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Vaccine-induced rabies in a red fox in Poland

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Abstract

Introduction: Rabies as a zoonosis threatens public health worldwide. Several thousand people die each year of infections by the rabies virus (RABV). Oral rabies vaccination (ORV) of wildlife was successfully implemented in many European countries and led to rabies being brought under control there. In Poland, ORV was introduced in 1993 using vaccines containing an attenuated strain of the rabies virus. However, attenuated rabies viruses may have residual pathogenicity and cause the disease in target and non-target animals. **Material and Methods:** A red fox carcass was tested as part of national rabies surveillance, and its brain was screened for RABV infection using two conjugates and a fluorescent antibody test (FAT). The rabies virus was isolated in mouse neuroblastoma cells by rabies tissue culture infection test (RTCIT), and viral RNA was detected by heminested reverse transcriptase PCR (hnRT-PCR) as well as by quantitative real-time RT-PCR (rtRT-qPCR). An amplicon of 600 bp was subjected to Sanger sequencing. To differentiate between vaccine and field RABV strains, PCR-restriction fragment length polymorphism (PCR-RFLP) using the Dra I, Msp I, Nla IV and Mbo II restriction endonucleases was performed. **Results:** The rabies virus was detected in the fox's brain using FAT, RTCIT and molecular tests. The PCR-RFLP revealed of vaccine-induced rabies, and full-length genome analysis showed 100% nucleotide sequence identity of the isolate with the reference sequences of Street Alabama Dufferin Bern (SAD Bern) vaccine strains and other vaccine-induced rabies virus isolates detected in animals and deposited in GenBank. **Conclusion:** We detected vaccine-induced rabies for the first time in Poland in a fox during routine rabies surveillance.

Keywords: fox, rabies, SAD Bern, vaccine-induced rabies, sequencing.

Introduction

Rabies as a zoonosis represents a threat to public health worldwide. Several thousand people die each year because of infections by the rabies virus (RABV) (24). Until the introduction of parenteral vaccination in the middle of the last century, rabies was transmitted in Europe mainly by dogs. While vaccination of dogs and accompanying veterinary control measures eventually eliminated dog-mediated rabies, rabies in wildlife sustained by the red fox (*Vulpes vulpes*) emerged in Europe in the late 1950s. Conventional methods of rabies control in foxes aimed to reduce the fox population below the threshold for the spread of the disease but were ineffective and could not halt its spread across Europe (1). Fortunately, the oral rabies vaccination (ORV) of wildlife against rabies developed in the USA offered a new approach to rabies control (2). After successful field trials, ORV was implemented in many European countries and led to progressive reduction of rabies cases and suppression of the disease in those countries (8). In Poland, ORV was introduced in 1993 in the western area bordering with Germany, and in subsequent years the ORV area was extended to the east (18).

All vaccines used for ORV contain either modified live or recombinant viruses. Attenuated rabies viruses used in vaccines may have residual pathogenicity and cause the disease in target and non-target animals, and in fact several vaccine-associated rabies cases were reported in Europe (10, 11, 20) and Canada (7). Although reduced immunocompetence was suspected in several cases, no subsequent transmission was observed and those cases did not have epidemiological relevance. Nonetheless, as a risk mitigating measure, characterisation of all rabies viruses from ORV areas is recommended in order to distinguish field from vaccine strains (5, 23). In Poland, monitoring of ORV is carried out in accordance with EU guidelines, including virus characterisation

(18). The aim of this study was to characterise rabies viruses isolated from a fox in a vaccination area to identify possible vaccine-induced rabies cases. The presented paper describes the initial findings from the first such case recorded in a red fox from Poland.

