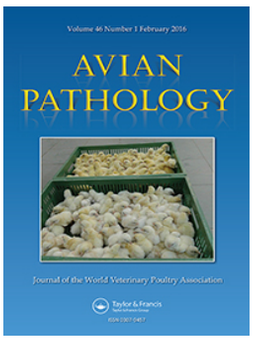

Avian Pathology

ISSN: 0307-9457 (Print) 1465-3338 (Online) Journal homepage: http://www.tandfonline.com/loi/cavp20

Clinicopathological characterization and genomic sequence differences observed in a highly virulent fowl *Aviadenovirus* serotype 4

Pedro F. Vera-Hernández, Andrés Morales-Garzón, Diana V. Cortés-Espinosa, Alejandra Galiote-Flores, Laura J. García-Barrera, Elizabeth T. Rodríguez-Galindo, Arnulfo Toscano-Contreras, Eduardo Lucio-Decanini & Angel E. Absalón

To cite this article: Pedro F. Vera-Hernández, Andrés Morales-Garzón, Diana V. Cortés-Espinosa, Alejandra Galiote-Flores, Laura J. García-Barrera, Elizabeth T. Rodríguez-Galindo, Arnulfo Toscano-Contreras, Eduardo Lucio-Decanini & Angel E. Absalón (2016) Clinicopathological characterization and genomic sequence differences observed in a highly virulent fowl *Aviadenovirus* serotype 4, Avian Pathology, 45:1, 73-81, DOI: 10.1080/03079457.2015.1125443

To link to this article: https://doi.org/10.1080/03079457.2015.1125443

AVIAN PATHOLOGY, 2016
VOL. 45, NO. 1, 73–81
http://dx.doi.org/10.1080/03079457.2015.1125443

Taylor & Francis
Taylor & Francis Group

Clinicopathological characterization and genomic sequence differences observed in a highly virulent fowl *Aviadenovirus* serotype 4

Pedro F. Vera-Hernández[a], Andrés Morales-Garzón[b], Diana V. Cortés-Espinosa[a], Alejandra Galiote-Flores[b], Laura J. García-Barrera[a], Elizabeth T. Rodríguez-Galindo[b], Arnulfo Toscano-Contreras[b], Eduardo Lucio-Decanini[b] and Angel E. Absalón[a]

[a]Instituto Politécnico Nacional, Centro de Investigación en Biotecnología Aplicada-Unidad Tlaxcala, México; [b]Investigación Aplicada S.A. de C.V., México

ABSTRACT

Highly virulent fowl aviadenoviruses (genus: *Aviadenovirus*) represent a significant risk in poultry farming that may contribute to increased mortality rates and may adversely affect the growth performance of poultry flocks. In this study, we performed the clinicopathological characterization of a FAdV strain SHP95 isolated from a commercial farm and its whole genome sequencing. The study revealed that the isolated strain is a highly virulent serotype 4 FAdV that can cause 100% mortality in day-old specific pathogen free (SPF) chickens with a dose of 2.5×10^5 $TCID_{50}$. At a lower viral dose (1.5×10^4 $TCID_{50}$), the infection in day-old SPF chickens caused 40% mortality and lesions characteristic for Hepatitis-hydropericardium syndrome (HHS). The viral strain was detectable by real time PCR in chicken organs, including the lymphoid organs until day 28 after infection. The whole genome assembly of strain SHP95 revealed a size of 45,641 bp, which encodes for 42 viral open reading frame (ORF). The comparative analysis in the genome shows 98.1% similarity between strain SHP95 and other FAdV-4 genomes reported. The major differences in the genome sequence between pathogenic and non-pathogenic fowl Adenovirus were identified in the right arm of the genome.

ARTICLE HISTORY
Received 5 June 2015
Accepted 21 September 2015

KEYWORDS
Thermocycler; Zymo Research; viral DNA; LMH cells; real time PCR

Introduction

Hepatitis-hydropericardium syndrome (HHS) is a severe condition that affects chickens and has the ability to reach mortality up to 100% (Ganesh & Raghavan, 2000; Mase *et al.*, 2010). The disease is characterized by the accumulation of a clear or amber coloured liquid of aqueous or gelatinous consistency in the pericardial sac and a deformed heart. In addition, the liver shows small multifocal areas of necrosis, the infiltration of mononuclear cells and the presence of basophilic intranuclear inclusions in its cells (Asthana *et al.*, 2013).

Similarly, other notable aspects of the disease are discoloured and swollen friable liver with focal necrosis, petechial bleeding in the heart muscles and other organs, lung congestion and oedema, and lastly, pale kidneys with prominent tubules. (Mazaheri *et al.*, 1998; Chandra *et al.*, 2000; Ganesh & Raghavan, 2000; Mittal *et al.*, 2014).

Studies to date indicate that the aetiologic agent of HHS is a Fowl Aviadenovirus (FAdV) species C and serotype 4 strain (Hess *et al.*, 1999; Balamurugan *et al.*, 2002; Dahiya *et al.*, 2002; Shivachandra *et al.*, 2003; Schonewille *et al.*, 2008; Mase *et al.*, 2010; Asthana *et al.*, 2013; Mittal *et al.*, 2014).

In addition, various members of the 12 FAdV serotypes reported to date can induce inclusion body hepatitis (IBH) syndrome; this condition is generally less serious and has effects on production parameters in affected farms (less than 30%) (Mittal *et al.*, 2014).

So far, the whole genome sequences of FAdV-4 strains KR5 (Marek *et al.*, 2012) and ON1 (Griffin & Nagy, 2011) have been reported, both of which are of low virulence (Cook, 1983; Grgic *et al.*, 2013). However, it should be noted that during the preparation of this manuscript, the complete genome of a highly pathogenic FAdV-4 was reported (Zhao *et al*, 2015).

