A simple method for detection of rabies viral sequences in archival brain specimens.

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Abstract

Archival, formalin-fixed, paraffin-embedded (FFPE) tissues of autopsy origin are important source for diagnosis and molecular analysis. However, nucleic acids are particularly vulnerable to degradation during tissue processing. Brain cutting process usually was performed after 1 week of brain storage in formalin followed by embedding of each particular neuro-anatomical specimen into paraffin. We described a simple method of deparafinnization, proteinase K digestion and RNA extraction using Boom technique in obtaining rabies RNA from non-buffered-FFPE brain tissues kept at 30 degree for 16 years. Reverse transcription polymerase chain reaction (RT-PCR) could identify rabies viral nucleocapsid (N) gene sequences of 150 bp in all patients, but not from every immunohistochemical positive specimens. Direct sequencing of 301 bp of N gene was achieved in 4 of 7 patients using 2 RT-PCR reactions with product sizes of 294 and 297 bp in sense and anti sense directions respectively. Result of sequencing of 1 431 bp of N gene from1 month old, 24 hour processed FFPE rabies infected brain tissue was in accord with that from fresh specimen analysis. We suggest that for further molecular analysis a piece of fresh brain tissue should be pre- collected prior to brain cutting process and stored in formalin no longer than 24 hours before embedding.

1. Introduction

A precise anatomical localization is required in order to correlate between clinical presentations and nature as well as degree of neuropathological changes. Therefore, brain cutting is usually performed 7 days after the whole brain has been fixed in formalin in order to maintain structural integrity which allows identification of individual structure possible. Each particular piece of tissue according to neuroanatomical region is subsequently embedded in paraffin. For molecular diagnosis and epidemiology, the major difficulty in using these tissues is the degradation of nucleic acids. Archival postmortem tissues in the past usually employ non-buffered formalin with storage duration of 1 week or longer. These induced extensive cross linking of tissue proteins and nucleic acid fragmentation. As a consequence, only very short sequences can be amplified (Lehmann and Kreipe, 2001). Nevertheless, these FFPE tissues remain an extraordinary source for molecular studies because the availability of large pathology archives of tissues related to clinical cases.

Rabies is still endemic in Thailand. There were 18 rabies deaths and more than 400,000 persons received rabies post exposure prophylaxis (PEP) in 2003 (Ministry of Public Health report, Thailand). Approximately 20-30% of animal brains submitted to rabies diagnostic laboratory were positive for rabies (Lumlertdacha et al., in press). Presence of rabies virus sequences in FFPE brain tissues ensures the accurate diagnosis of rabies (Warner et al., 1997). Characterization of rabies variant associated with particular vector also can be possible (Warner et al., 1999a; Nadin-Davis et al., 2003). We have tested extraction method using NucliSens isolation kit after the steps of deparaffinization and proteinase K digestion on at least 16 year old archival FFPE brain specimens from 7 confirmed rabies patients. Extracted nucleic acids were amplified and sequenced for rabies viral sequences. Accuracy of analyzed sequences of freshly prepared and 24 hour processed FFPE rabies infected brain tissues from rabies infected dog and human was also investigated.

2. Materials and methods

2.1. Samples

Archival autopsy materials were obtained from rabies patients admitted to Bamrasnaradura (Nonthaburi) and Chulalongkorn University (Bangkok) hospitals during 1987-1988. Autopsy was performed within 24 h of death. Brain tissue had been

fixed in non buffered-formalin for 7 days and embedded in paraffin and have been stored at room temperature (~30 °C) until examination. All samples were previously tested for the presence of rabies antigen by using avidin biotin immunohistochemical method (Tirawatnpong et al., 1989).

Brain stem tissues of rabies infected patient and dog (one of each; collected during 2000 and 2003 respectively) stored frozen at -80 degree were used as controls. These tissues were divided into 2 parts, one of which was kept frozen, whereas the other was fixed in non buffered-formalin for 24 h and embedded in formalin and stored at room temperature for 1 month. Both types of specimens were subjected to RNA extraction, amplification and analysis for rabies viral sequences.

2.2. RNA extraction

For frozen tissues, the RNA extraction was carried out using the NucliSens nucleic acid isolation kit (Biomerieux, Boxtel, The Netherlands) as previously described (Hemachudha et al., 2003).

For FFPE tissues, additional 2 pre-extraction steps were required.

Step1: Harvesting tissue and deparaffinization of embedded tissues.

5 pieces of scarped tissue were prepared by using sterile disposable scalpel to prevent cross contamination. They were carefully placed in sterile 1.5 ml microcentrifuge tube. Tissue scalping was deparaffinized 3 times by extraction with 1 ml of xylene for 15 min at room temperature and then centrifuged at 12000 g for 10 min. The resulting tissue pellet was subsequently extracted 3 times with 1 ml of absolute ethanol, an incubation time of 5 min each. The pellet was allowed to dry in laminar flow hood for 15 min. Dried pellet was ground using a sterile stick.

Step2: Proteinase K digestion.

The dried pellet was then processed for digestion step using proteinase K treatment; 100 ul of digestion buffer [10mMTris pH7.5,166.5 mM Nacl, 1.65mM $MgCl_2$, 0.65% NP40 containing 6 mg/ml of proteinase K (Roche Diagnostics GmbH, Basel, Switzerland)] was added to dried deparaffinized samples. Samples were incubated at 45°C for 6 h and 100°C for 7 min to inactivate proteinase K and left at room temperature.

Step3: RNA extraction.

Digested sample were extracted using NucliSens isolation kit as described above.

2.3 RT-PCR

The one step RT-PCR kit (Qiagen, GmbH) was used according to the manufacturer's instructions. Reverse transcription was performed at 50°C for 35 min

followed by HotStar Taq DNA polymerase activation at 95°C for 15 min. Thermal profiles were; 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. After 40 cycles, the final elongation step was 10 min at 72°C. 5-7.5 ul of purified RNA solution was used in the RT-PCR reaction for each set of primers. Amplified products were visualized by electrophoresis in a 2% agarose gel containing 0.5 ug/ml of ethidium bromide.

Six sets of primer pairs were designed to amplify 1431 bp of rabies nucleoprotein (N) gene (Table 1). The size of amplicon was no larger than 400 bp as shown. Another set of primer, P4, resulted in an amplified fragment of 150 bp (Table 1). This was used for screening purpose due to its higher sensitivity.

2.4. Sequencing

PCR products were separated by 2% agarose gel electrophoresis and purified using QIA quick gel extraction kit (Qiagen). Recovered PCR products were subjected to direct sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequences from control specimens, 24 h fixation were compared to those obtained from fresh tissue preparation.

2.5. Immunohistochemical (IHC) staining

Antigen was retrieved by pressure cooker with citrate buffer for 1 min. The sections were immunostained using DAKO EnVision System, HRP reagent (DAKO Corporation, CA, USA). Briefly, sections were incubated for 10 min with H_2O_2 to eliminate endogenous peroxidase, washed in phosphate-buffered saline (PBS), incubated for 20 min with horse serum to block nonspecific staining, and incubated for 60 min with anti-rabies nucleocapsid antibody prepared in rabbit (Biorad, France) at a dilution of 1:80 in 3% horse serum. After two 3-min rinses in PBS, sections were incubated in DAKO EnVision™ System, HRP reagent for 30 min, washed with PBS and incubated for 10 min with substrate of diaminobenzidine (50 mg/ml and 30%H_2O_2 in Tris-HCl buffer with 1 M imidazole). After rinsing with tap water, slides were counterstained with hematoxylin.

3. Results

3.1 Detection of rabies virus RNA and rabies antigen from FFPE tissues.

RT-PCR using P4 primer set yielded positive result in both 24 hr FFPE and fresh positive samples from 2 rabies infected human and dog.

Positive results were obtained by RT-PCR using P4 primer set in all 7 patients from archival FFPE specimens. However, not all samples were positive. Of 30 FFPE specimens (4 – 5 from various brain regions in each individual) from these patients, 20 were positive by RT-PCR using P4 primer set whereas 25 were IHC positive (Table 2). There was no correlation between RT-PCR and IHC results. Three IHC-negative specimens from parietal (H1), temporal and occipital (H6) regions were found positive by RT-PCR. On the contrary, 7 IHC-positive specimens were RT-PCR negative. Rabies virus antigen amount in each particular brain region remained comparable to that previously reported (Tirawatnpong et al., 1989).

3.2 Nucleotide sequencing

Nucleotide sequencing of 1432 bp of rabies N gene in 4 samples (2 fresh and another two 24 h processed FFPE samples) was achieved by using overlapping primers. The details of each primer set and amplicon sizes were shown in Table 1. Six PCR reactions were required for sequencing in 24 h FFPE samples . Sequences obtained from fresh and 24 h processed FFPE specimens were identical [GenBank accession numbers are AY219002 (HM88) and.....(D664/45)].

Direct sequencing of 301 bp nucleotide of N gene, position 1101-1401, from archival specimens were obtained from 2 RT-PCR reactions and succeeded in 4 cases. P1 and P9 or P10 primer sets (amplicon sizes of 294 297 and 180 respectively) in sense and anti sense directions were used for confirmation. This was re-confirmed by using another primer sets (data not shown).

4. Discussion

FFPE brain tissues are easier to obtain. They are not infectious and shipping is easy and simplified. Detection of viral RNA in formalin-fixed paraffin embedded brain tissues by RT-PCR has been widely used in the field of diagnostic pathology (Choi et al. 2003; Beaulieux et al. 2003; Bhudevi and Weinstock, 2003; Rekand et al. 2003) as well as in molecular epidemiologic study (Ding et al. 2003). In the case of rabies, diagnosis and characterization of virus strains can be done in these FFPE samples by IHC method using single polyclonal antibody or panel of monoclonal antibodies

(Tirawatnpong, et al. 1989; Warner, et al. 1997; Warner, et al. 1999a; Warner, et al. 1999b; Whitfield, et al. 2001). Nevertheless, a further detailed study of strain typing and molecular epidemiology requires nucleic acid detecting procedures such as in situ hybridization and direct sequencing of the amplified product of extracted RNA (Warner, et al. 1997; Warner, et al. 1999 b). Tissue fixation in buffered formalin in the dark for 24 hours before paraffin wax embedding is recommended to avoid nucleic acid fragmentation (Lehmann and Kreipe, 2001). Thus, average fragment length of DNA obtained in the case of biopsy tissue where fixation time is 24 hours, is 300-400 bases (Bonin et al., 2003) whereas this is much shorter in postmortem FFPE tissues with longer formalin fixation time, 7 days in the case of brain.

We have been able to demonstrate rabies viral RNA in archival materials from 7 rabies patients who died 16-18 years ago. Amplification was achieved by using P4 primer set which encompassed 150 bp RNA target. RT-PCR yielded positive result in 3 IHC negative specimens. Nevertheless, 7 IHC positive-FFPE brain samples were negative by using this screening primer set. This can be explained by the labile nature of RNA and the high ribonuclease content of brain tissues, and most importantly, degradation of RNA in non-buffered formalin with long fixation time (Choi et al., 2004). RNA amplification in FFPE tissues could be most consistently achieved by using primers with resultant amplicon size of less than 200 bp (Bresters et al., 1994; Guerrero et al., 1997; Goldsworthy et al., 1999; Godfrey et al., 2000). This is also true in our study even by using primers which resulted to only 150 bp target. Direct sequencing of 301 bp in PCR-positive samples with P4 primer set, however, was successfully obtained by using P1 (294 bp) and P9 (297) primers in sense and anti-sense directions.

A non-buffered formaldehyde solution used in this study oxidizes to formic acid and creates an acidic environment (Bonin et al., 2003), and cause cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methyl groups to the bases (Choi et al., 2004). Optimizing conditions and procedures by using proteinase K digestion resulted to degradation of proteins that were covalently cross-linked with each other and RNA as well as solubilizing tissue proteins and reversing monomehtyl nucleotide modification to RNA, thus allowing efficient RNA extraction (Jackson et al., 1990; Koopmans, et al. 1993; Specht et al., 2001; Weizsacker et al., 1991). We also used silica based instead of organic extraction phenol/chloroform (Bhudevi and Weinstock., 2003; Beaulieux et al., 2003; Ding et al., 2003). This method is based on binding of nucleic acids to activated silica (Boom et al., 1990). The extraction procedure using either the phenol/chloroform or NucliSens extraction allowed the same sensitivity using either P4 primer set as

screening or other primer sets in direct sequencing (data not shown). However the latter is applicable to all nucleic acids and broad range of sample types and volumes and can be completed within an hour.

Duration of formalin fixation is also crucial. Fixation of tissue in formalin for 1 week or longer prior to processing resulted in a more extensive crosslinking of tissue proteins and nucleic acid fragmentation than 24-48 h fixation. This jeopardizes the result of in situ hybridization and RT-PCR (Bhudevi and Weinstock, 2003; Lehmann and Kreipe, 2001; Warner et al., 1997, 1999b). Furthermore, the validity of sequence information obtained from chemically fixed tissue must be questioned in the absence of corroborating data from untreated tissue (Wong et al., 1998). They found that DNA alteration identified in chemically preserved tissues might be the result of chemical fixation. In formalin-fixed materials, up to one mutation artifact per 500 bases was reported (Williams et al., 1999). The artifacts are easily distinguished from the true mutations by starting the extraction step with higher number of target cells, thus adequate non-damaged templates dominate the amplification process and also by using direct sequencing, and confirmatory sequencing of independent PCR product (Williams et al., 1999). The PCR product sizes used for sequencing in our study in 24 h processed FFPE and archival specimens were approximately 300 bp. Direct sequencing with overlapping primers and 2 directional sequencing were used in order to ensure the corrected nucleotide sequence. Results of direct sequencing of 1 431 bp of rabies N gene was identical between freshly prepared and 24 h processed FFPE brain tissues despite the use of non-buffered formaldehyde solution. The nucleotide sequence obtained from at least 16 year old archival specimens showed some artifacts which were not found in 24 hr processed FFPE specimen, one month storage. Although it has been reported that RNA yield with the proteinase K method was not much affected by prolonged incubation in buffered formalin for 7 days and stored at 4 degree for 1 month, such prolonged fixation resulted in irreversible modification of RNA. (Masuda et al., 1999). In our case, fixation was done in non buffered formalin and storage time was much longer and at higher temperature (at 30 degree C) (Wong et al., 1998).

Limited sequence analysis of 200-300 bp of rabies N gene could characterize rabies virus into different genetic groups according to its transmitting vectors as well as geographical locations (Mcquiston et al., 2001; Smith et al.,1992; Lumlertdacha et al, in press). FFPE specimens, therefore, can be useful in such studies. Although not every FFPE sample was RT-PCR positive, cloning of amplified fragments may overcome this problem for sequencing purpose (Rekan et al, 2003).

In conclusion, type of formalin used, duration of fixation and storage in paraffin have to be considered in order to extract and amplify rabies viral RNA from FFPE brain tissues with good results. Routine brain cutting after 7 day is acceptable in terms of maintaining brain tissue integrity. However, a collection of small piece of brain, approximately 100 mg, followed by fixation in formalin of no longer than 24 h before paraffin wax embedding must be emphasized. Buffered formalin must be used in all cases. This is to further study in molecular biology and to avoid laborious work in obtaining good quality RNA and sequencing result.

References

Beaulieux, F., Berger, M.M., Tcheng, R., Giraud, P., Lina, B., 2003. RNA extraction and RT-PCR procedures adapted for the detection of enterovirus sequences from frozen and paraffin-embedded formalin-fixed spinal cord samples. J. Virol. Methods 107, 115-120.

Bhudevi, B., Weinstock, D., 2003. Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J. Virol. Methods 109, 25-30.

Bresters, D., Schipper, M.E., Reesink, H.W., Boeser-Nunnink, B.D., Cuypers, H.T., 1994. The duration of fixation influences the yield of HCV cDNA-PCR products from formalin-fixed, paraffin-embedded liver tissue. J. Virol. Methods 48, 267-272.

Boom, R., So,l C.J., Heijtink, R., Wertheim-van Dillen, P.M., van der Noordaa, J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495-503.

Bonin, S., Petrera, F., Niccolini, B., Stanta, G., 2003. PCR analysis in archival postmortem tissues. J. Clin. Pathol. Mol. Pathol. 56, 184-186.

