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Adenovirus of psittacine birds: Investigations on isolation and development of a real-time PCR for specific detection

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Adenovirus of psittacine birds: Investigations on isolation and development of a real-time PCR for specific detection

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Abstract

Liver samples of psittacine birds with a histological suspicion of an adenovirus infection, confirmed by electron microscopy examination, were subjected to virus isolation attempts using a heterologous and a homologous cell culture system in the form of chicken embryo liver (CEL) cells and psittacine embryo fibroblasts (PsEF), respectively. Whereas isolation in CEL cells failed, virus was isolated successfully in the PsEF cell culture system. Molecular investigations identified the virus as a specific psittacine adenovirus (PsAdV). Additionally, on the basis of the hexon gene sequence data obtained, a real-time PCR for specific detection of PsAdV was developed. To ensure an exclusive hybridisation with PsAdV selected primers were located within the variable L1 region of the hexon gene. Furthermore, the specificity of the real-time PCR was confirmed by investigation of a panel of different avian adenoviruses and unrelated DNA viruses. Using this PCR the threshold cycle values obtained support the propagation of PsAdV in the homologous cell culture system in comparison to the chicken cell culture system. Moreover, the developed PCR represents a reliable method for specific and sensitive detection of PsAdV in clinical samples.

Introduction

Avian adenoviruses are common infectious agents in a wide variety of avian species. They are members of the three genera *Aviadenovirus*, *Siadenovirus* and *Atadenovirus* within the family *Adenoviridae* (Benkő *et al.*, 2005). Most of the characterized adenoviruses isolated from chicken, turkeys, and other birds, which were previously referred as to group I avian adenoviruses, are currently classified into the genus *Aviadenovirus*. This genus comprised the five species *fowl adenovirus* (FAdV) *A*, *B*, *C*, *D*, and *E* with 12 serotypes as well as *goose adenovirus* (Benkő *et al.*, 2005). Further tentative species are duck adenovirus B, pigeon adenovirus and turkey adenovirus B. Adenoviruses of this genus are widely distributed all over the world and domestic avian species of all ages are susceptible (McFerran & Adair, 2003). However, their role in clinical diseases is not well defined. They have been associated amongst other conditions with inclusion body hepatitis in several avian species, hydropericardium syndrome in chicken and pigeons, and bronchitis in quail. However, they are also frequently present in apparently healthy birds (McFerran & Adair, 2003). In contrast, turkey adenovirus 3 (TAdV-3) and egg drop syndrome virus (EDSV), which are the most important adenoviruses causing significant diseases are members of the new genera *Siadenovirus* and *Atadenovirus*, formerly group II and III avian adenoviruses, respectively. Infections with adenovirus (Capua *et al.*, 1995; Gassmann *et al.*, 1981; Hess *et al.*, 2000; Ramis *et al.*, 1994; Soike *et al.*, 1998; Weissenböck & Fuchs, 1995) or adenovirus-like particles (Gomezvillamandos *et al.*, 1995; Mackie *et al.*, 2003; Mori *et al.*, 1989; Pass, 1987; Scott *et al.*, 1986) have been also described in a variety of psittacine birds. Most infections were associated with necrotizing hepatitis and basophilic inclusion bodies in hepatocytes caused by different serotypes of FAdV. Recently, an adenovirus detected from psittacine birds

was identified by its hexon loop 1 region sequence as a new avian adenovirus belonging to the group I and it was proposed to designate this virus as psittacine adenovirus (PsAdV) (Raue *et al.*, 2005). The diagnosis of adenovirus infection in psittacine birds is in most cases based on histological investigations and the observation of intranuclear inclusion bodies in hepatocytes. In the present study liver samples of 31 dead psittacine birds with a history of adenovirus-like infection were examined for virus isolation using chicken embryo liver (CEL) cell culture and psittacine embryo fibroblast (PsEF) cell culture. In addition, on the base of the sequence data obtained, which revealed a high degree of homology to the new adenovirus PsAdV, a molecular diagnostic method using real-time polymerase chain reaction (PCR) was developed for rapid, specific, and sensitive detection of PsAdV.