In 1995, on a farm from the state of Puebla, Mexico, an adenovirus that produces HHS symptoms was isolated, which was named SHP95. The strain is characterized by its high virulence with mortality of 60% in unprotected birds in the field. In this document, we describe a clinicopathological characterization of the virus in specific pathogen free (SPF) chickens and also present the first whole genome report of a highly virulent FAdV-4 capable of causing 100% mortality in SPF birds.

Materials and Methods

Cell culture and virus isolation

The SHP95 strain of FAdV-4 causing HHS was isolated in the state of Puebla, Mexico from a chicken broiler farm in 1995. The virus was isolated from the macerated livers of infected birds. FAdV-4 propagation was performed in

CONTACT Angel E. Absalón aabsalon@ipn.mx
Supplemental data for this article can be accessed here. [10.1080/03079457.2015.1125443]

primary culture of chick embryo liver cells cultured in medium 199 supplemented with 10% fetal bovine serum. After isolation, virus dilutions were made for single plaque purification in LMH cells. Briefly, virus dilutions from 10^{-3} to 10^{-7} were used for infection of LMH monolayers. One hour after infection the cell layer was overlaid with medium 199 containing agarose at 0.4%, and plates were incubated during 5–7 days. Isolated plaques were then picked and transferred to a new fresh culture of LMH cells.

Clinicopathological characterization of FAdV-4 SHP95 in chickens

Two experiments were conduced in SPF White Leghorn chickens at day 1of age in controlled-environment Horsfall-Bauer isolation cabinets.

The first experiment was conducted with 180 birds (1 day old) randomly divided in three groups of 60. One group was not challenged and was kept as a control group; the remaining two groups were infected with FAdV-4 SHP95 via the ocular route at one day of age and monitored for 28 days. The second group was infected with FAdV-4 SHP95 utilizing 2.5×10^5 median tissue culture infectious doses ($TCID_{50}$) while the third group was infected with 1.5×10^4 $TCID_{50}$ of the same virus.

For the clinicopathological characterization assay, a second experiment was conducted with 180 birds divided randomly in two groups. One group of 60 birds was not challenged and was kept as a control group. The second group of 120 birds was infected via the ocular route at day 1 of age using a 1.5×10^4 $TCID_{50}$ of the same virus and monitored for 28 days. From both groups, birds were sacrificed by cervical dislocation utilizing IACUC approved protocols (five at each time point) on days 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28. Necropsy was performed on all sacrificed birds to evaluate pathology. Chickens of group two that died due to infection were not utilized for histopathology or qPCR analysis. 20 birds that survived infection were maintained until day 50 and evaluated for clinical signs and mortality. Tissues samples from all of the sacrificed birds were taken from the liver, proventriculus, caecal tonsils, spleen, kidneys, bursa of Fabricius, thymus and large intestine with faecal content, and processed as described below for histopathology and real-time PCR analysis to determine the presence of the virus. At the end of the trial all surviving birds were euthanized as described above. All these experiments were evaluated and approved by ethical committees from Investigación Aplicada S.A. de C.V. and Instituto Politécnico Nacional.

Histopathological studies

A portion of 0.5–1 cm^2 from each tissue sample was placed in 10% formalin solution, embedded in paraffin by 24 h and stained with haematoxylin and eosin for microscopic examination as previously described (Kiernan, 2008).

Isolation of viral DNA

For the extraction of total DNA for real time PCR assays, 0.2–1 g of fresh tissue was taken and 4ml of PBS was added and macerated using a rotor homogenizer. The macerates were frozen and thawed three times to release the virus and the cellular remains were eliminated by centrifugation at 3000 g for 15 min. Total DNA was purified using the DNeasy blood and tissue Kit (Qiagen, Mexico City, Mexico).

Real Time PCR

Quantification of the FAdV-4 load in each of the analyzed organs was performed by Real Time PCR using a LightCycler 2.0 (Roche Applied Science, Indianapolis IN, USA) thermocycler. 25 µl reaction mixture was prepared with 50–200 ng of DNA extracted from each organ, 0.7 µM of each primer (52K-fw and 52K-rv) (Günes *et al.*, 2012) and the HotStart-IT SYBR Green qPCR (Affymetryx, Santa Clara, CA, USA) mixture. Amplification conditions were as previously reported (Günes *et al.*, 2012). The data analysis was carried out using LightCycler 4.1 (Roche Applied Science) software, automatically adjusting the threshold value. Serial dilutions of a plasmid vector containing a segment of gene 52K were used to generate the standard curve, starting at 6.7×10^8 copies per reaction. A number of copies of known DNA template were plotted against the corresponding CT values and the standard curve was built. Verification of the PCR product was done by analysis of the alignment curve (Tm of the PCR product).

Genome sequencing

For the isolation of viral DNA the supernatant from a primary culture of hepatocytes infected with FAdV-4 SHP95 was clarified and then concentrated by ultrafiltration using a membrane with a cutoff of 50 kDa (Millipore, Billerica, MA, USA). The retentate was resuspended in 1/10 volume of molecular biology degree water and subsequently treated with DNase I and RNase H to a final concentration of 100 U/ml each and incubated at 37°C for one hour. Subsequently, the adenovirus DNA was purified using DNeasy blood and tissue Kit (Qiagen) and then concentrated using genomic DNA clean and concentrator Kit (Zymo Research, Irvine, CA, USA). Whole genome sequencing of FAdV-4 was done by Next-Generation sequencing using an Illumina Hiseq 2500 system with paired reads of 150 bp in length, with average of 1.5×10^8 reads.

Genome assembly and analysis

The whole adenovirus genome was assembled using DNASTAR's Lasergene Genomics Suite software performing de novo assemblies and guided assemblies taking the genomes of FAdV-4 strains KR5 and ON1

as reference. The repeated sequences present in the genome were identified using Tandem Repeats Finder software (Benson, 1999). Genome pairwise alignments were done with mVISTA LAGAN (Brudno *et al.*, 2003).

