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Short communication

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New insights into the genetics of EBLV-1 from Germany

Neue Details zur Genetik von EBLV-1-Isolaten aus Deutschland

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Summary

Previous epidemiological studies on EBLVs indicated a distinct geographical distribution of EBLV-1 in Germany. In this study, 48 isolates were selected to further investigate the spatial and temporal distribution of EBLV-1 variants in Germany. The nucleoprotein-gene (N), the nucleoprotein-phosphoprotein spanning untranslated region (NP-UTR) and the UTR between G- and L-gene of each isolate were sequenced using direct cycle sequencing. Results of the subsequent phylogenetic analysis of the N-gene confirmed previous studies on EBLVs, showing a high sequence identity among German EBLV-1a isolates, and a correlation between genetic and temporal and spatial distance, respectively, was shown. Our results indicate that the GL-UTR is not suitable for phylogenetic analyses. Interestingly, 6 nt insertions in two isolates as well as a single nucleotide insertion in a different isolate were detected in the N-P UTR. Within the UTR between G- and L-gene one isolate showed a 35 nt deletion. The effect of those changes on viral properties remains elusive as such mutations have not been described for lyssaviruses before.

Keywords: EBLV-1, phylogeny, bat rabies Germany, insertion, deletion

Zusammenfassung

Vorherige epidemiologische Studien über EBLVs deuteten auf eine geografische Verteilung von EBLV-1 in Deutschland. Im Rahmen dieser Studie wurden 48 Fledermaustollwutvirusisolate aus Deutschland repräsentativ ausgewählt und durch die direkte Sequenzierung des N-Gens sowie der nicht-translatierte Bereiche zwischen dem N- und P-Gen (NP-NTR) bzw. dem G- und L-Gen (GL-NTR) vergleichend charakterisiert. Die Ergebnisse bestätigten vorangegangene Studien an EBLV-1a-Isolaten, wonach die Sequenzen eine hohe Identität aufweisen. Es konnte jedoch gezeigt werden, dass die genetische Distanz mit der räumlichen und zeitlichen korreliert ist. Zudem deuten die Ergebnisse darauf hin, dass die GL-NTR bei EBLV-1 nicht für vergleichende phylogenetische Studien geeignet zu sein scheint.
Interessanterweise konnten im NP-NTR bei zwei Isolaten eine 6 nt-Insertion bzw. bei einem Isolat eine 2 nt-Insertion nachgewiesen werden. Zudem wurde bei einem Isolat eine Deletion von 35 nt im GL-NTR festgestellt. Die Folgen dieser Mutationen auf virale Eigenschaften sind Gegenstand weiterer Untersuchungen, da diese bei Lyssaviren zuvor noch nicht beschrieben wurden.

Schlüsselwörter: Europäische Fledermaustollwut, EBLV-1, Deutschland, Insertion, Deletion

Introduction

Bats have been identified as vector or reservoir of a plethora of viruses including zoonotic diseases. The oldest known disease transmitted by bats, however, is rabies. Rabies is caused by various negative strand RNA-viruses in the genus *Lyssavirus*, family *Rhabdoviridae*. So far, four distinct bat lyssavirus species have been found in Europe; European bat lyssavirus (EBLV) type 1 & 2, West-Caucasian bat lyssavirus (WCBV), and Bokeloh bat lyssavirus (BBLV) (Fooks et al., 2003; Kuzmin et al., 2005; Freuling et al., 2011). In contrast to EBLV-1 and for reasons that are unknown yet, EBLV-2 have only sporadically been found in Europe (Banyard et al., 2011; Freuling et al., 2011). While EBLV-1 has a specific association with the Serotine bat (*Eptesicus serotinus*), EBLV-2 is associated with the Daubenton's (*Myotis* [*M.*] *daubentonii*) bat and the Pond bat (*M. dascyneme*) (Banyard et al., 2011). Single isolations were made with WCBV and BBLV from a *Miniopterus schreibersii* and *Myotis nattereri*, respectively (Kuzmin et al., 2005; Freuling et al., 2011).

