Jpn. J. Infect. Dis., 64, 391-396, 2011

Original Article

Molecular Epidemiology of Rabies Virus in Vietnam (2006–2009)

Anh K. T. Nguyen*, Dong V. Nguyen, Giang C. Ngo, Thu T. Nguyen, Satoshi Inoue[1], Akio Yamada[1], Xuyen K. Dinh, Dung V. Nguyen[2], Thao X. Phan[2], Bao Q. Pham[3], Hien T. Nguyen, and Hanh T. H. Nguyen

Department of Virology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam;
[1]Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo 162-8640, Japan;
[2]The Sub-Department of Animal Health of Ho Chi Minh City; and
[3]Center for Preventive Medicine of Gia Lai Province, Vietnam

(Received March 16, 2011. Accepted July 7, 2011)

SUMMARY: This study was aimed at determining the molecular epidemiology of rabies virus (RABV) circulating in Vietnam. Intra vitam samples (saliva and cerebrospinal fluid) were collected from 31 patients who were believed to have rabies and were admitted to hospitals in northern provinces of Vietnam. Brain samples were collected from 176 sick or furious rabid dogs from all over the country. The human and canine samples were subjected to reverse transcription-polymerase chain reaction analysis. The findings showed that 23 patients tested positive for RABV. Interestingly, 5 rabies patients did not have any history of dog or cat bites, but they had an experience of butchering dogs or cats, or consuming their meat. RABV was also detected in 2 of the 100 sick dogs from slaughterhouses. Molecular epidemiological analysis of 27 RABV strains showed that these viruses could be classified into two groups. The RABVs classified into Group 1 were distributed throughout Vietnam and had sequence similarity with the strains from China, Thailand, Malaysia, and the Philippines. However, the RABVs classified into Group 2 were only found in the northern provinces of Vietnam and showed high sequence similarity with the strain from southern China. This finding suggested the recent influx of Group 2 RABVs between Vietnam and China across the border. Although the incidence of rabies due to circulating RABVs in slaughterhouses is less common than that due to dog bite, the national program for rabies control and prevention in Vietnam should include monitoring of the health of dogs meant for human consumption and vaccination for workers at dog slaughterhouses. Further, monitoring of and research on the circulating RABVs in dog markets may help to determine the cause of rabies and control the spread of rabies in slaughterhouses in Vietnam.

INTRODUCTION

Rabies is a fatal zoonotic disease caused by rabies virus (RABV), which belongs to the genus *Lyssavirus* of family *Rhabdoviridae*. Rabies exists in more than 150 countries and territories worldwide. A recent estimate indicates that more than 55,000 people die of rabies every year. However, the actual incidence of human rabies may be 100 times higher than the officially reported numbers, and most of the fatalities due to rabies occur in African and Asian countries (6). In recent years, the number of cases of human rabies in Vietnam, the Philippines, Laos, Indonesia, and China has been rapidly increasing (2,4,8,10,11,13,15). In Vietnam, 362 cases of human rabies have been reported from 2007 to 2010, and the rabies epidemic has occurred in 25–27 provinces (4). The main reservoirs and transmitters of rabies in Vietnam are dogs rather than wild animals such as foxes, bats, and raccoons. Furthermore, most dogs are unvaccinated, and domestic transport and import/export of animals, including dogs and cats, are not well regulated (8). Therefore, determining the genotype of the circulating RABV is very important to understand its evolutionary relationship with the local as well as regional strains and to elucidate the dynamics of the widespread transmission of this disease. The objective of this study was to determine the molecular epidemiology of the RABV circulating in Vietnam.