Material and Methods

Sample collection, diagnostics and phylogenetic studies. Initially, a red fox carcass was submitted for routine rabies testing through a rabies surveillance programme to the regional veterinary laboratory in Kraków, where the brain was removed and subjected to rabies examination by the fluorescent antibody test (FAT). The initial positive FAT result was confirmed at the national reference laboratory for rabies at the National Veterinary Research Institute in Puławy using two conjugates (BioRad, Marnes-la-Coquette, France and Sifin, Berlin, Germany) according to the procedure in the OIE Manual of Standards for Diagnostic Tests and Vaccines (25). Additionally, the rabies virus was isolated in mouse neuroblastoma cells using the rabies tissue culture infection test (RTCIT) (25). Samples of salivary glands, tongue, and spinal cord were subjected to further molecular studies. Mandible and body fluid from the thorax cavities were collected in order to determine vaccine uptake, the age, and the serostatus of the rabid fox. For the latter, an indirect ELISA (BioPro ELISA kit; BioPro, Prague, Czech Republic) was used according to the manufacturer's instructions. Transversal sections of canine tooth were examined using epifluorescence microscopy to detect tetracycline as a marker for bait uptake. The age of the fox was determined on the basis of the number of cementum lines in the canine section under light microscope.

For molecular characterisation, total RNA was extracted from 10% (w/v) of brain tissue, spinal cord, salivary glands and tongue homogenates using a QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of viral RNA was verified by a heminested reverse transcriptase PCR (hnRT-PCR) (9) as well as a quantitative real-time RT-qPCR (rtRT-qPCR) (21). As a screening tool to differentiate between vaccine and field RABV strains, a PCR-restriction fragment length polymorphism (RFLP) using Dra I, Msp I, Nla IV and Mbo II restriction endonucleases was performed. Next, the product of RT-PCR was subjected to Sanger sequencing in both directions on an automated ABI PRISM 310 Genetic Analyser (Applied Biosystems) using a BigDye Sequencing Kit (Applied Biosystems, Waltham, MA, USA) with GeneScan Analysis Software. The nucleotide sequences of the 570 bp *N* gene were aligned using Clustal W Multiple Alignment 7.0.5.3 and a phylogenetic tree was generated using the maximum likelihood method with the appropriate evolutionary model and bootstrapped on the set of 1,000 replicates with MEGA 5 software (19).

Whole genome sequencing. The obtained RNA was converted into double-stranded cDNA using a combination of a SuperScript IV First-Strand cDNA Synthesis System (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and a NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA). The generated cDNA was fragmented using a Covaris M220 focused-ultrasonicator (Covaris, Woburn, MA, USA) and subsequently converted to Ion Torrent–compatible libraries using a GeneRead L Core Kit (Qiagen) and IonXpress barcode adaptors (Thermo Fisher Scientific) followed by size selection as previously described (22). After quality control with an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit, (Agilent Technologies, Santa Clara, CA, USA) and quantification (with a KAPA Library Quantification Kit for the Ion Torrent Personal Genome Machine Uni; KAPA Biosystems/Roche, Basel, Switzerland), libraries including the Ion S5 Calibration Standard were sequenced on an Ion 530 chip with an Ion S5 XL System (Thermo Fisher Scientific) in 400-bp mode according to the manufacturer's instructions. Full genome sequences were obtained by *de novo* assembly of full or partial matching reads from the forerun mapping (454 Sequencing System Software v3.0; Roche) and submitted to the European Nucleotide Archive under the study accession number PRJEB35810.

Results

Both the detection of virus antigen (FAT) and viable virus (RTCIT) confirmed rabies in this fox. The PCR-RFLP results indicated the presence of a vaccine strain of the rabies virus in the tested brain sample (Fig. 1), which was confirmed by Sanger sequencing of an RT-PCR amplicon of around 600 bp.

Multiple sequence alignments with reference RABV isolates using the basic local alignment search tool (BLAST) revealed the closest genetic relationships to the Street Alabama Duferrin Bern (SAD Bern) vaccine strain, which is used for the production of the Lysvulpen oral vaccines against rabies (Fig. 2).

