New Lyssavirus Genotype from the Lesser Mouse-eared Bat (Myotis blythi), Kyrgyzstan

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The Aravan virus was isolated from a Lesser Mouse-eared Bat (Myotis blythi) in the Osh region of Kyrgyzstan, central Asia, in 1991. We determined the complete sequence of the nucleoprotein (N) gene and compared it with those of 26 representative lyssaviruses obtained from databases. The Aravan virus was distinguished from seven distinct genotypes on the basis of nucleotide and amino acid identity. Phylogenetic analysis based on both nucleotide and amino acid sequences showed that the Aravan virus was more closely related to genotypes 4, 5, and—to a lesser extent—6, which circulates among insectivorous bats in Europe and Africa. The Aravan virus does not belong to any of the seven known genotypes of lyssaviruses, namely, rabies, Lagos bat, Mokola, and Duvenhage viruses and European bat lyssavirus 1, European bat lyssavirus 2, and Australian bat lyssavirus. Based on these data, we propose a new genotype for the Lyssavirus genus.

The Lyssavirus genus includes seven genotypes: rabies virus (RABV, genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus 1 (EBLV-1, genotype 5), European bat lyssavirus 2 (EBLV-2, genotype 6), and Australian bat lyssavirus (ABLV, genotype 7) (1,2). Lagos bat virus was isolated from frugivorous bats (Eidolon helvum) in Nigeria in 1956 (3) and in 1974 from another bat (Micropteropus pusillus) in the Central Africa Republic (4). Mokola virus was isolated from shrews (Crocidura sp.) and a child in Nigeria in 1968 (5,6), a girl in Nigeria in 1971 (7), and cats in Zimbabwe (8). Duvenhage virus was originally isolated from a human who died after being bitten by a bat in South Africa in 1970 (9) and from Miniopterus sp. bats in 1981 (10). EBLV-1 was isolated from bats (Eptesicus serotinus) in Germany in 1968 (11), in Poland in 1985 (12), in Denmark, Holland, and Spain in 1987, and in France in 1989 (13). Some isolates of EBLV-1 were obtained from bats in Ukraine and from one human case of bat origin in Russia in 1985 (14,15). EBLV-2 was isolated from a human in Finland in 1985 (16), and from bats in Holland, the Netherlands, Switzerland, and the U.K. EBLV-2 is mainly carried by bats of the Myotis genus (Myotis daubentonii and M. alicus) (17). ABLV was isolated from five species of flying fox bats, one species of an insectivorous bat, and two infected humans in 1996 (1,18,19).

Rabies viruses have been reported in Kazakhstan, central Asia (20). Terrestrial rabies viruses have been enzootic in all Central Asian countries and are mainly carried by dogs. Field rabies viruses have been isolated and characterized in Asia, specifically Pakistan, China, Indonesia, Thailand, the Philippines, Malaysia, India, and Sri Lanka (21–26). Isolation of lyssaviruses from bats has been reported only in India and Thailand; however, these viruses were reported as RABV (27,28). Recently, Arguin et al. detected neutralizing antibodies against ABLV in the serum of six bat species (Mineopterus schreibersi, Taphozous melanopogon, Philetor brachypterus, Scotophilus kuhli, Pteropus hypomelanus, and Rousettus aegyptiacus) in the Philippines (29).

Aravan virus was originally isolated from the brain of a lesser mouse-eared bat (Myotis blythi) in Kyrgyzstan in 1991. The antigenic profile of the virus was analyzed by using two panels of antinucleocapsid (N) gene monoclonal antibodies developed at the Wistar Institute of Anatomy and Biology (USA) and the Central Veterinary Laboratory of Great Britain (Weybridge, U.K.) (30–32). These results demonstrated that the virus differed from rabies and serotypes 2 (Lagos bat virus), 3 (Mokola virus), 4 (Duvenhage virus), 5 (EBLV-1), and 6 (EBLV-2). Furthermore, 386 nucleotides (nt) of the N gene were determined from reverse transcription-polymerase chain reaction (RT-PCR) product. Phylogenetic analysis suggested that the Aravan virus did not belong to the rabies virus group (33). In the present study, we determined the entire coding region of the N protein of Aravan virus and evaluated the phylogenetic relationships with other members of the Lyssavirus genus.

