

Determination of the rabies virus variants circulating in Poland in 2021–2023 and their phylogeny with analysis of the strains in the Mazowieckie and Podkarpackie voivodeships

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Abstract

Introduction: Rabies is endemic in Europe and red foxes are the vector and reservoir of the rabies virus (RABV). Based on classification established in the early 1990s, four variants of the rabies virus have been distinguished in Europe. Rabies broke out in January 2021 in the Mazowieckie voivodeship in central north-eastern Poland. The virus spread rapidly, reaching the Świętokrzyskie voivodeship in the central southern part and the Lubelskie voivodeship in the eastern part in the next months. Nine rabies cases were reported in the Podkarpackie voivodeship in south-eastern Poland between 2021 and 2023, mainly in red foxes but also in dogs and wildcat. The aim of the study was the identification of RABV variants in wildlife and domestic animals in Poland between 2021 and 2023. **Material and Methods:** The study involved 157 animal brains tested positive for rabies using a fluorescent antibody test. From 10% w/v brain homogenates, RNA was isolated and full-length RABV genomes were high-throughput sequenced with an RABV-enriched approach. Complete genomes of RABV isolates were phylogenetically analysed and the variants were estimated. **Results:** Molecular and phylogenetic studies revealed 147 (93.6%) of the RABV strains out of 157 which had rapidly spread in the wildlife of the Mazowieckie, Świętokrzyskie and Lubelskie voivodeships to be Central European strains. Nine RABVs (5.7%) detected in foxes, a wildcat and a dog in the Podkarpackie voivodeship were identified as North-Eastern European. A vaccine-induced rabies case was detected in a red fox in the Lubelskie voivodeship in May 2023. **Conclusion:** Central European and North-Eastern European RABVs were circulating in Poland between 2021 and 2023.

Keywords: rabies, North-Eastern European RABV, Central European RABV, vaccine-induced, sequencing.

Introduction

Rabies virus (RABV) belongs to the *Mononegavirales* order, the *Rhabdoviridae* family and the *Lyssavirus* genus (1, 5). Based on sequence comparison of nucleoprotein and glycoprotein lyssavirus genes, the International Committee on Taxonomy of Viruses (ICTV) has currently classified 17 *Lyssavirus* species; some remaining newly discovered lyssaviruses are awaiting classification (4). The occurrence of individual species is limited to certain geographical regions; however, the prototype *Lyssavirus* species, RABV, is the most widespread of all lyssaviruses and is found

worldwide except for in Antarctica and on a few islands. This virus causes infections in both humans and terrestrial animals including vampire bats and insectivorous bats in the Americas (17). The animal which is the main reservoir and vector of RABV may be the fox, skunk, raccoon or bat, depending on the region of the world. In the economically underdeveloped countries of Asia and Africa, dogs are still the principal reservoir of RABV.

Since the 1950s, red foxes have become a vector and reservoir of RABV. According to the classification established in the early 1990s, four variant RABVs are differentiated among European RABVs, related to their

distribution on the continent: (I) the Eastern-European (EE), (II) the Western-European (WE), (III) the Central-European (CE) and (IV) the North-Eastern-European (NEE) variants (1). Additional distinct RABV groups were established by McElhinney *et al.* (6) at the beginning of the 21st century, filling the last knowledge gaps regarding the variation of rabies virus strains and their distribution. Those groups are group A, including RABV strains from the Arctic region of Eurasia; group C, which includes RABV strains isolated in eastern Turkey, Georgia, Kazakhstan and south-west Russia, mostly isolated from foxes; group D, containing RABVs isolated from red foxes, dogs and people in the western European part of Russia at the Russian–Ukrainian and Russian–Belarusian borders, as well as in the western part of Turkey and Hungary; group G, composed of strains originating from north-eastern Turkey and Georgia; the SF group of rabies virus strains found in foxes and cats in Serbia; and the FRY group including RABVs isolated from cattle in 1978 and 1981 in the former Yugoslavia, particularly in Montenegro.

In the 1990s in Poland, all four RABV variants were reported. The predominant variants were CE (47.7% of 153 tested RABVs), detected in more than 60% of cases in red foxes and limited to the western and southern parts of the country; and NEE (43.8% of 153 tested), found mainly in red foxes on the eastern bank of the Vistula River, particularly in north-eastern Poland. The other two variants occurred less frequently and were limited to specific regions of Poland. The EE variant (4.6% of 153 tested) was only present in south-eastern Poland, while the WE variant (3.6% of 153 tested) was distributed in western and southern Poland (11).

