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Four genetically distinct types of rabies virus exist in Vietnam, including the SEA1 and SEA3 subclades within the Asian clade

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Rabies is a fatal zoonotic disease that causes encephalitis in almost all mammals. Vietnam remains endemic for rabies and shares borders with China, Laos, and Cambodia, where the disease also persists. Nucleoprotein and full-genome sequencing are valuable tools for investigating the genetic diversity and transmission dynamics of circulating rabies virus (RABV) strains. This study aimed to assess the current rabies situation in Vietnam and genetically characterize RABV strains using both sequencing approaches. Human and canine rabies cases are reported annually in Vietnam, where approximately half a million people receiving post-exposure prophylaxis each year, though this number has recently increased. Epidemiological data and RABVs from humans and rabid dogs were analyzed. Vietnamese RABVs were classified into four distinct genetic groups, all phylogenetically related to viruses circulating in neighboring countries. Full-genome analysis revealed regional differences in virus classification, suggesting that local factors may influence viral circulation between Vietnam and neighboring countries. The high genetic similarity between human- and dog-derived RABVs underscores the continued zoonotic threat and highlights the critical need for a One Health approach to rabies prevention and control in Vietnam and its neighboring regions.

Keywords Rabies virus, Epidemiology, Vietnam, Full-genome sequencing, Nucleoprotein

Rabies is one of the most serious zoonoses, causing fatal encephalitis in almost all mammalian species. It is caused by the rabies virus (RABV), a non-segmented, single-stranded, negative-sense RNA virus belonging to the order *Mononegavirales*, family *Rhabdoviridae*, subfamily *Alpharhabdovirinae*, and genus *Lyssavirus*[1]. The viral genome is approximately 12 kb and encodes five proteins: nucleoprotein (N protein), phosphoprotein, matrix protein, glycoprotein (G protein), and a large RNA-dependent RNA polymerase[2,3]. Rabies is estimated to cause 59,000 human deaths annually worldwide and is particularly prevalent in Asia and Africa[2,4]. Vietnam is among the rabies-endemic countries, with a monthly incidence rate of 117.2 cases per 100,000 population between 2011 and 2015[5,6]; at least 82 human deaths were attributed to dog-transmitted rabies in 2024[7], and approximately 500,000 people receive post-exposure prophylaxis (PEP) annually[8].

A national rabies control project in Vietnam was launched in 2009, incorporating surveillance for rabies-related data[9], guided by a One Health framework that emphasizes collaboration among the human, animal, and environmental health sectors[5,10,11]. To ensure accurate rabies surveillance, human and animal cases have been managed separately since 2015. The National Rabies Control Program coordinated by the National Institute of Hygiene and Epidemiology (NIHE) oversees human cases, while the National Centre for Veterinary Diagnosis (NCVD), Department of Animal Health (DAH) conducts routine surveillance for animal cases[12]. To achieve zero human deaths from dog-mediated rabies worldwide by 2030, the World Health Organization launched the

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"Zero by 30" plan in 2015, recommending the global use of the canine rabies vaccine[13–15]. In Vietnam, the dog population, including both stray and domestic dogs, was estimated at 7.7 million in 2016[16], with nearly all of the country's human rabies cases attributed to canine transmission[8]. Although Vietnam has also made significant efforts in its vaccination campaigns, coverage varies greatly between provinces, and the overall vaccination rate among dogs remains low (42.9% in 2015)[16]. As a result, the number of reported rabies cases has remained relatively stable, and the disease remains endemic.

Vietnam is geographically bordered by China to the north, Laos to the west, and Cambodia to the southwest[17]. Two Chinese provinces, Guangxi[18] and Yunnan[19], share a border with Vietnam. Because Guangxi borders only Vietnam, reported border-related infectious diseases have been primarily transmitted through human-to-human contact, with no transmission from animals or vectors. Diseases that are transmitted through human-to-human contact are primarily spread along this border[20,21]. Because Yunnan borders multiple countries, including Myanmar, Laos, and Vietnam, many infectious diseases are also transmitted by animals and vectors between it and neighboring countries[19,22].

Epidemiological RABV research in Vietnam has employed phylogenetic analysis based on gene sequences encoding the N or G proteins[23–25]. However, limited details from provinces in neighboring countries make the origin of RABV strains in Vietnam uncertain. Full-genome sequencing using next-generation sequencing (NGS) is suitable for identifying metadata such as the host, isolation date, prevalence, geography, and phylogenetic characteristics[26–28]. However, comparisons with neighboring countries are limited because of the scarcity of full-genome sequence registrations[29–31]. Using N protein analysis for broad comparisons across numerous registered sequences, along with full-genome sequencing providing high-resolution data capable of distinguishing individual strains, is beneficial for RABV tracking and research.

This study aimed to verify the phylogeny of RABV isolated from human and animal samples in Vietnam by analyzing both N protein and full-genome sequences.

