Antiviral Research 100 (2013) 84–89

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Genetic strain modification of a live rabies virus vaccine widely used in Europe for wildlife oral vaccination

Florence Cliquet *, Emmanuelle Robardet, Evelyne Picard Meyer

French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Nancy Laboratory for Rabies and Wildlife, European Union Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies Serology, WHO Collaborating Centre for Research & Management in Zoonoses Control, OIE Reference Laboratory for Rabies, Technopôle agricole et vétérinaire, BP 40 009, 54 220 Malzéville Cedex, France

ARTICLE INFO

Article history:
Received 24 May 2013
Revised 16 July 2013
Accepted 18 July 2013
Available online 27 July 2013

Keywords:
Rabies vaccine
Oral vaccination
Sequencing
Phylogeny
Nucleotide mutation

ABSTRACT

In Europe, the main reservoir and vector of rabies has been the red fox (*Vulpes vulpes*). Oral immunization of foxes with live vaccines, using attenuated rabies strains (SAD B19, SAD Bern), apathogenic mutants of an attenuated strain (SAG2) and the vaccinia-rabies glycoprotein recombinant virus vaccine (V-RG), has been shown to be the most effective method for the control and elimination of rabies. Among all vaccines currently used for wildlife oral vaccination, one vaccine (marketed as SAD Bern strain) has been widely used in Europe since 1992 with the distribution of 17 million of baits in 2011. Because of the potential environmental safety risk of a live virus which could revert to virulence, the full genome sequencing of this vaccine was undertaken and the sequence was characterized and compared with those of referenced rabies viruses. The vaccine showed higher similarity to the strains belonging to the SAD B19 vaccine virus strains than to the SAD Bern vaccines. This study is the first one reporting on virus strain identity changes in this attenuated vaccine.

1. Introduction

Rabies, a fatal zoonotic encephalitic disease first described in antiquity (Blancou, 2004), is caused by rabies or rabies-related viruses of the genus *Lyssavirus* in the family *Rhabdoviridea* of the order Mononegavirales (ICTV, 2011). The *Lyssavirus* genome consists of a single strand of negative-sense RNA (around 12 Kb) comprising five genes, each coding for a structural protein of the virion: the nucleoprotein (N), the phosphoprotein (P), the matrix (M), the glycoprotein (G) and the RNA-dependent RNA polymerase (L).

Rabies virus (RV) can affect all mammals, small to medium-sized carnivores throughout the world and bats in the Americas. In Europe, the epidemic was initially transmitted by dogs and wolves and started to disappear gradually at the beginning of the 20th century. In the 1940s, a new epizootic appears to have been maintained mostly in the red fox (*Vulpes vulpes*) on the Polish-Russian border (Blancou, 1988). This phenomenon, probably due to a spillover from domestic animals to wildlife, spread quickly southwards and westwards through Europe at a speed of approximately 30–60 km per year (Aubert, 1992).

Control measures aiming to reduce fox populations did not prevent the disease from spreading and raised serious ethical and ecological issues (WHO, 2013). In the 1980s European countries progressively implemented oral rabies vaccination (ORV) programmes for wildlife that were proven to be highly effective in controlling the disease. The first ORV field trial was conducted in 1978 in Switzerland (Steck et al., 1982) and was rapidly extended to the surrounding countries, leading several years later to the elimination of rabies in Western Europe and in some parts of Central Europe (Cliquet and Aubert, 2004; Matouch et al., 2006). Consequently, a drastic decrease in rabies cases was observed: while 20,999 terrestrial animal cases were detected in Europe in 1990, the number of cases was reduced fourfold to 5802 cases in 2011 (Rabies Bulletin Europe, 2012). In 2011, rabies was still endemic in several Eastern and South-Eastern European countries (Kirandjiski et al., 2012). All rabies vaccines used in Europe are modified live virus vaccines based on the Street Alabama Dufferin (SAD) strain isolated from a naturally-infected dog in the USA in 1935 (Cliquet and Aubert, 2004). The first field trial performed in Switzerland in 1978 was carried out with attenuated SAD Bern, a cell culture-adapted strain (Steck et al., 1982). SAD B19 and SAD

Abbreviations: EC, European commission; EDQM, European directorate for the quality of medicines & healthcare; ERA, Evelyn Rokitnicki Abelseth; G, glycoprotein; M, matrix; N, nucleoprotein; P, phosphoprotein; OIE, World Organization for Animal Health; ORV, oral rabies vaccination; L, RNA-dependent RNA polymerase; RV, rabies virus; SAD, Street Alabama Dufferin; V-RG, vaccinia glycoprotein recombinant; WHO, World Health Organization.

* Corresponding author. Address: French Agency for Food, Environmental and Occupational Health and Safety (ANSES), Technopôle agricole et vétérinaire, 54 220 Malzéville Cedex, France. Tel.: +33 (3) 83298950; fax: +33 (3) 83298958.

E-mail addresses: florence.cliquet@anses.fr (F. Cliquet), emmanuelle.robardet@anses.fr (E. Robardet), evelyne.picard-meyer@anses.fr (E. Picard Meyer).