Molecular characterization has increased the understanding of the epidemiology of lyssaviruses including EBLV-1. An early evolutionary analysis indicated that both EBLVs evolved into at least two genetically distinct lineages, EBLV-1a, 1b and EBLV-2a, 2b, respectively. (Amengual et al., 1997).

It was speculated that the different EBLV-1 lineages were introduced into parts of northern Europe from two directions, with EBLV-1b as the most recently introduced strain from a North African origin via the south of Spain. Thus, EBLV-1a was supposed to exhibit a west-east expansion, and EBLV-1b a north-south distribution, respectively (Amengual et al., 1997; Picard-Meyer et al., 2004). However, this hypothesis still lacks confirmation by molecular analysis of EBLVs from various parts of Europe due to inconsistent or still missing surveillance for EBLVs across Europe and incomplete phylogenetic analyses of existing isolates. In previous studies on bat rabies in Germany a distinct geographical distribution of EBLV-1 was observed (Müller et al., 2007; Freuling et al., 2008). The objective of this study was to analyze whether this spatio-temporal association is also evident using a statistical approach. Also, it was assessed whether the G-L region known for high variability among RABV is also suitable for phylogenetic analysis of EBLV-1 from Germany.

Material and Methods

A panel of 48 EBLV-1 isolates from the archive of the Friedrich-Loeffler-Institut were selected using a grid-based approach as described earlier (Freuling et al., 2008). Briefly, the distribution of all EBLV isolates was displayed on a map using map explorer of the ArcView (Esri, Redlands, USA) GIS software package. The selection of a representative panel of isolates for the subsequent phylogenetic analysis was performed by applying a random grid of 30 km edge length. One isolate per grid cell was selected. Following RNA extraction and RT-PCR, sequencing of the nucleoprotein-gene and the untranslated region (UTR) between the N- and P-gene was performed with a panel of IRD-800 labeled forward and reverse primers as described earlier (Freuling et al., 2008). Additionally GL-UTR was also sequenced. For the latter, a 775 nt fragment was amplified using the primers GL-for (5′-CATGTTGCAAAAGGTTC-3′) and GL-rev (5′-AATTGCTTCTCAGGTCT-3′) and subsequently sequenced using the same primers.

Sequence analysis was performed using the Lasergene 6 package (DNAstar Inc., USA), and phylogenetic and molecular evolutionary analyses were conducted using MEGA4 (Tamura et al., 2007).

The function implemented in MEGA was used to create a distance matrix (number of substitutions) for all tested isolates. Using the map explorer each origin of an EBLV-1 isolate was given coordinates in the Gauss-Krueger (PD) format. Based on X- and Y-values the Euclidian distance between two localities A and B was calculated. The formula was transferred in the spreadsheet program Excel, Office© (Microsoft, USA), and a geographical distance matrix between all selected isolates was generated. A similar matrix was built representing the temporal distance of the isolates in respect to the EBLV-1 reference isolate Hamburg (1968) (Wersching and Schneider, 1969).

To statistically test the different influences it was hypothesized that the genetic distance (Δ^G) between the isolates in relation to the reference isolate increases linearly with the temporal distance (Δ^t) or with the spatial distance (square-root) (Δ^S). Here, the temporal distance was measured as the distance between the time of isolation in years and the genetic distance as number of nucleotide differences in the N gene. Δ^t and Δ^S are random variables. The linear regression model $\Delta^{G^{i,j}} = \beta_0 + \beta_1\Delta^{t^{i,j}} + \varepsilon_{i,j}$ and $\Delta^{S^{i,j}} = \beta_0 + \beta_1\Delta^{G^{i,j}} + \varepsilon_{i,j}$ was created (Sokahl and Rohlf, 1994), to test the hypothesis $\beta_0 \leq 0$ against the alternative $\beta_1 \geq 0$ with a significance level of $\alpha = 0.5$. Δ^t and Δ^S is the distance in years and the geographic distance, respectively. In order to meet model requirements the square root of Δ^S has been drawn and the following formula was used $\sqrt{\Delta^{S^{i,j}}} = \beta_1 + \beta_0\Delta^{G^{i,j}} + \varepsilon_{i,j}$.

