ERA VACCINE-DERIVED CASES OF RABIES IN WILDLIFE AND DOMESTIC ANIMALS IN ONTARIO, CANADA, 1989–2004

Authors: Fehlner-Gardiner, Christine, Nadin-Davis, Susan, Armstrong, Janet, Muldoon, Frances, Bachmann, Peter, et al.

Source: Journal of Wildlife Diseases, 44(1) : 71-85

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-44.1.71

Journal of Wildlife Diseases, 44(1), 2008, pp. 71–85

ERA VACCINE-DERIVED CASES OF RABIES IN WILDLIFE AND DOMESTIC ANIMALS IN ONTARIO, CANADA, 1989–2004

Christine Fehlner-Gardiner,[1] Susan Nadin-Davis,[1] Janet Armstrong,[1] Frances Muldoon,[1] Peter Bachmann,[2] and Alexander Wandeler[1,3]

[1] Centre of Expertise for Rabies, Ottawa Laboratory–Fallowfield, Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, Ontario K2H 8P9, Canada
[2] Rabies Research and Development Unit, Ontario Ministry of Natural Resources, Trent University, Peterborough, Ontario K9J 7B8, Canada
[3] Corresponding author (email: wandelera@inspection.gc.ca)

ABSTRACT: A vaccination program for the control of terrestrial rabies in the province of Ontario, Canada, began in 1989. During the period between 1989 and 2004, over 13 million baits containing the live, attenuated rabies virus ERA-BHK21 were distributed across the province, with the aim of immunizing foxes by the oral route. Animals recovered from bait distribution areas were assayed by fluorescent antibody test for rabies virus infection. Immunoreactivity with a panel of monoclonal antibodies that discriminate between ERA and rabies virus variants known to circulate in Ontario, and molecular genetic analyses were used to identify animals infected with ERA. Nine cases of ERA variant rabies were identified over the 16-yr period of study; these did not appear to be stratified by species, year of discovery, or location of capture. The ERA-positive animals were found across the province in eight counties, all of which had been baited in the year of case discovery. The nine ERA-positive cases included four red foxes (*Vulpes vulpes*), two raccoons (*Procyon lotor*), two striped skunks (*Mephitis mephitis*), and one bovine calf (*Bos taurus*). Molecular phylogenetic analyses of the partial N gene sequences generated from these isolates indicated that these nine cases were due to infection with the ERA variant. The glycoprotein sequences were predicted from G gene sequencing of all nine field isolates and two laboratory stock ERA viruses. This revealed some heterogeneity at residue 120 (either arginine or histidine) in both field and laboratory stocks as well as a few other mutations in field isolates. The significance of this heterogeneity remains unclear. Our data demonstrate that the ERA vaccine distributed in Ontario carried residual pathogenicity; however, there does not appear to be any evidence of ERA establishment in wildlife populations over the 16-yr period. These results are consistent with previous reports of the rare detection of ERA vaccine–induced rabies and with laboratory studies of ERA pathogenicity.

Key words: ERA-BHK21 vaccine, Ontario, pathogenicity, rabies, raccoon, red fox.

INTRODUCTION

In 1960, Paul Fenje described the adaptation of the Street Alabama Dufferin (SAD) strain of rabies virus to grow in hamster kidney cell cultures (Fenje, 1960). The SAD virus, originally isolated from a rabid dog in Alabama, had a history of numerous passages through intracerebral inoculation of mice and was therefore considered to be a fixed virus. Abelseth (1964a) obtained the cell culture–adapted virus from Fenje and, after passage through chicken embryos, successfully propagated it in primary porcine kidney cells. In all subsequent publications, Abelseth (1964b, 1967) refers to this virus as Evelyn Rokitniki Abelseth (ERA) strain and describes its properties as a live attenuated vaccine. The ERA vaccine induces a long-lasting immunity by intramuscular (IM) inoculation (Lawson and Crawley, 1972). Until it was displaced by inactivated tissue culture vaccines, commercially produced ERA virus was used for IM vaccination of domestic animals in numerous countries around the world. Based on studies by Abelseth (1964b, 1967), it could be concluded that the ERA virus exhibited some residual pathogenicity, and this was supported by occasional observations of postvaccination rabies cases. That ERA virus occasionally induces rabies was finally proven by identifying the virus isolated from brains of suspect animals with a panel of monoclonal antibodies (Whetstone et al., 1984).

In the 1960s, Baer et al. (1963) demon-

strated that foxes could be immunized against rabies by oral application of the live ERA virus. This discovery gained much wider attention when it was presented to a European audience in 1970 at a conference sponsored by the World Health Organization (WHO) (Baer et al., 1971), and when Black and Lawson (1970) communicated similar findings. Consequently, the WHO facilitated the cooperation of American, Canadian, and European research groups to investigate oral immunization as a tool for wildlife rabies control. In 1972, the Lawrenceville Laboratory of the Center for Disease Control in Atlanta, Georgia, USA, provided ERA seed virus grown on baby hamster kidney (BHK)-21 cells to three European rabies laboratories. ERA grown on BHK-21 cells was referred to as ERA-BHK21 in order to distinguish it from the marketed ERA produced on primary porcine kidney cells. On request by the manufacturer of the commercial ERA, ERA-BHK21 had to be renamed to SAD. However, all SAD variants used in Europe for oral vaccination of wildlife are derivatives of ERA-BHK21.

Although live attenuated vaccines with reduced residual pathogenicity (Tidke et al., 1987; Lafay et al., 1994) and recombinant rabies vaccines (Kieny et al., 1984) were developed, ERA and SAD are still used in Ontario and in several European countries, and different oral vaccination systems (vaccine, bait, bait distribution) have been described (Aubert et al., 1994; Campbell, 1994; Wandeler, 1991, 2000). Baits that include a container with liquid vaccine are distributed at densities of no less than ten per square kilometer once or twice a year. Bait consumers rupture the container, which releases the vaccine into the oral cavity. The live attenuated or recombinant vaccine virus must infect host tissues in order to express rabies glycoprotein and elicit an immune response.

In 1989, the Ontario Ministry of Natural Resources (OMNR) began a fox rabies control program that continues to present day, utilizing ERA-laden baits as an oral vaccine (MacInnes et al., 2001). During the period between 1989 and 2004, over 13 million baits were distributed across the province. Active rabies surveillance programs were carried out in the bait distribution areas to assess disease prevalence in both target and nontarget species. In addition to the surveillance programs, suspect rabid animals submitted to Agriculture Canada, and, subsequently, to the Canadian Food Inspection Agency (CFIA) were assayed by fluorescent antibody test (FAT) for rabies virus infection. This paper describes the rabies cases diagnosed between 1989 and 2004 that were attributed to infection by vaccine-derived ERA virus. Rabies virus isolates were confirmed as ERA by both immunoreactivity with a variant-discriminating panel of monoclonal antibodies (mAb) and by molecular genetic analyses. All told, nine cases of ERA variant rabies in four species were identified over the 16-yr period of study. These data are consistent with previous reports of low-level residual pathogenicity of ERA.

MATERIALS AND METHODS

Vaccine and baits

ERA virus propagated on BHK-21 cells (Connaught Laboratories Ltd., Toronto, Ontario, Canada, 1989–1990; Langford Laboratories Ltd., Toronto, Canada, 1991; Cyanamid-Langford Ltd., Toronto, Canada, 1992–1994; Ayerst Ltd., Toronto, Canada, 1995–1997; Artemis Technologies Inc., Guelph, Ontario, Canada, 1998–2004) in titers ranging from $10^{7.00}$ to $10^{7.88}$ (median tissue culture infective doses [$TCID_{50}$]/ml) was used for oral immunization of wildlife. Virus-infected media (1.8 ml) were packaged within two bait matrices that differed in size: Ontario regular white bait (ORW; 35 mm × 35 mm × 16 mm) or Ontario slim green bait (OSG; 35 mm × 35 mm × 11 mm). Both bait types consisted of a blister pack of vaccine suspension embedded in an oleo-wax block that also contained 100 mg of tetracycline hydrochloride (a biomarker used to assess bait consumption) and an attractant to increase palatability; these were chicken (C), chicken-cod (CC), or vanilla-sugar (VS).

Baits were deployed by aerial distribution in rural areas (both forested and agricultural)

during September and October, and by hand distribution in urban centers during July and August of each year. Distribution sites were determined based on locations of previous outbreaks requiring control (see Fig. 1) and not every area was baited each year. The average bait distribution density ranged from 15 to 22 baits/km^2.

Selection of animals for study

Specimen animals were derived from two sources, either animals that were submitted for testing to the CFIA (Agriculture Canada, prior to 1997) by the general public or those that were submitted by the OMNR as part of an active surveillance program carried out in baited areas. The active surveillance program involved collection of specimens of both target and nontarget species (e.g., fox, skunk, and raccoon) trapped and hunted by licensed hunters and trappers. In addition to the collection of brain tissue, canine teeth were extracted from cadavers and examined for tetracycline deposition using ultraviolet light–fluorescence microscopy to determine whether baits had been consumed, as described previously (Bachmann et al., 1990).

Rabies diagnosis by FAT and monoclonal antibody (mAb) analysis of virus strain

Brain tissue, including where possible, samples of medulla oblongata, hippocampus, and cerebellum, were harvested from specimen animals and tested for the presence of rabies virus antigen by FAT as previously described (Dean et al., 1996). All FAT-positive specimens were examined in indirect immunofluorescence (Nadin-Davis et al., 2001) with a panel of 15 mAbs directed against rabies virus nucleoprotein that discriminates between ERA and all common rabies virus variants circulating in Ontario wildlife populations (Table 1). Staining patterns for each specimen were assessed by fluorescence microscopy and used to determine variant type.

Molecular genome analyses of virus isolates

For presumptive ERA specimens as determined by antigenic analysis, variant typing was confirmed by molecular phylogenetic analysis. The ERA virus maintained at the CFIA Ottawa Laboratory–Fallowfield was used for comparison with field isolates and was propagated by infection of mouse neuroblastoma cells (MNA) cells (maintained in minimal essential medium supplemented with 10% fetal calf serum (FCS), 5% tryptose phosphate broth, 1% antibiotic/antimycotic) at a multiplicity of infection (MOI) of 0.1. Tissue culture supernatant was harvested when 100% of cells in a monitor plate exhibited viral infection.

As recommended by the supplier (Invitrogen, Burlington, Ontario, Canada), total RNA was extracted from all brain specimens using TRIzol reagent, while TRIzol LS reagent was used for extraction of viral RNA from tissue culture supernatant. RT-PCR was performed on 2 μg of RNA to amplify a segment of the viral N gene using primers RabN1/RabN5 (Nadin-Davis, 1998); a second round of PCR was performed using internal primers RabNfor/RabNrev as required to produce sufficient yields of product for sequencing. The viral G gene was initially amplified as a single 1,749 base-pair (bp) fragment using universal G primers RabGfor (5′-CCGGGATCCTTTGRGCCTCCTGGATGTGA-3′) and RabGrev (5′-CGCGAGCTCTACCCTGTTGSAYGGAGT-3′), which target residues 3,260–3,279 and 4,889–5,008, respectively, of the Pasteur virus (PV) reference strain (Tordo et al., 1986). To generate sufficient amounts of amplicon for sequencing, this first-round product was reamplified by nested PCR using internal primers to generate overlapping products of 1,220 bp and 1,260 bp for the upstream and downstream portions of the G gene, respectively. Amplicons were purified using a Wizard PCR purification system (Promega, Madison, Wisconsin, USA) prior to nucleotide sequencing.

The N gene sequencing reactions were performed manually using either ^{32}P or ^{33}P 5′-terminal labeled oligonucleotides, Nseq1 and Nseq2, together with a fmol cycle sequencing system (Promega,) as described by Nadin-Davis et al. (1993, 1994). Reactions were electrophoresed through denaturing 6% acrylamide gels on a SA sequencing system, the gel was dried, and sequencing products were visualized by autoradiography. For all 9 ERA suspect specimens, a 410 base segment of the N gene was sequenced. Using a 384 base window, these data were aligned with corresponding sequences of other rabies virus strains using CLUSTALX v1.8 (Thompson et al., 1997). This alignment was analyzed by the PHYLIP v3.63 (Felsenstein, 1993) package of software programs to generate pairwise DNA distance values (DNADIST) and to generate a phylogenetic tree by a neighbour-joining (NJ) analysis, as described previously (Nadin-Davis et al., 2001). TREEVIEW (Page, 1996) was used to generate graphic outputs of the tree.

Subsequently, G gene sequencing was undertaken using an LI-COR 4200L automated sequencing system that employs custom pri-

