Journal of Virological Methods 183 (2012) 147–153

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E)

Ayse Günes, Ana Marek, Beatrice Grafl, Evelyn Berger, Michael Hess*

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria

Article history:
Received 20 December 2011
Received in revised form 3 April 2012
Accepted 18 April 2012
Available online 25 April 2012

Keywords:
Fowl adenoviruses
Real-time PCR
52K gene
Sensitivity
Virus shedding
Quantitation

A B S T R A C T

The present study describes the development of a SYBR Green based real-time polymerase chain reaction (real-time PCR) for detection and quantitation of all fowl adenovirus (FAdV) species. Primers were designed based on conserved nucleotide sequences within the 52K gene. Ten-fold serial dilutions of a vector DNA were used as standard for quantitation. The real-time PCR had an efficiency of 98%, a regression squared value of 0.999 and showed a range of 6.73–6.73 $\times 10^8$ copies of FAdV DNA per reaction. The assay was highly specific for FAdVs and an exact quantitation of all 5 FAdV species (FAdV-A to FAdV-E) could be demonstrated. It was shown, that twelve FAdV serotypes (FAdV-1 to 8a, and 8b to 11) were detectable and quantifiable. Other viral genomes as well as uninfected chicken embryo liver (CEL) cells did not produce positive signal. Cloacal swabs were taken during the animal experiment, which was performed with all FAdV species. Shedding of FAdVs was investigated in cell culture, by conventional PCR and by the developed real-time PCR. The real-time PCR was found more sensitive than cell culture and conventional PCR. Detection and quantitation of FAdVs in different type of samples was possible by the new real-time PCR.

1. Introduction

Fowl adenoviruses (FAdVs) belong to the family *Adenoviridae* and genus *Aviadenovirus*, which contains the worldwide distributed adenoviruses of chickens. Some strains are the causative agents of inclusion body hepatitis, hepatitis-hydropericardium syndrome and gizzard erosion, beside other less well defined disease conditions. FAdVs are transmitted horizontally as well as vertically. The virus could be transmitted in all excretions, yet the highest titers are found in feces (Adair and Fitzgerald, 2008). FAdVs have been isolated from healthy and diseased domestic fowl, and from other avian species (McFerran et al., 1972, 1976).

FAdVs are grouped into five different species (FAdV-A to FAdV-E) due to their molecular structure (Benkö et al., 2005), and additionally into twelve serotypes (FAdV-1 to 8a, and 8b to 11) as a result of cross-neutralization tests (Hess, 2000). Recently, at least twelve genotypes were identified within the five FAdV species based on the hexon gene sequences (Marek et al., 2010). The genome of non-enveloped FAdVs is a linear, double-stranded DNA molecule of approximately 45 kb (Chiocca et al., 1996; Ojkic and Nagy, 2000; Grgić et al., 2011; Griffin and Nagy, 2011; Marek et al., 2012).

FAdVs can be diagnosed by electron microscopy (McFerran, 1998), while histological examination was used to demonstrate typical intranuclear inclusions by inclusion body hepatitis with hematoxylin and eosin staining of tissues (Gallina et al., 1973). Many conventional PCR methods for detection of aviadenoviruses have been reported using different primer sets, which can amplify the different regions of the hexon gene (Raue and Hess, 1998; Meulemans et al., 2001; Raue et al., 2005a,b; Mase et al., 2009). In addition, different PCRs targeting other regions of the genome were developed (Hanson et al., 2006; Ivanics et al., 2010; Kajan et al., 2011). The virus is isolated in embryonated eggs or cell culture. Afterwards, PCR combined with restriction enzyme analysis or nucleotide sequencing can be used to determine the genotype (Raue and Hess, 1998; Meulemans et al., 2001).

Abbreviations: BLAST, basic local alignment search tool; bp, base pair; CEL cells, chicken embryo liver cells; CELO, chicken embryo lethal orphan; CPE, cytopathic effect; C_T, threshold cycle; d.p.i., days post-infection; FAdV, Fowl adenovirus; FAdV-A to FAdV-E, species *Fowl adenovirus A* to *Fowl adenovirus E*; FAdV-1 to 8a and 8b to 11, Fowl adenovirus serotypes 1 to 8a and 8b to 11; i.e., id est; L1, loop 1; min, minute; PBS, phosphate buffered saline; PCR, polymerase chain reaction; real-time PCR, real-time polymerase chain reaction; SPF, specific pathogen-free; TCID, tissue culture infective dose; T_m, melting temperature.

* Corresponding author at: Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinaerplatz 1, A-1210 Vienna, Austria. Tel.: +43 125075150; fax: +43 1250775192.

E-mail addresses: ayse.guenes@vetmeduni.ac.at (A. Günes), ana.marek@vetmeduni.ac.at (A. Marek), beatrice.grafl@vetmeduni.ac.at (B. Grafl), evelyn.schulz@vetmeduni.ac.at (E. Berger), michael.hess@vetmeduni.ac.at (M. Hess).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.04.005

Virus isolation requires special facilities and skilled personnel, as well as fresh specimens with viable viruses. Sometimes several passages must be done for isolating a FAdV in chicken embryo liver (CEL) cell culture (Hess et al., 1998). All described detection methods need a certain amount of time, especially titration in tissue culture is somewhat laborious.

Therefore, the aim of this study was to develop a specific and sensitive SYBR Green based real-time PCR assay for detection and quantitation of five FAdV species. The shedding of FAdVs by experimentally infected specific pathogen-free (SPF) chickens was detected and quantified from cloacal swab samples. The sensitivity of the real-time PCR was compared with virus isolation and conventional PCR assay, as well its specificity was tested on samples of other avian viral pathogens and uninfected CEL cells.

