Application of the Polymerase Chain Reaction to Detect Fowl Adenoviruses

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ABSTRACT

The possibility of using the polymerase chain reaction (PCR) for the detection of fowl adenoviruses (FAdV) was tested. The optimal reaction parameters were evaluated and defined for purified genomic DNA of type 8 fowl adenovirus (FAdV-8), and then the same conditions were applied for nucleic acid extracted from infected cells. One hundred picograms of purified viral DNA, or 250 FAdV-8-infected cells, were detected by ethidium bromide staining of the PCR products in agarose gels. The sensitivity was increased to 10 pg purified viral DNA, or 25 infected cells, when the PCR products were hybridized with a specific labeled probe. Several field isolates of FAdV and the CELO virus (FAdV serotype 1) could be amplified by the same primers and conditions, but the size of the amplicons was smaller than that for the FAdV-8 PCR product. Other avian viruses and uninfected cell cultures tested negative.

RÉSUMÉ

La réaction d'amplification en chaîne par la polymérase (ACP) fut utilisée pour détecter les adénovirus aviaires (AdVA). Les paramètres pour obtenir une réaction optimale ont été évalués et déterminés pour l'ADN génomique purifié de l'adénovirus aviaire de type 8 (AdVA-8), et par la suite les mêmes conditions ont été utilisées pour l'acide nucléique extrait de cellules infectées. Cent picogrammes d'ADN viral purifié, équivalent à 250 cellules infectées par l'AdVA-8, ont été détectés par coloration au bromure d'éthidium des produits de réaction d'ACP après électrophorèse sur gel d'agarose. L'utilisation d'une sonde spécifique marquée a amélioré la sensibilité de l'épreuve, permettant la détection de 10 pg d'ADN viral purifié, ou 25 cellules infectées. Plusieurs isolats de champ d'AdVA et le virus CELO (AdVA sérotype 1) ont été amplifiés en utilisant les mêmes amorces et conditions expérimentales, mais la taille des amplicons était plus petite que celle du produit d'ACP de l'AdVA-8. Aucune réaction ne fut observée avec d'autres types de virus aviaires de même qu'avec des cultures cellulaires non-infectées.

(Traduit par le docteur Serge Messier)

INTRODUCTION

Avian adenovirus infections may be asymptomatic or associated with a variety of clinical and pathologic conditions, including respiratory disease, marble spleen disease, inclusion body hepatitis, egg drop syndrome, enteritis and others that are found to occur in chickens, quails, turkeys, pheasants, geese, and guinea fowl (1–4).

The avian adenoviruses are non-enveloped, icosahedral, 70–90-nm particles, containing a double-stranded, linear, 30- to 45-kb DNA genome (5–8). These viruses are distributed worldwide. The Group I fowl adenoviruses (FAdV) can be placed into at least 12 distinct serotypes, based primarily on their immunological properties.

Detection of fowl adenovirus infections is usually accomplished by virus isolation with serotyping when fresh tissues are available, or histological techniques when formaldehyde-fixed tissues are submitted. Electron microscopy is also often used to confirm the presence of adenoviruses. Nuclear inclusion bodies in hematoxylin and eosin (H&E)-stained tissue sections are indicative of adenovirus infection. Immunoperoxidase staining of formaldehyde-fixed tissues and immunofluorescence staining of frozen unfixed tissues have been described (9,10). However, they all have some disadvantages. For example, excessive nonspecific staining may complicate the evaluation of the immunoperoxidase and immunofluorescent staining. Viral inclusions in H&E-stained tissue sections may vary both in size and in tinctorial properties, and they may range from enlarged and basophilic to small and eosinophilic (11–13). Aviadenovirus inclusions may be mistaken for those of herpesvirus, circovirus or polyomavirus, depending upon the species of bird. Furthermore, these techniques are time-consuming, tedious and expensive. DNA in situ hybridization was also evaluated for the diagnosis of avian adenovirus infections, but the method usually requires paraffin-embedded tissue (14).

PCR is a very sensitive and specific method that has been used for the detection of adenoviral infections of mammals. Since the complete nucleotide sequences of CELO virus (7) and FAdV type 8 (FAdV-8)

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(unpublished data) have already been determined, these were used to design PCR primers. This study describes the application of the PCR method for the detection of fowl adenoviruses.

MATERIALS AND METHODS

VIRUSES AND CELLS

FAdV-8, strain A-2A (ATCC VR-833) and FAdV-1, CELO virus (strain Phelps) were originally obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and maintained in the laboratory. FAdV field isolates, numbered from 1 to 8, were all obtained from materials submitted to the Veterinary Laboratory Services (AHL, University of Guelph,) and kindly provided by Dr. Doug Key.

The origin and propagation of fowlpox virus (FPV), infectious laryngotracheitis virus (ILTV), infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were described by Alexander and Nagy (15).

All of the viruses were grown in a chicken hepatoma cell line (CH-SAH, Solvay Animal Health, Mendota Heights, Minnesota, USA) as described by Alexander et al and Scholz et al (16,17).

EXTRACTION OF NUCLEIC ACID

The cells were infected with a multiplicity of infection (m.o.i.) of 1 with the appropriate viruses. The cells and supernatant were collected when the cytopathic effect in at least 75% of the cells became evident. After 3 cycles of freezing and thawing, the cell debris was pelleted and the virus was concentrated as described (18). The virus pellet was resuspended in TNE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4) and treated with 500 μg/mL proteinase K and 0.5% SDS at 37°C for 2 h, followed by incubation at room temperature overnight. Two phenol:chloroform (1:1) extractions were performed, followed by a chloroform wash, and the DNA was precipitated with ethanol overnight. The DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and the concentration was determined by spectrophotometry. DNA was also extracted directly from 25-cm² flasks of infected cell cultures, resuspended in 50 μL of sterile water, and 5 μL of this was used in the PCR. Uninfected cells were treated in the same manner and used as a negative control.

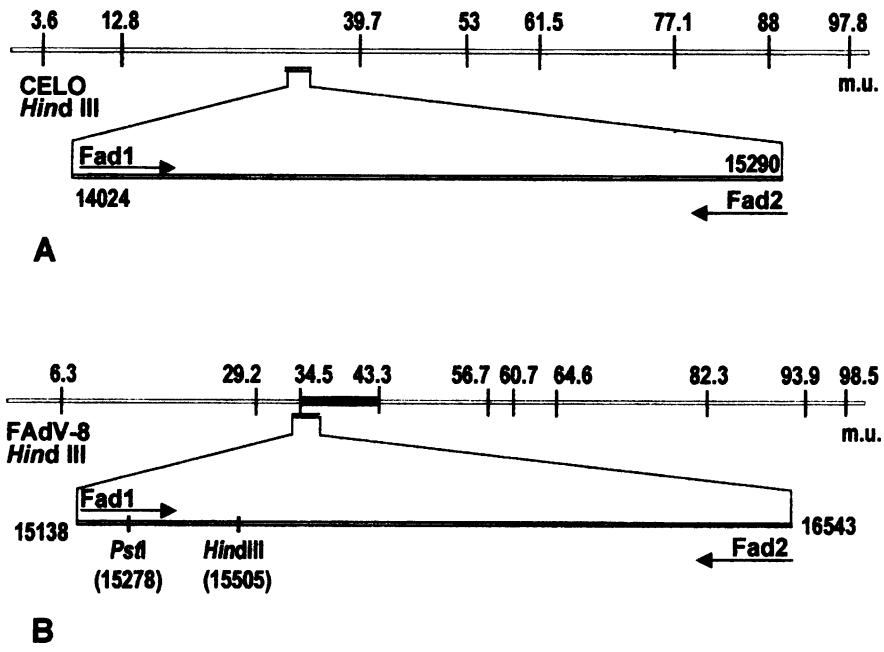

Figure 1. Locations of the primers on the CELO virus (panel A) and FAdV-8 (panel B) genomes. Bold area represents the FAdV-8 *Hind*III E fragment that was used as a probe for dot-blot and Southern blot hybridizations.