Results

Virus isolation

A virus from lysates of the samples collected from chicken livers caused cytopathic effect in LMH cell line. The presence of FAdV was confirmed by Real time PCR using specific oligonucleotides as previously described (Günes *et al.*, 2012). From the liver macerate, 10 fold dilutions were made. Dilution of 10^4 to 10^7 were plated on monolayers of LMH cells in agar media. All dilutions below 10^7 gave complete lysates, while dilutions of 10^{-7} produced separate plaque formation Three individual plaques were collected and used to infect separate plates of LMH cells to propagate the virus. After virus propagation a virus isolate was inoculated as described below into birds to confirm the development of HHS.

Mortality and lesions in birds caused by FAdV-4 SHP95

The pathogenicity of FAdV-4 SHP95 was determined in SPF chickens (1 day of age) at 2.5×10^5 $TCID_{50}$ and 1.5×10^4 ID. Those infected with 2.5×10^5 $TCID_{50}$, exhibited a mortality of 100% between days 4 and 7 post-infection, while at 1.5×10^4 $TCID_{50}$ they presented a mortality of 40% before day 7 (Figure 1). Of the surviving birds, 60% showed the characteristic lesions of HHS (Figure 2 and 3). One of the effects noted at necropsy was hydropericardium at day 7 post-infection (Figure 2A and 2B). Similarly, a discoloration of the liver was observed as the infection progressed. At day 3, small, local necrotic lesions appeared, which were characterized by a yellowish colouring (Figure 2C). These lesions continued to spread through the liver at day 4 (Figure 2D) until reaching the entire organ at day 5 (Figure 2E). The pathological effect was less noticeable in the remaining organs. However, a copious amount of a viscous liquid (oedema) in the thorax and abdomen of the birds was noted and was, not apparent in control birds.

The histopathological analysis of the liver showed the presence of inclusion bodies at day 5 and day 7; however, inclusion bodies were not detected in the liver histology at day 14 and 28 (Figure 3). Nevertheless, as the infection progressed, the liver became friable, inflamed and acquired a yellowish colour (Figure 2C, 2D and 2E).

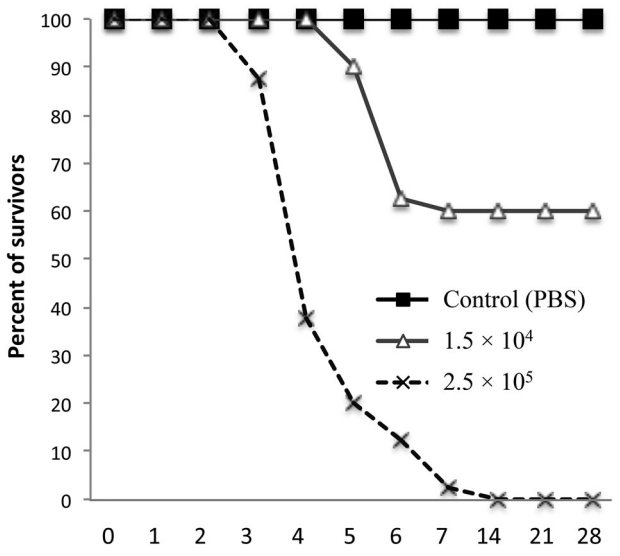

Figure 1. Survival curve. At 2.5×10^5 $TCID_{50}$, 100% of the birds died at first week post challenge. Only 40% (15 birds) of challenged birds died at 1.5×10^4 $TCID_{50}$ of the virus.

FAdV-4 SHP95 replicates in different organs

Organ samples (liver, thymus, spleen, bursa of Fabricius, intestine, caecal tonsils, kidneys and proventriculus) were taken from the birds infected at 1.5×10^4 ID and real time PCR was performed to quantify the viral load in each organ (Figure 4). SHP95 was detected in tissues at 3 days post-infection and reached maximum genome copies in the liver and intestine between 5–7 dpi. After the first week the genome copies decreased but viral persistence was noted through study until termination at 28 dpi.

Genome characteristics

The virus genome of SHP95 is 45,641 bp (Accession number at GenBank: KP295475) in size with a G + C percentage of 54.72%. The genome contains 42 encoded open reading frame (ORF) (Table 1), and like other *Aviadenovirus* the genes involved in the infection and replication process are located in the central region of the genome, while genes not conserved between the *Adenoviridae* family are grouped at both ends (Figure 5) (Griffin & Nagy, 2011; Marek *et al.*, 2012).

An important difference of the SHP95 genome compared to the reported genomes of FAdV-4 was identified in ORF19 (Lip). This gene in SHP95 has two stop codons caused by deletions of nucleotides (at positions 36,002 and 36,753 of the SHP95 genome), which cause changes in the reading frame. These nucleotide deletions in FAdV-4 SHP95 generate 3 ORF, which here we named lip1, lip2 and lip3. Of these, ORF19 (lip1) maintains the lipase region of the catalytic domain as well as the Kozak sequence that permits ribosome translation, although this transcript has low encoding potential (Griffin & Nagy, 2011). We did not identify Kozak sequences in region 5' at ORF 19 (lip2) and ORF19 (lip3).