*Corresponding author: Mailing address: Rabies Laboratory, Virology Department, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Tel: +84.4.39724819, Fax: +84.4.39717526, E-mail: nkanhhp@yahoo.com

MATERIALS AND METHODS

Study samples: We collected brain samples from 176 dogs, of which 100 were sick dogs found at slaughterhouses in the northern provinces. These 100 suspected rabies-infected dogs showed at least one of the following signs: refusal to eat or discontinuation of eating, excessive salivation, aggressiveness, and paralyses. The other 76, which were furious rabid dogs, were from the central, highland, and southern provinces in Vietnam. The diagnosis of rabies in those dogs was performed either using fluorescent antibody test (FAT) with the polyclonal antibody raised against the nucleoprotein (N) of RABV (Sanofi Diagnostics, Pasteur, France) or using reverse transcription-polymerase chain reaction (RT-PCR) with a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Intra vitam samples of saliva (SLV) and cerebrospinal fluid (CSF) were collected from 31 suspected rabies-infected humans who were admitted to national hospitals located in the northern provinces of Vietnam. RT-PCR analysis confirmed that these sam-

ples contained RABV.

Viral RNA extraction and RT-PCR analysis: Total RNA was extracted from 0.3–0.5 g of homogenized brain samples using the RNeasy Mini Kit (Qiagen) and from 0.5 ml of SLV and CSF using the QIAamp Viral RNA Mini Kit (Qiagen). The part of the N gene was then amplified using the QIAGEN OneStep RT-PCR Kit, using a sense primer N7 (nt. 15–34; 5′-ATG TAA CAC CTC TAC AAT GG-3′) and an anti-sense primer JW6E (nt. 601–619; 5′-CAG TTG GCA CAC ATC TTG TG-3′) in a 50-μl reaction mixture containing 400 μM of each dNTP, 10 μl of 5× buffer, 30 μM of each primer, 2 μl enzyme mix, 20 μl distilled water, and 10 μl of the template RNA. The reaction mixture was then subjected to reverse transcription at 50°C for 30 min, followed by heating at 95°C for 15 min, and then 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 1.5 min. The mixture was further incubated at 72°C for 15 min to complete elongation. The amplicon was then subjected to electrophoresis in 2% agarose gel and stained with ethidium bromide.

Nucleotide sequencing: The RT-PCR product was excised from the gel and purified using the QIA quick Gel Extraction Kit (Qiagen). Cycle sequencing was performed using N7 and JW6E primers with the ABI 3100 Genetic Analyzer. The nucleotide sequences of the N gene of RABV strains reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases (accession numbers AB614372–AB614393 and AB628210–AB628214).

Phylogenetic analysis: The partial nucleotide sequence of N gene (nucleotide position, 85–473) was determined and compared with the representative sequences obtained from the GenBank (www.ncbi.nlm.nih.gov). The accession numbers (the name of strain, country) of the representative sequences are as follows: AB299032 (HCM1, Vietnam); AB299033 (HCM2, Vietnam); AB299034 (HCM5, Vietnam); AB299035 (HCM6, Vietnam); AB299036 (HCM7, Vietnam); AB299037 (HCM8, Vietnam); AB299038 (HCM9, Vietnam); AB299039 (HCM10, Vietnam); AB116579 (VN3, Vietnam); AB116580 (VN52, Vietnam); U22653 (8738THA, Thailand); U22916 (8677MAL, Malaysia); AB070759 (PHI123–01, Philippines); AB 070761 (PHI127–03, Philippines); AB116581 (PHI103, Philippines); AB116582 (PHI114, Philippines); AB070817 (Mdn183/45, Philippines); EF555102 (CQQJDN06, China); EF555106 (SDJNCN01, China); EF555112 (GDZQDN45, China); U22918 (94260NEP, Nepal); EF555098 (CQQJDN02, China); EF555099 (CQQJDN03, China); and EF555100 (CQQJDN04, China). Mokola virus, AY333111 (Eth-16, Ethiopia), was used as an out-group. Complete alignment of nucleotide sequences was performed using ClustalX, version 2.0 (9). MegAlign software version 7.0 (DNASTAR, Madison, Wis., USA) was used to analyze homologies of the nucleotide and deduced amino acid sequences. The neighbor-joining method in MEGA4 version 4.0 (12) was used for constructing the phylogenetic tree with 1,000 bootstrap replications. Epidemiology map was constructed using HealthMapper software, version 4.2, which was supported by the World Health Organization.