Results

Epidemiology of rabies in Vietnam

Vietnam was divided into four main regions: North, Central, South, and Highland (Supplemental Fig. 1), and the annual number of people who received PEP vaccinations from 2018 to 2024 in each region was aggregated (Table 1 and Supplemental Table 1). During the global coronavirus disease 2019 (COVID-19) pandemic from 2020 to 2022, the number of people receiving PEP decreased compared with that in the years before the pandemic (2018–2019); however, after the pandemic (2023–2024), PEP recipients increased compared with pre-pandemic years. In 2024, the southern region had the highest PEP inoculation rate across Vietnam (Supplemental Table 1).

The annual number of human and canine cases tested and diagnosed as RABV-positive from 2018 to 2024 in North Vietnam was reported each year (Table 2). The annual number of human cases tested after the COVID-19 pandemic increased compared with the pre-pandemic and pandemic period.

Isolated strains clustered in the Asian clade

The isolated RABVs used for sequencing, derived from human patient samples (Table 3 and Supplemental Fig. 2 A) and suspected rabid dog samples (Table 4 and Supplemental Fig. 2B), were collected from North and Central Vietnam. Human samples consisted of saliva, cerebrospinal fluid (CSF), or both. The saliva positivity rate was 89.65%, while the CSF positivity rate was 57.89% (Supplemental Table 2). For each human case, samples with a positive saliva or CSF result were used for sequencing (Table 3). Virus isolation for some samples was attempted using suckling mice; however, only 8 of 20 saliva samples (isolation rate: 40%) and 1 of 4 CSF samples (isolation rate: 25%) were successfully isolated. Four of those isolates were used for full-genome sequencing (Table 3).

In the phylogenetic tree of partial N protein sequences (435 bp) alongside other strains registered in the National Center for Biotechnology Information (NCBI), the isolated strains were classified into the Asian clade (Fig. 1A). The Asian clade was further classified into five subclades, comprising SEA1 to SEA5. All strains isolated in this study belonged to the SEA1 subclade (Fig. 1B), except for H370 (Fig. 1C), which was classified into SEA3 with strains isolated in Laos and Cambodia. Eighteen other strains registered in the NCBI as strains isolated in Vietnam (AB299032–039, AB116579–80, EU086209–10, MH828450, MK790254–57, and MW055234) were also classified into SEA3.

The Vietnamese strains isolated in this study and classified into SEA1 were further divided into two distinct groups, designated as group (a) and (b) (Fig. 2A). Group (a) was composed of strains isolated in Guangxi, China, while group (b) was composed of strains mostly isolated in Yunnan, China. Within group (b), the Vietnamese isolates were found in several branches (labeled i–vii, Fig. 2A and 2B). Strain D182 was classified into group (a), which includes strains isolated in Guangxi with which > 98% sequence identity (Supplemental Table 3). Ten strains isolated from human patient samples (H231, H249, H292, H298, H339, H347, H360, H2504, H2540, and H2570) and nine strains isolated from dog samples (D085, D089, D098, D134, D159, D181, D190, D194, and D207) were classified into branch (iv) and shared 100% sequence identity with strains isolated in Yunnan. These strains were located on the west side of Northern Vietnam, including the border between Vietnam and Yunnan (Supplemental Fig. 3 A). Other strains matching those isolated in Vietnam (except those in this study) were also found in provinces including Phu Tho and Hoa Binh. Branch (vii) consisted solely of strains isolated in Vietnam, which were distributed without any specific bias (Supplemental Fig. 3B).

Full-genome sequences differences by province

To further investigate sequence variation by province, collection year, and host, we performed NGS. Phylogenetic tree analysis using 17 strains showed that D182 was classified as an outgroup (Fig. 3). D182 was closely related to the strains isolated in Guangxi (Fig. 2A), correlating with full-genome sequencing results; the nine strains isolated from Lang Son, excluding D182, were classified into the same subclade. D213 and D037 were identical