The virus was distributed in the spinal cord, salivary gland, and tongue samples as detected by FAT, RTCIT and rtRT-qPCR. Viable virus was isolated from brain tissue after the first cell passage, whereas a second passage was needed for the spinal cord, salivary glands and tongue. This corresponds to the viral load, *i.e.* the concentration of RABV RNA shown by the number of *N* gene copies of RABV determined in each of these tissue samples (Table 1).

With the blocking percentage established at 27.6, no antibodies against RABV were detected in the fox body fluid using the BioPro ELISA kit. Tetracycline lines identified in dentine indicate multiple uptakes of the vaccine baits by the animals over a very short time (Fig. 3). The age of the fox was estimated to be 1–2 years.

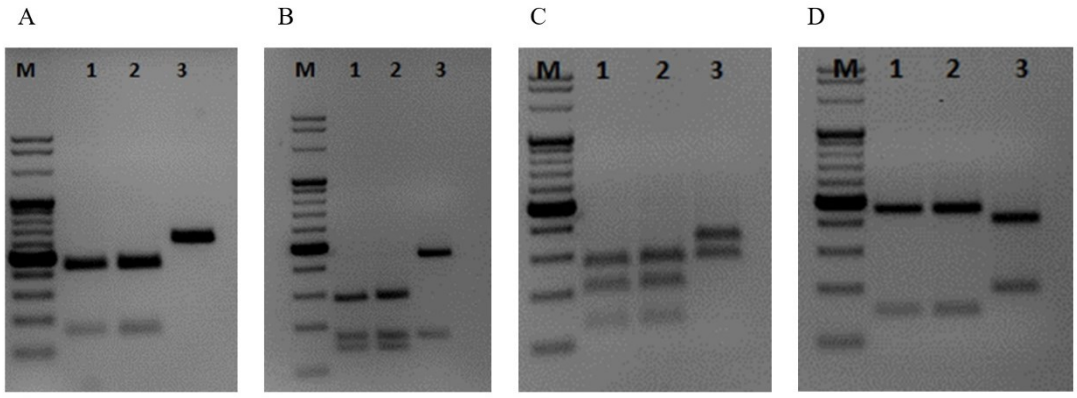

Fig. 1. PCR-RFLP results using Dra I (A), Mbo II (B), Msp I (C) and Nla IV (D) restriction endonucleases. M – DNA molecular mass ruler; 1 – vaccine-induced rabies, fox; 2 – rabies vaccine strain Street Alabama Duferrin Bern; 3 – rabies virus street/field strain

