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Detection and quantitation of fowl adenovirus genome by a real-time PCR assay

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A B S T R A C T

The purpose of the study was to develop a highly sensitive real-time polymerase chain reaction (PCR) assay to detect and quantitate fowl adenovirus (FAdV) DNA in chicken tissues, using FAdV-9 as a model. The assay had a dynamic range of 7 logs and minimum detection limit of 9.4 viral genome copies. It was shown to be highly specific, as tissues from uninfected chickens and other viral genomes, such as those of Marek's disease virus, fowlpox virus and infectious laryngotracheitis virus did not produce positive signal. The sensitivity of the real-time PCR was comparable with nested PCR and it was 100 times more sensitive than the conventional PCR.

The assay was validated by testing DNA from tissues of chickens infected with FAdV-9 collected at different days post-infection. FAdV-9 DNA was detected in liver, bursa of Fabricius and cecal tonsil tissues in a range of 10^2–10^7 copies per 100 ng of total DNA. High amounts of viral DNA were present in the cecal tonsils for a week after inoculation making this tissue an ideal sample source for the diagnosis of FAdV infection. This assay is an excellent research and diagnostic tool that provides high sensitivity, specificity and rapid post-PCR analyses.

1. Introduction

Fowl adenoviruses (FAdVs) are ubiquitous in poultry farms worldwide (Adair and Fitzgerald, 2008). However, some FAdV isolates, especially of serotypes 2, 7, 8 and 11 are responsible for inclusion body hepatitis (IBH) in chickens, an important disease in North America causing substantial economic losses to the poultry industry (Ojkic et al., 2008). Moreover, isolates belonging to serotype 4 have been described as the causative agent of hydropericardium syndrome (Mazaheri et al., 1998; Toro et al., 1999; Hess, 2000; Dahiya et al., 2002). In addition, respiratory disease and tenosynovitis in chickens have been associated with FAdVs (McFerran et al., 1971; Jones and Georgiou, 1984), and the pathogenic role of FAdV-1 in gizzard erosions has been also shown (Okuda et al., 2001; Ono et al., 2007). Nevertheless, the role of these viruses as primary pathogens is unclear (Toro et al., 2000, 2001). FAdVs are transmitted easily both horizontally and vertically (Grgic et al., 2006; Adair and Fitzgerald, 2008). FAdV specific antibodies can be detected by agar gel immunodiffusion test and enzyme-linked immunosorbent assay (ELISA) (Calnek et al., 1982; Ojkic and Nagy, 2003). IBH is diagnosed routinely by virus isolation in embryonated eggs or cell culture followed by electron microscopy or more recently by polymerase chain reaction (PCR) (Raue and Hess, 1998; Jiang et al., 1999). PCR followed by restriction enzyme digestion or sequencing of the products allows the differentiation of field isolates to species and presumptive serotypes (Meulemans et al., 2001, 2004). In general, PCR has proven to be very sensitive and specific, but it still requires time-consuming sample handling and post-PCR analysis. Real-time PCR has gained wide acceptance due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Mackay et al., 2002). Moreover, it has been introduced to diagnostic laboratories as a quantitative tool for a variety of human and animal pathogens (Watanabe et al., 2005; Willoughby et al., 2006; Abdul-Careem et al., 2006). The aim of this study was to develop a rapid and sensitive real-time PCR assay based on SYBR Green to detect and quantitate FAdV DNA in infected cells, and to follow the genome load in tissues of experimentally infected chickens over time.

2. Materials and methods

2.1. Chickens, sample collection and DNA extraction

A group of two-week-old White Leghorn chickens was inoculated intramuscularly with 10^6 plaque forming units (PFU) per chicken with fowl adenovirus 9 (FAdV-9). The group of the uninfected control chickens was kept in a separate room. Liver, bursa of Fabricius and cecal tonsils were collected aseptically at 3, 5, 7, 14 and 21 days post-infection (p.i.) and also for control birds, and placed into individual bags for storage at −70 °C until use. Total DNA was extracted with DNeasy® tissue kit (QIAGEN InC., Mississauga). DNA concentration was determined by spectrophotometry

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at 260 nm and adjusted to 50 ng/μl. Presence of viral DNA was determined by nested PCR and by real-time PCR. Presence of virus was determined by virus isolation.

2.2. Viruses

FAdV-1 (chicken embryo lethal orphan virus, CELO virus), FAdV-2, FAdV-8, FAdV-9 and FAdV-10 were propagated in a chicken hepatoma cell line (CH-SAH), kindly provided by Solvay Animal Health (Mendota Heights, MN). When cytopathic effect (CPE) was complete, the cells and supernatants were collected and subjected to three freeze-thaw cycles. Viral DNA was extracted from the concentrated viruses as described earlier (Ojkic and Nagy, 2001).

2.3. Virus isolation

Virus isolation was done in CH-SAH cells from tissue homogenates of liver, cecal tonsil and bursa of Fabricius taken from infected and uninfected chickens. Cell cultures that did not show CPE by day 5 p.i. were frozen and thawed once, and the lysates were inoculated into fresh CH-SAH cultures. A sample was considered negative if no CPE was observed after three blind passages.

