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

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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.

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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.

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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).

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