Virus Research 135 (2008) 144–149

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular epidemiology of rabies in Indonesia

Heru Susetya[a], Makoto Sugiyama[a,b,*], Akio Inagaki[b], Naoto Ito[a,b], Gatot Mudiarto[c], Nobuyuki Minamoto[a,b]

[a] The United School of Veterinary Sciences, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Laboratory of Zoonotic Diseases, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] Virology Unit, Animal Drug Assay and Certification Laboratory, Bogor, Indonesia

ARTICLE INFO

Article history:
Received 20 September 2007
Received in revised form 3 March 2008
Accepted 4 March 2008
Available online 16 April 2008

Keywords:
Rabies virus
Reverse transcriptase polymerase chain reaction
Molecular epidemiology
Indonesia

ABSTRACT

In order to clarify the genetic relationships and dynamics of rabies viruses that are epidemic in Indonesia, we determined and analyzed 1307 nucleotides of nucleoprotein genes of 34 rabies field isolates collected from Sumatra, Java, Kalimantan, Sulawesi and Flores islands. Results of phylogenetic analysis indicated that rabies isolates in Indonesia formed one cluster, were of Asian lineage, and were closely related to a rabies isolate in China rather than to rabies isolates in Thailand, India or Sri Lanka. Rabies isolates in Indonesia were divided into three phylogroups (ID1, ID2 and ID3) that included seven lineages. There was a correlation between phylogroup and geographical distribution of the isolates. Isolates in four lineages (SC1, SC2, SC3 and ST) of the ID1 phylogroup were mainly present in Sumatra. Isolates in the ST lineage were distributed widely in Sumatra, while isolates in the SC1, SC2 and SC3 lineages were limited to central Sumatra. ID2 and ID3 phylogroups included one lineage (JA) and two lineages (KS and SF), respectively. Results of phylogenetic analysis and historical background suggest that rabies viruses in China might have been transferred to Indonesia and spread to each island due to human activities.

1. Introduction

Rabies virus is distributed worldwide and causes lethal viral encephalitis in a wide range of host species. Although all mammals are susceptible to rabies infection, the vector species that transmit the virus to humans are limited to several canid and feline species and chiropteran species.

As a member of the *Lyssavirus* genus, rabies virus has a single-stranded RNA genome of approximately 12 kb containing N, P, M, G and L genes encoding nucleoprotein, phosphoprotein, matrix protein, glycoprotein and polymerase protein, respectively (Bourhy et al., 1992, 1993). The development in recent years of molecular methods of analysis such as reverse transcription and polymerase chain reaction (RT-PCR) and techniques for sequencing rabies genes has led to a better understanding of the distribution and genetic characteristics of rabies virus at both the global level (Smith et al., 1992; Kissi et al., 1995) and regional levels (David et al., 2000; Nadin-Davis et al., 1994; Nishizono et al., 2002; Liu et al., 2007).

Rabies has existed since at least the nineteenth century in Indonesia. Epidemiological form of rabies in Indonesia is urban rabies, in which domestic dogs act as the main reservoir. Despite the implementation of a campaign against dogs to eliminate rabies in 1989, rabies is still prevalent in Sumatra, Kalimantan, Sulawesi and Flores islands; there have been no reported cases of rabies in Java since 2000 (information from Directorate General of Livestock Production in Indonesia). In Indonesia, 135 cases of human deaths due to rabies and 3349 cases of post-exposure treatment for rabies were reported in 1999 (http://www.who.int/emcdocuments/rabies/whocdscsreph200210.html). However, there is little information on epidemics of rabies and dynamics of rabies viruses in Indonesia. Smith et al. (1992) reported that two rabies virus strains, one isolated from a dog and one from a human in Java, genetically belonged to the Asian group. However, this information is not sufficient for use in epidemiology of rabies in Indonesia because Indonesia consists of more than 13,000 islands and there is no genetic information on rabies viruses in other endemic areas of Indonesia. Genetic information on rabies viruses in endemic areas is important to trace the dynamics of rabies infection and eradicate rabies from Indonesia. In this study, we sequenced 1307 nucleotides of the N gene of rabies virus isolates from five islands in Indonesia and analyzed the phylogenetic relationships between these isolates and rabies strains originating

* Corresponding author at: Laboratory of Zoonotic Diseases, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Tel.: +81 58 293 2948; fax: +81 58 293 2948.
E-mail address: sugiyama@gifu-u.ac.jp (M. Sugiyama).