Choi, C., Ha, S.K., Chae, C., 2004. Development of nested RT-PCR for the detection of swine hepatitis E virus in formalin-fixed, paraffin-embedded tissues and comparison with in situ hybridization. J. Virol. Methods 115, 67-71.

Ding, X., Park, Y., Taltavull, T., Thung, S.N., Jin, X., Jin, Y., Trung, N., Edamoto, Y., Sata, T., Abe, K., 2003. Geographic Characterization of Hepatitis Virus Infections, Genotyping of Hepatitis B Virus, and p53 Mutation in Hepatocellular Carcinoma Analyzed by In Situ Detection of Viral Genomes from Carcinoma Tissues:Comparison among Six Different Countries. Jpn. J. Infect. Dis. 56, 12-18.

Godfrey, T.E., Kim, S.H., Chavira, M., Ruff, D.W., Warren, R.S., Gray, J.W., Jensen, R.H., 2000. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription- polymerase chain reaction. J. Mol. Diagn. 2, 84-91.

Goldsworthy, S.M., Stockton, P.S., Trempus, C.S., Foley, J.F., Maronpot, R.R., 1999. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol. Carcinog. 25. 86-91.

Guerrero, R.B., Batts, K.P., Brandhagen, D.J., Germer, J.J., Perez, R.G., Persing, D.H., 1997. Effects of formalin fixation and prolonged block storage on detection of hepatitis C virus RNA in liver tissue. Diagn. Mol. Pathol. 6, 277-81.

Hemachudha, T., Wacharapluesadee, S., Lumlertdaecha, B., Orciari, L.A., Rupprecht, C.E., La-Ongpant, M., Juntrakul, S., Denduangboripant, J., 2003. Sequence analysis of rabies virus in humans exhibiting encephalitic or paralytic rabies. J. Infect. Dis. 188, 960-966.

Jackson, D.P., Lewis, F.A., Taylor, G.R., Boylston, A.W., Quirke, P., 1990. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J. Clin. Pathol. 43, 499-504.

Smith, J.S., Orciari, L.A., Yager, P.A., Seidel, H.D., Warner, C.K., 1992. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. J. Infect. Dis. 166, 296-307.

Koopmans, M., Monroe, S.S., Coffield, L.M., Zaki S.R., 1993. Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. J. Virol. Methods 43, 189-204.

Lehmann, U., Kreipe, H., 2001. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 25, 409-418.

Lumlertdaecha, B., Wacharapluesadee, S., Denduangboripant, J., Ruankaew ,N., Hoonsuwan, W., Puanghat, A., Briggs D, Hemachudha, T., (in press) Complex genetic structure of rabies virus in Bangkok city and surrounding provinces: Implication for canine rabies control. Trans. R. Soc. Trop. Med. Hyg.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., Okubo, K., 1999. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 27, 4436-4443.

McQuiston, J.H., Yager, P.A., Smith. J.S., Rupprecht, C.E. 2001. Epidemiologic characteristics of rabies virus variants in dogs and cats in the United States. J. Am. Vet. Med. Assoc. 218, 1939-42.

Nadin-Davis, S.A., Sheen, M., Wandeler, A.I., 2003. Use of discriminatory probes for strain typing of formalin-fixed, rabies virus-infected tissues by in situ hybridization. J. Clin. Microbiol. 41, 4343-4352.

Rekand, T., Male, R., Myking, A.O., Nygaard, S.J.T., Aarli, J.A., Haarr, L., Langeland, N., 2003. Detection of viral sequences in archival spinal cords from fatal cases of poliomyelitis in 1951-1952. J. Virol. Methods 114, 91-96.

Specht, K., Richter, T., Muller, U., Walch, A., Werner, M., Hofler, H., 2001. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am. J. Pathol. 158, 419-29.

Tirawatnpong, S., Hemachgudha, T., Manutsathit, S., Shuangshoti, S., Phanthumchinda, K., Phanuphak, P., 1989. Regional distribution of rabies viral antigen in central nervous system of human encephalitic and paralytic rabies. J. Neuro. Sci. 92, 91-99

Warner, C.K., Whitfield, S.G., Fekadu, M., Ho, H., 1997. Procedures for reproducible detection of rabies virus antigen mRNA and genome in situ in formalin-fixed tissues. J. Virol. Methods 67, 5-12.

Warner, C., Fekadu, M., Whitfield, S., Shaddock, J., 1999a. Use of anti-glycoprotein monoclonal antibodies to characterize rabies virus in formalin-fixed tissues. J. Virol. Methods 77, 69-74.

Warner, C.K., Zaki, S.R., Shieh, W.J., Whitfield, S.G., Smith, J.S., Orciari, L.A., Shaddock, J.H., Niezgoda, M., Wright, C.W., Goldsmith, C.S., Sanderlin, D.W., Yager, P.A., Rupprecht, C.E. 1999b. Laboratory investigation of human deaths from vampire bat rabies in Peru. Am. J. Trop. Med. Hyg. 60, 502-507.

Weizsacker, F.v., Labeit, S., Koch, H.K., Oehlert, W., Gerok, W., Blum, H.E., 1991. A simple and rapid method for the detection of RNA in formalin-fixed, paraffin-embedded tissues by PCR amplification. Biochem. Biophys. Res. Commun. 174, 176-180.

Whitfield, S.G., Fekadu, M., Shaddock , J.H., Niezgoda, M., Warner, C.K., Messenger, S.L., The Rabies Working Group. 2001. A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. J. Virol. Methods 95, 145-151.

Williams, C., Ponten, F., Moberg, C., Soderkvist, P., Uhlen, M., Ponten, J., Sitbon, G., Lundeberg, J., 1999. A High Frequency of Sequence Alterations Is Due to Formalin Fixation of Archival Specimens. Am. J. Pathol. 155, 1467-1471.

Wong, C., DiCioccio, R.A., Allen, H.J., Werness, B.A., Prver, S., 1998. Mutations in BRCA1 from Fixed, Paraffin-Embedded Tissue Can Be Artifacts of Preservation. Cancer Genet. Cytogenet. 107, 21-27.

Table1.Oligonucleotide primers for RT-PCR (P) and sequence (S) reactions.

Primer pair	Forward 5'> 3'	Reverse 5'>3'	Product size
P1(CN8/110BT)	CN8: GT(TC) GGA TGT TAT ATG GG (1013-1029)	110BT: (G/A)TTCATCATGATTCGAGTAT (1306-1287)	294 bp
P4(509/110BT)	509: GAGAAGGAACT(C/T)CA(G/A)GAGTA (1157-1173)	110BT: (G/A)TTCATCATGATTCGAGTAT (1306-1287)	150 bp
P9(CN9/CN4)	CN9: TGACGAGGATTACTTCTCCG (1240-1259)	CN4: GGATTGAC(AG) AAG ATC TTG CTC AT (1536-1514)	297 bp
P10(CN9/CN2)	CN9: TGACGAGGATTACTTCTCCG (1240-1259)	CN2:GAG TCA CTC GAA TAT GTC (1419-1402)	180 bp
S1(CN1/CN10)	CN1: CTA CAA TGG ATG CCG AC (66-82)	CN10: AGGAGTGATCTTGTCTCCTTT (385-365)	320 bp
S2(KAM3F/P1.1R)	KAM3: ATCATGCCCTGA(A/G)GACTGG (319-337)	P1.1:TCAGAGTATGGTGTTCTACGAT (632-611)	315 bp
S3(CN11/Lis2R)	CN11: TGCAGACAGGATAGAGCAGA (565-584)	Lis2R: GAT CTC TTC CTC GAA GTT CTT (877-857)	313 bp
S4(CN12/CN13)	CN12: AATCTCACCGCGAGAGAGG (818-836)	CN13: GTGGCATTAAGAGACCTGAC (1034-1053)	236 bp

Table2. RT-PCR and IHC results in formalin-fixed, paraffin-embedded (FFPE) tissues

*Samples No.	Results (RT-PCR/IHC)**					Sequencing results (nucleotide position)
	a	b	c	d	e	
1	+/-	-/-	+/+	-/-	+/+	1053-1401
2	+/+	+/+	+/+	+/+	ND	NA
3	+/+	+/+	-/+	+/+	ND	1042-1401
4	-/+	+/+	-/+	-/+	ND	NA
5	-/+	+/+	+/+	+/+	ND	1056-1401
6	+/-	+/-	-/+	-/+	ND	NA
7	+/+	+/+	+/+	-/+	+/+	1033-1513

* Samples No.1a-e represent section of temporal, parietal, cerebellum, basal ganglia and thalamus, respectively.

Samples No.2a-d represent section of frontal, cerebellum, temporal /hippocampus and thalamus/basal ganglia, respectively.

Samples No.3a-d represent section of frontal, occipital, Medulla, and Basal ganglia, respectively.

Samples No.4a-d represent section of frontal, occipital, medulla, and pons, respectively.

Samples No.5a-d represent section of cerebellum, frontal, thalamus, and medulla, respectively.

Samples No.6a-d represent section of temporal, occipital, thalamus, and parietal, respectively.

Samples No7a-e represent section of frontal, temporal, thalamus, and medulla and midbrain, respectively.

ND is not done.

NA is not available.

** Positive (+) or negative (-) as examine by RT-PCR using P4 primers and immunohistochemistry technique.

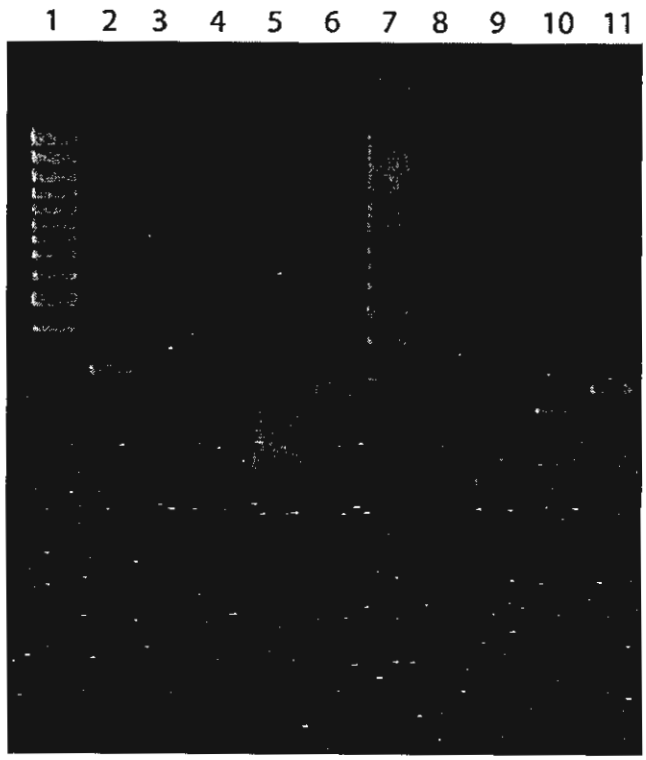

Figure 1

Figure 1. Ethidium bromide-stained agarose gel of PCR products derived from rabies RNA extracted from FFPE amplified with 9 primer pairs. Lane 2-6 and 8-11 was resulted from P1 to P9 primer pairs, respectively. Lane 1 and 7 contained the 100 bp DNA ladder (Fermentas,USA), starting with 100-bp marker. P4 primer pairs showed highest sensitivity and were chosen for screening diagnostic test.

เอกสาร 23

In Preparation

Suggested transmission dynamics of rabies virus in Thailand: Implications for disease control.

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Summary

Background In Thailand, rabies remains a neglected disease since authorities rely on human death statistics while ignoring an enormous increase in post-exposure prophylaxis. Past attempts to conduct a mass dog vaccination and sterilization program have been limited only to Bangkok city without success. We have used molecular epidemiology to define geographic localization of rabies virus genogroups and their pattern of spread.

Methods We analyzed 239 nucleoprotein gene sequences from animal and human brain samples collected from all over the country between 1998 and 2002. We then reconstructed a phylogenetic tree correlating these data with geographical information.

Findings All sequences formed a monophyletic tree of 2 distinct genogroups, TH1 and TH2. Three subgroups were identified in the TH1 subgroup and distributed in the middle part of the country. Eight subgroups of TH2 viruses were identified widely distributed throughout the country and overlapping the TH1 territory. There was a correlation between human-dependent transportation routes and the distribution of virus. Inter-regional migration paths of the viruses might be correlated with translocation of dogs associated with humans. Interconnecting factors between human socioeconomic and population density might determine the transmission dynamics of virus in a rural-to-urban polarity.

Interpretation Presence of 2 or more rabies virus groups in a location might be indicative of a gene flow, reflecting translocation of dogs within such region and adjacent areas. Different approaches may be required for rabies control based on the homo- or heterogeneity of the virus. Areas containing homogeneous virus populations should be targeted first. Control of dog movement associated with humans are essential.

Introduction

Rabies is not high on the list of the World Heath Organization's list of important infectious diseases, and is also often overlooked by regional, national, and local public-health professionals. The dog is the primary reservoir and vector of rabies transmission in Thailand and developing countries.[1]

To date, evaluation of the importance of rabies has been determined solely by estimating the number of human deaths and statistics on dog rabies infectivity, which may not be a reliable indicator in developing countries.[2] For example, an accurate assessment of the burden of rabies will never be completed without including the financial burden incurred due to human rabies post-exposure prophylaxis (PEP) and animal control.

In Thailand, the significant decline in human rabies deaths from almost 200 a decade ago to less than 20 in 2003, has occurred due to a huge and continuously escalating financial obligation in the annual budget to supply rabies biologicals for human PEP. More than 400,000 patients required PEP in 2003, as compared to approximately 90,000 in 1991 [Ministry of Public Health (MOPH) annual report]. Moreover, annual human rabies deaths in Bangkok, where diagnostic facilities and neurologists are readily available, rose from less than 5 in 1990-1994 to 5-10 in 1995-2001 (MOPH annual report).

There have been no reliable statistical analyses of dog populations that could be evaluated to determine the effectiveness of the current human rabies prevention methodologies used in Thailand. One figure of 6 million dogs in Thailand was undoubtedly an underestimate of the actual population present within the country. The Division of Disease Control and Ministry of Agriculture reported that between 60 to 78% of the dog population was vaccinated (based on estimated total population) in Thailand between 1995 and 2000. Experience in Latin America has shown that vaccination of a critical percentage of dogs, on the order of 40-70%, at least in major urban areas, was adequate to significantly interrupt canine rabies transmission and resulted in diminished human rabies deaths.[3] However, this has not been the case in Thailand. The percentage of rabies infectivity of samples sent to diagnostic laboratories all over the country remains significant, within the range of 30-40% (MOPH annual report).

A survey in 1999 by the Department of Livestock and the Bangkok Metropolitan Administration revealed that stray dog populations in Bangkok (an area of 1,565 sq km) have tripled in size, (from 40,756 in 1992 to 110,584 in 1999). Additionally, a 2002 survey suggested that dog populations tended to increase both in Bangkok and the countrywide, implying that the specific carrying capacity of canine habitats has not yet been saturated. Moreover, a significant number of dogs, especially stray and community dogs, are not vaccinated.

Due to budget limitation, an intensive dog vaccination and sterilization program has been in place only in Bangkok City since June 2002. Seventy-two million baht (approximately US$ 1,800,000) were spent during the first 2 phases of the program (June 2002-September 2003), with the third phase (October 2003-September 2004) costing an additional 31 million baht (approximately US$ 775,000). Although there were no human rabies deaths in Bangkok in 2002, 3 deaths were reported in 2003. Preliminary assessment revealed that less than 20 percent of the estimated dog population was sterilized and vaccinated.

Without reliable data on dog ecology and surveillance of rabies infection in dogs and humans, it is not possible to develop a strategic plan for rabies prevention and control and to assess program success. Therefore, our objective was to use molecular biological techniques to characterize the presence and movement of rabies virus according to geographical locations in Thailand and use this information as baseline data to design and implement rabies prevention programs in the country. Areas with evidence of continuous gene flow, and

presence of viruses of more than one genetic group or subclade, were characterized. The potential translocation of rabies virus from one area to another was evaluated in relation to natural barriers, transportation routes, human activity and socioeconomic factors.

Methods

Samples

Two hundred and thirty nine brain samples (dogs = _?_ , cats = _?_ , xxx = _?_ and humans = _?_) from 56 provinces were obtained from _?_ diagnostic laboratories all over Thailand between 1998 and 2002. Samples selected for analysis were chosen to be representative of the geographical location in each province down to the scale of small districts (subdivisions of a province). Samples were not available from 20 provinces. All samples were prescreened for evidence of rabies virus using the direct fluorescence antibody test and kept frozen at -80 degree C until genetic analysis was conducted.