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.07.012

P5/88, from SAD selections on BHK 21 cells, were used for the first time in 1983 and 1989, respectively (Schneider and Cox, 1983; Schneider et al., 1987) in Germany. Since the 1990s, SAG1, a mutated version of the SAD Bern virus (mutation on the arginine 333 codon linked to rabies pathogenicity) was initially used in France (Pastoret et al., 2004), but was then replaced by the SAG2 vaccine, a more genetically stable strain (Lafay et al., 1994). Another vaccine that was extensively used in Europe is a genetically engineered vaccine named V-RG (Vaccinia-Rabies Glycoprotein) recombinant. The vaccinia virus used (Copenhagen strain) was attenuated by replacement of the thymidin kinase gene with the glycoprotein cDNA from the Evelyn–Rokitnicki–Abelseth (ERA) strain derived from the original SAD isolate on various cell lines, inducing rabies immunity (Kieny et al., 1984; Wiktor et al., 1984).

This study describes the genetic characterization of a commercially available vaccine bait strain manufactured in the Czech Republic and widely used in Europe since 1992. Our institute is currently involved in independent batch testing (Servat and Cliquet, 2013) and this work was investigated as the name of the marketed virus strain was changed by the producer. Results were sent to the regulatory authorities of all member states and the company has also been informed on the results obtained. The genetic comparison of the vaccine liquid suspension was performed with several attenuated referenced RV vaccines, including ERA, SAD Bern and various laboratory derivatives of SAD Bern.

2. Material and methods

2.1. Viral samples and RNA extraction

Two vaccine strains, named Vaccine A (batch 5118 manufactured in 2011 in Czech Republic) and SAD Bern (numbered 09-99) underwent complete RV genome sequencing on coded samples. The Vaccine A contains a SAD Bern strain propagated in cell cultures, antibiotics and a stabilisation medium. The minimal titre is 1.8×10^6 TCID (http://www.bioveta.cz/en/veterinary-division/products/product-catalogue/lysvulpen-por-ad-us-vet.html). The liquid suspension is dispensed into plastic blisters (1.8 ml per bait). The SAD Bern 09-99 control virus was received in 2010 from Czech Republic and was propagated onefold in BHK-21 cells. This virus is provided as a positive control in the tests which should be carried out in order to check the viral titre of marketed oral vaccines prior to their release.

Total RNA was extracted from the supernatant of the first passage of SAD Bern 09-99 and from the Vaccine A suspension (batch 5118) using Iprep™ PureLink™ Total RNA kit (Invitrogen, France) according to the manufacturer's instructions. All extracted total RNA was stored at −70 °C until use.

2.2. Reverse transcription PCR and sequencing

Single cDNA was generated from 2 μg of extracted RNA using Superscript III (Invitrogen) as per manufacturer's instructions. The entire genome of SAD Bern 09-99 and Vaccine A (batch 5118) was amplified with two long-distance PCRs: N-G (4.5 kb) and G-L (6.6 kb) using specific rabies primers described in Table 1.

The long-distance PCR fragments (4.5 kb and 6.6 kb - PCR products) were amplified as follows. For 4.5-kb fragment generation (N-G), 2 μl of cDNA were amplified using high fidelity Taq Polymerase (Fermentas, France) at 94 °C for 30 s, 45 cycles at 94 °C for 30 s, 55 °C for 30 s, 68 °C for 5 min 30 s and a final cycle at 68 °C for 30 min. The same conditions were applied to the amplification of the 6.6-kb fragment (G-L) except for the annealing temperature (57 °C for 7 min 30 s).

Table 1
Description of nucleotide primers used for the PCR amplification of long distance fragments of Vaccine A and SAD Bern 09-99.

Long-distance PCRs	Primers	Sequence (5′ 3′)	Location*
4.5-kb	JW12F	ATGTAACACCYCTACAATG	55–73
	SAD1-03R	GAAAACGGTAGACGGGTCTGCCAG	4591–4568
6.6-kb	SAD1-01R	CAGGAAATCCTCTCACAAGATCCTG	11221–11197
	SAD1-02F	CTGGCAGACCCGTCTACCGTTTT	4568–4591
4.5-kb	JW12F	ATGTAACACCYCTACAATG	55–73
	PVN8R	AGTTTCTTCAGCCATCTC	1585–1568
	SAD04F	AGCCCGTCCAAACTCATTC	1366–1384
	SAD04R	ATTCCAACTCCTCCCCTTC	2907–2889
	SAD03F	GCACATTCTGAAATCATTCGAC	2714–2735
	SAD03R	TCCCCCCAACTCTTAAACAC	4421–4402
	SAD31F	GCTCCACTAACCACGATTAC	3879–3898
	SAD31R	GAAAACGGTAGACGGGTCT	4591–4573
6.6-kb	SAD16F	AGACGACAACATTCAACGAC	10178–10197
	SAD16R	AGCATCTCTGAAAAACTTCCC	11043–11063
	SAD07F	TTCTTCTGTCTGCCCGAACC	9176–9195
	SAD07R	ACTGAACCACTCTCAAGCCC	10430–10449
	SAD25F	TCTATTCCAAGTCGTCCCC	5785–5803
	SAD25R	TCAATCTTCAATTCCCGTTCC	7046–7066
	SAD23F	AACCTACGCAACACAATCTC	4777–4796
	SAD23R	GAACTTTACCAAGTCCTTCCC	6039–6059
	SAD11F	TCTAAGCCGCCTGTCAATCC	6954–6973
	SAD11R	ATTTTCCACCTTGTCCACCTC	8335–8405
	SAD18F	GCCAGTCCTACCATTCTACTC	8334–8354
	SAD18R	AGTCTCTTAGCCTTGTGTCTC	9322–9342
	SAD26F	CTGGCAGACCCGTCTACCGT	4569–4588
	SAD26R	TGACTGACACCTCCCTCCCT	4805–4824