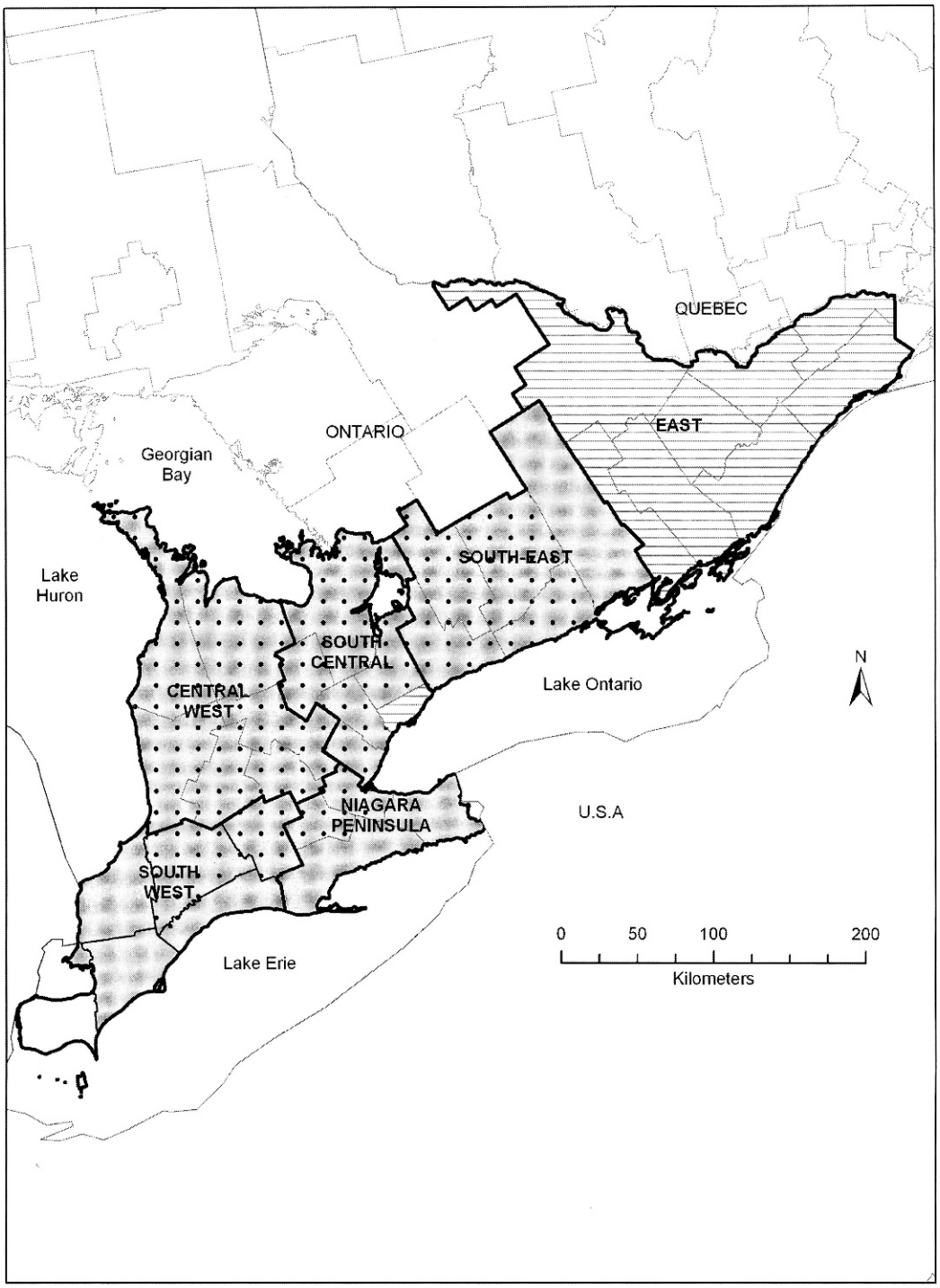

FIGURE 1. Map of Ontario illustrating vaccine-bait distribution areas in the early (1989–1993, stripes), middle (1994–1999, gray shading), and late (2000–2004, black dots) ERA vaccination campaigns. Ontario counties are outlined in gray. Provincial regions are outlined in black. In order to streamline the presentation and illustrate general trends, not all distribution data are shown in this figure. In some cases, there was baiting in regions not necessarily delineated on this map; see text for details.

TABLE 1. Staining patterns of Ontario rabies virus variants in indirect immunofluorescence with nucleoprotein-specific monoclonal antibodies (mAbs).

mAb	Immunizing virus	Rabies virus variant staining[a]									
		ERA	EON/ Arctic	MAC	BBB1	BBB2	BBB3	LBB	HRB	RDB	SHB
5DF12	SAD	+	+	+	+	+	+	+	+	+	+
11DD1	SAD	+	−	−	−	−	−	−	+	+	−
M992	Duvenhage	+	+	+	+	+		+	+	+	−
M1341	MAC	+	+	+	+	+	+	+	+	+	+
26BH11	SL dog	+	+	−	+	−	−	+	+	+	+
20CB11	SL dog	+	+	+	−	+	−	+	+	+	+
24FF1	SL dog	+	−	+	−	−	−	−	−	−	−
M993	Mokola	+	+	+	−	+	−	+	−	−	−
26AF11	SL dog	+	+	−	+	−	−	+	+/−	+	+
26BD6	SL dog	−	+	+	+	+	+	−	−	−	+
32FE10	Lima dog	+	+	+	+	+	+	+	−	+	+
32FF1	Lima dog	+	+	+	−	+	−	+	+	+	+
38FG5	EBL-1	+	+	+	−	+	−	+	+	+	+
M1347	MAC	−	−	+	−		−	+	+	+	+
7D2-7-4	BBB1	−	−	+	+	+	+	+	−	−	−

[a] Definitions: EON/Arctic = Eastern Ontario/Arctic Fox; MAC = mid-Atlantic raccoon; BBB = big brown bat; LBB = little brown bat; HRB = hoary bat; RDB = red bat; SHB = silver-haired bat; SL = Sri Lankan; EBL = European Bat Lyssavirus; SAD = Street Alabama Dufferin strain.

mers labeled with IR700 and IR800 dyes (LI-COR Biosciences, Lincoln, Nebraska, USA). Labeled primers internal to the amplicon sequence were employed with a thermosequenase cycle sequencing kit (Amersham Biosciences, Piscataway, New Jersey, USA). After electrophoresis for 10 hr through LI-COR 3% KB Plus gels, sequences were generated using the Eseq v2 software followed by manual editing. Nucleotide sequence translation to protein was performed by the DNAsis software package (Helixx Technologies, Scarborough, Ontario, Canada).

Nucleotide sequences generated in this study have been submitted to GenBank and assigned the following accession numbers: EF025113-4, partial N gene sequences of isolates 91-SS and 99-RF; EF025115–EF025123, partial G gene sequences of all nine field isolates, in sequential order of recovery; EF025124, partial G gene of tissue culture maintained stock ERA.FF; and EU119869, G gene of tissue culture maintained stock ERA.KK.

RESULTS

Vaccine distribution and rabies prevalence in Ontario from 1989 to 2004

For the sake of discussion, the period under review has been divided into three campaigns, early (1989 to 1993), middle (1994 to 1999) and late (2000 to 2004); areas of vaccine distribution are shown in Figure 1. In the early campaign, bait distribution occurred mainly in eastern Ontario counties from Lennox-Addington eastward to the Quebec border. Regions within some of these counties were also baited in 1994 and 1995. The early campaign also saw bait distribution in limited areas of some counties in southwestern and central-western Ontario, as well as in metropolitan Toronto. By the middle campaign (1994 to 1999), most baiting had ceased in eastern Ontario, and wide-scale baiting of southwestern and central-western Ontario had commenced. In addition, baiting was also carried out in counties within the Niagara Peninsula, south-central and southeastern Ontario. In the years 2000–2004, baiting continued in Middlesex and Oxford Counties of southwestern Ontario, as well as in the central-western, south-central, and southeastern regions of the province.

Table 2 details the number of baits distributed each year in addition to the

TABLE 2. Summary of ERA vaccine baiting activities and animals tested for rabies in Ontario from 1989 to 2004.