Genetic analysis

Genetic typing was based on nucleotide sequence differences in cDNA obtained by direct one step RT-PCR amplification of the nucleocapsid (N) gene fragment from the samples. The amplified products of 414 bp (nt 1101 - 1506) were characterized by sequencing. RT-PCR and sequencing procedures were done as previously described.[4] One set of primers was used for RT-PCR sequencing reaction. GenBank accession numbers of the N sequences in this study were AY849022-AY849260.

Eleven additional N sequences were retrieved from Genbank database to be outgroups for this study: a non-rabies lyssavirus Mokola Virus (S59448), 3 Australian Bat Lyssavirus (ABL) isolates (NC003243, AF081020, AF418014), a rabies strain Pasteur Virus (PV) (M13215), and 6 rabies viruses from other Asian countries (AY138550 from Sri Lanka, AY138551 Sri Lanka, AY138549 Sri Lanka, AF155039 China, AF374721 India, U22482 Iran). The sequences of all isolates were aligned together using program ClustalX.[5] Genetic relationships between these N gene sequences were calculated and a tree diagram was drawn using neighbor-joining (NJ) method, which was suitable to illustrate below species-level genetic relationships. These phylogenetic analyses were performed with program PAUP* version 4.0b10.[6] Robustness of the tree was accessed with branch supporting-values from bootstrap (BS) statistic analyses (1,000 replicates). The collecting provinces and districts of all virus samples were mapped on the trees. Geographical locations of samples were mapped (Arcview 3.2, ESRI) and compared among the subgroups.

Role of funding sources

The funding sources, Thailand Research Fund and National Science and Technology Development Agency, have no influence in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Results

Phylogenetic analyses of the 239 N rabies sequences collected from all over Thailand clearly demonstrated that all of the isolates formed a monophyletic group, separate from Mokola, ABL, PV and other rabies viruses circulating in Asian countries (Fig. 1). Neither the sequences of infected human nor non-canine animals (cats and other wildlife) were specifically clustered as a unique group, but rather paired with the dog rabies viruses analyzed in the study. Two major viral groups were clearly recognized from the tree and designated as TH1 and TH2 clusters, with 82% and 68% bootstrap supporting values, respectively.

Considering sampling locations of viral isolates, both Thai rabies genogroups were confined to certain geographical areas, though overlapping did occur in some areas (see the map of Thailand in Fig. 1). TH1 viruses were found mainly in the middle part of the country, from the lower northern region to the central region, Bangkok and surrounding provinces, and the upper southern region of Thailand. Based on BS supporting values on each branch, branch lengths (equally to numbers of substitutions/site), and the tree topology, the TH1 genogroup was divided into 3 minor subgroups (Fig. 2): TH1A isolates were identified in Bangkok and outskirts as well as some other provinces in the central region; TH1B isolates were identified in the northern and central regions, and TH1C isolates were identified in Bangkok, the central, and the upper southern regions.

The TH2 rabies genogroup was distributed in much wider areas than the TH1 genogroup (Fig. 1), from the northern to the southern-most regions of the country. The distribution areas of TH2 group covered almost every province in the northeastern region, all main provinces in the upper central and the central regions, Bangkok and 5 surrounding provinces, the eastern and western regions, and nearly the entire area of southern Thailand. Using similar criteria as in the TH1 group, TH2 was divided into 8 subgroups: TH2A (Fig. 3) with samples from the northeastern region, TH2B (Fig. 3) with samples from the south and some northeastern provinces, TH2C (Fig. 4) from a few provinces scattered in the east, upper central, and northeast, TH2D (Fig. 4) from western provinces, TH2E (Fig. 4) had a much wider distribution-range including the far north, northeast, central including provinces around Bangkok, to the upper south, TH2F (Fig. 4) and TH2G (Fig. 5) were mainly located in the northeastern regions, and TH2H (Fig. 5) was found in the lower north to the upper central.

Discussion

The use of molecular biological techniques to evaluate the epidemiology of viral diseases is being increasingly employed to complement conventional methods ([7-11] for rabies examples). These techniques can give a clearer understanding of the origination and transmission patterns of viral epidemics. Eventually, data produced from molecular epidemiological studies could lead to a better understanding of and a more effective strategy to control the spread of infectious diseases.

Our study revealed that all of the currently identified Thai rabies viruses share a common origin that is genetically distant from the PV, ABL, and Mokola outgroups. Additionally, the monophyletic tree of the Thai rabies viruses analyzed in this study was clearly distinguishable from other rabies N sequences from India, Sri Lanka, China, and Iran (Fig. 1). Thus, rabies viruses circulating in Thailand (or in Southeast Asia) could possibly have an exclusive evolutionary background that might be recognized as being unique, an hypothesis previously suggested by Susetya et al.[12] It will be necessary to analyze additional sequences of rabies viruses circulating in neighboring countries adjacent to Thailand

The NJ genetic distance tree also confirmed that the sequences obtained from non-canine sources (human, cats and other mammals) were very similar to those of rabid dogs. No specific grouping of sequences from rabies virus isolated from non-canine species was identified. Instead, these rabies virus sequences were scattered across the tree. This finding was in accord to our expectation that the dog is a prime reservoir and transmitting vector for rabies and causes spillover to human and domestic animals and wildlife. Nevertheless, we are also aware that there may be other vectors, such as bats, and other lyssaviruses, besides genotype 1, circulating in Thailand. In fact, our recent survey in Thai bats indicated that as many as 7.5% of the bat population had evidence of lyssavirus infection by an as yet unidentified genotype(s).[13]

The 2 major groups found in our Thai rabies phylogeny were judged to be significant with high bootstrap supporting values. Notably, these 2 major lineages resembled the putative

groups A and B found in our previous study[14] in which fewer numbers of samples from Bangkok and its surrounding provinces were analyzed. The 2 genogroups we identified had certain trends in their geographical distributions. The distribution areas of TH1 group were only found in the central part of the country - from Nakhon Sawan province, down along Choa Praya river to the capital city of Bangkok, ending at Ranong province in the upper southern region (Fig. 2). On the other hand, those of TH2 group were spread across more than three-quarters of the entire country – from Phayao province in the north (Fig. 4), to Ubol Ratchathani in the northeast corner (Fig. 5), and to the southern Yala province along the Thailand-Malaysia border (Fig. 3).

Although there are some overlapping areas shared between the TH1 and TH2 genogroups, the viral transmission dynamics and evolutionary background the sub-lineages may not be similar which could explain why both have different success levels in disease dispersals. It has been proposed that the degree of differences in compartmentalization mechanisms may influence the duration that each individual canine-associated rabies variant resides in certain geographical regions.[15] Relationships between dogs and humans within a community, dog population density, and relative dog-human population ratio are common explanations for such compartmentalization phenomenon.[16, 17] Local geographical barriers such as rivers and mountains are other important factors considered to have strong influences on the inhibition of the spread of vector-borne diseases.[18] This inhibition effect caused by natural barriers could, however, be compromised by human transportation routes, for instance bridges, or roads and railways through mountains.

To estimate the epidemiological characteristics of the TH1 and TH2 Thai rabies groups, a phylogeographical concept was introduced to infer their transmission dynamics.[18, 19] Comparison between the sampling localities and molecular phylogenetic-tree topology could determine how viruses in each and different group are genetically related. First, in Bangkok and the surrounding provinces both TH1 and TH2 were identified as occurring together (Fig. 1). This area is industrialized and highly populated. Hundreds of mainroads, highways, and railways have been built in the country heartland. Networks of transportation routes including bridges across waterways are an effective means for vector borne viral transmission. This is one explanation as to why some viral subgroups of both TH1 and TH2 were discovered along both sides of Choa Phraya and many other rivers (Fig. 6), as has been previously reported.[14]

Secondly, we suggest that transmission of rabies virus may be related to human activity, particularly human migration. Considering the phylogeographic areas of the 3 genetic subgroups of TH1 rabies virus (Fig. 2), genetic exchanges within the TH1B subgroup between Sukhothai in the north and Nonthaburi province near Bangkok, almost 500-kilometres apart, could not have been accomplished by migrations of animal virus-vectors alone. It is more likely that canine vectors of the TH1B genotype were translocated from areas around Bangkok to the north, and vice versa, simply by following movements of pet-owners via the national mainroads number 1 and 11 (Fig. 2). Similarly, the same translocation factors can be applied to a long-distance dispersal of the TH1C subgroup from central to southern Thailand, probably via the national mainroad number 4 (Fig. 2). Transmission dynamics of the TH1 subgroup might also have been influenced by a combination of factors including social and socioeconomic status, human and animal population density in addition to the availability of transportation routes.

The theory that the spread of canine rabies virus was instigated by pet-owner translocation via transportation routes was supported in this study by the results of the distribution pattern of each subgroup of TH2 (Figs. 3, 4, 5). Members of the TH2 group appeared to be scattered across the regions at a very distant range, and are unlikely to have occurred due to animal self-translocation. From our analyses, we propose that genetically linked viruses of each subgroup were localized in specific areas by utilizing transportation

routes throughout Thailand (as shown in Fig. 3, 4, and 5), and areas that have more than one viral group present are apparently local transportation, for instance, Mueng district (Khon Kaen province), Pa Kham district (Buri Ram province) and Phimi district (Nakhon Ratchasima province) (shown as black areas in Fig. 3).

The most convincing support for the human-facilitated rabies distribution hypothesis we propose herein is the geographical distribution of the TH2B subgroup in which all, except a few samples from the northeast, were from the southern region of the country. This phylogeographic subgroup with a 1300-1600 km spreading range, had a very strong bootstrap supporting-value (89%) on the genetic tree. We propose that this inter-regional migration path of the TH2 subgroup is explained by a rural-to-urban viral transmission polarity.[12, 16] The majority of people in the northeast have a relatively lower socioeconomic status than people living in other regions. Most of them are conventional crop farmers with low annual income [9,279 Baht (approximately US$ 230) average monthly household income versus national average of 13,736 Baht (approximately US$ 340), reported by National Statistical Office on 2002] and during the off-growing season they usually migrate to other regions to seek employment as common laborers. The strong economics in southern Thailand has been mainly supported by marine fishery as well as the rubber plant and oil palm agricultural industry, of which most workers originate from northeastern Thailand. Rabies virus infected canine pets accompanying the migratory workers from the northeastern therefore could be spread along their owners' travel routes. This would not only explain the northeast-to-south migration path of the TH2B viruses, but also could elucidate why most of the TH2 subgroups examined were closely linked with viral isolates from the northeastern region.

Selection of suitable areas using molecular epidemiological techniques should be considered as a powerful tool when planning disease control strategies. For decades, Thailand has invested vast sums of money and manpower on the effort to control and vaccinate the dog population in randomly selected districts and, recently, Bangkok capital city without success. Results of this research demonstrated that Bangkok and other metropolitan cities (such as Prathum Thani, Samut Sakhon, Nakhon Sawan, Khon Kaen, Ubol Ratchathani) contain various groups and subgroups of viruses, actively circulating to and from other surrounding provinces (Fig. 6). Therefore, developing a campaign for disease control in such city alone, without considering neighboring areas, is highly unlikely to be successful. We propose that the most appropriate place to initiate a rabies control campaign should be in a genetically isolated area, where there are either natural or artificial barriers to prevent further viral influxes.

On a national scale, we propose that rabies control can be successful if it is initiated in southern Thailand. This region contains only the TH2B rabies subgroup. Furthermore, it is an "island-like" area surrounding by Andaman Sea, Gulf of Thailand, and the Malaysian border. Influx from the TH1C subgroup has been restricted to an area around Ranong province, plausibly from high mountain ridges. Moreover, the majority of the population in southern Thailand are Muslims who do not keep pet dogs or feed stray dogs. Implementation of a rabies control in this region should therefore be effective in terms of a dog population reduction and vaccination campaign, and the enforcement of strict regulations regarding dog transfer.

In order to test this concept of "targeting a genetically defined area", a mass rabies control compaign should be conducted in a suitable-size province with a homogeneous virus population. The province of Kanchanaburi, 19,483 km sq and about 130 km westwards from Bangkok, appears to be a good choice since, according to our study, it contains only the TH2D rabies subgroups (Fig. 6) which are clustered mostly on the southernmost tambons (subdistricts; subdivisions of an district). The province is also island-like in that it is surrounded by the mountainous Thailand-Myanmar border and also has mountain ridges

along the eastern boundary to other provinces. Any strategic plan in this region should also include recommendations to control pet-dog movements via the national mainroad number 323, the primary transportation route into the province. Assessment of the success of such a program can be measured by a strict surveillance of rabies incidence in humans and animals and by analyzing genetic sequences of rabies virus as compared to others in adjacent provinces. This should also be correlated with transportation tracks on a local scale.

In conclusion, we have presented a novel approach to the development of a rabies control and prevention program through the utilization of genetic epidemiology. We believe that the implementation of such a disease control program utilizing existing information on the genetics of circulating rabies viruses in a country like Thailand could be successful if the campaign target areas have been carefully selected and limited to one circulating genogroup of virus and the movement of dogs along human transportation routes into the area is strictly enforced.

References

1 Mitmoonpitak C, Wilde H, Tepsumetanon W. Current status of animal rabies in Thailand. J Vet Med Sci 1997; 59:457-60.

2 Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. Lancet Neurol 2002; 1:101-9.

3 PAHO. 2000. Epidemiological surveillance of rabies in the Americas. Washington DC: Pan American Health Organization; 2000.

4 Hemachudha T, Wacharapluesadee S, Lumlertdaecha B, Orciari LA, Rupprecht CE, Laongpant M, Juntrakul S, Denduangboripant J. Sequence analysis of rabies virus in humans exhibiting encephalitic or paralytic rabies. J Infect Dis 2003; 188:960-6.

5 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25:4876-82.

6 Swofford DL. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4. Sunderland (MA): Sinauer Associates; 2002.

7 David D, Yakobson B, Smith JS, Stram Y. Molecular epidemiology of rabies virus isolates from Israel and other Middle- and Near-Eastern countries. J Clin Microbiol 2000; 38:755-62.

8 De Mattos CA, De Mattos CC, Smith JS, Miller ET, Papo S, Utera A, Osburn BI. Genetic characterization of rabies field isolates from Venezuela. J Clin Microbiol 1996; 34:1553-58.

9 Miksza LKC, Wadford DA, Schnurr PD. Molecular epidemiology of enzootic rabies in California. J Clin Virol 1999; 14:207-19.

10 Nadin-Davis SA, Casey GA, Wandeler AI. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. J Gen Virol 1994; 75:2575-83.

11 Teichman BFV, Thomson GR, Meredith CD, Nel LH. Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups. J Gen Virol 1995; 76:73-82.

12 Susetya H, Sugiyama M, Inagaki A, Ito N, Oraveerakul K, Traiwanatham N, Minamoto N. Genetic characterization of rabies field isolates from Thailand. Microbiol Immunol 2003; 47:653-9.

13 Lumlertdacha B, Boongird K, Wanghongsa S, Wacharapluesadee S, Chanhome L, Khawplod P, Hemachudha T, Kuzmin I, Rupprecht CE. Survey of bat lyssaviruses in Thailand. Emerg Infect Dis (in press).

14 Lumlertdacha B, Wachrapluesadee S, Denduangboripant J, Ruankaew N, Hoonsuwan W, Puanghat A, Sakarasaeranee P, Briggs D, Hemachudha T. Complex genetic structure of rabies virus in Bangkok city and its surroundings: implications for canine rabies control. Trans Roy Soc Trop Med Hyg (in press).

15 Smith JS. Molecular epidemiology. In: Jackson AC, Wunner WH, editors. Rabies. Amsterdam: Academic Press; 2002. p. 79-111.

16 De Mattos CC, De Mattos CA, Loza-Rubio E, Aguilar-Setien A, Orciari LA, Smith JS. Molecular characterization of rabies virus isolates from Mexico: implications for transmission dynamics and human risk. Am J Trop Med Hyg 1999; 61:587-97.

17 Holmes EC, Woelk CH, Kassis R, Bourhy H. Genetic constraints and the adaptive evolution of rabies virus in nature. Virology 2002; 292:247-57.