* Based on the PV genome (NC_001542). F and R correspond to forward and reverse primers.

A second round of PCR was performed with 2 μl of the first PCR products using 50 pmol of nested primers (Table 1) at 95 °C for 2 min, 35 amplification cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 10 min.

Following amplification, PCR products were purified from a 2% agarose gel and cloned into a plasmid vector pJET 1-2 blunt (Fermentas, France) and transformed in TOPO10 cells (Invitrogen, France). All purified plasmids were sequenced in both directions by Beckman Coulters Cogenics (Takeley, United Kingdom). All necessary safety measures were followed to prevent any cross-contamination or false positive results when performing the PCRs (Kwok and Higuchi, 1989).

2.3. Nucleotide sequence analysis and phylogenetics

At least two individual clones were analyzed for each insert using pJET primers and internal sequencing primers. For SAD Bern 09-99, 1–3 bacterial colonies were analyzed. Consensus sequences were derived from at least two independent forward and reverse sequences of independent clones, except for SAD Bern 09-99. Editing of the alignments and translations was performed using Genedoc (Nicholas et al., 1997). Percentage identities and similarity scores were determined using BioEdit (Hall, 1999).

A phylogenetic tree was generated by comparing the full-length genome of the Vaccine A strain (GenBank accession No. **KC178555**), the SAD Bern 09-99 control virus (GenBank accession No. **KC178556**) and referenced vaccine strains extracted from GenBank (seven SAD Bern sequences, six SAD-derived laboratory sequences and three vaccine strains) (Table 2). The bootstrap probabilities for each node were calculated using 1000 replicates to assess the robustness of the Neighbour Joining method. Bootstrap values over 70% indicated significant support for the tree topology (Baldauf, 2003). The trees' graphic representation was constructed with the MEGA program (Tamura et al., 2011).

Table 2
Description of the complete genome of vaccine strains retrieved from GenBank used in this study.

Rabies virus strain	Origin	Country	Accession No.	Source reference	Length
SAD-derived vaccine virus strains:					
SADP5/88			EF206715	Geue et al. (2008) (*21*)	11928
SAD VA1 original			EF206716	Geue et al. (2008) (*21*)	11928
SAD B19-1st$^{(fox)}$			EU877068	Beckert et al. (2009)	11886
SAD B19 Fuchsoral			EF206709	Geue et al. (2008) (*21*)	11928
SAG2			EF206719	Geue et al. (2008) (*21*)	11928
Rabies virus collection-date 2002 from Germany	Red fox	Germany	EU886632	Muller et al. (2009)	11907
SAD vaccines:					
SAD Bern Lysvulpen			EF206708	Geue et al. (2008) (*21*)	11928
SAD Bern Sanafox			EF206720	Geue et al. (2008) (*21*)	11928
SAD Bern original var1			EF206710	Geue et al. (2008) (*21*)	11930
SAD Bern original var2			EF206711	Geue et al. (2008) (*21*)	11930
SAD Bern original var3			EF206712	Geue et al. (2008) (*21*)	11928
SAD Bern original var4			EF206713	Geue et al. (2008) (*21*)	11928
SAD Bern original var5			EF206714	Geue et al. (2008) (*21*)	11928
Vaccine strain precursors:					
ERA (1978)			EF206707	Geue et al. (2008) (*21*)	11931
SAD1-3670 (1965)			EF206717	Geue et al. (2008) (*21*)	11931
SAD1-3670var2 (1965)			EF206718	Geue et al. (2008) (*21*)	11933

3. Results

Irrespective of the strains analysed, the two amplified full-length genome sequences of 11005 nucleotides (control SAD Bern 09-99 virus and Vaccine A) follow standard RV genome pattern with the five well-known proteins: Nucleoprotein (1353-nt), Phosphoprotein (894-nt), Matrix protein (609-nt), Glycoprotein (1575-nt) and Virion-associated RNA polymerase (partial 5646-nt).

The pairwise comparison of the full-length genome (≈11005-nt) (of Vaccine A and SAD Bern 09-99) against 25 vaccinal strains from GenBank showed 99.9% nucleotide similarity between Vaccine A and the SAD-derived laboratory vaccine virus strains (Table 3). 99.9% nucleotide identity was shown between the SAD Bern 09-99 isolate and the group forming the SAD vaccines (Table 3). Comparison of the 11-kb genome of Lysvulpen with the SAD Bern 09-99 showed a 99.6% nucleotide similarity (data not shown).

