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Complete genome sequence of a non-pathogenic strain of *Fowl Adenovirus* serotype 11: Minimal genomic differences between pathogenic and non-pathogenic viruses

Angel E. Absalón[a,*], Andrés Morales-Garzón[b], Pedro F. Vera-Hernández[a], Diana V. Cortés-Espinosa[a], Sara M. Uribe-Ochoa[a], Laura J. García[a], Eduardo Lucio-Decanini[b]

[a] *Instituto Politécnico Nacional, Centro de Investigación en Biotecnología Aplicada-Unidad Tlaxcala, Carr. Est. Santa Inés Tecuexcomac-Tepetitla Km. 1.5, Tepetitla, Tlaxcala CP 90700, Mexico*

[b] *Investigación Aplicada S.A. de C.V., 7 Norte No. 416 Col. Centro, Tehuacán, Puebla CP 75740, Mexico*

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ABSTRACT

In this study, we conducted the clinicopathological characterization of a non-pathogenic FAdV-D serotype 11 strain MX95, isolated from healthy chickens, and its entire genome was sequenced. Experiments in SPF chickens revealed that the strain is a non-pathogenic virus that did not cause death at challenge doses of 1×10^6 TCID50. Additionally, the infection in SPF chickens caused no apparent damage in most of the organs analyzed by necropsy and histopathology, but it did cause inclusion body hepatitis; nevertheless it did not generate severe infectious clinical symptoms. The virus was detected in several chicken organs, including the lymphoid organs, by real-time polymerase chain reaction (PCR) until 42 days. The genome of FAdV-11 MX95 has a size of 44,326 bp, and it encodes 36 open reading frames (ORFs). Comparative analysis of the genome indicated only 0.8% dissimilarity with a highly virulent serotype 11 that was previously reported.

1. Introduction

Fowl adenoviruses are found ubiquitously on poultry farms. Of the 12 serotypes reported, most of them are able to cause inclusion body hepatitis and represent a significant risk in poultry farming that may contribute to increased mortality rates and adversely affect farm performance.

Adenoviruses are non-enveloped, double-stranded linear DNA viruses. According to the more recent classification (ICTV, 2014), there are 5 genera within the family *Adenoviridae* including the genus *Aviadenovirus*, and eight species belonging to this genus. Actually, twelve serotypes of Fowl Adenovirus (FAdV) have been reported across five species named A, B, C, D and E.

In chickens the mortality and severity of FAdV infections may be influenced by the condition of the immune system of birds or by concurrent infection with other immunosuppressive infectious agents (Toro et al., 2000). However, some species of FAdVs are capable of inducing mortality as a primary agent in the absence of other infectious agents or immunosuppressive conditions, and they could cause Hydropericardium and Hepatitis Syndrome (HHS) (Mazaheri et al., 1998; Vera-Hernández et al., 2016). Additionally, several species of FAdVs have low pathogenicity and induce little or no symptoms of disease; however, they could induce Inclusion Body Hepatitis (IBH) in the liver. IBH is a condition characterized by necrotic and dystrophic changes in the liver accompanied by intranuclear inclusion bodies. This syndrome is generally not a serious disease and has very low or no mortality (Mase et al., 2012).

On the other hand, HHS is a severe disease that causes mortality up to 100% in unvaccinated birds (Asthana et al., 2013; Vera-Hernández et al., 2016). The main feature in infected animals is the accumulation of clear or amber liquid in the pericardial sac and damage to the heart. In addition, damage has been observed in other organs such as the liver, lungs and kidneys. The liver shows multifocal lesions of necrosis, mononuclear cell infiltration and the presence of intranuclear inclusion bodies (Mazaheri et al., 1998; Chandra et al., 2000; Ganesh et al., 2000).

Several studies indicate that most HHS causative agents belong to serotype 4 of FAdV-C, while IBH is caused by almost all species of FAdVs (Mazaheri et al., 1998; Chandra et al., 2000; Ganesh et al., 2000). However, recently the finding of a highly virulent FAdV-11 that cause HHS has been reported (Zhao et al., 2015); to our knowledge, this is the sole report that FAdV-11 is capable of reproducing this

* Corresponding author.
E-mail address: aabsalon@ipn.mx (A.E. Absalón).

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clinical illness.

In 1995, in a chicken farm in Mexico, a strain belonging to the family *Adenoviridae* was isolated from apparently healthy chickens; this strain was named MX95. In this study, we performed a clinicopathological characterization of the virus in specific-pathogen-free (SPF) chickens, and we also present the genome sequence of this non-pathogenic virus. We discuss the genomic differences between pathogenic and non-pathogenic strains and propose some questions not yet answered about the genetic determinants of virulence in Avian *Adenovirus*.

2. Material and methods

2.1. Cell culture and virus isolation

FAdV-11 strain MX95 was isolated from chickens from an apparently healthy farm in Mexico. The virus was isolated from the macerated livers of infected birds. Propagation of the virus was performed in primary cultures of chick embryo liver cells cultured in medium 199 supplemented with 10% fetal bovine serum. To discard the presence of contaminant infectious agents, dilutions of virus present in supernatant of cellular cultures were performed for single-plaque purification in LMH cells. Briefly, virus dilutions from 10^{-3} to 10^{-7} were used for infection of LMH monolayers with 80% confluence. One hour after infection, the infection medium was discarded and the cell monolayers were overlaid with medium 199 containing 0.4% ultrapure agarose. Plates were incubated at 37 °C and 5% CO_2 for 5–7 days; then the isolated plaques were picked and transferred to a new fresh culture of LMH cells for propagation.

2.2. Clinicopathological characterization of FAdV-11 MX95 in SPF chickens

The experiments for clinicopathological characterization were conducted in SPF White Leghorn chickens in controlled-environment Horsfall-Bauer isolation cabinets.

The experiment was conducted with 150 chickens randomly divided into two groups with the same number of birds. The first group of birds was not challenged and was used as a control group; the second group of birds was infected via the oral route (the natural infection route) at one-day-old with FAdV-11 MX95 at 1×10^{6} median tissue culture infectious dose (TCID50). All the experiments were monitored for 42 days.

From both groups, the birds were sacrificed by cervical dislocation using IACUC approved protocols (5 at each time point) on days 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35 and 42 of age, and necropsy was performed on all sacrificed birds to evaluate pathology. Twenty birds were maintained until day 52 and were evaluated for clinical signs. Tissues samples from all sacrificed birds were collected from the liver, proventriculus, cecal tonsils, spleen, kidneys, bursa of Fabricius, thymus and large intestine with fecal matter, and they were 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 animals were euthanized as described above. All of these experiments were evaluated and approved for ethical considerations by ethical committees from Investigación Aplicada S.A. de C.V. and Instituto Politécnico Nacional.

2.3. