Journal of Virological Methods 170 (2010) 147–154

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Protocols

Classification of fowl adenoviruses by use of phylogenetic analysis and high-resolution melting-curve analysis of the hexon L1 gene region

Ana Marek*, Ayse Günes, Evelyn Schulz, Michael Hess

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria

Article history:
Received 20 June 2010
Received in revised form
10 September 2010
Accepted 16 September 2010
Available online 24 September 2010

Keywords:
Fowl adenoviruses
Phylogenetic analysis
High-resolution melting-curve analysis
Hexon gene

A B S T R A C T

A total of 44 fowl adenovirus (FAdV) samples from 6 European countries, Pakistan, India, Kuwait, Mexico, Peru and Ecuador were used in this study and the phylogenetic analyses based on the loop 1 (L1) region of hexon gene were performed. For comparison, available hexon sequences of representatives of different FAdV species were also used. At least 12 genotypes within the five FAdV species (A–E) were revealed and the existence of these genotypes was supported by high bootstrap values. Furthermore, three primer pairs binding to the conserved pedestal regions (HexL1s/HexL1as and HexA/HexB) and pedestal (P1) region and loop 2 (L2) region (HexF1/HexR1) of the FAdV hexon gene were used for high-resolution melting (HRM)-curve analysis and results were compared with those of phylogenetic analyses. HRM-curve analysis based on the HexL1s/HexL1as region grouped all tested field isolates and reference strains into 22 subgroups, consistently with phylogenetic analysis. This method is a rapid and cost-effective alternative to existing serotype identification methods and offers a possibility to classify FAdV isolates more precisely. However, it has limitations such as need for extensive interpretation of results and potential for indeterminate results. Gaining of hexon sequences of further field isolates offers the potential for novel and additional information in analysis of the molecular epidemiology of FAdV.

1. Introduction

Fowl adenoviruses (FAdVs) belong to the family *Adenoviridae*, genus *Aviadenovirus*. The FAdVs are mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), respiratory tract disease and gizzard erosions (Adair and Fitzgerald, 2008).

The virus is diagnosed routinely by virus isolation in embryonated eggs or cell culture followed by PCR (Raue and Hess, 1998). FAdVs were grouped into five different species (A–E) and they are further subdivided into 12 serotypes (FAdV 1–8a and 8b–11) based on cross-neutralisation test (Hess, 2000; Benkö et al., 2005). Identification of the serotypes involved in infection is very useful for epidemiological tracing and important for vaccination in order to control a certain disease, mainly IBH. However, a relationship between serotype and virulence in FAdV has not been found (Dawson et al., 1980). Therefore, the designation of isolates into genotypes could be useful for analysis of the molecular epidemiology of FAdV.

PCR followed by restriction enzyme digestion or sequencing of the products allows the differentiation of field isolates to species and presumptive serotypes (Raue and Hess, 1998; Meulemans et al., 2001; Meulemans et al., 2004; Raue et al., 2005). Tests using PCR together with DNA sequencing are relatively time consuming, require extensive interpretation and are expensive to use as a routine typing tool. Recent research has found that the high-resolution melting (HRM)-curve analysis provides a useful and cost-effective alternative for large-scale research and diagnostic analyses. In addition, real-time PCR has gained wide acceptance due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. Very recently it was reported that HRM-curve analysis of HexL1s/HexL1as region could be used for the identification and differentiation of all 12 FAdV serotypes (Steer et al., 2009).

The aim of this study was to investigate the application of HRM-curve analysis of PCR amplicons from the hexon gene loop 1 (L1) region using a larger number of field isolates, and reference strains representing all species and different serotypes, and to establish a single closed-tube test method for FAdV typing. The results were compared with those of phylogenetic analysis based on the L1 region of hexon gene.

Abbreviations: FAdV, fowl adenovirus; FAdV 1-8a and 8b-11, fowl adenovirus serotypes 1-8a and 8b-11; L1, loop 1; L2, loop 2; P1, pedestal region 1; HRM, high-resolution melting; IBH, inclusion body hepatitis; CELO, chicken embryo lethal orphan; TAdV-3, turkey adenovirus 3; DAdV-1, duck adenovirus 1; PiAdV-1, pigeon adenovirus 1; NJ, neighbor-joining; HHS, hepatitis-hydropericardium syndrome.

* Corresponding author. Tel.: +43 1250775164; fax: +43 1250775192.
E-mail addresses: ana.marek@vetmeduni.ac.at (A. Marek), ayse.guenes@vetmeduni.ac.at (A. Günes), evelyn.schulz@vetmeduni.ac.at (E. Schulz), michael.hess@vetmeduni.ac.at (M. Hess).

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.09.019

2. Materials and methods

2.1. Source material

A total of 44 virus samples were used in this study. Some characteristics of samples used in the study are listed in Table 1. Reference FAdV strains CELO, SR48, SR49, KR5, 340, CR119, YR36, TR59, 764, A2–A, C2B and UF71 were also analysed.

2.2. Cell culture

The organ pools were homogenized in PBS containing Penicillin (100,000 IE/ml) and Streptomycin (1 mg/ml). Following 10 min centrifugation at 700 × *g* the supernatant was filtered using syringe filters with 0.2 μm pore size. Organ homogenates were used directly for DNA isolation and for inoculation of confluent monolayers of chicken embryo liver cells. The cells were incubated at

Table 1
List of samples used in this study.