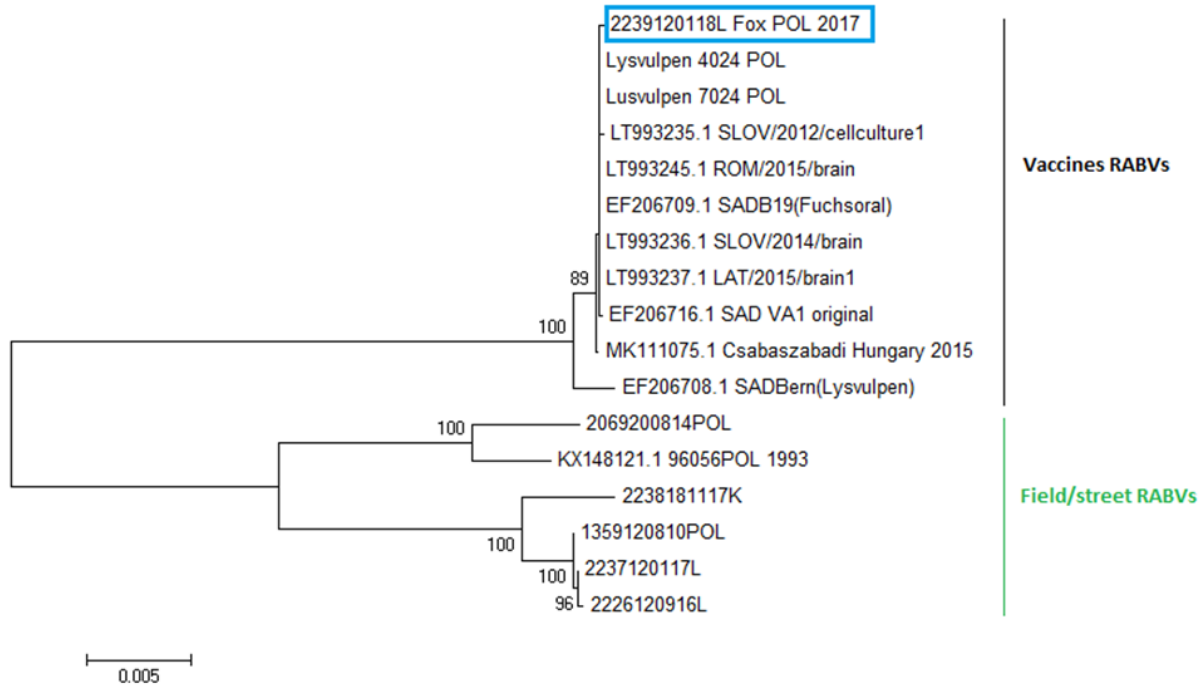

Fig. 2. Phylogenetic relationship of the vaccine-induced rabies virus isolated from a fox in Poland (indicated in the blue box) with reference rabies virus (RABV) isolates available in GenBank based on complete genomes. The phylogenetic tree was generated using the neighbour-joining method (Kimura2 parameter) in MEGA5 software. Bootstrap values (1,000 replicates) over 70% indicating significant support for the tree topology are shown next to the branches

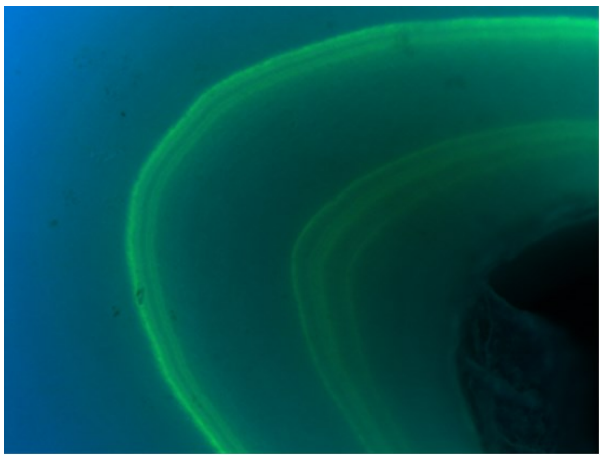

Fig. 3. Section of canine teeth showing yellow fluorescent lines of tetracycline

Table 1. Distribution of viral RNA in particular predefined organs

Organ	Threshold cycle	Copies/Reaction
Brain	19.37	9.01e+005
Spinal cord	23.03	7.25e +004
Salivary glands	36.79	5.62
Tongue	26.62	6.14e+003

Discussion

Oral vaccination of wildlife has been used as an effective method of rabies control and eventual elimination in Europe, with most countries in the EU having achieved rabies-free status (12). Irrespective of their efficacy and effectiveness, vaccines used for ORV must be safe for target and non-target animal species in

case of exposure in the field, as required by the World Health Organization (WHO), World Organization for Animal Health (WOAH) and the European Pharmacopoeia (6, 23, 26). The vast majority of oral vaccines against rabies that have been used across Europe are based on the attenuated SAD-related strain (14). The SAD Bern virus strain, the progenitor of several other vaccine derivatives, demonstrated some residual pathogenicity and was able to induce the disease under special circumstances (13). In fact, vaccine-induced rabies cases have been described in wild and domestic animals in Europe (Switzerland, Germany, Austria, Slovenia, Romania, Lithuania and Latvia) and Canada. However, the number of vaccine-induced rabies cases is still extremely low compared to the number of vaccine baits distributed in the field. Furthermore, since it is a sporadic event, the detection of vaccine-induced rabies cases is directly related to the intensity and quality of the prevailing rabies surveillance and monitoring system. It was reported that a few vaccine-induced rabies cases were initially not detected in the FAT, suggesting that the number of vaccine-induced rabies cases could be underestimated (11).