Materials and Methods

Field samples. Thirty-one liver samples originating from psittacine birds that died in a large aviary were used in this study. On *post mortem* examination livers of most birds were enlarged with multiple necrosis. Several livers demonstrated small white nodules scattered throughout the parenchyma. Histopathological examinations revealed hepatic damage. Large intranuclear inclusion bodies (IIB), typical for adenovirus infection were detected in the hepatocytes (Table 1). 16 liver samples were collected within 2 different time periods during an acute disease (GB 440-1 to -10 and GB 818-1 to -6). In addition, 15 liver samples from a third period were pooled with regard to the species and were examined in four pools (GB 943-pool 1 to -pool 4).

Virus detection by negative staining electron microscopy. For negative staining electron microscopy liver homogenates of ten birds were diluted 1:5 in phosphate buffered saline (PBS), sonicated for 40s at 20 kHz and clarified for 15 min at 3000 g. The supernatant was used for direct preparation and particle enrichment. A 400 mesh Zapon covered and carbon coated copper grid was placed on a drop of the supernatant for 7 min. Following removal of excess liquid and a washing step in distilled water the specimen was stained with 2% phosphotungstic acid (PTA) in PBS (pH 6.0) for 7 min and air dried. For particle enrichment the supernatant was centrifuged for 12 min at 120000g directly on the microscopic grid using an air-driven ultracentrifuge (Beckman Airfuge) with an A-110 Rotor. The enriched specimen was then stained as described above. Specimen grids were examined with a Jeol JEM 1010 electron microscope at 40 000-fold magnification and 80 kV.

Virus isolation. Monolayers of specific pathogen free (SPF) CEL cells as well as of PsEF cells were employed to attempt virus isolation. To prepare the specimens for inoculation liver pieces were homogenised in PBS as a 20% solution. The homogenised tissues were frozen and thawed three times, centrifuged at 1200 rpm for 15 min and the supernatants were filtered through 0.45 and 0.2 µm filters. Freshly prepared monolayers of CEL cells from 10-12 day-old embryonated SPF chicken eggs (Lohmann, Cuxhaven, Germany) were inoculated and incubated at 37°C under an atmosphere of 5% CO₂ (first passage-P1). The inoculated monolayers were examined daily for cytopathic effect (CPE) up to 7 days. Thereafter, inoculated cells were disrupted by three freeze - thaw cycles and centrifuged at 1200 rpm for 15 min. Supernatant was inoculated onto freshly prepared CEL cell monolayers (second passage-P2). A total of 11 blind passages was carried out in all this experiments. PsEF cell cultures cells were prepared from embryonated eggs of crimson rosellas (*Platycercus elegans*) and red-fronted parakeets (*Cyanoramphus novaeseelandiae*) on the 24th day of incubation. Cell

monolayer was inoculated as described for CEL cells. Cells were inspected daily for CPE up to 14 days and blind passages were also carried out as described for CEL cells. Due to restricted availability of embryonated psittacine eggs investigations were performed only for two selected specimens until the 6th passage.

Identification of the virus within CEL cells by the indirect immune fluorescence

technique (IIFT). The IIFT examination was carried out as described by Adair *et al.* (1980).

Primary CEL cells grown on cover slips within cell culture dishes were inoculated with the first passage of samples on CEL cells. Uninfected cells were used as controls. After 24h incubation at 37°C with 5% CO₂, inoculated monolayers were washed with PBS and then fixed with acetone. To each cover slip 100µl of specific antiserum was added, which was prepared in our institute in SPF chickens according to the method described (Mazaheri *et al.*, 1998) using FAdV-1 (CELO virus) antigen, and it was directed against group 1 avian adenoviruses. The cover slips were incubated for 60 min at room temperature and then washed three times with PBS. 100µl fluorescence isothiocyanate-labelled goat anti chicken antibody (1:40) was added and after 60 min the cover slips were washed three times with PBS. The presence of fluorescence was observed with a fluorescence microscope.

