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Source: Journal of Wildlife Diseases, 29(2) : 209-213

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-29.2.209

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Journal of Wildlife Diseases, 29(2), 1993, pp. 209–213
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DIFFERENTIATION OF TWO RABIES STRAINS IN ESTONIA WITH REFERENCE TO RECENT FINNISH ISOLATES

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ABSTRACT: We compared 24 rabies samples collected in Estonia in 1989 to 1992, to identify the kinds of rabies strains circulating in this country. Eleven of the strains came from the islands of Saaremaa, Hiiumaa and Muhu, off the Baltic coast; 13 came from the mainland. The mainland strains, like those from the 1988 to 1989 epizootic in Finland, were antigenically different from the 11 island isolates. The island isolates reacted negatively with monoclonal antibody W-187.5 as does the SAD B19 rabies vaccine strain, currently spread as baits to wildlife in Finland and other parts of Europe. In order to unambiguously distinguish the island isolates from the SAD B19 vaccine, we developed a polymerase chain reaction (PCR) protocol for rabies, followed by a single restriction enzyme digestion. This method enabled the island isolates to be differentiated with ease from the vaccine strain SAD B19 at the level of the nucleoprotein-coding region. Additionally, this method had the ability to distinguish other polar field isolates examined, as well as the laboratory challenge virus strain CVS, from SAD B19 vaccine. Modifications of the above PCR method may be used for epidemiological investigations of new outbreaks or of outbreaks involving different species.

Key words: Estonian islands, fox, *Vulpes vulpes*, raccoon dog, *Nyctereutes procyonoides*, polymerase chain reaction, restriction enzyme digestion, antigenic typing.

INTRODUCTION

Estonia and Finland are geographically at the convergence of the Arctic and European ecotypes. This makes the area interesting for studying the co-existence of dissimilar rabies virus strains, especially in free-ranging wildlife. These countries, with well-established fox (*Vulpes vulpes*), raccoon dog (*Nyctereutes procyonoides*) and wolf (*Canis lupus*) populations, offer a natural environment for the spread of several rabies strains with different origins.

Northern rabies strains have been characterized and investigated in several areas, such as Alaska (USA) (Ritter, 1981), the Northwest Territories, Quebec, and Ontario (Canada) (Webster et al., 1986); the Yamal Peninsula of Russia (Syuzyumova, 1968); and the Svalbard Islands of Norway (Prestrud et al., 1992). Rabies strains usually have been classified by monoclonal antibodies (Smith, 1989); using this technique there is variation even within the Arctic group at the level of some nucleocapsid epitopes (Webster et al., 1986).

Antigenic typing would be particularly helpful when the precise binding site of the monoclonal antibody is known (Dietzschold et al., 1987), and further, when this epitope can be related to a biologically meaningful function (Benmansour et al., 1992). This is not the case with most of the monoclonal antibodies widely used in typifying rabies strains. Therefore, analysis at the genomic level is often necessary, either when exact epidemiological typing is performed (Smith et al., 1991; Sacramento et al., 1991) or when the biological properties of certain virus strains are determined. Our objective was to determine the numbers and kinds of rabies strains circulating in Estonia.

MATERIALS AND METHODS

Twenty-four rabies-positive brain samples were collected from 1989 to 1992 from several areas in Estonia (Fig. 1; 21°30′ to 27°30′E, 57°30′ to 59°30′N). The exact locations and animal species of the cases are listed in Table 1. The corpses or animal heads were sent to the Veterinary Laboratory in Tallinn, Estonia, to be checked for rabies because they were from animals which had bitten people or domestic animals. All the domestic animals, which were checked for rabies, had clinical signs of disease. Some of the wild animals were found dead, but some were killed because they had behaved abnormally.

Portions of the brain specimens were transported in a physiological, 50% glycerol buffer,

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TABLE 1. Origin of the rabies specimens in Estonia, and their antigenic profile, 1990 to 1992.