Prior to the introduction of oral rabies vaccination (ORV), rabies was endemic in Europe. Campaigns of ORV targeting the principal reservoir, the red fox, led to a significant reduction of rabies cases in large parts of Europe (2, 7). In Poland ORV was introduced in 1993, and after that the number of rabies cases decreased from the 3,084 cases recorded in 1992 to a single rabies case in 2019, diagnosed in a red fox in the Lubelskie voivodeship in eastern Poland (14, 15). In the subsequent year, seven rabies cases were registered, four of which were diagnosed in red foxes in Lubelskie and comprised three rabies cases reported in the Tomaszów province and one rabies case in the Dorohusk province. The remaining three rabies cases detected in 2020 were diagnosed in a red fox, a cow and a dog in the southern Podkarpackie voivodeship. Since 2002, ORV has been conducted over the whole of Poland and has resulted in the elimination of the WE and EE RABV variants. In the first decade of the 20th century, only NEE RABV variants isolated on the eastern side of the Vistula River and CE RABV variants circulating on the western side were reported in Poland. The rabies outbreaks which occurred in the Małopolskie and Podkarpackie voivodeships in the south of the country between 2010 and 2017 were caused by the NEE RABV variant, the

major RABV variant that has been circulating in this area for several years (9).

In January 2021, a rabies outbreak was recorded in the Mazowieckie voivodeship in the central-eastern part of Poland, in a region which had been free of rabies since 2004 (13). In the subsequent months and until July 2022, RABV spread dynamically, mainly in the red fox population, towards the south of Poland, reaching the area of Świętokrzyskie (Samborzec and Sandomierz) and Lubelskie (Dęblin) voivodeships. Two separate rabies cases were reported in the western part of the Mazowieckie voivodeship near the border with the Kujawsko-Pomorskie voivodeship in the northern part of central Poland. Nine rabies cases were diagnosed in the Podkarpackie voivodeship between 2021 and 2023, one of which was detected in a red fox and one in a wildcat at the beginning of 2021. The remaining seven cases were reported in six red foxes and a dog in autumn and early winter 2023. All cases were located in the area close to the border with Ukraine.

The aim of the study is to present data on the distribution of RABV variants detected in Poland between 2021 and 2023. Variants were distinguished based on the degree of nucleotide sequence identity of RABV isolates to archival samples of RABV detected in Poland and reference strains of RABV circulating in Europe. Molecular epidemiological studies were also undertaken based on a phylogenetic analysis, to determine the most likely origin of the RABV responsible for the rabies outbreak in the Mazowieckie, Świętokrzyskie, Lubelskie and Podkarpackie voivodeships during the analysed period.

Material and Methods

Samples. The study involved 157 brain tissue samples collected between 2021 and 2023 in four voivodeships located in eastern Poland and the eastern part of central Poland. Regional laboratories approved for rabies diagnosis in suspect animal samples detected lyssavirus antigens in all brain tissue samples using fluorescent antibody tests (18, 19). Rabies was diagnosed in 135 red foxes, 6 dogs, 8 cats, 1 wildcat, 2 martens, 2 raccoon dogs, 2 roe deer and 1 badger. Thereafter the brains were sent to the National Reference Laboratory for Rabies at the National Veterinary Research Institute in Puławy for further studies.

Molecular studies. Ribonucleic acid was isolated from brain tissue (10% homogenates w/v) using a QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Next, full-length RABV RNA sequences were amplified with a previously described protocol (8). The amplicons were subjected to high-throughput sequencing with an RABV-enriched approach (8). The obtained complete genomes of RABVs were subjected to phylogenetic analysis in relation to reference RABV nucleotide (nt)

sequences. Rabies virus variants were identified after estimation of the closest relationship of the subjects to reference RABVs using the Basic Local Alignment Search Tool of the National Centre for Biotechnology Information. Phylogenetic analysis referred to archival RABVs isolated in Poland and was carried out with the MEGA 5 and BioEdit bioinformatics tools (3, 16).

