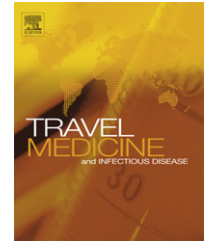




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The other rabies viruses: The emergence and importance of lyssaviruses from bats and other vertebrates

Charles H. Calisher^{a,*}, James A. Ellison^b

^a *Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 3195 Rampart Rd., Delivery Code 1690, Foothills Campus, Fort Collins, CO 80523-1690, USA*

^b *Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Centers for Disease Control and Prevention (CDC), 1600 Clifton Rd., N.E., Mailstop G-33, Atlanta, GA 30329, USA*

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Summary The recognition that viruses related to rabies virus cause rabies in humans has stimulated research into the relationships, geographic distribution and natural histories of these viruses. This paper reviews what is known of these fascinating viruses and the complexity of prevention and treatment of the disease they cause.

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Introduction

The taxonomic order *Mononegavirales* comprises the families *Rhabdoviridae*, *Bornaviridae*, *Filoviridae*, and *Paramyxoviridae*, each of which includes viruses pathogenic for humans and other vertebrates, certain plants or fish, and for other hosts. Within the family *Rhabdoviridae* six genera have been established: *Lyssavirus* (rabies virus and related viruses), *Vesiculovirus* (vesicular stomatitis viruses and related viruses), *Ephemerovirus* (bovine

ephemeral fever virus and related viruses), *Nov-irhabdovirus* (infectious hematopoietic necrosis and related viruses of aquatic vertebrates), *Cytorhabdovirus* (lettuce necrotic yellows and related viruses of plants), and *Nucleorhabdovirus* (potato yellow dwarf virus and related viruses of plants). Interestingly, as of yet there is no genetic evidence for a virus that constitutes a putative evolutionary link between plant and animal rhabdoviruses, or between lyssaviruses and viruses of any other genus of rhabdoviruses. However, comparative relationships between viral phylogeny and taxonomy remain incomplete, with numerous representatives awaiting further genetic characterization. Indeed the evolutionary pathways of viruses of particular genera may remain nearly impossible to recover.¹ One may begin to think of rabies virus as an extremely distant relative of eggplant mottled dwarf virus!

* Corresponding author. Tel.: +1 (970) 4912987; fax: +1 (970) 491 8707.

E-mail addresses: calisher@cybersafe.net (C.H. Calisher), hio6@cdc.gov (J.A. Ellison).

Within the past five decades, a few viruses have been shown to be related antigenically to each other and to what is known as “classical” rabies virus. Shope summarized these and several unpublished findings in a paper entitled “Rabies-related viruses”,² in which he laid out the previously unrecognized relationships between these viruses. At the time, Shope was Director of the Yale Arbovirus Research Unit, which housed the World Health Organization (WHO) World Centre for Arbovirus Reference and Research. Hundreds of viruses collected by arbovirologists and others around the world were sent there for identification, mainly using serologic testing of viral antigens and antibodies to them. This paper briefly describes rabies virus and its relatives, compares their relationships with each other, and discusses their possible medical, epidemiologic and evolutionary significances. Details regarding the general characteristics of these viruses have been published³ and will not be repeated in detail here so that the important biological characteristics and critical specific molecular properties of the viruses can be emphasized.

For organizational purposes, it is instructive to begin with the taxonomy of the family *Rhabdoviridae*. Within each genus are species, represented by viruses. The species *Rabies virus* is a member of the genus *Lyssavirus* and within that species is a single virus, rabies virus, the prototype virus of the genus *Lyssavirus* (λύσσα = lyssa = Lyssa was one of the Maniae [madnesses]. In mythology she was the goddess who drove mad the dogs of the youth Actaeon to kill their master, after the hunter had glanced upon a naked, bathing Artemis and did not look away). The origin of the word “rabies” is from the Sanskrit “rabhas”, meaning “to do violence”. The Latin word for madness, “rabere” means “to rave”. Whatever the origin of the words, the disease is a terrible one and, with exceedingly rare exceptions, fatal.

At this time, at least 11 distinct lyssavirus species have been formally recognized, viruses of 10 of which have been isolated from bats of various species and all appear to cause rabies encephalitis, consistent with that seen in rabies virus infections of humans (Table 1). Considering its global distribution, numerous terms have been unofficially assigned to lyssaviruses on the basis of their antigenic characteristics⁴ and will be used throughout this paper.

It is necessary (and instructional) to clarify two terms. First, the original term “rabies-related viruses” is incorrect. A virus cannot be related to a disease, only to a virus causing the disease. Therefore, we will use here the term “rabies virus-related virus” when making general mention of any lyssavirus other than rabies virus. The phrase is a bit awkward but it is better to be awkward and correct than to be graceful and incorrect. Second, with few non-standard exceptions, the names of all viruses end with the word “virus”. Rabies virologists have not always held to this standard, which has resulted in “European bat lyssavirus 1”, “European bat lyssavirus 2” and “Australian bat lyssavirus”. Were the word “virus” to be tacked onto the ends of these names we would have “European bat lyssavirus 1 virus”, “European bat lyssavirus 2 virus” and “Australian bat lyssavirus virus”, which is nonsense, so we are leaving them as is, incorrect or not. The name Australian bat lyssavirus was given to this virus because the Australian Government did not want to agree to use of terms indicating that a virus endemic to Australia causes rabies, which would have threatened its “rabies-free” status”.

Rabies is a disease, rabies virus is one of many etiologic agents of that disease, an acute, progressive, fatal encephalomyelitis caused by neurotropic lyssaviruses. This might seem obvious to some but in many book chapters, papers and certainly in oral presentations, cause and effect

Table 1 Recognized and proposed lyssaviruses.

