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In-depth genome analyses of viruses from vaccine-derived rabies cases and corresponding live-attenuated oral rabies vaccines

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ABSTRACT

Live-attenuated rabies virus strains such as those derived from the field isolate Street Alabama Dufferin (SAD) have been used extensively and very effectively as oral rabies vaccines for the control of fox rabies in both Europe and Canada. Although these vaccines are safe, some cases of vaccine-derived rabies have been detected during rabies surveillance accompanying these campaigns. In recent analysis it was shown that some commercial SAD vaccines consist of diverse viral populations, rather than clonal genotypes. For cases of vaccine-derived rabies, only consensus sequence data have been available to date and information concerning their population diversity was thus lacking.

In our study, we used high-throughput sequencing to analyze 11 cases of vaccine-derived rabies, and compared their viral population diversity to the related oral rabies vaccines using pairwise Manhattan distances. This extensive deep sequencing analysis of vaccine-derived rabies cases observed during oral vaccination programs provided deeper insights into the effect of accidental *in vivo* replication of genetically diverse vaccine strains in the central nervous system of target and non-target species under field conditions. The viral population in vaccine-derived cases appeared to be clonal in contrast to their parental vaccines. The change from a state of high population diversity present in the vaccine batches to a clonal genotype in the affected animal may indicate the presence of a strong bottleneck during infection. In conclusion, it is very likely that these few cases are the consequence of host factors and not the result of the selection of a more virulent genotype. Furthermore, this type of vaccine-derived rabies leads to the selection of clonal genotypes and the selected variants were genetically very similar to potent SAD vaccines that have undergone a history of *in vitro* selection.

1. Introduction

Rabies is one of the oldest known zoonotic infections, still causing several thousand human casualties each year [1]. The almost always fatal disease is mainly associated with rabies virus (RABV), a neurotropic single-stranded RNA virus of the family *Rhabdoviridae* in the genus *Lyssavirus* within the order *Mononegavirales*. While domestic dogs are responsible for the vast majority of human infections with RABV, with the highest burden in developing countries of Africa and Asia [2,3], RABV also circulates in various wild-life reservoirs [4]. This fact, however, makes a true disease eradication a challenge [5]. Since wildlife reservoir species are generally unavailable for parenteral vaccination, only with the advent of oral rabies vaccines (ORVs) did control of the disease in wildlife become a viable option [6]. Highly potent and safe ORVs based on live-attenuated, replication-competent viruses were

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subsequently developed [7]. In addition to the use of oral vaccines, baits and baiting strategies had to be developed. In combination, large-scale vaccination programs proved to be very efficient and successful in vaccination of red foxes (*Vulpes vulpes*) against rabies in Europe [8–11] and in Canada [12,13]. The majority of vaccines used in Europe and Canada contained one of the live-attenuated "Street Alabama Dufferin" (SAD) strains [14]. The present form of SAD vaccines originated from a diverse history of *in vivo*/*in vitro* passaging, plaque purification or escape mutant selection via monoclonal antibodies, that has been summarized by Höper et al. [15]. Briefly, the progenitor of all SAD vaccines was isolated from a rabid dog in 1935 and further propagated in mouse brains, followed by adaptation of the resulting strain to different cell culture systems [16,17]. As a result, the Evelyn-Rokitnicki-Abelseth (ERA) strain was the first vaccine strain that protected foxes reliably against rabies by oral immunization [6,18]. The ERA strain was further improved with respect to its thermostability and resulted in an early version of SAD Bern (SAD $Bern_{original}$) which was used in the world's first field trial in Switzerland [19]. SAD $Bern_{original}$ then served as source for several commercially available first generation vaccines, such as SAD Bern, SAD B19, SAD P5/88 and SRV9. Selection of escape mutants from SAD Bern in the presence of multiclonal antibodies against rabies glycoprotein led to the development of second generation SAD vaccines, called SAG1 and SAG2 [20,21].

The European ORV programs mainly applied SAD-related vaccines with approximately 665 million vaccine doses distributed between 1978 and 2014 in 30 countries, encompassing an area of about 2.5 million km^2. Of these, the ones used most frequently were the two first generation vaccines SAD B19 (40.3%) and SAD Bern (31.7%) [14]. For the Canadian ORV programs between 1989 and 2004, the ERA strain was propagated on BHK-21 cells and over 13 million baits were distributed in a total area of 0.75 million km^2 [12].