18 Smith JS, Orciari LA, Yager PA, Seidel HD, Warner CK. Epidemiologic and historical relationships among 87 isolates as determined by limited sequence analysis. J Infect Dis 1992; 166:296-307.

19 Holmes EC. The phylogeography of human viruses. Mol Ecol 2004; 13:745-56.

20 Avise, J.C. 2000. Phylogeography: the history and formation of species. London: Harvard University Press; 2000.

Figure legends

Fig. 1 Neighbor-joining (NJ) tree based on 414 bp nucleotide sequences of the N genes of all 239 Thai rabies virus isolates compared with other 11 lyssavirus outgroups. Numbers along tree branches are >50% bootstrap supporting-value (1,000 replicates). The map of Thailand indicates geographical distributions of the 2 major genogroups, TH1 and TH2, in a district-level (a subdivision of a province).

Fig. 2 A comparison between the NJ tree of 60 N gene sequences of TH1 rabies viruses (with the TH2 isolate 703KKm added as an outgroup) and the Thailand map indicates geographical distributions of the subgroups TH1A, TH1B, and TH1C.

Fig. 3 A comparison between the bottom part of the NJ tree of TH2 rabies viruses (with the TH1 isolate 125SSktb added as an outgroup) and the Thailand map indicates geographical distributions of the subgroups TH2A and TH2B.

Fig. 4 A comparison between the middle part of the NJ tree of TH2 rabies viruses and the Thailand map indicates geographical distributions of the subgroups TH2C, TH2D, TH2E, and TH2F.

Fig. 5 A comparison between the top part of the NJ tree of TH2 rabies viruses and the Thailand map indicates geographical distributions of the subgroups TH2G and TH2H.

Fig. 6 Geographical distribution of all Thai rabies virus subgroups. Kanchanaburi province was magnified to show province geography and roadmap. Red areas in the Kanchanaburi map indicate the collecting localities of rabies hosts in a tambon (a subdivision of a district)–level. The map was retrieved from www.thaitambon.com/Maps/Kanchanaburi.htm .

Authors' contributions and signatures

The first 3 authors (JD, SW, BL) contributed equally to this work.

Jessada Denduangboripant, PhD

"I declare that I participated in data analysis and interpretation, phylogenetic tree construction, and writing the paper, and that I have seen and approved the final version."

(Jessada Denduangboripant, PhD)

Supaporn Wacharapluesadee, MSc

"I declare that I participated in data collection and analysis, PCR primer design, sequencing rabies genes, and writing the paper, and that I have seen and approved the final version."

(Supaporn Wacharapluesadee, MSc)

Boonlert Lumlertdacha, DVM

"I declare that I participated in specimen collection, data analysis, and writing the paper, and that I have seen and approved the final version."

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"I declare that I participated in study design, data analysis and interpretation, and writing the paper, and that I have seen and approved the final version."

(Thiravat Hemachudha, MD)

Conflict of interest statement

No authors have any financial or personal relationships with other people or organizations that could inappropriately influence this research. All authors have access to all data in the study and held final responsibility for the decision to submit for publication.

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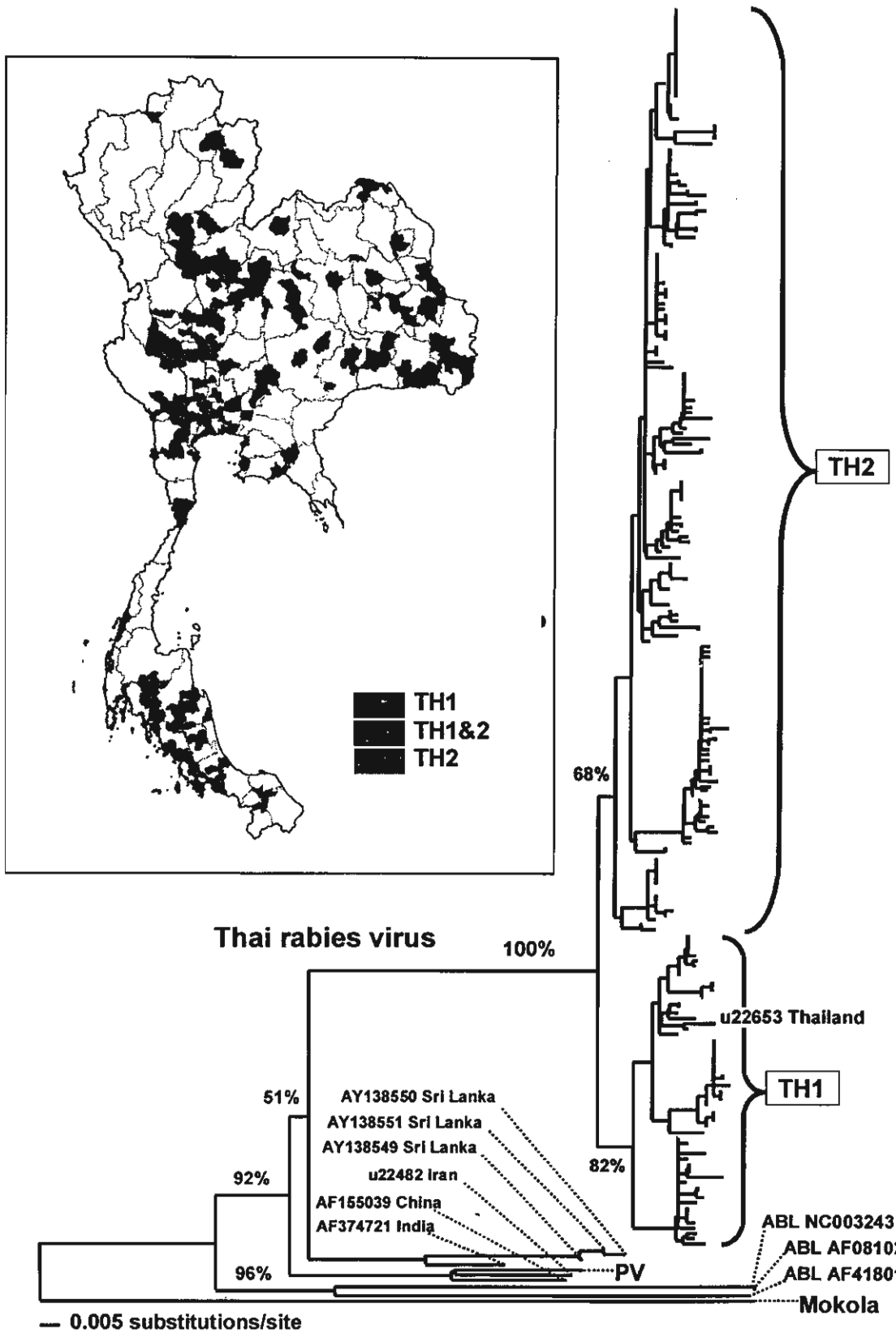
NJ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TH1&2
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TH2
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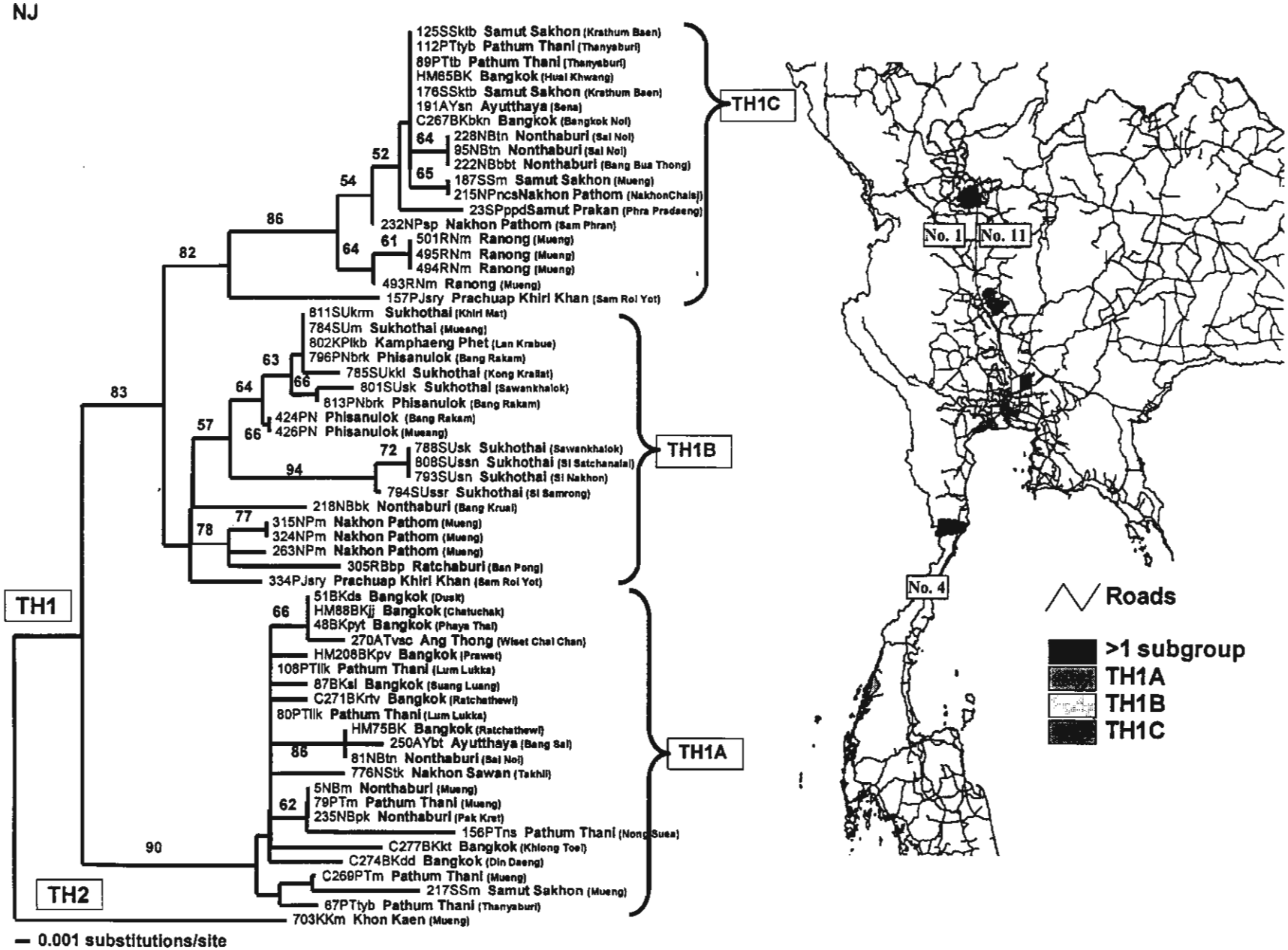
NJ
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HM85BK Bangkok (Huai Khwang)
176SSktb Samut Sakhon (Krathum Baen)
191AYsn Ayutthaya (Sena)
C267BKbkn Bangkok (Bangkok Noi)
228NBtn Nonthaburi (Sai Noi)
95NBtn Nonthaburi (Sai Noi)
222NBbbt Nonthaburi (Bang Bua Thong)
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494RNm Ranong (Mueng)
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157PJsry Prachuap Khiri Khan (Sam Roi Yot)
TH1C
811SUkrm Sukhothai (Khiri Mat)
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802KPlkb Kamphaeng Phet (Lan Krabue)
796PNbrk Phisanulok (Bang Rakam)
785SUkkl Sukhothai (Kong Krailat)
801SUsk Sukhothai (Sawankhalok)
813PNbrk Phisanulok (Bang Rakam)
424PN Phisanulok (Bang Rakam)
426PN Phisanulok (Mueang)
788SUsk Sukhothai (Sawankhalok)
808SUssn Sukhothai (Si Satchanalai)
793SUsn Sukhothai (Si Nakhon)
794SUssr Sukhothai (Si Samrong)
218NBbk Nonthaburi (Bang Kruai)
315NPm Nakhon Pathom (Mueng)
324NPm Nakhon Pathom (Mueng)
263NPm Nakhon Pathom (Mueng)
305RBbp Ratchaburi (Ban Pong)
334PJsry Prachuap Khiri Khan (Sam Roi Yot)
TH1B
51BKds Bangkok (Dusit)
HM88BKjj Bangkok (Chatuchak)
48BKpyt Bangkok (Phaya Thai)
270ATvsc Ang Thong (Wiset Chai Chan)
HM208BKpv Bangkok (Prawet)
108PTlk Pathum Thani (Lum Lukka)
87BKsl Bangkok (Suang Luang)
C271BKrtv Bangkok (Ratchathewi)
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776NStk Nakhon Sawan (Takhli)
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67PTtyb Pathum Thani (Thanyaburi)
703KKm Khon Kaen (Mueng)
TH1A
TH1
TH2
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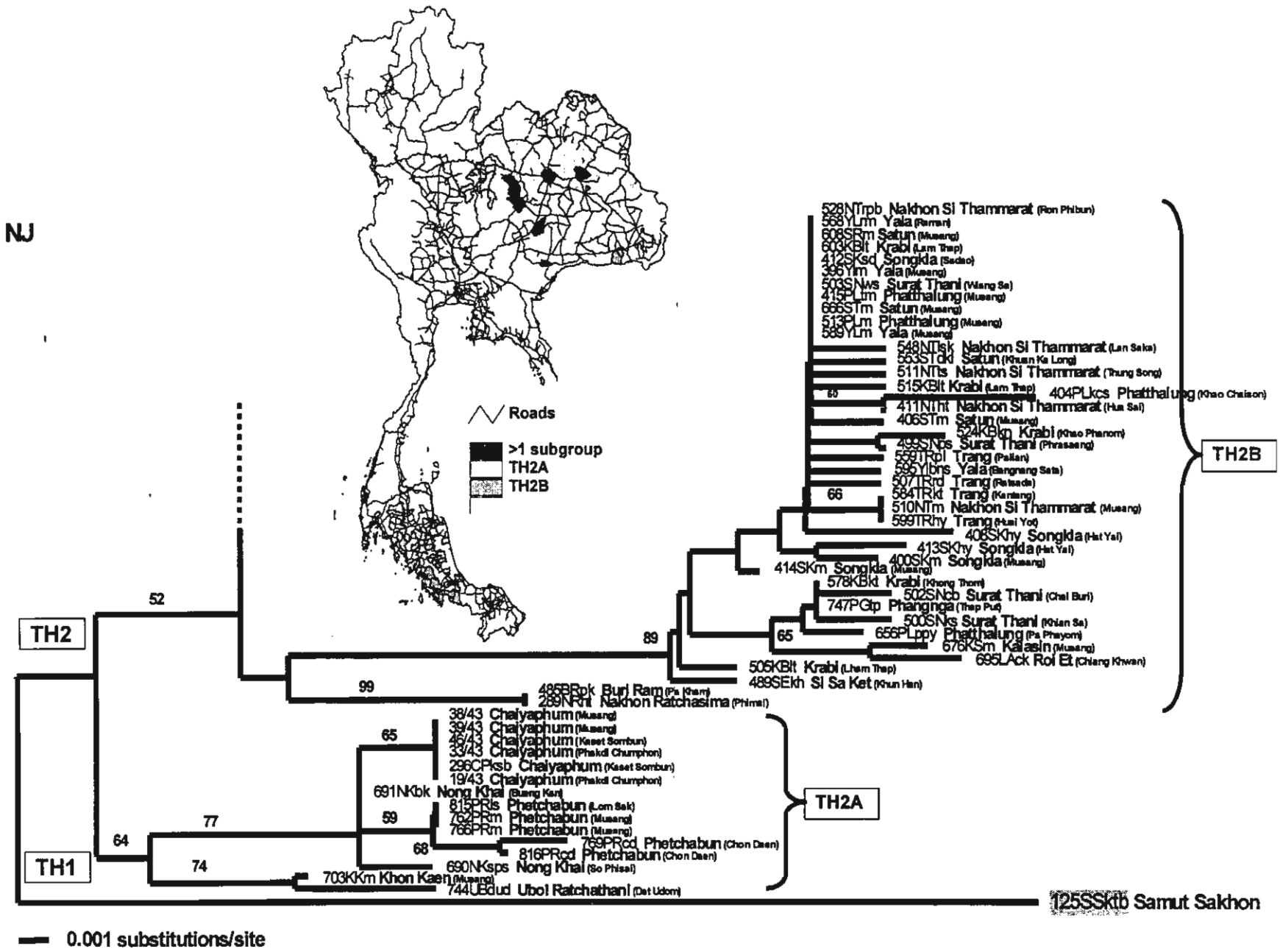
No. 11
No. 4
Roads
>1 subgroup
TH1A
TH1B
TH1C