DNA extraction. DNA for conventional as well as for real-time PCR was extracted from liver tissues and infected cells by the QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer with minor modifications as described previously (Lüschow *et al.*, 2004). For the four pooled liver samples GB 943 pool 1 to pool 4 liver pieces homogenised in 20% PBS solution were taken for DNA extraction. As template for real-time PCR DNA extracted from liver tissues GB 818 1-6 was diluted 1:200 in nuclease free water.

Detection of adenovirus DNA by conventional PCR system. For detection of adenovirus DNA a conventional PCR system employing the primer pairs H1/H2 and H3/H4 and amplifying fragments of approximately 1200 bp or 1320 bp, respectively, within the hexon gene of FAdV was used as described (Raue & Hess, 1998). For further characterisation the obtained H1/H2 PCR products were cleaved with *HaeII* restriction enzyme by following the manufacturer's instructions (New England Biolabs, Frankfurt am Main, Germany). The received restriction profiles were compared according to Raue & Hess (1998) with those of 12 reference strains of the different FAdV serotypes.

DNA sequencing. For sequencing the H1/H2 PCR product of one liver sample was gel purified using the Invisorb Spin DNA Extraction Kit according to the manufacturer's instruction (Invitek, Berlin, Germany). The purified PCR product was sequenced directly in a commercial DNA sequencing service (Agowa, Berlin, Germany) using the primers H1 and H2. Homology search was performed using the Blast program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence comparison and alignment was carried out using the program Clustal W (<http://www.ebi.ac.uk/clustalw/index.html>) (Thompson *et al.*, 1994). The obtained nucleotide sequence was compared with the following partial hexon gene sequences of avian adenoviruses retrieved from GenBank: FAdV species A-E comprising 12 serotypes (strain CELO: AF339914, strain SR48: AF508946, strain SR49: AF508948, strain KR5: AF508951, strain 340: AF508952, strain CR119: AF508954, strain YR36: AF508955, strain TR59: AF508956, strain 764: AF508958, strain C2B: AF508959, strain 380: AF339925), PsAdV1 (AY852270), EDSV (strain 127: Y09598), and turkey haemorrhagic enteritis virus (AF074946).

Specific PsAdV SYBR Green real-time PCR. Selection of specific PsAdV primers was supported by the software package Beacon Designer (Premier Biosoft, Palo Alto, CA). The chosen forward primer qPAV-1 (5' CACGAACGACACAAACACCAC 3') was located at nucleotides (nt) 168 to 190 of the PsAdV hexon gene (GenBank accession no: AY852270). The reverse primer qPAV-2 (5' CCGATACTCCTATGGCATTAAACG 3') corresponded to nt 313 to 291. Real-time PCR amplification was carried out in a total volume of 25 µl using the iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA) containing 0.4 µM of each primer and 2 µl DNA template extracted from liver samples or 5 µl DNA template extracted from cell culture material, respectively. The PCR was performed in the iCycler iQ™ Real-Time detection system (BioRad Laboratories, Hercules, CA) with the following protocol: initial denaturation for 3 min at 95°C followed by 40 cycles consisting of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 30 sec. The fluorescence data were collected during the elongation step. After termination of the reaction by a final extension step at 72°C for 5 min, a DNA melting curve was generated to verify the correct product by its specific melting temperature (T_m). Melting curve analysis was performed by heating at 95°C for 1 min, followed by cooling to 60°C and subsequent heating to 95°C in steps of 0.5°C. Samples were regarded as positive when both a measurable threshold cycle (C_t) and the expected T_m were seen. For preparation of a real-time PCR standard for determining assay analytic sensitivity and for use as positive control, liver sample GB 818-1 was amplified using Ready-To-Go™ PCR beads with the primer set qPAV-1/qPAV-2 according to the manufacturers instructions (GE Healthcare, Freiburg, Germany). The reaction conditions were the same as real-time PCR conditions. The PCR product was gel purified with the Invisorb Spin DNA Extraction Kit (Invitek, Berlin, Germany) and ligated into pGEM-T vector using pGEM-T Vector System II (Promega, Mannheim, Germany). After preparation of the recombinant plasmid with the QIAGEN Plasmid Midi Kit (Qiagen, Hilden,