Year and sample number	Country	Type of bird	Number of birds per pool	Material	Clinical/pathological observations	Accession number	Genotype
09/4889	Poland	broiler	10	intestine, cecal tonsil	increased mortality; fibrinous perikarditis, perihepatitis	FN869957	D3
08/10890	Germany	broiler	n.d.[a]	liver	IBH[b]	FN869958	D3
09/7498	Austria	broiler	8	liver	IBH	FN869959	D3
08/18735	Austria	broiler	2	liver	increased mortality; colispeticemia	s.i. to 09/7498[c]	D3
08/18926	Austria	broiler	6	liver	IBH	s.i. to 09/7498	D3
08/19507	Austria	broiler	1	liver	n.d.	s.i. to 09/7498	D3
08/18085	Poland	broiler breeder	3	liver	n.d.	FN869960	D3
09/8991	Hungary	chicken	4	intestine	lameness; swelling of tarsal joint	FN869961	D2
08/8872	Germany	broiler	5	liver	IBH	FN869962	D2
08/12809	Germany	broiler	4	liver	IBH	s.i. to 08/8872	D2
08/12811	Germany	broiler	n.d.	liver	IBH	s.i. to 08/8872	D2
08/12813	Germany	broiler	n.d.	liver	IBH	s.i. to 08/8872	D2
09/7190	UK	broiler	1	liver	IBH	FN869963	D2
08/9513	Germany	broiler	4	liver	IBH	s.i. to 09/7190	D2
Hungary6[d]	Hungary	n.d.	n.d.	n.d.	n.d.	FN869964	E3
09/8330	Hungary	chicken	3	joint swabs	necrosis of capitis femoris	FN869965	E3
09/8990	Hungary	chicken	4	intestine	necrosis of capitis femoris	FN869966	E2
08/17833	France	broiler	1	liver	IBH	FN869967	E2
08/17832	France	broiler breeder	1	liver	IBH	FN869968	E2
AG234[d]	Mexico	n.d.	n.d.	n.d.	HHS[e]	FN869969	C1
K388-95[d]	Mexico	broiler	n.d.	n.d.	HHS	s.i. to AG234	C1
K99-97[d]	Kuwait	broiler	n.d.	n.d.	HHS	FN869970	C1
Da60[d]	Germany	grey parrot	1	n.d.	IBH	FN869971	C1
K1013[d]	Ecuador	broiler	n.d.	n.d.	HHS	FN869972	C1
Peru53[d]	Peru	broiler	n.d.	n.d.	HHS	FN869973	C1
09/8846	Austria	chicken	n.d.	liver	n.d.	FN869974	C1
09/584	Austria	chicken	6	cecal tonsil	reduced growth; necrosis of capitis femoris	FN869975	C1
K31[d]	Pakistan	broiler	n.d.	n.d.	HHS	FN869976	C1
IV37[d]	India	broiler	n.d.	n.d.	HHS	s.i. to K31	C1
09/2602	Austria	chicken	10	joint swabs	swelling of tarsal joint	FN869977	C1
922/1[d]	Germany	chicken	n.d.	n.d.	IBH	FN869978	C1
09/1567	Germany	broiler	1	gizzard	gizzard erosion	FN869979	A1
09/7468	Hungary	chicken	1	liver, intestine	n.d.	FN869980	A1
09/7469	Hungary	chicken	1	liver, intestine	n.d.	FN869981	A1
09/7471-3	Hungary	chicken	5	cloacal swabs	n.d.	FN869982	A1
09/8234	Austria	chicken	4	cecal tonsil	increased mortality; swelling of tarsal joint	FN869983	A1
09/9962	Poland	broiler	n.d.	gizzard	gizzard erosion	FN869984	A1
09/9964	Poland	broiler	n.d.	gizzard	gizzard erosion	FN869985	A1
08/19729	Austria	broiler	3	liver	IBH	FN869986	A1
09/7470-2	Hungary	chicken	5	cloacal swabs	n.d	FN869987	B2
09/7473-2	Hungary	chicken	5	cloacal swabs	n.d.	FN869988	B2
08/21472	Austria	broiler	1	intestine	n.d.	FN869989	B2
08/8669	Austria	broiler	1	liver	IBH	FN869990	B2
09/6893	Hungary	chicken	1	joint swabs	lameness; swelling of tarsal joint	FN869991	B1

[a] n.d.: no data.
[b] IBH: inclusion body hepatitis.
[c] s.i.: sequence identical.
[d] Isolates were described earlier (Khanna, 1966; Hess et al., 1998, 1999, 2000; Schonewille et al., 2008).
[e] HHS: hepatitis-hydropericardium syndrome.

37 °C and 5% CO_2. After 4–5 days, the supernatants were collected and transferred onto new chicken embryo liver cells. The supernatants of obviously infected cell monolayers, showing cytopathic effect were also used for DNA isolation.

2.3. Extraction of viral DNA

Total DNA was isolated from 180 μl of tissue homogenates or cell culture supernatants using the DNeasy Blood and Tissue Kit (Qiagen, Vienna, Austria) according to manufacturer's instructions. DNA was eluted with 100 μl AE buffer.

2.4. Polymerase chain reaction

Diagnosis of FAdV was based on clinical signs and on detection of FAdV DNA in tissues using PCR for the L1 region of hexon gene (Meulemans et al., 2001). PCR was carried out using Taq-DNA-Polymerase (Invitrogen GmbH, Lofer, Austria) according to the manufacturer's recommendations. Each reaction contained 0.4 μM (final concentration) of each forward (HexA) and reverse (HexB) primer and 5 μl of DNA extract (corresponding to 20% of the total reaction volume). One cycle of 2 min at 94 °C was followed by 35 amplification cycles of 1 min at 94 °C, 1 min at 62 °C and 1 min 30 s at 72 °C and a final extension step for 2 min at 72 °C. Products of approximately 897 bp were visualized using UV light following electrophoresis and ethidium bromide staining.