RESULTS

Prevalence of rabies in humans and animals: From 2007 to 2009, 31 residents of the northern provinces of Vietnam who were suspected of having rabies were admitted to the Bach Mai Hospital or the Institute for Tropical and Infectious Diseases. We used the CSF and/or SLV samples of these patients for the laboratory diagnosis of rabies. Direct RT-PCR analysis of the patients' SLV and/or CSF samples confirmed that 23 of the 31 (74%) rabies suspected patients were infected with RABV (Table 1). Out of these 23 patients, 12 (52%) had been bitten by dogs or cats and 5 (22%) were involved in the butchering of sick cats or dogs, whereas 6 (26%) did not have any history of dog/cat bites or the butchering of sick animals. In this study, brain samples were collected from 100 sick dogs from slaughterhouses in the northern provinces. The findings of FAT and RT-PCR analysis confirmed that, out of those 100 dogs, 2 (2.0%) were infected with RABV. We also found that 15 (16.4%) of the 76 dogs from the southern and highland provinces were infected with RABV (Table 1).

Phylogenetic analysis: Phylogenetic analysis was performed on 27 strains of RABVs of canine and human origin (Table 2). In the phylogenetic tree, the RABVs

Table 1. Diagnostic results of rabies suspected cases of human and animals (2006–2009)

Location		Human	Animal
Region	Province	Positive/total (%)	Positive/total (%)
North	Ha Tay	10/12 (83.3)	2/72 (2.8)
	Hanoi	—	0/4
	Phu Tho	4/4	—
	Bac Ninh	1/1	—
	Hoa Binh	3/3	0/10
	Yen Bai	1/1	—
	Son La	1/1	0/1
	Nghe An	1/1	—
	Lang Son	1/1	0/6
	Tuyen Quang	1/1	—
	Vinh Phuc	0/1	0/1
	Thai Binh	0/1	—
	Unknown	0/1	—
	Ninh Binh	—	0/6
Subtotal	14 provinces	23/28 (82.1)	2/100 (2)
South and Highland	Gia Lai	0/3	3/3
	Lam Dong	—	1/1
	Ho Chi Minh	—	5/53
	Long An	—	1/4
	Soc Trang	—	2/3
	An Giang	—	1/1
	Dong Nai	—	1/4
	Tay Ninh	—	1/1
	Binh Duong	—	0/3
	Tra Vinh	—	0/1
	Tien Giang	—	0/1
	My Tho	—	0/1
Subtotal	12 provinces	0/3 (0)	15/76 (16.4)
Total		23/31 (74.2)	17/176 (9.7)

Table 2. Rabies virus strains used for the phylogenetic analysis isolated from rabies human and dogs, 2006–2009

Case	Location	History	Strain	Year of isolation	Genbank accession no.
Human	Ha Tay	Dog bite	H010607	2007	AB614379
			H040707	2007	AB614380
			H111007	2007	AB614385
			H240808	2008	AB614392
		Dog butchering	H020607	2007	AB628214
			H200608	2008	AB614390
			H280509	2009	AB614393
		Unknown	H140208	2008	AB614387
	Hoa Binh	Dog bite	H230808	2008	—
		Unknown	H170408	2008	AB614389
	Phu Tho	Dog bite	H060907	2007	AB628212
			H071007	2007	AB614382
			H210608	2008	AB614391
		Dog butchering	H091007	2007	AB614384
	Yen Bai	Dog butchering	H050707	2007	AB614381
	Tuyen Quang	Unknown	H150308	2008	AB614388
	Lang Son	Unknown	H130108	2008	AB614386
	Son La	Unknown	H080807	2007	AB614383
Dog	Gia Lai	Aggressive and human attacked	D156	2009	AB628211
			D157	2009	AB614377
			D158	2009	AB614378
	Ho Chi Minh	Aggressive and human attacked	D153	2007	AB614376
			D155	2007	—
	Lam Dong	Aggressive and human attacked	D154	2007	AB628213
	Soc Trang	Aggressive and human attacked	D150	2006	AB614373
	An Giang	Aggressive and human attacked	D151	2006	AB614374
	Long An	Aggressive and human attacked	D152	2007	AB614375
	Ha Tay	Collected from slaughterhouses	D010807	2007	AB614372
			D060807	2007	AB628210

circulating in Vietnam were classified into two major groups: Group 1 consisting of RABVs distributed in the northern, southern, and highland provinces and Group 2 consisting of the strains isolated in the northern provinces (Fig. 1).