For SAD derived ORV, residual pathogenicity was experimentally observed in rodents [22,23], skunks [24] and non-human primates [25]. However, SAD vaccine-associated rabies in other mammals is very rare, and only sporadic field cases have been reported so far [12,26–30]. The available sequence data are generally limited to only partial consensus sequences [12,26,28–30] with a single exception where full-length genome sequences are available [27]. These consensus sequences have been shown to be insufficient for addressing the complexity of viral populations, particularly in respect to SAD vaccines as previously demonstrated [15]. By using high-throughput sequencing it was also demonstrated that SAD-derived ORVs may be comprised of several genetic variants [15,31]. Given this background, this report describes the use of high-throughput sequencing analysis to elucidate the effect of an accidental *in vivo* replication of genetically diverse vaccine strains in the CNS of target and non-target species under field conditions. Moreover, we wanted to investigate whether virus population data can support ORV monitoring and pharmacovigilance of vaccine products.

2. Material and methods

2.1. Sample collection and preparation

Eleven vaccine-derived rabies cases were included in this study. Of those, eight cases were observed during surveillance following oral rabies vaccination campaigns in Europe between 2001 and 2015 and three cases during surveillance in Ontario (Canada) between 1991 and 1996 (Table 1).

Initially, vaccine associated cases were detected in the frame of routine rabies diagnostics and confirmed positive for RABV infection using either the fluorescence antibody test, virus isolation or PCR. Because virus typing, i.e. discrimination between vaccine and field strains is obligatory in vaccinated areas, all samples were subjected to further characterization using monoclonal antibodies and sequence analysis [12,26–30].

From these 11 cases, 14 different samples were available (Table 1) including original brain samples, and cell culture supernatant or inoculated mouse brain from virus isolation. Furthermore, three batches of each of the commercial SAD Bern and SAD B19 ORV, and an ERA vaccine production lot from Canada were included for comparison (Table 2).

2.2. RNA extraction, cDNA synthesis and sequencing

Total RNA was extracted from all samples. RNA extraction and cDNA synthesis were performed as described using the Sequencing protocol 1 of Hanke et al. [32] for the European samples. Briefly, 20 mg organ were homogenized using the Mikro-Dismembrator S (Sartorius, Göttingen, Germany) and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) including on-column DNAse I (Qiagen) digestion. RNA from Canadian samples was extracted as described previously [12]. For generation of cDNA we used the cDNA synthesis system kit (Roche, Mannheim, Germany) together with random hexamer primers (Roche). The resulting cDNA was subsequently prepared for sequencing with the Ion Torrent PGM (Life Technologies, Darmstadt, Germany) using Hi-Q chemistry, as described elsewhere [15]. Briefly, cDNA was sheared using the Covaris M220 (Covaris, Brighton, United Kingdom) and transformed into Ion Torrent compatible libraries using the GeneRead DNA Library L Core Kit along with corresponding barcoded adapters (Qiagen). After size selection (Agencourt AMPure XP magnetic beads; Beckman Coulter, Fullerton, USA) and quality control (High Sensitivity DNA kit; Agilent Technologies, Böblingen, Germany), the libraries were quantified (Ion Library Quantitation Kit; Life Technologies) and sequenced according to the manufacturer's instructions.

2.3. Sequence assembly

In order to create full-length RABV consensus sequences for all isolates we used the 454 Sequencing System Software v3.0 (Roche). The raw reads were initially quality trimmed, mapped along the NCBI reference sequence for SAD B19 (EF206709.1) and full or partial matching reads were further used for de-novo assembly. The full-length RABV consensus sequences obtained in this study were annotated in Geneious 10.0.9 [33] and submitted to the European Nucleotide Archive (ENA) under the study accession PRJEB21530.

2.4. Population-based analysis

In order to address genetic relationships between diverse viral populations that might not be reflected by consensus phylogeny we used a novel approach [15] to assess viral strain relationships. Initially all full-length SAD-related sequences obtained in this study were aligned using MAFFT [34] and a strict majority consensus sequence was deduced. This sequence and its reversed complement were used as reference for mapping of the datasets using the 454 Sequencing System Software v3.0 (Roche). Furthermore, sequence data, comprising SAD vaccine progenitors, commercial SAD vaccines and their derivatives, from a previous study [15] were included in the analysis (Table 2). In order to compare datasets with equal levels of sequence depth, we selected random subsets as necessary. Nucleotide counts for each position of the reference (reflecting the viral population) were derived from the resulting alignment (using the –nft option and the 454AlignmentInfo.tsv file) and results from the forward and reverse

Table 1
Vaccine-derived cases and respective samples included in the study and sequence data per sample.

