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

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ABSTRACT

This study focuses on the detection of chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) genomes in commercially available poultry vaccines. A duplex quantitative real-time PCR (dqPCR), capable of identifying genomes of both viruses in a single assay, was employed to determine the viral loads of these agents in commercially available vaccines. Thirty five vaccines from eight manufacturers (32 prepared with live and 3 with inactivated microorganisms) were examined. Genomes of CAV were detected as contaminants in 6/32 live vaccines and in 1/3 inactivated vaccines. The CAV genome loads ranged from 6.4 to 173.4 per 50 ng of vaccine DNA (equivalent to 0.07 to 0.69 genome copies per dose of vaccine). Likewise, AGV2 genomes were detected in 9/32 live vaccines, with viral loads ranging from 93 to 156,187 per 50 ng of vaccine DNA (equivalent to 0.28–9176 genome copies per dose of vaccine). These findings provide evidence for the possibility of contamination of poultry vaccines with CAV and AGV2 and they also emphasize the need of searching for these agents in vaccines in order to ensure the absence of such potential contaminants.

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 incidentally discovered by our group during a diagnostic search for CAV 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

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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′— CCAGCTTGCGTGCTATTCAT-3′; CAV reverse: 5′-CGAGCAACAGTACCCTGCTA-3′; AGV2 forward: 5′-CACGGGCAAGACACTAAATG-3′; AGV2 reverse: 5′- TATCGAGGTCGTTTCTGCTG-3′) and probes (CAV probe: 5′-JOE-93 ACAGCGCAAGGCACGCAAGT-BHQ-1-3′; and AGV2 probe: 5′-FAM - 97 CGCTCTCGCCGACAAGCAAC-BHQ-1-3′) 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 sampled. 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 $MgCl_2$ 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.

Table 1
Chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) genome loads in poultry vaccines.

Manufacture	Vaccine (agent)[a]	Vaccine (status)[b]	Country of production	Viral genome load[c]	
				AGV2	CAV
A	FPV	LV	Brazil	121	–
B	CAV	LV	United States of America	–	2,175381
B	IBV	LV	Hungary	–	–
B	IBV	LV	Hungary	–	–
C	IBV	LV	Brazil	–	–
C	AEV	LV	Brazil	–	–
C	NDV	LV	Brazil	–	–
C	TRTV/APV	LV	Brazil	–	–
C	TRTV/APV	LV	Brazil	–	–
D	IBV	LV	Brazil	–	103.2
D	IBV	LV	Brazil	2392	173.7
D	IBDV	LV	Brazil	–	–
D	IBDV	LV	Brazil	–	–
D	IBDV	LV	Brazil	–	–
D	FPV	LV	Brazil	–	18
D	FPV	LV	Brazil	–	–
D	NDV	LV	Brazil	–	–
D	NDV	LV	Brazil	–	–
D	NDV	LV	Brazil	93	8.7
D	MDV (HVT)	LV	Brazil	–	–
D	MDV (HVT)	LV	Brazil	1127	27
D	MDV (HVT)	LV	Brazil	156,187	–
D	MDV (HVT)	LV	Brazil	–	–
D	MDV (Rispens)	LV	Brazil	721	7
D	MDV (Rispens)	LV	Brazil	462	–
D	ACV	LV	Brazil	–	–
D	IC	IV	Brazil	–	–
D	EDS	OIV	Brazil	–	6.4
D	NDV/IBV/IBDV	OIV	Brazil	–	–
E	IBDV	LV	Brazil	–	–
E	ILTV	LV	Canada	27,418	–
E	NDV	LV	Netherlands	178	–
F	IBV	LV	Brazil	–	–
G	CAV	LV	Germany	–	54,238
H	CAV	LV	Germany	–	2386

[a] 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/Rispens or HVT); IC - Infectious coryza; ACV - Avian coccidiosis; ILTV - Infectious laryngotracheitis virus; EDS - Egg drop syndrome.
[b] LV - live virus; OIV - oil adjuvanted and inactivated; IV - inactivated virus.
[c] 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.

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