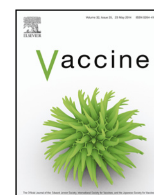
Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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



# High definition viral vaccine strain identity and stability testing using full-genome population data – The next generation of vaccine quality control

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ARTICLE INFO

*Article history:*
Received 9 June 2015
Received in revised form 19 August 2015
Accepted 30 August 2015
Available online xxx

*Keywords:*
Deep sequencing
Viral population analysis
Live-attenuated vaccines
Vaccine quality control
Population distance

ABSTRACT

*Background:* Vaccines are the most effective prophylactic public health tools. With the help of vaccines, prevention of infectious disease spread and, in concert with other measures, even eradication has become possible. Until now, licensing and quality control require the determination of consensus genome sequences of replication competent infectious agents contained in vaccines. Recent improvements in sequencing technologies now enable the sequencing of complete genomes and the genetic analysis of populations with high reliability and resolution. The latter is particularly important for RNA viruses, which consist of fluctuating heterogeneous populations rather than genetically stable entities. This information now has to be integrated into the existing regulatory framework, challenging both licensing authorities and vaccine producers to develop new quality control criteria.
*Methods:* Commercially available modified-live oral rabies vaccines and their precursor strains were deep-sequenced to assess strain identity and relations between strains based on population diversity. Strain relations were inferred based on the Manhattan distances calculated between the compositions of the viral populations of the strains.
*Results:* We provide a novel approach to assess viral strain relations with high resolution and reliability by deep sequencing with subsequent analysis of the overall genetic diversity within the viral populations. A comparison of our novel approach of inferring strain relations based on population data with consensus sequence analysis clearly shows that consensus sequence analysis of diverse viral populations can be misleading. Therefore, for quality control of viral vaccines deep sequencing analysis is to be preferred over consensus sequence analysis.
*Conclusions:* The presented methodology allows for routine integration of deep sequencing data in vaccine quality control and licensing for highly reliable assessment of strain identity and stability.

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## 1. Introduction

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Rabies is one of the most feared zoonotic diseases with tens of thousands of human deaths annually [1]. While canine rabies can be controlled by parenteral application of inactivated rabies vaccines, a breakthrough in the control of wildlife rabies was the development of efficacious oral rabies vaccines. Those live-attenuated rabies vaccines for bait-based oral immunization have been instrumental for the eradication of fox-mediated rabies in Europe and North America [2–4].

Fig. 1 summarizes the ancestry and passaging history of available live-attenuated rabies vaccines as presented in [5,6]. The ancestor strain of all European oral rabies virus vaccines is the “Street Alabama Dufferin” (SAD) field strain, isolated from a rabid dog in 1935, which after adaptation to cell culture was referred to as SAD [7,8]. This SAD field strain was serially passaged in hamster kidney cells, embryonated chicken eggs and pig kidney cells giving rise to the “ERA” strain [7–9], which was efficacious in foxes after oral vaccination [10]. Further adaptation on baby hamster kidney (BHK) cells and thermal stabilization yielded a virus that was

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http://dx.doi.org/10.1016/j.vaccine.2015.08.091
0264-410X/© 2015 Published by Elsevier Ltd.

Please cite this article in press as: Höper D, et al. High definition viral vaccine strain identity and stability testing using full-genome population data – The next generation of vaccine quality control. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.08.091

