Control of rabies: Epidemiology of rabies in Asia and development of new-generation vaccines for rabies

Makoto Sugiyama\textsuperscript{a,*}, Naoto Ito\textsuperscript{a,b}

\textsuperscript{a}United Graduate School of Veterinary Sciences, Gifu University, I-I Yanagido, Gifu 501-1193, Japan
\textsuperscript{b}Laboratory of Zoonotic Diseases, Faculty of Applied Biological Sciences, Gifu University, Japan

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Abstract

Rabies is an enzootic viral disease widespread throughout the world. Although it is a vaccine-preventable disease, the annual number of human deaths caused by rabies is estimated to be 32,000 in Asia. Phylogenetic analysis based on sequence data of the partial N gene of rabies viruses in Asia has shown that the viruses are divided into five genogroups, distributed in Middle East, South Asia, South East Asia, Malay, and Arctic regions. The genetic relationships among these rabies viruses agree basically with the results of previous studies. Meanwhile, new types of vaccines are being developed by applying gene manipulation techniques to rabies virus in order to overcome the disadvantages of current vaccines. This article reviews the molecular epidemiology of rabies in Asia and progress made in the development of new-generation rabies vaccines with the goal of elimination or control of rabies in Asia.

Keywords: Rabies virus; Molecular epidemiology; Asia; N gene; Vaccine; Reverse genetics; M gene
Résumé

La rage est une maladie virale enzootique très répandue dans le monde entier. Bien qu’il s’agisse d’une maladie évitable par vaccination, le nombre de décès imputé à la rage est estimé à 32,000 en Asie. Une analyse de la phylogénèse basée sur les données séquentielles du gène partiel N des virus de la rage en Asie a montré que les virus sont divisés en cinq gêognogroupes, répartis dans les régions du Moyen-Orient, de l’Asie du Sud, de l’Asie du Sud-Est, de la Malaisie et de l’Arctique. Les relations génétiques entre ces virus de la rage s’accordent fondamentalement avec les résultats des études précédentes. En attendant, des nouveaux types de vaccins sont développés en utilisant des techniques de manipulations génétiques au virus de la rage de façon à surmonter les désavantages des vaccins actuels. Cet article examine l’épidémiologie moléculaire de la rage en Asie et les progrès effectués dans le développement de vaccins contre la rage de nouvelle génération dans le but d’éliminer ou de contrôler la rage en Asie.

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Mots clés: Virus de la rage; épidémiologie moléculaire; Asie, Gêne N; Vaccin; génétique réverse; gêne M

1. Introduction

Rabies is an enzootic viral disease widespread throughout the world. It is transmitted to other animals and humans from infected domestic and wild animals. Once neurologic symptoms of the disease develop, rabies is fatal to both animals and humans. The annual number of human deaths worldwide caused by rabies is estimated to be 55,000 [1], mostly in rural areas of Africa and Asia. An estimated 10 million people receive post-exposure treatments each year after being exposed to rabies-suspect animals.

In Asia, rabies is one of the most important diseases because of the high human mortality rate and high costs of prevention and treatment. A survey has shown that Asia carries a larger part of the public health burden of rabies with an estimated 32,000 deaths and 96.5% of the economic burden of rabies in the developing world with US$ 560 million spent each year mostly on post-exposure prophylaxis [1]. At present, only a few countries, including Japan, Singapore, and Taiwan, are free of rabies. It has been reported that rabies spread from neighboring infected areas to rabies-free areas, the border region of South Korea and Flores island of Indonesia, in 1993 and 1997, respectively [2,3]. Attention must therefore be given to this disease as a potential emerging or re-emerging infectious disease even in rabies-free countries.