After gel electrophoresis, PCR products were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Vienna, Austria) according to the manufacturer's recommendations. Eluted PCR products were directly sequenced in both directions. Sequencing services were provided by Eurofins MWG Operon, Ebersberg, Germany and by LGC Genomics, Berlin, Germany. Unique sequences were submitted to the GenBank database and the accession numbers are given in Table 1.

2.5. Sequence analysis

For sequence analysis, primer binding sites were excluded from analysis and fragments of the hexon gene were analysed. Assembly and analysis of sequences as well as the nucleotide sequence alignments were performed using Accelrys Gene version 2.5 (Accelrys, San Diego, CA), Lasergene (DNASTAR Inc., Madison, WI) and BioEdit (Tom Hall, Ibis Biosciences, Carlsbad, CA) software.

2.6. Phylogenetic analysis

For phylogenetic analysis, a 687 bp region (corresponding to 1–687 bp of the GenBank sequence AF339914) of the hexon gene was used. All reference FAdV strains used in this study had identical sequences within the HexA/HexB region to the sequences already available in the GenBank. A number of FAdV sequences have been published (Meulemans et al., 2001; Raue et al., 2005). Sequence of the pigeon adenovirus 1 (PiAdV-1, GenBank FN824512) was also included (Hess et al., 1998). Two isolates representing siadenoviruses (turkey adenovirus 3, TAdV-3) and atadenoviruses (duck adenovirus 1, DAdV-1) were used for comparison (Hess et al., 1997; Pitcovski et al., 1998). Phylogenetic analysis was performed using Accelrys Gene version 2.5 (Accelrys, San Diego, CA) and Lasergene (DNASTAR Inc., Madison, WI) software. Phylogenetic tree was generated by the NJ method as implemented in Lasergene (DNASTAR Inc., Madison, WI) software. Robustness of the tree was determined by bootstrapping of multiple-sequence alignments (1000 sets). An effort was made to give a divergence criterion for FAdV typing based on serotyping data on FAdVs. Grouping into the genotypes was based on a threshold value of 95% for nucleotide sequence identity. Phylogenetic tree was also generated by the NJ method on uncorrected *p*-distances on 1000 bootstrapped data sets and values of >70% were shown on the tree as branches when Accelrys Gene version 2.5 software (Accelrys, San Diego, CA) was used.

2.7. Real-time PCR and HRM-curve analysis

The primers binding to the conserved pedestal regions (HexL1s/HexL1as and HexA/HexB) and pedestal (P1) region and loop 2 (L2) region (HexF1/HexR1) of the FAdV hexon gene were used in this study (Meulemans et al., 2001; Mase et al., 2009; Steer et al., 2009). Amplification of target sequences was carried out using a Rotor-Gene Q thermal cycler (Qiagen, Vienna, Austria) and Type-it HRM PCR kit (Qiagen, Vienna, Austria) according to the manufacturer's recommendations. A 25 μl reaction mix contained 0.4 μM (final concentration) of each forward and reverse primer and 5 μl of DNA extract. One cycle of 5 min at 95 °C was followed by 45 amplification cycles of 10 s at 95 °C, 30 s at 55 °C and 24 s, 36 s or 32 s at 72 °C (for HexL1s/HexL1as, HexA/HexB and HexF1/HexR1, respectively). Optical measurements in green channel were recorded during the extension step. HRM-curve analysis was performed using increasing temperatures from 65 to 95 °C at intervals (ramps) of 0.1 °C with a hold of 2 s at each step. The conventional melt curves were generated automatically and analysis performed using Rotor-GeneQ series software version 1.7. Genotyping was further performed using the normalized HRM data and for HexL1s/HexL1as normalization regions 84.50–85.00 and 90.50–91.00 were used. In addition, PCR products were visualized using UV light following electrophoresis and ethidium bromide staining.

3. Results

3.1. Detection of FAdV by PCR and virus isolation

The oligonucleotide primer pairs used in this study specifically amplified an 897 bp region of hexon gene. It was possible to amplify these fragments of hexon genes from all samples used in this study (data not shown). In addition, all samples were also positive in virus isolation in cell culture. The samples positive for presence of FAdV originated from birds coming from UK, Poland, Germany, Hungary, France and Austria. In addition, several positive samples originated from Pakistan, India, Kuwait, Mexico, Peru and Ecuador (Table 1).

3.2. Phylogenetic analyses based on hexon gene sequences

In the present study, at least 12 genotypes were identified within the 5 FAdV species (A–E) based on hexon gene sequences (Fig. 1). In general, each FAdV species was composed of 1–4 genotypes with the strains PiAdV-1 and TR22 forming separate clusters (putative species F and G). Species A consisted of 1 genotype (A1), species B of 2 genotypes (B1–B2), species C of 1 genotype (C1), species D of 4 genotypes (D1–D4) and species E of 4 genotypes (E1–E4). The same division of FAdV isolates was observed when using different phylogenetic analyses (data not shown), and the existence of these genotypes was supported by bootstrap values of >70% (Fig. 1). Genotypes identified in this study could be separated further into smaller clusters and these clusters were also supported by high bootstrap values (data not shown).