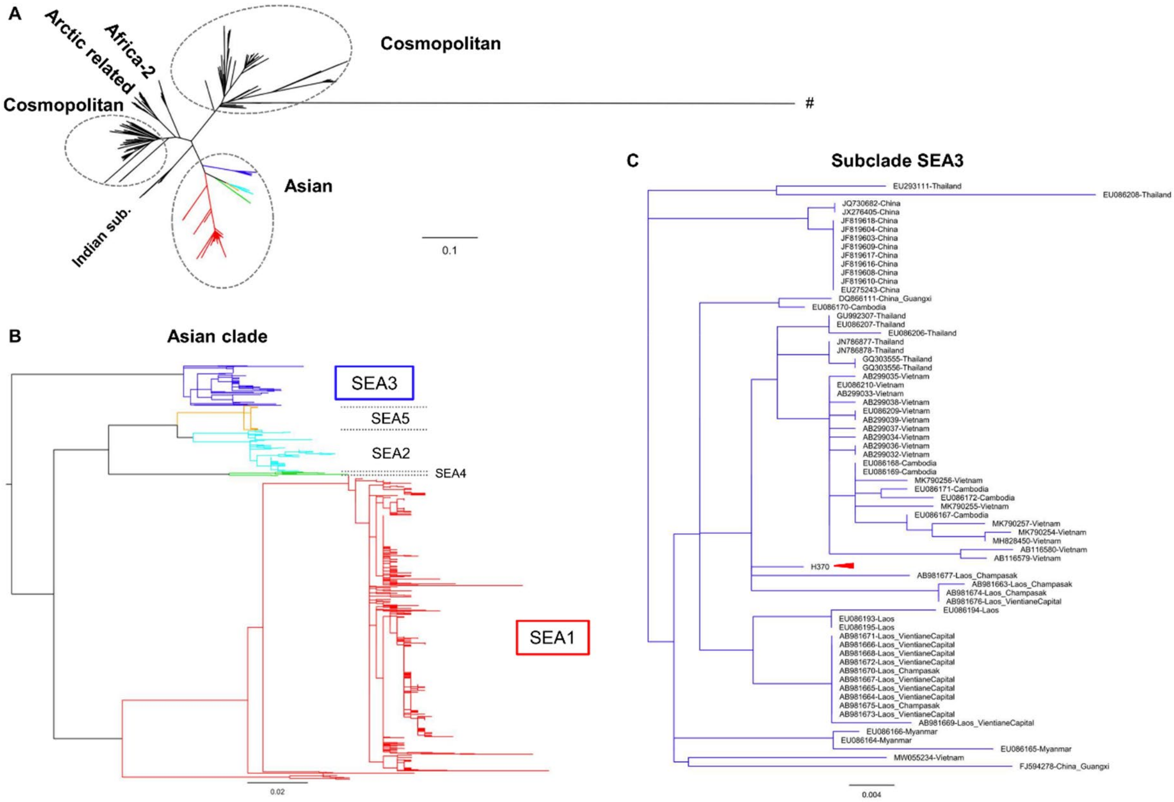

Fig. 1. Phylogenetic tree constructed based on partial N protein sequences (435 bp) using 1998 strains registered at NCBI and 76 strains isolated in this study. (**A**) The phylogenetic tree using all strains; (**B**) the tree focusing on the Asian clade; and (**C**) the expansion of the SEA3 subclade. Mokola lyssavirus (#) was used as an outgroup. Red arrowheads indicate the strains isolated in Vietnam in this study. The number at each branch indicates the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) value (%)/ultrafast bootstrap (UFBoot) value (%); these values are shown when the SH-aLRT value is ≥ 80% and UFBoot value is ≥ 95%. The values within the subclade are not shown.

in sequence and were both isolated from Bac Son. The remaining strains isolated from different provinces formed distinct subclades; their collection year and host did not differ.

Discussion

Rabies remains endemic in both humans and animals in Vietnam. In this study, data on individuals receiving PEP were collected and aggregated annually, showing that a large number of people continued to receive the vaccine. Additionally, the genetic analysis of samples collected from human patients and dogs with rabies in North and Central Vietnam classified circulating RABVs into four distinct genetic groups: one belonging to SEA3 and three to SEA1, while comparisons of full-genome sequences revealed differences between strains from each province. These results show that it is crucial to take preventive measures to stop the further spread of rabies and achieve the "Zero by 30" goal.

Three provinces in Vietnam (Cao Bang, Lang Son, and Quang Ninh) border Guangxi, China, while four others (Ha Giang, Lao Cai, Lai Chau, and Dien Bien) border Yunnan, China[32]. The border gates in these provinces are used by people to move between the two countries[32]; however, these were closed starting in January 2020 owing to the COVID-19 pandemic. During this period, strict nationwide lockdowns were implemented across Vietnam, leading to restricted mobility and reduced outdoor activities. Consequently, the number of dog bite incidents declined, resulting in fewer people requiring PEP. Additional pandemic-related challenges, such as disruptions in healthcare services and supply chains, diversion of resources toward pandemic response, and containment measures that may have limited access to immunization services, may also have contributed to the decline in PEP uptake during this period (Table 1). In January 2023, the Huu Nghi International Border Gate reopened, followed by the other gates in December 2023, re-establishing movement between China and Vietnam; this coincided with an increase in PEP administration and testing for both patients and animals (Tables 1 and 2). Since the decline of the COVID-19 pandemic, the number of individuals receiving PEP has risen compared with previous years[8,33], suggesting that renewed cross-border movement enhanced the mobility of humans and animals, contributing to an increase in dog bite cases. Additionally, rabies case caused by strains circulating near the border were increasingly detected within Vietnam, and genomic analysis indicated that several strains isolated in this study were closely related to those previously identified in Yunnan and Guangxi.