2. Materials and methods

2.1. Viruses

CEL cells were used to propagate the following FAdV strains: FAdV-A (CELO), FAdV-B (340), FAdV-C (KR5, C2B), FAdV-D (SR48, SR49, A2-A, UF71) and FAdV-E (CR119, YR36, TR59, Hungary6, 764) representing all 12 FAdV serotypes. When a cytopathic effect (CPE) was visible, the cells were harvested after three freeze–thaw cycles in a Falcon tube following 10 min centrifugation at $700 \times g$. The supernatants were collected and used for DNA extraction and inoculation of chickens. The titer of FAdV strains used for inoculation of chickens was determined according to the method of end point titration (Reed and Muench, 1938).

2.2. DNA extraction

Viral DNA was isolated from cloacal swabs, tissue homogenates of liver or intestine taken from infected SPF chickens or supernatants of cell culture using a DNeasy Blood and Tissue Kit (Qiagen, Vienna, Austria) according to manufacturer's instructions. DNA was stored at −20 °C until use.

2.3. Primers

Primers used in this study are listed in Table 1. Primer pair Hexon A/Hexon B (Meulemans et al., 2001) was utilized for the conventional PCR.

Primer sets 52K-F/52K-R (for conventional PCR) and 52K-fw/52K-rv (for real-time PCR) were designed in this study. In order to design these primers, the complete genome sequences of FAdVs (GenBank accession numbers U46933, AF083975, GU734104, GU188428 and HE608152) were aligned using the software Accelrys Gene, version 2.5 (Accelrys, San Diego, CA). Primers 52K-F and 52K-R were designed to anneal within the 52K and pIIIa genes, respectively. Additionally, PCR products of thirteen FAdV strains representing all 12 FAdV serotypes were sequenced and aligned to design the primer pair 52K-fw/52K-rv for the real-time PCR, which annealed within the highly conserved 52K region (Supplemental Material S.1).

The specificity of these primers was tested in a BLAST search. All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

2.4. Conventional PCR

Conventional PCR was performed using primer pair Hexon A/Hexon B for amplification of the loop 1 (L1) region of the hexon gene, as described before (Meulemans et al., 2001; Marek et al., 2010).

Part of the 52K and pIIIa genes were amplified using conventional PCR with primer pair 52K-F/52K-R from representatives of 12 FAdV serotypes. The 25 μl PCR reaction mixture contained 0.4 μM (final concentration) of each primer and 5 μl extracted DNA (corresponding to 20% of the total reaction volume). The initial denaturation was set at 94 °C for 2 min. Cyclic conditions consisted of 35 cycles at 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min 30 s followed by final extension at 72 °C for 2 min. Amplification products, which were between 755 and 794 bp depending on FAdV strain were run on a 1.5% agarose gel. After gel electrophoresis, PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Vienna, Austria) according to the manufacturer's recommendations.

2.5. Generation of standard and control vectors

For optimization of the real-time PCR and generation of the standard curve, the 52K-F/52K-R PCR product of FAdV-D (strain SR49) was used. To test the exact quantitation of all FAdV species, PCR products of FAdV-A, FAdV-B, FAdV-C and FAdV-E (strains CELO, 340, C2B and 764, respectively) were obtained. All five PCR products were cloned into the pCR4-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). All clones were checked for the integrated desired PCR product by restriction enzyme digestion using *Eco*RI. Positive clones were sequenced in both directions using M13 forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany).

The vectors were linearized using *Not*I restriction enzyme (Invitrogen, Carlsbad, CA) and separated on a 1% agarose gel for confirmation and purification. After gel electrophoresis, the correct size bands were extracted using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Purified plasmid DNAs were measured three times by a spectrophotometer (SmartSpec Plus; Bio-Rad Laboratories, Inc., Hercules, CA) and the copy numbers were calculated using the following formula: $[(g/\mu l \text{ DNA})/(\text{plasmid length in base pairs} \times 660) \times 6.022 \times 10^{23}]$.

2.6. SYBR Green based real-time PCR and standard curve

Real-time PCR was carried out on a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany) using the double-stranded DNA-binding dye method with a Rotor-Gene SYBR Green PCR kit (Qiagen, Hilden, Germany). Each 25 μl reaction mixture contained 5 μl template DNA and 0.7 μM (final concentration) of each primer (52K-fw and 52K-rv). Cyclic conditions included initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 5 s and combined annealing/extension step at 60 °C for 10 s and a melting step between 60 and 95 °C. The fluorescence data were collected during the annealing/extension step. Data analysis was performed using Rotor-Gene Q software version 1.7 (Qiagen, Hilden, Germany) by setting the threshold automatically. For generating standard curves, 10-fold serial dilutions of standard vector from 10^{-1} to 10^{-11} were prepared, starting at 6.73×10^8 copies per reaction and were run two times in duplicate. No template control (NTC) was used in each run to confirm that there was no contamination in the assay. A known copy number of target DNA was plotted against the corresponding C_T values and the standard curve was constructed. For quantitation of virus in samples of unknown concentration, their threshold cycle (C_T) values were compared with the standard curve, and the numbers of copies of FAdV DNA per reaction mixture were calculated. Melting curve analysis was done to verify PCR product specificity. The specificity of the real-time PCR was also confirmed by running the products on a 2% agarose gel.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Position	Reference
Hexon A	CAA RTT CAG RCA GAC GGT	144–161[a]	Meulemans et al. (2001)
Hexon B	TAG TGA TGM CGS GAC ATC AT	1041–1021[a]	Meulemans et al. (2001)
52K-F	TGT ACG AYT TCG TSC ARA C	12,788–12806[b]	This study
52K-R	TAR ATG GCG CCY TGC TC	13,542–13526[b]	This study
52K-fw	ATG GCK CAG ATG GCY AAG G	13,075–13093[b]	This study
52K-rv	AGC GCC TGG GTC AAA CCG A	13,250–13232[b]	This study

[a] Position is given for the FAdV-1 hexon gene (AF339914).
[b] Position is given for the FAdV-1 genome (U46933).