Rabies virus belongs to the genus *Lyssavirus* of the family *Rabdoviridae*. The genus is composed of rabies virus (genotype 1) and rabies-related viruses, including Lagos bat (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssaviruses 1 and 2 (genotypes 5 and 6, respectively), and Australian bat lyssavirus (genotype 7) [4]. The virus has a negative-sense single-stranded RNA genome of approximately 12 kb containing coding information for nucleocapsid (N), phosphoprotein (P), matrixprotein, glycoprotein (G), and RNA polymerase (L).
Although it is difficult to distinguish field isolates of rabies virus serologically because of a single serotype, rabies isolates can be distinguished by genetic analysis. 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 [5,6] and regional levels, such as in Asia [7–16]. The establishment of a database of rabies virus genes from molecular epidemiological analysis has enabled more precise definition of virus type and will be useful for tracing transmission of rabies virus from infected animals to humans or to other animals. Earlier, restriction fragment polymorphism analysis using PCR-amplified products actually enabled the origin of rabies virus infection among immigrants to the United States to be traced [17]. It is also important to establish a strategic plan for rabies prevention and control based on an understanding of the ecology and dynamics of rabies viruses in nature [15].

Humans become infected with rabies by the bite of an infected animal, mostly a rabid dog in Asia and Africa. Therefore, it is necessary to control rabid dogs by mass vaccination of dogs in epidemic areas for elimination of rabies. Development of fatal disease in humans bitten by infected animals can also be prevented by post-exposure vaccination. These indicate that rabies is a vaccine-preventable disease. However, many people are still becoming victims of the disease in the present situation, which is far from a situation of elimination or control of rabies in Asia and Africa as described above, despite the fact that safe and effective modern vaccines exist for both human and veterinary use. Since modern inactivated vaccines derived from tissue culture are too expensive to be used for immunization and treatment of humans or animals in developing countries, vaccines from nervous tissues of rabies-infected animals are still produced and used in some Asian countries. The premodern vaccines are less effective, require repeated visits to the hospital, and often cause severe side-effects [18,19]. To overcome these disadvantages of current vaccines, the development of new types of vaccines by applying gene manipulation techniques to rabies virus is in progress [20–23].

In this article, the molecular epidemiology of rabies in Asia and progress made in the development of new-generation rabies vaccines with the goal of elimination or control of rabies in Asia are reviewed.

2. Molecular epidemiology of rabies in Asia

Smith et al. [5] analyzed a 200-b region of the N genes from 87 rabies virus isolates from various parts of the world and showed for the first time that six unique genetic groups exist globally and that four of these groups are distributed in Asia. Interestingly, these data suggested that a historical reconstruction of events leading to the introduction of rabies into an area would be possible. From analysis of the complete nucleoprotein (N)-coding genes of 69 isolates from various parts of the world, it was also shown that at least 11 phylogenetic lineages could be identified in accordance with their geographical localization and species of origin [6].
Many genetic data on rabies virus, especially data on the N gene, have been deposited in a database. Phylogenetic analysis was therefore performed by using the sequence data of the partial 240 b corresponding to nucleotides at positions 39–278 of the N gene (open reading frame) of representative rabies viruses in Asia and its vicinity with Australian bat lyssavirus as an outgroup (Fig. 1). The results indicate that rabies viruses in Asia are divided into five genogroups, distributed in Middle East, South Asia, South East Asia, Malay, and Arctic regions (Fig. 2). These results are slightly different from those in the earlier analysis by Smith et al. The genetic relationships among these rabies viruses agree basically with the results of previous analyses at a regional level in Asia [7–16].

Fig. 1. A phylogenetic tree based on the nucleotide sequences of 240 b corresponding to the nucleotides at positions 39–278 of the N gene (open reading frame) of representative rabies viruses in Asia and its vicinity with Australian bat lyssavirus as an outgroup. Sequences were analyzed by the neighbor-joining method using Clustal X. Numbers at nodes are bootstrap probabilities calculated using 1000 replicates.
Kuzmin et al. [13] analyzed the N genes of 55 rabies virus isolates originating from different regions of the former Soviet Union and compared with them with N genes of isolates from various parts of the world. The isolates were divided to two clusters, clusters I and II, with two distinct groups and three distinct groups in clusters I and II, respectively. The isolates in the former genogroup belong to Arctic (-like) viruses originating from Eurasia and North America [24]. Arctic and Arctic-like rabies viruses are located in the polar circle and the border area with Mongol and in the border area with China and Far East Russia, respectively. Their analyses also showed that these Arctic viruses circulated mainly in wild animals: Arctic fox in the Arctic area and raccoon dog in the Far Eastern area.