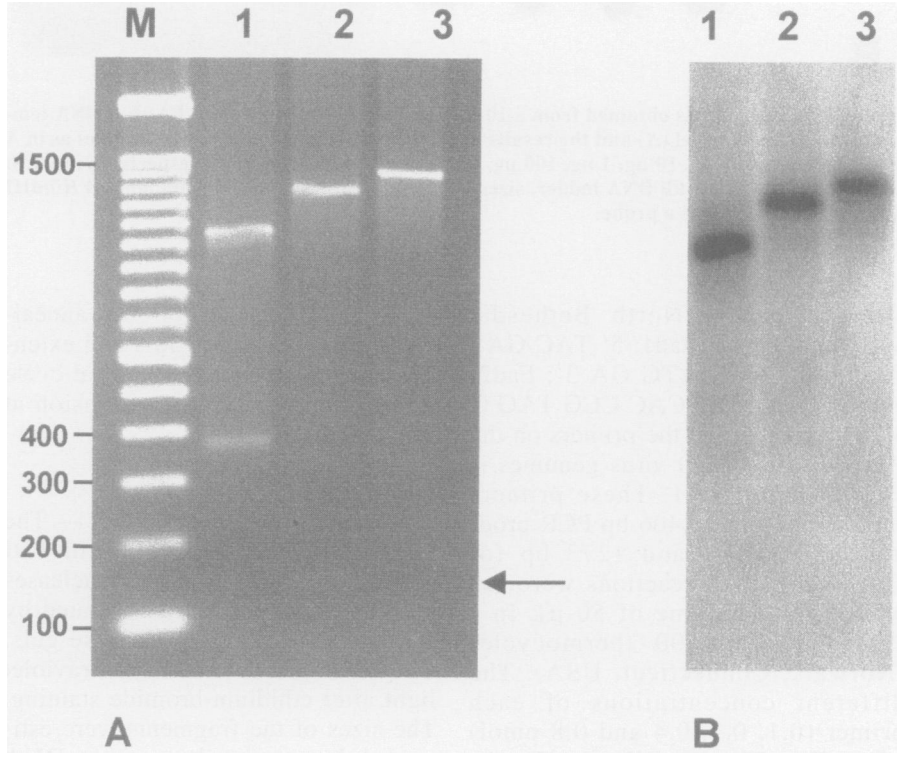

Figure 2. Agarose gel electrophoresis (panel A) and Southern blot hybridization (panel B) of the PCR product using purified FAdV-8 DNA. Lanes 1: digested with *Hind*III; lanes 2: digested with *Pst*I; lanes 3: undigested product; M: 100 bp DNA ladder. The arrow indicates the position of the faint 140 bp *Pst*I fragment. The digoxigenin-labeled *Hind*III E fragment was used as a probe.

POLYMERASE CHAIN REACTION

The primer set (Fad1: forward and Fad2: reverse) was designed from the sequence of FAdV-8 and CELO viral DNA using the Vector NTI software