2.4. Conventional and nested PCR

Primers directed to amplify a portion of the protein X gene were used for nested-PCR to detect viral DNA. The external primers were pX-For (CAGGAAGCGTCGCCAACATCAT) and pX-Rev (ACCGTTTCTCCTTCTCCTCGTTGA), for a 440 bp amplicon and the internal primers were pXin-For (CTTACGGGCGGGCGAACAGC) and pXin-Rev (CGGCACCTGAAACGGGAACC) for a 370 bp amplicon. The PCR reactions were made in 50 μl final volume with 1 × PCR buffer (200 mM Tris–HCl, 500 mM KCl, pH 8.4), 2 mM $MgCl_2$, 1 mM dNTPs, 20 pmol of each primer, 2 U Taq polymerase and 100 ng DNA, or 2 μl from the first round of PCR when using internal primers. The PCR conditions were as follows: initial denaturation at 94 °C for 4 min followed by 30 cycles and a 10-min final extension at 72 °C. For each cycle, the denaturation was at 94 °C for 30 s, annealing at 60 °C (for external primers) or 63 °C (for internal primers) for 30 s, and extension at 72 °C for 30 s. The PCR products were run in 1.5% agarose gels and stained with ethidium bromide.

2.5. Primer design for real-time PCR

Primers were designed (Rozen and Skaletsky, 2000) to amplify a region of 114 bp from nucleotide 32,733 to 32,846 of FAdV-9. The amplified region corresponded to part of ORF20A at the right end of the genome (GenBank accession no. AF083975). Forward and reverse primers were 5′-ATGGTGTTCTATTGGACGCA and 5′-TGTTTGGATGTTGCACCTTT 3′, respectively.

2.6. Reproducibility of real-time LightCycler® PCR

The 114-bp PCR product generated from the FAdV-9 DNA template was ligated into pCR®II-TOPO® vector using the TOPO TA cloning kit (Invitrogen, Life Technology). Subsequently, recombinant clones were screened by restriction enzyme digestion followed by sequencing with M13 forward and reverse primers.

The concentration of plasmids pCR®II-TOPO®-FAdV was measured spectrophotometrically. Copy number was calculated by the following formula:

$$\text{Amount(copies}/\mu\text{l)} = \frac{6 \times 10^{23}(\text{copies/mol}) \times \text{concentration(g}/\mu\text{l)}}{\text{MW(g/mol)}}$$

The quantified and serially diluted plasmid pCR®II-TOPO®-FAdV was the standard control. Standard curves were generated and run five times in triplicates.

2.7. LightCycler® real-time PCR

Real-time PCR assays were performed in glass capillaries in a LightCycler® instrument (Roche Diagnostics GmbH, Germany). Each reaction was carried out in 2 μl of DNA and 18 μl reaction mixture, containing 2.5 U of Tth polymerase (Roche Diagnostics), 1 × PCR buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.1 M KCl, 50 μg/ml bovine serum albumin, 0.05% Tween 20 (v/v), pH 8.9), 0.25 μM of each primer, 0.2 mM dNTPs, 10,000 times diluted SYBR Green I (Roche Diagnostics). Each amplification consisted of a denaturation step of 10 s at 95 °C, followed by 40 cycles of 15 s denaturation at 94 °C, 10 s annealing at 60 °C and elongation for 10 s at 72 °C, followed by a single fluorescence measurement. Amplification was followed by melting curve analysis between 65 and 95 °C and finally cooling for 1 min at 40 °C. In each run, a dilution series of plasmid standard was also included along with the DNA samples. The quantitation data, in terms of the crossing point value (C_p; which is expressed as the fractional cycle number and is the intersection of the log-linear fluorescent curve with threshold crossing line), were determined using the second derivative method of the LightCycler® software, version 3.5 (Roche Diagnostics GmbH, Germany).

DEPC-treated water was the negative control, and DNA from some avian viruses such as Marek's disease virus (MDV), fowlpox virus (FPV) and infectious laryngotracheitis virus (ILTV), obtained as described previously (Alexander and Nagy, 1997), were also tested to show the specificity of the method.

2.8. Data analysis

The efficiency of the real-time PCR was calculated by 10E (−1/slope of standard curve). Quantitation of FAdV-9 virus replication was done by the LightCycler® software version 3.5 (Roche Diagnostics GmbH, Germany).

2.9. One-step growth analysis

To examine replication kinetics, CH-SAH cells were infected with FAdV-9 at a multiplicity of infection (MOI) of 2 and incubated for 1 h at room temperature to allow virus adsorption. The cells were washed three times with sterile phosphate buffered saline (PBS), and then Dulbecco's modified eagle's medium nutrient mixture F-12 HAM (Sigma-Aldrich Company, Mississauga) containing 5% fetal bovine serum was added. Cells and supernatants were harvested at 6, 9, 12, 18, 24, 30 and 36 h p.i. Total virus titers were determined by the plaque assay in CH-SAH cells as described (Alexander et al., 1998).

3. Results

3.1. Optimization of real-time PCR conditions

Various PCR parameters such as cycling times, temperature, primer concentrations from 0.15 to 0.35 μM and $MgCl_2$ concentrations from 1 to 3 mM were examined. Among various combinations of concentrations of both components, only the combination of 1.5 mM $MgCl_2$ and 0.25 μM primers resulted in identical melting peaks for serial dilutions with no primer dimers. Therefore those concentrations were used for subsequent analyses.