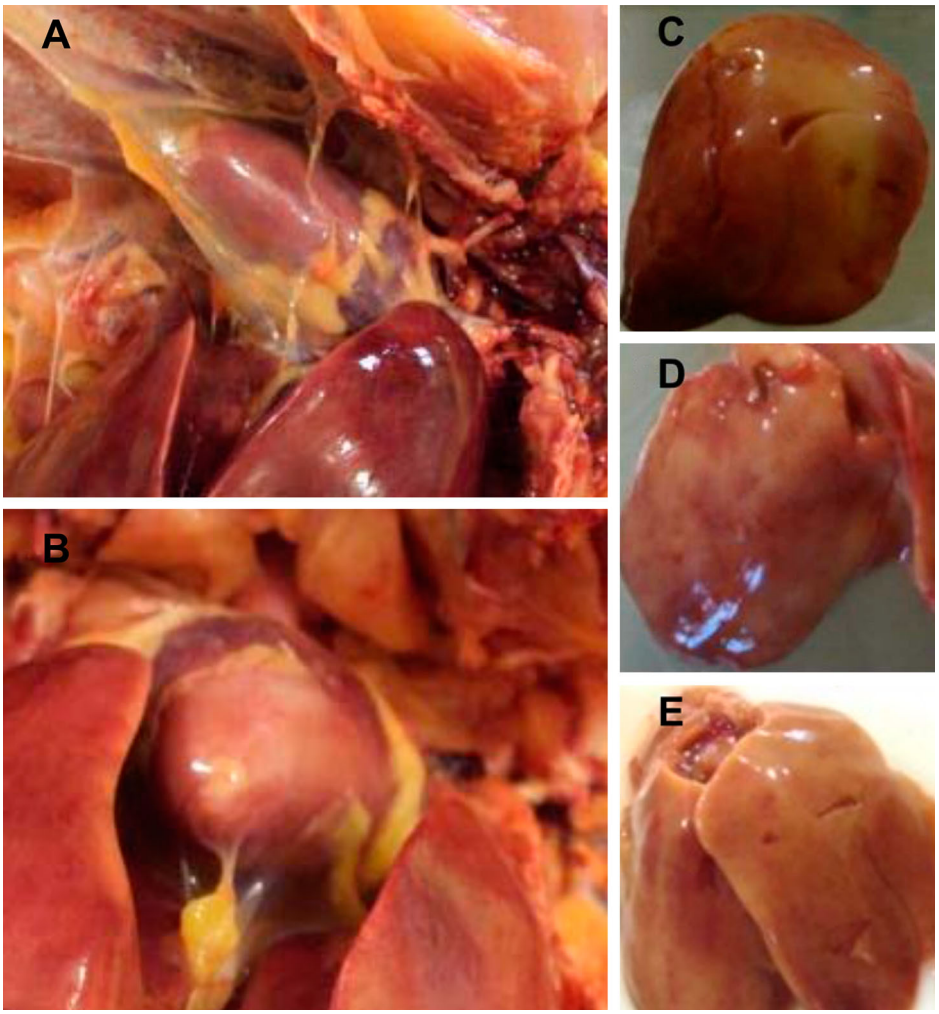

Figure 2. Macroscopic damage in the heart and liver of birds challenged with SPH95. 2A and 2B show hydropericardium at day 7 post-infection. 2C shows liver at 3 days post-infection with localized damage and slight yellowing; 2D shows liver at 4 days post-infection; and 2E shows liver at 5 days post-infection with a gross pathological damage.

The inverted terminal repeats (ITR) located at both ends of the genome are 56 bp in size. In addition, another eight repetitive sequences were identified in the genome of SHP95 (Table 2). The repetitive sequences TR-A, TR-E and TR-F are located in the regions with the lowest similarity percentage compared to FAdV-4 strains KR5 and ON1 (Figure 1). Although their role in the virus replication cycle has still to be determined, it has been postulated that these sequences may be regulating the transcription of some viral genes (Gruss *et al.*, 1981).

Fibre proteins

The size of fibre-1 in SHP-95 strain is 432 amino acid residues, the same as strain ON1 but one amino acid residue less than KR5 and the difference is in the poly G region. The fibre-2 protein of SHP-95 strain has 432 amino acids residues, the same number than ON1 but five amino acids less than KR5, the difference is in the deletion of five amino acid residues of SHP-95 at position 11 (Figure S1, supplemental data).

Similar to other members of FAdV-C the basic residues rich sequence KRPK/KKRP (position 22–25) and VYPF (position 36–40) in both fibre proteins of SHP-95 was identified. These motifs are possibly involved in nuclear localization and interaction with the penton base, respectively (Grgic *et al.*, 2011). The fibre-1 protein has a poly G at position 65–79 which was not identified in fibre-2 with non polar-polar uncharged (positions 50–66) (Figure S3).

Previous studies have shown the important role of fibre-2 in virulence of fowl aviadenoviruses (Pallister *et al.*, 1996). Some authors suggested some amino acids relevant for virulence based on the amino acid comparison of strains which induce HHS and non-virulent strains, positions located at 219 and 380 in the fibre-2 protein of KR5 (Marek *et al.*, 2012). At position 219(214 in SHP-95) the non-pathogenic strain KR5 has a glycine (G) as in all non-pathogenic strains and SHP-95 has aspartic acid (D) as in all pathogenic strains studied until now. In the position 380 (375 in SHP-95) the amino acid substitution is threonine (T) in SHP-95 and the pathogenic strains while in KR5 it is alanine (A). Additionally, we notice at

