FIRST CASE OF THE EUROPEAN BAT LYSSAVIRUS TYPE 1B IN BATS (EPTESISCUS SEROTINUS) IN POLAND IN RETROSPECTIVE STUDY

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Abstract

The paper describes the first case of the isolation of the European bat lyssavirus type 1b in the Serotine bat (Eptesicus serotinus) in Poland. The study relates to 52 samples, among them four were rabies isolates. Rabies was diagnosed by the fluorescent antibody test (FAT) as well as heminested RT-PCR. Further identification of European bat lyssaviruses was based on phylogenetic analysis of a 400 bp fragment of the nucleoprotein gene. Phylogenetic analysis of four viral isolates obtained from Polish bats and 22 nucleoprotein gene sequences previously described and available on the GenBank database showed that the Polish isolates were divided into two groups – EBLV-1a and EBLV-1b. Three out of four isolates were identified as EBLV-1a whereas one was recognised as EBLV-1b.

Key words: bats, lyssavirus, rabies, heminested RT-PCR, phylogenetic analysis, Poland.

Rabies is a widespread all over the world disease with the exception of a few geographical regions: Scandinavian Peninsula, Japan, and New Zealand. However, bat rabies was confirmed for the first time in Finland in 2009 and EBLV-2 was isolated from the bat. Rabies virus and rabies-like viruses are representatives of the Lyssavirus genus and Rhabdoviridae family. The classical rabies virus, which is isolated from terrestrial animals and haematophagous and insectivorous bats, belongs to genotype 1. Rabies-related viruses are classified as genotype 2 – Lagos bat, 3 – Mokola, 4 - Duvenhage, 5 - European bat lyssavirus 1, 6 - European bat lyssa virus 2, and 7 - Australian bat lyssavirus. Viruses isolated recently from bats (Aravan, Khujand, West Caucasian bat virus and Irkut virus) are waiting for classification and have been proposed as a new member of the Lyssavirus genus(1, 2, 5-7, 12).

In Europe, EBLV1 is mainly detected in Serotine bats (Eptesicus serotinus) while EBLV2 is recorded in Myotis species (M. daubentonii, M. dasycneme). Within EBLV-1 and EBLV-2, two independent lineages a and b are present, which differ in geographical distribution. Within the years 1977-2008, 861 bats were diagnosed as rabid in Europe. The highest number of positive bats was recorded in the Netherlands (303), Denmark (224), Germany (208), and Poland (68). The transmission of lyssavirus between bats and terrestrial animals is rare but it occurs. Human rabies cases due to EBLV-1 and EBLV-2 were also recorded and described (9, 10, 13, 15)

In Poland, the first case of bat rabies was reported in 1972 in a Serotine bat in Krakow. After many years, the next cases in bats were diagnosed in 1985 (Gdansk), 1990 (Kętrzyn), and in 1995 (Warszawa). Generally, till 1998, four rabid bats were diagnosed.

From 1998, when the next rabies case was diagnosed in a Serotine bat in Bydgoszcz city, an increase in the number of bats sent for rabies diagnosis to regional laboratories was reported. It was the beginning of wide passive surveillance of rabies in bats in Poland.

This paper describe the first isolation of EBLV-1b virus from bats in Poland and the comparison of its 400 bp nucleoprotein sequence with nucleoprotein sequences of EBLV-1 viruses available from the GenBank database.

Material and Methods

Virus isolates. On August 7, 1998, the Regional Veterinary Laboratory in Bydgoszcz received the carcass of a bat for routine laboratory investigation. The animal was found in the street. The bat showed clinical signs of paralysis and was not able to fly. The bat had been attacked by cats. The girl, who wanted to help had been bitten by the bat, and post-exposure treatment was applied to her.
The next case of rabies in a bat was diagnosed ten days later in the same area of the town. On August 19, 1998, a rabid bat with symptoms of paralysis was found in a garden in the Torunskie Pomorskie voivodeship (the present Kujawsko-Pomorskie voivodeship). The bat had been attacked by a dog.