Year	Baits dropped	Total area baited (km^2)	Animals tested	Positive animals (% of total tested)	ERA + cases (% of positives)
1989	310,772	16,115	9,234	1,904 (20.6)	0 (0)
1990	802,623	40,215	10,225	1,663 (16.3)	0 (0)
1991	746,593	35,710	12,465	1,237 (9.9)	1 (0.08)
1992	612,386	30,620	7,168	1,304 (18.2)	2 (0.15)
1993	706,377	36,085	8,023	1,252 (15.6)	0 (0)
1994	1,498,000	69,675	11,376	610 (5.4)	2 (0.34)
1995	1,680,201	78,210	9,021	328 (3.6)	0 (0)
1996	1,324,989	68,405	7,909	158 (2.0)	1 (0.63)
1997	1,184,572	71,630	7,454	95 (1.3)	1 (1.10)
1998	982,373	59,640	4,874	80 (1.6)	1 (1.30)
1999	942,864	57,741	6,171	100 (1.6)	1 (1.00)
2000	776,752	48,024	13,464	187 (1.4)	0 (0)
2001	713,186	35,489	9,701	213 (2.2)	0 (0)
2002	516,944	35,546	6,998	202 (2.9)	0 (0)
2003	584,172	38,313	6,306	126 (2.0)	0 (0)
2004	482,138	29,639	4,502	107 (2.4)	0 (0)
Total	13,864,942	751,057	134,891	9,566 (7.1)	9 (0.09)

number of animals tested (the result of both active and passive surveillance), the number of positive cases, and the number of cases attributable to infection with ERA strain rabies virus. The number of baits disseminated ranged from a low of 311,000 in 1989 to a high of 1.7 million in 1995. The total area baited within the province peaked during the middle part of the campaign when baiting was carried out in most of southern and central Ontario from Hastings County in the east to Kent County in the west (Fig. 1). The number of animals tested for rabies varied from year to year, with a low of 4,502 in 2004, to a high of 13,464 in 2000. On average, 8,430 animals were tested each year over the period studied. During the early campaign, the percent positive animals ranged between 10% and 21% of the total tested. Beginning in 1994, the detected cases decreased to less than 6% and continued to decrease each year in the middle campaign to less than 2%. Positive animals represented between 1.4% and 2.9% of animals tested during the later part of the campaign.

Identification of ERA virus infection by mAb typing and genetic analyses

Virus from all animals that tested positive in the FAT was typed using a discriminatory mAb panel (Table 1). Nine specimens exhibited indirect immunofluorescence staining patterns consistent with ERA virus. To confirm that these animals were infected with ERA, molecular phylogenetic analyses were carried out on RNA extracted from brain tissue. All nine ERA suspect samples generated a partial N gene amplicon from which a 410 base region was sequenced. Alignment of these nine sequences indicated that all were identical except for a single base substitution in the last case (99-RF) (data not shown); this clearly indicated that all cases were due to the same viral strain.

A 384 base window of the sequences of the first (91-SS) and last (99-RF) cases was compared to corresponding sequences for all other terrestrial and chiropteran rabies strains known to circulate in Ontario and neighboring provinces and states (USA) at that time, and to the published sequence

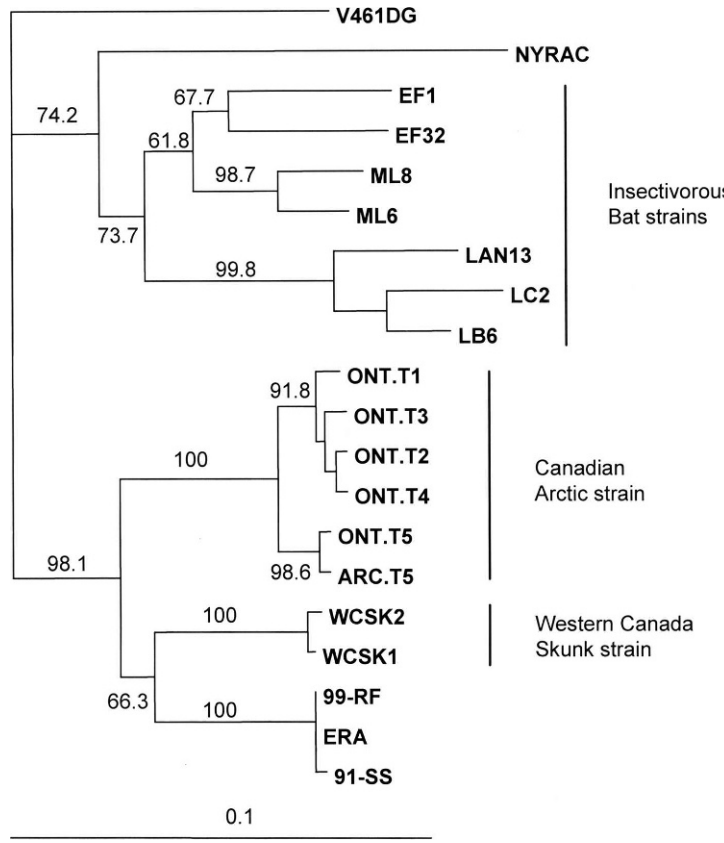

FIGURE 2. Phylogenetic analysis was performed by a neighbor-joining (NJ) algorithm using a 384 base segment of the rabies N gene. The 91-SS field isolate from a striped skunk and isolate 99-RF from a red fox were compared to the ERA strain (GenBank accession AF406695), a raccoon strain isolate recovered from New York state in 1992, and the following Canadian specimens: six isolates of the Arctic fox strain representative of all genetic variants identified in Ontario and the Arctic, two western skunk isolates, seven isolates representative of the bat rabies strains that circulate in Ontario. All of these Canadian isolates have been described elsewhere (Nadin-Davis et al., 2001). A Nigerian dog rabies isolate (V461DG) was also included as an outgroup. A scale indicating the genetic distances represented by the branch lengths is shown at the bottom of the figure. Bootstrap values for all clades were determined using 1,000 data set replicates and are expressed as a percentage to the left of most main branches.

for the ERA strain. Using the DNADIST program, the genetic distance between 91-SS and ERA was calculated as 0.0026, while 99-RF and ERA were genetically identical (0.0000 distance); values between 91-SS and other strains were much larger, ranging between 0.0800 and 0.1986 (data not shown). A phylogenetic tree, generated by NJ analysis using these DNA distance values, is shown in Figure 2. This tree clearly illustrates the very close association between specimens 91-SS and 99-RF and ERA, where a bootstrap value of 100 supports the clustering of these three viruses to a single monophyletic lineage. This confirms the identification of the strain responsible for these two cases as ERA, as well as the additional seven cases infected with a similar virus.

ERA positive cases

Table 3 outlines the details of each ERA-positive case discovered between 1989 and 2004. Two striped skunks (*Mephitis mephitis*), four red foxes (*Vulpes vulpes*), two raccoons (*Procyon lotor*), and

TABLE 3. ERA-positive cases discovered in Ontario, 1989–2004.