Case ID	Animal	Age	Sampling place	Date of finding	FAT	RT-PCR	Distributed vaccine[a]	Study	Available sample material	Sample ID	RABV reads[b]	RABV proportion[c]	Reference Coverage
1	Red fox *(Vulpes vulpes)*	Juvenile	Leverkusen, Germany	June 2001	–	+	SAD P5/88	Müller et al. [27]	Brain	GER/2001/brain	817	0.02%	99.83%
2	Red fox *(Vulpes vulpes)*	Juvenile	Limburg, Germany	Oct 2002	+	+	SAD B19	Müller et al. [27]	Cell culture: isolated from brain	GER/ 2002/cellculture	52,520	88.0%	100%
3	Red fox *(Vulpes vulpes)*	Juvenile	Völkermarkt, Austria	May 2004	+	+	SAD B19	Müller et al. [27]	Brain: mouse passage	AUS/2004/brain	1084	0.1%	99.37%
									Cell culture: isolated from brain	AUS/ 2004/cellculture	53,063	85.0%	100%
4	Red fox *(Vulpes vulpes)*	Juvenile	Mettmann, Germany	June 2004	–	+	SAD B19	Müller et al. [27]	Brain	GER/2004/brain	385	0.01%	99.79%
5	Red fox *(Vulpes vulpes)*	Juvenile	Slovenia	May 2012	+	+	SAD B19	Hostnik et al. [28]	Cell culture: isolated from brain	SLOV/ 2012/cellculture1	9931	17.6%	99.84%
									Cell culture passage: isolated from salivary gland	SLOV/ 2012/cellculture2	52,668	70.1%	100%
6	Stone marten *(Martes foina)*	n.d.	Slovenia	2014	+	+	SAD B19	Unpublished	Brain	SLOV/2014/brain	10,232	0.1%	100%
7	Badger *(Meles meles)*	n.d.	Aloja, Latvia	Apr 13	+	+	SAD Bern	Robardet et al. [29]	Brain	LAT/2015/brain1	17,370	0.2%	100%
									Brain suspension: mouse passage	LAT/2015/brain2	10,511	0.1%	100%
8	Cow *(Bos taurus)*	Adult	Romania	June 2015	+	+	SAD Bern	Vuta et al. [30]	Brain	ROM/2015/brain	13,115	2.4%	99.99%
9	Stripped skunk (*Mephitis mephitis*)	n.d.	Elderslie, Canada	1991	+	+	ERA	Fehlner-Gardiner et al. [12]	Brain	CAN/1991/brain	12,297	4.1%	99.98%
10	Cow (*Bos taurus*)	Juvenile	Gloucester, Canada	1994	+	+	ERA	Fehlner-Gardiner et al. [12]	Brain: mouse passage	CAN/1994/brain	10,806	0.4%	99.89%
11	Red fox *(Vulpes vulpes)*	n.d.	Ops, Canada	1996	+	+	ERA	Fehlner-Gardiner et al. [12]	Brain	CAN/1996/brain	6224	0.3%	99.82%

n.d.: not determined.
[a] Vaccine that was distributed at the time the case was detected.
[b] Number of sequencing reads representing RABV.
[c] Proportion of RABV reads in the complete dataset.

Table 2
Vaccines and respective batches/samples included in the study and sequence data per batch/sample.

Vaccine	Description	Batch ID	Data source	Sample ID	RABV reads[a]	RABV proportion[b]	Reference Coverage
ERA	ERA passaged in BHK21 cells	lot 016 (2005)	This study	ERA lot016	55,538	37.2%	99.95%
	First guinea pig passage of original strain ERA: isolated from brain	n.a.	Höper et al. [15]	ERA guinea pig passage	43,552	67.3%	100%
SAD	Ancestral SAD strain: passage history unknown	n.a.	Höper et al. [15]	SAD	45,638	52.4%	99.99%
SAD B19	Commercial product	7440311-A	Höper et al. [15]	SAD B19 744	54,893	90.4%	100%
		7930612-A	Höper et al. [15]	SAD B19 793	26,990	80.3%	100%
		8060712-A	Höper et al. [15]	SAD B19 806	54,727	96.7%	100%
		9580315-A	This study	SAD B19 958	11,376	66.1%	99.96%
		9590415-C	This study	SAD B19 959	33,729	89.6%	100%
		9690715-C	This study	SAD B19 969	7288	78.5%	100%
SAD $B19_{CS}$	Seed virus No 6. from Behring (1992-04-28): clonally selected	n.a.	Höper et al. [15]	SAD $B19_{CS}$	48,311	86.3%	100%
SAD $B19_{P1}$	First passage of original SAD B19 in BSR cells	n.a.	Höper et al. [15]	SAD $B19_{P1}$	49,501	60.7%	100%
SAD Bern	Commercial product	0213	Höper et al. [15]	SAD Bern 0213	60,240	70.5%	100%
		5023	This study	SAD Bern 5023	53,102	96.0%	100%
		5123	This study	SAD Bern 5123	52,397	95.6%	100%
		49235	This study	SAD Bern 49235	53,330	96.9%	100%
SAD $Bern_{original}$	Seed virus B from 1978: 7th passage (1983-02-25)	n.a.	Höper et al. [15]	SAD $Bern_{original}$	28,349	18.4%	100%
SAD P5/88	Commercial product	18708	Höper et al. [15]	SAD P5/88	45,792	19.1%	100%
SAD VA1	Patented vaccine strain	n.a.	Höper et al. [15]	SAD VA1	51,921	14.4%	100%
SAG2	Commercial product	F-04 92 08 75 79	Höper et al. [15]	SAG2	45,626	75.7%	100%

