

Quantifying Antigenic Relationships among the Lyssaviruses[∇]

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All lyssaviruses cause fatal encephalitis in mammals. There is sufficient antigenic variation within the genus to cause variable vaccine efficacy, but this variation is difficult to characterize quantitatively: sequence analysis cannot yet provide detailed antigenic information, and antigenic neutralization data have been refractory to high-resolution robust interpretation. Here, we address these issues by using state-of-the-art antigenic analyses to generate a high-resolution antigenic map of a global panel of 25 lyssaviruses. We compared the calculated antigenic distances with viral glycoprotein ectodomain sequence data. Although 67% of antigenic variation was predictable from the glycoprotein amino acid sequence, there are in some cases substantial differences between genetic and antigenic distances, thus highlighting the risk of inferring antigenic relationships solely from sequence data at this time. These differences included epidemiologically important antigenic differences between vaccine strains and wild-type rabies viruses. Further, we quantitatively assessed the antigenic relationships measured by using rabbit, mouse, and human sera, validating the use of nonhuman experimental animals as a model for determining antigenic variation in humans. The use of passive immune globulin is a crucial component of rabies postexposure prophylaxis, and here we also show that it is possible to predict the reactivity of immune globulin against divergent lyssaviruses.

Rabies remains a globally important zoonosis, despite being one of the oldest recognized infectious diseases (27, 55). The majority of rabies in terrestrial animals and humans is caused by classical rabies virus (RABV), a lyssavirus in the family *Rhabdoviridae*. Since the 1950s, many related lyssaviruses which are capable of causing clinical rabies have been identified. The majority of those viruses have been isolated from bats (Chiroptera), including four divergent viruses, which were isolated in separate geographic locations throughout Eurasia in the past 18 years (2, 29, 31). The Chiroptera, therefore, represent a global reservoir for lyssaviruses, creating the potential for “spillover” infection to terrestrial mammals, including humans. Occasionally transmission between members of a new host species will occur, with potential for a subsequent adaptation in that species (35). Phylogenetic evidence suggests that one or more host-switching events from bats into terrestrial mammals were originally responsible for the ongoing global epidemic of terrestrial RABV (6).

Pre- or postexposure prophylaxis, using vaccination and passive immune globulin administration according to World Health Organization (WHO) guidelines, is currently the only effective way to prevent rabies after infection with a lyssavirus (1). The efficacy of both active and passive immunization is

likely to be affected by antigenic differences between viruses. The lyssavirus trimeric glycoprotein is the primary surface antigen, the major target for neutralizing antibodies (8), and is involved in cell binding and entry (34, 36, 53). Antigenic sites on the glycoprotein have been described using monoclonal antibody escape mutants (8, 16, 47, 51). These studies have elucidated two major sites (sites II and III) and multiple minor sites. Although estimates of antigenic differences can be made using information regarding these known antigenic sites, protein structure, and amino acid properties, predictions of the relative importance of those sites and specific mutations within those sites cannot be quantitatively tested without a method to reliably measure antigenic effect.

The use of serological cross neutralization data to measure antigenic difference is limited by the reliability of the serological test and, more importantly, by paradoxes, or irregularities in the data. These irregularities include higher heterologous than homologous titers and individual variations between sera raised against the same antigen (22, 52). Hence, serological data are considered to have low resolution, and they are often used only qualitatively. Despite these difficulties, studies have attempted to further quantify antigenic differences among lyssaviruses. Badrane et al. (5) showed a linear correlation between the glycoprotein amino acid identity and four cross neutralization titers. Other studies have demonstrated variable serological cross-reactivity between European bat lyssaviruses (EBLV) and RABVs (10, 11) and suggested that antigenic relationships between EBLV-1 and EBLV-2 may not be fully