Case no.	Year	Species[a]	Time from baiting to capture (days)	Age	Tet deposition in canine	Bait type[b]	Vaccine titer[c]	Capture location (Town)	Capture location (County)	Baited previous year
91-SS	1991	SS	90	U	ND	ORW-C	7.00–7.88	Elderslie	Bruce	Y
92-RF	1992	RF	25	U	ND	ORW-CC	7.06–7.65	Carleton	OC-RM	Y
92-Rac	1992	RC	24	U	ND	ORW-CC	7.06–7.65	Tyendinaga	Hastings	N
94-Bov	1994	BOV	29	C	ND	ORW-CC	7.00–7.60	Gloucester	OC-RM	Y
94-SS	1994	SS	23	J	2 lines in dentin	ORW-VS	7.00–7.60	Hullett	Huron	N
96-RF	1996	RF	45	U	ND	ORW-CC	7.00–7.60	Ops	Victoria	Y
97-RF	1997	RF	29	Y	2 lines in cementum	ORW-CC	7.12–7.54	Middleton	H-N	Y
98-Rac	1998	RC	27	Y	bone deposits	ORW-VS	7.05–7.47	Normanby	Grey	Y
99-RF	1999	RF	92	U	ND	OSG-VS	7.27–7.51	Mulmur	Dufferin	Y

[a] Definitions: SS = striped skunk; RF = red fox; RC = raccoon; BOV = bovine; U = unknown; C = calf; J = juvenile; Y = yearling; Tet = tetracycline hydrochloride; ND = not determined; OC-RM = Ottawa-Carleton Regional Municipality; H-N = Haldiman-Norfolk; Y = yes; N = no.

[b] See Materials and Methods section for descriptions.

[c] Range of titers in bait lots distributed in that year (in $\log_{10}$ median tissue culture infective doses [$TCID_{50}$]/ml).

one bovine calf (*Bos taurus*) were identified. Of these animals, seven (91-SS, 92-RF, 92-Rac, 94-Bov, 96-RF, 98-Rac, 99-RF) were submitted for testing as suspect rabid; the remaining two (94-SS and 97-RF) were the result of active surveillance programs carried out by the OMNR in baited areas. The first case was discovered in 1991 (2 yr after baiting programs had commenced in the province), and the last case was discovered in 1999. No ERA-positive cases were found in the years 1989, 1990, 1993, 1995, and 2000 to 2004. The time elapsed between baiting and the capture of the animals ranged from 23 to 92 days. In most cases (seven of nine), the animals were captured in areas that had been baited in the year of capture as well as in the previous year. Animals for which age was determined were yearlings or juveniles (four of nine).

In the years in which positive animals were found, the bait composition did differ with respect to the attractant that was present in the matrix. Positive cases were found for C-, CC-, and VS-containing baits. In all the cases except for 99-RF, the ORW bait was distributed; in 1999, the OSG bait was distributed (Table 3).

The presence of tetracycline deposits in the dentition was determined for the two positive animals captured as part of the OMNR active surveillance programs and for 98-Rac, identified from passive surveillance activities. These data indicate that all three animals consumed baits containing the tetracycline biomarker. Specimen 94-SS exhibited two tetracycline lines in the dentin, indicating the consumption of baits on two different days in 1994. Specimen 97-RF exhibited two lines in the cementum, one attributed to bait consumption in 1996 and the second to bait consumption in 1997. For specimen 98-Rac, tooth analysis was not possible; however, tetracycline deposits were detected in the bone surrounding the canine teeth. Salivary glands were also recovered from 97-RF, but virus was not detected (data not shown).

ERA-positive animals were found across the province in eight different counties (Fig. 3). Two animals were discovered in the Ottawa-Carleton Regional Municipality (92-RF, 94-Bov); however these did not occur in the same year. In years in which two positive animals were discovered, 1992 and 1994, the cases occurred in different counties; in 1994, the positive animals were captured in counties on opposite ends of the province. Thus, the ERA-positive cases did not appear to be stratified by species, year of discovery, or location of capture.

Examination of the G gene of the ERA strain rabies viruses

The possibility that the cases of clinical rabies were due to a virus that had undergone mutation to increase its pathogenic capability was considered. Since changes in the amino acid encoded at certain residues of the glycoprotein are known to affect the pathogenicity of the virus, most notably at residue 333 (Dietzschold et al., 1983; Tuffereau et al., 1989), the sequence of the G gene of all nine rabies virus isolates was determined. The sequences of the mature, processed glycoprotein thereby predicted from these nucleotide sequence data were compared to the predicted protein sequence of ERA determined on three separate occasions: a published sequence (Anilionis et al., 1981) and two sequences recently determined independently from stocks of ERA both originating from the Connaught seed stock virus, ERA.FF (obtained in 1988) and ERA.KK (vaccine production lot from 2004). The sequence of the closely related SADB19 strain was also included for comparison. The ERA.FF and ERA.KK sequences were identical with the exception of an Arg/His replacement at position 120, while the previously published ERA G protein sequence differed from both ERA.FF and ERA.KK at position 8 (Pro/Leu) but was similar to ERA.FF at residue 120. All but one of the field isolates exhibited the histidine replacement at position 120, and some also exhibited other differences. The isolate that retained Arg120 (94-SS) was distinctive in having an arginine at residue 256 in place of glutamine. Two isolates (91-SS and 92-RF) exhibited a substitution at residue 182, where serine replaced the normal asparagine. Three isolates (both 1992 cases and the 1996 case) substituted threonine for isoleucine at residue 449.

DISCUSSION

Although ERA is an attenuated rabies virus, its residual pathogenicity is well documented (Bijlenga and Joubert, 1974; Wachendörfer et al., 1978; Black and Lawson, 1980; Esh et al., 1982; Wandeler et al., 1982; Whetstone et al., 1984; Lawson et al., 1987). The clinical symptoms caused by ERA virus that progresses to the central nervous system are indistinguishable from infections with street rabies virus, and routine postmortem diagnostic tests do not differentiate between them. The residual pathogenicity of ERA, particularly for rodents, raised a number of concerns when the vaccine was considered for wildlife rabies control. Though such concerns cannot be fully invalidated, a field trial on a small river island in Switzerland indicated that establishment of ERA-derived SAD in central European wild rodent populations was unlikely (Wandeler et al., 1982). However, the risk remains that individual animals ingesting the live vaccine may succumb to ERA rabies.

The effectiveness of the Ontario ERA vaccination campaign and the prevalence of vaccine-induced rabies were monitored via both active and passive surveillance activities. More than 100,000 animals were tested for rabies virus from 1989 to 2004. Fluorescent antibody tests (FAT) on brain tissue from these animals revealed 9,566 that were positive for rabies virus antigen. Variant typing was subsequently performed on FAT-positive specimens using a mAb panel that discriminates well