Group 1 consisted of RABVs from Thailand, Malaysia, the Philippines, and China, as well as the 11 viruses isolated from humans in the northern provinces and all RABVs from dogs in the highland and southern provinces of Vietnam. Group 1 was further divided into 2 subgroups as shown in Fig. 1. Subgroup 1A consisted of RABVs from Thailand and Malaysia, and all RABVs isolated from dogs located in the southern provinces of Vietnam, and the strains of Ho Chi Minh City, as reported by Yamagata et al. (14). All highland virus strains clustered together (virus strains from D1560609VN to D1580609VN). Subgroup 1B consisted of RABVs from the Philippines and China as well as the 11 RABVs of human origin that were isolated from residents of the northern provinces. Interestingly, the RABV strain H130108VN was clustered with the strain SDJNCN01 from a rabid cow from China. The virus strain H130108VN isolated from a human in Lang Son province located at the Vietnam-China border.

Group 2 consisted of the strains of both human and canine origin isolated in the northern provinces of Vietnam as well as the strains from southern China.

Geographical distribution: The distribution of RABV strains circulating in Vietnam is shown in Fig. 2. The RABV strains in Group 1 were found in 11 of northern, southern, and highland provinces/cities. In contrast, the RABV strains in Group 2 were isolated exclusively in the northern provinces of Vietnam. The circulating of RABV strains belonging to both Group 1 and Group 2 were found in Ha Tay and Phu Tho provinces that experienced a major rabies epidemic (Fig. 2).

DISCUSSION

In Vietnam, the Ministry of Health has devised a system for human rabies surveillance. Thus far, this system has reported the annual data on the number of human deaths due to rabies on the basis of clinical diagnosis alone and not laboratory confirmation. To the best of our knowledge, this is the first report of human rabies confirmed by laboratory diagnosis.

According to the annual reports of the human rabies surveillance program in Vietnam in recent years, human rabies cases have mainly occurred in the northern and highland provinces, particularly in Ha Tay and Phu Tho