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TABLE 1. Virus panel^a

Designation	Species	Host	Country	Yr isolated	Original designation	GenBank accession no.
CVS-11	RABV	— ^b	—	—	—	EU352767
PM	RABV	—	—	—	—	AJ871962
LEP	RABV	—	—	—	—	GU936866
RV51	RABV	Fox	USA (Michigan)	1986	86-1393	GU936880
RV108	RABV	Bat	Chile	1985	85/120	GU936868
RV313	RABV	Red fox	Germany	1990	G1455	GU936875
RV410	RABV	Mongoose	South Africa	1990	332/90	GU936878
RV437	RABV	Raccoon dog	Estonia	—	269	GU936879
RV1237	RABV	Deer	Serbia	1999	329/99	GU936869
RV102	RABV	Fox	Morocco	1988 ^c	RV102	GU936867
RV334	RABV	Vaccine	China (Beijing)	—	SRFV 289	GU936876
RV1	LBV	Bat	Nigeria	1956	LBV	EF547431
RV4	MOKV	Shrew	Nigeria	1968	MOKV	GU936877
RV131	DUVV	Bat	Zimbabwe	1986	RS16(Z12)	GU936870
RV1423	EBLV1	Bat	Germany	1998	934	GU936872
RV20	EBLV1	Bat	Denmark	1986	RA552(86)	GU936874
RV9	EBLV1	Bat	Germany	1968	RV9	EU352768
RV1332	EBLV2	Bat	UK	2002	105/02	GU936871
RV628	EBLV2	Bat	UK	1996	96/18	GU936882
RV1787	EBLV2	Bat	UK	2004	603-04	EU352769
RV634	ABLV	Bat	Australia	1996	96-0648	GU936883
IRKV	—	Bat	Siberia	2002	—	EF614260
KHUV	—	Bat	Tajikistan	2001	—	EF614261
ARAV	—	Bat	Kyrgyzstan	1991	—	EF614259
WCBV	—	Bat	Russia	2002	—	EF614258

^a Abbreviations: CVS, challenge virus standard; LEP, Flury low egg passage; PM, Pittman Moore; RABV, rabies virus; LBV, Lagos bat virus; MOKV, Mokola virus; DUVV, Duvenhage virus; EBLV, European bat lyssavirus; ABLV, Australian bat lyssavirus; IRKV, Irkut virus; KHUV, Khujand virus; ARAV, Aravan virus; WCBV, West Caucasian bat virus.

^b —, data not available.

^c Year first passed.

reflected in the genetic relationships (41). Recent investigations into the efficacy of biologics against the Eurasian lyssaviruses showed an array of relatedness between lyssavirus species, with, for example, a murine anti-Aravan virus (anti-ARAV) serum neutralizing Khujand virus (KHUV) and ARAV equally but an anti-KHUV serum being less effective at neutralizing ARAV than KHUV (22). Until recently, however, there were no established tools for the quantitative analysis of antigenic data.

Here we resolve the issue of quantitative interpretation of antigenic data using antigenic cartography. Antigenic cartography is a theory and associated computational method that resolves the paradoxes in the interpretation of antigenic data and makes possible high-resolution quantitative analyses and visualizations of binding assay data (15, 20, 25, 44, 49, 52).

Integrating antigenic data with direct sequencing data, here we quantify the antigenic and genetic variation among a global panel of lyssaviruses, including representatives from all lyssavirus species. Furthermore, we address two key issues in the development of antilyssavirus biologics: the appropriateness of animal models and the development of efficacious alternatives to human rabies immune globulins (HRIGs).

MATERIALS AND METHODS

Virus isolates. A panel of 25 lyssaviruses (Table 1) were grown in baby hamster kidney (BHK-21) or murine neuroblastoma (N2A) cell culture. Viruses were passaged in tissue culture between two and eight times to obtain satisfactory titers. Tissue culture supernatant (TCSN) was harvested, aliquoted, and stored at -80°C .

Sequence data. Complete glycoprotein ectodomain sequences were obtained for each virus. Nine of 25 sequences were obtained from GenBank, and the remaining 16 samples were sequenced as part of this study as previously described (39). RNA was extracted from the brains of mice infected with the TCSN used in the neutralization tests, or directly from the TCSN, to minimize the effect of any amino acid mutations that may have occurred during passage in tissue culture (51) and to ensure consistent comparison between antigenic and genetic data. Primer sequences were designed using published virus sequences (39) and are available on request.

Phylogenetic analyses. The most appropriate model of nucleotide substitution was determined to be the general time-reversible model, with the gamma distribution of rate variation among sites determined by the data (ModelTestv3.7). Phylogenetic trees were then inferred using maximum-likelihood (ML) methods (PAUP, v4.0), and tree bisection-reconnection branch swapping was used to search for the optimal tree. The sequences were bootstrap resampled 100 times to assess the robustness of each tree node.

Production of antisera. Polyclonal antisera were raised in 29 naïve adult male New Zealand White rabbits against 18 viruses (one or two rabbits per virus) using beta-propiolactone-inactivated TCSN and either Freund's incomplete adjuvant or Montanide ISA50V(Seppic). TCSN combined with adjuvant was injected at multiple sites subcutaneously and intramuscularly on days 0, 21, 28, and 35. Sedation, euthanasia, and exsanguination were carried out on day 42. The inactivation of viruses prior to inoculation was confirmed with a mouse inoculation test (MIT) (28). Blood samples were taken from selected mice used in the MIT by cardiac puncture under anesthesia at the time of euthanasia ($n = 7$). After centrifugation of blood samples, serum was removed from the blood pellet, heat inactivated at 56°C for 30 min, and then stored at -20°C . Human rabies immune globulin (HRIG) (Bio Products Laboratory, Herts, United Kingdom) was diluted to 20 IU/ml in phosphate-buffered saline.