The variable β_0 = b0 is the y-intercept, β_1 is the slope of the regression line and is the error coefficient. To perform the test, the statistical program SAS 9.1 (SAS Institute Inc., USA) was used.

Results

The sequence alignment showed that the coding region of the N-gene of all investigated EBLV-1 isolates was 1356 nt long. The UTR between the N- and P-gene consisted of 90 nt. In four isolates, deviations in the form of insertions were found in this part of the genome. The additional nucleotides are insertions in the transcription-termination

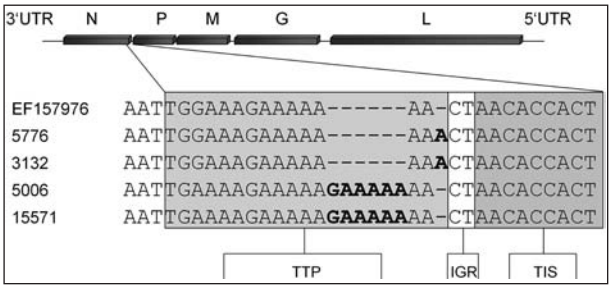

FIGURE 1: *Alignment of the NP-untranslated region (UTR). Boxes show the transcription termination and polyadenylation (TTP) motif, the intergenic region (IGR) and the transcription initiation signal (TIS). The relative position within the genome is indicated.*

and polyadenylation (TTP) signal (Fig. 1). The EBLV-1a isolates 3132 and 5778 had a singular A-insertion. Also, isolates 5006 and 15771 from the EBLV-1b group had a 6 nt insertion (Fig. 1). The GL-UTR comprised 560 nt. One single exception was isolate 5782 with a length of just 525 nt due to a 35 nt deletion at positions 5321-5355 (according to EF157976) in this part of the genome.

The phylogenetic tree using the N-gene sequences revealed a separation of the isolates into the two lineages, EBLV-1a and 1b, with geographic clustering within EBLV-1a (Fig. 2). When using sequences of the NP and GL-UTR the phylogenetic tree was less structured and a separation into similar clusters was not possible (data not shown).

When the year of isolation was correlated to the number of substitution, the separation into two clusters was evident (data not shown). Only the isolates 12868 (1970) and 12871 (1983) were isolated before 1985. With respect to the reference isolate 12865 (Hamburg, 1968) all isolates tested differed in fewer than 10 nucleotides within the 1356 nucleotide sequence of the N gene (data not shown). Exceptions were three isolates of subtype EBLV-1b, which had more than 50 nucleotide exchanges compared to isolate 12865.

When the isolates of subtype EBLV-1a were considered separately, a statistically significant increase ($p < 0.05$) in the genetic differences with increasing temporal distance was evident. The H_0 hypothesis is rejected, therefore, the genetic distance increases linearly with increasing distance in time.

When all geographic and genetic distances of the studied isolates were displayed three clusters were found. Representatives of the EBLV-1a subtype showed a maximum of 14 nucleotide differences with a geo-

TABLE 1: *EBLV-1 isolates used in this study, their details and GenBank accession numbers*