The pairwise comparison of the five rabies genes (i.e. N, P, M, G and L genes) performed on the two isolates of this study in comparison with 25 published sequences from GenBank is exposed in Table 4. A perfect nucleotide identity of 100% was shown among the N, P, M and G genes from the Vaccine A and SAD-derived laboratory vaccines (i.e. SAG2, SAD VA1 original, SAD B19 Fuchsoral, SAD B19-1st$^{(mouse)}$, SAD B19-1st$^{(fox)}$ and SAD B19-10th) (Table 4). The same perfect identity was shown for the SAD Bern 09-99 control virus and the group of SAD vaccines for the N, P and L genes (Table 5). The mutations between SAD Bern 09-99 and the SAD Bern original variant 5 (EF206714) were shown on the G gene on positions 223 (A → G), 602 (G → A), 823 (C → A) and 1009 (C → T), whereas the single nucleotide mutation between Vaccine A and the SAD B19-1st$^{(mouse)}$ variant (EU877069) was shown on the L gene (C → T) in position 4214.

As expected, the phylogenetic analysis of the dataset ($n = 27$) from the 11 kb genome showed that Vaccine A and the SAD Bern 09-99 control virus belong to the groups representative of SAD B19 vaccine virus strains and SAD Bern vaccines, respectively, with a bootstrap probability of 100. The phylogenetic analysis with the Neighbour Joining tree is shown in Fig. 1. The first group, made up of 15 referenced SAD-derived laboratory vaccine virus strains and Vaccine A, formed a solid cluster with a high significant bootstrap support value (100%). The latter is made up of seven different SAD Bern vaccines and the SAD Bern 09-99 control virus (bootstrap of 100).

Table 3
Percentage of nucleotide identity between the amplified RV genome (≈11 kb) of the two isolates Vaccine A (KC178555) and SAD Bern 09-99 (KC178556) and 25 vaccine strains from GenBank representative of SAD Bern and SAD-derived laboratory strains.

Description of sequences	Accession number	SAD Bern 09-99	Vaccine A
SAD-derived vaccine virus strains:			
SADP5/88	EF206715	99.6	99.9
SAD VA1 original	EF206716	99.6	99.9
SAD B19-1st$^{(fox)}$	EU877068	99.6	99.9
SAD B19-1st$^{(mouse)}$	EU877069	99.6	99.9
SAD B19 Fuchsoral	EF206709	99.6	99.9
SAG2	EF206719	99.6	99.9
Rabies virus collection-date 2002 from Germany	EU886632	99.6	99.9
SAD vaccines:			
SAD Bern Lysvulpen	EF206708	99.9	99.7
SAD Bern Sanafox	EF206720	99.9	99.6
SAD Bern original var1	EF206710	99.9	99.6
SAD Bern original var2	EF206711	99.9	99.6
SAD Bern original var3	EF206712	99.9	99.6
SAD Bern original var4	EF206713	99.9	99.7
SAD Bern original var5	EF206714	99.9	99.7
Vaccine strain precursors:			
ERA	EF206707	99.5	99.5
SAD1-3670	EF206717	99.4	99.3
SAD1-3670var2	EF206718	99.4	99.3

The SAD Bern 09-99 control virus sample showed the highest nucleotide similarity with the group made up of the SAD Bern sequences, while Vaccine A showed more similarity to the strains belonging to the SAD B19-linked vaccine virus strains.

4. Discussion

The objective of this work was to analyze the full-length genome of a commercial oral bait vaccine by comparing it with the control virus, provided together with the vaccine for virus vaccine titration, prior to its release in the field. The sequences were also compared with those of other SAD strains.

This study is the first to report on the viral changes in an attenuated live rabies vaccine currently known as the SAD Bern strain for oral fox vaccination. Genetic analysis of Vaccine A derived from the original SAD Bern (Geue et al., 2008) revealed that it belongs to the group formed by SAD B19 strains while the current control SAD

Table 4
Pairwise comparison of nucleotide and amino acid sequence identities of Vaccine A (batch 5118, GenBank accession No. KC178555) with representative SAD Bern and SAD-derived laboratory vaccine strains. The percentage of nucleotide identity for the five rabies virus genes is shown. Sequence analysis has been performed by using BioEdit software.