NJ
Roads
>1 subgroup
TH2A
TH2B
TH2
TH1
TH2B
TH2A
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568YLm Yala (Raman)
608SRm Satun (Muaang)
603KBlt Krabi (Lam Thap)
412SKsd Songkla (Sadao)
396Ylm Yala (Muaang)
503SNws Surat Thani (Wiang Sa)
415PLtm Phatthalung (Muaang)
666STm Satun (Muaang)
513PLm Phatthalung (Muaang)
589YLm Yala (Muaang)
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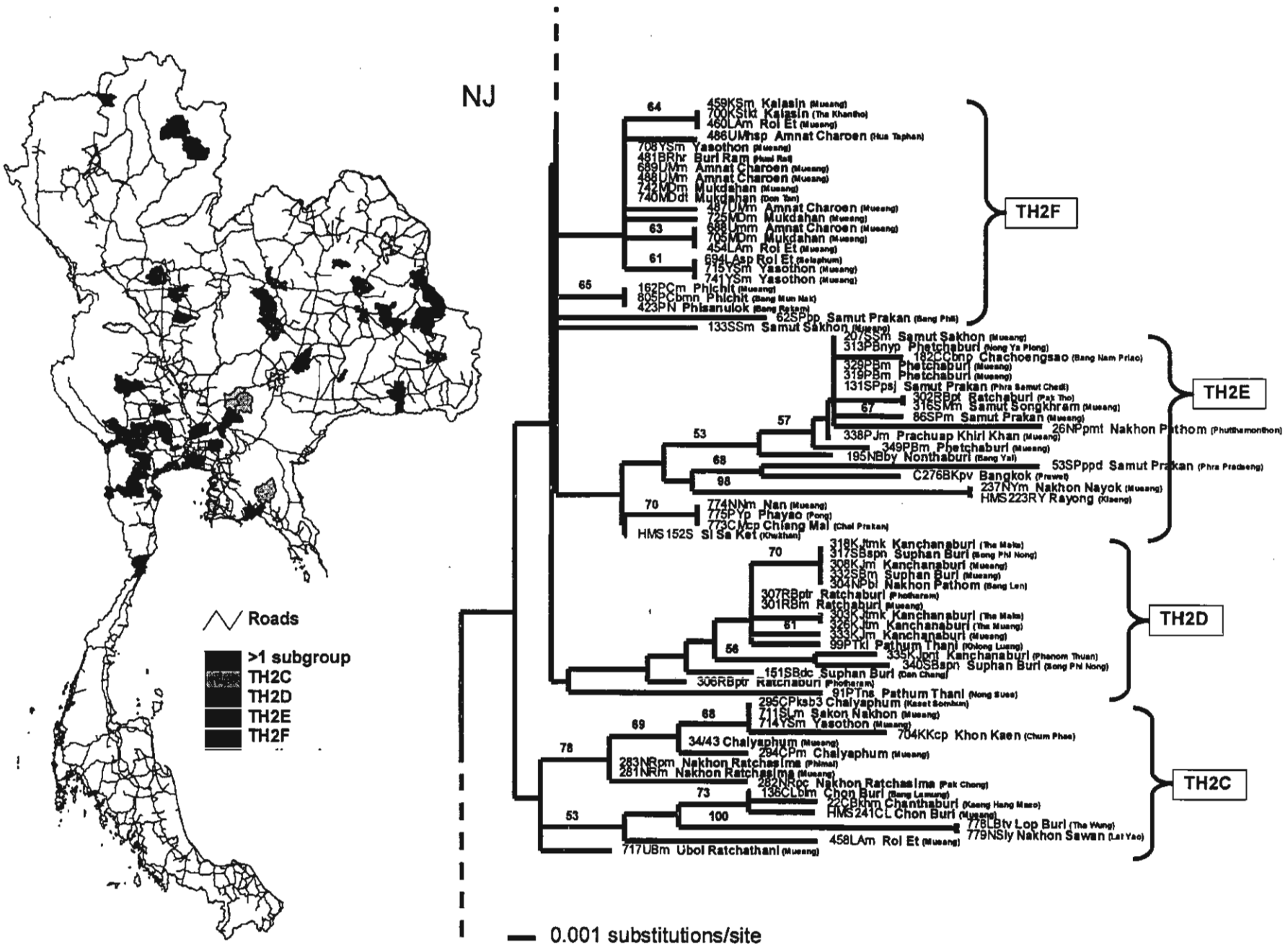
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313PBnyp Phetchaburi (Nong Ya Plong)
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319PBm Phetchaburi (Mueang)
131SPpsj Samut Prakan (Phra Samut Chedi)
302RBpt Ratchaburi (Pak Tho)
316SMm Samut Songkhram (Mueang)
86SPm Samut Prakan (Mueang)
26NPpmt Nakhon Pathom (Phutthamonthon)
338PJm Prachuap Khiri Khan (Mueang)
349PBm Phetchaburi (Mueang)
195NBby Nonthaburi (Bang Yai)
53SPppd Samut Prakan (Phra Pradaeng)
C276BKpv Bangkok (Prawet)
237NYm Nakhon Nayok (Mueang)
HMS223RY Rayong (Klaeng)
TH2E
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773CMcp Chiang Mai (Chai Prakan)
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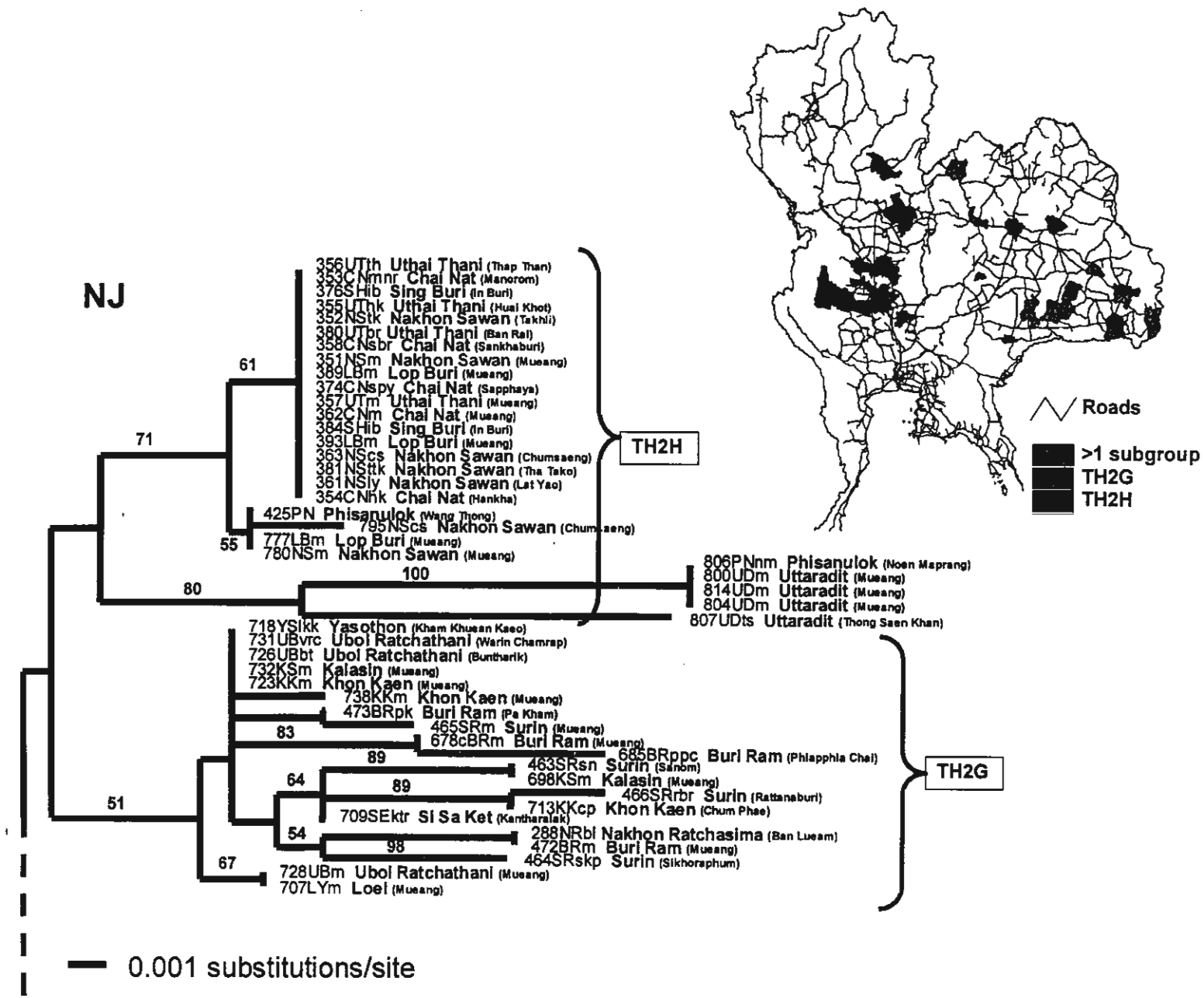
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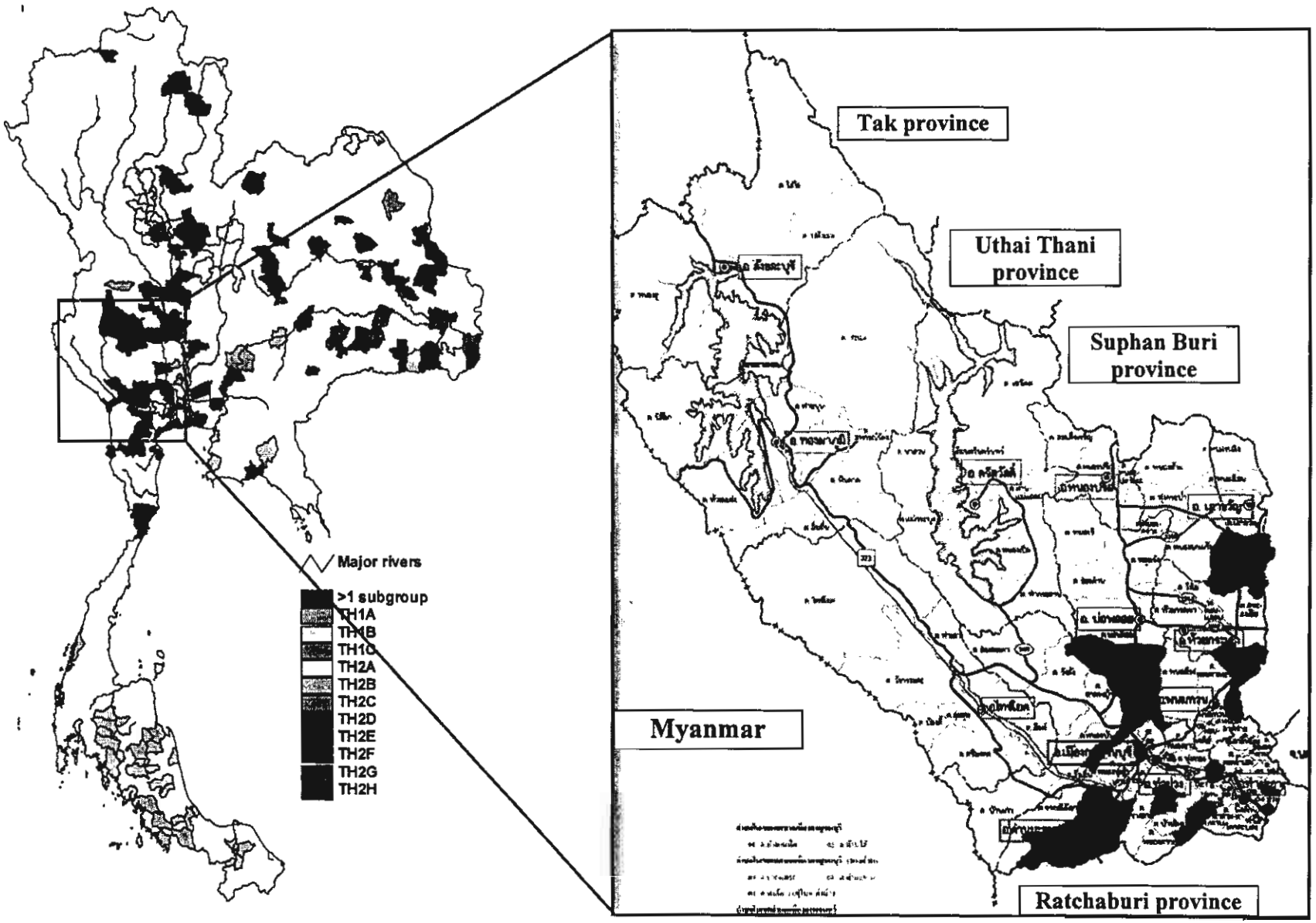
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Suphan Buri province
Myanmar
Ratchaburi province

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Rabies Control in Asia

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Clinical aspects of human rabies

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Rabies is a widely feared illness. The rate of death after a bite of a rabid animal is 35-57%, depending on the severity of the wound and the virus content of the saliva [1]. Although transdermal bites (especially with bleeding) on the head, face, neck and hand carry the highest risk, a bite on the leg and foot must be treated with the same urgency. The risk can also be high in the case of rabid bats, even from a scratch, because of the unique property of this rabies variant to replicate effectively in non-neural cells, such as fibroblasts and epithelial cells [2]. Other non-bite exposures include inhalation of aerosolized rabies virus, corneal transplant, handling and skinning of infected carcass and contaminated an open wound, scratch, abrasion or mucous membrane by infected saliva or neural tissue.

Classical and non-classical forms

The clinical features of human rabies can be differentiated as classical and non-classical. Classical encephalitic (furious) and paralytic (dumb) rabies is almost always associated with the canine rabies variant of genotype 1 [1, 3, 4]. Non-classical rabies occurs in patients exposed to viruses of insectivorous or frugivorous bat origin (genotype 1, 5, 6 or 7) and, recently, in Thai patients infected with canine rabies variant [1, 3, 5].

In classical forms the clinical features can be divided into five stages, the incubation period, the prodrome, the acute neurological phase, coma and death or recovery.

Incubation period and prodrome

The incubation period is usually 1-2 months but can range from less than 7 days to 6 years [6]. In classical presentations after exposure to canine rabies variant, local sensory prodromes (paraesthesia, itching and burning) reflecting ganglioneuronitis can be found in as many as 30% of patients [Mitrabhakdi *et al.*, submitted]. They are equally common in furious and dumb rabies. These symptoms occur at the bite site and may gradually spread to involve the whole limb or ipsilateral side of the face.

Acute neurological phase

Within hours or a few days after the prodrome, rabies patients enter an acute neurological phase. Two-thirds of patients suffer from the furious form, and the remaining present with paralysis. Furious rabies patients generally die within 7 days (average, 5 days) after the clinical onset, although survival may be as long as 2-3 weeks. The average length of survival in paralytic cases is 13 days.

In furious rabies, the earliest neurological abnormalities are fever and hyperactivity brought on by internal (thirst, fear, nervousness) or external (light, noise) stimuli. These are followed by typical features consisting of fluctuating consciousness, phobia (aero- and hydrophobia) and spontaneous inspiratory spasms and autonomic dysfunction (hypersalivation, nonreactive pupils, piloerection). Seizures and hallucination are rare in both forms of rabies but are occasionally seen in patients with fully developed disease, often at the preterminal stage.

In paralytic rabies, the cardinal features of furious rabies appear late and are usually mild. Phobic spasms, seen in all furious rabies patients, occur in only one-half of these patients; however, inspiratory spasms occur in all patients during the preterminal phase. Weakness may start initially at the bitten limb and progressively involve all limbs, and pharyngeal, facial and respiratory muscles. However, most have ascending paralysis similar to Guillain-Barré syndrome. Fever, pure motor, predominantly proximal weakness with loss of deep tendon reflexes and urinary incontinence are universal findings. Sensory function remains intact in all modalities. Percussion myoedema can be elicited in all dumb rabies patients; however, this sign can be observed in other conditions such as hyponatraemia, renal insufficiency, hypothyroidism and severe cachexia.