Virus (phylogroup)	Geographical Distribution	Source	Available biologics	Genbank accession number used to generate Fig. 1
Rabies virus (I)	Essentially global	Carnivores, bats in the Americas	Yes	ABX46663
Lagos bat virus (II)	Africa	Frugivorous and insectivorous bats	No	ABU87631
Mokola virus (II)	Africa	Shrews, small rodents, cats	No	AAA67271
Duvenhage virus (I)	Africa	Insectivorous bats	Yes	ACF32425
European bat lyssavirus 1 (I)	Europe	Insectivorous bats	Yes	AAX62819
European bat lyssavirus 2 (1)	Europe	Insectivorous bats	Yes	AAX62813
Australian bat lyssavirus (I)	Australia	Frugivorous and insectivorous bats	Yes	AAN63532
Aravan virus (I) ^a	Asia	Insectivorous bats	Yes	AAP86775
Khujand virus (I) ^a	Asia	Insectivorous bats	Yes	AAP86779
Irkut virus (I) ^a	Asia	Insectivorous bats	Yes	AAR03480
West Caucasian bat virus (n/a) ^a	Asia	Insectivorous bats	No	AAR03484
Shimoni virus (II) ^{a,c}	Africa	Insectivorous bats	No ^b	ADD84510
Bokelho virus (I) ^{a,c}	Europe	Insectivorous bats	Yes ^b	AEL79468

n/a, not applicable.

^a Single isolations only.

^b Based on relative degree of cross-reactivity to commercial rabies virus vaccines and immune globulin, additional testing required.

^c Putative lyssavirus species not formally recognized by ICTV as of 9th Edition.

are confused. Here we define the differences between the disease and the viruses that can cause that disease.

All lyssaviruses share certain characteristics, including a negative-sense single-stranded RNA genome about 12 kb long, common genome organization, antigenic properties, virion morphology (bullet-shaped, 60–110 × 130–250 nm in size), two structural units (an internal helical nucleocapsid, about 50 nm in diameter, and a lipid envelope which is derived from the host cytoplasmic membrane during budding), as well as physicochemical and physical properties, and so on.³ The genome of lyssaviruses consists of five genes, each of which encodes a structural protein (N, P, M, G and L). The glycoprotein (G) is embedded in the viral envelope and plays a pivotal role in pathogenicity. The G protein has been extensively studied, and is the only viral antigen shown to induce virus-neutralizing antibody.⁵

Genetic distances between lyssaviruses are significantly less than the distances between viruses in other rhabdovirus genera, a property which has been attributed to evolutionary constraints, possibly imposed by their unique pathobiology or by their requirement for unique vector-reservoirs. The rabies virus-related viruses are transmitted directly from virus-infected mammals to susceptible mammals by bites, scratches, saliva, or mucosal exposure. Arthropods do not appear to serve as vectors for biological transmission of lyssaviruses causing rabies, and let us hope they do not. Their rates of evolution are slower than those of most rhabdoviruses, providing opportunities for studies of this feature and for our further understanding not only of virus evolution in general but of the geographic distribution of lyssaviruses in particular.

Most lyssaviruses have bats (Chiroptera) as reservoir hosts but "terrestrial" carnivores and bats maintain rabies virus in natural cycles and are critical for transmission of the virus. Lyssaviruses have been found on all continents except Antarctica (where there are no bats) and on certain isolated islands. Not all lyssaviruses are found everywhere and their geographic ranges differ considerably.

Rabies virus

The first description of the disease dates from the 23rd century B.C.E. in the Eshuma Code of Babylon and rabies was long recognized as having some relationship between human disease and animals, particularly dogs, but, it was the physician Girolamo Fracastoro of Verona who described the disease in some detail and its connection to routes of transmission in 1530, nearly 300 years before Louis Pasteur was born.

Rabies is characterized by central nervous system involvement leading to progressive encephalitis. The first signs of rabies may be non-specific, such as general weakness or ill-defined distress or irritation, headache and fever, and may persist only for a few days. An itchy or tingling sensation (parasthesia) at the site where the rabies virus-infected animal bit the patient is followed by symptoms of cerebral dysfunction, including anxiety, agitation and confusion, followed by abnormal behavior, hallucinations, delirium, insomnia or coma, dementia, and other indications of severe cognitive impairment. This acute stage may last only 2–10 days but nearly always leads to fatal outcome. Rabies is one of the few viral diseases in which

post-exposure vaccination is routinely practiced and, if the patient has been exposed to a rabid animal, prompt wound care and post-exposure prophylaxis (PEP) according to approved regimens will prevent rabies caused by rabies virus.^{6,7} PEP includes both active and passive immunization with anti-rabies immune globulin of either human (HRIG) or equine origin (ERIG), and potent cell culture vaccines.

To make sense of the various terms used by those who study rabies virus, it must be understood that rabies virus, the prototype virus of the group, is not a single, genetically homogeneous and unvarying virus but a virus that has undergone genetic changes in adapting to local hosts and local habitats. From the work by Wiktor and Koprowski showing that monoclonal antibodies to rabies (vaccine) virus, and other lyssavirus antigens could be used to differentiate various "street" rabies strains,⁸ to the elegant work of Jean Smith and others at CDC (summarized in reference 9 and elsewhere), who used this technique to study the geographic distribution, molecular epidemiology, genetics, antigenic variation, transmission dynamics, and human risk, our ability to definitively type rabies viruses in nature has improved considerably. One example, a rabies virus isolate from a ground-dwelling vertebrate (human, raccoon, skunk, other) might clearly be shown to be the same as a rabies virus from Mexican free-tailed bats (*Tadarida brasiliensis*) in the same area. This sort of advancement has led to a better understanding of the epidemiology of rabies.