Description of sequence	Accession number		N (%)	P (%)	M (%)	G (%)	L (%)
SAD-derived vaccine virus strains:							
SAD P5/88 Rabifox	EF206715	Nucleotide	99.8	100	100	99.8	99.9
		Amino acid	99.7	100	100	99.8	99.9
SAD VA1 original	EF206716	Nucleotide	100	100	100	99.9	99.9
		Amino acid	100	100	100	99.8	99.9
SAD B19-1st$^{(fox)}$	EU877068	Nucleotide	100	100	100	100	99.9
		Amino acid	100	100	100	100	99.9
SAD B19-1st$^{(mouse)}$	EU877069	Nucleotide	100	100	100	100	99.9
		Amino acid	100	100	100	100	99.9
SAD B19 Fuchsoral	EF206709	Nucleotide	100	100	100	100	99.9
		Amino acid	100	100	100	100	99.9
SAG2	EF206719	Nucleotide	100	100	100	99.8	99.9
		Amino acid	100	100	100	99.6	99.9
Rabies virus collection-date 2002 from Germany	EU886632	Nucleotide	100	100	100	100	99.9
		Amino acid	100	100	100	100	99.9
SAD vaccines:							
SAD Bern Lysvulpen	EF206708	Nucleotide	99.6	99.1	98	99.8	99.9
		Amino acid	99.3	98.3	96	99.6	99.9
SAD Bern Sanafox	EF206720	Nucleotide	99.4	99.1	98	99.9	99.9
		Amino acid	99.1	98.3	96	100	99.9
SAD Bern original var1	EF206710	Nucleotide	99.6	99.1	98	99.8	99.9
		Amino acid	99.3	98.3	96	99.6	99.9
SAD Bern original var2	EF206711	Nucleotide	99.6	99.1	98	99.9	99.9
		Amino acid	99.3	98.3	96	99.8	99.9
SAD Bern original var3	EF206712	Nucleotide	99.6	99.1	98	99.8	99.9
		Amino acid	99.3	98.3	96	99.6	99.9
SAD Bern original var4	EF206713	Nucleotide	99.6	99.1	98	99.8	99.9
		Amino acid	99.3	98.3	96	99.6	99.9
SAD Bern original var5	EF206714	Nucleotide	99.6	99.1	98	99.9	99.9
		Amino acid	99.3	98.3	96	99.8	99.9
Vaccine strain precursors:							
ERA	EF206707	Nucleotide	99.7	99.3	98.1	99.6	99.6
		Amino acid	99.5	98.6	96	99.4	99.3
SAD1-3670	EF206717	Nucleotide	99.5	98.9	97.8	99.3	99.5
		Amino acid	99.5	97.9	95	98.6	99.3
SAD1-3670var 2	EF206718	Nucleotide	99.5	98.9	97.8	99.3	99.5
		Amino acid	99.5	97.9	95	98.6	99.3

Bern 09-99 belongs to the original SAD Bern vaccine group. Previous studies undertaken on inactivated human rabies vaccine (Finke et al., 2012) and a live oral rabies vaccine different from Vaccine A (Geue et al., 2008) partially or fully sequenced showed some discrepancies in the genome identification.

In contrast to the previous report by Geue et al. (2008), the Vaccine A RNA isolated here from a 2011 commercial batch numbered 5118 was shown to be genetically distinct from the referenced SAD Bern sequence (EF206708) isolated in 2006 from a commercial vaccine batch (No. 0213) (Geue et al., 2008).

The modified oral rabies vaccines are replication-competent live viruses which could therefore induce the disease (Bingham et al., 1992; Fehlner Gardiner et al., 2008; Muller et al., 2009; Ruprecht et al., 1990; Steck et al., 1982; Vos et al., 1999; Wandeler, 1988). The G gene responsible for the immune response of the host is the major determinant of rabies pathogenesis (Faber et al., 2005). It was previously shown that virus variants with an Arg → Glu mutation at position 333 affecting antigenic site III of the glycoprotein results in the loss of the RV's ability to kill adult immunocompetent mice, regardless of the site of infection or the dose used (Badrane et al., 2001). By comparing the Vaccine A with the original SAD Bern and SAD B19 strains, we have demonstrated that amino acid Arg_{333} is well conserved in antigenic site III of the KC178555 sequence (data not shown), making it possible to distinguish SAD Bern and SAD B19 strains from the SAG2 strain.

Substantial amounts of rabies vaccine baits are dropped in areas near human habitats in infected countries. In 2012, ORV programmes have been implemented in 17 European countries (http://ec.europa.eu/food/animal/diseases/eradication/legisl_en.htm). In 2011, the majority of manufactured vaccines purchased in Europe for oral vaccination of wildlife were SAD Bern vaccines from Czech Republic, SAD B19 vaccines from Germany and SAG2 vaccines from France, used in nine, five and three countries, respectively. For SAD Bern vaccines only, approximately 17 million baits were distributed throughout Europe in 2011. During the production process, live rabies vaccines should be tested to meet EDQM (European Pharmacopoeia, 2011a, b), WHO (WHO, 2013), EC (Anonymous, 2002) and OIE (World Health Organisation for Animal Health, 2012a, b) requirements to guarantee their identity and stability as well as their efficacy and safety in target and non-target species. The manufacturing process, from production procedures to final product testing, should guarantee the release of a high quality product through appropriate controls and an effective quality assurance system. More specifically, the two European monographs (European Pharmacopoeia, 2011a, b) provide several verifications to be performed by the vaccine producer to avoid any mutations or contamination of the master seed virus strain by extraneous agents. The vaccine strain should be clearly identified using a suitable method in order to distinguish it from related strains. To avoid any genetic changes, it is also specified (European Pharmacopoeia, 2011b; World Health Organisation for Animal Health, 2012a) that a maximum of 5 passages from the initial master seed virus is allowed. Susceptible inoculated cells should be checked for the presence of contaminating rabies virus and then stained with a panel of monoclonal antibodies reacting with rabies virus strains different from the vaccine strain.