The last case was recorded on September 8 the same year in small town - Osieck - on the bank of the River Noteck. A sick bat with signs of paralysis (the bat was unable to fly and hissed) was found on the ground by a citizen of the town during the daytime.

Rabies-routine investigations using fluorescent antibody test (FAT) were done in the Regional Veterinary Laboratory in Bydgoszcz. Next, all the samples were delivered to the National Veterinary Research Institute in Pulawy as original bat materials. Between August 7 and October 20, 1998, 52 different bat species were submitted for rabies diagnosis (Table 1).

Detection of rabies virus antigen. Impressions from the bat brains and salivary glands were made on a microscope slide. The impressions were air-dried, fixed in cold acetone for 30 min, and dried. After the fixation, the smears were stained with FITC conjugate diluted 1:1 and then incubated at 37°C for 30 min in a humid chamber. The slides were washed twice in PBS without Mg and Ca ions, rinsed in distilled water, and dried. Positive and negative control for rabies was prepared for FAT. Slides were read under a fluorescence microscope in a dark room.

RNA extraction. RNA was extracted directly from the rabid animal brains or mouse brains inoculated previously with bat brain homogenates. Brain samples were homogenised in water for injection and RNA extraction was performed with the commercial kit QiAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was re-suspended in RNase-free water in a final volume of 50 µl and used immediately for PCR. The remaining RNA was stored frozen at -20°C for further investigation.

RT-PCR assay. Reverse transcription (RT) and first PCR was performed using a OneStep RT-PCR Kit (Qiagen). Two microlitres of RNA was added to the mixture containing: 3 µl of 5x OneStep RT-PCR buffer, 0.6 µl of dNTPs each at the concentration of 10 mM, 0.5 µl of RNase inhibitor, 0.6 µl of enzyme mix, 1 µl of each primers: JW12 (5’-ATG TAA CAC CYC TAC ATT TTG ATC – 3’)) and JW6DPL (5’-CAA TTC TCA CAC ATT TTG TG-3’) at the concentration of 10 mM, and 7.3 µl of RNase-free water in the final volume of 15 µl. Amplification was performed in a Personal Cycler (Biometa) using the following programme: one cycle of RT at 50°C for 30 min, followed by denaturation at 95°C for 15 min, 35 cycles with denaturation at 94°C for 30 s, annealing at 49°C for 30 s and elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

hnRT-PCR assay. Heminested RT-PCR was carried out with an Enhanced Avian HS RT-PCR Kit, (Sigma). One microlitre of 10-fold diluted RT-PCR product was added to the mixture containing: 2.6 µl of accuTaq buffer, 1.5 µl of dNTPs each at the concentration of 2 mM, 1 µl of each primers JW 12, and for genotype 5: Jebl1 (5’- GTC CCG AGT GAG ATC TTG A – 3’) at the concentration of 10 µM, 0.25 µl of JumpStart polymerase, and 13.65 µl of PCR water to the final volume of 20 µl. Amplification was done in the same thermocycler as RT-PCR was performed. The following programme was set: one cycle of polymerase activation 95°C for 5 min, 30 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

Sequencing and phylogenetic analysis. hn-RT-PCR products were purified with the commercial kit QIAquick PCR purification kit (Qiagen) and were sequenced in both directions on an automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystem) by using a BigDye Sequencing Kit (Applied Biosystem) with GeneScan Analysis Software. The sequencing primers were the same as used for hn RT-PCR.

The nucleotide sequence of antisense strand after sequencing was reverted to, using a Reverse-Complement programme available on the Internet.

The multiple-sequence alignment of 26 EBLV-1 sequences was generated by using Clustal W Multiple alignment and visualised with the BioEdit software v. 7.0.5.3 based on the 400 bp region of nucleoproteine gene. A phylogenetic tree was generated using the maximum likelihood method with the BioEdit software. For graphic presentation and printing of the tree, the TreeView (Win 32) programme was applied.