Coma

It is extremely difficult to diagnose rabies at this stage, as the two forms of rabies are indistinguishable once the patient becomes comatose. Inspiratory spasms are the only helpful sign in diagnosis. In dumb rabies, alveolar hypoventilation and ventilatory failure develop before the patient becomes obtunded. In furious rabies, abnormal breathing patterns and depression of consciousness appear simultaneuously. Regular breathing, interpersed with inspiratory spasms, is replaced by tachypnoea and then by apneutic respiration and terminally by ataxic rial tachycardia, a wandering atrial pacemaker and premature ventricular ectopic beats. Prior to or at the time of hypotension, a reduced ejection fraction is almost always observed on echocardiographic evaluation. Viral involvement at the autonomic ganglia or at the sinus or atrioventricular node and myocarditis are likely to be the underlying mechanisms. Coma almost always precedes circulatory insufficiency, which is the prime cause of death. Haematemesis is seen in 30-60% of patients 6-12 h before death.

The clinical manifestations in rabies patients exposed to viruses of insectivorous or frugivorous bat origin differ in many respects from those of the classical forms of furious and dumb rabies due to canine rabies variant [3]. Local prodromes were reported in 25 out of 40 patients infected with bat rabies variant documented between 1951 and 1977. One patient had radicular pain along C8-T2 accompanied by an inability to perform fine movements and by chorea. Two patients had objective sensory loss in one

arm and in the face on the same side. Ataxia was demonstrated in at least five patients and vertigo or dizziness in two others.

During the acute neurological phase, unexpected focal neurological signs are found, and weakness of the bitten extremity was recorded in eight patients infected with bat rabies variant. None progressed to develop dumb rabies. One had nerve conduction abnormalities of the weak limb. Myoclonus was found in six patients, which was generalized in two and was confined to both arms in one, to the left arm in one, to the left leg and trunk in one and to both legs in the other. Segmental myoclonus progressed to a generalized pattern in two patients. Hemiparesis was demonstrated in two patients, with hemisensory loss with Horner syndrome in one and asymmetry of deep tendon reflexes in the other. Nine patients might have had involvement of the brainstem or cranial nerves, as they presented with one or more of the following: anisocoria, bilateral ptosis, diplopia, nystagmus, pinpoint pupils, intermittent facial nerve palsy and tremor, paralysis of extraocular muscles and unilateral vocal cord paralysis. *Post mortem*, one was found to have extensive demyelination.

Convulsive and non-convulsive seizures were observed in eight patients who remained alert and coherent. One presented with status epilepticus. The patient infected with bat rabies variant in Australia in 1996 had pain and numbness in her left arm and later developed dizziness, diplopia, complete ophthalmoplegia and progressive weakness of all limbs. The most recent case had paraesthesia in the left hand, painful muscle spasms and rabies-like encephalitis. In the USA, where half of the cases are associated with variants found in insectivorous bats, hydro- and aerophobia were found in only 50% of cases. Phobic spasms were not described in four patients infected with bat rabies variant in 1997.

With canine rabies variant, weakness of the bitten extremity was observed only in patients who subsequently developed dumb rabies. Myoclonus, tremor, oculomotor abnormalities and cerebellar deficit were not observed, and neither hemisensory loss nor hemiparesis was found. Horner syndrome and loss of sweat on the right side of the face and trunk were seen in one patient infected with canine rabies variant. Seizures of the non-convulsive type during the early neurological phase were seen in one patient. Hallucinations were common in patients infected with bat rabies variant but in only one infected with canine rabies variant.

Since 1997, unusual manifestations have been seen in Thai patients infected with canine rabies variant [1]. Of 14 patients, five had atypical clinical features. One had ocular myoclonus and hemichorea. Another had spontaneous repeated ejaculations with pleasure and did not show the cardinal signs of rabies until the pre-terminal phase. One boy with paralytic rabies preserved deep tendon reflexes in all limbs until coma. A patient with a 2-month history of dog bite on the left wrist, presenting with severe pain in his left arm and later with loss of pinprick and joint position sense and weakness of the left hand, showed only nocturnal agitation, remaining calm and rational during the day. He had no phobic spasms, signs of autonomic stimulation or difficulty in swallowing. Monoclonal antibody analysis implicated a variant not commonly found in rabid dogs in Thailand. Another patient with severe itching on his right leg as a prodrome subsequently developed paralysis of both legs and facial and bulbar musculature with sparing of both upper extremities. Loss of deep tendon reflexes was confined to the legs. Three of the 14 patients who remained fully awake or were still arousable died

suddenly. The most recent case (November 2000, Hemachudha, unpublished) had bilateral arm paralysis accompanied by loss of pinprick sensation on both upper extremities and allodynia confined to the chest region. Weakness progressed to involve all the limbs, with loss of deep tendon reflexes. He subsequently lapsed into coma without exhibiting phobic spasms or agitation.

Laboratory findings

Routine laboratory examination is not adequate for diagnosis [1, 4]. The complete blood count is usually normal or shows mild leukocytosis with neutrophilia. Hyponatraemia is present in approximately one-third of cases, regardless of the clinical type or stage of the disease, owing to inadequate water intake due to dysphagia and hydrophobia or inappropriate secretion of antidiuretic hormone. Hypernatraemia with polyuria has also been described but occurs rarely. The cerebrospinal fluid (CSF) is normal in most cases, but a profile of mild pleocytosis with lymphocytic predominance and slightly elevated protein concentration has been found occasionally. Pleocytosis of > 100 cells/dL is rare.

An 8-h video-electroencephalographic recording of a 24-year-old patient with furious rabies showed well developed, low-to-medium amplitude, posteriorly dominant, 8-10 Hz, irregular background alpha activity [1]. Intermittent low-to-medium amplitude, 2-5 Hz mixed delta activity over the left and right temporal areas was noted as the patient became more confused. When the patient was obtunded 1.5 h before death, the background showed increasing runs of medium-to-high amplitude, diffusely distributed, 2-5 Hz mixed delta-theta activity. Approximately 6 min before death, a burst suppression pattern appeared, which was then followed by an electrocerebral silence. At this stage, with an electroencephalographic pattern mimicking brain death, the latencies of visual and brainstem auditory evoked potentials were within normal limits.

The electromyographic pattern correlates with the stages and forms of the disease [Mitrabhakdi *et al.*, submitted]. Diminished sensory action potentials were found in the affected limb, regardless of the clinical type of rabies, when the patients had a local prodrome. In cases of furious rabies, acute denervation potentials indicative of anterior horn cell damage in the affected limb segments were found earlier and progressed diffusely to the contralateral limb and rostrally and caudally along the spinal cord. This was found very late in dumb rabies. F and H reflexes were markedly prolonged or absent early in the course in dumb rabies. These symptoms were followed by other demyelinating features like those in Guillain-Barré syndrome. During the pre-terminal stage, the electromyographic patterns did not distinguish nerve and anterior horn cell damage.

Computerized tomography of the brain usually does not reveal abnormalities, with either the plain or contrast enhancement technique. Rarely, multiple bilateral areas of decreased density involving both the grey and white matter can be found. Magnetic resonance imaging of two recent patients with paralytic rabies showed gadolinium-enhancing lesions mainly in the brainstem, thalamus, hypothalamus and grey matter of the cervical spinal cord and roots [1, 7, 8]. Imaging in two patients with furious rabies showed a similar predilection of midline structures [Laothamathas *et al.*, submitted]. Similar abnormalities in the brainstem, thalamus and basal ganglia have been described

in arboviral encephalitides, such as eastern equine and Japanese encephalitis, and enterovirus 71 [9-11]. The absence of oedema, haemorrhage, negative computerized tomography findings and preferential involvement of the brainstem can reliably differentiate rabies from other arboviral encephalitides and enterovirus infections. The brainstem was found to be abnormal by non-contrast magnetic resonance imaging in two cases of encephalitis and paralysis associated with bat and canine rabies variants [12, 13].

Differential diagnosis of furious rabies

Acute hepatic porphyria with neuropsychiatric disturbances, such as psychosis, seizures, signs of autonomic dysfunction and involvement of the peripheral nervous system, can be mistaken for rabies. Fluctuating consciousness is observed in both conditions, but phobic and inspiratory spasms are seen only in rabies. A family history of the disease, ingestion of porphyrinogenic agents, abdominal pain and dark urine with elevated delta-aminolaevulinic acid and porphobilinogen concentrations, aid the diagnosis.

Other disease conditions that mimic rabies during the acute neurological phase include intoxication by a variety of substances, such as atropine-like compounds, cannabis, alcohol and tetanus. Acute serotonin syndrome in the form of encephalopathy or seizures described in individuals taking serotonin re-uptake inhibitors can be misdiagnosed as rabies, but may be reliably excluded by the absence of other cardinal signs. Tetanus resembles rabies only in the form of reflex spasms [14]. All tetanus patients have a clear sensorium, whereas rabies patients do not have persistent rigidity or sustained contraction of axial musculature such as the jaw, neck, back and abdomen, as seen in tetanus. Spasms in rabies predominantly involve the accessory respiratory muscles and diaphragm, whereas in tetanus, spasms occur in muscles of axial structures. Opisthotonos is far more frequent in tetanus than in rabies.

In parts of the world where nervous tissue rabies vaccine is still widely used, allergic encephalomyelitis must be considered in the differential diagnosis. These "accidental" complications develop in approximately one in 400 vaccinees and appear between 6 and 14 days after the first injection in over 75% of affected individuals [15, 16]. Delayed onset and a picture of chronic progressive encephalitis have also been observed. Neither phobic spasms, paraesthesia at the bite site nor fluctuating consciousness are present in these post-vaccination reactions.

Differential diagnosis of dumb rabies

Acute motor axonal neuropathy, an axonal form of Guillain-Barré syndrome, shares many similar clinical features with paralytic rabies. Acute motor axonal neuropathy after *Campylobacter jejuni* infection may be preceded by diarrhoea, which may be mistakenly diagnosed as a prodromal symptom of rabies. Quadriparesis, bilateral facial weakness and areflexia without sensory deficits are observed in both conditions. Urinary incontinence is common from the beginning in paralytic rabies. Inspiratory spasms with abnormal behaviour may appear late in the disease and may be masked by severe generalized paralysis and superimposed electrolyte and metabolic disturbances, which

may occur in both conditions. An acute inflammatory demyelinating form of Guillain-Barré syndrome or acute motor sensory axonal neuropathy and Guillain-Barré syndrome-like syndrome after administration of nervous tissue rabies vaccine results in some degree of sensory deficit, which is usually absent in paralytic rabies [15, 16]. Further, local symptoms on any limb or one side of the face, even without a history of bite, initial autonomic dysfunction, especially hypersalivation, abnormal pupils and piloerection, suggest paralytic rabies.

Electrophysiological studies of peripheral nerves cannot distinguish paralytic rabies from Guillain-Barré syndrome. Demyelinating features may be found early, followed by findings suggestive of both axonal and myelin damage.

Asymmetrical weakness in an unimmunized patient in an epidemic setting suggests paralytic poliomyelitis or atypical forms of Japanese encephalitis. The outbreak of paralytic rabies in Trinidad was initially thought to be poliomyelitis.

Establishing the diagnosis

Ante-mortem diagnosis of human rabies is extremely important. Delays in diagnosis result in a potential spread of contamination and an unnecessary need for post-exposure prophylaxis. Although there is no established evidence of human-to-human transmission, saliva and tracheal secretions frequently contain virus.

On the basis of clinical grounds alone, furious rabies can be diagnosed with confidence when three classical or major cardinal signs are present together: fluctuating consciousness, phobic spasms and autonomic dysfunction. However, in areas where canine rabies is not endemic, such as North America, and where bats become the principal vector of rabies to humans, these clinical expressions may be variable. Phobic spasms were found in only half of cases. However, either phobic spasms alone or the presence of three or more of the following – agitation, confusion, seizures or dysphagia, hypersalivation, limb pain, paresthesia, limb weakness, paralysis or ataxia – were significantly associated with ante-mortem diagnosis [17]. Nevertheless, these clinical features may overlap with those associated with enterovirus 71 or Nipah virus encephalitis [11, 18]. Local prodromal symptoms alone, although considered by the patients as severe, must be interpreted with caution, since they may be modified by the patientís anxiety, fear of rabies and wound infection. A definite history of a bite, although commonly found in cases of canine rabies variant infection, is not helpful in cases associated with bat rabies variant. Of the 21 cases of infection with bat rabies variant reported since 1980 in the USA, only one had a definite history of a bat bite.

Serological testing of serum and CSF should be the easiest and most practical method but is of limited value [1, 17, 19]. Low rabies antibody titres, as determined by the rapid focus fluorescent inhibition test in serum, were detected in six of 31 unvaccinated rabies patients tested within 1-26 days after onset of the disease. None of the 27 unvaccinated Thai patients infected with canine rabies variant had antibodies against rabies virus in their CSF [1]. All serum samples containing antibody were obtained within 9 days after onset, which differs from cases in the western hemisphere. Analysis of 102 samples from 39 cases in the USA and 16 cases in France since 1960 showed that

antibody usually develops if the patients survive more than 8 days; antibodies appeared in CSF later [17, 19].

Rabies virus may be isolated from saliva specimens in mouse neuroblastoma cells [17]. This cell culture method is sensitive and specific, and the results are available within 4-5 days; however, all samples tested must be maintained frozen after collection, with no preservatives. The success rate also depends on the status of rabies antibody [87% (13 of 15) positive in antibody-negative and none (0 of 17) positive in antibody-positive patients] and the intermittence of rabies virus shedding in the saliva. False-negative results may be obtained in decomposed brain, in the case of biopsy specimens or post-mortem material.

Rabies viral antigen may be detected by the fluorescent antibody technique in frozen sections of nuchal skin obtained by biopsy. At least 20 sections must be examined to detect rabies nucleocapsid inclusions around the base of hair follicles. The result is unrelated to the antibody status. The proportion of positive results tends to increase as the disease progresses. However, in another 26 rabies patients studied, antigen could be detected in as many as five of six patients within 4 days after onset. This number dropped to 6 of 10 between days 5 and 8 and to 7 of 10 from day 9 [19]. Detection of rabies viral antigen in corneal and salivary impression smears is unreliable [1, 4].

We have developed the nucleic acid sequence-based amplification technique to detect rabies-specific RNA in saliva and cerebrospinal fluid [20]. This technique involves avian myeloblastosis virus reverse transcriptase, *Escherichia coli* RNase H and T 7 RNA polymerase enzymes to amplify an RNA template under isothermal conditions. By using an appropriate pair of primers, one of which contains the T 7 RNA polymerase binding site to the target RNA, a large number of RNA copies can be generated and can be detected with an automated reader, provided that an electrochemiluminescence detection region is attached to the 5' end of the other primer.

Nucleic acid sequence-based amplification detection of rabies-specific RNA in saliva and CSF allowed a correct diagnosis in four patients *(Table I)*. Three had antibodies in saliva (five of eight samples); two saliva samples without antibody were collected from one dumb rabies patient (on days 3 and 7), but this patient had antibodies in the CSF. A negative sample was also obtained from another patient (on day 6), whose remaining samples (on days 2, 3 and 7) were positive. Two of three CSF samples were positive. All 21 CSF-negative control samples were negative.

A previous report of the use of reverse transcriptase polymerase chain reaction on saliva allowed a diagnosis of rabies in five of nine patients [19]. A sensitivity of 100% (10 of 10) was achieved in one series only after the virus variants responsible for the infections were known and appropriate primers could be identified [17]. The nucleic acid sequence-based amplication technique is relatively easy, and the whole process, from extraction, to amplification to detection requires approximately 4 h. The sensitivity might be improved if the samples were collected in lysis buffer to prevent degradation of RNA.

Table I. Diagnosis of rabies with the nucleic acid sequence-based amplification technique.

Form of rabies	Time of sample collection (days after onset)	Saliva	Cerebrospinal fluid
Furious	3	+(2,614)	–
Dumb	3	–	+(156,203)
	7	–	
Furious	2	+(18,329)	
	3	+(195,347)	+(384,365)
	6	–	
	7	+(8,704)	
Furious	3	+(3,764)	

In parentheses, electrochemiluminescence signal.
–, cut-off level of 520.