Neutralization data. The neutralizing abilities of the sera were assessed using fluorescent-antibody virus neutralization (FAVN) tests on BHK-21 cells (13), modified for each virus under test. A constant volume of virus (100 50% tissue culture infective doses [TCID₅₀]/50 μl) was added to serial 2-fold dilutions of serum in duplicate. The 50% endpoint dilution, where neutralization of the virus ceased, was calculated with the Spearman-Kärber method (3). The virus dose

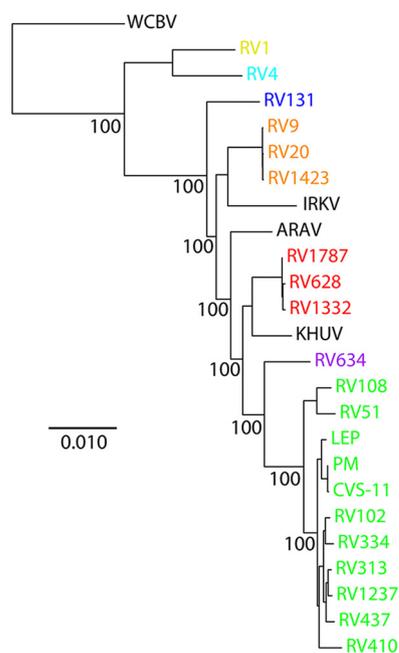


FIG. 1. Phylogenetic tree of 25 lyssavirus glycoprotein ectodomain nucleotide sequences. Sequences were aligned using Clustal X2. Trees were produced using a maximum-likelihood algorithm with a GTR+I+ Γ_4 model; the consensus was generated and is presented with bootstrap values at key nodes. Trees were rooted using WCBV as an outgroup. Isolates are colored by species (Table 1 and Fig. 3).

was checked by back titration on every test, and results were rejected if the virus dose was <64 or >256 TCID₅₀. Serum from a rabbit inoculated with uninfected, beta-propiolactone-treated BHK cell TCSN was used as a negative control.

Antigenic cartography. Lyssavirus antigenic maps were generated from the FAVN data using antigenic techniques described previously (15, 52). Briefly, a target distance from a serum to each virus is derived by calculating the difference between the logarithm (\log_2) reciprocal neutralization titer for that particular virus and the \log_2 reciprocal maximum titer achieved by that serum (against any virus). Thus, the higher the reciprocal titer, the shorter the target distance. As the \log_2 of the reciprocal titer is used, a 2-fold change in titer will equate to a fixed change in target distance whatever the magnitude of the actual titers (denoted one antigenic unit [AU]). Antigenic cartography (52) was then used to optimize the positions of the viruses and sera relative to each other on a map, minimizing the sum-squared error between map distance and target distance. Each virus is therefore positioned by multiple sera, and the sera themselves are also positioned only by their distances to the viruses. Hence, sera with different neutralization profiles to the virus panel are in separate locations on the map but contribute equally to positioning of the viruses. To increase the likelihood of obtaining the best fit of map distances to the neutralization data, multiple random restart optimizations (25 to 100) were carried out, creating multiple maps which we ranked in order of total error and quantitatively compared for self-consistency. The positions of points on the map can have more than three coordinates, and therefore mathematically it is possible for the maps to occupy more than the conventional three dimensions. Although one cannot easily visualize maps in more than three dimensions, all quantitative measurements, such as the Euclidean (“straight-line”) distance between points, are as straightforward in more than three dimensions as they are in three dimensions or fewer (see the supplemental material of reference 52 at http://www.who.int/rabies/PEP_prophylaxis_guidelines_June10.pdf). To determine the optimum dimension (D) for these data, the resolution of the maps in each dimension was determined by first making maps in 1D, 2D, 3D, 4D, and 5D with a random 10% of titers omitted and then predicting those omitted titers using the distances from the maps.