Germany) a tenfold dilution series from 10^8 to 10^1 copies per reaction was tested in duplicate to determine the analytic sensitivity of the real-time PCR. To verify the specificity of the constructed primers DNA of the following 12 FAdV reference strains was used: strain Celo (FadV-1), strain SR48 (FadV-2), strain SR49 (FadV-3), strain KR5 (FadV-4), strain 340 (FadV-5), strain CR119 (FadV-6), strain YR36 (FadV-7), strain TR59 (FadV-8a), strain 764 (FadV-8b), strain A2 (FadV-9), strain C2B (FadV-10), strain UF71 (FadV-11). In addition, DNA of the avian adenoviruses TAdV-3 and EDSV-127, as well as of unrelated avian DNA viruses represented by infectious laryngotracheitis virus strain ASL, Marek's diseases virus strain CU-2, and fowlpox virus strain HP-B was included.

Accession number. The obtained partial hexon gene sequence of GB 818-3 was deposited in GenBank with accession number EF442329.

Results

Detection of adenovirus by electron microscopy. Ten original liver samples were investigated by electron microscopy. Adenovirus particles were detected in all samples (Table 1).

Virus isolation on chicken embryo liver (CEL) cells. 12 samples were negative over all passages in CEL cells (Table 1). Eight samples showed CPE after the first passage in CEL cells. These samples reacted with antibodies to adenovirus group I in the IIFT. However, in the second passage the CPE of these samples was clearly weaker, cells were rounded off, but

degeneration was not seen any more. After the third passage this CPE disappeared completely and until the eleventh passage no visible CPE was detected.

Virus isolation on psittacine embryo fibroblast (PsEF) cells. Original supernatants of the two specimens GB 818-3 and GB 818-4, which showed a CPE in the first passage in CEL cells, were selected for propagation on psittacine cell cultures. One of these samples (GB 818-3) developed adenovirus-typical changes within the second passage and cell degeneration was observed from the third passage. The other sample (GB 818-4) showed weak changes within the first and the second passage and a clear CPE appeared one passage later. CPE of both samples became stronger in the further passages (Table 2), indicating successful virus isolation from both investigated samples using psittacine cell cultures. Investigations by real-time PCR confirmed the identification as adenovirus for both isolates (see results below).

Unfortunately, no further passages or isolation attempts from other samples could be tried using psittacine cell cultures, because the availability of embryos for preparation of the cultures was very restricted. In order to produce larger virus quantities for further characterization, we tried to adapt the GB 818-3 virus isolated in PsEF cells to CEL cells. After 11 passages, no visible CPE could be observed and real-time PCR revealed negative results from the seventh passage onwards (Table 3). The isolated adenovirus could not be adapted on CEL cells.

Detection of adenovirus DNA by conventional PCR system. Initially we investigated whether adenovirus DNA could be detected in 6 original liver samples (GB 818-1 to -6) by the use of the conventional PCR system originally designed for detection of FAdV. Using the primer set H1/H2 five samples reacted positive, in some cases with a weak signal, and one sample reacted negative. Digestion of the obtained H1/H2 PCR products revealed for all

tested samples no *Hae*II restriction site. The use of primer set H3/H4 delivered very weak signals for two and negative results for four tested samples. Summarizing these results, adenovirus DNA was demonstrated in some original liver samples, but the detected adenovirus was distinguishable from FAdV reference strains (data not shown).

Therefore, the H1/H2 PCR product of liver samples GB 818-3 was sequenced and a fragment of 700 nucleotides (nt) of the obtained sequence was compared with partial hexon gene sequences (688 nt -715 nt) of other avian adenoviruses. This analysis revealed a similarity of 63% to 68% to sequences obtained from FAdV serotypes. Clearly lower similarities were found, with 15% for turkey hemorrhagic enteritis virus and 6% for EDSV. In contrast, sequence comparison of a fragment of 550 nt showed a very high (99%) degree of nucleotide homology to a recently detected specific psittacine adenovirus (Raue *et al.*, 2005).