Kissi et al. [6] reported that an isolate from Nepal was grouped in the Arctic viruses. As shown in Fig. 1, this is supported by our phylogenetic analysis using partial N genes of rabies viruses in Asia. It has also been reported that two isolates from the northeastern area in Iran were shown by phylogenetic analyses based on the nucleotide sequence of the P gene to be closely related to Arctic viruses [12].

In South Korea, rabies re-emerged in 1993, although the disease had been eradicated in 1985 (Fig. 2). It was reported that isolates in the current outbreak of rabies are closely related to Arctic viruses on the basis of results of phylogenetic analyses [14]. The fact that the raccoon dog is the main epidemic carrier of rabies in Korea and the results of these studies supported the conclusion of the study in the former Soviet Union [13] that raccoon dogs take part in the circulation of rabies virus within their natural territories in the Far East. Interestingly, an isolate from a dog in Japan in the 1940s, when rabies was still endemic, was also closely related to
isolates in the Far Eastern area of Russia and Korea, indicating that it belongs to Arctic-like viruses (Fig. 1) [7].

2.2. Rabies viruses in the Middle East

As shown in Fig. 1, rabies viruses in the Middle East form one genogroup. These viruses are related more closely to viruses distributed in Europe and Africa rather than those in Asia. This is supported by the results of phylogenetic analysis based on the partial N gene in a previous study showing that many isolates from Israel were included in the same genetic group of isolates from Middle-Eastern countries, South Lebanon, Iran, Oman, and Saudi Arabia [8]. It was also shown that the isolates throughout Iran belonged to the same lineage disseminated widely in the Middle East, Europe, Africa, and America except for two isolates from the northeastern area, which belonged to the Arctic group on the basis of results of genetic analysis of the partial P gene sequences as described above.

2.3. Rabies viruses in South Asia

Rabies viruses from Sri Lanka and India form one lineage independently based on the partial N gene sequence (Fig. 1). It was shown by comparing partial sequences of the N gene that the isolates from Sri Lanka formed a specific cluster that included an isolate from India [9]. Recently, Nagarajan et al. [16] showed by analyses of the cytoplasmic domain of the G gene and the G-L intergenic region that the isolates from India were included in one lineage.

2.4. Rabies viruses in South East Asia

In an earlier study, it was shown that rabies viruses from Philippines formed one of six genetic groups uniquely based on partial sequences of the N gene [5]. It has also been reported that many isolates from Philippines belonged to a different lineage from other Asian isolates [10]. As shown in Fig. 1, phylogenetic analysis of the partial N gene shows that an isolate from the southern part of China [7] is included in the same genogroup as that of Philippines isolates. We have recently analyzed N genes of isolates from Indonesia and have obtained results showing that those isolates formed one lineage and were closely related genetically to the isolates from Philippines and China (Fig. 1). Rabies was introduced to Flores Island from Sulawesi Island by sailors in 1997 (Fig. 2), and its outbreak resulted in at least 113 human deaths from 1998 to 2002 [3]. The isolates from both islands were included in the same genetic lineage (submitted for publication).

2.5. Rabies viruses in Malay Peninsula

We revealed that isolates from Thailand belonged to the same genogroup consisting of two genetic clusters and were genetically different from isolates from other Asian areas [7,11]. Based on results of detailed genetic analyses of these isolates
and on the historical background of Thailand, we hypothesize that one of rabies viruses that were prevalent in central and northeastern areas might have been introduced to the lower southern parts of Thailand (Fig. 2). This hypothesis is supported by recent results of more detailed analyses of N genes of rabies virus in Thailand [15]. The present phylogenetic analysis also shows that this genogroup includes an isolate from Malaya [6], although it was collected from an unknown part of Malay Peninsula.

