Chicken anemia virus and avian gyrovirus 2 as contaminants in poultry vaccines

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1. Introduction

Chicken anemia virus (CAV) and avian gyrovirus type 2 (AGV2) are members of the family Circoviridae, genus Gyrovirus [1,2]. CAV is the causative agent of chicken infectious anemia, an important avian disease characterized by anemia, immunosuppression and mortality in young chickens [3–5]. The virus is widely distributed in virtually all countries with significant poultry production [1]. Regarding AGV2, it was initially discovered by our group in 2008 in chickens [2]. Infections with AGV2 have been identified in several different locations in Brazil and Netherlands, suggesting a worldwide distribution [6].

Nevertheless, to date, no evidence of AGV2 causing disease has been produced.

Since the first isolation of CAV from contaminated vaccines in Japan in 1979 [7], its role as a vaccine contaminant has been investigated [8–10]. The introduction of CAV into vaccines usually takes place vertically by infected embryonated hen’s eggs. Improved vaccine production practices, such as the use of CAV-free specific pathogen-free (SPF) eggs did not eliminate the possibility of vaccine contamination [11–13]. As AGV2 bears significant similarity with CAV and it is possibly transmitted by similar routes, it might be expected that this virus could also potentially infect vaccines produced in embryonated eggs. However, as AGV2 has only recently been discovered, no studies have been performed to examine such possibility.

In view of the potential role for vaccines in the transmission and dissemination of extraneous pathogens—particularly relevant when most poultry vaccines are based on live attenuated or modified
pathogens—this study was set up in order to detect CAV and AGV2 genomes in commercially used vaccines. With this aim, a duplex quantitative real-time PCR (dqPCR), capable of detecting the genomes of both viruses in a single assay, was employed [14]. The specificity of the amplified products was confirmed by cloning and sequencing.

2. Materials and methods

2.1. Vaccine samples

Thirty-five commercially available vaccines, 32 prepared with live and 3 with inactivated microorganisms, were purchased commercially or obtained by donation from eight different laboratories from different countries (1 from United States of America, 2 from Hungary, 1 from Canada, 1 from Netherlands, 2 from Germany and 28 from Brazil). All vaccines are largely employed in poultry farming and are aimed to immunize birds against several pathogens. A list with all vaccines evaluated in the present study is provided on Table 1.

2.2. DNA extraction

Total DNA was extracted from 500 μl of each of the vaccine suspensions using the PureLink™ Genomic DNA Mini Kit (Invitrogen), following the manufacturer’s instructions. The DNA extracted from the vaccines was quantified with a fluorometer (Qubit® 2.0; Life technologies) and it diluted up to 17 ng/μl of DNA. All samples were stored at −20 °C until testing.

2.3. Duplex quantitative real time PCR (dqPCR)

The DNA extracted from the vaccines under test was evaluated by dqPCR as previously described [14]. Two plasmids were used as positive controls and to generate the dqPCR standard curves: a plasmid containing the CAV complete genome (pcR2.1CAV) [14] and a second plasmid containing the full AGV2 genome (pcR2.1AGV2) [2]. The CAV- and AGV2-specific primers [CAV forward: 5’- CCAGCTTGCCGCTATCTAT-3’; CAV reverse: 5’-CACGGCAAAGACTAATAG-3’; AGV2 forward: 5’-CACCCGCGAAAGACATTAATG-3’; AGV2 reverse: 5’-TATCT-GAGTCTGGTTCCTGCTG-3’] and probes (CAV probe: 5’-JOE-93 ACAGCGCAAGGACACATBHQ1-3’; and AGV2 probe: 5’-FAM-97 CGCTCTGGCCAAGGCACAC-BHQ1-3’) used in the assay were previously described [14]. The standard curves constructed with the plasmids mentioned above were used to estimate the number of genome copies in each vaccine sample. The assay was capable of detecting at least 5 copies of CAV genomes. The minimum sensitivity for AGV2 genomes was previously determined and consisted of 50 copies of genome per assay.