Comparison of sera. In addition to the rabbit sera used to make the antigenic map, seven mouse sera (from MITs) and HRIG were tested against a subset of viruses. To compare the mouse and rabbit sera, a separate antigenic map was made using only mouse serum titers against a subset of viruses ($n = 9$). Virus-

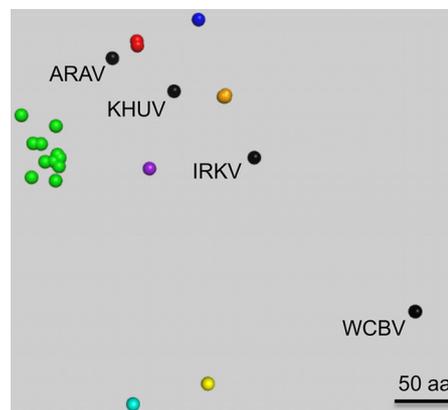


FIG. 2. Amino acid sequence variation map. Viruses are positioned relative to each other using multidimensional scaling and target distances derived from the number of amino acid substitutions between viruses (Table 2). The map is in two dimensions and is oriented and colored according to the antigenic map (Fig. 3). The scale bar represents 50 amino acid (aa) substitutions.

to-virus distances measured from that mouse map were compared with virus-to-virus distances on the rabbit serum map to quantify any difference between antigenic relationships determined using mice and rabbits. There were too few sera to make separate maps from HRIG, so we used an indirect method to compare HRIG with the rabbit sera. HRIG was tested for its ability to neutralize a subset of the viruses. An antigenic map was then constructed using the rabbit serum titers and the HRIG titers. In this case, however, the virus positions were determined by the rabbit titers only and not the HRIG titers. When distances are measured from the map, we can compare how effectively the HRIG titers fit with the antigenic positions of the viruses as determined by the rabbit serum titers. If HRIG and rabbit sera have the same breadth of neutralization titers and therefore determine antigenic differences at the same scale, then the rabbit serum antigenic map should be a good characterization of the HRIG titers. This characterization was assessed quantitatively by plotting target distance as a function of map distance, allowing comparison of the slopes and intercepts of linear models applied to the data. This indirect method was then also applied to compare mouse and rabbit sera.

“Genetic” maps of the viruses based on their amino acid sequence variation were also made. The same multidimensional scaling techniques used for antigenic data were applied to a distance matrix generated from the number of glycoprotein ectodomain amino acid substitutions between viruses (52).

RESULTS

Genetic variation. Phylogenetic analysis of the glycoprotein ectodomains of the panel of viruses (Fig. 1) shows evolutionary relationships among the lyssaviruses, inferred using maximum-likelihood methods. The majority of evolutionary relationships are well supported by bootstrap analysis. The close relationship of the vaccine strains Flury-LEP, Pittman Moore (PM), and challenge virus standard (CVS) is clear, as is the distinction between New World (RV108 and RV51) and Old World (RV102, RV313, RV410, RV437, and RV1237) RABV. ABLV (RV634) has the closest of the lyssavirus species to RABV, followed by EBLV-2s (RV628, RV1332, and RV1787) and then EBLV-1 (RV9, RV1423, and RV20). Mokola virus (MOKV) (RV4) and Lagos bat virus (LBV) (RV1) are approximately equally distinct from the remaining viruses. The tree suggests that Irkut virus (IRKV) is most closely related to EBLV-1 and that KHUV is most closely related to EBLV-2.

The lyssavirus amino acid map (Fig. 2) shows the relationships of all viruses based on the glycoprotein amino acid ho-

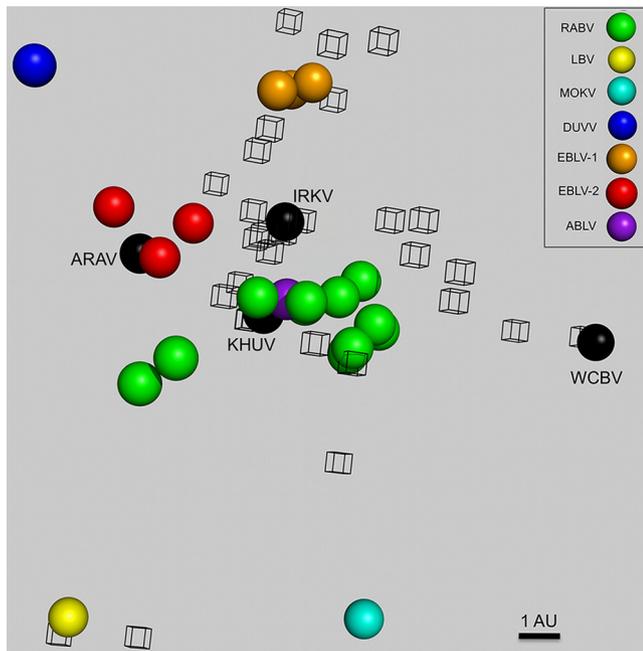


FIG. 3. Three-dimensional antigenic map. Viruses (spheres) and sera (open boxes) are positioned such that the distance from each serum to each virus is determined by the neutralization titer. Multidimensional scaling is used to position both sera and viruses relative to each other, so orientation of the map within the axes is free. The scale bar shows one antigenic unit (AU), which is equivalent to a 2-fold dilution in antibody titer. (Viewing was with PyMOL, DeLano Scientific LLC, San Carlos, CA).

mology. All 11 lyssavirus species are distinct from each other. A good correlation between table distance and map distance ($r = 0.96$, $P < 001$) indicates that two dimensions are adequate for representing the amino acid distances.