Results

Two variants of RABV were distinguished between 2021 and 2023 in Poland; the dominant one was CE RABV, which caused 147 rabies cases. The NEE variant was the agent of only 9 rabies cases. On May 2022 rabies was detected in the Lubelskie voivodeship in a red fox. The molecular study of the relevant isolate revealed that case to be vaccine-induced rabies and attributed the highest nt sequence identity of the isolate to the Street Alabama Dufferin (SAD) Bern strain. Figure 1 presents the phylogenetic analysis of RABVs isolated in Poland between 2021 and 2023 in relation to reference nt sequences, and Fig. 2 presents the distribution of RABV cases in Poland between 2021 and 2023.

The Central-European variant was detected in 2021–2022 in the Mazowieckie voivodeship during the rabies outbreak, and some rabies cases caused by CE RABVs were detected in the Lubelskie and Świętokrzyskie voivodeships. This variant was detected mainly in red foxes (n = 127, 86.4%). The North-Eastern-European variant was identified in the Podkarpackie voivodeship in 2022 and 2023 in red foxes (n = 7, 77.8%), a wildcat (n = 1, 11.1%) and a dog (n = 1, 11.1%). Details of individual rabies cases are presented in Table 1. All the CE RABV variants detected in the Mazowieckie, Świętokrzyskie and Lubelskie voivodeships belonged to a cosmopolitan group of RABVs forming a common cluster on the phylogenetic tree and showing a high degree of nucleotide sequence identity ranging between 99.8 and 99.9%. The NEE RABV variants formed a separate group and revealed sequence nt identity with archival RABVs circulating in the last decade in the southern Podkarpackie voivodeship of 98.3–99.8%. The nt sequence identity of CE and NEE RABV isolates detected in Poland between 2021 and 2023 ranged between 93.8 and 94.1% (genetic distance 5.9–6.2%).