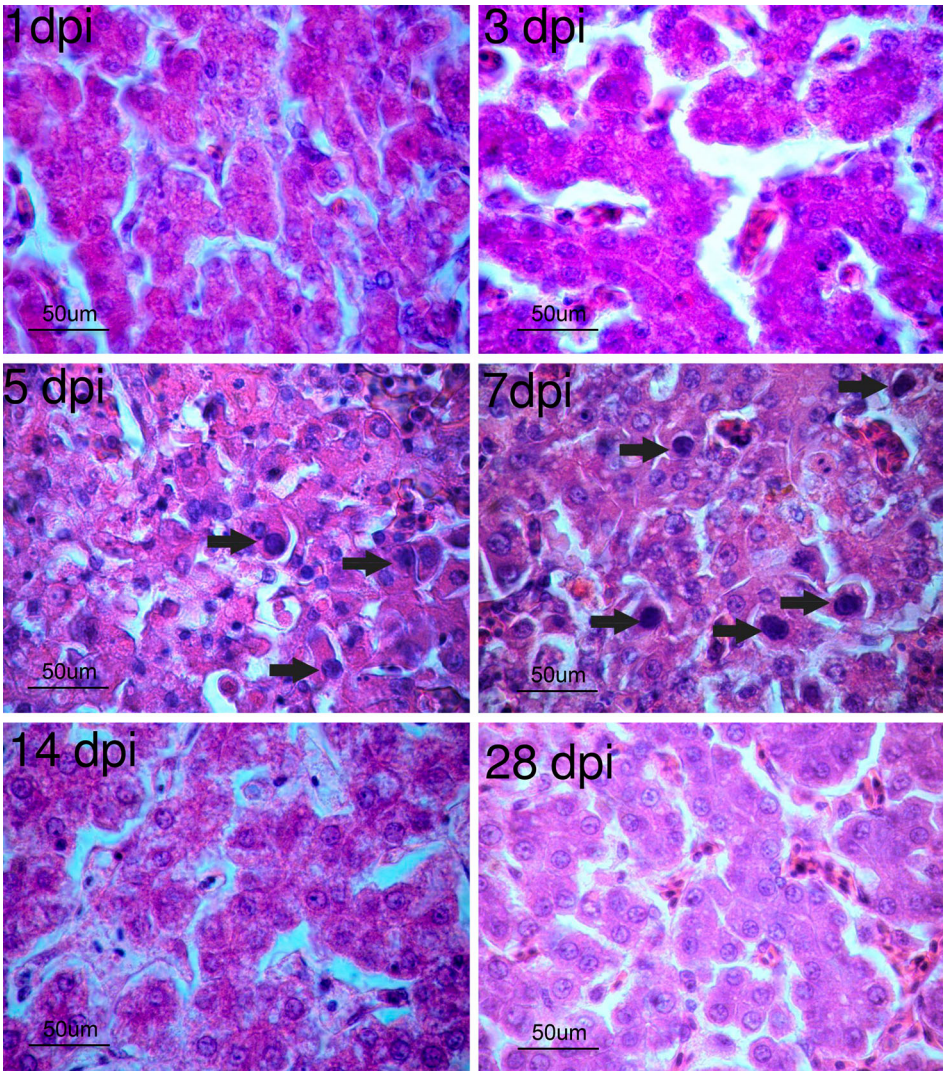

Figure 3. Histological changes in liver of birds infected with SPH95**.** Inclusion bodies were observed at day 5 and 7 post-infection (arrows). However, at 2, 3 and 4 weeks post-infection, no obvious inclusion bodies in liver were seen.

position 295 in SHP-95 and the pathogenic strains, threonine (polar uncharged amino acid), while in non-pathogenic strains this amino acid is methionine or isoleucine (non-polar aliphatic amino acids).

Discussion

Fowl aviadenoviruses (FAdVs) are present in chicken farms all over the world. The most severe form of disease associated with FAdV is HHS, which has been attributed exclusively to serotype 4 (Hess *et al.* 1999; Ganesh & Raghavan, 2000; Balamurugan *et al.*, 2002; Asthana *et al.*, 2013; Kim *et al.*, 2014).

In this study, FAdV-4 SHP95 was isolated from a commercial farm in the state of Puebla in Mexico and clinicopathologically characterizated in SPF chickens to reproduce the development of HHS and high mortality in unprotected birds. The strain SHP95 caused up to 100% mortality in unprotected SPF birds at 8 dpi (at a dose 2.5×10^5 $TCID_{50}$); and 40% mortality when a lower infective dose was used (1.5×10^4 $TCID_{50}$). This result confirms that the mortality in chickens under the evaluated conditions has a dose-dependent relation. Compared to other highly virulent strains, the challenge dose which caused 100% mortality in SPF birds is lower or similar to other experimental studies with highly pathogenic FAdV-4 strains (Mazaheri *et al.*, 1998; Mase *et al.*, 2010).

At a low dose of infection, the histopathology of the liver revealed the presence of inclusion bodies in early stages of infection similar to other FAdVs-4 that cause HHS (Chandra *et al.*, 2000; Ganesh & Raghavan, 2000).

The highest copy number of viral DNA was detected in all organs between 3 and 7 days post-infection, highlighting an increased quantity in liver, intestine and spleen with respect to the other analyzed tissues. Changes in the quantity of viral DNA correlated with the time of appearance and disappearance of inclusion bodies in the liver of birds as noted in other studies (Chandra *et al.*, 2000; Ganesh & Raghavan, 2000). Following the critical stage of infection during the first