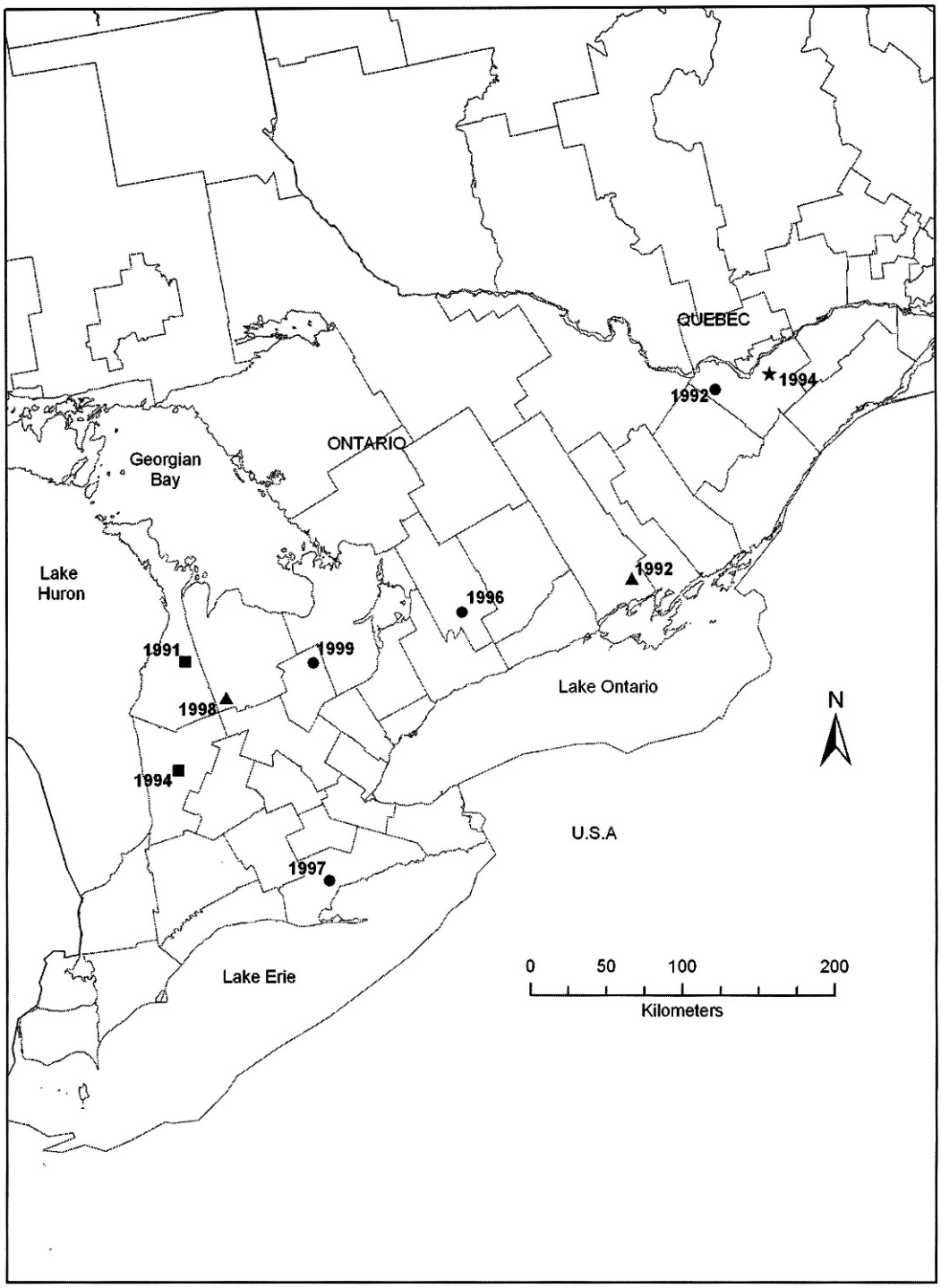

FIGURE 3. Map of Ontario illustrating the location and date of capture of the nine ERA-positive animals identified between 1989 and 2004. Species legend: circle, red fox (*Vulpes vulpes*); triangle, raccoon (*Procyon lotor*); square, striped skunk (*Mephitis mephitis*); star, bovine calf (*Bos taurus*).

between ERA and all rabies virus variants known to circulate in Ontario. Cases exhibiting staining patterns consistent with ERA were confirmed by molecular phylogenetic analyses of RNA extracted from brain tissue. ERA-positive animals were found across the province in eight different counties over a period of nine years. Four red foxes, two raccoons, two striped skunks, and one bovine calf were among the positive cases discovered. Molecular phylogenetic analyses of the partial N gene sequences generated from these isolates clearly support the conclusion that these nine cases were due to infection with the ERA strain.

In the present study, tetracycline deposition data were available for three of the nine ERA cases (94-SS, 97-RF, 98-Rac), and these indicated that these animals all consumed at least one bait. These data support the conclusion that the ERA infections probably resulted from bait consumption and were not due to lateral transmission from ERA-infected animals. While in some laboratory studies virus was found in salivary glands and trigeminal ganglion of rodents that ate baits (Winkler et al., 1976; Nicholson and Bauer, 1981), in others, ERA virus could not be isolated from submandibular salivary glands in either rodents or skunks that developed ERA vaccine–induced rabies (Lawson et al., 1987, 1989; Tolson et al., 1988). Furthermore, transmission of ERA from experimentally infected rodents to healthy littermates could not be detected in other studies (Steck et al., 1982; Baer, 1988). It is therefore likely that all the Ontario ERA cases resulted from infection following bait consumption.

Many areas of the province were baited in multiple, consecutive years; however, no ERA-positive animals were found in regions that were baited in the preceding year but not in the year of case discovery. These data suggest that the disease development in the positive animals occurred in the 1- to 3-mo period subsequent to bait distribution and not over a period of 12–16 mo. The positive red fox from 1997 (97-RF) is curious in that the tetracycline deposition analysis indicated consumption of baits by this animal in both 1996 and 1997. It is unclear whether the incubation period was 29 days or 13 mo in this case. However, based on the lack of detection of ERA-positive animals in areas not baited in the year of case discovery, as well as the incubation periods observed in animals experimentally infected with ERA virus (Tolson et al., 1988, 1990; Lawson et al., 1989), it is likely that the first bait failed to immunize the fox, and the second bait caused the disease.

At least one ERA-positive case was discovered in most years between 1991 and 1999; none has been found since 1999. The incidence of ERA-induced rabies may depend on numerous factors, such as vaccine titer, bait density, differential susceptibility of different species or individuals within a species, the number of baits they consume, as well as the presence of immunocompromising conditions. Furthermore, the rate of discovery of vaccine rabies cases depends on both the thoroughness of surveillance and the sensitivity and specificity of virus identification methods used. None of these factors would appear to account for the lack of positive cases between 2000 and 2004. Over this period, the total number of animals tested each year did not decrease significantly, and the methods for virus identification were consistent over the 16-yr study period. The vaccine titer and volume did not change from year to year, although the bait matrix did change with respect to size and in the attractant used. While the eastern region of the province was baited only in the early years of the vaccination campaign (1989 to 1994), there was significant overlap in areas that were baited between 1994 and 1999 and those baited subsequent to 1999. Thus, possible differences in susceptible wildlife populations or in geographic or environmental features in varying regions

of the province cannot account for the lack of ERA-positive cases in the years 2000–2004. Similarly, the number of baits distributed during this time does not seem to be a likely factor, as the number of baits distributed from 2000 to 2002 was similar to that in years when cases were found. It is possible that after multiple, consecutive years of baiting in certain areas, many baits were consumed by animals that were already immunized. These animals would thus be unlikely to develop vaccine-induced rabies.