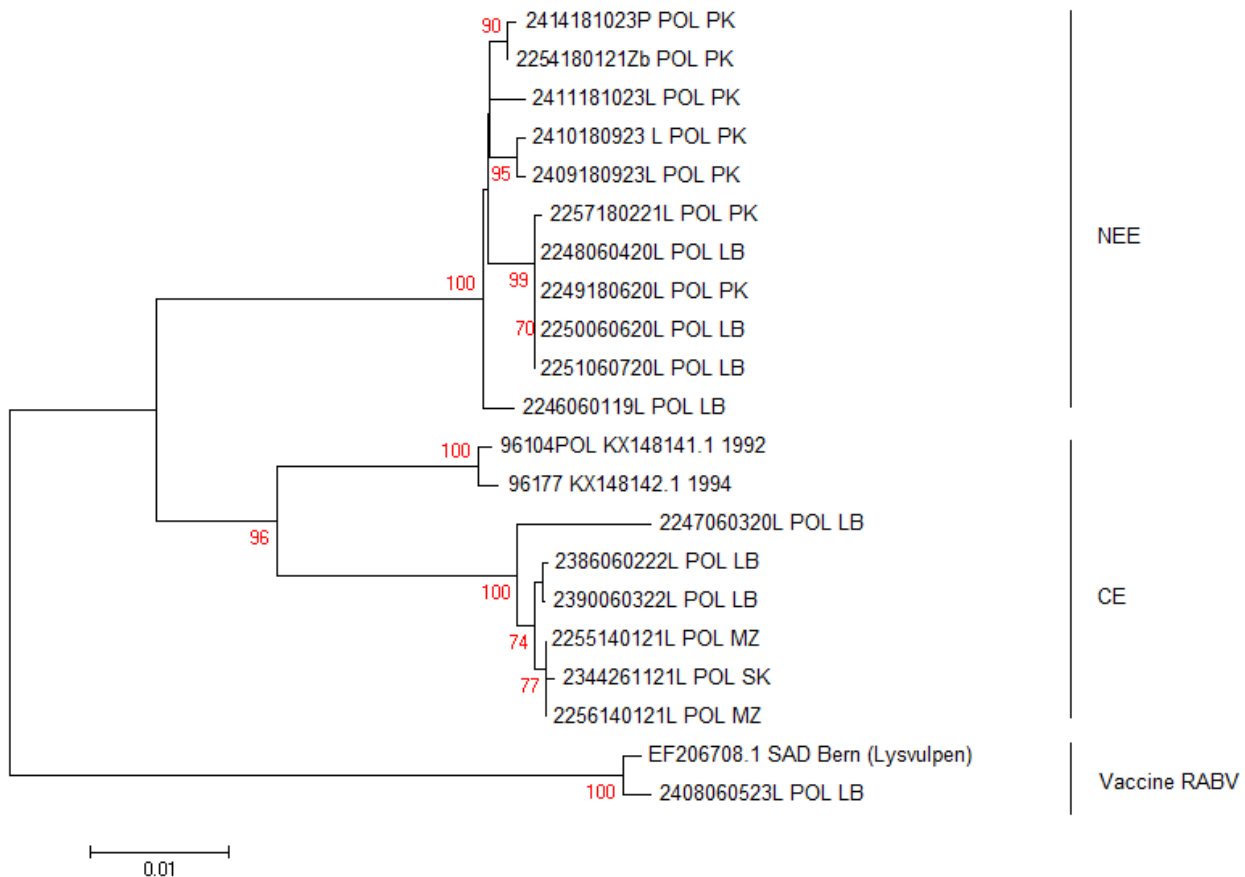


Fig. 1. Phylogenetic relationships of representative rabies virus (RABV) isolates collected in Poland between 2021 and 2023 to complete RABV reference genomes available in GenBank. The phylogenetic tree was generated using the neighbour-joining method (Kimura2 parameter). Bootstrap values (1,000 replicates) over 70% indicating significant support for the tree topology are shown next to the branches. NEE – North-Eastern European variants; CE – Central European variants

Discussion

The last case of rabies in the Mazowieckie voivodeship prior to the rabies outbreak in 2021 was recorded in 2004 in a cat and was caused by the NEE variant of RABV. In line with current law concerning rabies elimination, since 2017 ORV campaigns have not been conducted in the Mazowieckie voivodeship. Seventeen years after the cat rabies case, rabies was diagnosed in the region again in a red fox in January 2021 in the municipality of Wiązowna. Between 2021 and 2023, 140 rabies cases were diagnosed in the Mazowieckie voivodeship. Subsequent to the first new outbreaks in Mazowieckie, five rabies cases were also detected in the Świętokrzyskie voivodeship, three in Lubelskie and nine in Podkarpackie. The cases in the Podkarpackie voivodeship were located in close proximity to the Polish–Ukrainian border.

The RABVs detected during the rabies outbreak in the Mazowieckie voivodeship were CE variants and all shared nearly 100% nt sequence identity. This indicates that one variant of RABV was responsible for the rabies outbreak in Mazowieckie and that it was different from archival rabies virus isolates collected from 2000 to 2004 in the same voivodeship (NEE RABV) and an RABV isolated from a red fox in Stalowa Wola in the Podkarpackie voivodeship, the geographically closest rabies case detected one year before the Mazowieckie outbreak (Fig. 1). The genetic distance at approximately 5% and the results of phylogenetic analyses clearly ruled out that the rabies outbreak in the Mazowieckie voivodeship was caused by the RABV that circulated in this area at the beginning of the 21st century.

It is difficult to indicate unambiguously the source of the RABV that caused the rabies outbreak in the Mazowieckie voivodeship. Most likely, the CE RABV variant was introduced to this voivodeship from a geographically distant area. It should be noted that the last case of rabies caused by the CE variant before the rabies outbreak in Mazowieckie was found in June 2020 at the Polish–Ukrainian border in the Dorohusk region of the Lubelskie voivodeship. Two RABVs detected in February and March 2022 in Dęblin in Lubelskie also revealed affiliation to the CE RABV variant and were almost 100% genetically identical to RABVs detected during the rabies outbreak in the Mazowieckie region. It suggests that both rabies cases detected in Dęblin in 2022 were caused by RABVs that had spread from Mazowieckie. The third rabies case detected in May 2023 in the Trawniki municipality in the Świdnik region, Lubelskie voivodeship, showed 100% similarity of full-length nucleotide sequences to the SAD Bern RABV strain used for the production of the Lysvulpen oral rabies vaccine distributed in ORV campaigns. This was a second confirmed vaccine-induced rabies case following the first reported in a red fox in the Małopolska voivodeship in 2018 (12).