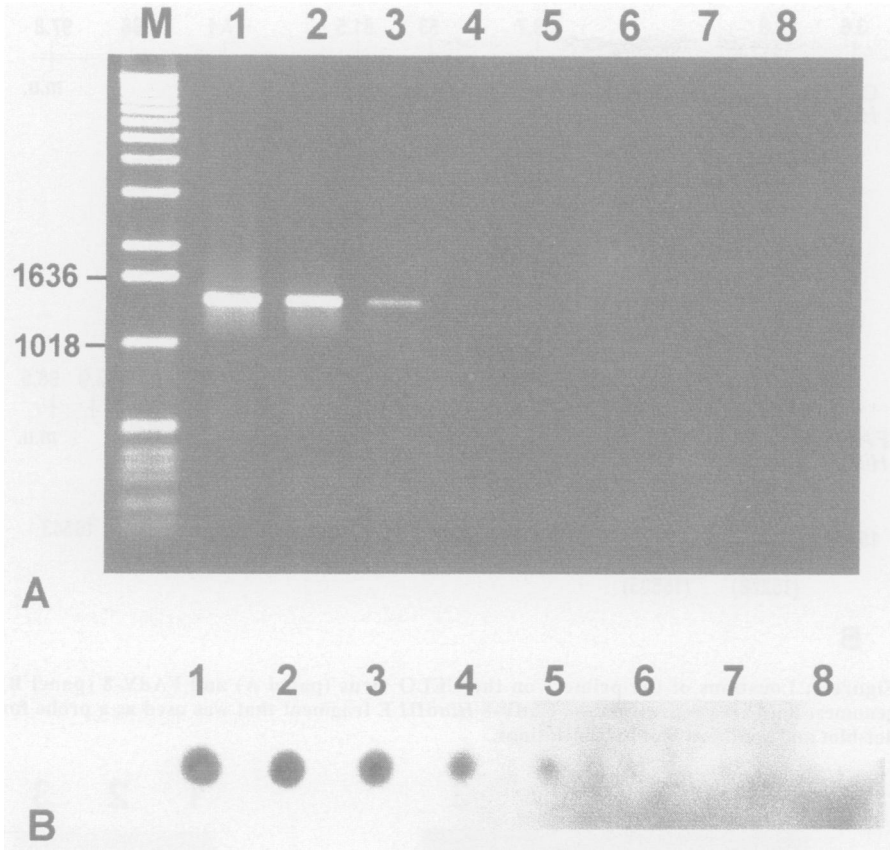

Figure 3. PCR products obtained from a 10-fold serial dilution of purified FAdV-8 DNA template, in 1% agarose gel (A) and the results of DNA hybridization of the same samples as in A (B). Lanes 1-7: 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg and 100 fg DNA, respectively; lane 8: negative control. M: 1 kb DNA ladder, sizes are given in bp. The digoxigenin labeled *Hin*dIII E fragment was used as a probe.

(Informax Inc., North Bethesda, Maryland, USA): Fad1: 5′ TAC GAT TAC GGG GCT ATG GA 3′; Fad2: 5′ TGT TCC GTC CAC CCG TAG G 3′. The location of the primers on the FAdV-8 and CELO virus genomes is shown in Figure 1. These primers should result in a 1406 bp PCR product for FAdV-8 and 1273 bp for CELO virus. All reactions were run with a total volume of 50 μL in a Perkin-Elmer 9600 thermocycler (Norwalk, Connecticut, USA). The different concentrations of each primer (0.1, 0.2, 0.4 and 0.8 pmol) (GenoSys, The Woodlands, Texas, USA), each deoxynucleoside triphosphate (dNTP) (0.1, 0.2 and 0.4 mM) (Boehringer Mannheim, Laval, Quebec), $MgCl_2$ (1, 2, 3 and 4 mM) and *Taq* DNA polymerase (1.5, 3 and 4.5 U) (GIBCO-BRL, Burlington, Ontario) were tested and optimized. The cycling parameters were as follows: an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56 or 60°C for 30 s and extension at 72°C for 60 s. The final cycle was followed by a long extension at 72°C for 10 min.

DETECTION OF PCR PRODUCTS

Agarose gel electrophoresis — The PCR products were digested with *Pst*I and *Hin*dIII restriction endonucleases and the fragments were separated by electrophoresis in 2% agarose gels. The DNA was detected by ultraviolet light after ethidium bromide staining. The sizes of the fragments were estimated by comparison to a DNA molecular weight marker (100 bp Ladder or 1 kb DNA Ladder; GIBCO-BRL).

The DNA extracted from purified FAdV-8 was serially diluted from an initial concentration of 50 ng/μL and amplified as previously described. The products were visualized by ethidium bromide staining of 1% agarose gels.

Hybridization — The probe was the 4 kb *Hin*dIII E fragment of the FAdV-8 genomic DNA, located between 34.5 and 43.3 map units on the FAdV-8 genome (Fig. 1). The DNA fragment was separated by agarose gel electrophoresis and purified with the aid of the GeneClean kit (BIO/CAN Scientific, Mississauga, Ontario) and labeled with a Digoxigenin Labeling Kit (Boehringer Mannheim), according to the manufacturers' instructions. Both dot-blot and Southern blot hybridizations were used for the detection of the PCR amplified DNA fragments. In dot blotting, 3 μL of the PCR product was incubated for 10 min in boiling water and loaded directly onto a Nytran membrane (Schleicher and Schuell, Keene, New Hampshire, USA). The membrane was air-dried and the DNA was cross-linked at 254 nm in an UV cross-linker (Fisher Scientific, Ottawa, Ontario). In Southern blotting, 10 μL of the PCR product was separated by electrophoresis in a 1% agarose gel and transferred from the gel to Nytran membranes bidirectionally, as described by Smith and Summers (19). The hybridization and the colorimetric and chemiluminescence detections were done using the Digoxigenin Detection Kit (Boehringer Mannheim) following the manufacturer's instructions.