2.7. Sensitivity of the real-time PCR assay

One sample of FAdV DNA (strain SR48) was diluted serially 100-fold and 10-fold. Undiluted sample and its serial dilutions were run in real-time PCR and in conventional PCR (using primer set Hexon A/Hexon B) and the results were compared.

2.8. Specificity of the real-time PCR assay

In order to verify the specificity of the real-time PCR assay, DNA samples from the following 13 FAdV strains were tested: CELO (FAdV-1), SR48 (FAdV-2), SR49 (FAdV-3), KR5 (FAdV-4), 340 (FAdV-5), CR119 (FAdV-6), YR36 (FAdV-7), TR59 (FAdV-8a), Hungary6 (FAdV-8a/8b), 764 (FAdV-8b), A2-A (FAdV-9), C2B (FAdV-10) and UF71 (FAdV-11). Additionally, DNA samples from other avian viruses such as poxvirus, chicken anemia virus, Marek's disease virus, hemorrhagic enteritis virus and egg drop syndrome virus were examined in this assay. DNA sample isolated from uninfected CEL cells was also tested.

2.9. Quantitation of all FAdV species

Ten-fold serial dilutions of control vectors from FAdV-A, FAdV-B, FAdV-C and FAdV-E were made and were run in replicates. Control vectors were quantified using standard vector (FAdV-D) by different runs of the real-time PCR. The results were compared to the spectrophotometric measurements. Their mean values and standard deviations were calculated.

2.10. Animal experiment and preparation of samples

Specific pathogen-free (SPF) eggs were obtained from Lohmann Tierzucht, Cuxhaven, Germany. One-day-old birds were placed in isolator units for 70 days at the Clinic for Avian, Reptile and Fish Medicine, Vienna, Austria. One group of 8 birds was kept uninfected as control. Five groups each consisting of 8 one-day-old chicks were infected orally with 0.5 ml of 10^5 tissue culture infective dose ($TCID_{50}$)/ml per chicken with FAdV-A to FAdV-E (strains CELO, 340, KR5, SR48 and YR36, respectively). Reinfection was carried out at 21 and 42 days of age. Cloacal swabs were taken from 5 birds at 3, 7, 10 and 14 days post-infection (d.p.i.) and weekly until termination of the animal trial. Blood samples were taken weekly and investigated by serum neutralization test to confirm infection. Liver and intestine tissue samples were collected from chickens that died unexpectedly during the experiment. The animal experiment was discussed and approved by the institutional ethics committee under the licence number GZ 68.205/0217-C/GT/2007.

Cloacal swabs were placed in 1 ml PBS containing penicillin (100,000 IU/ml) and streptomycin (1 mg/ml). Samples were filtered using syringe filters with 0.2 μm pore size (VWR, Austria). One hundred microliters of sample were used for DNA extraction followed by conventional PCR and by real-time PCR. Also, 100 μl of sample were inoculated on CEL cells for virus isolation.

Tissue samples were homogenized with PBS containing penicillin (100,000 IU/ml) and streptomycin (1 mg/ml). Homogenates were used for DNA extraction. DNAs were examined by the real-time PCR.

2.11. Virus isolation from cloacal swabs

Primary CEL cells were prepared from 13- to 14-day-old SPF chicken embryos (Lohmann Tierzucht GmbH, Cuxhaven, Germany) following a standard procedure (Schat and Purchase, 1998). A sample was determined as positive when a cytopathic effect (CPE) appeared up to three passages.

2.12. Serum neutralization test

Prior to testing, all sera were inactivated for 30 min at 56 °C in a thermomixer (Eppendorf, Austria). CEL cells were prepared as described in Section 2.11. The serum neutralization test was performed in microtiter plate according to a constant virus dilution method using 100 μl $TCID_{50}$/50 μl of respective FAdV reference strain. The plates were kept for 5 days after inoculation at 37 °C in 5% CO_2 and were investigated for CPE.

2.13. Nucleotide sequence accession numbers

The sequences of 52K-F/52K-R PCR products for ten FAdV strains were submitted to the GenBank with accession numbers HE602018 to HE602021 and HE603114 to HE603119 (Supplemental Material S.1).

3. Results

3.1. Optimization of real-time PCR assay

Thirteen FAdV strains that belong to five different FAdV species (FAdV-A to FAdV-E) and twelve serotypes (FAdV-1 to 8a, and 8b to 11) were examined in the conventional PCR using primer set 52K-F/52K-R. All samples were positive and showed an amplification product of the expected length (data not shown). The PCR products of representatives of all FAdV species were sequenced (Supplemental Material S.1) and the primer pair for the real-time PCR (52K-fw/52K-rv) was designed.

Different primer concentrations were tested. The primer concentrations higher than 0.7 μM resulted in fluorescence measurement of primer dimers (data not shown). Therefore, 0.7 μM (final concentration) of each primer was chosen for a reaction. The temperature-time profiles as described in manufacturer's instructions for the Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) were chosen.