Case no. (refers to map in Fig. 1)	Species	Location	Reactivity with monoclone W-187.5
1	Fox	Kehtna, Rapla	+
2	Badger (*Meles meles*)	Saue, Harju	+
3	Dog	Muhu	−
4	Fox	Haapsalu, Läänemaa	+
5	Raccoon dog	Ravila, Harju	+
6	Cow	Jõelähtme, Harju	+
7	Raccoon dog	Viimsi, Harju	+
8	Sheep	Sõrve, Saaremaa	−
9	Cow	Sõrve, Saaremaa	−
10	Raccoon dog	Valjala, Saaremaa	−
11	Dog	Lümanda, Saaremaa	−
12	Fox	Sõrve, Saaremaa	−
13	Fox	Kihelkonna, Saaremaa	−
14	Fox	Kõrgessaare, Hiiumaa	−
15	Dog	Kõrgessaare, Hiiumaa	−
16	Raccoon dog	Sõmerpalu, Võru	+
17	Raccoon dog	Haanja, Võru	+
18	Fox	Kuldre, Võru	+
19	Raccoon dog	Lasva, Võru	+
20	Raccoon dog	Kuressaare, Saaremaa	−
21	Fox	Mässa, Saaremaa	−
22	Dog	Anija, Harju	+
23	Fox	Paide, Türi-Jäneda	+
24	Fox	Paide	+

and stored at −20 C or −70 C for further use. Suspensions of ground tissue were inoculated into murine neuroblastoma cell cultures in four parallel wells of a 24-well tissue culture plate (Nunc, Roskilde, Denmark) and one 25-cm² tissue culture flask as previously described (Kulonen et al., 1991). The inoculated cell cultures were stained with two anti-rabies conjugates: Centocor FITC anti-rabies monoclonal globulin (Centocor Inc., Malvern, Pennsylvania, USA) and polyclonal anti-rabies fluorescent conjugated serum (Behringwerke, Marburg, Germany). They then were typed with monoclonal antibodies W-239, W-187.5 and P-41, which had been prepared by the World Health Organization Reference Laboratory, Tübingen, Germany (Schneider et al., 1985). Monoclone W-239 reacts with all rabies strains. Monoclone W-187.5 does not react with SAD B19 vaccine, but reacts with most terrestrial rabies field strains. Monoclone P-41 reacts with polar rabies strains, but does not react with SAD B19 vaccine nor with field strains from temperate regions of Europe. Some of the samples were passed three to five times before there were enough positive cells for typing.

Ribonucleic acid was extracted directly from the brains or from the cell culture fluids using the method of Ermine et al. (1990), with the exception that we performed the following additional RNA precipitation step with lithium chloride (LiCl). The samples were incubated at a concentration of 2 M LiCl overnight at 4 C, and then centrifuged 1,000 × g at 4 C. The precipitate was diluted with distilled water, and then precipitated with ethanol as described by Ermine et al. (1990). The reverse transcription and polymerase chain reaction (PCR) were induced according to Coen (1991). Restriction enzyme digestion was performed with *Mbo*I (Promega, Madison, Wisconsin, USA) and *Nsi*I (Promega) at 10 U/3 μg DNA in a 20 μl reaction. The digestions were incubated for 2 hr at 37 C. The products underwent electrophoresis in a 4% NuSieve 3:1 gel (Rockland, Maine, USA).

The primers used at the PCR reaction were chosen from a group of oligonucleotides preselected with a computer program (Lowe et al., 1990) by Dr. Carl Dieffenbach, on the basis of the published sequence of SAD B19 rabies virus strain (Conzelman et al., 1990). The following pair was used: the anti-genomic primer 5′GAAGCCTGAGATTATCGTGG3′ from nucleotide at position 121 to 140, and the anti-messenger primer 5′CCCTTCTACATCAGTACG3′ from nucleotide at position 424 to 407,