Antigenic variation. Antigenic maps were made in multiple dimensions (1D, 2D, 3D, 4D, and 5D). To determine the appropriate number of dimensions and the resolution of the map, 25 antigenic maps were made, each with a different random 10% of titers omitted, and those titers predicted using the maps. The average prediction error was 1.36 (standard error

[SE], 0.16) antigenic units in 2D, 1.22 (SE, 0.17) in 3D, 1.20 (SE, 0.16) in 4D, and 1.20 (SE, 0.16) in 5D. Resolution therefore increases with increasing dimension, but the incremental increase in precision becomes negligible beyond three dimensions for this data set, suggesting that 3D maps are sufficient for visualizing the lyssavirus antigenic data.

Figure 3 shows a three-dimensional antigenic map showing the antigenic relationships among the panel of 25 lyssaviruses, color coded according to species. Sera are represented by open boxes. Antigenic distances can be measured from the antigenic map. Table 2 shows the means and ranges of antigenic distances between representatives of the genotypes (upper right triangle) and the number of amino acid substitutions between viruses (bottom left triangle) for comparison.

Despite their wide host range and geographical origin (Table 1), the wild-type (WT) RABV strains included in the study are antigenically similar. However, the laboratory-adapted strains CVS and PM are antigenically distinct from WT RABV (Fig. 3), despite being derived from WT-RABV strains and genetically closely related to them. Conversely, ABLV is antigenically indistinguishable from the WT RABV strains but is genetically divergent. Of the remaining genotypes, EBLV-1 and EBLV-2 are closest to RABV, followed by Duvenhage virus (DUVV). Previously, EBLV-1 and -2 were suggested to be equally antigenically distinct from RABV (41), which is reflected in our findings. However, EBLV-1 appears to be antigenically further than EBLV-2 from CVS and PM (Table 2). The viruses IRKV and ARAV are antigenically more similar to EBLV-2 than EBLV-1, and KHUV is closer to RABV than either of the EBLVs.

LBV and MOKV have been placed in a separate phylogroup based on genetic distance, limited cross neutralization, and differences in pathogenicity (5, 7). In our study, as in others (22), we saw a low but detectable degree of cross-reactivity between phylogroups I and II for a small number of sera, and hence we were able to position the phylogroup II viruses relative to the others on the antigenic maps. Both LBV and MOKV are twice as far from RABV as the EBLVs on the antigenic map. Neutralization of West Caucasian bat virus (WCBV) by all sera raised with both phylogroup I and II viruses was very weak, indicating that WCBV is distinct from

TABLE 2. Intergenotype antigenic and glycoprotein amino acid distances

Virus	Distance ^a from:											
	CVS-11	RABV	LBV	MOKV	DUVV	EBLV1	EBLV2	ABLV	IRKV	KHUV	ARAV	WCBV
CVS-11		5.1	7.4	8.5	7.1	8.9	5.0	4.5	7.8	4.6	5.4	13.1
RABV	30		9.3	7.1	8.3	5.9	4.7	1.6	4.5	2.2	5.1	8.3
LBV	162	159		6.6	12.4	13.0	9.0	8.8	10.3	8.2	8.3	14.2
MOKV	162	160	87		14.1	12.1	9.9	7.5	9.3	7.3	9.6	9.1
DUVV	101	101	167	168		6.6	4.7	7.3	7.7	7.3	5.4	15.4
EBLV1	101	99	161	160	72		4.8	5.3	3.3	5.4	5.2	9.8
EBLV2	86	84	164	166	71	71		3.4	3.9	3.1	1.5	11.5
ABLV	74	72	155	161	92	84	65		3.8	0.9	3.8	9.0
IRKV	113	111	158	156	92	71	87	102		3.4	3.7	8.2
KHUV	79	76	165	168	78	71	39	59	85		3.2	9.1
ARAV	87	85	159	164	69	52	50	69	79	51		11.4
WCBV	197	200	182	192	201	196	198	195	197	198	195	

^a The antigenic distance (in antigenic units) measured from the antigenic map is shown above the diagonal. The number of amino acid substitutions in the glycoprotein ectodomain is shown below the diagonal. Where there are multiple representatives for each genotype on the antigenic map, the mean antigenic distance and number of amino acid substitutions are given.

