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Screening for viral extraneous agents in live-attenuated avian vaccines by using a microbial microarray and sequencing

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ABSTRACT

The absence of extraneous agents (EA) in the raw material used for production and in finished products is one of the principal safety elements related to all medicinal products of biological origin, such as live-attenuated vaccines. The aim of this study was to investigate the applicability of the Lawrence Livermore Microbial detection array version 2 (LLMDAv2) combined with whole genome amplification and sequencing for screening for viral EAs in live-attenuated vaccines and specific pathogen-free (SPF) eggs.

We detected positive microarray signals for avian endogenous retrovirus EAV-HP and several viruses belonging to the *Alpharetrovirus* genus in all analyzed vaccines and SPF eggs. We used a microarray probe mapping approach to evaluate the presence of intact retroviral genomes, which in addition to PCR analysis revealed that several of the positive microarray signals were most likely due to cross hybridization with the EAV-HPΔpol and ALV-E ev1, ev3 and ev6 loci sequences originating from the chicken genome.

Sequencing of the vaccines on a MiSeq instrument verified the microarray findings and showed similar cross hybridization. Our results suggest that genomic microarrays and sequencing of avian attenuated vaccines may be applied in tests for EA.

1. Introduction

During the production of live-attenuated vaccines, there is a risk of carry-over of extraneous agents (EA) originating either from the cell cultures, the specific pathogen-free (SPF) eggs or additives used during the production. Viral EAs are defined as any viral agent other than the agent of the virus present on the label of the vaccine. Several cases of viral EAs in animal and human vaccines have previously been reported [1–5]. Endogenous retrovirus (ERVs) are ancient previously infectious exogenous retroviruses that have integrated into germ-line cells [6]. ERVs originate from the biological material used in the production of live-attenuated vaccines and carry an inherent risk of viral contamination. The Crandell-Rees feline kidney cell line used to grow some feline and canine viruses express the feline infectious ERV (RD-114) [7], which has also been identified in vaccines for cats and dogs [8]. In the human live-attenuated vaccines against measles, mumps, rubella (MMR-vaccine) and yellow fever virus (YFV), avian endogenous retrovirus (EAV) and endogenous Avian leukosis virus subgroup E (ALV-E) have been identified. The viruses originated from the chicken embryonic fibroblasts (CEFs) used in the production [9–12]. There has been no evidence of the transmission of the ERV to human recipients or infectivity into susceptible chicken cells [11,13]. SPF eggs are also used in the production of many veterinary and human vaccines. The high-throughput sequencing of chicken embryos and allantoic fluids identified only contigs corresponding to EAV and ALV-E [14]. ALV-Es are also termed ev loci, and at least 50 different ev loci have been identified in chicken DNA, of which approximately half have been characterized [15,16]. Several of these ev loci are structurally complete, transcriptionally expressed and can produce infectious virus particles, e.g., ev2, ev18 and ev19, while others are transcriptionally silent or structurally defective, e.g., ev3 and ev6, which have deletions in the *gag-pol* or the LTR-*pol* region [17,18]. Evidence suggests that ALV-J emerged from a recombination event between EAV *env* sequences and exogenous ALVs [19].

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A regulatory framework describes procedures and quality control testing of biological products through all production steps to assure that final products are safe and do not contain infectious extraneous agents (EA). A part of the regulations describes that the use of animal models for screening for infectious EA and consequently animals are widely used for this purpose. Since the mid-eighties, great progress in implementing the 3R principles (replacement, reduction and refinement) has been achieved, and the regulatory frameworks are continuously revised for further improvements to make it possible to exploit newer technologies that are in accordance with 3R.

Traditionally, real-time and PriProET PCR approaches have shown potential for EA nucleic acids (NA) screening [20–22], but these PCR-based methods are often only able to screen for one or a few EA targets at a time. Thus, screening for all potential EA viruses using these techniques in live-attenuated avian vaccine may not be feasible, and furthermore, unforeseen EAs would not be found [23]. The Livermore Microbial detection array contains probes corresponding to all sequenced viruses and bacteria [24]. This microbial array has previously been used as a tool to screen for extraneous agents in human live-attenuated vaccines [5], and porcine circovirus-1(PCV1) DNA was identified in a rotavirus vaccine. This contamination was not detected using established tests, emphasizing the benefit of introducing still more sensitive and broader tests for solving issues related to EA.

The aim of this study was to evaluate the use of the of the Lawrence Livermore Microbial detection array version 2 (LLMDAv2) [24] combined with whole-genome amplification [25,26] and sequencing for the unbiased screening of viral EAs in live-attenuated avian vaccines and in SPF eggs.

2. Materials and methods

2.1. Vaccines and allantoic fluid from SPF eggs

The avian vaccines investigated in this study are the vaccines routinely used to prevent avian infections with infectious bursal disease virus (IBDV), avian encephalomyelitis virus (AEV), infectious bronchitis virus (IBV), chicken infectious anemia virus (CIAV) and Newcastle disease virus (NDV). The vaccines were purchased in 2012–13 and assigned an anonymized code defined by the letters AV (avian vaccine) and a chronological number (e.g., AV-1, AV-2, etc.). The live-attenuated virus components of the vaccines are listed in Table 1. Allantoic fluid from three non-inoculated SPF eggs was also analyzed. The SPF eggs were all from the same flock but not from the same batch used to produce the various vaccines investigated in this study. For simplicity, allantoic fluid from non-inoculated SPF eggs is referred to as SPF eggs throughout the remainder of the text. The Diphtheria vaccine, containing inactivated diphtheria toxoid, was used as a negative control.