Design of a PsAdV real-time PCR. On the basis of alignment data of the obtained sequence of GB 818-1 and Genbank sequences of different avian adenoviruses the primers qPAV-1 and qPAV-2 were constructed for specific detection of PsAdV. Both primers were located in the variable L1 region of the hexon gene for exclusive hybridisation with PsAdV and amplified a fragment of 146 bp. Using these primers the T_m value of real-time PCR product obtained from liver samples GB 818-1 was about 85°C. To ascertain the detection limit of the assay a tenfold dilution from 10^8 to 10^1 copies of the plasmid DNA GB 818-1 was tested. With the system used we were able to amplify DNA from about 10^8 to 10^2 copies per reaction with an efficiency of about 86 % (Figure 1). No amplification was observed by investigation of 12 FAdV strains representing the five species A to E, TAdV-3, and EDSV as well as of other avian DNA viruses.

Detection of PsAdV DNA by real-time PCR. Six original liver samples from the second time period (GB 818-1 to -6) as well as the four pooled samples from the third time period (GB 943-pool 1 to -pool 4) were investigated by real-time PCR. PsAdV DNA was amplified successfully in all tested samples, independently from the bird species (Table 1). In addition, for selected specimens (GB 440-4 to -6 and GB 818-3 to -4) different passages in CEL and PsEF cells, respectively, were investigated. In CEL cells which were used for propagation as well as for adaptation attempts PsAdV DNA was detected in most of the examined passages (Table 3), although a visible CPE was mostly not apparent. However, in early passages samples reacted positive at a low cycle threshold, indicative that high amounts of viruses were present in these samples. With an increasing number of passages the Ct values at which samples reacted positive increased also (Table 3), demonstrating an obvious decrease of virus amounts with each subsequent passage. In consequence, investigation of the latest passages revealed only very weak positive or negative results, respectively. Examination of PsEF cells showed amplification of PsAdV DNA in all tested passages with relative constant or alternating Ct values (Table 3), suggestive of a virus replication in these cells.

Discussion

The present study describes for the first time the successful isolation of a specific psittacine adenovirus using a homologous cell system as well as the development and application of a real-time PCR for specific and sensitive detection of PsAdV. Adenovirus infections in psittacine birds have been described many times. In most cases the laboratory diagnosis was carried out using conventional methods like histopathology (Desmidt *et al.*, 1991; Ramis *et al.*, 1992; Tsai *et al.*, 1994) or electron microscopy examination (Pass, 1987). These

techniques only provide the diagnosis of adenovirus infections, but they allowed no further characterization of the detected virus. In consequence, data about the biological properties and the classification of adenoviruses infecting psittacines are rare.

The first adenovirus isolation from such birds was described by McFerran *et al.* (1976). The viruses isolated from two exotic birds in chick kidney cell culture were identical with known FAdV serotypes 2 and 8, indicating the ability of avian adenoviruses to pass species boundaries. Further adenovirus isolates from psittacines were classified as FAdV 3 and 4 serotypes (Capua *et al.*, 1995; Soike *et al.*, 1998). Recently, a psittacine adenovirus belonging to group I avian adenovirus, but distinguishable from known FAdVs was described on the basis of molecular characterization by sequencing of the L1 region of the hexon gene, however attempts to isolate the virus failed (Raue *et al.*, 2005). A further psittacine adenovirus was identified by consensus PCR and sequence analysis of a region within the DNA polymerase gene (Wellehan *et al.*, 2005). Phylogenetic and comparative sequence analysis indicated also a classification of this virus as a member of the genus *Aviadenovirus* (former group I avian adenovirus) and it was termed as Meyer's parrot adenovirus 1 (Wellehan *et al.*, 2005).