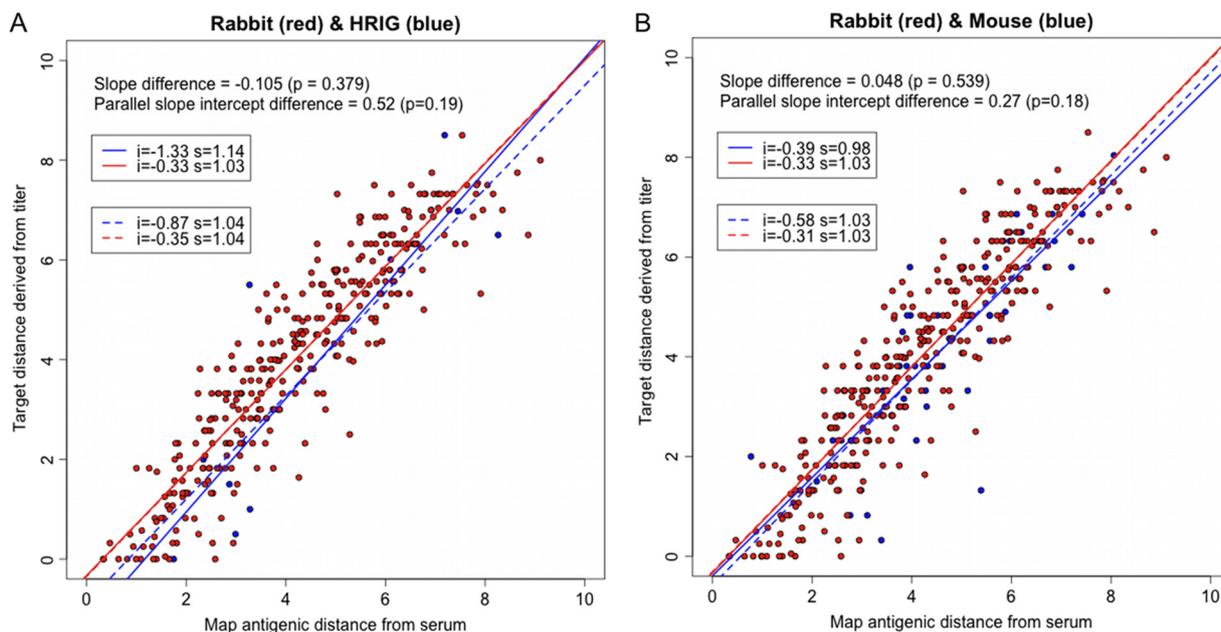


FIG. 4. Comparison of responses of different species. Target distance derived from titers of rabbit sera (red) (A and B), HRIG (blue) (A), and mouse sera (blue) (B) is shown as a function of antigenic map distance determined only by the rabbit sera. The correlation between rabbit map distance and target distances from sera from other species gives an indication of whether antigenic relationships determined by each species are similar. Correlations between the target distances and rabbit map distances for the mouse sera ($r = 0.83$ [95% CI, 0.72 to 0.90]; $P < 0.001$; residual standard error, 1.09) and HRIG ($r = 0.88$ [95% CI, 0.55 to 0.97]; $P < 0.001$; residual standard error, 1.60) are similar to that for the rabbit sera ($r = 0.92$ [95% CI 0.90 to 0.93]; $P < 0.001$; residual standard error, 0.82), and neither the slopes (s) nor intercepts (i) of linear models applied to the data are significantly different from those applied to the rabbit data.

all other viruses (on average 9 AU from RABV, equivalent to approximately a 500-fold difference in neutralizing antibody titer).

Serum comparisons. Distances from virus to virus on a map made using mouse sera (not shown) correlated closely with distances from the rabbit serum antigenic map ($r = 0.86$ [95% confidence interval {CI}, 0.74 to 0.92]; $P < 0.01$; residual standard error, 0.76). The correlation between the target distances and map distances for the mouse sera and HRIG are all similar to that for the rabbit sera. Neither the slopes nor intercepts of linear models applied to the data are significantly different from those applied to the rabbit data (Fig. 4), showing that for the data tested, antigenic distances determined using sera from animal models are the same as those determined using human sera.

Predicting antigenic distance from amino acid sequence homology. Pairwise comparison of all antigenic distances with genetic distances shows a statistically significant correlation (Pearson product-moment correlation coefficient, $r = 0.82$ [95% CI, 0.78 to 0.85]; $P < 0.001$; residual standard error, 1.75) (Fig. 5). In addition, a linear regression model predicts on average, one unit of antigenic change (95% CI, 0.93 to 1.07; $P < 0.001$), equivalent to a 2-fold change in antibody titer, for every 21 amino acid substitutions (4.8%) in the glycoprotein ectodomain. The correlation between antigenic and genetic distances when calculated only for viruses in the same phylogroup is lower than the correlation for all viruses but remains statistically significant ($r = 0.64$ [95% CI, 0.56 to 0.71]; $P < 0.001$; residual standard error, 1.63).