As actions forming part of ORV monitoring and surveillance for rabies which started in parallel to ORV in 1997, all indicator animals, and foxes shot for ORV monitoring purposes, were tested for the presence of rabies antigen using the FAT. Between 2014 and 2018, 1,791 animals were tested in the rabies surveillance programme and 4,888 animals were tested as a part of ORV monitoring in the Lesser Poland voivodeship, where the case reported in this article was identified. The percentage of rabies-positive animals detected during ORV monitoring and rabies surveillance ranged between 0.1% and 4.6% of all tested animals per year. FAT-positive samples were subsequently typed using PCR-RFLP to determine if they were vaccine-induced or field/street rabies virus. Until December 2017, all Polish virus isolates detected during rabies surveillance and monitoring of ORV were characterised as field RABV strains. This first reported vaccine-associated rabies case in Poland was initially found using PCR-RFLP. This is a very robust and easy screening method. Prior to molecular detection coming into use, vaccine variants were identified using typing with monoclonal antibodies (17).

While it was initially assumed that there was a change in the vaccine strain used in Lysvulpen in the past (4), it was later verified that some of the SAD-derived vaccines comprise virus populations rather than a single homogeneous variant (3, 15), making it impossible to distinguish between SAD Bern and SAD B19. Partial sequence analysis using the PCR fragment already indicated the presence of a SAD-based vaccine virus strain, *i.e.* SAD Bern, the virus strain used for the production of Lysvulpen. In the present research, both consensus sequence and sub-genomic population analysis of viral reads from next generation sequencing clearly identified the virus as SAD Bern (3), which corresponds to the fact that this vaccine was used for ORV in this area. The reasons why the SAD Bern strain induced rabies in this fox remain elusive. One aspect that was discussed elsewhere is that affected animals may have been immunocompromised (11). In fact, despite the consumption of several vaccine baits, the fox did not demonstrate detectable levels of antibodies against RABV. On the other hand, the age of 1–2 years cannot be considered a factor for immunosuppression, and there was no evidence of external or internal parasitic invasion (data not shown) that could also suppress the immune system.

Theoretically, as attenuated RABVs used in oral vaccines consist of replication-competent virus particles, they may revert to virulence. In spite of the content of only safe, attenuated virus strains in live oral rabies vaccines, vaccine-induced rabies cases were reported in both target and non-target species caused by SAD Bern, SAD B19, SAD P5/88 and ERA vaccine strain infections (3, 7, 11 16, 20). In our case, neither full genome consensus sequence analysis nor subgenomic population-based analyses as discussed before provided any evidence for a genetic change that could lead to reversion to virulence.

The presence of tetracycline in the teeth and bones of the fox with vaccine-induced rabies supports the conclusion that the animal's SAD Bern infection was the result of the vaccine bait consumption rather than a transmission of vaccine-originated rabies virus in the animal population. The presence of an infectious virus in the salivary gland of the rabid fox represents the theoretical possibility of onward transmission. However, similarly to previously reported cases, no further rabies cases have been reported from this area ever since, confirming that the infection is self-limiting.

In conclusion, we report a vaccine-induced rabies case in a fox in Poland that was successfully detected during routine rabies monitoring and surveillance efforts. While such cases may occur when attenuated oral rabies vaccines are distributed in the field, the sporadic nature and their limited impact do not diminish the reputed safety of ORVs and their general utility for the successful elimination of fox-mediated rabies in Europe.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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