Lab-No	Year	Species*	Location	County	Federal state
915	1996	*E. serotinus*	Osnabrück	Osnabrück	Niedersachsen
933	1993	*E. serotinus*	Oldendorf	Stade	Niedersachsen
976	1992	*P. nathusii*	Marienhafen	Aurich	Niedersachsen
992	1998	*E. serotinus*	Moordorf/Südbrookmer	Aurich	Niedersachsen
2976	1999	?	Hassbergen	Nienburg a. d. Wese	Niedersachsen
3131	1999	?	Winsen (Luhe)	Harburg	Niedersachsen
3132	1999	*E. serotinus*	Jena, Stadt/THÜ	Jena, Stadt	Thüringen
4644	1997	?	Plate	Schwerin	Mecklenburg-Vorpommern
4895	2000	?	Freyburg	Burgenlandkreis	Sachsen-Anhalt
5006	2000	?	Wadgassen	Saarlouis	Saarland
5185	2000	*E. serotinus*	Hitzhausen	Osnabrück	Niedersachsen
5226	1996	*P. auritus*	Lingen	Emsland	Niedersachsen
5248	2000	*E. serotinus*	Bremen Grohn	Bremen	Bremen
5250	1994	*P. pipistrellus*	Ricklinger Holz	Hannover	Niedersachsen
5254	2000	*E. serotinus*	Osterode	Osterode	Niedersachsen
5776	2001	?	Halle/Saale,St.	Halle, Stadt	Sachsen-Anhalt
5778	2001	?	Bernburg (Saale)	Leipziger Land	Sachsen-Anhalt
5782	2001	?	Horburg-Maßlau	Merseburg-Querfurt	Sachsen-Anhalt
6795	2002	*E. serotinus*	Luckenwalde	Teltow-Fläming	Brandenburg
8624	2003	*E. serotinus*	Osterholz-Schar	Osterholz	Niedersachsen
10924	2004	?	Ostrhauderfehn	Leer	Niedersachsen
10925	2004	?	Emden,Stadt	Leer	Niedersachsen
10927	2004	?	Hesel/NDS	Leer	Niedersachsen
11647	2005	*E. serotinus*	Kyritz	Ostprignitz-Ruppin	Brandenburg
12865	1968	?	Hamburg	Hamburg	Hamburg
12868	1970	?	Stade	Stade	Niedersachsen
12871	1983	?	Bremerhaven	Bremerhaven	Bremen
13394	1978	?	Klixbüll	Nordfriesland	Schleswig-Holstein
13401	1986	?	Cuxhaven	Cuxhaven	Niedersachsen
13403	1986	?	Neunkirchen	Neunkirchen	Saarland
13406	1985	*E. serotinus*	Nienburg	Nienburg	Niedersachsen
13407	1985	*E. serotinus*	Rodenberg/Deister	Grafschaft Schaumb	Niedersachsen
13408	1986	*E. serotinus*	Langwedel-Etelsen	Verden	Niedersachsen
13409	1986	*E. serotinus*	Negenborn	Holzminden	Niedersachsen
13414	1988	*E. serotinus*	Bad Bramstedt	Segeberg	Schleswig-Holstein
13415	1988	*E. serotinus*	Gleschendorf	Ostholstein	Schleswig-Holstein
13416	1988	*E. serotinus*	Lübeck	Lübeck, Stadt	Schleswig-Holstein
13418	1988	*E. serotinus*	Tetenhusen	Schleswig-Flensbur	Schleswig-Holstein
13422	1989	?	Reher	Steinburg	Schleswig-Holstein
13423	1989	*E. serotinus*	Berlin Sadt	Berlin	Berlin
13428	1990	?	Aurich	Aurich	Niedersachsen
13438	1990	?	Harsewinkel	Gütersloh	Nordrhein-Westfalen
13439	1990	?	Itzehoe	Steinburg	Schleswig-Holstein
13445	1996	*E. serotinus*	Hinte	Aurich	Niedersachsen
13450	1998	*E. serotinus*	Berlin Steglitz	Berlin	Berlin
13452	1999	*E. serotinus*	Borna	Borna	Sachsen
13453	1999	*E. serotinus*	Riepsdorf	Ostholstein	Schleswig-Holstein
15548	2006	*E. serotinus*	Rostock	Rostock	Mecklenburg-Vorpommern
15571	2006	*E. serotinus*	Wadern	Merzig	Saarland
15730	2004	*E. serotinus*	Hartmannsdorf	Oberspreewald-Laus	Brandenburg