At this time, the recognized rabies virus-related viruses are:

Lagos bat virus

Boulger and Porterfield isolated a virus from a straw-colored fruit bat (*Eidolon helvum*) at Lagos Island, Nigeria.¹⁰ They named it Lagos bat virus but were unable to determine an antigenic relationship with any other virus, so they sent it to Shope and to other colleagues asking for help in identifying it. Lagos bat virus was the first rabies virus-related virus (i.e., the second lyssavirus) to be identified but has not been shown to cause human illness. Additional isolations of Lagos bat virus have been obtained from cats, dogs, and mongoose from throughout Africa, including isolations in South Africa.^{11,12} Several of the Lagos bat virus-infected cats had been vaccinated against rabies virus, but still succumbed to infection. One isolate was obtained in France from a fruit bat (*Rousettus aegyptiacus*) which had been displaying signs of aggression. The bat had been imported from Africa. Recently, improved molecular techniques have suggested greater genetic diversity within Lagos bat virus isolates, supporting the proposed existence of four geographically clustered independent lineages.¹³

Mokola virus

Kemp et al. isolated Mokola virus from shrews (*Crocidura* spp.) and, later, from Nigerian children with central nervous system disease.¹⁴ None of these viruses was classified easily or soon after discovery. By 1969 Shope had shown that Lagos bat, Mokola and Obodhiang viruses¹⁵ were related antigenically, to each other, but a relationship to

rabies virus was not investigated. First, Lagos bat and Mokola viruses were shown to cross-react in complement-fixation tests. Simultaneous collaborative studies with Frederick Murphy, a veterinary pathologist and expert electron microscopist then at CDC in Atlanta, had been intended to classify vertebrate rhabdoviruses. David Simpson, in England, had sent Lagos bat virus to Murphy, asking him to determine the morphology of that virus. Murphy related to Simpson and Shope that Lagos bat virus was a rhabdovirus and, because of similarities between that virus and rabies virus in regard to virus shape and location of intracytoplasmic inclusions, suggested that Shope determine whether it was antigenically related to rabies virus. It was, and an antigenic group was established to include these viruses. Independently, Dorothy Moore, also at the Ibadan laboratory, found that kotonkan virus was related to Mokola virus and kotonkan virus was subsequently shown to be morphologically similar to Obodhiang virus.¹⁶ Mokola virus was later isolated from a dog and a cat in South Africa. Presently the principal reservoir host of Mokola virus remains unknown.

Serendipity? Not quite. Lee was an entomologist interested in culicoids and Kemp was a veterinarian interested in the rhabdovirus (genus *Ephemerovirus*) causing bovine ephemeral fever, a virus transmitted by culicoids, as are bluetongue and many other viruses (family *Reoviridae*, genus *Orbivirus*). It is interesting that Kemp eventually showed that kotonkan virus causes a bovine ephemeral fever-like disease of cattle.

These confounding findings were surprising to investigators involved in those studies as well as to arbovirologists (a virus from culicoids related to rabies virus? a virus causing a bovine ephemeral fever-like disease related to rabies virus?), clinicians, virus taxonomists, evolutionists, and others. Nonetheless, physicians, veterinarians, academics and others had now established that viruses from bats and dogs, and from mosquitoes, culicoids, and shrews were somehow evolutionarily associated and that at least some of them were the etiologic agents of central nervous system infections in humans and other vertebrates, only then realizing that "rabies" likely did not have a single cause and that rabies virus is simply a member of a group of related viruses with origins in the mist of time, as are so many viruses.

Duvenhage virus

In South Africa, Meredith, Prossow and Koch had published on what they called an unusual case of rabies.¹⁷ A patient died after having been bitten on the lip by a bat. From his brain they obtained a virus isolate that did not react in the rabies immunofluorescence test. This virus was named Duvenhage virus and eventually was shown to be another relative of classical rabies virus. Two additional human infections with this virus have been diagnosed, another one from South Africa,¹⁸ and the second from the Netherlands in a tourist who was infected with the virus while in Kenya.¹⁹

European bat lyssavirus 1

Another rabies-like virus was first isolated in Europe in 1954 when a diagnosis of rabies was determined with tissue from

a child who was bitten on the finger by a bat.²³ What was called "rabies-like virus", then "European bat lyssavirus", was isolated from a serotine bat (*Eptesicus serotinus*) in Denmark.²⁴ Using monoclonal antibodies and inoculations of experimental hosts, the virus was compared with an isolate from a New York bat and with Mokola virus from a human in South Africa. Ten Danish isolates from serotine bats reacted identically with a panel of monoclonal antibodies. By immunofluorescence, reaction with monoclonal antibodies, and histopathologic studies, these isolates were similar to the exclusively African Duvenhage virus and, only somewhat similar to classical rabies virus. The Danish isolates produced fatal infections in laboratory mice inoculated intracranially, as well as by footpad and oral routes. Domestic dogs and cats inoculated intracerebrally but not intramuscularly or intravenously with these viruses died within 10 days. The dogs inoculated intramuscularly or intravenously developed neutralizing antibodies to the virus and resisted challenge with a lethal dose of street rabies virus. Cats inoculated intramuscularly died of an illness compatible with rabies within 15 days. At necropsy, rabies viral antigens were detected in several organs, including brain and salivary glands. Subsequent reports from Europe indicated that rabies virus-like viruses were usually associated with serotine bats. Eventually (*vide infra*) this virus was named European bat lyssavirus 1 (EBLV-1). EBLV-1 has been divided into two lineages, EBLV-1a and EBLV-1b, based on phylogenetic analyses supported by geographic distribution.²⁵

European bat lyssavirus 2

With the refinement of the use of monoclonal antibodies and improved ability to distinguish between very closely related rabies viruses and rabies virus-like viruses, this second European bat lyssavirus was identified. Both European bat lyssaviruses have been shown to be more closely related to Duvenhage virus of Africa than to classical rabies virus; however, it soon became apparent that they were not at all simply variants of the same virus. Using an expanded panel of monoclonal antibodies it was observed that the differences between them were considerable. Indeed, EBLV-1 is antigenically and genetically more closely related to Duvenhage virus than it is to EBLV-2.²⁶