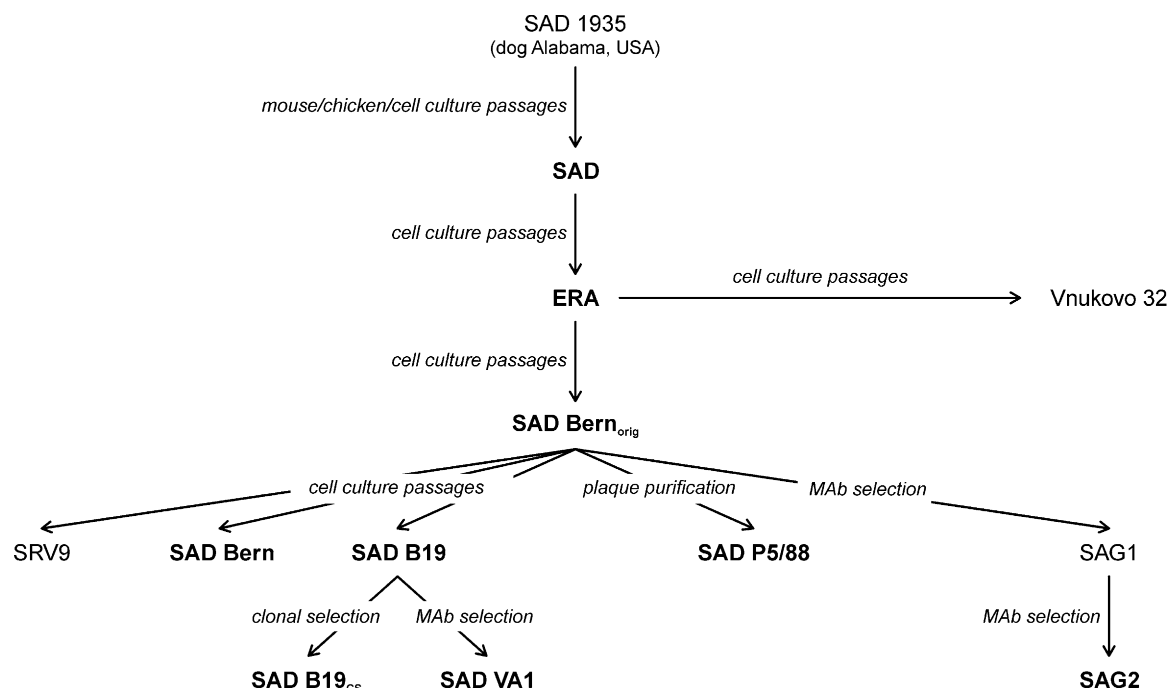


**Fig. 1.** Supposed passage history of SAD derived oral rabies virus vaccines (modified from [5,6]). Vaccine strains investigated in this study are highlighted in bold.

subsequently renamed "SAD Bern" [11], hereinafter named "SAD Bern$_{orig}$". This progenitor virus was transferred to various European laboratories for further developments of oral rabies vaccines for foxes to increase stability and efficacy while reducing residual pathogenicity. Continued passages and adaptation of "SAD Bern$_{orig}$" on original BHK cells, cloned BHK cells and selection after plaque purification on BHK/BSR cells resulted in the first generation commercial vaccines Lysvulpen, Fuchsoral, and Rabifox. According to the licenses, these three vaccines contain "SAD Bern" (Lysvulpen) [12], hereinafter named "SAD Bern", "SAD B19" (Fuchsoral) [13], and "SAD P5/88" (Rabifox) [14], respectively. Subsequent passage of "SAD Bern$_{orig}$" in BHK cells in the presence of monoclonal antibodies (MAb) led to the so-called second generation vaccine virus strains "SAD VA1" [15] and the "SAG" lineage, i.e. "SAG1" and "SAG2". The latter are MAb-escape mutants with alterations in the glycoprotein [16], of which SAG2 was commercialized as Rabigen [17].

The current understanding of ancestry, diversity, and genetic stability of those oral rabies virus vaccines is based either on laboratory documentations, or partial or full-genome consensus sequences obtained via conventional sequencing techniques. Classical whole-genome Sanger sequencing revealed a total of 120 single nucleotide variants in eight SAD derived vaccine strains compared to the common ancestor, differentiating them into SAD Bern and SAD-B19-linked viruses [18]. Since RNA viruses can have the possibility to interact with the host both via genomic RNA secondary structures which may also involve non-coding regions (reviewed for positive-strand RNA viruses in Ref. [19]) and of course by expression of the coded proteins, those differences in genome sequences are of interest for researchers and regulatory authorities alike. Based on the assumption that viruses with identical genomes should have identical properties, genome sequences may be useful for identity and stability testing in vaccine quality control. While phenotypical stability as required by the regulatory authorities has been tested in vivo for most available oral rabies vaccines [20], unfortunately, their genetic stability testing after serial passages has been largely incomplete. The only genetic stability study for an oral rabies virus vaccine (SAD B19) after serial in vivo passaging done so far was performed by classical Sanger full genome sequencing [21]. This enables the determination of the consensus genome sequence representing the majority of viral genomes present in the viral population, but disregards existing minority variants. However, RNA viruses are generally assumed to consist of diverse, fluctuating populations rather than genetically stable entities. Due to the error-prone genome replicases of RNA viruses, with estimated error-rates of approximately $10^{-5}$–$10^{-3}$ mutations per site per replication cycle [22,23], diverse viral populations arise during replication of RNA viruses. These so-called quasispecies (reviewed in [24,25]) are also referred to as "fitness landscape" [26].

Existence of rabies virus quasispecies is long known and studies of these have been conducted using Sanger sequencing [27,28]. However, these studies were limited by the restriction to partial genome sequences and by the low depth that could be achieved. Since the advent of next-generation sequencing (NGS) technologies, a number of protocols have been proposed for RNA virus genome sequencing in general and for rabies viruses in particular [29,30], enabling the determination of viral quasispecies at the full-genome level. This now allows the dissection of the viral population at the single base level. Hence, it is possible to gain insight into the genetic complexity beyond the consensus sequence level. However, while numerous approaches have been proposed to detect single nucleotide variants with ever improving reliability and sensitivity [31–33], there is to date no straightforward methodology described to benefit from population data for enhanced resolution for the detection of strain relations.

A recent publication reporting an unexpected strain modification compared to this consensus sequence in one of the oral rabies virus vaccines marketed in Europe [34] raised the question whether this observation reflected a vaccine strain replacement or fluctuating virus populations. Here we propose a novel and innovative bioinformatics approach for the analysis of deep sequencing data to elucidate phylogenetic relations between viral populations and strain diversity at a high resolution. Moreover, we suggest a feasible procedure to integrate the results in the existing regulatory framework for Marketing Authorization.

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