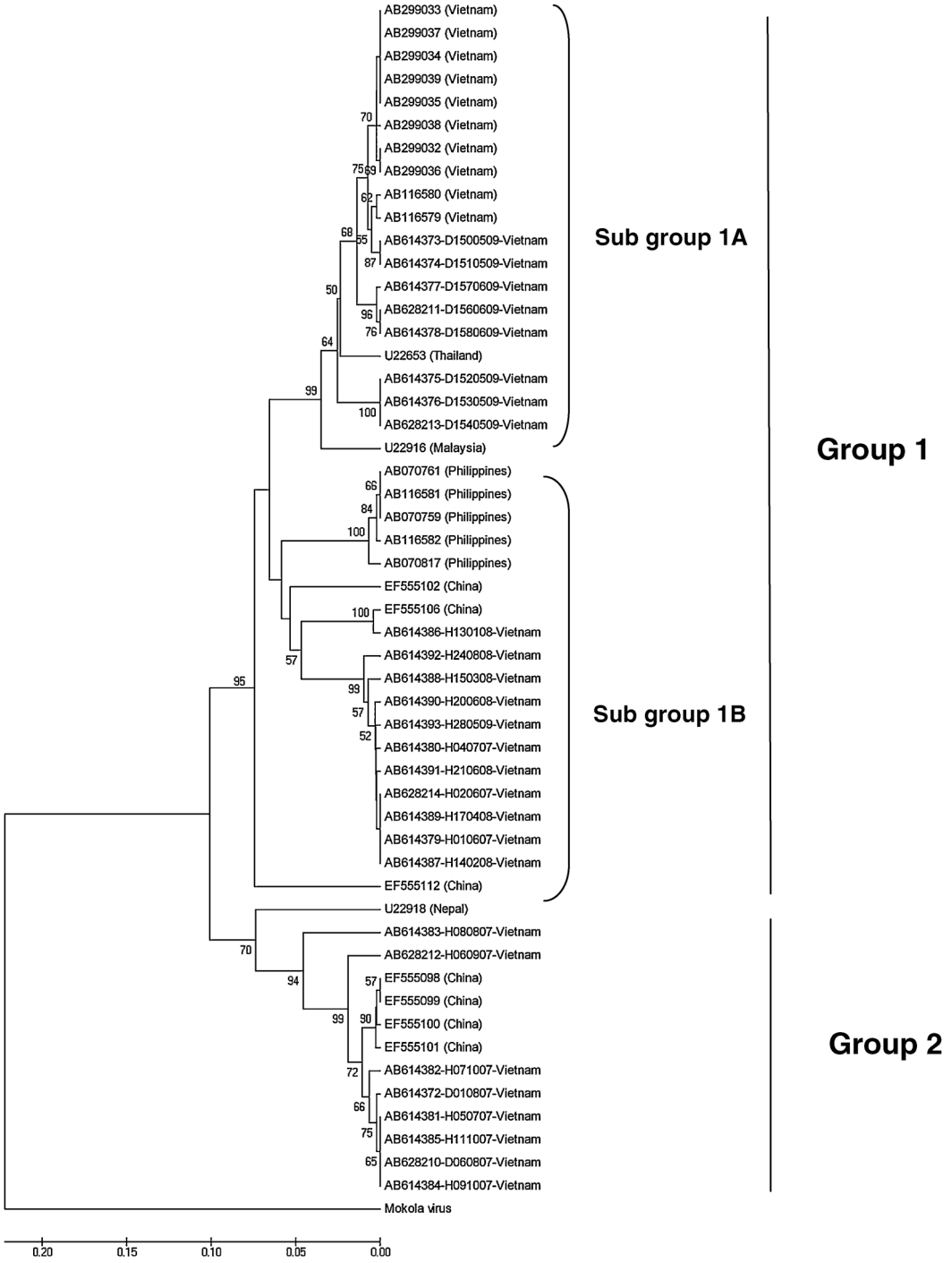

Fig. 1. Neighbor-joining phylogenetic tree based on partial N gene sequences (388 bp) of 27 RABV strains from humans and animals in Vietnam, 2006–2009. Partial nucleotide sequence of N gene (position from 85 to 473) was determined and compared with the representative sequences achieved in the GenBank (www.ncbi.nlm.nih.gov) described as the accession numbers (country). Mokola virus, AY333111 (Eth-16, Ethiopia), was used for an outgroup. Bootstrap values expressed as percentage of 1,000 replicates are shown at tree nodes.

provinces (4). From 2007 to 2009, RABV infection was confirmed in 23 of the 31 rabies suspected patients from 13 provinces. Out of these 23 patients with rabies, 10 were reported in Ha Tay province and 4 were reported in the Phu Tho province. The findings showed that 5 of the 23 rabies patients did not have any history of dog or cat bites, but they had an experience of butchering dogs or cats, or consuming their meat. RABV was detected in 2 of 72 (2.8%) dogs from the slaughterhouses in northern provinces.

Vietnamese communities commonly raise stray dogs, but the dogs are not vaccinated; vaccination coverage is only 10–20% (8). Furthermore, domestic and international transportation of animals is not well regulated in Vietnam. This lack of control has led to the spread of rabies from one region to others, and consequently, to the increase in the number of cases of human rabies. Therefore, strict measures for the control of rabies must