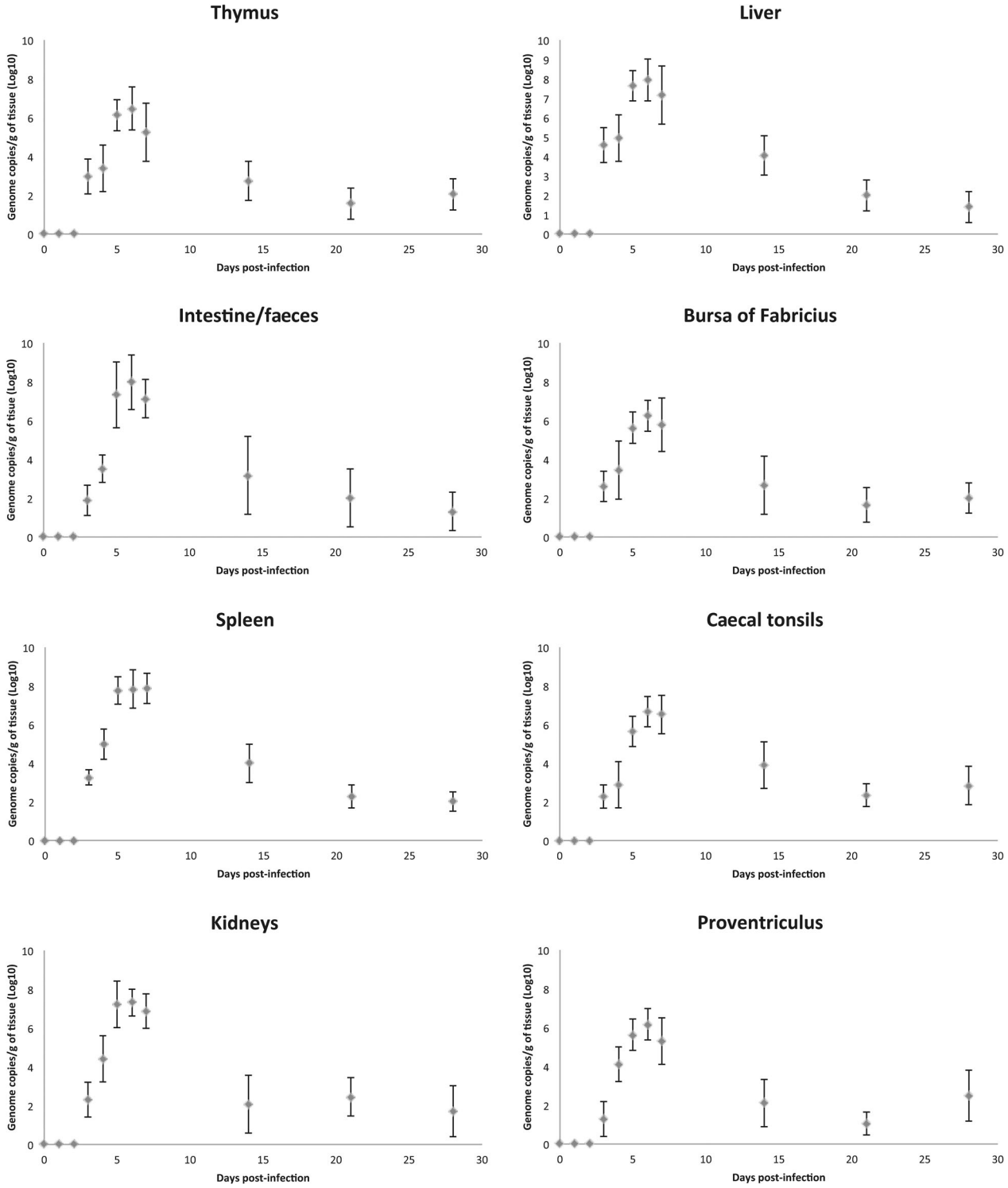

Figure 4. Tissue tropism and genome copies of SPH95 in different organs from day 1 until day 28 post-infection. Three organs of different birds were analyzed every day until day 7; after that, the virus quantification was performed at days 14, 21 and 28. Viral genome copies are expressed in Log_{10} of genome copies. The mean (small rhombus) and standard deviation (vertical lines) are provided for virus genome copies.

week after infection, copies of the viral genome decreased and the inclusion bodies disappeared, although the virus persisted in birds until the end of experiments.

In addition to non-lymphoid tissues, the presence of SHP95 in lymphoid organs (bursa of Fabricius, thymus, caecal tonsils, spleen) was also confirmed by real time PCR. The tropism in lymphoid tissues might be linked to the mortality effect in birds at a high viral dose and to the manifestation of HHS, as has been hypothesized previously (Naeem *et al.*, 1995). A particularly high load of viral DNA was detected in spleen with respect to the other lymphoid organs. Previous studies (Schonewille *et al.*, 2008) reported a drop of lymphocytes and a generalized suppression of the humoral and cellular immune system

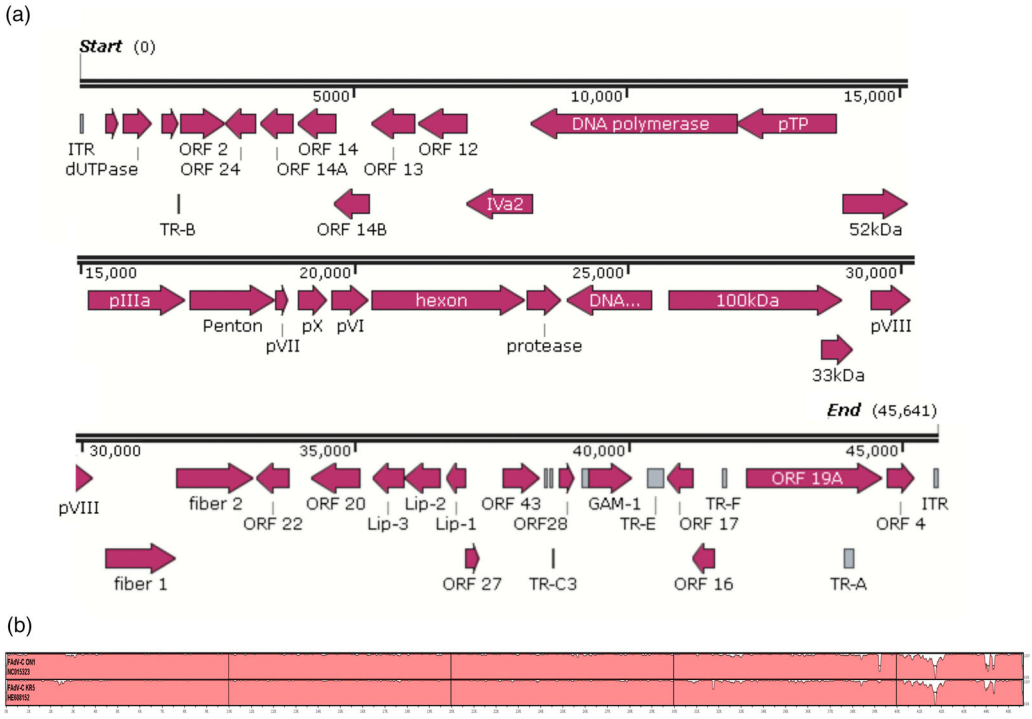

Figure 5. Map of the complete genome of Fowl Adenovirus serotype 4 strain SHP95. The graphs represent the comparison between the SHP95 genome and genomes of low pathogenicity fowl aviadenovirus serotype 4. This comparative analysis reveals that the largest differences are located on the right arm of the genome.

after infection with a virulent FAdV-4 in chickens. In this trial, we found adenoviral DNA in the main lymphoid organs, which could explain the previously reported effect.