The determination of CAV and AGV2 genome loads in DNA extracted from vaccines was performed in total volumes of 12.5 μl using 50 ng of DNA extracted from vaccines, 6.25 μl of 2 × Platinum® quantitative PCR SuperMix-UDG (Invitrogen - Life Technologies), 200 nM of each forward and reverse primers (IDT) and 5 μM of each probe. A final concentration of 3 mM MgCl2 was used to each reaction. Amplification and detection were performed in a StepOne™ Real-Time PCR system (Life Technologies) under the following conditions: uracil DNA glycosylase (UDG) incubation at 50 °C for 2 min; initial denaturation and Platinum® Taq activation at 95 °C for 2 min, followed by 40 cycles of amplification (15 s at 95 °C and 30 s at 60 °C). All real-time assays were performed in triplicate. The results presented are the averages of such triplicates. Fluorescent measurements were carried out during the elongation step. From each amplification plot, a threshold cycle (Ct) value was calculated representing the PCR cycle number in which the reporter dye fluorescence was detectable above an arbitrary threshold. Data analysis was performed with the StepOne software v2.2.2. All vaccine samples that were found to contain CAV or AGV2 genomes were submitted to fresh DNA extractions and were re-tested in triplicate by dqPCR to confirm the results obtained.

Standard precautions were taken to avoid the risk of cross-contamination; each step of the handling was carried out in different rooms. In addition, different displacement micropipettes and filter tips were used throughout.

2.4. Amplification by conventional PCR

DNA samples from vaccines which were found to contain CAV and/or AGV2 DNA were submitted to amplification by conventional PCR with the same primers used in the dqPCR. The amplification was performed in an Eppendorf Master Cycler apparatus, in reaction mixtures containing 50 ng of vaccine DNA extract, 4 μl of 5× Phusion HF Buffer, 5 pmol of each primer, 1 U Phusion High-Fidelity

Table 1

<table>
<thead>
<tr>
<th>Manufacture</th>
<th>Vaccine (agent)</th>
<th>Vaccine (status)</th>
<th>Country of production</th>
<th>Viral genome load</th>
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<tr>
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<td>LV</td>
<td>Brazil</td>
<td>–</td>
</tr>
<tr>
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<td>AEV</td>
<td>LV</td>
<td>Brazil</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
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<td>LV</td>
<td>Brazil</td>
<td>–</td>
</tr>
<tr>
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CAV - Chicken anemia virus; IBV - Infectious bronchitis virus; IBDV - Infectious bursal disease virus; FPV - Fowlpox virus; AEV - Avian encephalomyelitis virus; NDV - Newcastle disease virus; TRTV/APV - Turkey rhinotracheitis virus and Avian pneumovirus; MDV - Marek’s disease virus (CVI-988/Respirs or HVT); IC - Infectious coryza; ACV - Avian coccidiosis; ILTV - Infectious laryngotracheitis virus; EDS - Egg drop syndrome.

b LV - live virus; OLV - oil adjuvanted and inactivated; IV - inactivated virus.

d Data presented are the averages of the results obtained by duplex quantitative real time PCR (dqPCR) performed in triplicate and expressed as number of CAV or AGV2 genome copies per 50 ng of vaccine DNA.
DNA polymerase (Thermo Scientific), 0.8 mM each dNTP and sterilized Milli-Q water q.s.p. 20 μL. The temperature cycling conditions consisted of 1 cycle at 98 °C for 3 min followed by 35 cycles of 98 °C for 15 s, 57 °C for 15 s and 72 °C for 15 s, plus a final extension step at 72 °C for 3 min. Five microliters of the PCR products were separated in 2% agarose gels, stained with ethidium bromide and photographed on an UV transilluminator. The specific bands were identified in comparison with a DNA ladder (Thermo Scientific GeneRuler 50 bp DNA Ladder).

2.5. Cloning and sequencing

All amplifications products obtained by conventional PCR were cloned into plasmid pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Scientific) following the manufacturer’s protocol. At least three recombinant plasmids of each reaction were sequenced on both strands using pJET1.2-forward and pJET1.2-reverse oligonucleotides as primers in a MegaBACE 500 apparatus with the Dyenamic ET terminator cycle sequencing kit (Amersham Biosciences).