At a time when environmental questions are being raised, the viral changes in the SAD Bern oral vaccine raise questions. We can hypothesize natural genetic evolution of the original SAD

Table 5
Pairwise comparison of nucleotide and amino acid sequence identities of SAD Bern 09-99 sample (GenBank accession No. KC178556) with representative SAD Bern and SAD-derived laboratory vaccine strains. The percentage of nucleotide identity for the five rabies virus genes is shown. Sequence analysis has been performed by using BioEdit software.

Description of sequence	Accession number		N (%)	P (%)	M (%)	G (%)	L (%)
SAD-derived vaccine virus strains:							
SAD P5/88 Rabifox	EF206715	Nucleotide	99.7	99.1	98	99.4	100
		Amino acid	99.5	98.3	96	99	100
SAD VA1original	EF206716	Nucleotide	100	99.1	98	99.5	100
		Amino acid	99.3	98.3	96	99	100
SAD B19-1st$^{(fox)}$	EU877068	Nucleotide	99.6	99.1	98	99.6	100
		Amino acid	99.3	98.3	96	99.2	100
SAD B19-1st $^{(mouse)}$	EU877069	Nucleotide	99.6	99.1	98	99.6	100
		Amino acid	99.3	98.3	96	99.2	100
SAD B19 Fuchsoral	EF206709	Nucleotide	99.6	99.1	98	99.6	100
		Amino acid	99.3	98.3	96	99.2	100
SAG2	EF206719	Nucleotide	99.6	99.1	98	99.4	100
		Amino acid	99.3	98.3	96	98.8	100
Rabies virus collection-date 2002 from Germany	EU886632	Nucleotide	99.6	99.1	98	99.6	99.9
		Amino acid	99.3	98.3	96	99.2	100
SAD vaccines							
SAD Bern Lysvulpen	EF206708	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99	100
SAD Bern Sanafox	EF206720	Nucleotide	99.7	100	100	99.6	100
		Amino acid	99.7	100	100	99.2	100
SAD Bern original var1	EF206710	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99.2	100
SAD Bern original var2	EF206711	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99	100
SAD Bern original var3	EF206712	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99.2	100
SAD Bern original var4	EF206713	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99.2	100
SAD Bern original var5	EF206714	Nucleotide	100	100	99.8	99.6	100
		Amino acid	100	100	99.5	99	100
Vaccine strain precursors:							
ERA	EF206707	Nucleotide	99.7	99.1	99.3	99.6	99.6
		Amino acid	99.3	98.3	99.5	99.4	99.4
SAD1-3670	EF206717	Nucleotide	99.6	98.7	98.8	99.3	99.5
		Amino acid	99.3	97.6	98	98.6	99.3
SAD1-3670var 2	EF206718	Nucleotide	99.6	98.7	98.8	99.3	99.5
		Amino acid	99.3	97.6	98	98.6	99.3