In conclusion, the analyses show that there are at least five genetic groups of rabies viruses distributed in Asia. Rabies viruses in four of the five groups are prevalent locally and those in the remaining group, the Arctic (-like) rabies viruses, are distributed widely in the Northern Hemisphere from Canada to the northeastern area of the Middle East (Fig. 2). Further genetic analyses of rabies viruses, especially in countries and areas with little genetic information on rabies, such as Iraq, Afghanistan, Bhutan, Bangladesh, Myanmar, Laos, Cambodia, Vietnam, and China, are needed to elucidate the dynamics of rabies in Asia.

3. Development of new-generation rabies vaccines by gene manipulation of rabies virus

3.1. Current rabies vaccines

Currently, inactivated rabies vaccines are most popularly being used for prevention of rabies around the world. However, inactivated vaccines, especially vaccines derived from tissue culture, are too expensive for vaccination of people and animals in developing countries. The high production cost of the vaccines is mainly due to the requirement of large amounts of viral antigen to sufficiently induce a protective immune response in the inoculated animal. On the other hand, inactivated vaccines from nerve tissues of rabies virus-infected animals (e.g., Semple rabies vaccine) can be produced at a lower cost. However, such vaccines can cause serious side-effects such as autoimmune encephalomyelitis in inoculated animals [18,19]. Furthermore, such vaccines require a needle-tipped syringe for delivery, hindering vaccination in developing countries, where a shortage of syringes and needles has continuously been a serious problem.

Attenuated live vaccines, on the other hand, efficiently elicit a protective immune response with a smaller amount of the virus, because the vaccine virus propagates and synthesizes viral antigen in the inoculated animal. These vaccines can generally be produced at a lower cost than inactivated vaccines and can be delivered by needle-free methods such as oral inoculation [25–27]. However, attenuated live vaccines have a serious problem: the vaccine virus sometimes causes rabies in the inoculated animal [28,29] by its residual virulence or pathogenic mutation during viral propagation in the body. This is the main reason for generally using inactivated rabies vaccines rather than attenuated live vaccines. Also, development of attenuated live vaccines needs much time because the vaccine virus is generally established by serial passages of the parental virus in cultured cells. During the passages, the virus acquires mutations on its genome, which are related to
its adaptation to the cultured cells and some of which are coincidentally associated with its attenuation.

3.2. Gene manipulation of rabies virus for vaccine development

The problems of the current rabies vaccines described above indicate the type of new-generation rabies vaccines that are needed. Attenuated live rabies vaccines that are safer and more effective than the currently available vaccines are required for prevention of rabies in the world, especially in developing countries. In order to accomplish this purpose, a gene manipulation system (also called a reverse genetics system or infectious cDNA) of rabies virus has recently been utilized in many studies.

Fig. 3 shows the principle of the gene manipulation system of rabies virus, which was first established in the SAD B19 strain by Schnell et al. [30] in 1994. Using almost the same principle, systems for the other five strains have been reported [31–35]. This system can be rephrased as “a method for recovering infectious virus from cloned cDNA”. Briefly, a plasmid expressing full-length anti-genomic RNA (genome plasmid) and three plasmids expressing N, P, and L proteins of the virus (helper plasmids) are transfected into a cell. Afterward, the anti-genomic RNA and the proteins form an anti-genomic ribonucleoprotein (RNP) complex. Since the anti-genomic RNP complex has the same biological activity as that generated in rabies virus-infected cells, genomic RNA is synthesized using this anti-genomic RNP as a template, followed by synthesis of mRNA from the genomic RNP and expression of viral protein. Assembly of the genomic RNP and the other viral proteins, M and G proteins, results in generation of an infectious recombinant rabies virus.