0168-1702/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2008.03.001

Table 1
Rabies field viruses from Indonesia used in this study

Island	Area	Location	Identification number	Year	Animal origin	Accession number
Sumatra	Northern	Medan	SN00-14	2000	Dog	AB154235
		Deli Serdang	SN00-03	2000	Dog	AB154234
	Central	Bukittinggi	SC02-90	2002	Dog	AB154243
		Bukittinggi	SC97-01	1997	Dog	AB154233
		Bukittinggi	SC02-89	2002	Dog	AB154231
		Bukittinggi	SC01-66	2001	Civet cat	AB154213
		Bukittinggi	SC01-74	2001	Cat	AB154209
		Bukittinggi	SC02-79	2002	Dog	AB154229
		Bukittinggi	SC02-82	2002	Cat	AB154211
		Bukittinggi	SC01-70	2001	Tiger	AB154242
		Bute	SC01-63	2001	Dog	AB154228
		Bukittinggi	SC02-87	2002	Monkey	AB154224
		Bukittinggi	SC01-65	2001	Deer	AB154214
		Bukittinggi	SC01-73	2001	Cattle	AB154212
		Bukittinggi	SC01-68	2001	Cat	AB154208
		Padang	SC00-36	2000	Dog	AB154226
		Tj. Mutiara	SC00-45	2000	Dog	AB154227
		Bukittinggi	SC02-83	2002	Dog	AB154230
		Bukittinggi	SC01-75	2001	Cat	AB154210
		Bukittinggi	SC02-91	2002	Dog	AB154232
		Pesisir Selatan	SC00-12	2000	Dog	AB154225
	Southern	Rejanglebong	SS01-21	2001	Dog	AB154238
		Bengkulu	SS01-13	2001	Dog	AB154237
Java	Western	Cirebon	JA97-05	1997	Dog	AB154220
Kalimantan	Eastern	unknown	KL00-18	2000	Dog	AB154221
		unknown	KL97-03	1997	Dog	AB154223
		Tapin	KL01-27	2001	Dog	AB154222
Sulawesi	Northern	Manado	SW97-04	1997	Dog	AB154241
	Southern	unknown	SW02-22	2002	Dog	AB154240
		Pangkep	SW01-11	2001	Dog	AB154239
Flores		Ende	FL01-06	2001	Dog	AB154215
		Flores	FL97-01	1997	Dog	AB154218
		Flores timur	FL02-08	2002	Dog	AB154216
		Sikka	FI02-10	2002	Dog	AB154217

from other regions in the world. Based on the results, the dynamics of rabies viruses in Indonesia are discussed.

2. Materials and methods

2.1. Sample collection

A total of thirty-four brain samples from twenty-five dogs, four cats, a cow, a civet cat, a deer, a monkey and a tiger were used for genetic analysis in this study (Table 1). The brain samples from dogs were collected in Sumatra, Java, Kalimantan, Sulawesi and Flores islands of Indonesia. The samples from the other animals were collected in central Sumatra. The civet cat was a wild animal and the others were domestic animals. After these animals had bitten humans or had shown signs of rabies, they were diagnosed as having rabies from the results of fluorescent antibody tests (Dean et al., 1996) carried out by the Animal Diseases Investigation Centers of Indonesia.