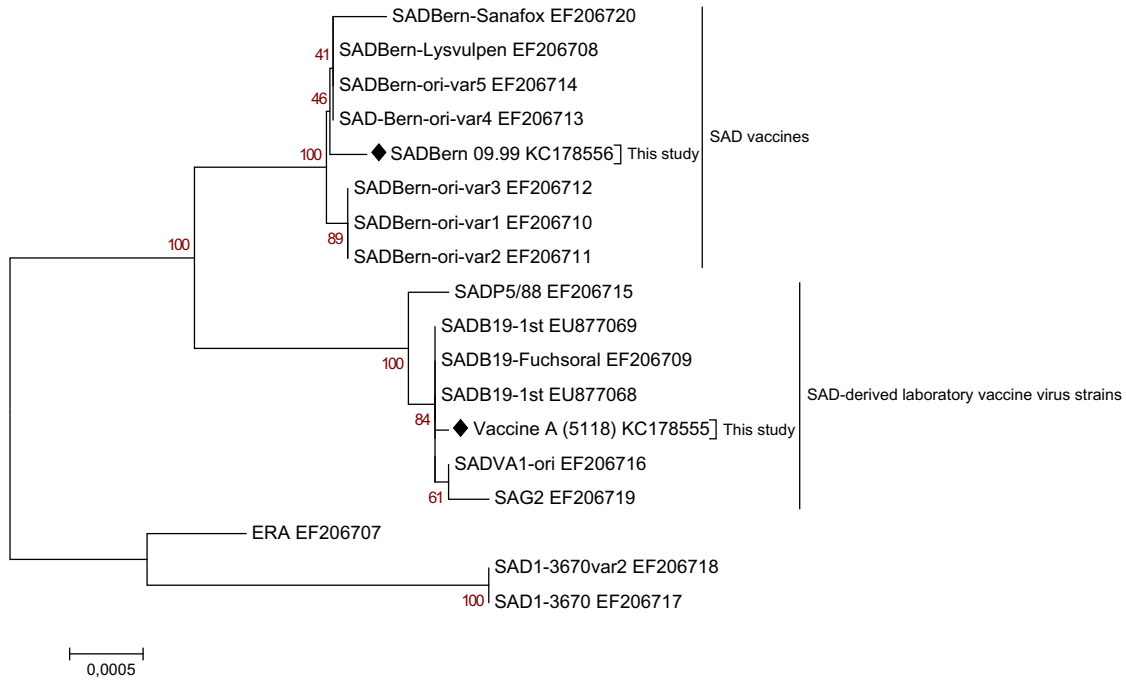

Fig. 1. Phylogenetic tree comparing the two isolates of this study (Vaccine A GenBank accession No. **KC178555** and SAD Bern 09-99 control, **KC178556)** and reference vaccine sequences. Phylogenetic relationships were determined by comparing the full-length genome sequences using MEGA5 software (Neighbour Joining method, Tamura 3-paramater method, 1000 replicates).

strain due to serial cell culture passages or a possible change or replacement of the master seed virus and/or the working seed virus. Further investigations on the master seed virus, the working seed virus and on a larger number of vaccine batches over a longer period of time are needed to clarify the reason for these variations. If those additional investigations confirm the results of this study, it could suggest that Vaccine A is more attenuated and safer than the original SAD Bern strain. Additionally, all available attenuated oral rabies vaccines currently used in Europe should be similarly investigated.

Conflict of interest

The authors declare that we have no competing interests.

Acknowledgments

The authors thank Mélanie Biarnais and Marie José Duchêne for their excellent technical contribution. In addition, we thank the four anonymous reviewers for their very valuable comments on the manuscript.

This research was funded by Anses (French Agency for Food, Environmental and Occupational Health & Safety).

References

Anonymous, 2002. The oral vaccination of foxes against rabies. Report of the Scientific Committee on Animal Health and Animal Welfare, European Commission, p. 55.

Aubert, M., 1992. Epidemiology of fox rabies. In: Bögel, L., Meslin, F.X., Kaplan, M. (Eds.), Wildlife Rabies Control. Wells Medical Ltd, Kent, pp. 9–18.

Badrane, H., Bahloul, C., Perrin, P., Tordo, N., 2001. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J. Virol. 75, 3268–3276.

Baldauf, S.L., 2003. Phylogeny for the faint of heart: a tutorial. Trends Genet. 19, 345–351.

Beckert, A., Geue, L., Vos, A., Neubert, A., Freuling, C., Muller, T., 2009. Genetic stability (in vivo) of the attenuated oral rabies virus vaccine SAD B19. Microbiol. Immunol. 53, 16–21.

Bingham, J., Foggin, C.M., Gerber, H., Hill, F.W., Kappeler, A., King, A.A., Perry, B.D., Wandeler, A.I., 1992. Pathogenicity of SAD rabies vaccine given orally in chacma baboons (*Papio ursinus*). Vet. Rec. 131, 55–56.

Blancou, J., 1988. Ecology and epidemiology of fox rabies. Rev. Infect. Dis. 10, S606–S609.

Blancou, J., 2004. Rabies in Europe and the Mediterranean basin: from antiquity to the 19th century. In: King, A.A., Aubert, M., Wandeler, A.I. (Eds.), Historical Perspective of Rabies in Europe and the Mediterranean Basin. OIE, Paris, pp. 15–24.

Cliquet, F., Aubert, M., 2004. Elimination of terrestrial rabies in Western European countries. Dev. Biol. 119, 185–204.

European Pharmacopoeia, 2011a. Rabies vaccine (Live, Oral) for foxes, . seventh ed.. In: European Pharmacopoeia, Europe (Ed.), General Notices Apply to All Monographs and Other Text seventh ed. EDQM, Strasbourg, pp. 936–937.

European Pharmacopoeia, 2011b. Vaccines for veterinary use. European Pharmacopoeia. General notices apply to all monographs and other texts,, EDQM, Strasbourg, pp. 590–595.

Faber, M., Faber, M.L., Papaneri, A., Bette, M., Weihe, E., Dietzschold, B., Schnell, M.J., 2005. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. J. Virol. 79, 14141–14148.

Fehlner-Gardiner, C., Nadin-Davis, S., Armstrong, J., Muldoon, F., Bachmann, P., Wandeler, A., 2008. ERA vaccine-derived cases of rabies in wildilfe and domestic animals in Ontario, Canada, 1989–2004. J. Wildl. Dis. 44, 71–85.

Finke, S., Karger, A., Freuling, C., Muller, T., 2012. Assessment of inactivated human rabies vaccines: biochemical characterization and genetic identification of virus strains. Vaccine 30, 3603–3609.