3.2. Standard curve

The real-time PCR assay showed a detection range of 6.73–6.73 × 10^8 copies per reaction. The lower detection limit was

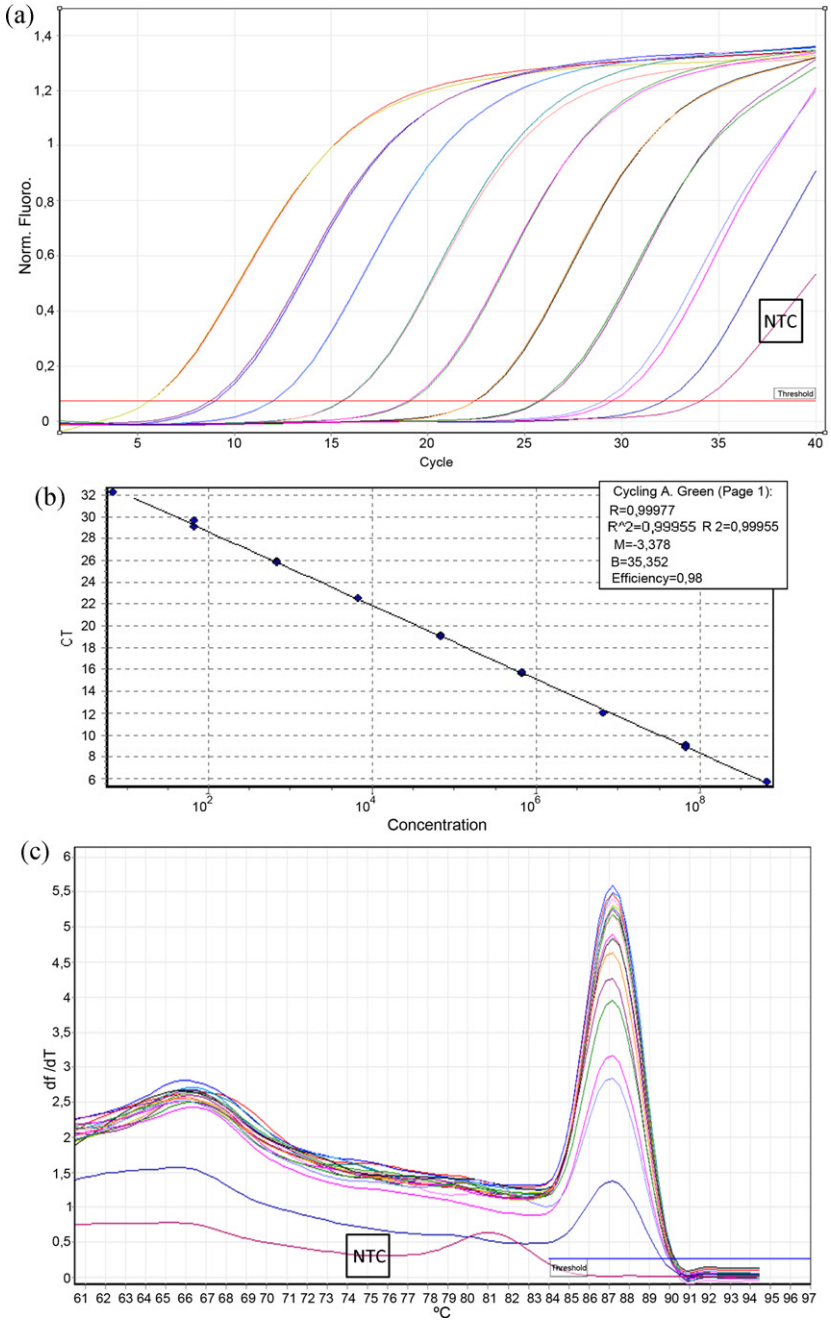

Fig. 1. SYBR Green based real-time PCR using primers 52K-fw and 52K-rv and 10-fold serial dilutions of standard FAdV-D vector. (a) Amplification plot. (b) Standard curve. The efficiency was 0.98 (98%), and the regression squared value was 0.999. (c) Melting curve. The specific melting temperature is 87.1 ± 0.1 °C.

10^{-9} dilution of the standard vector and was corresponding to a threshold cycle (C_T) of 32.2 in this assay (Fig. 1a). The samples with a $C_T \leq 32.2$ were taken as positive and quantifiable within the range of the standard curve. The assay showed linearity with a R^2 value (a regression squared value) of 0.999, a slope (M) value of −3.378, and a reaction efficiency of 98% (Fig. 1b). The specific real-time PCR products had an average specific melting temperature (T_m) of 87.1 ± 0.1 °C (Fig. 1c). No template control (NTC) showed a positive signal in the amplification plot due to primer dimer formation as shown in the melting plot (Fig. 1a and c). The specificity of the real-time PCR products was confirmed and visualized by agarose gel as a 176 bp band (data not shown).

3.3. Sensitivity of the real-time PCR assay

Undiluted, one hundred- and ten-fold serial dilutions of a DNA sample SR48 (FAdV-D) were investigated by the conventional PCR and the real-time PCR. In conventional PCR with Hexon A/Hexon B primer pair, samples up to 10^{-2} (1:100) were detected (Fig. 2a). When the real-time PCR was performed, the last detectable dilution was 10^{-6} (1:1,000,000) (Fig. 2b). The undiluted sample gave a C_T value of 12.48. For the 10^{-2} dilution, a C_T value of 19.07 was measured. The C_T of the last dilution (10^{-6}) was 31.33. Therefore, the new developed SYBR Green based real-time PCR was approximately 10^4 times more sensitive than the conventional PCR.