EBLV-2 was isolated from a zoologist who died in Finland with what was diagnosed as rabies.²⁷ Similar viruses were isolated from pond bats (*Myotis dasycneme*) in The Netherlands.²⁸ Sequencing of the genomes of these viruses confirmed that at least two bat lyssaviruses circulated in Europe. The lyssavirus from serotine bats then was renamed European bat lyssavirus 1 and the newly recognized lyssavirus from Daubenton's bat (*Myotis daubentonii*) was named European bat lyssavirus 2 (EBLV-2²⁹;). It is now recognized that EBLV-2 is widely distributed in European bats of the genus *Myotis*, exclusively pond bats and Daubenton's bats, and that EBLV-2 has been the etiologic agent of rare, geographically scattered but inevitably fatal human infections. Whereas EBLV-1 is not uncommonly detected in mainland European bats and is not found in the United Kingdom, EBLV-2 has been detected only in the United Kingdom, The Netherlands, Finland and near the border

between Switzerland and Germany.³⁰ The possibility that there are pathogenetic differences between EBLV-1 and EBLV-2 has been considered. EBLV-2 has been shown to be less virulent in animal models than has EBLV-1 and both are less virulent than is rabies virus. Unprovoked (depending on your definition of "provoked") biting has been associated with bats infected with EBLV-2, whereas apparently healthy bats in Spain have been shown to excrete EBLV-1. Thus, bats infected with one or both of these lyssaviruses might occur. As summarized by Calisher et al.³¹ and others, bats having inapparent infections with any of many viruses may serve as convenient and cryptic reservoirs of pathogens of humans, livestock and other vertebrates. This field of research is attracting increasing attention.

Clearly, the take away lessons from all this are that insectivorous bats can become infected with and transmit viruses that cause rabies, that rabies acquired from bats infected with any lyssavirus can be fatal, that anyone handling or otherwise in close contact with bats should at least be immunized against rabies virus (until evidence to the contrary is available, this seems reasonable), and that post-exposure prophylactic treatment for rabies should be considered for anyone bitten by a bat when that bat is unavailable for rabies testing. In addition, patients with acute flaccid paralysis or clinically diagnosed encephalitis should be asked, or their relatives and friends should be asked, whether they had been bitten by a bat. Finally, diagnosis of compatible illnesses should include virus isolation or virus detection by polymerase chain reaction assays.

Australian bat lyssavirus

Increased surveillance for Hendra virus, a paramyxovirus that causes fatal human and equid infections in eastern Australia and for which the reservoir hosts are fruit bats ("flying foxes"), aided in the discovery of yet another lyssavirus. The so-called Australian bat lyssavirus was detected in a sick black flying fox (*Pteropus alecto*) by virus isolation, immunohistochemical means and pathology.²⁰ The virus has now been shown to occur in bats of all flying fox species (black flying fox, gray-headed flying fox (*Pteropus poliocephalus*)), little red flying fox (*Pteropus scapulatus*) and spectacled flying fox (*Pteropus conspicillatus*) found in Australia. Currently two distinct lineages are recognized as being maintained in insectivorous and frugivorous bats.²¹

Two fatal human infections with Australian bat lyssavirus have been recorded. The first was in 1996, when a 39-year old animal handler who had been scratched and possibly bitten five weeks earlier by a yellow-bellied sheath-tailed bat (*Saccolaimus flaviventris*) died. The second, a 37-year old bat-carer who had been bitten by a flying fox 27 months previous to the onset of her illness in 1998 also died.²² Vaccination against rabies virus is effective against infection with Australian bat lyssavirus and rabies vaccine will prevent clinical disease caused by this virus.

Thus, although the Australian government claims that country is free of rabies, what is meant is that "street rabies" virus, classical rabies virus, is not present there, irrespective of a few importations of the virus in people

who acquired it elsewhere. Australia may be free of street rabies virus but it is not completely free of "rabies". That, however, is an issue to be taken up by those who protect international trade matters, not those interested in virological facts.

Aravan virus

In 1991 Kuzmin et al.³² isolated a virus from an insectivorous lesser mouse-eared myotis, also known as the lesser mouse-eared bat (*Myotis blythi*, sic: *Myotis blythii*) in Kyrgystan (Central Asia). Using a panel of monoclonal antibodies, they compared this virus, which they named Aravan virus, to other lyssaviruses and determined that the virus was not classical rabies virus. Comparing the complete sequence of the nucleoprotein gene of this virus with nucleoprotein genes of 26 other lyssaviruses, this virus was differentiated from them on the basis of both nucleotide and amino acid sequences. Phylogenetic analyses indicated that Aravan virus is most closely related to Duvenhage virus and EBLV-1 and, to a lesser extent, EBLV-2, than to other lyssaviruses but should not be considered a member of any of the seven recognized species.³³

Khujand virus

Isolated from an insectivorous whiskered myotis bat (*Myotis mystacinus*) collected in northern Tajikistan in 2001, monoclonal antibody testing indicated that this virus is distinct from other lyssaviruses. Subsequent nucleotide sequencing of the viral genome supported this conclusion and indicated that Khujand virus is yet another distinct virus, related to Aravan virus but most closely related to EBLV-2.³⁴

Irkut virus

The source of this lyssavirus was an insectivorous bat, a greater tube-nosed bat (*Murina leucogaster*) captured in the Irkutsk region of eastern Siberia in 2002. One human fatal illness caused by this virus has been documented.³⁵

West Caucasian bat virus

This lyssavirus was isolated from a Schreibers's long-fingered bat (*Miniopterus schreibersii*) captured in 2002 near Krasnodar, near the east coast of the Black Sea in southern Russia.³⁶ As with other rabies virus-related lyssaviruses, antibody to this virus has been found in Kenyan bats of various *Miniopterus* species. Note that, because of cross-reactivity among closely related lyssaviruses, the presence of antibody in an individual does not necessarily indicate that individual had been infected with the virus with which the test was conducted. The need for infectious virus or viral RNA is essential if specific characteristics of the virus are desired and whenever a bat bites or otherwise contacts a person, collection of that bat also is essential for the same reasons.