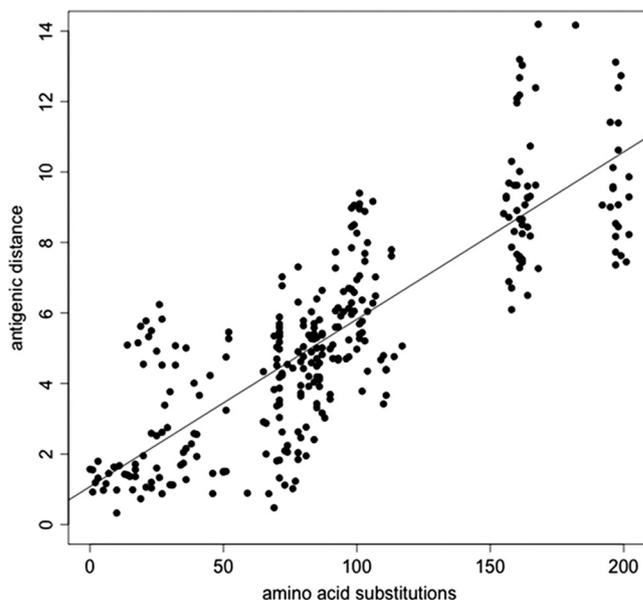


FIG. 5. Plot of pairwise antigenic and genetic distances between viruses. Antigenic distances are measured from the antigenic map, in antigenic units. Genetic distances between viruses are represented as the number of amino acid substitutions in the glycoprotein ectodomain. The line represents a linear regression model ($r^2 = 0.67$; $P < 0.01$), which predicts, on average, an antigenic distance change of one antigenic unit, equivalent to a 2-fold change in neutralization titer, per 21 amino acid substitutions (4.8% of the glycoprotein ectodomain).

DISCUSSION

We have described the antigenic relationships among a panel of 25 lyssaviruses using serological binding assay data and antigenic cartography. This approach has quantified clinically important antigenic differences between lyssaviruses; shown that those differences are equivalent for mouse, rabbit, and pooled human sera; and allowed integration of quantitative antigenic data with genetic distances.

These data give a precise estimate for the correlation between genetic and antigenic distances (95% CI for $r = 0.81$ to 0.88), an improvement in accuracy over the current estimate (95% CI for $r = 0.39$ to 1.00 and $P = 0.08$ calculated from published data [5]). Increased accuracy allows the evaluation of glycoprotein amino acid sequence homology as a predictor of antigenic difference. Fitting a linear regression model to our data demonstrates that on average, a 4.8% change in the glycoprotein ectodomain amino acid sequence will cause one antigenic unit of difference between viruses (equivalent to a 2-fold change in antibody titer) (95% CI, 0.93 to 1.07 AU; $P < 0.001$) (Fig. 5). A linear regression model applied to the \log_2 of previously published data gives a similar mean of a 2-fold change in antibody titer per 5.5% change in glycoprotein amino acid sequence homology, but with a much larger confidence interval (-0.8 to 4.4 2-fold dilutions; $P = 0.296$). Despite a good correlation between genetic and antigenic distances among the lyssaviruses, over 30% of the variance in antigenic distance cannot be predicted by the number of amino acid substitutions between viral glycoprotein ectodomains, illustrating the difficulty in interpreting antigenic differences using the gene sequence alone. Although these and previous studies have used the entire glycoprotein sequence, the techniques applied here could be applied to specific regions of the glycoprotein, for example, previously reported antigenic sites.

Integration of genetic and antigenic data allows identification of viruses where there are differences between genetic and antigenic relationships. For example, the antigenic positions of IRKV and ARAV, which are closer to EBLV-2, and KHUV, which is closer to RABV would not be expected from genetic relationships. Phylogenetic analysis using the glycoprotein ectodomain here, similar to that using the entire glycoprotein and entire nucleoprotein gene previously (29), suggests that KHUV is more closely related to EBLV-2 than ARAV or IRKV is to EBLV-2. However, both genetic and antigenic studies are limited by the existence of only one representative of some viruses, a problem that also applies to divergent classified lyssavirus species such as LBV (38).