To genetically manipulate the rabies virus, it is only necessary to change the viral cDNA sequence on the genome plasmid by a conventional molecular cloning method and to transfect the modified genome plasmid, together with helper plasmids, into a cell in the same manner as that described above. Using this gene

![Fig. 3. Principle of the gene manipulation system of rabies virus.](image-url)
manipulation system of rabies virus, an attenuated live vaccine virus can be established much more quickly than that using the classical method by cell culture passages of the virus. Also, in this system, it is possible to discretionarily change biological characters of the vaccine virus in order to increase its safety and immunogenicity, whereas the classical method relies on attenuation-related mutations coincidentally occurring during the passages.

3.3. New-generation rabies vaccines

The candidates for attenuated live vaccines generated by gene manipulation can be distinguished into the four categories. First, insertion of a foreign gene into the viral genome, which causes attenuation of the virus and effective immune response, was utilized for generation of attenuated vaccine viruses. Pulmanausahakul et al. [36] generated a recombinant rabies virus expressing a proapoptotic protein, cytochrome c (SPBN-Cyto c(+)) strain, and showed that the SPBN-Cyto c(+) strain strongly induced apoptosis in infected cells and, consequently, was more attenuated than the negative control virus carrying inactivated cytochrome c gene (SPBN-Cyto c(−) strain). The SPBN-Cyto c(+) strain also induced a protective immune response in inoculated mice more effectively than did the SPBN-Cyto c(−) strain. Very recently, the same group reported that a recombinant virus expressing tumor necrosis factor alpha was also attenuated and strongly induced inflammation in the brains of infected mice [37].

Second, it has also been reported that an attenuation-related mutation was introduced into the viral genome to obtain an attenuated virus. Since an arginine or lysine residue at position 333 in the G protein is well known as a pathogenic determinant [38–40], this residue was chosen as a target to generate the attenuated virus. For example, Morimoto et al. [20] constructed many recombinant viruses harboring the G gene from various rabies virus strains with/without a mutation at the position and showed that alteration of the amino acid residue is useful for generation of the attenuated virus. On the other hand, Mebatsion [41] reported that deletion of the dynein light-chain binding site in P protein, which is thought to be important for axonal transport of the virus, reduces peripheral infectivity of the virus, as shown by intramuscular inoculation of the mutant virus into suckling mice.

Third, in order to enhance immunogenicity of the attenuated vaccine virus, an attempt has been made to increase the expression level of G protein in infected cells, the most important viral antigen to elicit protective immunity, by insertion of an additional G gene into the genome, as reported by Faber et al. [42]. In that study, a recombinant rabies virus (SPBNGA-GA strain) carrying two identical G genes was generated. Both of the G genes contained the attenuation-related mutation with a change in the amino acid residue at position 333 in the G protein as described above. Faber et al. showed that cultured cells infected with the SPBNGA-GA strain produced twice as much G protein as did cells infected with the virus carrying only a single G gene (SPBNGA strain) and that the strain induced apoptosis more strongly. They also showed that immunization of mice with the SPBNGA-GA strain resulted in more efficient protective immunity than that with the SPBNGA strain.
Finally, there have been some reports of recombinant rabies viruses lacking an entire gene in the genome, in order to completely attenuate the virus. Shoji et al. [43] established a recombinant virus lacking the P gene, of which the product, P protein, is known as a cofactor of viral RNA polymerase. Although the genome did not contain the P gene, the P gene-deficient (def-P) virus could be recovered from the genome plasmid with supplementation of the P protein from helper plasmid. They showed that the def-P virus propagated in cell lines stably expressing P protein. On the other hand, the def-P virus did not effectively grow in normal cells that did not express the protein. Because of this property, the def-P virus was completely apathogenic for adult and suckling mice even when inoculated intracranically. It was also shown that the def-P virus was capable of primary transcription by virion-associated P protein and, consequently, transient expression of viral proteins other than P protein from the viral genome. Immunization of mice with the def-P virus resulted in high-level induction of virus-neutralizing antibody (VNA) and conferment of protective immunity to inoculated mice against a lethal rabies virus infection. Interestingly, it has recently been reported that the P protein blocks both type I interferon production [44] and the signaling pathway [45], thereby inhibiting host innate immunity. The loss of this function in the def-P virus may contribute to the high immunogenicity of the virus.