Phylogenetic analysis did not show strict clustering of isolates according to clinical signs of disease. Within species A, for example, CELO strain, which does not induce gizzard erosions, was grouped within genotype A1 together with several isolates sampled from birds with gizzard erosions (Fig. 1, Table 1). No clear link could be observed between the geographical regions where the samples came from and phylogenetic relationship (Fig. 1, Table 1).

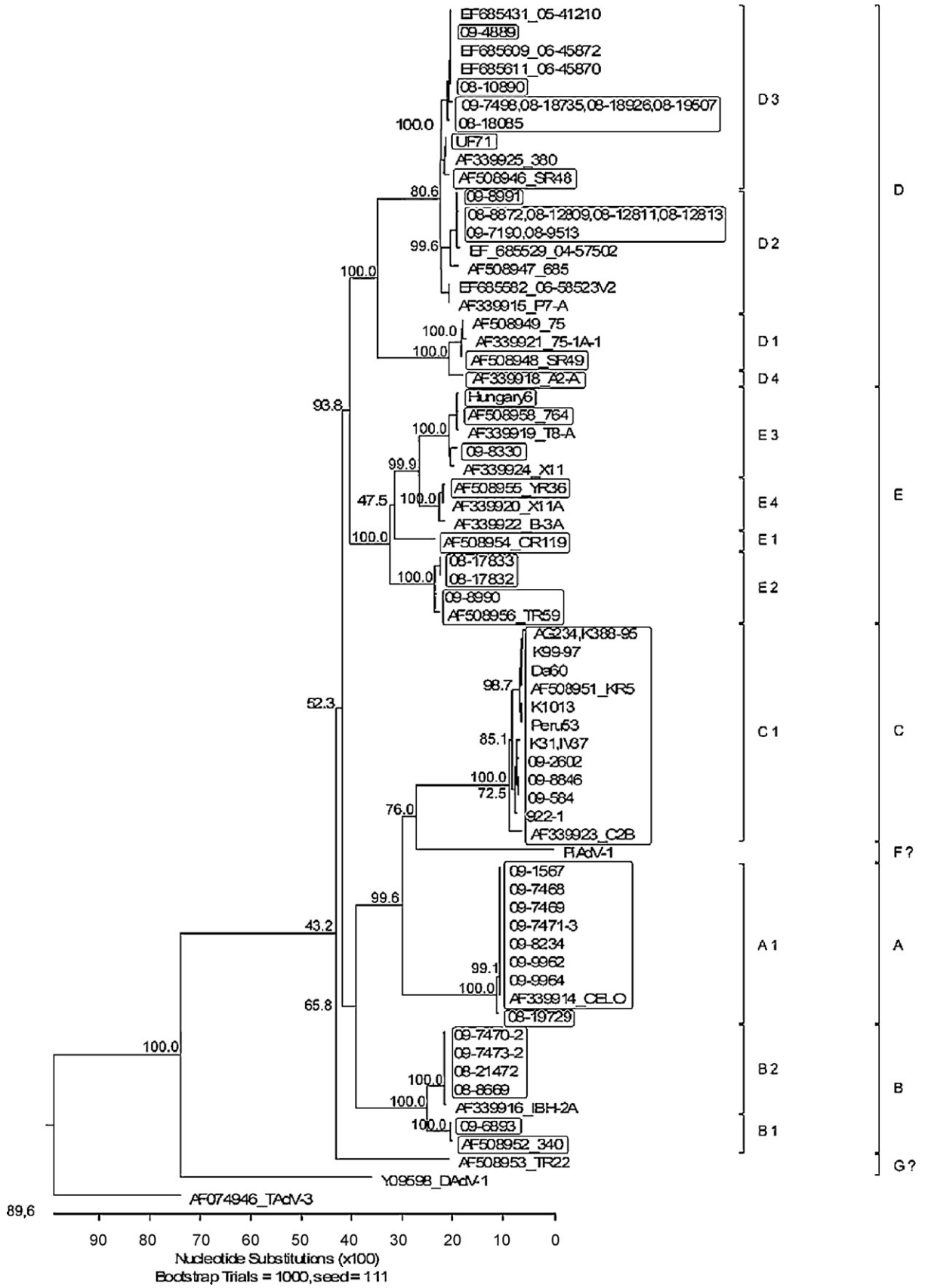

Fig. 1. Phylogenetic tree of FAdV isolates based on a 687 bp region of the hexon gene. The sequences used for comparison are already published sequences of FAdV strains, pigeon adenovirus 1 (PiAdV-1) and two isolates representing siadenoviruses (turkey adenovirus 3 (TAdV-3)) and atadenoviruses (duck adenovirus 1 (DAdV-1)) (Hess et al., 1997, 1998; Pitcovski et al., 1998; Meulemans et al., 2001; Raue et al., 2005). Phylogenetic tree was generated by the NJ method as implemented in Lasergene (DNASTAR Inc., Madison, WI) software. Robustness of the tree was determined by bootstrapping of multiple-sequence alignments (1000 sets). Bootstrap values are indicated for the major nodes. FAdV species are labelled with letters A–G, genotypes with A1, B1–B2, C1, D1–D4 and E1–E4 and 22 subgroups detected with HRM-curve analysis are circled.

3.3. HRM-curve analysis

With HexF1/HexR1 primer pair, representatives of 15 tested subgroups generated one or more major peaks and were visually distinct from each other in conventional melt curve profiles of 800 bp products. However, the amplification efficiencies were low compared to other two PCRs (<1.4) (data not shown).