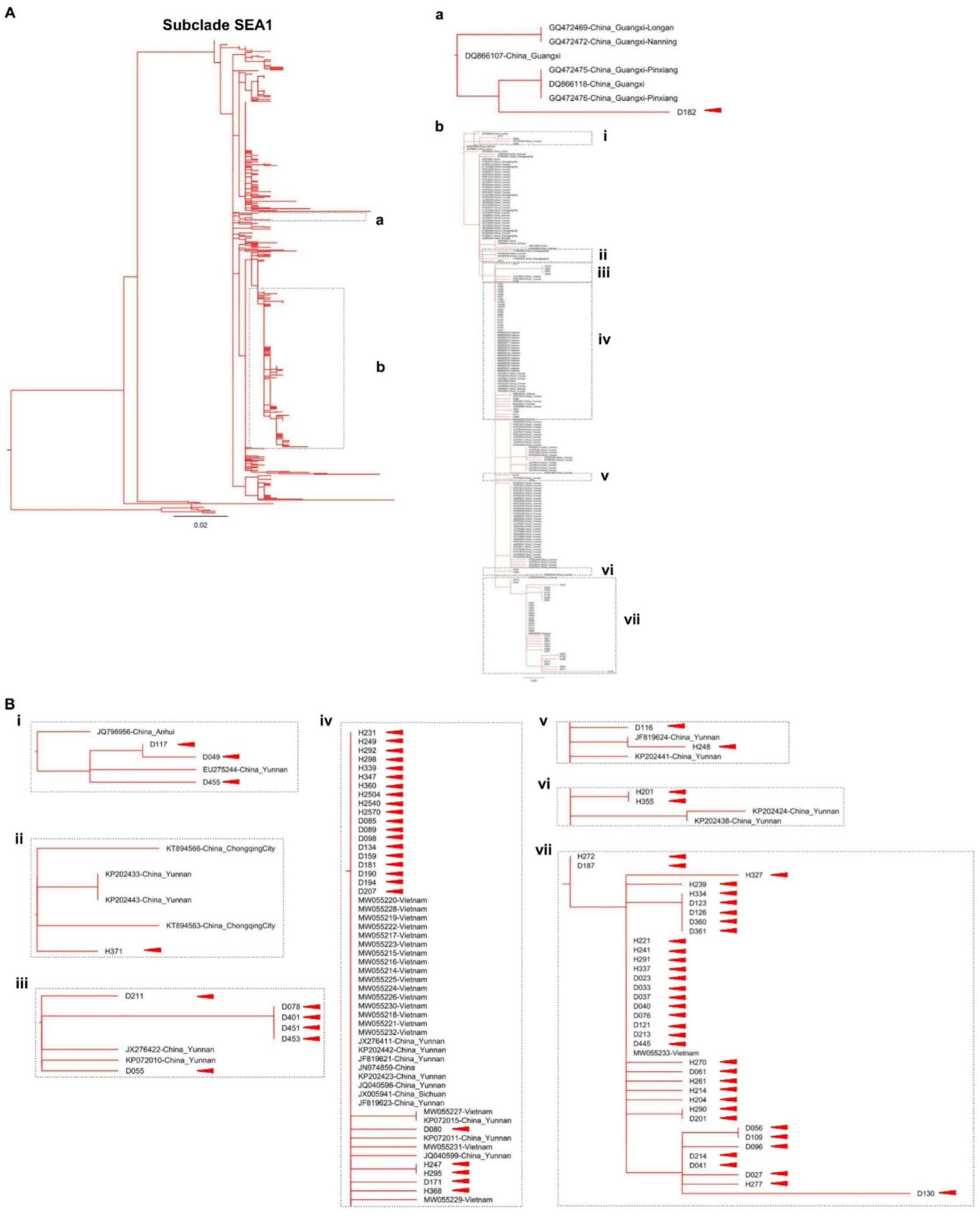

Fig. 2. Expanded phylogenetic tree of the SEA1 subclade. The phylogenetic branching is broadly organized for convenience, and the Vietnamese isolates are highlighted for further analysis. (**A**) The Vietnamese strains isolated in this study and classified into SEA1 are divided into two distinct groups (a and b). Within group (b), the Vietnamese isolates were found in several branches (labeled i–vii). (**B**) The individual branches are further expanded. Red arrowheads indicate the strains isolated in Vietnam in this study.

These findings suggest that preventing the spread of rabies requires protective measures, including surveillance and dog vaccination, across extensive border regions between China and Vietnam.

The strains in branches (iv) and (vii) showed different geographic distribution patterns across provinces (Supplemental Fig. 3A and 3B). The strains in branch (iv), which matched those isolated from Yunnan, tended to spread from border provinces to Central Vietnam (Supplemental Fig. 3 A). The route between Yunnan and