Shimoni bat virus

Kuzmin et al. collected 616 bats representing 22 species in Kenya.¹³ They isolated a previously unrecognized lyssavirus from the brain of a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*) found in a cave. Thorough phylogenetic studies of this virus, which they named Shimoni virus, substantiated by antigenic evaluations, demonstrated that it is not identical to any presently recognized lyssavirus and suggested that it be considered a new virus representing a new species within phylogroup 2, most closely related to Lagos bat virus. No evidence has yet been found of association of Shimoni virus with disease in humans, livestock or other vertebrates.

Bokeloh virus

In November 2009, a Natterer's bat (*Myotis nattereri*) was found on the ground in Bokeloh, Lower Saxony, Germany. In February 2010 the bat began to display clinical signs of rabies. Ten days after recognition of the first clinical signs, the bat died. Lyssaviral antigen was detected in numerous brain neurons. However, the salivary glands did not contain lyssaviral antigens. Sequence analysis of the nucleoprotein gene showed that Bokeloh virus differs from all other published lyssavirus sequence. Subsequent phylogenetic analysis using concatenated N-P-M-G-L nucleotide sequences Bokeloh virus is most closely related to Khujand virus and, to a lesser extent, to EBLV-2.³⁷ We have placed this virus in phylogroup 1 (Table 1) but that is not yet an official placement.

Other lyssaviruses in bats

In 1999, 300 bats not identified to species were captured in Nanning, the capital city of Guangxi province, China, and their brain tissues tested for virus and by reverse transcription-polymerase chain reaction for lyssaviral RNA; three were positive, but no information regarding further identification of the viruses isolated was presented in the publication.³⁸ The same publication reported that in 2002 there had been a first report of human rabies in China after the bite of a bat. A person in northeast Jilin Province was bitten by a bat on his face the evening of July 17, 2002. Twelve days later he reported facial numbness and severe headache. On July 31, the patient had a fever, was nauseated and faint and had pain in the upper torso. He also was observed to have signs of rabies, including a fear of wind and light. He was hospitalized August 1 with a clinical diagnosis of rabies and died the next day, 16 days after the exposure and 4 days after he first showed clinical signs. "This was the first reported case of bat rabies virus in China, although the species of the bat was never identified; the tissue samples from the bat and the viral isolates were discarded." In 1977, human bat-associated rabies cases were reported to have occurred in the town of Voroshilovgrad, Ukraine. Neither the virus nor the bats were identified (I. Kuzmin, pers. comm., 2011).

It is unknown as to how many infections with rabies virus and rabies virus-like viruses go unreported. Bats comprise more than 1000 species and represent approximately 25% of

all species of mammals. Categories of risk of extinction of bats of these 1000 species range from rare (or extinct) to threatened, endangered, or not at risk (i.e., safe for now). These vertebrates are critical elements in terrestrial biotic communities, including helping control insects, reseeding cut or burned forests, and pollinating plants that provide food for humans and others. Their guano is used as fertilizer and for manufacturing soaps, gasohol, and antibiotics. Bat echolocation and signal processing have provided models for sonar systems. Nonetheless, negative public perceptions of bats, human land use patterns, intentional habitat destruction, and economic development not tempered by adequate concern and conservation, and intentional extirpation of entire colonies of bats, as well as adoption of bats as food sources in less well-developed areas have had profound effects on populations of bats of certain species. Still, the sizes of colonies and clouds of certain bats can be enormous and bats represent about 25% of all the mammals of the world, so if they are important it is likely that they are very important.

The peculiarities of bats with respect to their physiologies, feeding patterns, food sources, colonial behaviors, breeding behaviors, that some migrate, hibernate, have periods of torpor, co-colonize, and otherwise are remarkably variable provide considerable opportunities for virus transmission, either between bats or from bats. In addition, the more than 100 viruses that have been detected in apparently asymptomatic bats at least suggest that bats may serve as perfect hosts for viruses.³¹

Two recent publications may be used as exemplars of the recent trends in bat virus research. Wright et al. have hypothesized that Lagos bat virus is endemic in straw-colored fruit bats (*Eidolon helvum*) in Ghana and speculated that this rabies virus-related virus may have co-evolved with African megachiroptera.³⁹ Also in 2010, Donaldson et al. reported that metagenomic analyses of the viromes of bats of three North American species provided data suggesting that those bats encounter and perhaps disseminate a large and diverse assemblage of viruses capable of infecting many different vertebrates, insects, and plants.⁴⁰ If these and other studies were to be expanded, perhaps we would soon have a better perspective on the role of bats as virus reservoirs and transmitters than we do now.

The persistent question as to whether a newly discovered virus is an emergent one or whether better techniques or coincidence played a role in its recognition cannot be answered for the rabies virus-related viruses or for many other viruses. Clearly, improved techniques and increased efforts by investigators in a few key laboratories have revealed the existence of additional lyssaviruses and we should expect that more will be discovered. Thus far, rabies virus-related viruses have been detected in bats of only a relatively few species, so we could reasonably expect that hundreds more rabies virus-related viruses could be found, were this to be a scientific priority. However, because of the expense of such studies, laws, non-legal decisions to protect bats and for other reasons, this is not being done and likely will not be done. Therefore, the only way that other rabies virus-related viruses will be discovered will be if informed physicians and biologists pay particular attention to patient reports and personal

observations, obtain an accurate patient history, including asking questions regarding animal exposure, themselves be aware of the need for detailed diagnosis of rabies-compatible illnesses in humans and bats and the need to collect and retain germane samples.