* *E. serotinus = Eptesicus serotinus, P. nathusii = Pipistrellus nathusii, P. pipistrellus = Pipistrellus pipistrellus, P. auritus = Plecotus auritus.*

graphical distance of up to 500 km to each other. All geographical distances associated with more than 40 exchanges showed relationships between representatives of each subtype EBLV-1a and 1b. Since the three EBLV-1b isolates shared a narrower geographical origin in the southwest of Germany (Saarland), the distance to EBLV-1a isolates ranged between 300 km and 700 km. Because of the higher genetic heterogeneity among the EBLV-1b isolates 5006, 15571 and 13403, a genetic distance of 20–30 nucleotide differences was associated with a geographic distance of < 50 km. Among the EBLV-1a group the correlation between geographic and genetic distance of the studied German EBLV-1a isolates was significant ($p < 0.05$).

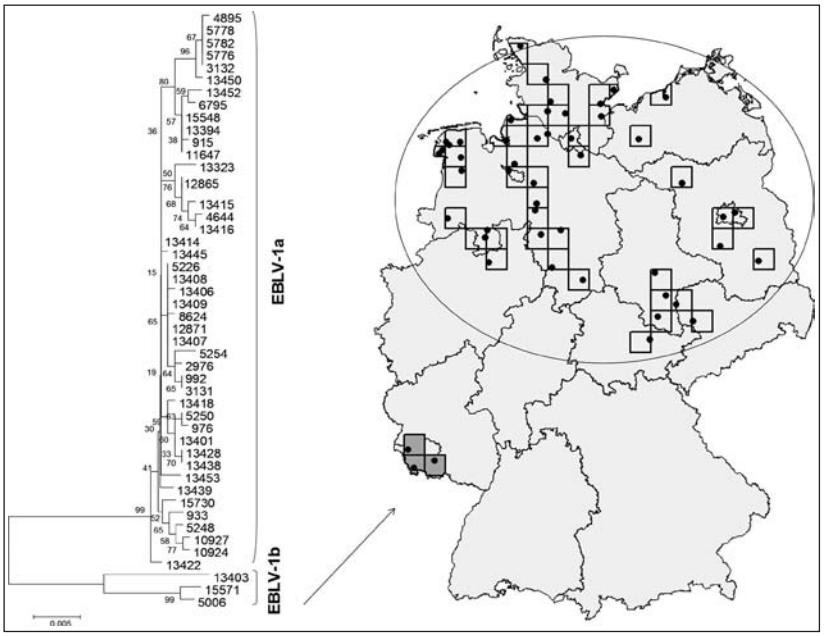

FIGURE 2: *Left: Phylogenetic analysis based on the N-gene using Neighbor-Joining (Kimura-2). Bootstrap values (> 70) are included in the tree. Right: geographical origin of EBLV-1 isolates and general distribution of subtypes 1a and 1b in Germany.*

Discussion

Using a larger panel of EBLV-1 isolates and a larger part of the genome we corroborate previous preliminary findings on the occurrence of EBLV-1b in Germany and the high genetic homogeneity among German EBLV-1a (Müller et al., 2007).

Phylogenetic analyses divided the EBLV-1 sequences into the sublineages a and b, irrespective of the part of the genome used. The best differentiation, however, was seen when analyzing sequences of the complete N-gene. The variability of the GL-region was not sufficient for the discrimination of clusters, an unexpected finding in respect to studies on RABV (Coetzee and Nel, 2007; Nel et al., 2005; Paez et al., 2003).