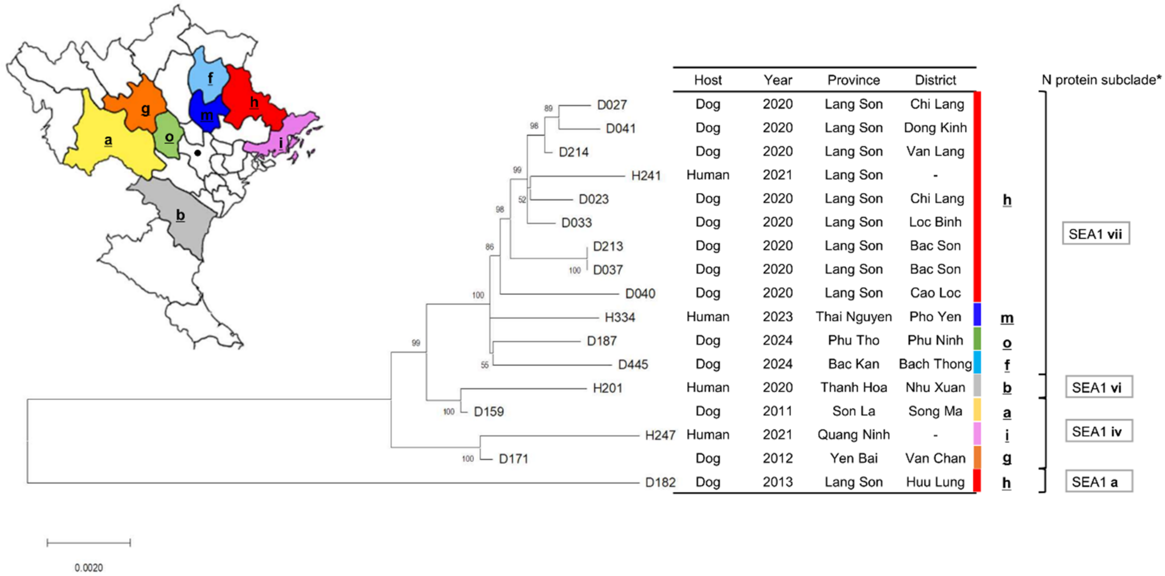

Fig. 3. Phylogenetic tree constructed using 17 strains isolated in this study, based on full genome sequences (11859 bp). The isolated provinces are mapped on the left side, while the host, collection year, province, and district are shown on the right side. The phylogenetic tree was created using MEGA 10 with 1,000 bootstrap replicates. Each province on the map is represented by the same letter as in Supplemental Fig. 2. *: The N protein subclade was referenced in Fig. 2B.

Year	North	Central	South	Highland	Total
2018	115,465	100,178	299,491	20,657	535,791
2019	130,416	90,546	305,727	19,826	546,515
2020	108,142	86,298	288,278	19,101	501,819
2021	79,446	71,086	235,345	15,948	401,825
2022	87,246	77,835	286,813	13,930	465,824
2023	112,976	108,678	435,736	18,833	676,223
2024	156,634	148,797	548,703	27,997	882,131

Table 1. Number of people who received post-exposure prophylaxis (PEP) after a dog bite in Vietnam.

	Human		Dog	
Year	Number of tests	Number of RABV positive	Number of tests	Number of RABV positive
2018	26	13	5	3
2019	18	8	11	8
2020	22	9	42	22
2021	21	12	1	0
2022	29	17	1	0
2023	41	15	4	3
2024	38	14	36	36

Table 2. Number of cases tested and rabies virus (RABV)-positive results in humans and dogs in North Vietnam from 2018 to 2024.

Hanoi is connected by both highway and train, and both routes pass through Lao Cai, Yen Bai, Phu Tho, Vinh Phuc, and Hanoi (Supplemental Fig. 3 A). The highway also passes through Thanh Hoa, Nghe An, Ha Tinh, and Quang Binh (Supplemental Fig. 3 A), ultimately reaching Ca Mau. The provinces where strains circulating in regions bordering Yunnan were isolated tended to align with the highway route, though not perfectly. This suggests that animal movement and RABV spread may occur along the highway route as well as through other border-crossing movements. Only H204 was collected from Hanoi (Supplemental Fig. 2 A), suggesting that there may be evidence of further expansion into the area with increased sampling. In contrast, strains in group

No.	Code	Kind of sample	Year of collection	Province	Area of map	RT-PCR (606 bp)	NGS (11,875 bp)
1	H239	CSF	2021	Son La	a	+	-
2	H355	Saliva	2024	Son La	a	+	-
3	H201	Mouse brain inoculated with patient saliva	2020	Thanh Hoa	b	+	+
4	H347	CSF	2024	Thanh Hoa	b	+	-
5	H221	CSF	2020	Nghe An	c	+	-
6	H370	CSF	2024	Nghe An	c	+	-
7	H2540	CSF	2025	Nghe An	c	+	-
8	H360	Saliva	2024	Hoa Binh	d	+	-
9	H371	Saliva	2024	Hoa Binh	d	+	-
10	H204	Saliva	2020	Ha Noi	e	+	-
11	H214	Saliva	2020	Bac Kan	f	+	-
12	H290	Saliva	2020	Bac Kan	f	+	-
13	H231	Saliva	2021	Yen Bai	g	+	-
14	H241	Mouse brain inoculated with patient saliva	2021	Lang Son	h	+	+
15	H247	Mouse brain inoculated with patient saliva	2021	Quang Ninh	i	+	+
16	H337	Saliva	2023	Quang Ninh	i	+	-
17	H249	Saliva	2021	Dien Bien	j	+	-
18	H368	Saliva	2024	Dien Bien	j	+	-
19	H2504	CSF	2025	Ha Tinh	k	+	-
20	H248	Saliva	2021	Lao Cai	l	+	-
21	H298	Saliva	2023	Lao Cai	l	+	-
22	H2570	CSF	2024	Lao Cai	l	+	-
23	H261	Saliva	2022	Thai Nguyen	m	+	-
24	H334	Mouse brain inoculated with patient saliva	2023	Thai Nguyen	m	+	+
25	H270	Saliva	2022	Tuyen Quang	n	+	-
26	H291	Saliva	2022	Tuyen Quang	n	+	-
27	H327	Saliva	2023	Tuyen Quang	n	+	-
28	H272	CSF	2022	Phu Tho	o	+	-
29	H295	Saliva	2022	Phu Tho	o	+	-
30	H277	Saliva	2022	Bac Giang	p	+	-
31	H292	CSF	2022	Quang Binh	q	+	-
32	H339	Saliva	2023	Lai Chau	r	+	-

Table 3. Details of rabies virus strains detected in human patients. CSF; cerebrospinal fluid, RT-PCR; reverse transcription-PCR, NGS; next-generation sequencing.