RESULTS

Different concentrations of the main chemical components of the PCR and the cycling parameters were compared using genomic FAdV-8 DNA. The results showed that the best conditions for this PCR were as follows: 50 μL of reaction mix containing 0.8 pmol of each primer, 3 mM $MgCl_2$, 0.2 mM dNTP, 3 U Taq DNA polymerase and 1 × PCR buffer (GIBCO-BRL). The optimal cycling parameters were: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s, and the final cycle followed by a long extension at 72°C for 7 min. The size of the amplified DNA fragment from FAdV-8 was approximately 1.4 kb (Fig. 2A) which was in good agreement with the calculated size of 1406 bp based on the sequence data.

When the PCR product was digested with *Hin*dIII 2 fragments of 1039 bp and 367 bp in size were detected by ethidium bromide staining (Fig. 2A, lane 1). Digestion of the amplicon with *Pst*I resulted in 2 fragments of 1266 and 140 bp in size (Fig. 2A, lane 2). The DNA fragments were transferred onto a Nytran membrane and probed with the digoxigenin-labeled *Hin*dIII E fragment. The probe hybridized to the undigested PCR product and to the larger fragments generated by these restriction enzymes (Fig. 2B).

Serial 10-fold dilutions of the purified FAdV-8 DNA (from 100 ng to 100 fg) were amplified and one fifth of each product was run in a 1% agarose gel and visualized by ethidium bromide staining. The bands of the expected size (1406 bp) were visible from 100 to 0.1 ng (100 pg) amount of the template (Fig. 3, panel A). When the PCR products were detected by a chemiluminescent reaction in a dot-blot assay, the lowest amount of the purified DNA detected was 0.01 ng (10 pg) (Fig. 3, panel B).

Serial 10-fold dilutions of DNA extracted from FAdV infected cells were also subjected to PCR. The results indicated that 250 infected cells could be detected in an agarose gel stained with ethidium bromide and 25 infected cells could be detected with the PCR followed by chemiluminescent detection (Fig. 4A).

The nucleic acids from FPV, ILTV, NDV and IBV infected cell lysates were extracted and amplified by the same PCR protocol as done for FAdV DNA. Only the FAdV DNA was amplified, whereas the other 4 viral nucleic acids and the uninfected cell lysate did not give visible bands and did not hybridize to the labeled probe (data not shown).

The CELO virus and 8 field isolates of FAdV were studied with these PCR primers and conditions. The agarose gel picture of the PCR products amplified from total DNA extracted from infected and uninfected cells is shown in Figure 4B. The field isolates of FAdV, except No. 6 (Fig. 4B, lane 8), all resulted in PCR products of similar size as the CELO virus product, about 1270 bp. No visible amplicon was detected from the uninfected CH-SAH cell culture. In Southern blot hybridization analysis, the bands of the PCR products could be detected using the *Hin*dIII E fragment of FAdV-8, as a digoxigenin-labeled probe (data not shown), but no hybridization signal was noticed for isolate No. 6.