Comparison with other RNA viruses illustrates the close antigenic relationships among the lyssaviruses. Studies of human influenza A(H3N2) viruses showed a similar correlation between the amino acid sequence of the main antigenic component of the virus (the hemagglutinin HA1 domain) and the antigenic distance between viruses ($r = 0.81$), but a different slope. On average, only 2.9 amino acid substitutions ($<1\%$ of the hemagglutinin HA1 domain) caused a 1-unit change in antigenic difference (52). Antigenic distances measured by different binding assays and for different pathogens may not be directly comparable, meaning that one antigenic unit (2-fold dilution) derived for influenza virus will not equal one antigenic unit (2-fold dilution) from lyssavirus studies. Influenza

viruses and lyssaviruses both have trimeric surface glycoproteins. However, an average of 13 amino acid substitutions in influenza virus hemagglutinin will cause lack of cross-reactivity, compared with lyssaviruses, where viruses with as many as 100 amino acid substitutions still show significant cross-reactivity. Such conservation in the phenotype of the key antigenic determinant of lyssaviruses is consistent with a low immune selective pressure upon lyssaviruses (9, 14, 24) in comparison with pathogens such as influenza virus that are under large selection pressure (42, 52). Although low immune selective pressure could be expected from the natural history of RABV in terrestrial animals, where infection classically leads to death, the dynamics of RABV in terrestrial animals may not be applicable to bats, particularly in light of evidence of high seroprevalence against RABV and other lyssaviruses in apparently healthy conspecifics (23, 30, 54).

A limitation of all *in vitro* antigenic studies is the potential effect of adaptation to cell culture on the viral genomic sequence. Sequences generated for this study were taken from cell culture supernatant used in neutralization assays or after single passage of supernatant in mouse brain, to ensure valid comparison of genetic and antigenic data. Although we cannot rule out potential differences between those viruses in cell culture and the original isolate, previous studies using rabies virus have shown no change in the glycoprotein consensus sequence despite 20 passages in cell culture (26).

All current rabies vaccine virus strains are based on classical rabies viruses (18). Evidence suggests these vaccines are fully effective against virtually all RABVs tested to date (4, 11, 33, 37, 46, 50) but not against distantly related viruses in phylogroup II or WCBV (5, 43). In addition, the reported variable efficacy of vaccines against the EBLVs and DUVV (11, 17, 32) and recent evidence of variable efficacy against ARAV, KHUV, and IRKV (22) suggest that there may be a gradual loss of protection as viruses become antigenically distant from vaccine strains. This gradation in protection is the case for other viruses (42, 52). Although protection can ultimately be tested only in challenge models, reliably quantifying the antigenic differences among divergent lyssaviruses is an important step toward predicting differences in vaccine protection (5).

In addition to active vaccination, passive immunization against rabies remains a critical part of postexposure prophylaxis (1). The gold standard, human rabies immune globulin (HRIG), is expensive and in short supply, prompting attempts to find less expensive alternatives, including cocktails of monoclonal antibodies (21, 40). In some studies dose-dependent survival has been demonstrated following immune globulin administration in animal models (21, 48), suggesting that the potency of passive immune globulin may be related to the neutralizing titer. If this is true, antigenic differences as measured by neutralizing antibodies are of direct relevance to protection provided by passive immune globulin treatments. HRIG titers correspond to rabbit titers and are therefore predictable using antigenic maps. Further investigation is necessary to determine whether candidate monoclonal antibody cocktails can be predicted with similar accuracy.

Sera from animal models are widely used to investigate antigenic differences between lyssaviruses (5, 22). However, all require extrapolation to species of clinical interest, which has thus far been largely unsubstantiated. Here we have demon-

strated that antigenic differences between viruses determined by a variety of species are equivalent. These findings validate the use of nonhuman experimental animals as a model for determining antigenic variation that is relevant to humans.

The Chiroptera are increasingly implicated as reservoirs for many zoonotic viral diseases (12). The lyssaviruses provide one globally widespread example, with at least one antigenically distinct lyssavirus having been isolated from bats on all continents except Antarctica. The possibility of future elimination of lyssaviruses from bats by human intervention is at best very optimistic. The threat posed by lyssaviruses in bats is therefore global and likely to be continuous. Recent human deaths due to DUVV (45) and EBLV-2 (19) highlight the significance of spillover into humans. More alarming is the possibility of a bat lyssavirus adapting to a terrestrial host, as is hypothesized to have created the current global epidemic of terrestrial RABV (6), with widespread and prolonged consequences. A recent example of a bat RABV switching its host to skunks (35) illustrates the ease with which this adaptation can occur. With knowledge of the alarming clinical manifestations and extremely high mortality rates caused by lyssaviruses, along with increasing globalization and the altered interface between humans and wildlife, a better understanding of the antigenic as well as genetic relatedness among lyssaviruses is vital. The methods presented here provide a quantitative method to test predictions regarding the antigenic effects of amino acid substitutions, and those antigenic effects in turn can be used to make predictions regarding the efficacies of biologicals.

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