Such molecular techniques have proved to be effective and common for identification of new viruses in recent times, nevertheless a definitive diagnosis of viral infection has relied upon virus isolation. Although the use of a homologous cell culture system was reported to be preferable (McFerran & Adair, 2003) avian adenoviruses have also been isolated successfully from different species of birds using chicken cells (Bouquet *et al.*, 1982; Gassmann *et al.*, 1981; Hess *et al.*, 1998; McFerran *et al.*, 1976; Vereecken *et al.*, 1998). In the present study the suspicion of adenovirus infection was confirmed by electron microscopic examination. However, attempts to isolate the adenovirus in CEL cells failed. Nevertheless, reaction with antibodies to adenovirus group I in the IIFT showed that virus from some original samples

was able to infect the cells, but no visible virus propagation was found after further passages in CEL cells. On the other hand in a homologous cell culture system in the form of PsEF cells, virus was isolated from both investigated samples, which was demonstrated by the production of a CPE which became consequently stronger with subsequent passage. Such a poor replication capability of avian adenoviruses in heterologous cell culture systems was also found for a falconid adenovirus, which was isolated only in cell cultures of falcon origin (Oaks *et al.*, 2005). Also Zsivanovits *et al.* (2006) reported unsuccessful isolation attempts of an adenovirus from raptors in CEL cells, whereas PCR gave positive results.

Altogether the findings obtained from virus isolation in this study were suggestive for an adenovirus naturally occurring in psittacine birds and closely related to group I avian adenoviruses. Unfortunately, attempts to produce larger virus quantities by adaptation of one of the isolates in CEL cells failed. This would be an important prerequisite for further virological characterisation as well as for performance of serologic assays. Nevertheless, by molecular investigations the virus was identified in agreement with Raue *et al.* (2005) as specific PsAdV, which infects different species of psittacine birds. The data from the newly established real-time PCR analysis demonstrated the presence of PsAdV specific DNA in most of the investigated cell culture passages and the evaluation of Ct values supports the successful propagation of the virus in the homologous cell culture system in comparison to the chicken cells. Furthermore, due to the restricted availability of embryonated psittacine eggs and the failure of propagation in cells of chicken origin, virus isolation is not practicable for routine diagnosis.

The electron microscopic examination, which is in principle a suitable diagnostic method, has a relative low sensitivity in comparison with other techniques like cell culture detection or nucleic acid amplification (Biel & Gelderblom, 1999). However, the real-time PCR developed in this study proved to be a reliable method for specific and sensitive

detection of PsAdV in clinical samples. As shown, PsAdV specific DNA was amplified successfully in all the original liver samples examined. To ensure the specificity of this assay the chosen primers were located within L1 region, representing the most variable region of the hexon gene (Raue *et al.*, 2005; Raue & Hess, 1998). In addition, analysis of a panel of different avian adenoviruses as well as of further unrelated avian DNA viruses, which revealed in all cases negative results, confirmed the high specificity of the primers used. In conclusion, the established real-time PCR has been shown to be a useful method for rapid and sensitive detection of PsAdV which can be used for diagnostic purposes as well as for epidemiological studies in the future.

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Table 1. Preliminary report of samples used in the study and results of adenovirus detection in original liver tissue as well as of virus isolation in chicken embryo liver cells