The rabies viruses detected in the Świętokrzyskie voivodeship in 2021–2022 also belonged to the CE

RABV variant and formed a common phylogenetic group with RABVs collected in Mazowieckie, with a full-length nt sequence identity between 99.82% and 99.95% and genetic distances ranging from 0.05% to 0.18% (Fig. 1). This high value of nt identity indicates a close phylogenetic relationship between these RABVs. Based on the date of the RABV detection in the Świętokrzyskie voivodeship, it can be assumed that rabies cases in that area were caused by the virus circulating in Mazowieckie in 2021, which subsequently spread within the population of susceptible animals into Świętokrzyskie.

All rabies viruses detected in 2021–2023 in the Podkarpackie voivodeship clustered with the NEE reference variant in the phylogenetic tree and showed no phylogenetic relationship to the RABVs from the rabies outbreaks in Mazowieckie, Świętokrzyskie or Lubelskie (Fig. 1). The sequence similarity of RABVs isolated in the Podkarpackie, Mazowieckie and Świętokrzyskie voivodeships was estimated at 93.8–94.1%, and the genetic distance at 5.9–6.2%. It should be emphasised that the NEE variant has been persistently present in the southern Małopolska and Podkarpackie voivodeships since at least 2009 (9), but was circulating in these voivodeships in the 1990s. This variant was also the cause of rabies in animals in the last decade in Slovakia, Romania, Moldova and Hungary (10) and currently seems to be the dominant variant in the European Union. Unfortunately, data is lacking on rabies surveillance in Belarus and Ukraine and no molecular characterisation has been published of RABVs circulating there, particularly in the areas bordering Poland. These shortfalls in data limit tracking the spread of rabies virus in Europe and make epidemiological investigation difficult. At the same time, the current geopolitical situation of ongoing war in Ukraine makes it impossible to distribute oral rabies vaccines along the Polish–Ukrainian border. Moreover, the shelling, air raids and mass evacuations in Ukraine may frighten animals and thus increase their movement, and if some of the displaced animals are in the rabies incubation period, this raises the risk of the spread of RABV to Poland. Therefore, because of the buffer zone nature of the Podkarpackie and Lubelskie voivodeships for the spread of the rabies virus westward from Ukraine and the current political situation's consequences, it is necessary to carry out extremely deep rabies surveillance in the provinces located in eastern Poland, especially in their border areas.

Phylogenetic analysis performed for RABVs detected in red foxes, a wildcat and a dog in the Podkarpackie voivodeship between 2021 and 2023 and analysis of archival rabies viruses detected in this area in 2018–2020 revealed high nt sequence identity of between 98.3% and 99.8%. It suggests that closely related NEE-variant rabies viruses have been circulating in Podkarpackie over the last decade (Fig. 1).

Conclusion

Molecular and phylogenetic studies conducted on RABVs detected in Poland between 2021 and 2023 revealed that 147 out of the 157 rabies cases (93.6%) were caused by the CE RABV variant, which rapidly spread within a naive population of wildlife in 2021 and the first six months of 2022 in the Mazowieckie voivodeship, but also caused a few rabies cases in the Świętokrzyskie and Lubelskie voivodeships. The CE RABV variant was detected in foxes as well as in other wild and domestic animal species. The source of the RABV that triggered the outbreak in Mazowieckie in 2021 has not been definitively identified. The circulation of the CE variant in Poland in the last decade was limited only to the areas of Podlaskie and Lubelskie close to the Polish–Belarusian and Polish–Ukrainian borders, and in that time, this variant rarely caused rabies cases; the NEE variant predominated in Poland. There is a lack of data in the GenBank database on the nucleotide sequences of RABV circulating in Ukraine and Belarus, which makes it impossible to carry out phylogenetic studies on the distribution of RABV variants in this part of Europe. Vaccine –induced rabies (0.6%) detected in a red fox in May 2023 in the Lubelskie voivodeship was induced by an RABV vaccine strain used for oral immunisation of foxes against rabies. Nine further rabies cases (5.7%) detected in foxes, a wildcat and a dog at the beginning of 2021 and also at the end of 2023 in the Podkarpackie voivodeship were caused by the NEE RABV variant, which has recently been the causative agent of rabies cases in south-eastern European countries besides also being the rabies agent in this region of Poland.

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