2.2. RT-PCR

Total RNA was extracted from each brain specimen using a Mag Extractor RNA Kit (TOYOBO, Osaka, Japan). The complementary DNA (cDNA) of the rabies genome was synthesized by using Ready-To-GoYou-Prime First-Strand Beads (Amersham Pharmacia, NJ, U.S.A) with positive sense primer RHN-19 (5′-AAAATGTAACACCT CTACAATG-3′) acting at positions from 52 to 73 (based on the nucleotide number of the viral genome of Nishigahara strain [Ito et al., 2001]). In the first step, the cDNA of N gene was amplified by PCR using PCR Beads (Amersham Pharmacia) with primer RHN-19 and negative sense primer RHNS-8 (5′-AGGGAGACTGTCCACTTCTATAGG-3′) acting at positions from 1641 to 1664. This first PCR amplified a DNA fragment of 1613 bp. The second PCR was performed by using positive sense primer RHN-17 (5′-TTCAAAGTCAATAATCAGGTGG-3′, at positions from 92 to 113) and negative sense primer RHNS-3 (5′-TCAGGATTGACAAACATTTTGCTC-3′ at positions from 1517 to 1540), which gave a DNA fragment of 1454 bp. The profiles of the first and second PCRs consist of an initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation for 30 s at 95 °C, reannealing for 1 min at 50 °C, and elongation for 2 min at 72 °C, and a final incubation for 5 min at 72 °C.

2.3. Direct sequencing

Amplified cDNA was employed for direct sequencing with Cy5-labeled primers RTNSEQ1 (5′-AGAATCAGGAGTGATCTTGTCTCC-3′, negative sense, at positions from 368 to 391), RTNSEQ2 (5′-CTATTTGGGAGAAGAGTTTTTTGG-3′, positive sense, at positions from 1099 to 1122), RTNSEQ 3 (5′-GCTGGAACCTACGACATGTTTTTC-3′, positive sense, at positions from 689 to 712), and RTNSEQ4 (5′-GCCCTGAGCAGTCTTCATAAGCAG-3′, negative sense, at positions from 762 to 785). After purification of the second PCR product using an UltraClean kit (MO BIO, CA, U.S.A), a cycle sequencing reaction was carried out by using an AutoCycle Sequencing Kit (Amersham Pharmacia). Sequencing products were analyzed by