Materials and Methods

Viruses

Aravan virus was isolated from the brain of one lesser mouse-eared bat (Myotis blythi) during a survey of 269 bats collected in the Osh region of Kyrgyzstan from 1988 to 1992 (30,32). A direct fluorescent antibody test was conducted. Aravan virus–infected mouse brains were impressed on glass slides, air-dried, and fixed with acetone. To detect the lyssavirus antigen, specimens were stained with fluorescein isothiocyanate (FITC)–labeled anti-rabies globulin (BBL,
Cockeysville, MD) or FITC-labeled anti-rabies monoclonal globulin (Centocor Inc., Malvern, PA). FITC-labeled anti-nucleoprotein monoclonal antibodies (NC-MAbs, W502) cross-reactive to lyssaviruses were also used (19).

**Amplification of Nucleoprotein cDNA and Direct Sequencing**

Total RNA was extracted from virus-infected mouse brain emulsions with a commercial reagent (RNasy Mini Kit, QIAGEN, Germany). cDNA was obtained with a T-Primed First-Strand kit (Amersham Biosciences Corporation, Piscataway, NJ). PCR amplification and sequencing of the N gene were performed by using the sense primer AraN-S01 (5’-ATGTACCACCTCTCAATGG-3’, nt 55–74) and an antisense primer AraNC-1400 (5’-TCATGCTCAATTGTAAAAC-3’, nt 1456–1474). The cDNA template (2 µL) was amplified by using primers (AraN-S01 and AraNC-1400), according to the manufacturer’s instruction (Super Taq Premix Kit, Sawady Technology, Tokyo, Japan). PCR reactions were incubated at 94°C for 2 min, subjected to 40 cycles of 94°C for 30 s, 48°C for 20 s, and 68°C for 2 min, and a final extension at 68°C for 7 min in a DNA thermal cycler (GeneAmp PCR System 9700 Applied Biosystems, Perkin-Elmer Corporation, Japan) (24,25). PCR products were purified by using a commercial kit (QIAquick PCR Purification Kit, QIAGEN). The sequences of the purified DNA products were determined on an automated sequencer (ABI model 310, Applied Biosystems, Foster City, CA) by using a PRIMS Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

**Phylogenetic Analysis**

The 1350-nt and the deduced 450 amino acid (aa) sequences of the N gene of the Aravan virus were aligned with 26 lyssaviruses by using Clustal W program (34). A phylogenetic tree was constructed with the computer software MEGA 2 (35). Pairwise evolutionary nucleotide distances, including both transitions and transversions, were estimated according to Kimura’s two-parameter method. Phylogenetic trees were constructed by the neighbor-joining method with 1,000 replicates to generate bootstrap probabilities at each node (36).

**Results and Discussion**

**Direct Fluorescent Antibody Assay**

The three stains used in this study reacted against the Aravan virus infected mouse brain impressions. Fluorescence showed more scattered inclusions than those of the challenge virus standard in the acetone-fixed mouse brain smear (data not shown). The results confirmed that the Aravan virus is a lyssavirus.

**Nucleotide and Deduced Amino Acid Sequence Identities among the Aravan Virus and Other Lyssaviruses**

The 1350-nt and the deduced 450 aa sequences of the Aravan virus were compared with 26 representative lyssaviruses belonging to seven genotypes (Table 1). We selected 16 representative rabies variants from the eight diverse groups, including rabies variants from geographic areas of Asia near