2.2. Pretreatment and nucleic acid purification

The freeze-dried avian vaccines were resolved in nuclease-free water to a concentration of 10 doses pr. 200 µl. The diphtheria vaccine was diluted to a concentration of 0.5 dose pr. 200 µl. Allantoic fluid from SPF eggs was not diluted. A volume of 230 µl sample material was centrifuged at 13,200 rpm for 10 min, and supernatant was passed through 0.22 µm Corning® Costar® Spin-X® centrifuge tube filters (Sigma Aldrich, CLS8169-200EA). Viral NA was extracted from either nuclease-digested vaccines or undigested samples. Nuclease treatment was performed with a combination of 5 U DNase I (NEB) and 1 U RiboShredder™ RNase Blend (Epicentre, Tebu-bio, RS12500) according to the manufacturer's protocol. Viral NA was extracted using the PureLink® Viral RNA/DNA mini kit (Invitrogen, ThermoFisher Scientific, 12,280,050) according to the manufacturer's instruction with the modification of no carrier RNA in the lysis buffer. Undigested samples used for PCR or NGS analysis were subjected to DNase/RNase treatment after NA purification using the Invitrogen™ Ambion™ RNase Cocktail Enzyme Mix (Invitrogen, ThermoFisher Scientific, AM2286) or Invitrogen™ DNase I (Invitrogen, ThermoFisher Scientific, 18,047,019) according to the manufacturer's protocol.

2.3. LLMDAv2 microarray analysis

cDNA synthesis was performed with SuperScript III Reverse Transcriptase (Invitrogen, ThermoFisher Scientific, 180,800,93) and 5′-phosphorylated random hexamer (Hex-P) primers as previously described (24). cDNA and DNA was amplified at 30 °C for 2 h using the QuantiTect® Whole Transcriptome kit (Qiagen, 207043) and purified using QIAamp® DNA mini kit (Qiagen, 51304). Prior to hybridization on the microarray, the amplified DNA was labeled with Cy3 fluorescent dye using the One-Color DNA labeling kit (Nimblegen, Roche). Eight micrograms of Cy3 labeled DNA was loaded on the LLMDAv2 and allowed to hybridize for 16–20 h at 42 °C. The array was washed and scanned according to the manufacturer's protocol. The obtained data were analyzed with the previously described Excel-based analysis

Table 1
Microarray detection of NA from vaccine virus and NA from retrovirus.

Vaccine/sample[a]	Vaccine type	Vaccine virus[b]	Positive signal for viral NA in untreated vaccines[c]	Positive signal for viral NA in treated vaccines[d]
AV-1	Live-attenuated	**IBDV**	**IBDV**, EAV-HP	**IBDV**
AV-2	Live-attenuated	**IBDV**	**IBDV**, EAV-HP	**IBDV**
AV-3	Live-attenuated	**IBDV**	**IBDV**, EAV-HP, ALV, RSV, AMV	**IBDV**, EAV-HP
AV-4	Live-attenuated	**IBDV**	**IBDV**, EAV-HP, ALV, RSV, FSV, AMV	**IBDV**, EAV-HP
AV-5	Live-attenuated	**AEV**	**AEV**, EAV-HP, ALV, RSV	**AEV**, EAV-HP
AV-6	Live-attenuated	**IBV**	**IBV**, EAV-HP	–
AV-7	Live-attenuated	**IBV**	**IBV**, EAV-HP, ALV, RSV	**IBV**, EAV-HP, ALV, RSV, FSV, AMV
AV-8	Live-attenuated	**CIAV**	**CIAV**, EAV-HP, ALV, RSV	**CIAV**, EAV-HP, ALV, RSV
AV-9	Live-attenuated	**NDV**	**NDV**, EAV-HP, ALV, RSV, AMV	**NDV**
AV-10	Inactivated	**NDV**	**NDV**, EAV-HP	–
SPF-1	–	–	EAV-HP, ALV, RSV, AMV, FSV	EAV-HP, ALV, RSV, AMV, FSV
SPF-2	–	–	EAV-HP, ALV, RSV, AMV, FSV	–
SPF-3	–	–	EAV-HP, ALV, RSV, AMV, FSV	EAV-HP, ALV, RSV, AMV, FSV
Neg. ctrl	Toxoid	–	–	

[a] Vaccines were anonymized by the abbreviation AV (avian vaccine) and a chronological number. SPF; Allantoic fluid from non-inoculated SPF eggs. The Diphtheria vaccine, containing diphtheria toxoid, was used as a negative control.

[b] Vaccine virus IBDV, infectious bursal disease virus; AEV, avian encephalomyelitis virus; IBV, infectious bronchitis virus; CIAV, chicken infectious anemia virus; NDV, Newcastle disease virus.

[c] Detection of viral NA in untreated vaccines. Bold font represents the vaccine virus, and normal font represents viral EAs.

[d] Detection of viral NA in nuclease-treated vaccines. Bold font represents the vaccine virus, and normal font represents viral EAs. EAV-HP, avian endogenous retrovirus; ALV, avian leukosis virus; RSV, Rous sarcoma virus; FSV, Fujinami sarcoma virus; AMV, avian myelocytomatosis virus; and AMV, avian myelocytomatosis virus.