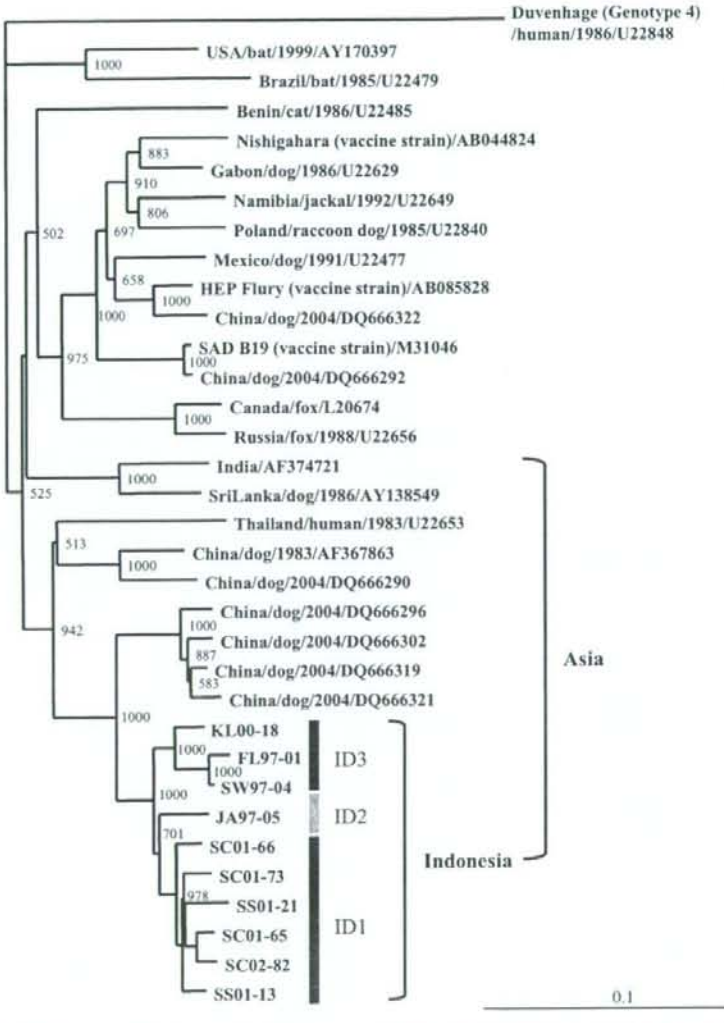

Fig. 1. A phylogenetic tree based on sequences of 1307 nucleotides of N genes from rabies viruses. Nucleotide sequences of 34 Indonesian isolates were determined in this study. The sequences of ten representative isolates were used for this phylogenetic tree. The remaining sequences were obtained from the GenBank database (country or strain/animal origin/year, accession number). The tree was constructed by using the Clustal X program. The genetic distances between nodes are proportional to branch lengths. Numbers at nodes are bootstrap probabilities that were calculated using 1000 replicates. Horizontal lines indicate degree of nucleotide difference. ID1, 2 and 3 indicate each phylogroup classified in Fig. 2.

using an ALF DNA Sequencer (Amersham Pharmacia). The N gene target sequence was 1307 b, corresponding to the nucleotides at positions 114 to 1420 of the viral genome of Nishigahara strain (Ito et al., 2001).

2.4. Genetic analysis

A 1307-nucleotide sequence of the N gene of the isolates from Indonesia was compared phylogenetically with those of strains from other regions in the world, the information being obtained from the GenBank database. Phylogenetic analysis was performed by the neighbor-joining method using Clustal X (Larkin et al., 2007). The bootstrap probabilities were calculated using 1000 replicates.

3. Results

3.1. RT-PCR and nucleotide sequence of N gene (nt 114–1420)

A specific PCR product (1454 bp) was amplified from all of the samples used in this study (data not shown). Nucleotide sequences (1307 b) of the N genes (nt 114–1420) of 34 rabies isolates (Table 1) were determined and compared with those of 20 isolates from other regions or three vaccine strains (see Fig. 1). Rabies isolates in Indonesia showed 88.4–90.2%, 86.1–88.4%, 85.9–87.4% and 86.2–87.4% nucleotide homologies to those in China, Thailand, India and Sri Lanka, respectively. They showed comparatively low nucleotide similarities (85.5–86.7%, 85.2–86.6%, 85–86.6% and 84.9–86.0%) to rabies isolates from Mexico, USA and Africa and the

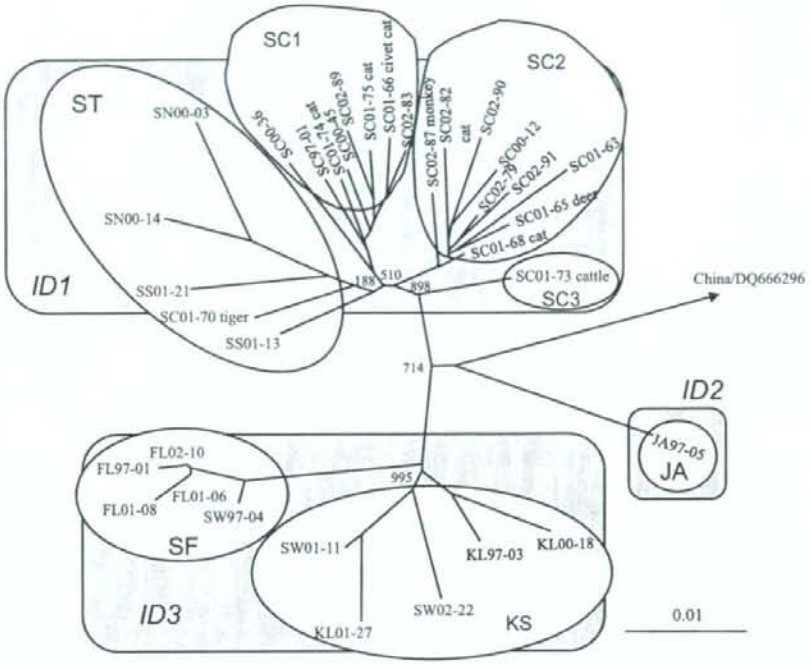

Fig. 2. A radical phylogenetic tree based on sequences of 1307 nucleotides of N genes of rabies field isolates in Indonesia. Thirty-four isolates in Indonesia determined in this study and one isolate in China obtained from the Gen Bank database were used for analysis. The tree was generated using the Clustal X program. The genetic distances between nodes are proportional to branch lengths. Numbers at nodes are bootstrap probabilities that were calculated using 1000 replicates. Horizontal lines indicate degree of nucleotide difference. ID1, 2 and 3 indicate each phylogroup. SC1, SC2, SC3, ST, JA, SF and KS represent each lineage. Animal species from which each isolate derived were indicated with identification number. All isolates without indication of the species were derived from dogs.