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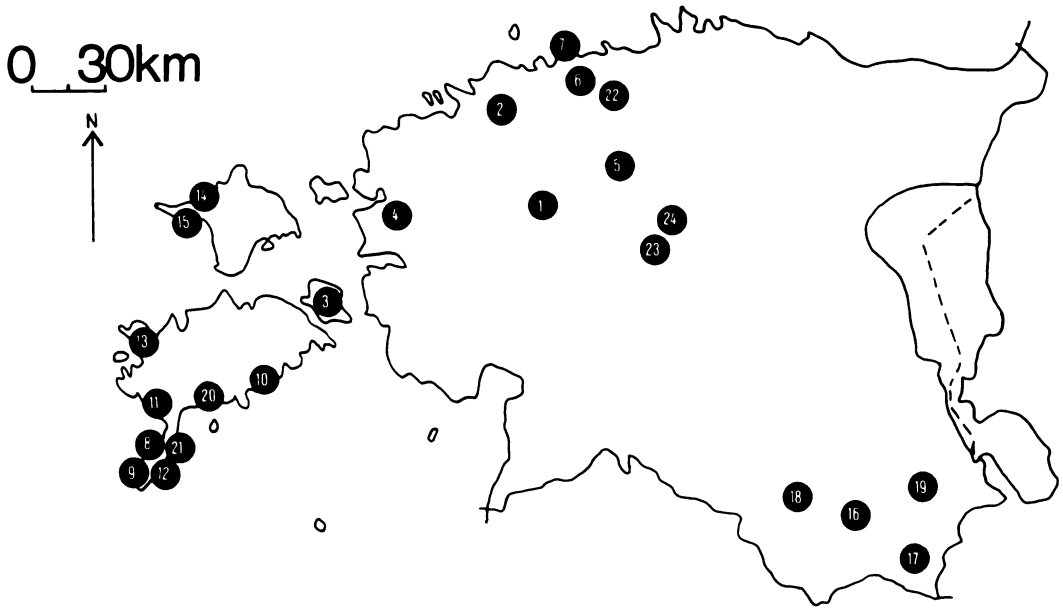


FIGURE 1. Map of Estonia showing locations from which the rabies samples were obtained. The numbers refer to case numbers listed in Table 1.

where G represents guanine, A adenine, C cytosine and T thymine. The former primer has also been documented by Ermine et al. (1990). We chose the primers to ensure that those sites in the nucleoprotein-coding region which, at the amino acid level, were different in SAD B19 (Conzelman et al., 1990) from PV (Pasteur Virus; Tordo et al., 1986) and CVS (Challenge Virus Strain; Mannen et al., 1991) would be included. The origin of the strains has been described previously (Sacramento et al., 1992). The restriction enzymes were selected following the same principle, to indicate variation at the exact sites where the former strains differ at the amino acid level. We included SAD B19 vaccine and two pathogenic strains in the comparison to select for differences which may later prove to be of a more generalized value.

RESULTS

All the samples examined reacted positively with the Centocor monoclonal conjugate and monoclones W-239 and P-41. Eleven of the samples were negative with W-187.5, and 13 isolates were positive with W-187.5. Variations in reactions with W-187.5 were not related to any vector species (Table 1). The samples which were positive with W-187.5 came from the mainland; those which were negative from the large islands (Fig. 1).

In the PCR, the primer pair chosen was capable of amplifying genomes of both of the virus types examined within a wide scale of magnesium concentrations (Fig. 2). By using the restriction enzyme *Mbo*I, we were able to distinguish the island type and the Estonian and Finnish mainland strains from the SAD B19 vaccine strain (Fig. 3). Thus this differentiation was not based on the absence of any specific polar marker in the SAD B19 vaccine.

The *Mbo*I restriction enzyme cut the 304-base pair (bp) PCR-fragment of SAD B19 into one major (236 bp) and two smaller pieces (49 bp and 19 bp). The PCR-fragment of the Estonian island and Finnish strains was cut by *Mbo*I into two larger pieces (126 bp and 110 bp) and two minor pieces (49 bp and 19 bp). The 236-bp fragment (Fig. 3, lane 3) and the 126- and 110-bp fragments (Fig. 3, lanes 6 and 9) were easily differentiated in the gel. The *Mbo*I digestion products of the Estonian mainland strain and the Challenge Virus Strain (CVS) were similar in size to those of the

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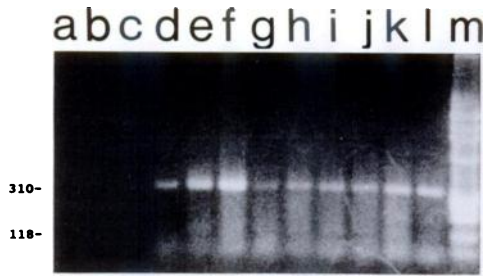


FIGURE 2. Analysis of the SAD B19 vaccine (lanes d to f), Finnish (no. R1631/88, lanes g to i) and Estonian "island type" (no. 15, lanes j to l) rabies viruses. Amplification of a 304-base pair PCR-fragment in the coding region of the rabies nucleoprotein. Polymerase chain reactions were performed with the following $MgCl_2$ concentrations: 6 mM (lanes d, g, j); 4 mM (lanes e, h, k); 2 mM (lanes f, i, l). The marker is *Hae*III-digested ΦX174-RF (lane m). The size markers of base pairs are shown on the left.