### Table 1. Lyssavirus isolates used in this study

| Genotype | Yr isolated | Virus (strain) | Country of isolation | Host | Accession no. |
|----------|-------------|----------------|----------------------|------|---------------|
| 1 (Rabies) | ? | CTN | China | ? | AF367863 |
| 1 (Rabies) | 1983 | 8738THA | Thailand | Human | U22653 |
| 1 (Rabies) | ? | ? | India | ? | AF374721 |
| 1 (Rabies) | 1996 | SRL1032 | Sri Lanka | Jackal | AB041964 |
| 1 (Rabies) | 1992 | 9218TCH | Chad | Dog | U22644 |
| 1 (Rabies) | 1988 | 9141RUS | Russia | Arctic fox | U22656 |
| 1 (Rabies) | ? | 9196FX | Canada | Vulpes vulpes | L20676 |
| 1 (Rabies) | 1987 | 1500AFS | Rep.South Afr. | Yellow mongoose | U22628 |
| 1 (Rabies) | 1985 | 9142EST | Estonia | Raccoon dog | U22476 |
| 1 (Rabies) | 1986 | 8681IRA | Iran | Dog | U22482 |
| 1 (Rabies) | 1985 | 8611BRE | Brazil | Vampire bat | U22479 |
| 1 (Rabies) | 1992 | BBCAN | Canada | Eptesicusfuscus | AF351833 |
| 1 (Rabies) | 1992 | MYCAN | Canada | Myotis lucifugus | AF351839 |
| 1 (Rabies) | ? | ? | Chile | Tadarida brasilensis | AF070450 |
| 1 (Rabies) | 1988 | Insectivorous bat | Chile | Insectivorous bat | AF351850 |
| 1 (Rabies) | 1989 | PA R89 | USA | Raccoon | U27221 |
| 2 (Lagos bat) | 1958 | 8619NGA | Nigeria | Eidolon helvum | U28242 |
| 3 (Mokola) | ? | Y09762 | ? | ? | Y09762 |
| 3 (Mokola) | 1981 | MOK | Zimbabwe | Cat | U28243 |
| 4 (Duvenghage) | 1986 | 86132AS | Rep.South Africa | Human | U28248 |
| 5 (EBLV-1) | 1985 | 8615POL | Poland | Eptesicus serotinus | U28244 |
| 5 (EBLV-1) | 1989 | 8918FRA | France | E. serotinus | U28245 |
| 6 (EBLV-2) | 1986 | 9007FIN | Finland | Human | U28246 |
| 6 (EBLV-2) | 1986 | 9018HOL | Holand | M. dasyneume | U28247 |
| 7 (ABLV) | 1996 | Ballina | Australia | Pteropus alecto | AF006497 |
| 7 (ABLV) | 1996 | Insectivorous isolate | Australia | Insectivorous bat | AF081020 |
| 7 (ABLV) | 1991 | Aravan | Kyrgyzstan | M. blythi | AB094438 |

*EBLV-1, European bat lyssavirus 1; EBLV-2, European bat lyssavirus 2; ABLV, Australian bat lyssavirus; MOK, strain name in Mokola virus*
Aravan virus, based on the 1350-nt sequence of the N gene was proposed of a new genotype (1, 23, 38). Hence, the nucleotide and amino acid sequence identities of 79.8% and 93.3%, respectively. ABLV (genotype 7) was closely related to SRL1032 (genotype 1, Sri Lankan rabies virus) with a 93.1% aa sequence identity. These values were almost same as maximum variation of genotype 1. Based on our present data, we determined that isolates sharing <79.8% nt and 93.1% to 93.3% aa sequence identities belonged to different genotypes. In several studies, thresholds of <80% nt and 92% or 93% aa sequence identities warranted the proposal of a new genotype (1, 23, 38). Hence, the nucleotide and amino acid percent identity values demonstrated that Aravan virus should be regarded as a new lyssavirus genotype.