While all lyssavirus genes seemed equally suitable for phylogenetic analysis (Wu et al., 2007), our results indicate that this may not hold true for untranslated regions.

The alignment of the nucleoprotein-phosphoprotein UTR did not reveal a higher resolution of the phylogeny (data not shown) but showed short genomic insertions. While Johnson and co-workers (2007) identified two isolates (15571 and 5006) of the EBLV-1b group with a 6 nt insertion in the 3′UTR of the N-gene, we have found single nucleotide insertions (A) in isolates 3132 and 5776 stemming from a cluster in the east of Germany. Both isolates have otherwise identical sequences in the N-gene and GL-UTR. This insertion was absent in other closely related members of this cluster (5782, 5778). Within the UTR between G- and L-gene the isolate 5782 showed a 35 nt deletion.

EBLV-1 has a very low genetic diversity (Davis et al., 2005), however, several of the isolates tested showed insertions or deletions (indels) in UTRs. Such alterations had only been described for single isolates of EBLV-1 and two before (Johnson et al., 2007), presumably as only coding regions of specific genes were used for sequence analysis. It was speculated that such alterations could modify the N mRNA transcription efficiency resulting in changes in viral properties for virulence or virus-host interaction (Johnson et al., 2007). Also, the length of the intergenic regions plays an important role in regulating gene expression (Finke et al., 2000). It is also possible that the length of the UTRs has a similar influence and that the 35 nt deletion in the GL-UTR changes viral properties. Further studies are needed to elucidate those features in more detail. With the advent of high throughput sequencing at lower costs it is likely that more of such indels are found in lyssavirus genomes (Höper et al., 2012).

The demonstrated relation between the nucleotide divergence and temporal distance (Fig. 1) is an effect of the mutation and substitution patterns of virus evolution (Duffy et al., 2008). However, all isolates of EBLV-1a tested had fewer than ten substitutions within the 1356 nt of the N-gene, so that the overall divergence is considered low. In fact, Amengual et al. (1997) could not find a linear correlation between the time of isolation and the number of substitutions when analyzing isolates from northern Germany and the Netherlands.

Our study supports previous investigations that have shown a high sequence identity among EBLV-1, particularly in subtype 1a (Amengual et al., 1997; Davis et al., 2005; Freuling et al., 2008; Van der Poel et al., 2005). Also, our results complement previous studies on the relationship between geographic and genetic distances. Both when using a hypothetical introduction point, i. e. Gibraltar (Amengual et al., 1997) or a model based origin of a putative common ancestor a significant positive correlation between geographic and genetic distances was found for EBLV-1b (Davis et al., 2005). The number of EBLV-1b isolates in our study was too limited as to allow an analysis. For EBLV-1a although low bootstrap-values were observed in the phylogenetic analysis (Fig. 3), a previous cluster analysis indicated a geographical segregation (Freuling et al., 2008). By the analysis in this study it was demonstrated that this was not an artifact.

While we found a significant positive correlation when assessing all German EBLV-1a isolates based on the N-gene, no correlation was observed with lineage 1a using a hypothetical introduction point in a previous study (Amengual et al., 1997). Davis and co-workers (2005) found a correlation, but less pronounced than in EBLV-1b. This genetic homogeneity of isolates across geographic

regions led to the suggestion that there has been some spatial mixing and an established viral traffic among bat populations in northern Europe (Davis et al., 2005). While this may be a sporadic event, our observations in Germany (Freuling et al., 2008) as well as from neighboring Netherlands (Van der Poel et al., 2005) rather suggest that transmission of EBLV-1a following long distance movement of bats within the country does not seem to play a major role in EBLV-1 epidemiology. Almost identical sequences of EBLV-1a within certain geographical regions indicate little geographic spread or intermixing as would be expected from the migration pattern of *Eptesicus serotinus* (Hutterer et al., 2005). Our data therefore support previous assumptions (Davis et al., 2005) for adaptation of EBLV-1a to its host.

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