Whether these viruses really matter in the overall epidemiology of rabies in dog rabies-endemic areas has been called into question by Weyer et al.⁴¹ Results of their retrospective 25-year (1983–2007) study of laboratory-confirmed human rabies in the Republic of South Africa suggested the importance of domestic dogs as reservoirs and transmitters of rabies virus. These authors also concluded that, by comparison, there is an almost negligible contribution to wildlife vectors in the total cases of rabies in at least that particular dog rabies-endemic area. Perhaps this is so, which would be a good thing, because dog rabies is much easier to control than is wildlife rabies. Nonetheless, people who die of wildlife rabies viruses do not suffer any less than those dying of rabies virus acquired from dogs.

Usefulness of prophylactic application of rabies virus vaccine against infection with a rabies virus-related virus

As additional newly recognized rabies virus-related viruses continue to be discovered, it has become even more obvious that there is a need to know whether vaccination with rabies vaccine will prevent infection in those exposed to these viruses and whether post-exposure treatment, as used to prevent classical rabies, is sufficient. The literature contains numerous papers reporting smaller studies with this intent but publications of a rigorous study of vaccine efficacy in humans are missing because morality demands that. A paper by Hanlon et al. reported the effect of pre-exposure rabies vaccination using two laboratory hosts, Syrian hamsters and ferrets.⁴² The hamsters were administered a commercial human or veterinary vaccine or an experimental vaccinia-rabies glycoprotein recombinant vaccine by the intramuscular route. Five weeks after vaccination, animals were challenged with Aravan, Khujand, Irkut or West Caucasian bat virus, or with a traditional rabies virus of dog/coyote origin. Previously vaccinated and unvaccinated ferrets also were challenged with the four new isolates. In addition, the combined effects of rabies immunoglobulin and vaccine after exposure of hamsters to each of the four isolates were investigated using commercially available human products or an experimental monoclonal antibody. Results showed reduced efficacy of pre-exposure vaccination with conventional rabies post-exposure prophylaxis against each of the four new bat viruses. In general, and interestingly, efficacy was inversely related to the genetic distance between the new isolates and traditional strains of rabies virus. For example, using West Caucasian bat virus, the most divergent of these lyssaviruses, no significant difference in mortality was found after either pre-exposure vaccination or conventional post-exposure prophylaxis, as compared with naïve controls. The potential impact of these newly recognized on human and domestic animal health and the impact on populations of the presumed bat

reservoir will require further field and laboratory investigations.

Methods used to determine phylogroups and antigenic (sero)types

The classification of lyssaviruses has evolved over time. Initially lyssaviruses were subdivided into serotypes based on their assorted cross-reactivities in classical serologic assays.² However the introduction of monoclonal antibody techniques and later molecular typing provided a much improved level of resolution of this genus. Presently, lyssavirus species can be grouped into phylogroups based on sequence and phylogenetic analysis, each with unique genetic, immunogenic and pathogenic properties.⁴³ Within the lyssavirus genus, phylogroup I includes all lyssavirus species except Lagos bat virus, Shimoni bat virus, Mokola virus, and West Caucasian bat virus.^{13,36,43} The phylogroup II viruses diverge more at the amino acid level on the glycoprotein gene (Fig. 1). West Caucasian bat virus may represent a new phylogroup; however, only a single isolation of this virus has been reported, thus precluding official designation.³⁶

The operational term “genotype” has been used for lyssavirus classification since the time when molecular techniques replaced serotyping for classification purposes. Demarcation of genotypes has been based largely on genetic distances between members of the genus and on the bootstrap support of phylogenetic constructions.^{44,45} However, the International Committee on Taxonomy of Viruses (ICTV) does not recognize operationally defined genotypes, and only recognizes viral species for taxonomy at this level. Comparison of all five concatenated lyssavirus genes has been shown to provide the most robust level of resolution thus far, with threshold for differentiation between 76.4% and 81.6% nucleotide sequence identity.^{13,46}

Summary of the natural history of lyssaviruses and their capacity to cause rabies

Rabies virus is well known to most as being transmitted between bats (virus maintenance) and from bats to other vertebrates, which acquire the virus and die, either before or after transmitting it to other individuals. Bats of many species have been shown to be involved in the natural cycle (bat-to-bat or bat-to-other vertebrate-to-other vertebrate, etc.) and in the dead-end road represented by a vertebrate which dies before transmitting the virus.

Since 1953, rabies virus has been reported to occur in insectivorous bats in North America.⁴⁷ Subsequently, increased surveillance revealed rabies virus infection in insectivorous bats of the majority of species throughout the U.S.A. and Canada.⁴⁸ Estimates of rabies virus prevalence in North American bats have varied between species and across years within species.⁴⁹ These include not only the commonly recognized vampire (hematophagous) bats but bats that feed on insects, fruits, flowers, pollen, or nectar, fish, frogs, lizards, small rodents, small birds, and other bats. Bats of many species are colonial in nature and spend