n.a.: not applicable.
[a] Number of sequencing reads representing RABV.
[b] Proportion of RABV reads in the complete dataset.

mapping were combined for homopolymer correction using R [35] (version 3.3.3, http://www.R-project.org) and R studio [36] (version 0.98.983, https://www.rstudio.com). Only basecalls present in both datasets were considered for further analysis. The minimum assessed variant frequency was dependent on the respective sequence coverage and ranged from 0.01 for a local depth of at least 400 to 0.1 in case of a local depth lower than 100. These settings matched the criteria for the detection of minor sequence variants defined by Wang et al. [37]. Pairwise Manhattan-distances were calculated between the populations and visualized using a multidimensional scaling and plotting as described in detail [15].

2.5. Consensus based analysis

For phylogenetic analysis of consensus sequences, the consensus sequences were deduced from all datasets included in this study and aligned using MAFFT [34]. A phylogenetic tree was inferred using the maximum-likelihood method implemented in IQ-TREE [38] (version 1.5.4) under options for automated model selection and statistical support from 100,000 ultra-fast bootstrap replicates [39]. Sequences of all vaccine-derived cases were compared to batches of the corresponding vaccine (Table 1) in order to identify unique nucleotide exchanges.

3. Results and discussion

For this study, a total of 21 samples (Tables 1 and 2, Supplemental Figure S1) including brain material, cell culture supernatants, and commercial vaccine batches were sequenced. The proportion of RABV reads in the complete dataset depended on sample type and preservation, ranging from 0.01% to 96.9% (Tables 1 and 2). The highest virus to host ratios were achieved for cell culture-propagated viruses (commercial vaccine batches, cell culture isolation), while direct sequencing of RABV from organ material resulted in substantially lower virus to host ratios. This is most likely caused by the fact that in original tissue samples compared with cell cultures a lower portion of the cells is infected and the use of supernatant enriched for viral particles is not possible. Nevertheless, assembly of complete coding RABV sequences was successful for all samples. These results show that high coverage sequencing is feasible directly from any sample without the need for prior amplification (For more information on site-specific coverage please see Supplemental Figure S2.). This is especially important for analyzing low abundance variants within viral populations, since prior amplification may result in biased data due to non-random selection of viral variants [40].

3.1. Viruses from vaccine-derived cases show a very low genetic heterogeneity

Interestingly, previous analyses using high-throughput sequencing revealed substantial genetic heterogeneity within some commercial SAD-derived oral rabies virus vaccines [15,31]. It was shown that in particular SAD Bern and SAD B19 exist as diverse viral populations, whereas other SAD derivatives had nearly clonal genotypes [15]. In contrast, previous analyses of vaccine-derived cases were based on consensus sequences [12,26–30] which did not provide any information about viral populations.

In this study, sequencing of 11 samples representing 9 different cases and all vaccine batches yielded sufficient data for the analysis of nucleotide variants while coverage for samples GER/2001/brain, AUS/2004/brain and GER/2004/brain was insufficient for variant analysis. Including the dataset of Höper et al., initially only the occurrence of high confidence nucleotide variants with frequencies above 5% was analyzed (Fig. 1). Our analysis clearly revealed characteristic variant patterns for all analyzed vaccine strains (Fig. 1A and B). The results confirmed that batches of SAD B19 and SAD Bern comprise a large number of high frequency nucleotide variants appearing as barcode-like patterns, especially in the N, P, M