HexA/HexB PCR products of the approximately 897 bp generated 23 different conventional melt curves containing one to three peaks between 84.8 °C and 88.8 °C. Thirty-nine tested field isolates and 12 reference strains were grouped within 23 FAdV subgroups

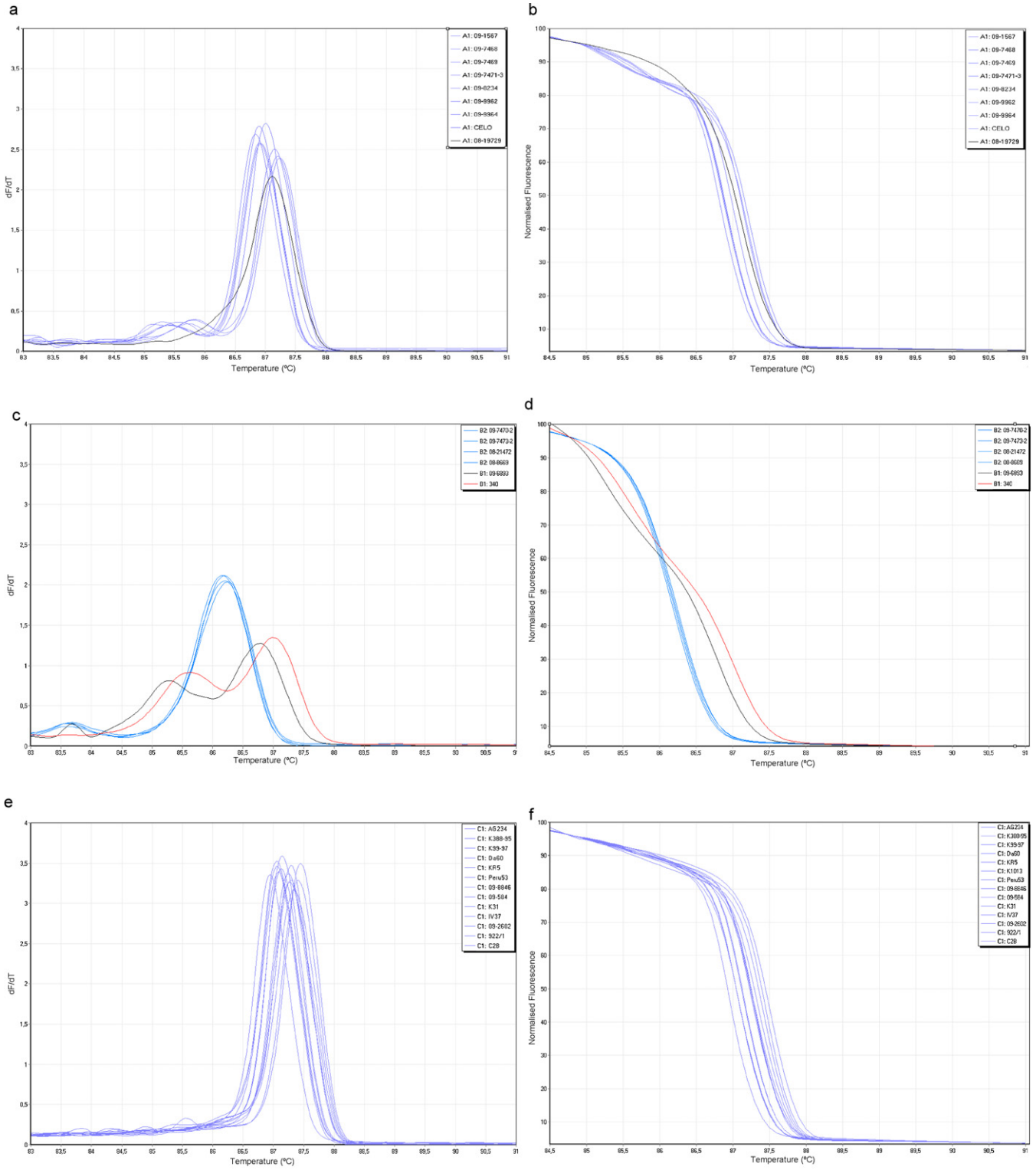

Fig. 2. (a, c, e, g, i) Conventional melt curves and (b, d, f, h, j) normalized HRM curves of HexL1s/HexL1as PCR products of FAdV species A–E. Genotypes are labelled with A1, B1–B2, C1, D1–D4 and E1–E4 and 22 subgroups detected with HRM-curve analysis are presented in different colours. (a, b) Species A, (c, d), species B, (e, f) species C, (g, h) species D and (i, j) species E.

