data, the discovery emphasized biosafety challenges. Arbovirus laboratory acquired infections, including dengue, have been reported from aerosol-generating steps and sharps accidents (Artika & Ma'roef, 2017), reinforcing the importance of enhanced containment and sharps safety. The absence of inventory control in this case posed risks similar to those noted in high-profile incidents involving forgotten pathogens (CDC, 2014). Immediate implementation of access-controlled storage, digital inventory systems, and annual training refreshers addressed these hazards. Immediate implementation of access-controlled storage, digital inventory systems, and periodic training refreshers addressed these hazards. These corrective actions exemplify the WHO Laboratory Biosafety Manual (4th ed.) core principles of biorisk management, particularly the hierarchy of controls (e.g., substitution of unsafe practices with engineering controls) and the institutional commitment to sustainable biosafety (WHO, 2020). This also echoes preparedness frameworks recommending harmonized diagnostics and whole-genome sequencing for JEV (Mansfield et al., 2025). In summary, MY8662 demonstrates close genetic similarity to the Nakayama strain with minor substitutions, while its discovery underscores the critical biosafety lesson that undocumented stocks pose dual scientific and safety challenges.

Acknowledgements

This work was supported by the Ministry of Higher Education, Malaysia under the Higher Institution Centre of Excellence (HICoE) program for niche area research (TIDREC-2023 and MO002-2019).

Conflict of interest statement

The authors declare no conflicts of interest.

References

Arroyo, J., Guirakhoo, F., Fenner, S., Zhang, Z. X., Monath, T. P., & Chambers, T. J. (2001). Molecular basis for attenuation of neurovirulence of a yellow fever virus/Japanese encephalitis virus chimera vaccine (ChimeriVax-JE). *Journal of Virology*, *75*(2), 934–942. https://doi.org/10.1128/JVI.75.2.934-942.2001

Artika, I. M., & Ma'roef, C. N. (2017). Laboratory biosafety for handling emerging viruses. *Asian Pacific Journal of Tropical Biomedicine*, *7*(5), 483–491. https://doi.org/10.1016/j.apjtb.2017.01.020

Bause, E. (1983). Structural requirements of N-glycosylation of proteins: Studies with proline peptides as conformational probes. *Biochemical Journal*, *209*(2), 331–336. https://doi.org/10.1042/bj2090331

Campbell, G. L., Hills, S. L., Fischer, M., Jacobson, J. A., Hoke, C. H., Hombach, J. M., Marfin, A. A., Solomon, T., Tsai, T. F., Tsu, V. D., & Ginsburg, A. S. (2011). Estimated global incidence of Japanese encephalitis: A systematic review. *Bulletin of the World Health Organization*, *89*(10), 766–774. https://doi.org/10.2471/BLT.10.085233

Centers for Disease Control and Prevention. (2014, July 8). *CDC media statement on newly discovered smallpox specimens*. https://www.cdc.gov/media/releases/2014/s0708-NIH.html

Khor, C. S., Mohd-Rahim, N. F., Hassan, H., Tan, K. K., Zainal, N., Teoh, B. T., Sam, S. S., Khoo, J. J., Lee, H. Y., Lim, Y. A. L., & AbuBakar, S. (2020). Serological evidence of DENV, JEV, and ZIKV among the indigenous people (Orang Asli) of Peninsular Malaysia. *Journal of Medical Virology*, *92*(6), 956–962. https://doi.org/10.1002/jmv.25651

Kim, J. M., Yun, S. I., Song, B. H., Hahn, Y. S., Lee, C. F., Oh, H. W., & Lee, Y. M. (2008). A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. *Journal of Virology*, *82*(16), 7846–7862. https://doi.org/10.1128/JVI.00783-08

Kulkarni-Kale, U., & Kolaskar, A. S. (2003). Prediction of 3D structure of envelope glycoprotein of Sri Lanka strain of Japanese encephalitis virus. *Conferences in Research and Practice in Information Technology*, *19*, 87–96.

Le, K. K., & Blacksell, S. D. (2025). Dengue virus biosafety: An analysis of evidence, global inconsistencies, and risk gaps. *Applied Biosafety*. Advance online publication. https://doi.org/10.1089/apb.2024.0023

Loong, S. K., Teoh, B. T., Johari, J., Khor, C. S., Abd-Jamil, J., Nor'e, S. S., Samsudin, N. I., Azizan, N. S., Yaacob, C. N., Che Mat Seri, A. A., Mahfodz, N. H., & AbuBakar, S. (2017). Penicillin-susceptible, oxidase-negative, nonhemolytic, nonmotile *Bacillus megaterium* in disguise of *Bacillus anthracis*. *Case Reports in Infectious Diseases*, *2017*, Article 2578082. https://doi.org/10.1155/2017/2578082

Mansfield, K. L., Parekh, I., Rasmussen, T. B., Lohse, L., Olesen, A. S., Dheilly, N. M., Gonzalez, G., Migné, C. V., Gondard, M., Helle, T., Lilja, T., Lindahl, J. F., van der Poel, W. H. M., Harders, F., Bron, G. M., & Holwerda, M. (2025). European preparedness for Japanese encephalitis virus through alignment of animal health laboratory diagnosis. *Transboundary and Emerging Diseases*, *2025*, Article 5516160. https://doi.org/10.1155/2025/5516160

Singha, H., Gulati, B. R., Kumar, P., Singh, B. K., Virmani, N., & Singh, R. K. (2013). Complete genome sequence analysis of Japanese encephalitis virus isolated from a horse in India. *Archives of Virology*, *158*(1), 113–122. https://doi.org/10.1007/s00705-012-1462-y

World Health Organization. (2020). *Laboratory biosafety manual* (4th ed.). https://www.who.int/publications/i/item/9789240011311

Yamaguchi, Y., Nukui, Y., Tajima, S., Nerome, R., Kato, F., Watanabe, H., Takasaki, T., & Kurane, I. (2011). An amino acid substitution (V3I) in the Japanese encephalitis virus NS4A protein increases virulence in mice, but not its growth rate in vitro. *Journal of General Virology*, *92*(7), 1601–1606. https://doi.org/10.1099/vir.0.030585-0