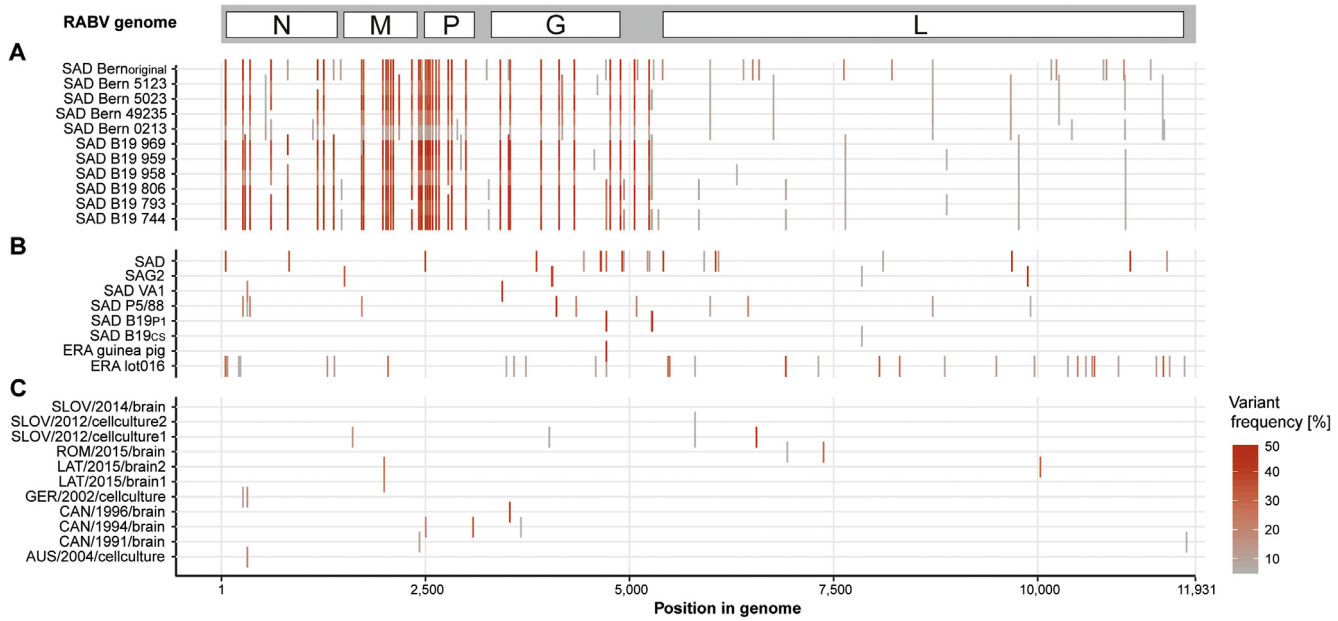

Fig. 1. Positions of nucleotide variants with frequencies >5%. The positions of nucleotide variants, as indicated by bars, are mapped along the RABV genome for all samples. The variant frequency is indicated by the bar color. (A) Vaccine batches of SAD B19 and SAD Bern; (B) Ancestral SAD strain, ERA vaccine samples and selection variants of SAD $Bern_{original}$; (C) Samples from vaccine-derived rabies cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and G gene region (Fig. 1A). Only the polymerase gene (L) seemed to be less variable, in accord with previous findings [15,31,41]. In contrast, samples from other SAD vaccine derivatives contained only a few characteristic nucleotide variants, scattered over the genome (Fig. 1B) and the guinea pig brain passage of ERA vaccine (ERA guinea pig) even appeared to be clonal (Fig. 1B).

Interestingly, similar to those vaccine strains that have undergone a history of clonal selection, the population analysis of vaccine-derived cases indicated a much lower variability of viral populations (Fig. 1C) compared to their related vaccine strains, i.e. ERA, SAD B19 and SAD Bern. Viruses from vaccine-derived cases had between none and four nucleotide variants (Supplemental