A sequence identity matrix (percentage identity) calculated from the multiple alignment was constructed using the Lasergene programme.

Results

In four out of 52 examined samples from bats, lyssavirus antigen was detected by FAT in brain tissue and salivary glands, as a brilliant fluorescing apple green particle variable in shape and size. The geographical origin of EBLV 1 positive bats is presented in Fig. 1. Forty-four out of 52 bats sent to the Regional Veterinary Laboratory were from the Bydgoszcz area. Only eight bats were outside Bydgoszcz city.

For the phylogenetic analysis, 400 bp fragment of the nucleoprotein gene of four field isolates (see map Fig. 1) obtained from hnRT-PCR, and a set of 22 sequences of the homologous region of bat rabies strains available in GeneBank, were used. Nucleotide sequences of positive hnRT-PCR products and the 22 sequences from the Genebank were compared by using Clustal W multiple alignment with the BioEdit software (Fig 4). The maximum-likelihood tree showed that four examined isolates were divided into two independent genogroups, creating two clusters (Fig. 2). 400 bp long sequences of the nucleoproteine gene of three isolates were strictly similar to the sequences falling in the cluster EBLV-1a. The sequence obtained from one positive bat tagged as Bat19.PL presented high homology (majority 98.7% and more) with EBLV-1b
cluster. The phylogenetic tree, as well as percentage of identity (Fig. 3), showed the highest similarity of Bat19.PL to French isolates AY245832.1 (98.9%) and AY245837 or AY863400 (98.7%), respectively. The lower similarity to the bat called Bat19.PL presented Spanish isolates U89442.1 and U89443.1 (97.6%) but they all fell in the same EBLV-1b cluster (Fig. 3). Three EBLV-1a isolates showed the highest similarity to Denmark isolates (99.7%).

Four bats out of 52 were diagnosed as rabies positive and all of them were the Serotine bat (*Eptesicus serotinus*) – three males and one female.

Table 1

<table>
<thead>
<tr>
<th>Lp</th>
<th>Species</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serotine (<em>Eptesicus serotinus</em>)</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>Parti-coloured bat (<em>Vespertilio murinus</em>)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Brown long-eared bat (<em>Plecotus auritus</em>)</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Daunenton’s bat (<em>Myotis daubentonii</em>)</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Brandt’s bat (<em>Myotis brandtii</em>)</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Ponds bat (<em>Myotis dasycneme</em>)</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Nathusius pipistrelle (<em>Pipistrellus nathusii</em>)</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Common pipistrelle (<em>Pipistrellus pipistrellus</em>)</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td><em>Myotis</em> sp.</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Pipistrellus</em> sp.</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>Not determined species</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>52</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution of diagnosed bats with positive and negative test results.
Fig. 2. The phylogenetic relationship between four examined EBLV-1 isolates and 22 sequences of EBLV-1 extracted from the GenBank database. EBLV-1b referred to AY 245840 sequence. EBLV referred to the Polish European Bat Lyssavirus 1 isolate N8615POL available in GenBank as AY 863369.

Fig. 3. Distance matrix obtained by MegAlin. Percentage of identity in upper triangle.
Discussion

Rabies in bats has a long history. The first description of the disease comes from a Spanish colonist in the 16th Century. They reported rabies cases in humans and cattle due to vampire-bat bites. The first case of rabies in insectivorous bats was recorded in Trinidad and South Africa at the beginning of the 20th Century. In 1953, a man died in Florida after exposure to a bat. Cases of bat rabies were also reported in Europe, Africa, and Russia, as well as, in 1996, in Australia (3, 4).