Nishigahara vaccine strain, respectively. The nucleotide homologies dropped to 74.2–75.4% compared to Duvenhage virus, a genotype IV rabies related virus. Nucleotide and amino acid sequences among isolates in Indonesia were 94.4–100% and 97.5–100% homologous, respectively.

3.2. Phylogenetic analysis

Phylogenetic analysis based on the N gene sequences (1307 b) of Indonesia isolates and street viruses from various parts of the world revealed that all rabies isolates in Indonesia formed one cluster and belonged to Asian lineage (Fig. 1). Rabies isolates in Indonesia are more closely related to four isolates in China, which were found in Jiangsu, Henan, Hunan and Guizhou provinces in 2004 (Zhang et al., 2006), than to isolates from Thailand, India or Sri Lanka. The rabies viruses in Indonesia were divided into three phylogroups designated as ID1, ID2 and ID3 (Fig. 2). Phylogroup ID1 consists of three lineages (SC1, SC2 and SC3) and one lineage (ST) formed by isolates from Sumatra. Phylogroup ID2 consists of one lineage (JA) formed by an isolate from a dog in Java. Phylogroup ID3 consists of two lineages (KS and SF) formed by isolates from eastern areas of Indonesia, Kalimantan, Sulawesi and Flores islands. The bootstrap

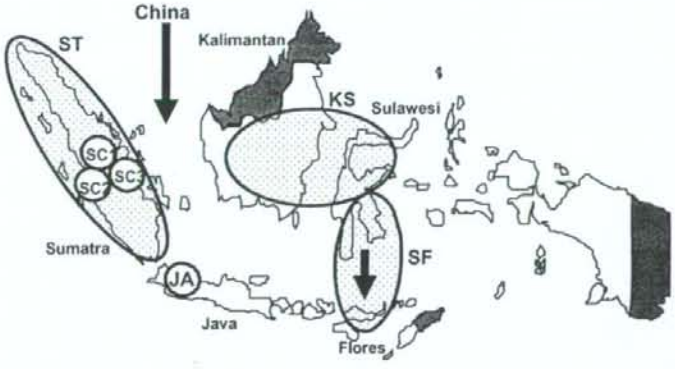

Fig. 3. Geographical distribution and proposed dynamics of rabies field isolates in Indonesia. SC1, SC2, SC3, ST, JA, SF and KS represent each lineage determined by phylogenetic analysis based on nucleotide sequences (see Fig. 2).

values supported the lineage association except for the node of ST and SC1, of which value was only 188. Isolates from two cats, one deer and one monkey belonged to lineage SC1. Isolates from two cats and one civet cat belonged to lineage SC2. An isolate from a cow belonged to lineage SC3. There was no specific genetic relationship among isolates obtained from domestic and wild animals.

3.3. Geographic distribution

The geographical distribution of the genetic lineages of rabies virus in Indonesia is shown in Fig. 3. Isolates of ST lineage in the ID1 phylogroup showed a large geographic distribution in Sumatra. Isolates of SC1, SC2 and SC3 lineages in the ID1 phylogroup were distributed restrictedly in central Sumatra. An isolate of JA lineage in the ID2 phylogroup was present in Java. Two remaining lineages (KS and SF) in the ID3 phylogroup were found in the eastern area of Indonesia. Isolates of KS lineage were distributed in Kalimantan and Sulawesi and those of SF lineage were distributed in Sulawesi and Flores islands.