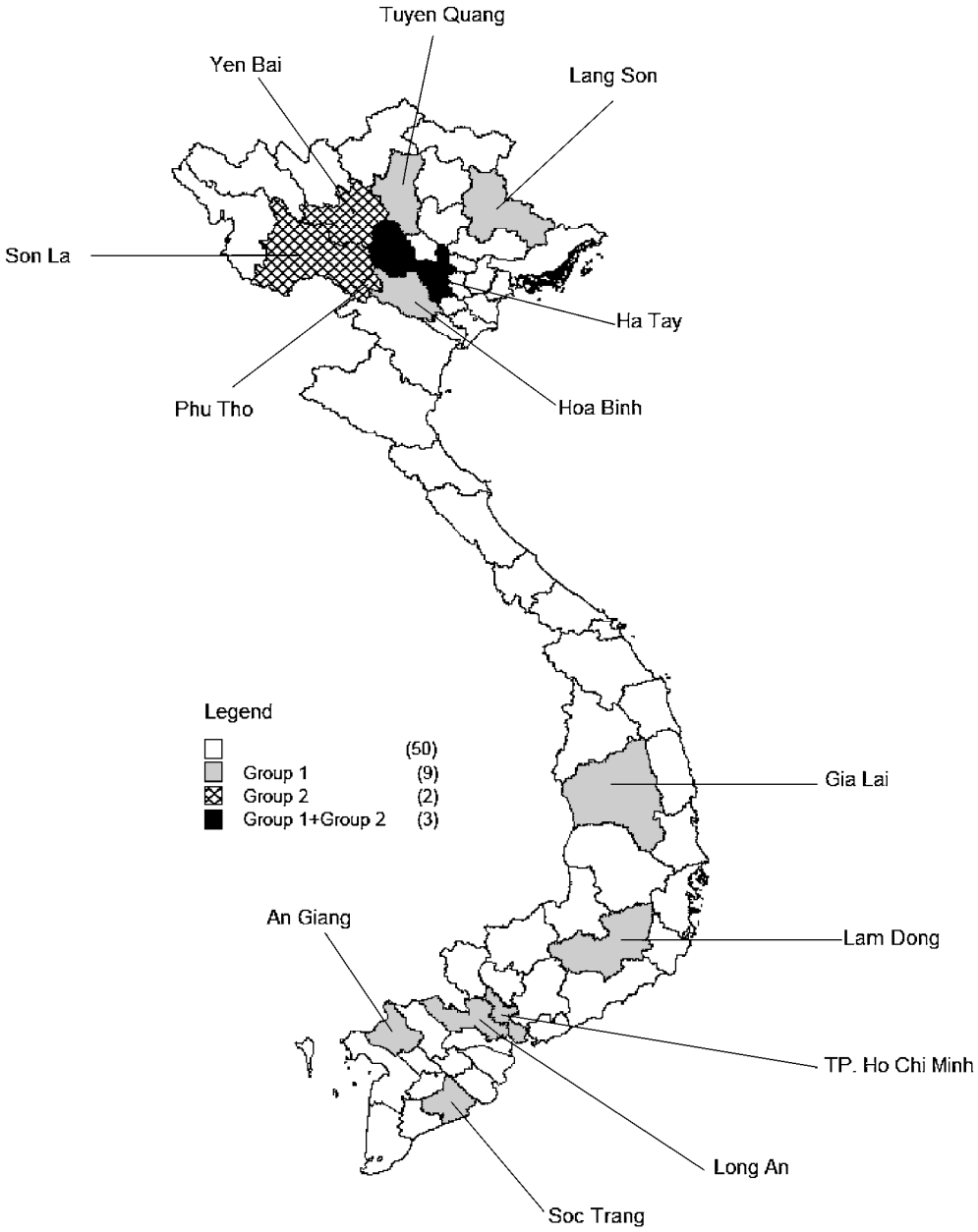

Fig. 2. Topography of RABV strains isolated in Vietnam, 2006–2009. Topographical chart was constructed by HealthMapper software, version 4.2 which is supported by World Health Organization.

be implemented throughout the country. RABV was detected in 2 of the 100 sick dogs (2%) from slaughterhouses located in the northern provinces of Vietnam (Table 1). Previous studies suggest that since some of the infected patients were never bitten by dogs or cats, they must have been infected while butchering the sick dogs or cats (1,3,5,7,15).