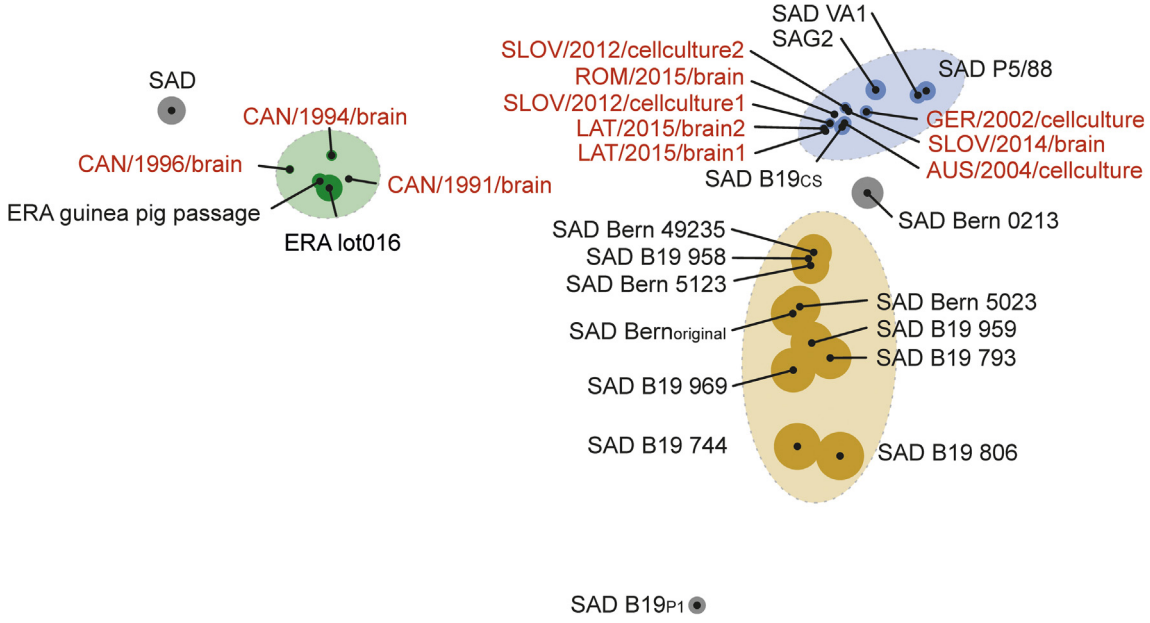

Fig. 2. Population phylogeny based on fitted pairwise Manhattan distances. Nucleotide counts for each position of the genome were derived for each sample using deep sequencing and corrected nucleotide frequencies were deduced. Pairwise Manhattan distances between each sample were calculated based on these frequencies and visualized using multidimensional scaling. Each sample is represented by a dot and their population diversity, calculated as distance between the population and the respective consensus data, is indicated by circle size. Vaccine-derived cases (indicated in red) associated with ERA, clearly clustered together with an ERA vaccine batch (green cluster). Vaccine-derived cases from Europe cluster separately from most of their associated commercial SAD Bern and SAD B19 vaccine batches (orange cluster). Their population properties seem to be rather associated to highly *in vitro* selected derivatives of these vaccines (blue cluster). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table S1). This suggests that infection of the affected hosts with those genetically heterogeneous vaccine strains represents a strong genetic bottleneck that selected against many individual variants as shown for other RNA viruses [42–45].

All viruses except one virus from a vaccine-derived case, which had no variants at the given thresholds, showed few but distinct genetic alterations widely distributed across the genome. Altogether, in vaccine-derived cases 20 nucleotide variants reaching frequencies above 5% were found, of which 8 resulted in amino acid changes. While in a number of ERA-derived cases non-synonymous variants were detected, the SAD $Bern_{original}$-derived viruses obtained from original brain samples comprised only synonymous variants (Supplemental Table S1). In vaccine-derived viruses isolated and subsequently propagated *in vivo* (mice) and *in vitro* (cell culture) both scenarios were found. For example, in original material and brain tissue from the first mouse passage of a vaccine-derived case from Latvia, a synonymous variation at residue 161 (ccc → cct) of the P gene was present in both samples at comparable frequencies. The mouse-passaged isolate had one additional variation that was not present in the original brain sample (Supplemental Table S1). Similarly, vaccine-derived viruses isolated from different organs from the Slovenian case and subsequently propagated in cell culture showed different synonymous variants (Supplemental Table S1). These genetic alterations may indicate early cell culture adaptation or another bottleneck effect [46]. However, the number of variations in passaged viruses is very limited and therefore is assumed not to influence the analysis significantly. Hence, original brain material or low passaged viruses preferentially should be used for population analysis.

3.2. SAD Bern and SAD B19 induced cases are phylogenetically related to clonally selected SAD variants

The plot of the Manhattan distances and population diversities calculated from variant frequencies (Fig. 2), together with the previously sequenced vaccine batches, confirmed the high genetic diversity [15,31] and stability of the populations [15] within the vaccine batches of SAD Bern and SAD B19. The ERA vaccine also showed a substantial genetic diversity. However, because only a single ERA vaccine batch was available, no conclusions could be drawn regarding the stability of the ERA vaccine virus population. In contrast, this analysis (Fig. 2) revealed a clear loss of viral population diversity in all analyzed vaccine-derived cases, resulting in a nearly clonal virus genotype in the affected animals. The closest genetic relationship of the ERA-derived vaccine cases was found with their progenitor viruses. In contrast, the SAD $Bern_{original}$-derived rabies cases were located distantly from their progenitors. Interestingly, these cases clustered closely with the *in vitro* selected attenuated rabies vaccine strains P5/88, VA1, SAG2 and SAD $B19_{CS}$ (Fig. 2). The observed reduction of the population diversity in vaccine-derived cases therefore implies that only a small proportion or even only a single haplotype of the complete original vaccine population is selected and able to replicate efficiently in the affected individual during the course of infection