In Europe, the first case of rabies in bats was reported in Hamburg, Germany, in 1954. From that time extensive surveillance of rabies in bats started in some European countries. Two genotypes of lyssaviruses are recognised in European bats that are related to, but genetically and antigenically distinct from, rabies virus (genotype 1) present in terrestrial animals. There are EBLV type 1 and EBLV type 2. Phylogenetic analysis of nucleoprotein, as well as the glycoprotein gene sequences of EBLV-1 genotype, has revealed two independent lineages – EBLV-1a and EBLV-1b. They have different patterns of geographical distribution and possibly have different places of introduction to Europe. EBLV type 1a has been found across Northern and Central Europe whereas EBLV type 1b has been isolated in some Western-European countries. France, the Netherlands, and Germany are the countries in which both EBLV-1a and EBLV-1b have been detected. EBLV type 2, however, has been noticed in North-Western European countries, mainly in Daubenton’s bat. In total, there are 19 records of this virus from Denmark, Finland, the Netherlands, Ukraine, Switzerland, some cases in United Kingdom, and the last isolation of EBLV 2 after 50 years of rabies surveillance of bats in Germany (7-9, 14).

Before 1985, only 14 cases of bat rabies had been reported in Europe. An increase in the number of reported cases from 15 in 1985 to 122 in 1986, and 142 in 1987 indicated a possible spread of the rabies virus among European bats. The increase was not sustained and in the recent years the number of recorded cases has stabilised at approximately 20 cases per year. The most common bat species infected by EBLV-1 is the Serotine bat (Eptesicus serotinus). More than 95% of EBLV-1 infection in bats in Europe has been diagnosed in Serotine bats (11, 14). The Serotine bat follows a passage between summer and winter roosts. It is the most numerous species in summer, distributed evenly throughout Poland, but also regularly spends winter here. It is a non-migratory bat and usually performs passages up to 5 km between summer and winter roosts. However, the greatest distance of 330 km has been recorded. In Poland, the greatest distance of Serotine migration was 88 km. It is therefore possible that long-distance transmission is facilitated by migratory bat species that roosts with E. serotinus (16, 17). The other thing is that in Poland bat species sent for rabies diagnosis have not been confirmed.

The phylogenetic analysis of a short genomic fragment (400 bp) of the nucleoprotein gene coding sequence of viruses isolated in Poland and rabies viruses isolated from bats in Europe clearly demonstrate that Polish isolates are members of genotype 5 of lyssaviruses – EBLV-1. The maximum-likelihood tree showed that EBLV-1 isolates were subdivided into two clusters: EBLV-1a and EBLV-1b. The majority of isolates clustered with the EBLV-1a subgroup. However, one out of four Polish isolates was related to the cluster of subgenotypes EBLV-1b. Phylogenetic analysis followed by percentage of homology revealed
that EBLV-1a isolates were the most similar or almost identical to Denmark isolates extracted in 1987, which confirms significant genetic stability within this subgroup. It can also suggest that the virus is highly adapted to the principal host *E. serotinus*. In Germany sequences from isolates separated by a number of years were often identical (14). Polish EBLV-1b isolate, however, was closely related to the EBLV-1b viruses present especially in France and Spain.

Until now, the EBLV-1b genotype has been reported in the Netherlands, France, and recently in Germany (9, 11, 14). This paper reports for the first time the presence of EBLV-1b in Poland. In Poland, rabies in bats was mainly caused by EBLV type 1a with the recent identification of a single case of EBLV-1b. The geographic origin of the analysed Polish EBLV-1b isolate was the North-central part of the country. A recent epidemiological study of EBLV 1 viruses in Germany reported the cases of type 1b localised in the village of Wadgassen near Saarbrücken in the Federal State of Saarland bordering with France where the EBLV 1b in bats had been detected previously. The distance between Saarland and the Kujawsko-Pomorskie voivodeship is more than 800 km and it seems to be too long a distance for Serotine bats to travel. However, further investigation of phylogenetic and epidemiological relationships among European bat lyssaviruses type 1 should be carried out. The most important phylogenetic relationships are especially between isolates coming from neighbouring countries like Germany.

In conclusion, we would like to summarise that the distribution of the EBLV-1b subgroup is wider than it was established so far. EBLV-1b was found not only in Western Europe but also in Central Europe. More epidemiological data is needed to compare all lyssavirus isolates from bats to revise present distribution of lyssaviruses in bats in Europe.

References