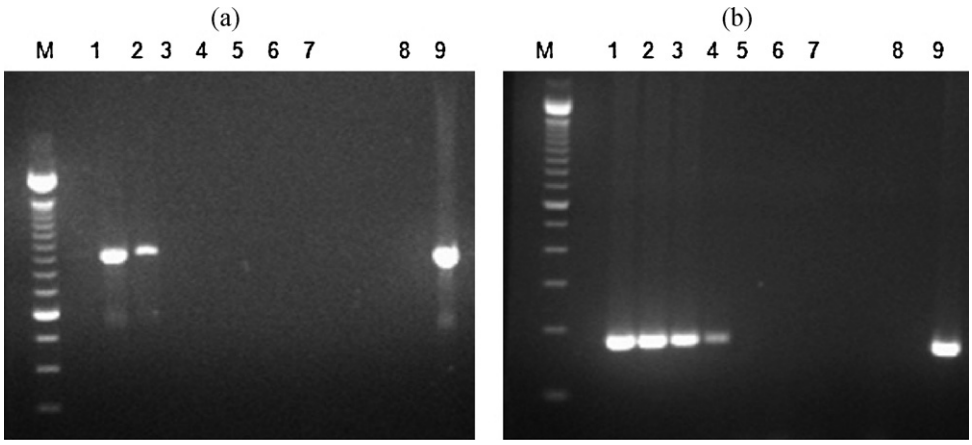

Fig. 2. Gel electrophoresis of PCR products of serial dilutions from DNA sample SR48. Lane M, 100-bp DNA ladder (Invitrogen); lane 1, undiluted sample; lanes 2–7, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-9} and 10^{-10}, respectively; lane 8, negative control; lane 9, positive control. (a) Conventional PCR with primers Hexon A/Hexon B. 10^{-2} was the lowest dilution detected. (b) Real-time PCR with primers 52K-fw/52K-rv. 10^{-6} was the lowest dilution detected.

3.4. Specificity of the real-time PCR assay

All 13 tested FAdV strains were detected and quantified. The specificity of the real-time PCR products was confirmed by gel electrophoresis, whereby bands of approximately 176 bp were visualized (data not shown).

The real-time PCR was performed with positive samples for poxvirus, chicken anemia virus, Marek's disease virus, hemorrhagic enteritis virus and egg drop syndrome virus, and a sample from the uninfected CEL cells. All samples were negative; i.e., no specific melting peak was observed in the melting curve analysis and no specific band was visible after gel electrophoresis (data not shown).

3.5. Quantitation of all FAdV species

Serial dilutions of control vectors from FAdV-A, FAdV-B, FAdV-C and FAdV-E were quantified using a standard curve (obtained with FAdV-D) in the real-time PCR. Results of the real-time PCR agreed with the results from the spectrophotometric measurements. It was confirmed that all FAdV species could be quantified accurately in the developed real-time PCR assay. The results for four measurements of 10^{-3} dilutions are shown in Table 2.

3.6. Comparison of virus isolation, conventional PCR and real-time PCR

Cloacal swabs obtained from SPF chickens experimentally infected with FAdV-A to FAdV-E (strains CELO, 340, C2B, SR48 and YR36, respectively) were investigated by three detection methods: cell culture, conventional PCR and real-time PCR. The sensitivity of these methods was compared (Table 3). Depending on FAdV species, between 65% and 80% of samples were positive in the real-time PCR assay, whereas 27.5–60% were positive in virus isolation and only up to 22.5% of samples were positive in the conventional PCR.

Samples from liver and intestine taken from chickens that died unexpectedly at different time points during the animal experiment were examined by the real-time PCR assay. All samples were found positive (data not shown).

3.7. Viral genome copy number in cloacal swabs

All cloacal swab samples taken up to 42 days post infection were examined by real-time PCR. Viral genome copy numbers were determined and mean values for each time point were calculated (Fig. 3). The highest shedding of FAdV-A, FAdV-B and FAdV-D were found at 10 d.p.i. (3.84 ± 0.4, 3.25 ± 0.9 and 5.89 ± 0.6 viral genome copies per reaction in logarithmic scale ($\log_{10}$), respectively). FAdV-C had a shedding peak at 7 d.p.i. (an average of 3.27 ± 0.5 copies per reaction in $\log_{10}$) and FAdV-E at 21 d.p.i. (an average of 4.54 ± 0.9 copies per reaction in $\log_{10}$). The highest genome copy number was observed in samples of FAdV-D in comparison with other FAdV species. No shedding was detected from FAdV-B at 3 d.p.i. and FAdV-D at 35 days of life.

3.8. Serum neutralization test

The antibody response against viruses representing five FAdV species could be detected initially at 1 week after infection. The antibody levels increased following experimental re-infection at 21 and 42 days of life, with maximum titers of 14 $\log_2$. Depending on the FAdV strain, the peak titers were seen at 3 or 4 weeks after the first infection, ranging from 3 $\log_2$ to 14 $\log_2$ (data not shown).

4. Discussion

Detection of fowl adenoviruses is accomplished usually by virus isolation in cell culture or by histological investigations. Electron microscopy is often used to confirm the presence of adenoviruses in tissues (Adair and Fitzgerald, 2008). However, these techniques are time-consuming, tedious and do not allow further characterization of viruses regarding their classification into a specific group. Many PCR methods for detection of FAdV DNA described in the literature use primers designed to anneal within the different regions of FAdV genome (Raue and Hess, 1998; Jiang et al., 1999; Meulemans et al., 2001; Ganesh et al., 2002; Raue et al., 2005a,b; Hanson et al., 2006; Mase et al., 2009; Ivanics et al., 2010; Kajan et al., 2011). Great variation exists between individual PCRs with regard to sensitivity and specificity. Conventional PCR is a sensitive tool for detecting different viruses, but the technique is unable to detect viral loads quantitatively (Watanabe et al., 2005).