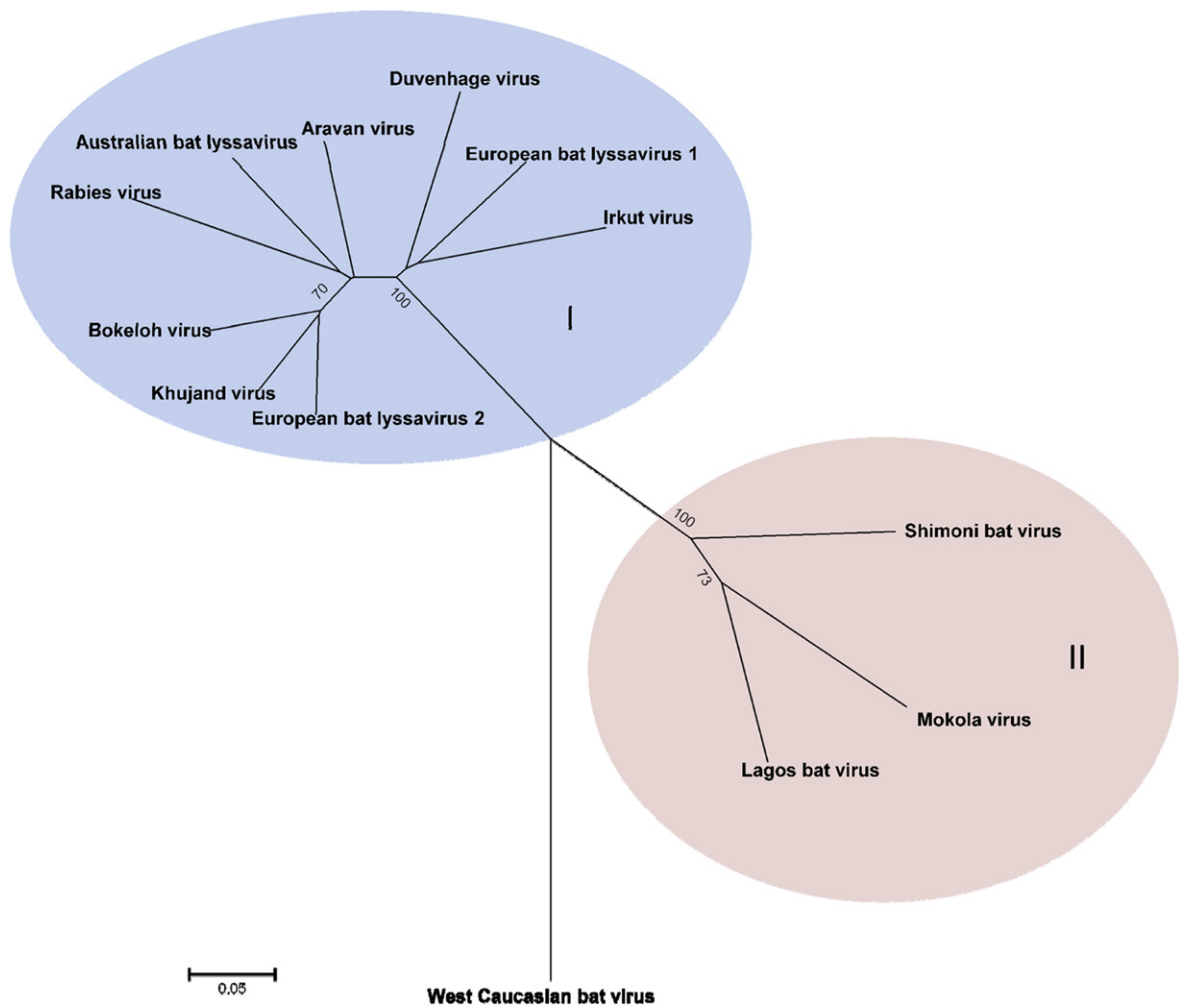


Figure 1 Phylogenetic analysis of characterized and putative lyssavirus isolates based on 524 amino acids of the glycoprotein gene. Sequences were aligned using Clustal W, and the tree was visualized using MEGA 5, bootstrap values are presented for key nodes. The differentiation into phylogroups according to lyssavirus biologic properties is labeled and silhouetted.

a great deal of their time in close proximity to one another, either daily while resting, seasonally while in hibernation, when breeding, or in maternal colonies. No matter the reason for their close proximity, infected bats are able to transmit rabies virus to their cohorts and to their offspring, perhaps vertically, although whether the latter is an important means of virus maintenance is unknown.⁵⁰

The epidemiology of rabies virus appears simple (infected vertebrate-to uninfected vertebrate) but is complex. The virus can be transmitted directly from bats (virus-infected bat to uninfected human, equid, bovid, canid, raccoon, skunk, or other terrestrial vertebrate) but the terrestrial vertebrate that becomes infected may serve as a temporary (until it dies) source of virus which can then infect other terrestrial vertebrates, thus establishing a cycle not involving bats. It is instructive to recognize the correlation between rabies virus variants in terrestrial vertebrates and rabies virus variants in bats in the same area. Clearly, the rabies virus-infected bats above them somehow infect the terrestrial vertebrates below, whether by scratch or bite by the bat or by the terrestrial vertebrate eating a virus-infected bat.

Tests recommended for diagnosis

Considering the severity of rabies and the often non-specific spectrum of neurological signs and symptoms, it is important to make an initial clinical diagnosis followed by a laboratory-based confirmation to distinguish the cause from other etiologies of viral encephalitis.^{6,51} For this reason, a number of rabies diagnostic techniques have been internationally standardized by the World Health Organization (WHO) and the World Organization for Animal Health (OIE).^{6,52}

Lyssaviruses show broad antigenic cross-reactivity at the nucleocapsid level, mainly because of sequence conservation of the N protein. The standard diagnostic test consists of direct fluorescent antibody (DFA) testing of impressions made from fresh brain tissue (i.e., cerebellum, hippocampus and brain stem). When performed properly, no other laboratory test for the diagnosis of rabies is as simple, sensitive, specific, inexpensive and rapid as the DFA test performed on fresh brain tissue.⁵³ This allows the use of similar reagents for diagnosis by immunofluorescence and is the method of choice used by U.S. public health laboratories for routine diagnosis of rabies virus infection.⁹

Fresh brain tissue may not be routinely collected, obviating diagnosis by DFA. In such cases, application of experimental diagnostic techniques, including DFA on formalin-fixed tissue, immunohistochemistry or polymerase chain reaction assay and sequencing are used. Modern molecular techniques have been essential in clarifying the role of diverse rabies virus reservoirs in wildlife in the U.S.A.