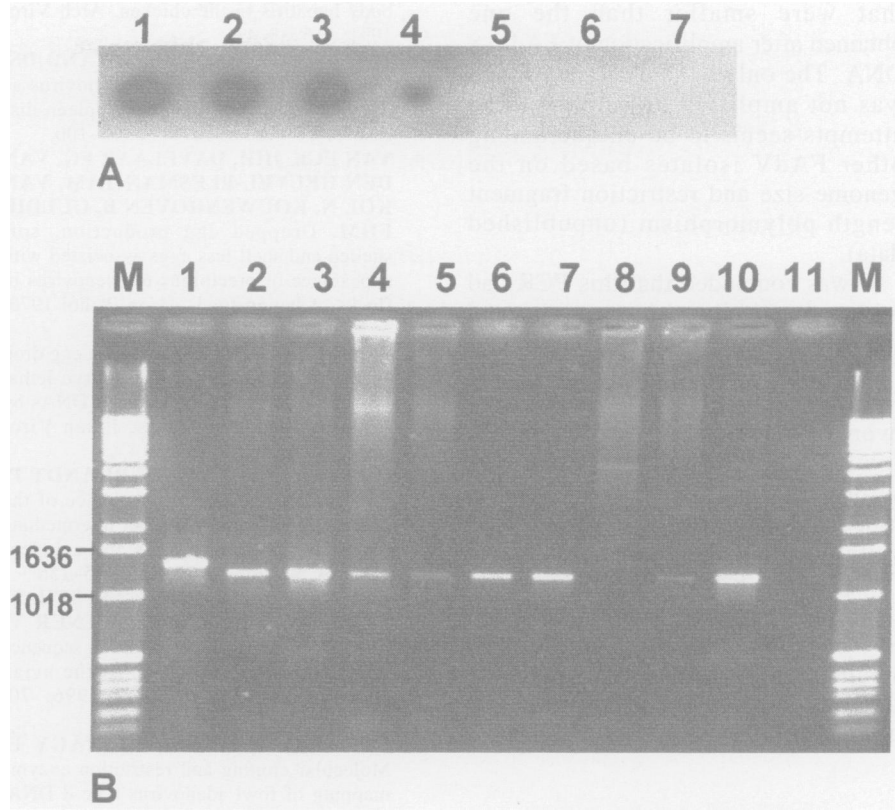

Figure 4. Panel A. Dot blot hybridization of PCR products from 10-fold serial dilution of DNA from FAdV-8 infected cell lysate; dots 1–6 represent: 25 000, 2500, 250, 25, 2.5, and 0.25 infected cells, respectively; dot 7 represents uninfected cells. The digoxigenin-labeled *Hin*dIII E fragment was used as a probe. Panel B. Results of PCR amplification of DNA samples extracted from cells that were infected with different fowl adenovirus isolates. Lane 1: FAdV-8; lane 2: CELO virus; lanes 3- 10: field isolates from 1 to 8, respectively; lane 11: uninfected cells. M: 1 kb DNA ladder, sizes are given in bp.

DISCUSSION

PCR is a simple method for amplifying a specific DNA fragment. It has been widely used for the diagnosis of avian virus infections (15,20–22), but it has not been reported so far for fowl adenoviruses. The primers selected for this study were designed to amplify a fragment of viral DNA located within pIIIa and pIII gene sequences in both FAdV-8 and CELO virus genomes.

This study demonstrated that the primer set designed from the FAdV-8 and CELO virus DNA was suitable for the detection of a number of different FAdV isolates. This primer set was also shown to be specific for FAdV, because it did not amplify other avian viruses or uninfected cellular DNA, and the product from FAdV-8 carried the *Pst*I and *Hin*dIII restriction enzyme sites at the positions which were expected according to the physical map of FAdV-8 (8,18). The labeled FAdV-8 *Hin*dIII E fragment also hybridized specifically to the amplified DNA fragments.

The limit of detection of this PCR was in the picogram range, but it could be increased by one log, if it was combined with dot-blot DNA hybridization using the digoxigenin-labeled probe. The lowest number of infected cells which could be detected with this PCR and DNA hybridization was 25, which may be important in the diagnosis of subclinical cases.

This PCR could also distinguish FAdV-8 from the CELO virus, because the size of the PCR product generated from FAdV-8 was larger than that from the CELO virus. The field isolates of FAdV included in this study were different from FAdV-8 based on the size of the PCR products

that were smaller than the one obtained after amplification of FAdV-8 DNA. The only isolate (No. 6) which was not amplified despite repeated attempts seems to be unique among other FAdV isolates based on the genome size and restriction fragment length polymorphism (unpublished data).

It was concluded that this PCR had a high specificity and sensitivity and it could be used for the detection of fowl adenoviruses, especially for FAdV-8 and the CELO virus. However, because there are many other fowl adenovirus serotypes, other serotypes should be tested with this procedure, to determine if this PCR is suitable for distinguishing FAdV-8 from other FAdV strains besides CELO virus. It will also be important to determine in the future which tissue samples of the infected chickens are the most appropriate for the detection of FAdV DNA.

ACKNOWLEDGMENTS

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