**Phylogenetic Analysis**

A phylogenetic tree of 27 lyssaviruses, including the Aravan virus, based on the 1350-nt sequence of the N gene was constructed by using the vesicular stomatitis Indiana virus (VSIV, tsW16B/U13898) as an outgroup (Figure, a). The lyssaviruses divided into two groups: one group consisted of genotypes 2 and 3, and the other consisted of genotypes 1, 4, 5, 6, 7, and the Aravan virus. The latter group was divided into six distinct clusters corresponding to genotypes 1, 7, 6, and 5 (high bootstrap values of 98%, 99%, 100%, and 100%, respectively), then Aravan virus and genotype 4. Moreover, the Aravan virus clustered with genotypes 4, 5, and 6 (low bootstrap value of 59%). Duvenhage virus (genotype 4) and EBLV-1 (genotype 5) formed the same cluster (high bootstrap value of 91%), and are therefore closely related. The Aravan virus occupied the phylogenetic position between genotype 6 and the cluster of genotypes 4 and 5. We also constructed a phylogenetic tree based on the deduced 450-aa sequences of the N gene (Figure, b). Similar to the nucleotide data, the amino acid sequences divided into two large groups and further subdivided into eight groups. One group consisted of genotypes 2 and 3 (bootstrap value of 89%), and the other group consisted of genotypes 1, 7, 6, 4, and 5, and the Aravan virus (high bootstrap value of 100%). The latter group had three distinct clusters corresponding to genotypes 1, 7, and 6 (high bootstrap values of 100%, 99%, and 100%, respectively), genotypes 4 and 5 (same cluster with a high bootstrap value of 98%), and the Aravan virus. The Aravan virus did not group with any other genotypes and is located at a position close to the cluster of genotypes 4 and 5 (bootstrap value of 66%).

These results, along with those in Table 2 and the Figure, suggest that the Aravan virus does not belong to any of the seven Lyssavirus genotypes (rabies, Lagos bat, Mokola, Duvenhage, EBLV-1, EBLV-2, and ABLV). Thus, we propose that the Aravan virus forms an independent cluster and is a new member of the Lyssavirus genus.

In this article, we have reported the first lyssavirus distinct from rabies virus originating on the Asian continent. The Aravan virus was more closely related to genotypes 4, 5, and, to a lesser extent, 6, which circulates among insectivorous bats in Europe and Africa. The lesser mouse-eared bat, from which

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**Table 2. Comparison of nucleotide and deduced amino acid sequences of Aravan virus with other 13 lyssaviruses**

| Amino acid sequence identity (%) | Nucleotide sequence identity (%) |
|---------------------------------|---------------------------------|
| Aravan                          | Genotype 1 (rabies virus)       |
| SRL1032                         | Genotype 2                      |
| U22479BRE                       | Genotype 3                      |
| U22628AFS                       | Genotype 4                      |
| U22644CHAD                      | Genotype 5                      |
| U22653THA                       | Genotype 6                      |
| U22644CHAD                      | Genotype 7                      |
| AF351850                        |                                 |
| U22721                          |                                 |
| MKU22843                        |                                 |
| U9007FIN                         |                                 |
| AF006497                        |                                 |

**Notes:**
1. The values were shown as maximum variation of nucleotide sequence identities (%) within genotype 1.
2. The values were shown as maximum variation of amino acid sequence identities (%) within genotype 1.
3. Thresholds of nucleotide sequence identities % as different genotypes.
4. Thresholds of amino acid sequence identities % as different genotypes.
the Aravan virus was isolated, is widely distributed in northern Africa, the Mediterranean, southern Europe, Crimea, Caucasus, Palestine, southwest Asia, and parts of central and eastern Asia. This information should be considered in the discussion of lyssavirus classification and evolution, as it suggests the possibility of a broader geographic distribution of the Aravan virus. We have no information about human rabies caused by bat exposure from central Asia, and rabies surveillance in this area is not known well. Based on this information and the virus’ misdiagnoses as rabies, we consider that transmission of Aravan virus to humans is possible. Indeed, this finding stimulates interest in new genotypes of lyssaviruses and is important from the viewpoint of public health, necessitating further lyssavirus surveillance of bats on the Asian continent.

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