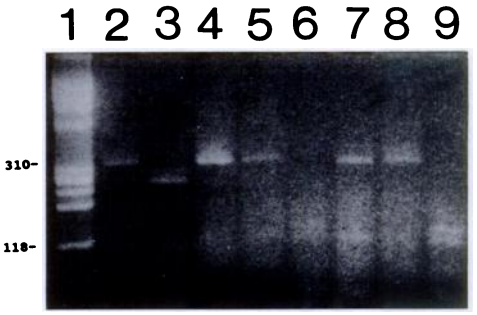


FIGURE 3. Analysis of the SAD B19 vaccine (lanes 2 to 4), Finnish (no. R1631/88, lanes 5 to 7) and Estonian "island type" (no. 15, lanes 8 and 9) rabies viruses. Lanes 2, 5 and 8 were digested with *Nsi*I; lanes 3, 6 and 9 digested with *Mbo*I; lanes 4 and 7 were undigested. The marker is *Hae*III-digested ΦX174-RF (lane 1).

Estonian island type (data not shown). The *Nsi*I restriction enzyme did not cut any of the PCR-fragments (Fig. 3, lanes 2, 5 and 8).

DISCUSSION

Based on our results, Estonia is a region where two different rabies types meet. One strain bears similarity to the northern, Finnish strain. With the limited monoclonal antibody panel used, the profile of the Estonian isolates from the mainland was the same as that of all of the Finnish isolates from the 1988 to 1989 epizootic (Nyberg et al., 1992). In contrast, the Estonian island isolates had a pattern similar to the vaccine strain SAD B19 in reactivity to W-187.5, but dissimilar to SAD B19 in reactivity to P-41. The rabies strains examined from polar areas are known to react with monoclone P-41 (Schneider et al., 1985). We were surprised to detect a rabies strain from terrestrial animals with the combination of P-41 positivity and of W-187.5 negativity. Monoclone W-187.5 had been used as a preliminary marker of a vaccine strain.

Owing to the results of antigenic typing, the unusual geographic distribution, the poor growth in cell cultures and the low number of cases, we had to rule out the possibility that the island type was a strain with unusually low virulence or a mutated vaccine strain. The island type could be differentiated from SAD B19 by the reaction with monoclone P-41. Our aim, however, was to find a definable site at the genome which would differentiate not only arctic, but also other field rabies isolates from the SAD B19 vaccine on an epizootiological basis and, if possible, on a more general functionally related basis. We found that PCR followed by one restriction enzyme digestion was a convenient and precise way of characterizing the rabies isolates of northern Europe. It also offers prospects for further comparison by sequencing.

Because the *Mbo*I cuts before the GATC sequence, it appears that both of the Estonian strains and the Finnish strain are cut by *Mbo*I at position 266; thus, there is guanine in that position. This is not the case with SAD B19, which cannot be cut at position 266. On the basis of the published sequence of SAD B19 (Conzelman et al., 1990), this implies a change from aspartic acid (Asp) (Estonian and Finnish strains) to asparagine (Asn) (SAD B19) at the amino acid level.

Despite the close contact between the islands and mainland in Estonia, there seems to be little or no spatial overlap between the two types. In both types, how-

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ever, the main wildlife vectors are the fox and the raccoon dog. Therefore, it is probable that, before long, the geographical distribution will change and that either one of the types will die out or the two will co-exist in the same areas. If the coexistence takes place within a species, even mixed infections may occur. However, the data on skunk rabies (Hill and Beran, 1992) support the idea that strong species-specific susceptibility differences may influence the distribution of rabies strains, if they spread at the same geographical area.

ACKNOWLEDGMENTS

We are grateful to Drs. Harry Peil and Tõnu Ostrat for valuable advice and support in collecting the specimens.

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Received for publication 29 May 1992.

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