(vii) were distributed across various locations without bias (Supplemental Fig. 3B) and were only isolated from Vietnam. As these strains have taken root in Vietnam, it is crucial to prevent their cross-border transmission.

The D182 strain closely related to strains isolated in Guangxi were sporadically detected in the border province of Lang Son (Fig. 2A). A previous study reported that a strain isolated from a human in Lang Son was closely related to strains found in China, suggesting the possibility of the cross-border transmission[34]. The border between Guangxi and Lang Son is mountainous, with no rivers physically blocking movement; therefore, these strains may spread further within Vietnam.

The H370 strain isolated from a human patient in Nghe An province in 2024, while the other reference strains were isolated mainly from dogs and one bovine in Tay Ninh, Ho Chi Minh, Quang Nam, Kien Giang, Ca Mau, Ca Tho, and Quang Nam in 2001 to 2019 (Fig. 1C). These strains differ in host, collection year, and province; however, all of these provinces are found near the borders between Vietnam, Laos, and Cambodia. Laos and Cambodia, which are geographically close to Vietnam, have been linked to the SEA3 subclade strains. This suggests that the strains isolated from Vietnamese provinces bordering Laos and Cambodia circulated along the border and may have the potential to spread throughout Vietnam. It is essential to implement preventive measures against their spread, such as physical barriers at border crossings and canine vaccinations.

A comparison of the full-genome sequences isolated in this study shows that they were closely related, but the province from which they were isolated affected their classification into different subclades (Fig. 3). Strains D037 and D213 have identical sequences and were isolated from the same district from locations only 5–6 km apart, suggesting that they are circulating among the dog population in this closely connected area. Relying solely on the N gene limit resolution, as RABV strains are typically grouped within the same clade, which makes it difficult to detect provincial-level variation or finer genetic divergence. At present, the number of available full-genome sequences in Vietnam remains limited, restricting comprehensive comparisons across provinces or host species. Expanding full-genome sequencing in future surveillance efforts will enable the identification of

No.	Code	Kind of sample	Year of collection	Province	Area of map	RT-PCR (606 bp)	NGS (11,875 bp)
1	D159	Brain tissue	2011	Sơn La	a	+	+
2	D455	Brain tissue	2024	Son La	a	+	-
3	D360	Brain tissue	2024	Nghe An	c	+	-
4	D361	Brain tissue	2024	Nghe An	c	+	-
5	D049	Brain tissue	2024	Bac Kan	f	+	-
6	D076	Brain tissue	2024	Bac Kan	f	+	-
7	D445	Brain tissue	2024	Bac Kan	f	+	+
8	D171	Brain tissue	2012	Yen Bai	g	+	+
9	D182	Brain tissue	2013	Lang Son	h	+	+
10	D023	Brain tissue	2020	Lang Son	h	+	+
11	D027	Brain tissue	2020	Lang Son	h	+	+
12	D033	Brain tissue	2020	Lang Son	h	+	+
13	D037	Brain tissue	2020	Lang Son	h	+	+
14	D040	Brain tissue	2020	Lang Son	h	+	+
15	D041	Brain tissue	2020	Lang Son	h	+	+
16	D213	Brain tissue	2020	Lang Son	h	+	+
17	D214	Brain tissue	2020	Lang Son	h	+	+
18	D130	Brain tissue	2024	Lang Son	h	+	-
19	D056	Brain tissue	2024	Quang Ninh	i	+	-
20	D061	Brain tissue	2024	Quang Ninh	i	+	-
21	D109	Brain tissue	2024	Quang Ninh	i	+	-
22	D126	Brain tissue	2024	Quang Ninh	i	+	-
23	D134	Brain tissue	2024	Quang Ninh	i	+	-
24	D085	Brain tissue	2024	Dien Bien	j	+	-
25	D089	Brain tissue	2024	Dien Bien	j	+	-
26	D098	Brain tissue	2024	Dien Bien	j	+	-
27	D123	Brain tissue	2024	Dien Bien	j	+	-
28	D401	Brain tissue	2024	Lao Cai	l	+	-
29	D451	Brain tissue	2024	Lao Cai	l	+	-
30	D453	Brain tissue	2024	Lao Cai	l	+	-
31	D055	Brain tissue	2024	Phu Tho	o	+	-
32	D080	Brain tissue	2024	Phu Tho	o	+	-
33	D116	Brain tissue	2024	Phu Tho	o	+	-
34	D187	Brain tissue	2024	Phu Tho	o	+	+
35	D190	Brain tissue	2024	Phu Tho	o	+	-
36	D201	Brain tissue	2024	Phu Tho	o	+	-
37	D207	Brain tissue	2024	Phu Tho	o	+	-
38	D211	Brain tissue	2024	Phu Tho	o	+	-
39	D078	Brain tissue	2024	Lai Chau	r	+	-
40	D181	Brain tissue	2024	Lai Chau	r	+	-
41	D121	Brain tissue	2024	Ha Giang	s	+	-
42	D117	Brain tissue	2024	Cao Bang	t	+	-
43	D194	Brain tissue	2024	Vinh Phuc	u	+	-
44	D096	Brain tissue	2024	Quang Tri	v	+	-