Detection by SYBR Green is the simplest and least expensive approach since it does not require the design of fluorogenic probes against the viral genome (Segura et al., 2010). SYBR Green based real-time PCR methods for detection of FAdVs were developed earlier (Romanova et al., 2009; Grgić et al., 2011). Those methods are based on primers binding within different regions of FAdV genomes applying either vector or genomic DNA as standard. However, the real-time PCR detection of all twelve FAdV serotypes was shown for the first time in the present study. Furthermore, design of primers

Table 2
Quantitation of FAdV-A, FAdV-B, FAdV-C and FAdV-E control vectors in the real-time PCR using a standard FAdV-D vector.

FAdV species	Given concentration[a] ±SD[b] (copies/reaction)	C_T value[c] ±SD[b]	Calculated concentration[c] ± SD[b] (copies/reaction)
A	1.85E+07 ± 5.57E+05	10.08 ± 0.04	2.54E+07 ± 1.51E+07
B	6.31E+06 ± 4.95E+05	15.33 ± 0.4	5.11E+06 ± 1.40E+06
C	1.90E+07 ± 3.21E+05	12.79 ± 0.05	2.28E+07 ± 1.01E+07
E	4.93E+06 ± 1.03E+05	15.26 ± 0.12	4.18E+06 ± 1.85E+06

[a] Given concentrations were calculated on the basis of spectrophotometric measurements.
[b] Standard deviation.
[c] The results of quantitation in the real-time PCR.

Table 3
Cloacal swab samples from chickens infected with all five FAdV species were investigated by virus isolation, conventional PCR and new real-time PCR. Results are shown as number of positive samples/number of tested samples.

d[a]	FAdV-A			FAdV-B			FAdV-C			FAdV-D			FAdV-E		
	VI[b]	PCR	Real-time PCR	VI	PCR	Real-time PCR	VI	PCR	Real-time PCR	VI	PCR	Real-time PCR	VI	PCR	Real-time PCR
3	0/5	0/5	2/5	0/5	0/5	0/5	5/5	0/5	3/5	5/5	1/5	5/5	0/5	0/5	2/5
7	3/5	0/5	5/5	4/5	0/5	5/5	5/5	0/5	5/5	5/5	3/5	5/5	3/5	0/5	4/5
10	5/5	0/5	5/5	4/5	1/5	5/5	4/5	0/5	4/5	5/5	5/5	5/5	2/5	0/5	3/5
14	4/5	0/5	5/5	3/5	0/5	4/5	1/5	0/5	3/5	5/5	0/5	5/5	4/5	0/5	3/5
21	0/5	0/5	4/5	0/5	0/5	1/5	2/5	0/5	4/5	3/5	0/5	1/5	5/5	0/5	5/5
28	0/5	0/5	2/5	0/5	0/5	4/5	1/5	0/5	2/5	1/5	0/5	2/5	1/5	0/5	3/5
35	0/5	0/5	4/5	0/5	0/5	3/5	0/5	0/5	3/5	0/5	0/5	0/5	0/5	0/5	2/5
42	0/5	0/5	5/5	0/5	0/5	5/5	1/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	4/5
Total	12/40 (0,12,0)[c]	0/40	32/40	11/40 (2,8,1)	1/40	27/40	19/40 (0,17,2)	0/40	29/40	24/40 (9,12,3)	9/40	28/40	15/40 (0,9,6)	0/40	26/40

[a] Day of life.
[b] Virus isolation on CEL cells.
[c] Positive samples obtained in the 1st, 2nd or 3rd passage in cell culture.

was based on the five available complete genome sequences of FAdV strains (Chiocca et al., 1996; Ojkic and Nagy, 2000; Grgić et al., 2011; Griffin and Nagy, 2011; Marek et al., 2012) and on the 52K-F/52K-R PCR products from thirteen FAdVs. Consequently, primers binding within conserved regions of the 52K gene (52K-fw/52K-rv) were designed and used in the new real-time PCR. It was demonstrated that the primer pair 52K-fw/52K-rv was suitable for detection of all FAdV species and serotypes.

Genomes of other avian viruses and uninfected CEL cells did not produce positive signal confirming the specificity of the real-time PCR assay. Additionally, viruses of other adenovirus genera: *Siadenovirus* (hemorrhagic enteritis virus of turkeys) and *Atadenovirus* (egg drop syndrome virus) were not detectable in the new real-time PCR (data not shown).