With respect to the genomic analysis, some nucleotide sequences of genes and partial genome of highly virulent FAdV-4 has been described. However, to date there are no reports describing the complete genome of a FAdV-4 capable of inducing HHS. The fact that SHP95 can readily induce HHS and induce high mortality underscores the importance of determining what genomic variation may exist in comparison to other complete genomes of FAdV-4 isolates, e.g., strains ON1 and KR5, which are non-virulent (Griffin & Nagy, 2011; Marek *et al.*, 2012; Asthana *et al.*, 2013; Kim *et al.*, 2014).

The genome of SHP95 is 45,641 bp in length, and has a high degree of homology (98.1%) with the genome of others previously reported FAdVs-4 (Marek *et al.*, 2012; Griffin & Nagy, 2011). Additionally, the SHP95 genome differs in more than 50% from other FAdVs of different serotypes (Table S1).

In the central region of the SHP95 genome we noticed similar organization and a high percent of homology with respect to other FAdV4 strains and FAdV-s in general. Similarly as reported previously for FAdV-C strains, SHP95 genome has two fibre genes: fibre-1 and fibre-2 (Chiocca *et al.*, 1996; Griffin & Nagy, 2011; Marek *et al.*, 2012).

In the left part of the SPH95 genome, 11 ORFs (ORF0-IVa2) prior to the start codon of the viral DNA polymerase showed a high homology with the same region present in KR5 and ON1 (Griffin & Nagy, 2011; Marek *et al.*, 2012).

Also, at the right end the SHP95 genome showed a similar organization to genomes of KR5 and ON1. However, the percent of similarity was low, particularly in ORF27, ORF16, ORF19A and ORF4 which differed more than 4% until 25% (Table S2). The SHP95 genome differed also in size with respect to non-pathogenic FAdVs-4, due to deletions in the repetitive region (TR-F). Repetitive regions are present in genomes of FAdVs but until now no relevant function has been reported. Nevertheless, these TR sequences are dispensable in FAdV (Ojkic & Nagy, 2001).

Also at the right end, another remarkable difference was noted in ORF19 (lip) of SHP95. This gene has an earlier stop codon, which generates a shorter protein than the one in KR5 and ON1. There have been previous reports, which we corroborated into a comparative alignment (data not shown), stating that ORF19 (lip) has similarity to triacylglycerol lipase of the Marek disease virus (MDV) (Corredor *et al.*, 2008). The role of Lip in the viral cycle of FAdVs has not been studied; but in MDV this protein enhances the replication and pathogenic potential (Kamil *et al.*, 2005). Although there is no evidence about the function of orf19 (lip) in FAdVs, studies should be performed to confirm a possible participation of this gene in virus replication.

With regard to the virulence association of fibre-2, previous studies reported that this protein has an effect

Table 1. Position of ORFs found in the genome of FAdV-4 SHP95 encoding potential proteins.

Gene	Strand	Location	No. of bp	No. of aa
ORF0	R	477–716	240	79
dUTPase (ORF1)	R	789–1313	525	174
ORF1B	R	1484–1807	324	107
ORF2	R	1849–2664	816	271
ORF14C	I	2666–3202	537	178
ORF14B	L	3314–3901	588	195
ORF14A	L	3995–4681	687	228
ORF14	L	4665–5294	630	209
ORF13	L	5328–6128	801	266
ORF12	L	6205–7074	870	289
IVa2	L	7088–8272	1185	394
DNA pol	L	8256–12014	3759	1252
pTP	L	12019–13827	1809	602
52K	R	13957–15156	1200	399
pIIIa	R	15143–16915	1773	590
Penton base (III)	R	16987–18564	1578	525
pVII	R	18573–18806	234	77
pX	R	18978–19517	540	179
pVI	R	19607–20290	684	227
Hexon	R	20336–23149	2814	937
Protease	R	23167–23796	630	209
DBP	L	23917–25437	1521	506
100K	R	25777–28944	3168	1055
22K	R	28559–29149	591	196
pVIII	R	29464–30207	744	247
U exon	L	30152–30439	288	95
Fibre1	R	30438–31736	1299	434
Fibre2	R	31720–33144	1425	474
ORF22	L	33197–33784	588	195
ORF20A	L	33787–34054	267	88
ORF20	L	34204–35091	888	295
Lip3	L	35334–35897	564	187
Lip2	L	35912–36556	645	214
Lip1	L	36673–37014	342	113
ORF27	R	37013–37294	282	93
ORF43	R	37716–38381	666	221
ORF28	R	38735–39028	294	97
GAM1	R	39276–40091	816	271
ORF17	L	40707–41183	477	158
ORF16	L	41176–41586	411	136
ORF19A	R	42164–44653	2490	829
ORF4	R	44747–45244	498	165

R = Rightward transcribed strand.
L = Leftward transcribed strand.

on the virulence of fowl adenovirus (Pallister *et al.*, 1996). Additionally, aligning fibre-2 amino acid sequences of several FAdVs-4 which induce HHS and apathogenic strains, we noted particular amino acid substitutions between both kinds of strains (Marek *et al.*, 2012). According to this, fibre 2 of SHP95 has changes in amino acids D214 and T375 like other strains which induce HHS (all polar amino acids). In comparison, apathogenic strains have nonpolar amino acids at the same positions. Additionally, we observed a similar polar-nonpolar substitution at (T→I/M)295. Nevertheless any functional consequences of amino acid substitutions in fibre-2 protein of pathogenic strains (inducing HHS) is still speculative.

Table 2. Position of repetitive sequences in the genome of FAdV-4 SPH95.