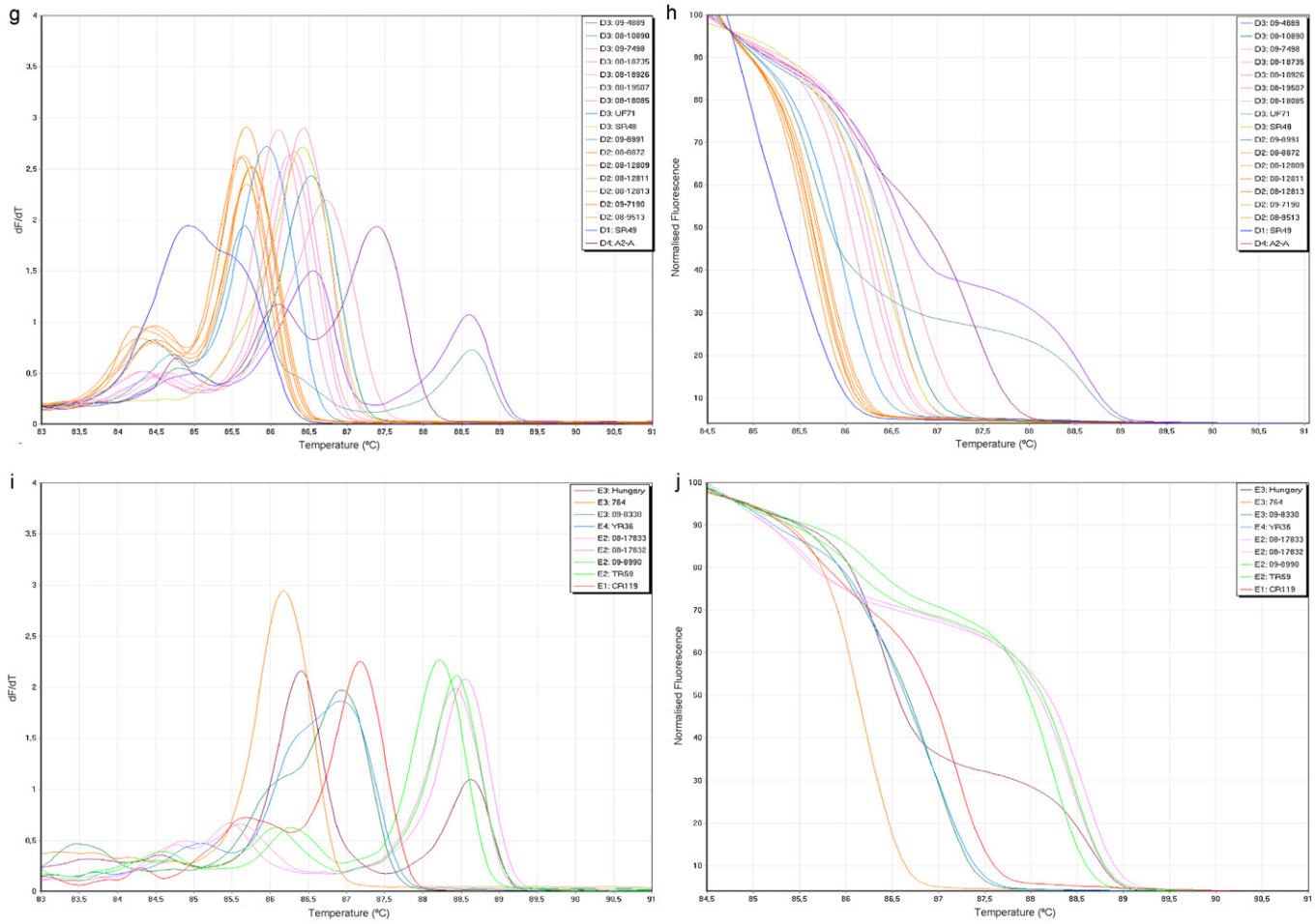

Fig. 2. *(Continued.)*

and in the normalized HRM graph, each subgroup displayed a distinct curve profile (data not shown). However, strain 340 showed low-level of amplification in the HexA/HexB PCR compared to other two PCRs and several additional field isolates belonging to species B were also analysed and showed the same result (data not shown).

In the conventional melt curve analysis of the approximately 590 bp HexL1s/HexL1as PCR product, all tested isolates generated 22 different curve forms with one to three peaks between 83.33 °C and 88.63 °C (Fig. 2). Examination of the PCR products by agarose gel electrophoresis resulted in a single stained band indicating that a single pure product was amplified (data not shown). In general, the melt curve profiles for each individual sample were consistently produced from PCR products generated on different days and from templates originating from different DNA extractions. HRM-curve analysis for both regions of the hexon gene (HexL1s/HexL1as and HexA/HexB) showed slight shifts in melting temperatures between PCRs that ran on different days using templates from different DNA extractions, however the conventional melt curve shapes were unchanged. For one subgroup maximal difference between the peak temperatures measured from different samples was 0.53 °C (data not shown). Using 44 field isolates and 12 reference strains, 22 visually distinct curve profiles were generated, making each subgroup easy to distinguish from others. In the normalized HRM graph, each subgroup displayed a distinct curve profile (Fig. 2). Differences in melt curve profiles correlated with phylogenetic analysis (Fig. 1). However, analysis of the conventional melt curves revealed that viruses belonging to the same genotype did not always generate identical patterns. Examination of the normalized HRM curves also revealed that the curves generated from viruses belonging to same genotype did not always resemble each other (Figs. 1 and 2). In addition, all analysed isolates belonging to species C showed the same curve profiles (Fig. 2e and f). No clear link could be established between the serotype and HRM-curve analysis. Therefore, HRM-curve analysis of HexL1s/HexL1as region could be used for the identification and differentiation of many more subgroups (22) than 12 FAdV serotypes or genotypes.

4. Discussion

This is the first report detecting and characterizing genetically FAdVs from larger number of field samples from various countries. More importantly, phylogenetic analyses indicate the existence of at least 12 genotypes within FAdV species A–E based on hexon gene sequences (Fig. 1). The existence of these genotypes was supported by high bootstrap values of >70%. Earlier, six clusters supported by high bootstrap values with the Japanese strain TR22 as a sole member of a seventh cluster were identified using phylogenetic analysis based on amino acid sequence of the L1 region of the hexon gene (Meulemans et al., 2004). Similar results were reported in other studies based on amino acid or nucleotide sequences (Raue et al., 2005; Ojkic et al., 2008). However, only a limited number of isolates were investigated, or they were not genetically diverse. The designation of FAdV isolates into at least 12 genotypes offers the potential for novel and additional information in analysis of FAdV molecular epidemiology. Phylogenetic analysis supports the distinction of PiAdV-1 from other FAdV species

(A–E), as reported earlier by cross-neutralisation tests (Hess et al., 1998).