For the first time an exact quantitation of all FAdV species was demonstrated. For this, part of the 52K and the pIIIa genes were amplified and cloned to establish FAdV-D as a standard vector and the FAdV-A, FAdV-B, FAdV-C and FAdV-E as control vectors. The exact quantitation of control vectors was tested by the real-time PCR assay using the FAdV-D standard vector. Their calculated concentrations in the real-time PCR confirmed the spectrophotometric measurements. This comparison showed that the primer pair

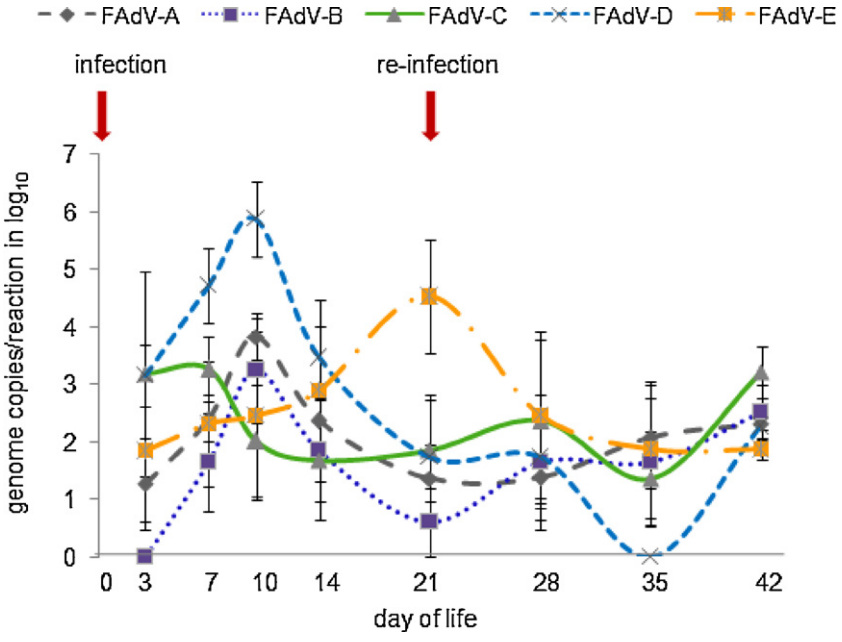

Fig. 3. Shedding of all five FAdV species was calculated by the real-time PCR in cloacal swab samples obtained from experimentally infected SPF chickens.

52K-fw/52K-rv was suitable for an exact quantitation of all FAdV species.

In the present study it could be demonstrated that the SYBR Green based real-time PCR for detection of FAdVs was more sensitive than conventional PCR using Hexon A/Hexon B primer pair. Not all dilutions of the FAdV-D (strain SR48) sample were detected by conventional PCR, which were positive in the new real-time PCR.

Cloacal swab samples from chickens infected with representative strains belonging to all FAdV species were examined by a real-time PCR in order to quantify the virus shedding, in the presence of neutralizing antibodies. While FAdV-A (FAdV-1, strain CELO), FAdV-B (FAdV-5, strain 340), and FAdV-D (FAdV-2, strain SR48) showed maximum excretion at 10 d.p.i., the shedding of FAdV-C (FAdV-4, strain KR5) peaked at 7 d.p.i. The highest virus shedding for FAdV-E (FAdV-7, strain YR36) was found at 21 d.p.i. Our study confirmed earlier investigations that the shedding of FAdVs depends on the strain used for infection (Cook, 1983).

Presence of virus was investigated in cloacal swab samples by virus isolation. Presence of viral DNA was examined by conventional PCR and by the developed real-time PCR. The comparison of three detection methods showed that the detection rate of FAdV in samples could be improved substantially by the developed real-time PCR in contrast to conventional PCR using Hexon A/Hexon B primers and to virus isolation in cell culture.

Furthermore, it was possible to quantify virus in different type of samples. All C_T values for the samples were in the range of the standard curve, and the amount of FAdV present in each sample could be determined as the number of copies of FAdV DNA per reaction mix. If quantitation of virus is done, it must be considered that the values are theoretical and need normalization based on equal quantity and quality of the starting material (Gunson et al., 2006). In this study, the quantitation of FAdVs in different samples was performed mainly to verify that the real-time PCR could be used in different type of sample materials such as supernatant of FAdV infected cell culture, cloacal swab or tissue sample.

In conclusion, the presented SYBR Green based real-time PCR is suitable for diagnosis of FAdVs and allows for rapid and simple detection and exact quantitation of all FAdV species. Also, it has the advantage of reduced risk of contamination due to a single closed tube procedure. It is an applicable method for diagnosis and research of FAdVs, since it allows quantitation in different experimental and clinical samples. The new real-time PCR has a higher sensitivity compared to virus isolation in cell culture and to conventional PCR.

Acknowledgements

The authors thank Günther Landsmann and Katja Zidek who helped with the animal experiment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2012.04.005.

References

Adair, B.M., Fitzgerald, S.D., 2008. Group 1 adenovirus infections. In: Saif, Y.M., Fadly, A.M., Glisson, J.R., et al. (Eds.), Diseases of Poultry. , 12th ed. Iowa State University Press, Ames, IA, pp. 260–286.

Benkö, M., Harrach, B., Both, G.W., Russell, W.C., Adair, B.M., Ádám, É., de Jong, J.C., Hess, M., Johnson, M., Kajon, A., Kidd, A.H., Lehmkuhl, H.D., Li, Q.G., Mautner, V., Pring-Akerblom, P., Wadell, G., 2005. Family *Adenoviridae*. In: Fauquet, C.M., Mayo, M.A., Maniloff, J. (Eds.), Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, New York, NY, pp. 213–228.

Chiocca, S., Kurzbauer, R., Schaffner, G., Baker, A., Mautner, V., Cotton, M., 1996. The complete DNA sequence and genomic organization of the avian adenovirus CELO. Journal of Virology 70, 2939–2949.

Cook, J.K., 1983. Fowl adenoviruses: studies on aspects of the pathogenicity of six strains for 1-day-old chicks. Avian Pathology 12, 35–43.

Gallina, A.M., Winterfield, R.W., Fadly, A.M., 1973. Adenovirus infection and disease. II. Histopathology of natural and experimental disease. Avian Diseases 17, 343–353.