Previous studies have identified residues in the rabies glycoprotein that affect viral pathogenicity, with varying mechanisms of action (Tuffereau et al., 1989; Dietzschold et al., 2004; Faber et al., 2005). Residue 333, located in antigenic site III, is likely involved in virus attachment to cellular receptors (Seif et al., 1985; Tuffereau et al., 1998). Mutation of codon 333 so as to encode an amino acid other than the normal arginine or lysine at this position reduces virulence in adult mice (Tuffereau et al., 1989). However, this is not the basis for the attenuation of ERA since Arg333 is maintained in this strain. Recently, a change at residue 194 from asparagine to lysine was reported to modestly increase the pathogenicity of certain recombinant rabies viruses, due to an increase in viral spread, faster internalization of virus into cells, and a shift in the pH threshold for membrane fusion (Dietzschold et al., 2004; Faber et al., 2005). It is possible that other residues of the glycoprotein may also affect viral virulence. We compared the sequence of the predicted glycoprotein from the ERA virus isolates obtained from the Ontario cases with that of the ERA strain. It was notable that two separate aliquots of virus that both originated from seed stock used to generate the virus used in baits differed at one position, residue 120, which was histidine (His) in ERA.KK and arginine (Arg) in ERA.FF, an observation suggesting the presence of both variants in these stocks. Eight of the field isolate sequences also contained histidine at residue 120. This residue is not contained within any known antigenic site of the rabies glycoprotein, and any consequence of this replacement upon viral pathogenicity is presently unknown; of several rabies virus G genes that have been characterized (Benmansour et al., 1992), Arg120 was found only in the ERA, ERA.FF, and SAD strains. However, given that it appears to be a significant variant in stocks of ERA, a role for His120 in increased pathogenicity would appear unlikely. The substitution Thr449 to Ile449 found in three of the field isolates occurs within the rather poorly conserved transmembrane domain and thus may not be functionally significant, though replacement of threonines in this region with nonpolar amino acids would help to increase the domain's hydrophobic nature and hence its affinity for membrane lipids. A single field isolate carried a replacement (Gln to Arg) at position 256; again the potential role of this substitution in modifying viral pathogenicity is unknown.

The discovery of ERA-variant rabies in Ontario wildlife is consistent with previous reports of the rare detection of ERA/SAD vaccine–induced rabies, as well as with laboratory studies of ERA pathogenicity (Bijlenga and Joubert, 1974; Wachendörfer et al., 1978; Black and Lawson, 1980; Esh et al., 1982; Wandeler et al., 1982; Whetstone et al., 1984; Lawson et al., 1987). The evidence that the positive cases were identified over a 9-yr period, in four different species, and in eight counties dispersed across the province indicates that the disease incidence was not related to a particular lot of vaccine/bait distributed in a given year. Furthermore, the small number of positive cases, the observed case distribution, and the inability to recover virus from the salivary glands of 97-RF support the conclusions from earlier European studies that ERA-derived SAD virus does not become established in wildlife populations (Wandeler et al., 1982). Although nine ERA-

positive cases were diagnosed in the 16-yr period under review, it is likely that other cases went undetected given the large geographic areas that were baited. However, even assuming that the number of ERA-positive cases has been underestimated, the fact that over 13 million baits were distributed over this time period indicates that the incidence of vaccine-induced rabies was extremely low. It is clear that the vaccination campaigns have been successful in reducing the number of rabid animals in Ontario; in the eastern counties, the Arctic fox variant of rabies has been eliminated (MacInnes et al., 2001, unpubl. data). Thus, when balanced against the success of the Ontario vaccination campaign, it would appear that the small risk of individual disease development due to the residual pathogenicity of the ERA vaccine is acceptable.

ACKNOWLEDGMENTS

We wish to thank the following individuals who contributed to this work: D. Johnston and C. MacInnes were instrumental in the establishment of the Ontario ERA vaccination program that provided the basis for this study; K. Bennett, L. Calder, and M. Pedde helped to collect specimens from trappers and hunters and compile respective information; L. Brown helped assign the bait distribution data; sequencing of the ERA stocks was undertaken by F. Fallahi (ERA.FF) and M. Sheen (ERA.KK); A. Clark prepared Figures 1 and 3; and numerous hunters and trappers participated in the active rabies surveillance programs.

LITERATURE CITED

Abelseth, M. K. 1964a. Propagation of rabies virus in pig kidney cell culture. Canadian Veterinary Journal 5: 85–87.

———. 1964b. An attenuated rabies vaccine for domestic animals produced in tissue culture. Canadian Veterinary Journal 5: 279–286.

———. 1967. Further studies on the use of ERA rabies vaccine in domestic animals. Canadian Veterinary Journal 8: 221–227.

Anilionis, A., W. H. Wunner, and P. J. Curtis. 1981. Structure of the glycoprotein gene in rabies virus. Nature 294: 275–278.

Aubert, M. F., E. Masson, M. Artois, and J. Barrat. 1994. Oral wildlife rabies vaccination field trials in Europe, with recent emphasis on France. Current Topics in Microbiology and Immunology 187: 219–243.

Bachmann, P., R. N. Bramwell, S. J. Fraser, D. A. Gilmore, D. H. Johnston, K. F. Lawson, C. D. MacInnes, F. O. Matejka, H. E. Miles, M. A. Pedde, and D. R. Voight. 1990. Wild carnivore acceptance of baits for delivery of liquid rabies vaccine. Journal of Wildlife Diseases 26: 486–501.

Baer, G. M. 1988. Oral rabies vaccination: An overview. Reviews of Infectious Diseases 10 (4 suppl): S644-8.

———, S. B. Linhart, and D. J. dean. 1963. Rabies vaccination of foxes. *In* Annual Report of the Division of Laboratories and Research, New York State Department of Health, Albany.

———, M. K. Abelseth, and J. G. Debbie. 1971. Oral vaccination of foxes against rabies. American Journal of Epidemiology 93: 487–490.

Benmansour, A., M. Brahimi, C. Tuffereau, P. Coulon, F. Lafay, and A. Flamand. 1992. Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogeneous nature of the viral population. Virology 187: 33–45.

Bijlenga, G., and L. Joubert. 1974. Haute pathogénicité pour la souris par la voie digestive d'un composant viral du vaccin antirabique … virus vivant modifié ERA/BHK. Bulletin de l'Académie Vétérinaire 47: 424–435.

Black, J. G., and K. F. Lawson. 1970. Sylvatic rabies studies in the silver fox (*Vulpes vulpes*). Vaccination by the oral route. Canadian Veterinary Journal 14: 206–211.

———, and ———. 1980. The safety and efficacy of immunizing foxes (*Vulpes vulpes*) using baits containing attenuated rabies virus vaccine. Canadian Journal of Comparative Medicine 44: 169–176.

Campbell, J. B. 1994. Oral rabies immunization of wildlife and dogs: Challenges to the Americas. Current Topics in Microbiology and Immunology 187: 245–266.

Dean, D. J., M. K. Ableseth, and P. Atanasiu. 1996. The fluorescent antibody test. *In* Laboratory Techniques in Rabies, 4th Edition, F. X. Meslin, M. M. Kaplan and H. Koprowski (eds.). World Health Organization (WHO), Geneva, pp. 88–95.

Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proceedings of the National Academy of Sciences of the USA 80: 70–74.

———, M. Faber, J. A. Mattis, K. Y. Pak, M. J. Schnell, and B. Dietzschold. 2004. In vitro growth and stability of recombinant rabies

viruses designed for vaccination of wildlife. Vaccine 23: 518–524.

ESH, J. B., J. G. CUNNINGHAM, AND T. J. WIKTOR. 1982. Vaccine-induced rabies in four cats. Journal of the American Veterinary Medical Association 180: 1336–1339.

FABER, M., M.-L. FABER, A. PAPANERI, M. BETTE, E. WEIHE, B. DIETZSCHOLD, AND M. J. SCHNELL. 2005. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. Journal of Virology 79: 14141–14148.

FELSENSTEIN, J. 1993. PHYLIP: Phylogeny inference package. Version 3.52c. University of Washington, Seattle, Washington,

FENJE, P. 1960. Propagation of rabies virus in cultures of hamster kidney cells. Canadian Journal of Microbiology 6: 479–484.

KIENY, M. P., R. LATHE, R. DRILLIEN, D. SPEHNER, S. SKORY, D. SCHMITT, T. WIKTOR, H. KOPROWSKI, AND J. P. LECOCQ. 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163–166.

LAFAY, F., J. BÉNÉJEAN, C. TUFFEREAU, A. FLAMAND, AND P. COULON. 1994. Vaccination against rabies: Construction and characterization of SAG2, a double avirulent derivative of SAD (Bern). Vaccine 12: 317–320.

LAWSON, K. F., AND J. F. CRAWLEY. 1972. The ERA strain of rabies vaccine. Canadian Journal of Comparative Medicine 36: 339–344.

———, J. G. BLACK, K. M. CHARLTON, D. H. JOHNSTON, AND A. J. RHODES. 1987. Safety and immunogenicity of a vaccine bait containing ERA strain of rabies virus. Canadian Journal of Veterinary Research 51: 460–464.

———, R. HERTLER, K. M. CHARLTON, J. B. CAMPBELL, AND A. J. Rhodes. 1989. Safety and immunogenicity of ERA strain of rabies virus propagated in a BHK-21 cell line. Canadian Journal of Veterinary Research 53: 438–444.

MACINNES, C. D., S. M. SMITH, R. R. TINLINE, N. R. AYERS, P. BACHMANN, D. G. BALL, L. A. CALDER, S. J. CROSGREY, C. FIELDING, P. HAUSCHILDT, J. M. HONIG, D. H. JOHNSTON, K. F. LAWSON, C. P. NUNAN, M. A. PEDDE, B. POND, R. B. STEWART, AND D. R. VOIGT. 2001. Elimination of rabies from red foxes in eastern Ontario. Journal of Wildlife Diseases 37: 119–132.

NADIN-DAVIS, S. A. 1998. Polymerase chain reaction protocols for rabies virus discrimination. Journal of Virological Methods 75: 1–8.

———, G. A. CASEY, AND A. WANDELER. 1993. Identification of regional variants of the rabies virus within the Canadian province of Ontario. Journal of General Virology 74: 829–837.

———, ———, AND ———. 1994. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. Journal of General Virology 75: 2575–2583.

———, W. HUANG, J. ARMSTRONG, G. A. CASEY, C. BAHLOUL, N. TORDO, AND A. I. WANDELER. 2001. Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. Virus Research 74: 139–156.

NICHOLSON, K. G., AND S. P. BAUER. 1981. Enteric inoculation with ERA rabies virus: Evaluation of a candidate wildlife vaccine in laboratory rodents. Archives of Virology 67: 51–56.

PAGE, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357–358.

SEIF, I., P. COULON, P. E. ROLLIN, AND A. FLAMAND. 1985. Rabies virulence: Effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. Journal of Virology 53: 926–934.

STECK, F., A. WANDELER, P. BICHSEL, S. CAPT, U. HAFLIGER, AND L. SCHNEIDER. 1982. Oral immunization of foxes against rabies: Laboratory and field studies. Comparative Immunology, Microbiology, and Infectious Diseases 5: 165–171.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

TIDKE, R., C. PREHAUD, P. COULON, J. BLANCOU, AND A. FLAMAND. 1987. Characterization of a double avirulent mutant of rabies virus and its potency as a vaccine, live or inactivated. Vaccine 5: 229–233.

TOLSON, N. D., K. M. CHARLTON, K. F. LAWSON, J. B. CAMPBELL, AND R. B. STEWART. 1988. Studies of ERA/BHK-21 rabies vaccine in skunks and mice. Canadian Journal of Veterinary Research 52: 58–62.

———, ———, R. B. STEWART, G. A. CASEY, W. A. WEBSTER, K. MACKENZIE, J. B. CAMPBELL, AND K. F. LAWSON. 1990. Mutants of rabies viruses in skunks: Immune response and pathogenicity. Canadian Journal of Veterinary Research 54: 178–183.

TORDO, N., O. POCH, A. ERMINE, G. KEITH, AND F. ROUGEON. 1986. Walking along the rabies genome: Is the large G-L intergenic region a remnant gene? Proceedings of the National Academy of Sciences of the USA 83: 3914–3918.

TUFFEREAU, C., H. LEBLOIS, J. BÉNÉJEAN, P. COULON, F. LAFAY, AND A. FLAMAND. 1989. Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. Virology 172: 206–212.

———, J. BÉNÉJEAN, D. BLONDEL, B. KIEFFER, AND A. FLAMAND. 1998. Low-affinity nerve growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO Journal 24: 7250–7259.

WACHENDÖRFER, G., P. FARRENKOPF, W. LOHRBACH,

U. Förster, J. W. Frost, and W. A. Valder. 1978. Passageversuch mit einer Variante des Tollwut-Impfstammes ERA bei wildlebeneden Species (*Ondatra zibethica* und *Rattus norvegicus*). Deutsche TierärztlicheWochenschrift 85: 273–285.

Wandeler, A. I. 1991. Oral immunization of wildlife. *In* The natural history of rabies. 2nd Edition, G. M. Baer (ed.). CRC Press, Boston, Massachusetts, pp. 485–503.

———. 2000. Oral immunization against rabies: Afterthoughts and foresight. Schweizer Archiv für Tierheilkunde 142: 455–462.

———, W. Bauder, S. Prochaska, and F. Steck. 1982. Small mammal studies in a SAD baiting area. Comparative Immunology, Microbiology, and Infectious Diseases 5: 173–176.

Whetstone, C. A., T. O. Bunn, R. W. Emmons, and T. J. Wiktor. 1984. Use of monoclonal antibodies to confirm vaccine-induced rabies in ten dogs, two cats, and one fox. Journal of the American Veterinary Association 185: 285–288.

Winkler, W. G., J. H. Shaddock, and L. W. Williams. 1976. Oral rabies vaccine: Evaluation of its infectivity in three species of rodents. American Journal of Epidemiology 104: 294–298.

Received for publication 15 February 2007.