Lab no	Preliminary report				Detection in liver tissue		CPE [†] in CEL cells		
	Species	Gender	Age	Histopathological findings for the liver	EM ^d	PCR ^e	P1	P2	P3 - P11
440-1	<i>Psittacidae</i> ^a	Unknown	Unknown	No detail data available	+	ND ^g	-	-	-
440-2	<i>Poicephalus robustus</i>	Female	Adult	Multiple necrotic foci basophilic IIB ^c	+	ND	?	?	-
440-3	<i>Poicephalus cryptoxanthus</i>	Male	Adult	Many IIB	+	ND	?	?	-
440-4	<i>Poicephalus senegalus</i>	Female	6 weeks	Hyperaemia, multiple necrotic foci, basophilic IIB	+	ND	+	?	-
440-5	<i>Cacatua laedbeateri</i>	Unknown	Unknown	Hyperaemia, multiple necrotic foci, basophilic IIB	+	ND	+	?	-
440-6	<i>Poicephalus ruePELLI</i>	Female	Adult	Hyperaemia, multiple necrotic foci, few basophilic IIB	ND	ND	++	+	-
440-7	<i>Poicephalus robustus</i>	Male	Adult	Inclusion bodies in the liver	ND	ND	?	?	-
440-8	<i>Psittacidae</i> ^a	Unknown	Unknown	No detail data available	+	ND	+	?	-
440-9	<i>Cacatua laedbeateri</i>	Female	4 weeks	Hyperaemia, parenchyma heavily destroyed, many IIB	ND	ND	+	?	-
440-10	<i>Poicephalus meyeri</i>	Female	8 weeks	Hyperaemia, multiple necrotic foci, basophilic IIB	ND	ND	++	?	-
818-1	<i>Psittacidae</i> ^a	Unknown	Unknown	No detail data available	+	+	?	-	-
818-2	<i>Pyrrhura rupicola sandiae</i>	Male	6 weeks	Hyperaemia, very few small necrotic foci, few IIB, perivascular infiltrations with mainly mononuclear cells	+	+	?	-	-
818-3	<i>Psittacula alexandri abbotti</i>	Unknown	6 weeks	Hyperaemia, multiple necrotic foci, basophilic IIB	ND	+	+	?	-
818-4	<i>Amazona aestiva xanthopteryx</i>	Male	8 weeks	Hyperaemia, multiple necrotic foci, basophilic IIB	+	+	++	?	-
818-5	<i>Psittacidae</i> ^a	Unknown	Unknown	No detail data available	ND	+	?	-	-

818-6	<i>Psittacidae</i> ^a	Unknown	Unknown	No detail data available	+	+	?	-	-
943-pool 1	<i>Psittacula</i> ^b	Unknown	Unknown	No detail data available	ND	+	-	-	-
943-pool 2	<i>Psittacula</i> ^b	Unknown	Unknown	No detail data available	ND	+	-	-	-
943-pool 3	<i>Amazona</i> ^b	Unknown	Unknown	No detail data available	ND	+	-	-	-
943-pool 4	<i>Amazona</i> ^b	Unknown	Unknown	No detail data available	ND	+	-	-	-

^a Belonging to the family *Psittacidae*, genus and species unknown

^b Belonging to the genus *Psittacula* or *Amazona*, respectively, species unknown

^c IIB, intranuclear inclusion bodies

^d Detection of adenovirus by electron microscopy

^e Detection of PsAdV specific DNA by real-time PCR

^f Judgement of CPE: - = no difference to the negative control; ? = cells are not destroyed but different to the negative control; + = rounded cells without cell lyses; ++ = rounded cells with detachment

^g ND, not done

Table 2. *Results of virus isolation in psittacine embryo fibroblast cells*

Lab no.	Appearance of cytopathic effect ^a in PsEF cells					
	P1 ^b	P2 ^b	P3 ^b	P4 ^b	P5 ^b	P6 ^b
GB 818-3	?	+	++	++	++	++
GB 818-4	?	?	+	++	++	++

^a Judgement of CPE: ? = cells are not destroyed but different to the negative control; + = rounded cells without cell lyses; ++ = rounded cells with detachment

^b Passage no.

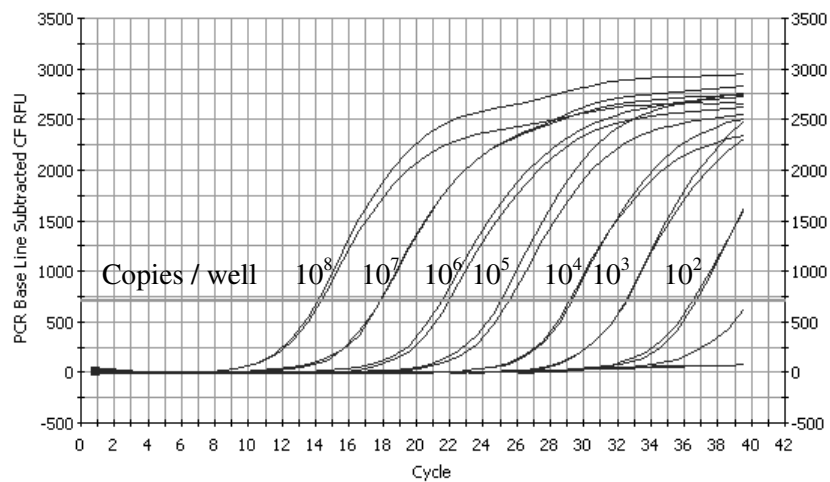
Table 3. *Amplification of psittacine adenovirus specific DNA by real-time PCR in selected passages of different cell cultures*