Acknowledgements

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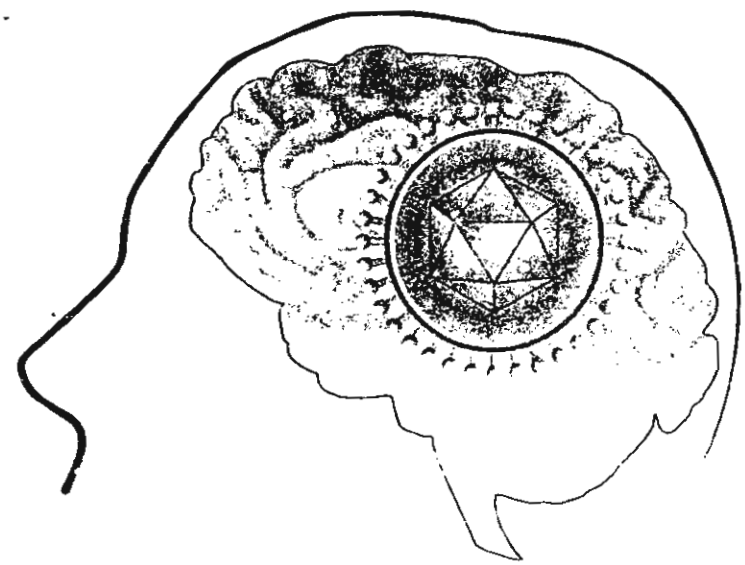
References

1. Hemachudha T, Mitrabhakdi E. Rabies. In: Davis LE, Kennedy PGE, eds. *Infectious diseases of the nervous system.* Oxford: Butterworth-Heinemann, 2000: 401-44.
2. Morimoto K, Patel M, Corisdeo S, *et al.* Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. *Proc Natl Acad Sci USA* 1996; 93: 5653-8.
3. Hemachudha T, Phuapradit P. Rabies. *Curr Opin Neurol* 1997; 10: 260-7.
4. Hemachudha T. Rabies. In: Vinken PJ, Bruyn GW, Klawans HL, eds. *Handbook of clinical neurology,* Revised series. Amsterdam: Elsevier Science Publishers, 383-404.
5. Hanna JN, Carney IK, Smith GA, *et al.* Australian bat lyssavirus infection: a second human case, with a long incubation period. *Med J Austr* 2000; 172: 597-9.
6. Smith JS, Fishbein DB, Rupprecht CE, *et al.* Unexplained rabies in three immigrants in the United States: a virologic investigation. *N Engl J Med* 1991; 324: 205-11.
7. Laothamatas J, Hemachudha T, Tulyadechanont S, Mitrabhakdi E. Neuroimaging in paralytic rabies. *Ramathibodi Med J* 1997; 20: 149-56.
8. Pleasure SJ, Fischbein NJ. Correlation of clinical and neuroimaging findings in a case of rabies encephalitis. *Arch Neurol* 2000; 57: 1765-9.
9. Dereswicz RI, Thaler SJ, Hsu I, Zamani AA. Clinical and radiographic manifestations of eastern equine encephalitis. *N Engl J Med* 1997; 336: 1867-74.

10. Kalita J, Misra U.K. Comparison of CT scan and MRI findings in the diagnosis of Japanese encephalitis. *J Neurol Sci* 2000; 174: 3-8.
11. Ho M, Chen ER, Hsu KH, *et al.* An epidemic of enterovirus 71 infection in Taiwan. *N Engl J Med* 1999; 341: 925-35.
12. Roine RO, Hillbom M. Valle M, *et al.* Fatal encephalitis caused by a bat-borne rabies-related virus. Clinical findings. *Brain* 1988; 111: 1505-16.
13. Hantson PH, Guerit JM, de Tourtchaninoff M, *et al.* Rabies encephalitis mimicking the electrophysiological pattern of brain death. *Eur Neurol* 1993; 33: 212-7.
14. Srikiatkachorn A, Hemachudha T. Tetanus. In: Gilman S, ed. *MedLink Neurology* CD (and www.medlink.com). Arbor Publishing Corp, 2001.
15. Piyasirisilp S, Hemachudha T, Griffin DE. B-cell responses to myelin basic protein and its epitopes in autoimmune encephalomyelitis induced by Semple rabies vaccine. *J Neuroimmunol* 1999; 98: 96-104.
16. Piyasirisilp S, Schmeckpeper BJ, Chandanayingyong D, Hemachudha T, Griffin DE. Association of HLA and T-cell receptor gene polymorphisms with Semple rabies vaccine-induced autoimmune encephalomyelitis. *Ann Neurol* 1999; 45: 595-600.
17. Noah DL, Drenzek CL, Smith JS, *et al.* Epidemiology of human rabies in the United States, 1980 to 1996. *Ann Intern Med* 1998; 128: 922-30.
18. Goh KJ, Tan CT, Chew NK, *et al.* Clinical features of Nipah virus encephalitis among pig farmers in Malaysia. *N Engl J Med* 2000; 342: 1229-35.
19. Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J Clin Microbiol* 1998; 36: 1117-21.
20. Watcharapluesadee S, Hemachuda T. Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. *Lancet* 2001 (in press).

เอกสาร 25

Clinical Neurovirology



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14

Rabies

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1 INTRODUCTION

Rabies is one of the most dramatic infections of the nervous system owing to its horrifying clinical presentations and almost invariably fatal outcome. It is also a complex disease, and the mechanism for the diverse presentations has not yet been clarified. The clinical symptomatology can vary considerably, particularly in cases associated with bats. Atypical clinical presentations resembling other viral encephalitides have been increasingly observed in Thailand since 1997, after exposure to canine rabies variants (crv). Differences in cellular tropism either at the inoculation site or in the nervous system, differences in routes of spread or host response, or differences in viral strains may account for this diversity.

With rapid movement of people among continents, human and animal cases can appear in regions where rabies was once eradicated or where it has never been recorded. It is crucial that healthcare providers be able to diagnose and differentiate rabies from other neurological disease and know how to provide postexposure prophylaxis.

2 HISTORY

The disease has been associated with the supernatural and the dog since antiquity. An early record appeared in the pre-Mosaic Eshnunna Code of Mesopotamia about 2300 BCE [1]. The name "rabies" has its roots in Sanskrit, rabhas, which refers to the god of death

and his dog, from the Vedic period of India (thirtieth century BCE). In ancient Egypt, the god Sirius was pictured as a furious dog. *Rabhas*, *Lyssa* or *Lytta* (Greek), and *rage* (French) refer to the cause of violence or madness. The term "hydrophobia" was coined by Cornelius Celsus, a Roman of the first century CE who provided a classic clinical description of this disease. The infectivity of saliva and urine from rabid dogs was suspected as early as Roman times, and the disease was attributed to a poison (*virus* in Latin). In 1804, transmission of infection by saliva was demonstrated. Burning and cupping of the wounds of those bitten by rabid dogs had been practiced since antiquity.

Girolamo Fracastoro (1478–1553) an Italian scientist, accurately determined the incubation period of rabies in humans. Virus advance to the central nervous system (CNS) via the nerves was postulated in 1769 by Giovanni Battista Morgagni on the basis of symptoms of paresthesia at the bite site. Pasteur demonstrated the CNS as a prime target and proved that the disease mainly affected the brainstem [2]. He also showed that nervous tissue was infectious as well as saliva. Moreover, the virus can be attenuated by serial passages. On July 6,1885, he used his rabbit spinal cord rabies vaccine in two patients who had been exposed to rabies [1,2]. The development of this vaccine involved over 90 serial intracerebral passages of rabies virus in rabbits, followed by air drying, which resulted in loss of infectivity. Although Pasteur's treatment saved the lives of countless victims, it carried some serious neuroparalytic risks, an immune-mediated encephalitis and neuritis [3–5]. The discovery of "endocellular Negri bodies" was made by Adelchi Negri in 1903. The diagnostic value of this was demonstrated in 1913 by Negri's wife, Lina Negri-Luzzani [6].

Modern cell culture rabies vaccines have proved to be close to ideal immunogens because of their efficacy, the few injections required, and their relative lack of side effects. Human diploid cell rabies vaccine and antirabies serum produced in mules protected all but one of 45 persons who were severely bitten by rabid dogs and wolves in northern Iran in 1975 [7]. Other equally safe and effective vaccines include purified Vero cell, chick embryo cell, and duck embryo rabies vaccines [5]. Experimental vaccines being developed include oral and parenteral poxvirus- and adenovirus-vectored recombinant and parenteral plasmid rabies vaccines and edible vaccine, a purified virus protein from tobacco plants infected with recombinant alfalfa mosaic virus displaying rabies glyco- and nucleoproteins [8].

It has become clear that aggressive wound care and the administration of purified rabies immunoglobulin (RIG) plus vaccine have saved more severely exposed patients than vaccine alone [5].

3 EPIDEMIOLOGY

Rabies is a zoonosis of domestic and wild mammals. About 50,000 people die of rabies every year. However, with deaths in India alone reaching 30,000 annually, this is likely to be a gross underestimate. Underestimation is undoubtedly a contributory factor to rabies being ranked low on the priority lists for disease control programs of the World Health Organization (WHO) and developing countries [9]. The domestic dog is the principal but not exclusive reservoir host in Asia, Africa, the Pacific islands, and South America. Wildlife such as the mongoose, jackal, and meerkat of South Africa; the pariah dog and jackal of Southeast Asia; the vampire bat of South America; and the fox of eastern Europe also play a significant role in epizootic transmission [8,9].

In North America, there is an epizootic in raccoons in the Mid-Atlantic and northeastern states. Other reported rabid animals include skunks, foxes, insectivorous bats, cats,

horses, and dogs [8]. Transmission from bats was the most common cause of human cases of rabies. In the United States and Canada, during 1980 and 2000, bat rabies variants (brv) were identified in 27 of 42 patients [8,10]. Twenty of these 27 cases had evidence of infection with a variant found primarily in the silver-haired bat (*Lasionycteris noctivagans*) or eastern pipistrelle bat (*Pipistrellus subflavus*). Only two gave a definite history of bat bite. Australia, a previously "rabies-free" continent, had become a lyssavirus-endemic area by 1996 [8,9]. This new variant pteropid lyssavirus or Ballina virus (for Ballina, New South Wales, Australia, where the first human infection was contracted) has been found in fruit bats (flying foxes, genus *Pteropus*) and has also been identified in other bats, including insectivorous species. Since November 1996, two human cases of rabies-like illness have occurred. Virtually all of Asia's human deaths from rabies were of people who did not receive postexposure treatment. Of 13 treatment failure cases between 1992 and 2000 in Thailand, all but three had treatment flaws or deviation from WHO recommendations [9,11; personal experience (TH)].

Although legally enforced canine rabies immunization, a practice of strict quarantine, and rigid control of stray dogs have virtually eliminated canine rabies in the western hemisphere, Australasia, and Japan, rabies has shifted to wild or sylvan carnivorous animals in some of these areas. In many Asian countries, cultural and religious customs still prevent reduction of the stray dog population.

4 VIROLOGY

Rabies virus with its distinct bullet shape belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family. The classical rabies virus, isolated from terrestrial mammals including dogs and hematophagous and insectivorous bats, is of sero- and genotype 1 which is the most prevalent worldwide [8]. Comparison of the viral nucleoprotein gene (N) allowed delineation of seven genotypes. Serotype classification is based on differences in viral antigens and antibodies produced by the host (Table 1). The rabies virus contains a single-stranded, antisense, nonsegmented RNA molecule of 11,932 nucleotides of negative polarity. It measures approximately 180 mm × 75 nm and has regularly spaced knoblike spikes on a cylindrical envelope, except for the flat end of the "bullet." The rabies genome contains a leader of 50 nucleotides, followed by genes that encode five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L). All rhabdoviruses have two major structural components: a helical ribonucleoprotein core (RNP) and a surrounding envelope. In the RNP, the genome RNA is tightly encased by the N protein. Two other viral proteins, P and L, are associated with the RNP. Transcription and replication of the virus are ensured by the RNP complexes of these N, P, and L proteins. The glycoprotein forms approximately 400 trimeric spikes, which are tightly arranged on the surface of the virus. Changes in the G protein amino acid sequence have a strong influence on viral virulence. The M protein is associated with both the envelope and the RNP and may be the central protein of rhabdoviral assembly. The arrangement of these proteins and the RNA genome determine the structure of the rabies virus.

5 PATHOPHYSIOLOGY

Human rabies is almost always attributed to a rabid animal bite. The risk of rabies due to bite is about 50 times that of scratches (5–80% versus 0.1–1%) [9]. Besides severity of the bite, efficient transmission also depends on the number of acetylcholine receptors at the site and the amount of virus in the saliva of the rabid animal [1,9]

Table 1 Seven Putative Genotypes in the Genus *Lyssavirus*

Genotype[a]	Serotype	Virus[b]
1	1	Classical rabies virus
2	2	Lagos bat
3	3	Mokola
4	4	Duvenhage
5	5	EBL-1
6	5	EBL-2
7		Australian bat lyssavirus (Ballina virus)

[a] Genotypes 1 and 2 belong to phylogroup 2; the remainder belong to phylogroup 1.
[b] EBL = European bat lyssavirus.

Although rabies usually follows bite exposures, it can be acquired via inhalation from aerosolized virus in caves inhabited by rabid bats and in laboratory accidents with infected aerosolized tissues [8,9]. Transmission of rabies is also associated with handling and skinning of infected carcasses and exposure of the conjunctiva, oral mucous membranes, genitalia, and skin abrasions to the saliva of rabid animals. Human-to-human transmission other than by corneal transplantation has not been well documented [1,8,9]. Transplacental transmission has been rarely reported in humans, and infants born to mothers with rabies encephalitis were found to be healthy [9].

Binding of viral glycoprotein to the alpha subunit of nicotinic AchR (nAchR) leads to multiplication in the muscle cells [9]. Following primary infection, the virus undergoes an "eclipse" phase. This silent phase is variable and may be explained by localization of virus within the muscle, which in turn provides an opportunity for host immune clearance and for postexposure treatment. Rabies antigen and genome may exist for as long as 2 months after inoculation into the muscle [12]. It is not known which factors control the length of this silent delay period.

After budding from plasma membranes of the muscle cells, virus or its genome is taken up into unmyelinated nerve endings at the neuromuscular junctions or at the muscle spindles. Rabies virus is then transported to the CNS via retrograde axoplasmic flow. The virus then infects and replicates again in the dorsal root ganglia and anterior horn cells [13,14]. At the dorsal root ganglia, it is presumed that viral replication can then be recognized and attacked by immune effectors, giving rise to clinical prodromal symptoms at the bite site [9]. Direct viral entry into the nerves without prior replication in the muscles may explain the short incubation period of less than 7 days demonstrated in a patient who had bite injury to the brachial plexus [15].

In the case of cryptic bat rabies, where a history of exposure is rarely obtained, the epidermis and dermis, rather than muscle, may serve as portals of entry [16]. Local sensory prodromes reflecting ganglioneuritis are present in 30% of crv cases (versus 70% in bat-related cases). This suggests that in brv cases, a sensory pathway may be preferential [9].

Travel time from the peripheral nerve to the CNS is relatively constant at a rate of 8–20 mm/day and depends on the proximity of the inoculation site to the CNS [14]. Studies of a preparation of challenge virus standard (CVS) in chick nerve–muscle cocultures have shown that the neuromuscular junction is the major site of entry to neurons. Colocalization of virus and endosome tracers within the nerve terminals, along with progressive accumula-

tion of virus and tracers in axons and nerve cells, indicated retrograde transport of endocytosed virus from motor nerve terminals [17]. Because nAchR is not present on all categories of neurons susceptible to rabies virus, it is unlikely to be the only receptor that mediates viral entry into neurons. Rabies virus may also use carbohydrates, phospholipid, gangliosides, neural cell adhesion molecule (NCAM or CD 56), and low-affinity nerve growth factor receptor-P75 neurotrophin receptor (P75NTR) to gain entry into the cells [9].