2.6. Analysis of sequence data

Viral DNA sequences were initially evaluated by BLAST and subsequently aligned using the programs MEGA 5 [15] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) programs [16]. Previously published CAV and AGV2 sequences available at GenBank were used as references (AB031296.1 for CAV and JQ690763.1; HM590588.1; JQ308212.1; FR823283.1 for AGV2).

3. Results

3.1. dqPCR analysis

The results of the dqPCR performed in this study are summarized in Table 1. As expected, CAV genomes were detected in the three vaccines aimed to immunize for CAV (manufactured by laboratories B, G and H; Table 1) albeit with highly variable viral genome loads (copy numbers ranging from 2386 to 2175.381/50 ng; equivalent to 32 to 27,192.263 genome copies per dose of vaccine). As contaminants, CAV genomes were detected in vaccines from one producer laboratory only; six of those were live vaccines (Table 1, manufacturer D). In total, CAV viral genomes were detected in 7 out of the 35 vaccines tested (Table 1). The vaccines where CAV genomes were identified included two vaccines to Marek’s disease, two vaccines to infectious bronchitis, one vaccine to fowlpox, one vaccine to egg drop syndrome (EDS-76) and one vaccine to Newcastle disease. In such vaccines, CAV genome loads ranged from 6.4 to 173.7 copies per 50 ng of vaccine DNA, equivalent to 0.07 to 0.69 genome copies per dose of vaccine, respectively.

Regarding AGV2, such genomes were detected in nine vaccines manufactured by three laboratories (A, D and E; Table 1). These included one vaccine to fowlpox (manufacturer A), two vaccines to Newcastle disease (manufacturers D and E), one vaccine to infectious laryngotracheitis (manufacturer E), one vaccine to infectious bronchitis and four vaccines to Marek’s disease (manufacturer D). Among the Marek’s disease vaccines, two were prepared with serotype 1 (CVI988/Rispens) and two with serotype 3 (turkey herpesvirus – HVT) viruses. The detectable levels of AGV2 DNA varied from 93 to 156,187 genome copies per 50 ng of vaccine DNA (equivalent to 0.28–9176 genome copies per dose of vaccine). All vaccines that contained AGV2 DNA were live vaccines.

In addition, four of the 35 examined vaccines contained DNA of both CAV and AGV2 (infectious bronchitis, Newcastle disease and Marek’s disease vaccines of two serotypes; Table 1). All these vaccines were live vaccines and all were produced by a same manufacturer (Table 1).

3.2. Sequence analyses

At least three recombinant plasmids, containing either CAV or AGV2 DNA, amplified from vaccine DNA, were sequenced on both strands. These were compared with similar sequences available at GenBank (as detailed in methods). All sequences displayed a high degree of similarity to equivalent regions of CAV or AGV2 genomes (Fig. 1) and they confirmed the specificity of the results obtained at dqPCR.

In the putative CAV sequences amplified from the vaccines DNA (Fig. 1, A/B), two nucleotide changes occurred at positions 849 (G/A) and 860 (G/C), some of them (CAV vaccines D; F; J; O; U) resulting in a discrete aminoacid change (S/T). In sequenced clones from putative AGV2 genomes amplified from the vaccines, (Fig. 1, C/D) nucleotide changes were identified at positions 153 (A/G) and 168 (G/A). However, these did not reflect in aminoacid substitutions.

4. Discussion

The present study aimed to identify genomes of CAV and AGV2 in commercially available avian vaccines. The findings obtained revealed that genomes of both agents may be detected in poultry vaccines; the specificity of the detection method was confirmed by sequence analysis. These results indicate the need to implement appropriate quality control measures to ensure absence of contamination of vaccines with such agents. Of eight manufacturer laboratories from which vaccines were tested, three had one or more vaccines that contained DNA of either CAV or AGV2, or both. One of the laboratories (manufacturer D) had the highest number of contaminated vaccines among those tested; besides, this was the only manufacturer whose vaccines were found to contain CAV genomes, besides AGV2 genomes. However, this was the manufacturer from which the greatest number of vaccines was examined; as the choice of vaccines was based essentially on the availability of the products for testing, these proportions may have been biased by the fact that 20 out of the 35 vaccines tested were from the same laboratory. Nevertheless, it points out that this particular manufacturer seems to have a problem of CAV contamination along its vaccine production line. Regardless, these results do highlight the need for improving quality control practices to ensure the absence of such contaminants in all vaccines, particularly those where embryo-nated eggs are used as substrate for vaccine production, since these seem to be the main source of this type of contamination.