4. Discussion

Based on the N gene sequence, phylogenetic relationships among rabies viruses could be examined and distinguished more easily and closely than by using monoclonal antibodies or other conventional methods. We previously employed 550 b (Ito et al., 1999) or 512 b (Susetya et al., 2003) of the rabies N gene for examining genetic relationships of rabies isolates in Thailand and reported that rabies field isolates in Thailand have a close genetic relationship to an isolate from China as a member of an Asian lineage. In this study, 1307-nucleotide sequence of N gene was used to determine the genetic relationships of rabies field isolates in Indonesia. Better results for bootstrap values were obtained by using 1307 b of the gene than by using 550 b or 512 b of the N gene (data not shown). The results of this study confirmed that all isolates from Indonesia belong to *Lyssavirus* genotype 1, the genotype of classical rabies virus.

When compared with the same gene regions of rabies isolates from various countries in the world, isolates from Indonesia made a cluster with an Asian lineage and were more closely related to an isolate from China than to isolates from Thailand, India or Sri Lanka. This genetic relationship between the isolates from Indonesia and China is supported by 88.4–90.2% nucleotide homologies. Based on limited sequence analysis of the N gene, it has been reported that one isolate from Anhui of China and two isolates from Java of Indonesia belonged to the same group (Smith et al., 1992), and isolates in Indonesia were closely related genetically to the isolates from Philippines and China (Sugiyama and Ito, 2007). It is known that many Chinese people had migrated to Indonesia from China for work or trading. The genetic data and historical facts suggest that rabies street viruses in China might have been transferred to Indonesia through dogs brought by humans who had migrated (Fig. 3).

We showed by phylogenetic analysis that there are three rabies phylogroups that have been maintained endemically in Indonesia. There was a correlation between phylogroup and geographical distribution of the isolates (Fig. 3). Isolates in the ID1, ID2 and ID3 phylogroups were distributed in Sumatra, Java and the three islands (Kalimantan, Sulawesi and Flores), respectively. In the ID1 phylogroup, while the rabies viruses belonging to ST lineage were pandemic throughout Sumatra, the viruses belonging to the other three lineages (SC1, SC2 and SC3) were endemic in central Sumatra. The viruses of the latter three lineages, which had been maintained among dogs in central Sumatra, spread not only to domestic animals but also to a wild civet cat. Many people have kept dogs for hunting wild boar in the jungles of central Sumatra. Since there is a local superstition that dogs injected with rabies vaccine become less aggressive for hunting, some people do not want their dogs to be vaccinated. This may be the reason for the maintenance of rabies viruses in dogs in some areas of central Sumatra. We found that domestic animals other than dogs, including cats, a cow, a deer, a monkey and a tiger, were infected with rabies virus in central Sumatra. This means that rabies virus in rabid dogs easily disseminated to other animals and humans in central Sumatra. The detection of rabies virus in a wild civet cat raises the possibility that rabies may appear as a sylvatic type in Indonesia in the future.

The ID2 phylogroup consisted of an isolate collected in 1997 in Java, where rabies was thought to have been almost eradicated. Smith et al. (1992) reported the nucleotide sequences of part of the N gene of two isolates from Java collected in 1989. In this study, phylogenetic analysis using the limited region of the N gene revealed that these two isolates belonged to a JA lineage (data not shown). These results suggest that rabies viruses in a JA lineage were endemic in Java from 1989 until at least 1997.

The ID3 phylogroup consisted of isolates from Kalimantan, Sulawesi and Flores islands. Rabies viruses in KS and SF lineages of the ID3 phylogroup were found in eastern areas of Indonesia. An SF lineage is formed by isolates from Sulawesi and Flores islands. No cases of rabies had been reported in Flores until 1997. It is thought that a fisherman brought infected dogs from southeast Sulawesi to East Flores island (Windiyaningsih et al., 2004). The results of sequencing show a close genetic relationship between isolates from the two islands, supporting the above report. Results obtained in Kalimantan and Sulawesi also raise the possibility that rabies viruses were transferred between the two islands by human activities in a manner similar to that in the case of Sulawesi and Flores islands.