Lab no.	PsAdV DNA amplification in CEL cells (Ct ^b value)						
	P1 ^a	P5 ^a	P6 ^a	P7 ^a	P9 ^a	P10 ^a	P11 ^a
GB 440-4	14.9	ND ^c	ND	22.9	ND	ND	35.8
GB 440-5	15.5	ND	ND	24.8	ND	ND	37.9
GB 440-6	14.7	ND	ND	23.7	ND	ND	37.4
GB 818-3	10.9	15.6	20.1	ND	31.5	36.3	ND
GB 818-4	15.4	29.8	35.6	ND	Negative	Negative	ND
PsAdV DNA amplification in PsEF cells (Ct value)							
	P1 ^a	P4 ^a	P5 ^a	P6 ^a			
GB 818-3	13	12.2	15.1	14.1			
GB 818-4	16.1	20.6	24.4	22.8			
PsAdV DNA amplification after adaption on CEL cells (Ct value)							
	P1 ^a	P2 ^a	P5 ^a	P7/P9/P11 ^a			
GB 818-3	17.8	22.7	30.8	Negative			

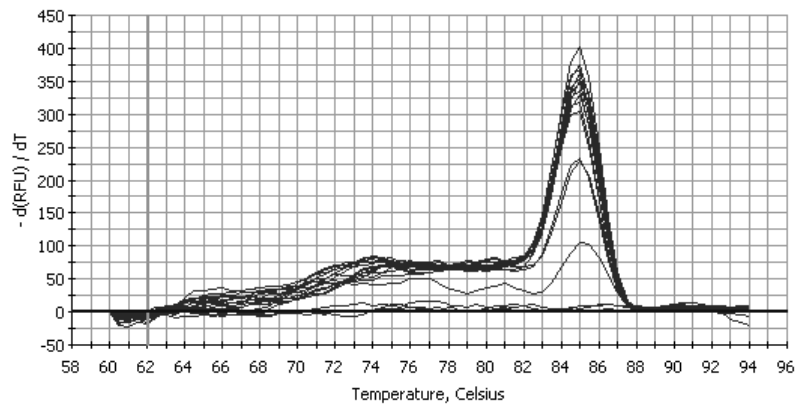
^a Passage no.

^b threshold cycle

^c ND, not done



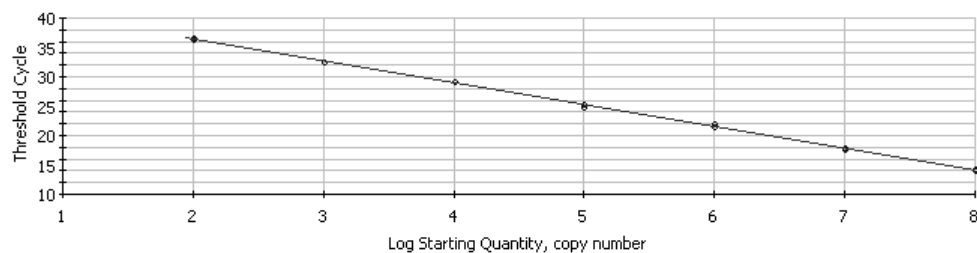
(a)



(b)

Correlation Coefficient: 1.000 Slope: -3.715 Intercept: 43.897 $Y = -3.715 X + 43.897$
 PCR Efficiency: 85.9 %

□ Unknowns
 ○ Standards



(c)

Figure 1. Determination of analytical sensitivity of PsAdV real-time PCR based on a tenfold dilution series of recombinant plasmid-DNA. (a) Amplification plot. (b) Melting curve. (c) Associated standard curve graph.