Ganesh, K., Suryanarayana, V.V.S., Raghavan, R., 2002. Detection of fowl adenovirus associated with hydropericardium hepatitis syndrome by a polymerase chain reaction. Veterinary Research Communications 26, 73–80.

Grgić, H., Yang, D.H., Nagy, E., 2011. Pathogenicity and complete genome sequence of a fowl adenovirus serotype 8 isolate. Virus Research 156, 91–97.

Griffin, B.D., Nagy, E., 2011. Coding potential and transcript analysis of fowl adenovirus 4: insight into uORFs as common sequence features in adenoviral transcripts. Journal of General Virology 92, 1260–1272.

Gunson, R.N., Collins, T.C., Carman, W.F., 2006. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. Journal of Clinical Virology 35, 355–367.

Hanson, A.L., Rudis, M.R., Vasquez-Lee, M., Montgomery, D.R., 2006. A broadly applicable method to characterize large DNA viruses and adenoviruses based on the DNA polymerase gene. Virology Journal 3, 28.

Hess, M., 2000. Detection and differentiation of avian adenoviruses: a review. Avian Pathology 29, 195–206.

Hess, M., Prusas, C., Monreal, G., 1998. Growth analysis of adenoviruses isolated from pigeons in chicken cells and serological characterization of the isolates. Avian Pathology 27, 196–199.

Ivanics, E., Palya, V., Markos, B., Dan, A., Ursu, K., Harrach, B., Kajan, G., Glavits, R., 2010. Hepatitis and hydropericardium syndrome associated with adenovirus infection in goslings. Acta Veterinaria Hungarica 58, 47–58.

Jiang, P., Ojkic, D., Tuboly, T., Huber, P., Nagy, E., 1999. Application of the polymerase chain reaction to detect fowl adenoviruses. Canadian Journal of Veterinary Research 63, 124–128.

Kajan, G.L., Sameti, S., Benkö, M., 2011. Partial sequence of the DNA-dependent DNA polymerase gene of fowl adenoviruses: a reference panel for a general diagnostic PCR in poultry. Acta Veterinaria Hungarica 59, 279–285.

Marek, A., Günes, A., Schulz, E., Hess, M., 2010. Classification of fowl adenoviruses by use of phylogenetic analysis and high-resolution melting-curve analysis of the hexon L1 gene region. Journal of Virological Methods 170, 147–154.

Marek, A., Nolte, V., Schachner, A., Berger, E., Schlötterer, C., Hess, M., 2012. Two fiber genes of nearly equal lengths are a common and distinctive feature of *Fowl adenovirus C* members. Veterinary Microbiology 156, 411–417.

Mase, M., Mitake, H., Inoue, T., Imada, T., 2009. Identification of group I–III avian adenovirus by PCR coupled with direct sequencing of the hexon gene. Journal of Veterinary Medical Science 71, 1239–1242.

McFerran, J.B., 1998. Adenoviruses. In: Swayne, D.E., John, R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens. University of Pennslyvania, Kennnet Square, PA, pp. 100–105.

McFerran, J.B., Clarke, J.K., Connor, T.J., 1972. Serological classification of avian adenoviruses. Archiv fur Die Gesamte Virusforschung 39, 132–139.

McFerran, J.B., Connor, T.J., McCracken, R.M., 1976. Isolation of adenoviruses and reoviruses from avian species other than domestic fowl. Avian Diseases 20, 519–524.

Meulemans, G., Boschmans, M., Berg, T.P., Decaesstecker, M., 2001. Polymerase chain reaction combined with restriction enzyme analysis for detection and differentiation of fowl adenoviruses. Avian Pathology 30, 655–660.

Ojkic, D., Nagy, E., 2000. The complete nucleotide sequence of fowl adenovirus type 8. Journal of General Virology 81, 1833–1837.

Raue, R., Hess, M., 1998. Hexon based PCRs combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. Journal of Virological Methods 73, 211–217.

Raue, R., Gerlach, H., Müller, H., 2005a. Phylogenetic analysis of the hexon loop 1 region of an adenovirus from psittacine birds supports the existence of a new psittacine adenovirus (PsAdV). Archives of Virology 150, 1933–1943.

Raue, R., Schmidt, V., Freieck, M., Reinhardt, R., Johne, R., Kamphausen, L., Kaleta, E.F., Müller, H., Krautwald-Junghanns, M.E., 2005b. A disease complex associated with pigeon circovirus infection young pigeon disease syndrome. Avian Pathology 34, 418–425.

Reed, L.J., Muench, H., 1938. A simple method for estimating 50 percent endpoints. American Journal of Hygiene 27, 493–497.

Romanova, N., Corredor, J.C., Nagy, E., 2009. Detection and quantitation of fowl adenovirus genome by a real-time PCR assay. Journal of Virological Methods 159, 58–63.

Schat, K.A., Purchase, H.G., 1998. Cell culture methods. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens. , 4th ed. University of Pennslyvania, Kennnet Square, PA, pp. 223–234.

Segura, M.M., Monfar, M., Puig, M., Mennechet, F., Ibanes, S., Chillon, M., 2010. A real-time PCR assay for quantification of canine adenoviral vectors. Journal of Virological Methods 163, 129–136.

Watanabe, M., Kohdera, U., Kino, M., Haruta, T., Nukuzuma, S., Suga, T., Akiyoshi, K., Ito, M., Suga, S., Komada, Y., 2005. Detection of adenovirus DNA in clinical samples by SYBR Green real-time polymerase chain reaction assay. Pediatrics International 47, 286–291.