Lyssaviruses are antigenically distinct from most other rhabdoviruses and little cross-reactivity has been shown by serum neutralization and complement-fixation tests. Nonetheless, a quandary has arisen regarding observations that antigens of certain genetic members of the genus *Ephemerovirus* (bovine ephemeral fever virus and its close relatives), including Obodhiang virus, kotonkan virus and Puchong virus, all of which cause a bovine ephemeral fever-like disease in cattle, and Berrimah virus, Kimberley virus and Adelaide River virus, which are not known to cause disease, are detected by antibody to lyssaviruses and vice versa. Schmidt, at the U.S. Naval Medical Research Unit in Cairo, had isolated Obodhiang virus from *Mansonia uniformis* mosquitoes in Sudan,¹⁵ Kemp, Lee, Moore, Shope, Causey and Murphy, at the Virus Research Laboratory of the University of Ibadan, isolated kotonkan virus from culicoids (midges) collected from cattle at the University farm¹⁶ and Puchong virus was isolated from *Mansonia uniformis* mosquitoes collected in Selangor State, Malaysia, by unspecified workers. Adelaide River and Berrimah viruses each were isolated from bloods of apparently healthy bovids in the Northern Territory of Australia, Kimberley virus was isolated from *Culex annulirostris* mosquitoes in the Northern Territory as well as from *Culicoides brevitarsis* and bovid blood in Queensland, Australia. It is not at all clear why certain ephemeroviruses share antigens with certain lyssaviruses but, according to Peter J. Walker, CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia, a sequence of six to eight conserved amino acids can be recognized as a cross-reactive epitope. Walker has shown that such sequences occur in Adelaide River virus N protein at sites homologous to rabies virus N sequences to which even commercial rabies virus monoclonal antibodies bind. The rhabdovirus N protein is relatively, if minimally, conserved in some regions, so it is not unlikely that such short peptides could be the source of this confusion. Nevertheless, it is clear that Obodhiang, kotonkan, Puchong, Berrimah, Kimberley and Adelaide River viruses are ephemeroviruses, not lyssaviruses, and therefore are not rabies virus-related viruses in the true sense of that term, notwithstanding the work of Shope²¹ and Calisher et al.⁵⁴

Monoclonal antibodies for characterization of rabies viruses are useful in distinguishing rabies virus and its close relatives. Once a monoclonal antibody is raised against a particular virus, it can be used to probe related viruses to determine how specifically it binds. An antigenicity profile can be generated using a panel of monoclonal antibodies raised against different isolates. These studies have demonstrated that rabies virus is distinguishable from related lyssavirus genotypes and that rabies virus isolates from a given geographic area or those belonging to different species have unique reactivity patterns, both due to common epitopes within the viral ribonucleoprotein core and to the glycoprotein components of the virion.⁵⁵

Recently, molecular methods have been adopted as confirmatory assays or as alternative methods for cases in which diagnosis is required while the patient is alive. Once viral RNA has been extracted from a sample, there are a number of molecular protocols dedicated to RNA viral detection based on nested and real-time reverse transcription PCR⁵⁶ or nucleic acid sequence-based amplification.^{57,58} Positive results obtained using these methods should be confirmed by sequencing to avoid false positives, and is also critical for epidemiological surveillance through strain typing. Diagnosis of human rabies in patients with symptoms suggestive of rabies can be achieved with the following tests¹: DFA test on skin punch biopsy from the back of the neck,² virus isolation from saliva,³ virus neutralization assay with serum and cerebrospinal fluid for evidence of antibody to rabies virus and⁴ reverse transcription PCR for rabies virus RNA. As the diagnosis of human rabies is a diagnosis of exclusion, all four tests are required to rule out an ante-mortem diagnosis of rabies.

These techniques currently are adopted as diagnostic tools in suspect human rabies cases and are useful for testing samples not appropriate for DFA, or fluid samples such as saliva. However, because viral shedding may be intermittent, multiple different samples (saliva, skin biopsy, CSF) must be collected and analyzed for an intravital rabies diagnosis.

Research needs

As suggested above, additional and geographically extended, long-term field studies of bats and their viruses are necessary if we are to approach an understanding of the actual virus load of bats. Increasingly, this is being done, but done for specific purposes. Investigators who are interested in a particular virus or group of viruses collect samples and test them specifically for that virus or those viruses. Not surprisingly, these have been quite successful and more than 100 viruses or viral nucleic acid sequences have been detected to date, with many more expected to be discovered. However, what is not being tested for is not being detected, so that much information is not being gathered, even though a considerable amount of field work has been done and so many potentially useful samples have been tested – a wasteful use of resources. Methods are needed to survey for nucleic acid sequences of multiple viruses; sample sharing would be helpful.

The success of recent work with viruses of bats promises even more successes. Cell cultures prepared from bat cells show promise in regard to teasing out viruses that have not heretofore been shown to be adapted to propagation in vitro. Using such cultures could provide tools for biological studies useful in understanding lyssaviral phenotypic characteristics, such as titer, plaque size, mechanisms of viral attachment to cells, etc. In addition, these cells might reveal the existence of lyssaviruses that infect insects or vertebrates other than bats.

Evolutionary studies of lyssaviruses also are likely to be productive. Given the relatively short genetic distances between these viruses, it may be not only that they are closely related but that understanding the development of

lyssaviruses might be fruitful. Have the rabies virus-related viruses developed from rabies virus itself or was rabies virus derived from a less pathogenic, less insidious progenitor? Highly pathogenic viruses find themselves at risk when they kill or disable their hosts, as do all parasites. Therefore, we might obtain clues as to the origin of rabies virus by having a greater understanding of lyssaviral evolution.

Does a peculiarity of bat immunology lead to virus persistence? Do bat migratory pathways lead to reassortment (and evolution) of viruses with segmented genomes or to exposure to newly emerging viruses? Are bats the natural sources of viruses infecting humans (e.g. severe acute respiratory syndrome-like coronavirus being an example)? Do diverse feeding patterns of bats lead to diverse exposures to viruses? Are there important differences in virus transmission between bats that colonize and bats that are more solitary in nature? Given the more than 1000 species and the remarkable and fascinating peculiarities of bats, it is likely that we have not scratched the surface of what is yet to be known and that many more viruses, including at least some of the lyssaviruses, will be recognized in the near future.⁵⁹ The full significance of these viruses for human and veterinary medicine has yet to be determined.

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