We studied in detail the molecular epidemiology of rabies in Indonesia for the first time by using many rabies isolates originating from several animals and geographical areas. The findings suggest that rabies viruses in China might have been transferred to Indonesia and spread to each island with human activities. The results should be useful for tracing the route of rabies transmission and for establishing measures to eliminate rabies in Indonesia.

Acknowledgments

This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (nos. 16658120, 17255010 and 17405044) and a grant-in-aid for scientific research (The 21st Century center-of-Excellence Program) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (E-1).

References

Bourhy, H., Kissi, B., Lafon, M., Sacramento, D., Tordo, N., 1992. Antigenic and molecular characterization of bat rabies virus in Europe. J. Clin. Microbiol. 30, 2419–2426.

Bourhy, H., Kissi, B., Tordo, N., 1993. Taxonomy and evolutionary studies on Lyssaviruses with special reference to Africa. Onderstepoort J. Vet. Res. 60, 277–282.

David, D., Yakobson, B., Smith, J.S., Stram, Y., 2000. Molecular epidemiology of rabies virus isolates from Israel and other middle and near eastern countries. J. Clin. Microbiol. 38, 755–762.

Dean, D.J., Abelseth, M.K., Atanasiu, P., 1996. The fluorescent antibody test. In: Meslin, F.X., Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies, 4th ed. World Health Organization, Geneva, pp. 88–95.

Ito, N., Kakemizu, M., Ito, K.A., Yamamoto, A., Yoshida, Y., Sugiyama, M., Minamoto, N., 2001. A comparison of complete genome sequences of the attenuated RC-HL strain of rabies virus used for production of animal vaccine in Japan, and the parental Nishigahara strain. Microbiol. Immunol. 45, 51–58.

Ito, N., Sugiyama, M., Oraveerakul, K., Piyaviriyakul, P., Lumlertdacha, B., Arai, Y.T., Tamura, Y., Mori, Y., Minamoto, N., 1999. Molecular epidemiology of rabies in Thailand. Microbiol. Immunol. 43, 551–559.
Kissi, B., Bourhy, H., Tordo, N., 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 209, 526–537.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and X Version 2.0. Bioinformatics 23, 2947–2948.
Liu, Q., Xiong, Y., Luo, T.R., Wei, Y.C., Nan, S.J., Liu, F., Pan, Y., Feng, L., Zhu, W., Liu, K., Guo, J.G., Li, H.M., 2007. Molecular epidemiology of rabies in Guangxi Province, south of China. J. Clin. Virol. 39, 295–303.
Nadin-Davis, S.A., Casey, G.A., Wandeler, A., 1994. A molecular epidemiology study of rabies virus in central Ontario and western Quebec. J. Gen. Virol. 75, 2575–2583.
Nishizono, A., Mannen, K., Ellio-Villa, L.P., Tanaka, S., Li, P.S., Mifune, K., Arca, B.F., Cabanban, A., Martinez, B., Rodriquez, A., Atienza, V.C., Cambra, R., Ronostoc, N., 2002. Genetic analysis of rabies virus isolates in Philippines. Microbiol. Immunol. 46, 1413–1417.
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 analysiST. Infect. Dis. 166, 296–307.
Sugiyama, M., Ito, N., 2007. Control of rabies: epidemiology of rabies in Asia and development of new-generation vaccines for rabies. Comp. Immunol. Microbiol. Inf. Dis. 30, 273–286.
Susetya, H., Sugiyama, M., Inagaki, A., Ito, N., Oraveerakul, K., Traiwanatham, N., Minamoto, N., 2003. Genetic characterization of rabies filed isolates from Thailand. Microbiol. Immunol. 47, 653459.
Windiyaningsih, C., Wilde, H., Meslin, F.X., Suroso, T., Widarso, H.S., 2004. The rabies epidemic on Flores Island Indonesia (1998–2003). J. Med. Assoc. Thai. 87, 1389–1393.
Zhang, Y.Z., Xiong, C.L., Zou, Y., Wang, D.M., Jiang, R.J., Xiao, Q.Y., Hao, Z.Y., Zhang, L.Z., Yu, Y.X., Fu, Z.F., 2006. Molecular characterization of rabies virus isolates in China during 2004. Virus Res. 121, 179–188.