The comparison of phylogenetic analysis to serotyping (cross-neutralisation test) was not conducted in this study as it is not applicable for typing of numerous clinical samples. In addition, cross neutralisation study for large number of FAdV field isolates is lacking until now. Since cross neutralisation tests are time consuming and can give indeterminate results (McFerran and Connor, 1977), it would be very useful to determine the prototype sequences for each FAdV type and precise genetic divergence criteria for FAdV typing. Therefore, efforts were made to determine the threshold values for nucleotide sequence identities for both FAdV species and genotypes. The lowest percent of nucleotide sequence identities between the analysed samples within the same species was 73%, and the highest percent of nucleotide sequence identities between the samples belonging to two different species was 71.7% (data not shown). Therefore, we would like to propose the nucleotide sequence identity of approximately 72% as a criterion for molecular identification of FAdV species. Based on this criterion, both PiAdV-1 and TR22 are members of new putative species (F and G) within FAdVs (data not shown). For PiAdV-1 this separation is also supported by serological data (Hess et al., 1998). Since the sequence of hexon region of isolate TR22 (Meulemans et al., 2004) shows $\leq$67.2% nucleotide sequence identity with other species B isolates used in this study (data not shown), and the serological studies showed contradictory results (McFerran et al., 1972; Grimes and King, 1977), serotyping of this isolate requires further investigations. Based on serotyping data on FAdVs (Benkö et al., 2005) an effort was made to give a divergence criterion for FAdV genotypes. The close relationship between FAdV-4 and FAdV-10 was also taken into consideration (Erny et al., 1995). Grouping into the genotypes was based on phylogenetic analyses and a threshold value of 95% for nucleotide sequence identity was implemented to differentiate genotypes since the highest percent of nucleotide sequence identities between samples representing two different serotypes (A2–A representing FAdV-9 and 75 representing FAdV-3) was 94.9% within the analysed region. However, 12 genotypes identified in this study overlap only partially with the reported 12 serotypes (Benkö et al., 2005). For example, species B comprises only 1 serotype (FAdV-5), but contains 2 genotypes (B1–B2). Species C is composed of 2 serotypes (FAdV-4 and 10), but contains only 1 genotype (C1). Interestingly, it was already suggested that FAdV serotypes 4 and 10 could be considered as subtypes of the same serotype (Erny et al., 1995). It is important to note that the genotypes identified in this study could be further separated into smaller clusters and these clusters were also supported by high bootstrap values. It is possible that new serotypes and genotypes will be determined when new samples are investigated and additional cross neutralisation study for FAdVs is conducted. Further studies could also lead to identification of the new threshold value for nucleotide sequence identities for FAdV genotypes.

In addition, this study provides a direct comparison between nucleotide sequencing coupled with phylogenetic analysis and HRM-curve analysis for detection of differences in the L1 region of the FAdV hexon gene for the identification and differentiation of FAdV genotypes. HRM-curve analysis was applied to amplicons of three different regions of the FAdV hexon gene, using a commercial PCR kit. Previously published primer pair H1/H2 (Raue and Hess, 1998) was not used since the amplified product contains both L1 and L2 regions and would probably contain numerous melting domains and therefore, the product would probably be unsuitable for HRM analysis (Steer et al., 2009).

Primer pair HexF1/HexR1 was shown to amplify DNA fragments from all groups (I–III) of avian adenoviruses (*Aviadenovirus*, *Siadenovirus* and *Atadenovirus*), nevertheless only a small number of FAdV strains were analysed (Mase et al., 2009). However, in this study, it was possible to amplify the expected fragment from representatives of all FAdV serotypes (data not shown), hence proving the universality of these primers. In addition, HRM-curve analysis of approximately 800 bp PCR products could differentiate 15 tested FAdV subgroups. However, as the amplification efficiencies were low compared to other two PCRs (data not shown), HRM-curve analysis of the HexF1/HexR1 PCR product was shown not to be suitable for typing of FAdV isolates.

With HexA/HexB primer pair, 23 subgroups were detected with one or more major peaks and were visually distinct from each other in conventional melt curve profiles. This result is in agreement with the recent study where representatives of 12 FAdV serotypes were used (Steer et al., 2009). Nevertheless, in that study, strain 340 showed low-level or no amplification in the HexA/HexB PCR, and the amount of product obtained was not sufficient for further analysis. On the contrary, in this study, a PCR product was successfully amplified using strain 340 and this is further supported by examination of the nucleotide sequence of the strain 340, which revealed no mismatch with the primers used. In addition, when using other isolates belonging to species B the amount of product obtained was sufficient for further analysis (data not shown). However, since strain 340 showed low-level of amplification in the HexA/HexB PCR compared to other two PCRs and several additional field isolates belonging to species B showed the same result, we decided not to use HRM-curve analysis of the HexA/HexB PCR product for genotyping of FAdV isolates.