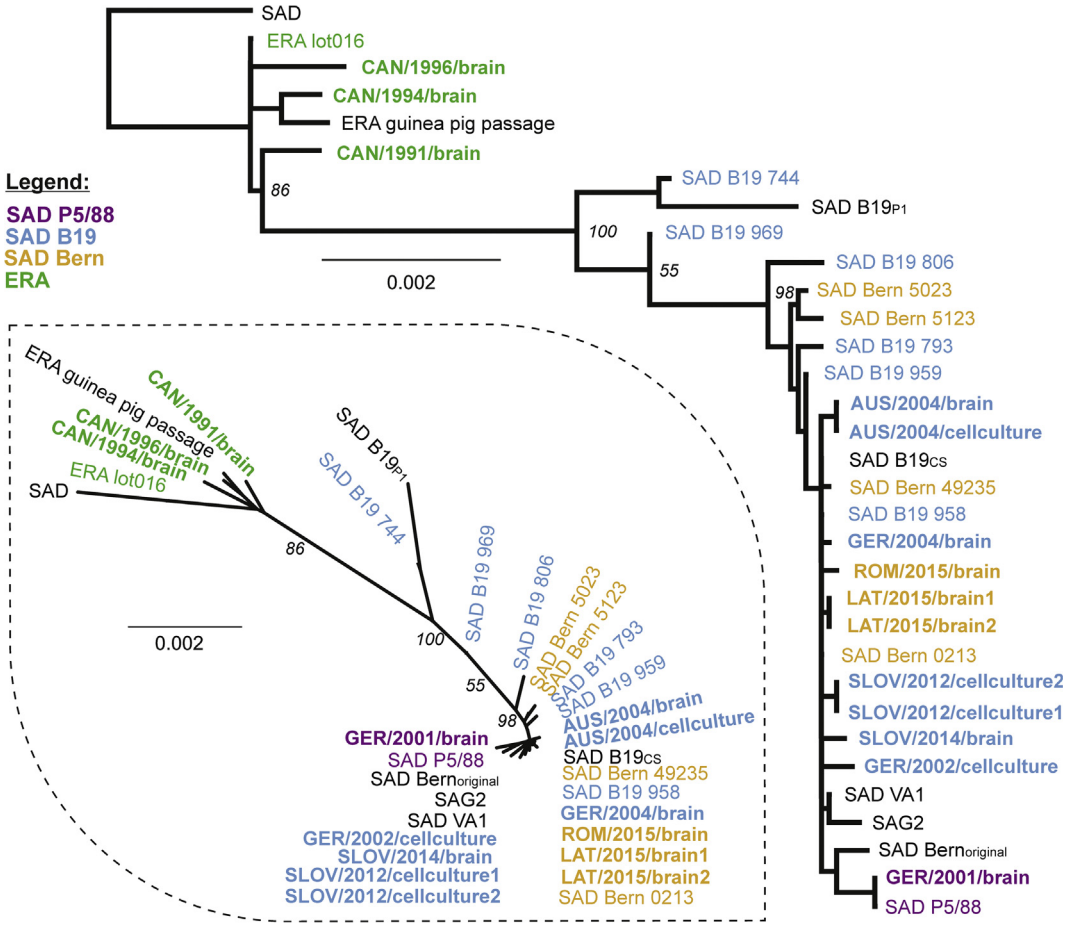

Fig. 3. Maximum-likelihood phylogeny of SAD vaccines and vaccine-derived cases calculated from consensus sequences. The presented tree is rooted at the ancestral SAD strain. Vaccine-derived cases are highlighted in boldface (purple: SAD P5/88, blue: SAD B19, orange: SAD Bern, green: ERA). While SAD $Bern_{original}$-derived and ERA-derived viruses were separated from each other, there is no unambiguous association between SAD B19 and SAD Bern vaccine-derived cases and their respective vaccine batches. The inlay (dotted box) presents an unrooted tree of the same computation. Bootstrap values are indicated for main branches and the scales represent substitutions per position. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[42–45]. It appears that during *in vivo* selection in the infected animals and *in vitro* selection of attenuated SAD strains similar variants were able to pass the respective genetic bottleneck. The observed differences between ERA and SAD induced cases (Fig. 2), i.e. the clustering of SAD induced cases with clonal vaccine strains on the one hand and the clustering of the ERA induced rabies cases with their progenitor virus on the other hand, may be a result of (i) inherent lower overall heterogeneity of the ERA vaccine and (ii) differences between SAD and ERA in the number of replication competent haplotypes. In case there is a higher number of replication competent virus variants present in the vaccine, a random selection of any these variants could result in a vaccine induced rabies case, the sequence of which can shift in any direction in the Manhattan distance analysis. In contrast, if only a single replication competent haplotype is present in the progenitor, then any selection regardless of *in vitro* or *in vivo* will result in the same shift in the Manhattan distance analysis. So, based on this understanding the first situation could apply to ERA while the second would explain the phenomenon seen in SAD $Bern_{original}$-derived rabies cases (Fig. 2). However, to prove this, additional haplotype analysis would be required.