Geue, L., Schares, S., Schnick, C., Kliemt, J., Beckert, A., Freuling, C., Conraths, F.J., Hoffmann, B., Zanoni, R., Marston, D., McElhinney, L., Johnson, N., Fooks, A.R., Tordo, N., Muller, T., 2008. Genetic characterisation of attenuated SAD rabies virus strains used for oral vaccination of wildlife. Vaccine 26, 3227–3235.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Series 41, 95–98.

ICTV (2011) ICTV Official taxonomy: updates since the 8th report. Vertebrate. <http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/vertebrate-official/4188.aspx> (accessed 30.04.13.).

Kieny, M.P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H., Lecocq, J.P., 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312, 163–166.

Kirandjiski, T., Mrenoski, S., Celms, I., Mitrov, D., Dzadzovski, I., Cvetkovikj, I., Krstevski, K., Picard-Meyer, E., Viviani, P., Malinovski, D., Demerson, J.M., Cvetkovikj, A., Davcheva, K., Nakova, E., Tomeska Mickova, S., Cliquet, F., 2012. First reported cases of rabies in the Republic of Macedonia. Vet. Rec. 170, 312.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Lafay, F., Benejean, J., Tuffereau, C., Flamand, A., Coulon, P., 1994. Vaccination against rabies: construction and characterization of SAG2, a double avirulent derivative of SADBern. Vaccine 12, 317–320.

Matouch, O., Vitasek, J., Semerad, Z., Malena, M., 2006. Elimination of rabies in the Czech Republic. Dev. Biol. 125, 141–143.

Muller, T., Batza, H.J., Beckert, A., Bunzenthal, C., Cox, J.H., Freuling, C.M., Fooks, A.R., Frost, J., Geue, L., Hoeflechner, A., Marston, D., Neubert, A., Neubert, L., Revilla-Fernandez, S., Vanek, E., Vos, A., Wodak, E., Zimmer, K., Mettenleiter, T.C., 2009. Analysis of vaccine-virus-associated rabies cases in red foxes (*Vulpes vulpes*) after oral rabies vaccination campaigns in Germany and Austria. Arch. Virol. 154, 1081–1091.

Nicholas, K.B., Nicholas, H.B., Deerfield, D.W., 1997. GeneDoc: analysis and visualization of genetic variation. Embnew News 4, 14.

Pastoret, P.P., Kappeler, A., Aubert, A., 2004. European rabies control and its history. In: King, A.A., Aubert, M., Wandeler, A.I. (Eds.), Historical Perspective of Rabies in Europe and the Mediterranean Basin. OIE, Paris, pp. 337–350.

Rabies Bulletin Europe, 2012. Rabies Information System of the WHO Collaboration Centre for Rabies Surveillance and Research. WHO, Wusterhausen (Germany).

Rupprecht, C.E., Charlton, K.M., Artois, M., Casey, G.A., Webster, W.A., Campbell, J.B., Lawson, K.F., Schneider, L.G., 1990. Ineffectiveness and comparative pathogenicity of attenuated rabies virus vaccines for the striped skunk (*Mephitis mephitis*). J. Wildl. Dis. 26, 99–102.

Schneider, L.G., Cox, J.H., 1983. Ein Fedelversuch zur oralen Immunisierung von Füchsen gegen die Tollwut in der Bundesrepublik Deutschland. Tierärztl. Umsch. 38, 315–324.

Schneider, L.G., Cox, J.H., Müller, W.W., 1987. Der Feldversuch zur oralen Immunisierung von Füchsen gegen die Tollwut in der Bundesrepublik Deutschland (BRD). Eine Zwischenbilanz. Tierärztl. Umsch. 42, 184–198.

Servat, A., Cliquet, F., 2013. Official batch control of rabies veterinary vaccines: current situation and perspectives in the European Union. Altern. Lab. Anim. 41, 10–11.

Steck, F., Wandeler, A., Bichsel, P., 1982. Oral immunization of foxes against rabies. Laboratory and field studies. Comp. Immunol. Microbiol. Infect. Dis. 5, 165–171.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Vos, A., Neubert, A., Aylan, O., Schuster, P., Pommerening, E., Muller, T., Chivatsi, D.C., 1999. An update on safety studies of SAD B19 rabies virus vaccine in target and non-target species. Epidemiol. Infect. 123, 165–175.

Wandeler, A.I., 1988. Control of wildlife rabies. In: Campbell, J.B., Charlton, K.M. (Eds.), Rabies. Kluwer Academic Publishers, Boston, pp. 365–380.

WHO, 2013. WHO expert consultation on rabies - second report. WHO Technical Report Series 982, p. 139.

Wiktor, T.J., Macfarlan, R.I., Reagan, K.J., Dietzschold, B., Curtis, P.J., Wunner, W.H., Kieny, M.P., Lathe, R., Lecocq, J.P., Mackett, M., et al., 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc. Natl. Acad. Sci. USA 81, 7194–7198.

World Health Organisation for Animal Health, 2012a. Principle of veterinary vaccine production. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), seventh ed. World Organization for Animal Health, Paris, pp. 52–63.

World Health Organisation for Animal Health, 2012b. Rabies. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), seventh ed. World Organization for Animal Health, Paris, pp. 263–282.