We reported generation of an M gene-deficient rabies virus (RC-HL\textsubscript{D}M strain) as a vaccine strain [47] using the gene manipulation system of the RC-HL strain [32,46], which is a highly attenuated strain used for production of animal rabies vaccine in Japan. The gene product, M protein, is known to be necessary for viral assembly and budding [48] (Fig. 4). Therefore, it is expected that the RC-HL\textsubscript{D}M strain infects cells and synthesizes viral proteins other than M protein in infected cells, thus eliciting protective immunity more effectively than an inactivated vaccine. However, no particle formation of the progeny virus can occur, because the M protein is not expressed in infected cells due to deletion of the coding gene in the genome. Hence, it is thought that this property greatly reduces the risk of vaccine-caused rabies in inoculated animals.

In the presence of M protein supplied from an additional helper plasmid, the RC-HL\textsubscript{D}M strain was recovered from the genomic plasmid (Fig. 4). Infection of the RC-HL\textsubscript{D}M strain in cultured cells was confined to single cells, in contrast to that of the parental RC-HL strain, which extensively spread in a few days after infection. The infectious progeny virus was not detected in a culture supernatant from RC-HL\textsubscript{D}M strain-infected cells, whereas the infectious virus was effectively produced in the supernatant from RC-HL strain-infected cells. A baby hamster kidney cell line expressing M protein of the RC-HL strain has been established as a supporting cell line for propagation of the RC-HL\textsubscript{D}M strain.

The body weights of adult mice intracerebrally inoculated with RC-HL strain transiently decreased, whereas those of mice inoculated with RC-HL\textsubscript{D}M strain continued to increase without any clinical signs of rabies. RC-HL\textsubscript{D}M strain also did not cause lethal infection in the mice in contrast to RC-HL strain, which killed suckling mice by intracerebral inoculation. These findings showed that RC-HL\textsubscript{D}M strain is completely apathogenic for both adult and suckling mice. The RC-HL\textsubscript{D}M
strain induced effective VNA in adult mice by intramuscular inoculation, whereas the UV-inactivated RC-HL strain failed to induce it. It was also shown that intranasal inoculation with RC-HLAM strain much more efficiently induced anti-rabies virus antibody than did that of the UV-inactivated RC-HL strain, indicating the possibility that the intranasal route is useful for inoculation of RC-HLAM strain as a needle-free delivery method. We clearly showed that the RC-HLAM strain is a good candidate for new-generation rabies vaccine. Further studies are needed to determine whether immunization with the RC-HLAM strain protects inoculated mice against challenge with lethal rabies virus infection.

As mentioned above, the gene manipulation system of rabies virus has opened up the possibility of development of new-generation rabies vaccines. On the other hand, we need to carefully investigate whether these genetically modified rabies viruses have sufficient aptitude for the vaccine. In this aspect, the viruses mentioned above still have problems to be resolved. For example, safety of a recombinant virus with a foreign gene has to be cautiously examined since we cannot exclude the possibility that expression of the foreign gene will cause serious side-effects in inoculated animals and humans. Furthermore, in the case of a recombinant virus with a foreign gene or an attenuation-related mutation, there is a possibility that the virus will become pathogenic during viral propagation in the body of the inoculated animal due to a certain mutation hindering expression of the foreign gene or the attenuation property of virus. This possibility might be ruled out by development of a virus that has multiple attenuation-related mutations in the genome. We believe that
accumulation of fundamental information about the molecular function of rabies virus is important not only for solutions of these problems, but also for further development of attenuated live rabies vaccines using the gene manipulation system of the virus.

References