Since vaccine-derived cases of rabies appear to be nearly clonal, we also included full-length sequences from samples with insufficient depth (GER/2001/brain, AUS/2004/brain, GER/2004/brain) for phylogenetic analysis. In the resulting ML-tree (Fig. 3) the ERA-derived viruses were clearly separated from all SAD $Bern_{original}$-derived vaccine cases and vaccines. While the ERA-derived viruses did not form a monophyletic clade, all SAD $Bern_{original}$-derived vaccine cases including those with insufficient depth were located in a relatively uniform clade. The genetic diversity of the ERA-derived viruses suggests that the inherent heterogeneity of the ERA vaccine may be higher than shown (Fig. 2). To prove this assumption, the investigation of additional ERA batches and other ERA vaccines, e.g. ERA333 [47], is necessary. Analogous to the population-based analysis, there is no unambiguous association between vaccine-derived cases and their respective vaccine batches, if the latter comprise high genetic diversity.

3.3. Changes in aa sequences are rare

A number of non-synonymous major and minor variants were detected in the complete population sets comparing vaccine-derived cases and corresponding vaccine strains. Interestingly, the number of nucleotide exchanges at consensus level was higher in the ERA-derived than in the SAD $Bern_{original}$-derived cases. The reasons for this remain elusive and no common SNP pattern was observed. Unique amino acid substitutions that were not present in any of the corresponding vaccine batches were detected in all but the P encoding gene (Supplemental Table S2). As none of them were found in any of the known pathogenicity determining sites as compiled by Eggerbauer et al. [48], it is unlikely that these mutations caused a reversion to a more virulent phenotype, as observed for live-attenuated polio vaccines [49,50]. Moreover, only nearly clonal viral populations were identified within the vaccine-derived cases; hence, it seems unlikely that population diversity was the basis for virulent phenotypes in the analyzed vaccine derived rabies cases. This assumption is supported by experimental studies showing that a virus isolate from a SAD derived case did not induce disease when inoculated in juvenile foxes [27]. Although the residual pathogenic potential of the individual vaccine (highly or less attenuated) does play a more important role, it rather appears that sporadic vaccine-derived cases could still be the result of specific host factors, such as host age, immune status or primary infections and parasitic burden [27]. These circumstances may enable certain variants of the viral population of the attenuated strains to reach and replicate in the CNS of those animals, while under normal conditions the self-limiting and immunogenic infection with these vaccines will be controlled by the host [51]. This, however, cannot be addressed by the presented data but would require additional investigations.

4. Conclusion

This extensive deep sequencing analysis of vaccine-derived rabies cases in the frame of ORV programs provided deeper insights into the effect of accidental *in vivo* replication of genetically highly diverse vaccine strains in the CNS of target and non-target species under field conditions. The results showed that viruses from these cases comprise a viral population with a very limited diversity approaching clonality. This is in sharp contrast to the highly diverse populations observed within their progenitor vaccines. There is clear indication that the viral vaccine populations have passed an *in vivo* bottleneck during infection and only a small number of variants had been selected. Genetically, the selected variants were nearly indistinguishable from potent SAD vaccines. For the latter, an analysis of vaccine-derived rabies cases solely based on sequence data may be misleading as the great majority of currently used live attenuated rabies virus vaccines are genetically nearly identical, since they all originate from a single progenitor [41]. Therefore, an analysis of SAD-derived rabies cases in the frame of ORV monitoring and pharmacovigilance of vaccine products always requires additional epidemiological information.

5. Authors contributions

Drafted the concept and design of the study: MB, CMF, DH, TM, TCM; acquisition of sample material: SND, CMF, CFG, PH, TM, ER, VV, FC; sample preparation for sequencing and sequencing data analysis: FP; coordination of sequencing experiments: DH; interpreted data: CMF, DH, FP, TM; drafted the article and revised it critically for important intellectual content: all; all authors have approved the final manuscript to be submitted.

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Conflict of interest

TM and CMF received funding from IDT Biologika GmbH (Dessau, Germany), a company producing oral rabies vaccines mentioned in this study, for research into mechanisms of oral rabies vaccination and serological response. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.01.083.

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