Once rabies reaches the CNS, rapid amplification occurs. Virus disseminates via plasma membrane budding and direct cell-to-cell transmission or by trans-synaptic propagation [14]. The G protein is required for attachment to neuronal receptors and trans-synaptic spread [18]. Following stereotactic inoculation into the striatum, rabies virus has been shown to travel by retrograde fast axonal transport. This transport involves an interaction between viral capsid P protein and microtubule dynein [19,20]. Neurons are the CNS cells selectively and dominantly involved. However, infection of astrocytes and glial cells in animals and humans has also been reported [1]. Negri bodies, a pathological hallmark of rabies, result from excessive accumulation of ribonucleoprotein (RNP) in the cytoplasm [8]. In human rabies, clinical and laboratory evidence suggests that differential response at various CNS regions may contribute to the diversity of clinical manifestations [9,15]. Regional CNS rabies antigen distribution as well as magnetic resonance imaging (MRI) of the brain are similar in both encephalitic and paralytic forms [9,21] (Figs. 1A, 1B). Brainstem, thalamus, basal ganglia, and spinal cord are preferential sites in both forms. Minimal or absence of rabies viral antigen was found in the hippocampus and neocortex. Despite a similar MRI localization in brainstem in both brv and crv rabies patients, brainstem signs and myoclonus are usually lacking in the latter [9,22]. Inflammatory reactions are usually scant and when they are present do not correlate with clinical manifestations. The presence of virus in the CNS does not determine the clinical severity of the disease. High titers of virus in the brain and spinal cord can be found in animals long before clinical signs appear [1]. The degree of muscarinic AchR function modification in the hippocampus of rabid dogs was not dependent on the viral load [23]. Rabies virus antigen was readily demonstrable by immunofluorescence in the frontal area of one paralytic patient who remained fully conscious and had quadriplegia and respiratory failure requiring ventilatory support [1].

The animal host and viral strain may not be the major determinants of clinical manifestations, although rabies after a vampire bat bite is almost always of the paralytic form [1]. A recent outbreak of human rabies in the Peruvian jungle that was transmitted by vampire bats, however, presented as the furious (encephalitic) form [24]. Furthermore, the same dog that transmitted paralytic rabies to one patient also caused classical encephalitic rabies in another [9]. Incomplete immunization was not associated with any certain clinical form of human rabies [1].

It remains uncertain why rabies in patients with intact cellular immunity to rabies virus tends to manifest as encephalitic rabies. In theory, rabies infection of the CNS, particularly the brainstem, leads to the production of cytokines and proinflammatory molecules such as IL-1, alpha/beta, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), interferons (IFN), and NO and to the secretion of chemokines [9]. These cytokines can activate the TNF-α p55 kDa receptor, resulting in the recruitment of T and B cells. This may therefore lead to the promotion of immune recognition against rabies virus at such an "immune-privileged" site. In addition, these cytokines can modify hippocampus and other limbic system functions, including the electrical cortical and HPA axis activities and serotonin metabolism. Immune activation also leads to further cytokine production, thus accentuating limbic symptomatology. Delayed mortality was observed in mice deficient

Figure 1 (A) Sagittal T1-weighted MRI with gadolinium. Enhanced lesions at the brainstem in a paralytic rabies patient.

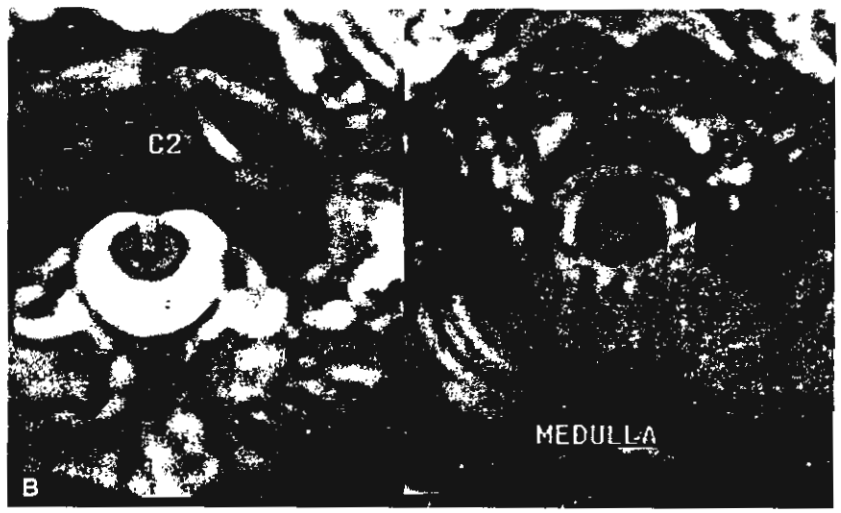

Figure 1 (B) Axial T1-weighted MRI with gadolinium. Enhanced lesions at the level of cervical cord and medulla in a paralytic rabies patient. (Courtesy of Dr. Jiraporn Laothamatas, Ramathibodi Hospital, Bangkok, Thailand.)

for the p55 kDa TNF-α receptor as a result of an increase in IFN-γ and IL-10 concentration and a reduction in inflammatory cell infiltration in the CNS. This may indicate that cytokines, which signal via p55 kDa TNF-α receptor, have a negative effect on survival of the host [9]. Furious rabies patients die faster, on the average in 5 days (versus 13 days in dumb rabies). In paralytic rabies, the exaggeration of cytokine production might not occur due to the lack of immune recognition of rabies virus in the brainstem. This possibly explains the relative paucity of limbic dysfunction and the absence of cellular immune activity to rabies virus in the paralytic patients.

The nature of weakness in paralytic rabies has not yet been fully explained. Although spinal cord motor neurons are likely to be involved at some point, it is still not known whether anterior horn cells or peripheral nerve dysfunction contribute to such clinical weakness, particularly in the early phase of clinical illness. There is also no conclusive information regarding the neuropathic pain at the bitten region, which is thought to be related to sensory ganglioneuropathy. Our preliminary studies of nerve conduction do not differentiate paralytic rabies from Guillain-Barré syndrome (GBS).

Eventual centrifugal spread from the CNS along neural pathways to the heart, skin, and other organs, especially salivary and serous glands of the tongue, is an important component of the complete rabies cycle [8]. Peripheral tissues such as the nape of the neck and cornea can serve as diagnostic tools [5]. Because all neural and non-neural organs, except for blood, may contain viable virus, transplant organs obtained from patients with an unexplained neurological disease may transmit rabies [5].

6 CLINICAL FEATURES

Not every rabid animal bite results in clinical rabies. Rabies mortality after untreated bites by rabid dogs varies from 35% to 57%, depending on the severity and location of the wound and presumed virus concentration in the saliva [8,9,15]. Transdermal bites, particularly with bleeding, on the head, face, neck, and hand carry the highest risk and are usually associated with a shorter incubation period. Nevertheless, all bites should be treated with the same urgency. In the case of rabid bats, the risk is present even from a scratch, owing to the unique ability of these agents to replicate in the skin.

The clinical features of rabies can be classified as classical and nonclassical. The classical encephalitic (furious) and paralytic (dumb) forms are almost always attributed to the canine rabies variant (crv) of genotype 1. Nonclassical rabies is found in patients exposed to bats (genotype 1, 5, 6, or 7) and has recently been found in Thai crv rabies – infected patients [9]. Atypical presentations were also seen in rare rabies survivors [1,9] (see Sec. 6.5).

The clinical features of classical rabies can be divided into five stages: the incubation period, the prodrome, the acute neurological phase, coma, and death. The first four are discussed briefly in Secs. 6.1–6.4.

6.1 Incubation period

The incubation period for rabies is usually 1–2 months but may range from less than 7 days to more than 6 years [9,25,26]. The cases with unusually long incubation periods (27 months; 4 and 6 years) were associated with Australian bat lyssavirus and have been found in immigrants to the United States from southeast Asia. An incubation period of less than a week has been seen with direct inoculation of the virus into nervous tissue, as

in patients with brachial plexus injury from dog bites [15]. Rabies cannot be excluded by the absence of a history of rabid animal bite, particularly in rabies-endemic areas where unrecognized exposures are common.

6.2 Prodrome

The prodromal stage begins when the virus enters the dorsal root ganglia and subsequently the CNS. At this stage, symptoms are vague and nonspecific such as fever, generalized muscle aches, and gastrointestinal disturbances. However, approximately one-third of patients with dog-related infections (regardless of clinical types) and three-fourths of those with bat-related disease experience local neuropathic symptoms at the bite site. The intense local prodrome of burning, itching, or piloerection, which starts at the wound and gradually spreads to the entire limb or the same side of the face in a nonradicular pattern, is a reliable indicator of rabies [1]. Rarely, these symptoms occur at locations remote from the bite site. For instance, two patients developed severe itching on the ears as a prodrome after having been bitten on their toes [1,15].

6.3 Acute Neurological Phase

Within hours or days after the prodrome, rabies patients enter the acute neurological phase. Two-thirds suffer from an encephalitic (furious) form, and the remainder present with paralysis resembling GBS [1,15]. The average length of survival is 5–7 days in furious cases and 13 days in paralytic cases. Mental dysfunction can be seen in furious cases as well as in some paralytic patients but to a much greater degree.

Encephalitic (Furious) Rabies

The earliest neurological feature resembles an intense anxiety reaction or acute psychosis. This can be aggravated by thirst, fear, bright light, or loud noise. Fever is a constant finding and may have started after the prodromal phase. There are three major cardinal signs of furious rabies: (1) fluctuating consciousness, in which the mental state alternates between periods of severe agitation and periods of normality or depression; (2) phobic and inspiratory spasms, which result from spasms of the accessory respiratory muscles of the neck and diaphragm followed by neck flexion or extension and end with perception of dyspnea; and (3) signs of autonomic dysfunction, with hypersalivation, pupillary abnormality, piloerection, excessive sweating, priapism, repeated ejaculation, and neurogenic pulmonary edema.

In classic furious rabies, there are usually no cranial nerve deficits or hemitract signs (i.e., hemiparesis or hemianesthesia). Seizures are rare but may occasionally be seen in fully developed rabies or in the preterminal phase. As the disease progresses, fluctuation of mental state is no longer observed, and the period of irritability is followed by deterioration of consciousness and coma.

Aero- and hydrophobia can be incited by blowing or fanning air on the face or chest wall of the patient and by encouraging the patient to swallow or by merely offering a cup of water. During the induced spasms, patients are extremely aroused and may display a fearful facial expression. Pharyngeal spasms may not necessarily be present, but when evident the patients may spit abundant saliva. This creates another characteristic and terrifying image of rabies. The first attack of hydrophobia may occur suddenly without prior swallowing difficulties or pain on swallowing. This argues against this being a conditioned reflex. One patient had his first experience of hydrophobia while taking a bath. Soft palate

and pharyngeal sensation remains intact, but there is a hyperactive gag reflex. Once the patients lapse into coma, phobic spasms are replaced by intermittent inspiratory spasms that occur spontaneously every few minutes. These inspiratory spasms may actually present in the early stage but may not be identified because of their nonintense character and infrequent occurrence.

Paralytic (Dumb) Rabies

Paralytic (dumb) rabies can be difficult to diagnose due to a lack of aggression and relative sparing of consciousness. The major cardinal signs of rabies appear late or may be mild in paralytic cases. Phobic spasms occur in only half of the patients, whereas inspiratory spasms occur in all cases during the preterminal phase and may not be recognized as such. Weakness usually starts in the bitten extremity and progresses to all limbs and bulbar and respiratory muscles. Facial diaparesis is as common as in sporadic GBS. In the case of facial bites, weakness may initially involve ipsilateral facial and oculomotor muscles. However, no correlation between the development of paralytic rabies and the site of the bite has been found [21]. Presentations mimicking ascending myelitis with fasciculation, loss of joint position sense, or hypoesthesia to pinprick up to the thoracic level have been rarely observed [1].

The following features suggest paralytic rabies and serve to differentiate this disorder from GBS: persistent fever from the onset of limb weakness, intact modalities of sensory functions except at the bitten region, percussion myoedema, and bladder dysfunction [1,9]. It is unknown why percussion myoedema is present in paralytic rabies but not in encephalitic rabies or in neuroparalytic accidents following neural tissue vaccination. Myoedema has also been found in extreme cachexia, hyponatremia, hypothyroidism, renal failure, and syndrome of inappropriate secretion of antidiuretic hormone (SIADH) and therefore needs to be interpreted with caution.

Clinical manifestations in rabies patients after exposure to virus of insectivorous or frugivorous bat origin (genotype 1, 5, 6, or 7) differ in many ways from the classic forms of crv furious and dumb rabies. Local prodromes are much more common, as reported in 30 out of 46 brv-rabies patients documented between 1951 and 2000 [9,15]. There have been reports of radicular pain, objective sensory or motor deficits, and choreiform movements of the bitten extremity during the prodromal phase. Both focal brainstem signs and myoclonus are common. These brv-rabies manifestations correlate with MRI findings of abnormalities in the brainstem [22]. It is intriguing that despite the similar MRI abnormalities in crvrabies, obvious brainstem signs are usually not noted (Fig. 1). Other patients have been described as having hemiparesis or hemisensory loss, ataxia, vertigo, or Horner's syndrome. Convulsive and nonconvulsive seizures and hallucinations are frequent. Phobic spasms were described in only one out of six brv-rabies patients during 1997–2000 [1,9,10].

In crv rabies, weakness of the bitten extremity was usually observed only in patients who subsequently developed dumb rabies. Myoclonus, tremor, oculomotor abnormalities, and cerebellar signs were not present. Neither hemisensory loss nor hemiparesis was observed in crv patients. Horner's syndrome or loss of sweat on one side of the face and trunk was noted in one crv patient. Nonconvulsive seizures during the early neurological phase were seen in one patient [1]. Hallucinations were seen in only two crv patients in our experience.

Nonclassic presentations have been noted in at least six patients with dog-related rabies since 1997 at Chulalongkorn University Hospital alone [1]. One patient presented

with ocular myoclonus and hemichorea. The other had spontaneous repeated pleasurable ejaculations and did not exhibit cardinal signs of rabies until the preterminal phase. Other manifestations included paraparesis, facial and bulbar weakness with preserved arm strength, or bilateral arm weakness. No patients had phobic spasm or autonomic hyperactivity.

6.4 Coma

It is extremely difficult to diagnose rabies at the coma stage. The two forms of rabies are indistinguishable once the patients become comatose. Inspiratory spasms are useful in diagnosis at this stage but are difficult to detect in paralytic rabies due to weakness. In encephalitic rabies, abnormal breathing patterns and depression of consciousness appear simultaneously. Regular breathing interspersed with inspiratory spasms is replaced by tachypnea, apneustic respiration, and, finally, ataxic respiration. These patterns are not observed in paralytic rabies, in which alveolar hypoventilation and ventilatory failure develop before the patients become obtunded. Sinus tachycardia, disproportionate to the fever, is evident in most cases even when adequate hydration is maintained. Coma precedes circulatory insufficiency, the prime cause of death in most cases. Hematemesis is seen in 30–60% of patients 6–12 h before death [1,9].

6.5 Recovery

Seven patients with atypical rabies presentations have been reported to survive [9]. None of them had phobic spasms or other cardinal features of rabies. The first patient (1972), who was bitten by a bat, had unsteady gait, dysarthria, and hemiparesis. The second patient (1976), bitten by a rabid dog, had quadriparesis and generalized myoclonus at the early stage and later developed cerebellar signs (ataxia, dysmetria, and dysdiadochokinesia), frontal lobe dysfunction, and bibrachial weakness. The third patient (1977) had aerosol exposure to a highly concentrated fixed rabies virus strain. Of the remaining four patients, three were bitten by rabid dogs and one by a vampire bat. Each patient received prophylaxis promptly with cell culture vaccine but not rabies immunoglobulin. All the children developed encephalitis within a month of exposure, with high concentrations of neutralizing antibodies to rabies virus detected in the CSF. Acute signs persisted for months, and there were chronic sequelae.

7 LABORATORY FINDINGS

Routine laboratory studies are nondiagnostic. Complete blood counts are usually normal or show mild leukocytosis with neutrophilia. Hyponatremia is present in approximately one-third of the patients regardless of the clinical type or stage of the disease [1]. This can be explained by inadequate intake from dysphagia and hydrophobia or SIADH. Hypernatremia with polyuria is rare. CSF examination is normal in most cases. However, mild CSF pleocytosis (less than 30 cells/dL) with lymphocytic predominance and slightly elevated protein level (less than 100 mg/dL) in GBS-like patients who are HIV-seronegative should alert the clinician, particularly when fever, hyponatremia, and bladder dysfunction occur early in the course of illness. A pleocytosis of over 100 cell/dL (110–950) is rare and suggests other diagnosis. Magnetic resonance imaging can be helpful in antemortem diagnosis of rabies [9]. Paralytic and encephalitic rabies patients had similar distributions of abnormal, ill-defined, mildly hypersignal T2 images involving the brainstem, hippocam-