Table 4. Details of rabies virus strains detected in dogs.

additional genetic traits, improve resolution of geographic sub-structures, and strengthen the overall capacity for epidemiological tracking and cross-border comparison.

There have been numerous reports of infectious pathogens spreading across borders[19,22,35]. Closely related RABV strains have also spread across the border between Tanzania and Kenya[26]. Additionally, genetic surveillance using full-genome sequencing has previously reported differences in the RABV sequences across states in the USA, as well as cross-state transmission among wild animals[36]. These reports indicate an area-dependent sequence of infectious diseases that have crossed state and provincial borders to spread across countries, contributing to the global expansion of rabies.

This study only reported cases in which RABV was detected by reverse transcription-PCR (RT-PCR); those based on clinical diagnosis were excluded, meaning the actual number of rabies cases in Vietnam may be higher than reported. For some cases (H248, H261, and H295), the initial saliva sample collected on the first hospital visit was negative, but retesting a few days later confirmed RABV, indicating that a single-time-point collection may miss some diagnoses. Because of this, it is advisable to collect additional samples a few days after the first collection in suspected cases. Additionally, since we only collected samples from Vietnam, whether strains isolated in Vietnam have already spread to or circulated among neighboring countries remains unknown; to address this, expanded surveillance efforts including neighboring countries will be necessary. Overall, RABV strains isolated from human patients and dog samples were closely related and exhibited province-dependent patterns. These findings highlight the need for integrated control measures targeting both animals and humans, as part of a One Health approach, to reduce rabies-related death.

Conclusion

Rabies not only poses significant public health risks but also causes economic losses, including costs of prevention (such as PEP inoculation and immunoglobulin), labor for treatment and diagnosis, and damage to livestock. These problems can be addressed through pre-exposure vaccination, canine vaccination campaigns, and measures to prevent the virus from spreading between Vietnam and neighboring countries. In this study, RABVs in Vietnam were genetically classified into four distinct groups, with some crossing borders and spreading within the country. There is a risk that strains unique to Vietnam could spread to neighboring countries. Regional and multinational cooperation, together with proactive control strategies that integrate both human and animal health perspectives, are essential to reduce RABV spread and to mitigate its public health and economic impacts.

Materials and methods

Ethical consideration

Human rabies surveillance at the NIHE in Vietnam is conducted under the Prime Minister's directives in the National Program on Rabies Control and Elimination (NPRCE), as outlined in Decision 193/QD-TTg and 2151/QD-TTg. The murine experimental protocol was approved by the Ethic Committee of the NIHE (Approval number: 278/QD-VSDTTU). All methods were carried out in accordance with relevant guidelines and regulations. Every effort was made to minimize the suffering of laboratory animals. During this portion of the study, mice were housed in the animal facility of NIHE under appropriate conditions. All methods are reported in accordance with the ARRIVE guidelines.

Rabies virus strains and clinical samples from dogs and humans were obtained through the National Rabies Surveillance Program, stored at NIHE, and approved for use under official surveillance decisions issued annually from 2018 to 2024 (Decision numbers: 305/QD-VSDTTU, 685/QD-VSDTTU, 561/QD-VSDTTU, 559/QD-VSDTTU, 842/QD-VSDTTU, 529/QD-VSDTTU, and 421/QD-VSDTTU). All procedures were performed in accordance with relevant guidelines and regulations. All reasonable efforts were made to minimize the suffering of rabid dogs, from which samples were collected post-mortem as part of routine surveillance.

Informed consent was obtained from all individual participants included in this study, including consent for the collection and use of saliva and CSF samples.

Sample collection

Patients with suspected rabies symptoms (such as hydrophobia, hypersalivation, seizures, and paralysis) were transferred to provincial or national hospitals. The Centers for Disease Control collaborated with hospitals to collect and transport specimens to designated laboratories. Saliva and CSF samples were obtained under physician supervision with the patient's family's consent.Human samples were submitted to NIHE for analysis.

Brain samples from dogs with suspected rabies symptoms, such as aggression, unprovoked biting, frequent drooling, or illness, were collected by the local Sub-Department of Animal Health and sent to the NIHE or NCVD. Dog samples associated with human rabies cases were also sent to NIHE for testing. The program for collecting human and animal specimens was part of the rabies surveillance activities and is approved by the NPRCE. The use of the samples for research was authorized by the sample management authority, NIHE and NCVD. Epidemiological data on humans who received PEP after dog bites and reported cases were aggregated through the NPRCE. The population of each province in 2024[37] was used as a reference in Supplemental Table 1.