In the search for CAV, as expected, the vaccines meant to have CAV as immunogens did in fact contain CAV genomes, though with a considerable variation in the numbers of genome copies detected. One can speculate on how such variation might influence the vaccine’s potential to induce protection. However, the main concern of the present report was the detection of CAV (and, as discussed below, AGV2) as contaminant. So, in this study, CAV genome was detected in 7 vaccines, all from the same laboratory. Contamination of vaccines with CAV has been known since 1979 [7], when the agent was first described. In Brazil, a retrospective study with commercial vaccines detected CAV contamination in vaccine batches from some laboratories until 1997, but not in 13 batches of distinct vaccines produced by five distinct laboratories from 2001 onwards [8]. However, the results obtained in the present study demonstrate the presence of CAV genomes in recently produces vaccines, substantiating the need for improved control measures to avoid CAV contamination. Although in this study only
viral genomes were searched for, it is very likely that infectious virus might be present in such vaccines, as most of them were prepared with live microorganisms. This opens the possibility of potentially infectious virus to be present in the end product. It has been suggested that contamination of live vaccines with CAV may have influenced the epidemiology of chicken anemia in Brazilian and Argentinian poultry flocks [9]. In Brazil, a high prevalence of antibodies to CAV (89%) was detected in all States with intensive commercial broiler flock production [17]. Although this hypothesis might give rise to debate, it is clear that the presence of unwanted CAV might potentially introduce the virus and/or disease in flocks vaccinated with contaminated immunogens. In addition, it may lead to misleading attempts to trace the origin and evolution of the virus’ apparently wide distribution in chicken populations. As stated above, CAV can be transmitted vertically [18]; vertical transmission of AGV2 has not yet been demonstrated, but AGV2 genomes were detected in embryonic culture cells obtained from either by ingestion or handling of poultry and related products; yet it may be possible that such introduction — if it indeed happened — might have taken place by the administration of AGV2-contaminated vaccines to humans. Clearly, though, such hypothesis would require further investigation on additional vaccines, particularly on modified live attenuated vaccines for human use prepared in embryonated eggs.

The search for contaminants in vaccines was initially based on traditional techniques such as detection of antibodies or virus isolation [23]. In the course of time, genome detection technology was introduced with advantages over classical methods. Such advantages include the speed in which results can be obtained, improved accuracy and sensitivity [24]. Additionally, the possibility of detection of different contaminants in a same reaction as well as to quantify such contaminants has opened new perspectives for vaccine quality control. Genome detection technology, however, does not detect infectious virus; therefore, the results obtained here must be interpreted with caution, as must be cautious extrapolations. Nevertheless, the sole presence of extraneous viral genomes in such biological products provides in itself substantial findings highlight the need of including the search for CAV and AGV2 in quality control testing by manufacturer laboratories, not only to improve the quality of the immunogens, but also to avoid unwanted side effects.

**Contributors**

Ana Paula Muterle Varela participated in all stages of the study. Helton Fernandes dos Santos participated in execution of the work. Samuel Paulo Cibulski participated in execution of the work. Camila Mengue Scheffer participated in execution of the work. Candice Schmidt participated in drafting of the paper.
Francisco Esmaile Sales Lima participated in drafting and review of the paper.
Alessandra D’Avila Silva participated in data interpretation.
Paulo Augusto Esteves participated in analysis and interpretation of data, drafting and review of the paper.
Ana Cláudia Franco participated in review of the paper.
Paulo Michel Roehe provided intellectual contribution to the study and participated in the review of the paper.

Conflict of interest

All authors contributed to the study, have approved the final version of the manuscript and declare that no competing interests exist.

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References