The melt curve profiles generated using HexL1s/HexL1as primers were highly reproducible and all tested isolates consistently segregated into the same subgroups when analysis was based on HRM-curve analysis. Differences in melt curve profiles correlated with phylogenetic analysis (Figs. 1 and 2). Amplicons of 590 bp HexL1s/HexL1as region from FAdV genotype C1 (serotypes 4 and 10) could not easily be discriminated from each other by analysis of their HRM curves. However, recently it was reported that HRM-curve analysis of HexL1s/HexL1as region could be used for identification of all 12 FAdV serotypes and could differentiate isolates KR5 and C2B from each other (Steer et al., 2009). This difference may have resulted from different chemistries and different thermal cyclers used in PCRs. It should also be noted that melt curve profiles generated in this study were different to those obtained previously with the same primer pair and the same reference strains (Steer et al., 2009). In addition, while in the previous study only one reference strain belonging to each serotype (4 and 10) was used, in this study several additional field isolates were also analysed and showed the same result (Fig. 2e and f). It was suggested previously that FAdV serotypes 4 and 10 could be considered as subtypes of the same serotype (Erny et al., 1995).

Altogether, 44 field isolates and 12 reference strains representing 12 different serotypes were used in this study in order to analyse the usability of the method. Unfortunately, several reference strains, which clustered in separate genotypes based on phylogenetic analyses and the strain TR22, which formed a separate cluster from other species (A–E) were not available (Fig. 1), thus were omitted in HRM-curve analysis. However, the other techniques used in laboratories for FAdV typing at the moment have limitation such as lengthy of processes, higher costs or potential for carry-over contamination compared to HRM-curve analysis. Therefore, this method is a rapid and cost-effective addition to existing serotype identification methods and offers a possibility to classify FAdV isolates more precisely. However, the limitations of this method are the need for extensive interpretation of results and obtaining possible indeterminate results. Consequently, at the present stage it remains questionable if this method could completely substitute sequencing coupled with phylogenetic analysis.

To our knowledge, this is the first study that presents a comparison of genotyping technique based on a closed-tube method

of real-time PCR and HRM-curve analysis and sequencing coupled with phylogenetic analysis using a larger number of field isolates and reference strains representing all FAdV serotypes. The designation of FAdV isolates into at least 12 genotypes offers the potential for novel and additional information for the molecular epidemiology of FAdV.

Acknowledgements

The authors are thankful to all the colleagues who supplied us with tissue samples of fowl adenoviruses.

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. 252–266.

Benkö, M., Harrach, B., Both, G.W., Russell, W.C., Adair, B.M., Adam, E., 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.

Dawson, G.J., Orsi, L.N., Yates, V.J., Chang, P.W., Pronovost, A.D., 1980. An enzyme-linked immunosorbent assay for detection of antibodies to avian adenovirus and avian adenovirus-associated virus in chickens. Avian Dis. 24, 393–402.

Erny, K., Pallister, J., Sheppard, M., 1995. Immunological and molecular comparison of fowl adenovirus serotypes 4 and 10. Arch. Virol. 140, 491–501.

Grimes, T.M., King, D.J., 1977. Serotyping avian adenoviruses by a microneutralization procedure. Am. J. Vet. Res. 38, 317–321.

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

Hess, M., Blocker, H., Brandt, P., 1997. The complete nucleotide sequence of the egg drop syndrome virus: an intermediate between mastadenoviruses and aviadenoviruses. Virology 238, 145–156.

Hess, M., Prusas, C., Bergmann, V., Mazaheri, A., Raue, R., 2000. Epizootiology of fowls adenoviruses. Berl Munch. Tierarztl. Wochenschr. 113, 202–208.

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

Hess, M., Raue, R., Prusas, C., 1999. Epidemiological studies on fowl adenoviruses isolated from cases of infectious hydropericardium. Avian Pathol. 28, 433–439.

Khanna, P.N., 1966. Occurrence of avian adenoviruses in Hungary. Acta Veterinaria Academiae Hungaricae 16, 351–356.

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. J. Vet. Med. Sci. 71, 1239–1242.

McFerran, J.B., Clarke, J.K., Connor, T.J., 1972. Serological classification of avian adenoviruses. Arch. Gesamte Virusforsch. 39, 132–139.

McFerran, J.B., Connor, T.J., 1977. Further studies on the classification of fowl adenoviruses. Avian Dis. 21, 585–595.

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 Pathol. 30, 655–660.

Meulemans, G., Couvreur, B., Decaesstecker, M., Boschmans, M., van den Berg, T.P., 2004. Phylogenetic analysis of fowl adenoviruses. Avian Pathol. 33, 164–170.

Ojkic, D., Martin, E., Swinton, J., Vaillancourt, J.P., Boulianne, M., Gomis, S., 2008. Genotyping of Canadian isolates of fowl adenoviruses. Avian Pathol. 37, 95–100.

Pitcovski, J., Mualem, M., Rei-Koren, Z., Krispel, S., Shmueli, E., Peretz, Y., Gutter, B., Gallili, G.E., Michael, A., Goldberg, D., 1998. The complete DNA sequence and genome organization of the avian adenovirus, hemorrhagic enteritis virus. Virology 249, 307–315.

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

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. J. Virol. Methods 73, 211–217.

Schonewille, E., Singh, A., Göbel, T., Gerner, W., Saalmüller, A., Hess, M., 2008. Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in specific pathogen-free chickens following experimental infection. Vet. Immunol. Immunopathol. 121, 130–139.

Steer, P.A., Kirkpatrick, N.C., O'Rourke, D., Noormohammadi, A.H., 2009. Classification of fowl adenovirus serotypes by use of high-resolution melting-curve analysis of the hexon gene region. J. Clin. Microbiol. 47, 311–321.