RNA extraction

RABV RNA was extracted from the saliva or CSF of rabid patients using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and from the brain tissue of suspected rabid dogs using the QIAzol Lysis Reagent (QIAGEN) following the manufacturer's protocol. RNA was dried using the RNAstable Tube Kit (Biomatrica, La Jolla, CA, USA) and dissolved in 50 µL DEPC-treated water (Nippon gene, Tokyo, Japan).

RT-PCR

RT-PCR was performed on viral RNA using the QIAGEN OneStep RT-PCR Kit (QIAGEN) with previously described conditions and primers[38]. Electrophoresis was performed on a 1% agarose gel, followed by purification using the QIAquick PCR Purification Kit (QIAGEN) for DNA sequence analysis.

Viral isolation using suckling mice

Saliva samples were diluted with Minimum Essential Medium supplemented with 5% fetal bovine serum and antibiotics (penicillin at 500 IU/mL and streptomycin at 1500 IU/mL). Samples (10–15 µL) were inoculated into 2–3-day-old Swiss suckling mice using a 0.5 mL insulin syringe. The mice were observed for 21 days; those showing clinical signs (including tremor, hind-limb paralysis or quadriplegia) were euthanized using carbon dioxide for samples collection. Brain samples were tested using either fluorescent antibodies or RT-PCR to detect

RABV. The direct fluorescent antibody test was performed according to a previously described protocol[39] using FITC-conjugated Anti-Rabies Monoclonal Globulin (FUJIREBIO, Tokyo, Japan).

Full-genome sequencing

The NEBNext Ultra Ⅱ RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) along with NEBNext Multiplex Oligo for Illumina (New England Biolabs), were used to prepare the NGS library. Ribosomal RNA and globin were removed through fragmentation and priming with 4 µL RNA, 4 µL Next First Strand Synthesis Reaction Buffer, 1 µL random primer, 1 µL QIAseq FastSelect-rRNA HMR (QIAGEN), and 1 µL QIAseq FastSelect-Globin (QIAGEN). The mixture was incubated under the following conditions: 94 °C for 7 min, 75 °C for 2 min, 70 °C for 2 min, 65 °C for 2 min, 60 °C for 2 min, 55 °C for 2 min, 37 °C for 2 min, and 25 °C for 2 min. After fragmentation and priming, cDNA libraries were constructed following the manufacturer's instructions. Library purification was performed using AMPure XP beads (Beckman Coulter, Brea, CA, USA). All starting DNA samples were quantified using a Qubit 2.0 Fluorometer (Thermo Fisher, Waltham, MA, USA). Sequencing was performed using either the iSeq 100 (Illumina, San Diego, CA, USA) or MiSeq (Illumina), and sequence assembly and analysis were performed using CLC Genomics Workbench 23.0.2 (QIAGEN) and GENETYX Ver.15 (GENETYX, Tokyo, Japan).

The complete genomic sequences of the 17 strains were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC867577 to LC867593.

Phylogenetic analysis

RABV N protein and full-genome sequences were obtained from NCBI GenBank and used as reference sequences. The full-genome sequences of the reference strains and those isolated in this study were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) tools. Phylogenetic analysis was performed with MEGA 10 (Molecular Evolutionary Genetics Analysis) software (Pennsylvania State University, State College, PA, USA). For N protein sequences, both reference strains and isolates from this study were aligned using AliView (Systematic Biology, Uppsala University, Uppsala, Sweden). A maximum likelihood phylogenetic analysis was then carried out using IQ-TREE 2.3.6[40], and a phylogenetic tree was generated using FigTree v1.4.4[41]. The number at each branch indicates the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) value (%)/ultrafast bootstrap (UFBoot) value (%).

Data availability

Sequence data that support the findings of this study have been deposited in the DNA Data Bank of Japan with Accession Numbers LC867577 to LC867593 (LC867577, LC867578, LC867579, LC867580, LC867581, LC867582, LC867583, LC867584, LC867585, LC867586, LC867587, LC867588, LC867589, LC867590, LC867591, LC867592, and LC867593). All data generated or analyzed during this study are included in this published article and its Supplementary Information.

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Conceptualization, Michiko Harada; resources, Thu Tuyet Nguyen, Dong Vinh Nguyen, Giang Chau Ngo, Huong TT. Nguyen, Phuong TM. Nguyen, and Tho Dang Nguyen; methodology, Michiko Harada, Thu Tuyet Nguyen, Dong Vinh Nguyen, Giang Chau Ngo, and Keita Ishijima; investigation, Thu Tuyet Nguyen; data analysis, Michiko Harada and Keita Ishijima; writing—original draft preparation, Michiko Harada; writing—review and editing, Michiko Harada, Thu Tuyet Nguyen, Akiko Okutani, Satoshi Inoue, and Ken Maeda; supervision, Ken Maeda; project administration, Akiko Okutani; funding acquisition, Akiko Okutani and Ken Maeda. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Consent to participate

Informed consent was obtained from all individual participants included in the study, including for the providing of saliva and CSF samples used in this study.

Additional information

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