Repetitive sequence	Location	Period size	Copy number	% GC
ITR	1–56, 45586–45641	56	2.0	54
TR-B	1796–1810	10	2.5	32
TR-C1	38457–38523	18	3.7	15
TR-C2	38568–38610	16	2.6	29
TR-C3	38605–38644	17	2.4	27
TR-D	39153–39264	33	3.4	65
TR-E	40354–40644	123	2.4	44
TR-F	41733–41801	33	2.1	37
TR-A	43956–44112	48	3.4	51

Note: All TR regions were named by similitude at the position of repetitive sequence according to sequence GU188428 (Griffin & Nagy, 2011).

Currently, the majority of ORFs of FAdVs are still not characterized and it is necessary to continue studies about their function on viral infection, replication, and virulence. In this report, we present the first whole genome of a highly virulent strain and therefore it represents an important starting point to initiate studies on the molecular basis of the virulence and genetic determinants that trigger the onset of HHS.

Acknowledgments

We are grateful to Roberto Ortega for technical support. Thanks to Dr. Claudio L. Afonso for his advice and help in review the manuscript.

Funding

This study was supported by CONACYT (Grant FINNOVA-2012–191919). PFVH receive a scholarship from CONACYT (287820).

References

Asthana, M., Chandra, R. & Kumar, R. (2013). Hydropericardium syndrome: current state and future developments. *Archives of Virology*, 158, 921–931.

Balamurugan, V., Kataria, J.M., Kataria, R.S., Verma, K.C. & Nanthakumar, T. (2002). Characterization of fowl adenovirus serotype-4 associated with hydropericardium syndrome in chicken. *Comparative Immunology, Microbiology and Infectious Diseases*, 25, 139–147.

Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research*, 27, 573–580.

Brudno, M., Do, C.B., Cooper, G.M., Kim, M.F., Davydov, E., Green, E.D., Sidow, A., & Batzoglou, S. (2003). LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. *Genome Research*, 13, 721–731.

Chandra, R., Shukla, S.K. & Kumar, M. (2000). The hydropericardium syndrome and inclusion body hepatitis in domestic fowl. *Tropical Animal Health and Production*, 32, 99–111.

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

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

Corredor, J.C., Garceac, A., Krell, P.J. & Nagy, E. (2008). Sequence comparison of the right end of fowl adenovirus genomes. *Virus Genes*, 36, 331–344.

Dahiya, S., Srivastava, R.N., Hess, M. & Gulati, B.R. (2002). Fowl adenovirus serotype 4 associated with outbreaks of

infectious hydropericardium in Haryana, India. *Avian Diseases*, 46, 230–233.

Ganesh, K. & Raghavan, R. (2000). Hydropericardium hepatitis syndrome of broiler poultry: current status of research. *Research in Veterinary Science*, 68, 201–206.

Grgic, H., Poljak, Z., Sharif, S. & Nagy, E. (2013). Pathogenicity and cytokine gene expression pattern of a serotype 4 fowl adenovirus isolate. *PLoS One*, 8, e77601.

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

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

Gruss, P., Dhar, R. & Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. *Proceedings of the National Academy of Sciences USA*, 78, 943–947.

Günes, A., Marek, A., Grafl, B., Berger, E. & Hess, M. (2012). Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). *Journal of Virology Methods*, 183, 147–153.

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

Kamil, J.P., Tischer, B.K., Trapp, S., Nair, V.K., Osterrieder, N. & Kung, H.J. (2005). vLIP, a viral lipase homologue, is a virulence factor of Marek's disease virus. *Journal of Virology*, 79, 6984–6996.

Kiernan, J.A. (2008). *Histological and Histochemical Methods: Theory and Practice*, 4th Edn (pp. 103–131). Bloxham: Scion Publishing.

Kim, M.S., Lim, T.H., Lee, D.H., Youn, H.N., Yuk, S.S., Kim, B.Y., Choi, S.W., Jung, C.H., Han, J.H. & Song, C.S. (2014). An inactivated oil-emulsion fowl Adenovirus serotype 4 vaccine provides broad cross-protection against various serotypes of fowl *Adenovirus*. *Vaccine*, 32, 3564–3568.

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

Mase, M., Nakamura, K. & Imada, T. (2010). Characterization of Fowl adenovirus serotype 4 isolated from chickens with hydropericardium syndrome based on analysis of the short fibre protein gene. *Journal of Veterinary Diagnostic Investigation*, 22, 218–223.

Mazaheri, A., Prusas, C., Voss, M. & Hess, M. (1998). Some strains of serotype 4 fowl adenoviruses cause inclusion body hepatitis and hydropericardium syndrome in chickens. *Avian Pathology*, 27, 269–276.

Mittal, D., Jindal, N., Tiwari, A.K. & Khokhar, R.S. (2014). Characterization of fowl adenoviruses associated with hydropericardium syndrome and inclusion body hepatitis in broiler chickens. *Virus Diseases*, 25, 114–119.

Naeem, K., Niazi, T., Malik, S.A. & Cheema, A.H. (1995). Immunosuppressive potential and pathogenicity of an avian adenovirus isolate involved in hydropericardium syndrome in broilers. *Avian Disease*, 39, 723–728.

Ojkic, D. & Nagy, É. (2001) The long repeat region is dispensable for fowl adenovirus replication in vitro. *Virology*, 283, 197–206.

Pallister J., Wright P.J. & Sheppard M. (1996). A single gene encoding the fibre is responsible for variations in virulence in the fowl Adenovirus. *Journal of Virology*, 70, 5115–5122.

Schonewille, E., Singh, A., Gobel, T.W., Gerner, W., Saalmuller, 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. *Veterinary Immunology and Immunopathology*, 121, 130–139.

Shivachandra, S.B., Sah, R.L., Singh, S.D., Kataria, J.M. & Manimaran, K. (2003). Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAV-4) associated with hydropericardium syndrome. *Veterinary Research Communications*, 27, 39–51.

Zhao, J., Zhong, Q., Hu, Y. & Zhang, G.Z. (2015). Pathogenicity and complete genome characterization of fowl adenoviruses isolated from chickens associated with inclusion body hepatitis and hydropericardium syndrome in China. *PLoS One*, 10, 7.