The findings of the molecular study showed that at least two different genetic groups of RABV are circulating in Vietnam. Group 1 included strains isolated in Vietnam, and these strains were closely related to those isolated in China, Malaysia, Thailand, and the Philippines. Group 2 consisted of RABVs isolated from humans and dogs in the northern provinces of Vietnam, and these strains were genetically similar to those isolated in southern China (Fig. 1). This finding suggests that the recent increase in cases of infections with Group 2 RABV can be attributable to a constant influx of RABVs from China into Vietnam or vice versa. A previous molecular study conducted by Yamagata et al. (14) in Ho Chi Minh City, Vietnam found that the RABV strains from Vietnam belonged to the South East Asia 1 genotype and were similar to the isolates from Thailand and strain N11 from Guangxi province of China. Further, evidence on the presence of the distinct subgroups in Group 1 (Fig. 1) suggests that RABV may have independently entered Vietnam from several countries such as China, the Philippines, Thailand, and Malaysia and may have established and spread throughout Vietnam.

Strict border control should be implemented to mitigate further influx of RABV from neighboring countries. Although the incidence of rabies due to circulating RABVs in slaughterhouses is less common than

that due to dog bite, the national program for rabies control and prevention in Vietnam should include monitoring of the health of dogs meant for human consumption and vaccination for workers at dog slaughterhouses. Further, monitoring of and research on the circulating RABVs in dog markets may help to determine the cause of rabies and control the spread of rabies in slaughterhouses in Vietnam.

Acknowledgments We would like to thank Dr. Huong Thanh Nguyen (Epidemiology Department, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam) for her help on construction of rabies epidemiology map.

This study was financially supported by the Ministry of Health, Welfare and Labour, Japan and the Ministry of Health, Vietnam.

Conflict of interest None to declare.

REFERENCES

1. Aguilar, E. (2008): Rabies Feared in 30 Dog Meat Eaters. Philippines Daily Inquirer. January 13, 2008.
2. Bounlay Phommasack, et al. (2008): ''Country Report on Rabies Control and Prevention in LDR'', ASEAN + 3 Conference on Sharing Information on Rabies and Prevention. Halong, Vietnam, April 2008.
3. Wertheim, H.F.L., Nguyen, T.Q., Nguyen, K.A.T., et al. (2009): Furious rabies after an atypical exposure. Plos Med., 6(3), 1–5.
4. Nguyen, H.T. (2009): Rabies in Vietnam. 2nd International Rabies in Asia Conference (RIACON) Proceeding. Hanoi, Vietnam, September 2009.
5. Childs, J.E. (2002): Rabies. Routes of Rabies Virus Transmission to Humans. p. 125–126. Academic Press.
6. Knobel, D., L., Cleaveland, S., Coleman, P.G, et al. (2005): Re-evaluating the burden of rabies in Africa and Asia. Bull. World Health Organ., 83, 1–11.
7. Kureishi, A., Xu, L.Z., Wu, H., et al. (1992): Rabies in China: recommendations for control. Bull. World Health Organ., 70, 443–475.
8. Van, K.D. (2008): ''Animals Rabies Control and Prevention in Vietnam'', ASEAN + 3 Conference on Sharing Information on Rabies and Prevention. Halong, Vietnam, April 2008.
9. Larkin, M.A., Blackshields, G., Brown, N.P., et al. (2007): Clustal W and Clustal X version 2.0. Bioinformatics, 23, 2947–2948.
10. Deray, R.A. (2008): ''Human Rabies in the Philippines'', ASEAN + 3 Conference on Sharing Information on Rabies and Prevention. Halong, Vietnam, April 2008.
11. Heng, S. (2008): ''Rabies Situation in Cambodia'', ASEAN + 3 Conference on Sharing Information on Rabies and Prevention. Halong, Vietnam, April 2008.
12. Tamura, K., Dudley, J., Nei, M. et al. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24, 1596–1599.
13. Zhen, X. (2008): ''Situation, Surveillance and Control of Rabies in Mainland, China'', ASEAN + 3 Conference on Sharing Information on Rabies and Prevention. Halong, Vietnam, April 2008.
14. Yamagata, J., Ahmed, K., Khawplod, P., et al. (2007): Molecular of rabies in Vietnam. Microbiol. Immunol., 51, 833–840.
15. Wallerstein, C. (1999): Rabies cases increase in the Philippines. Br. Med. J., 318, 1306.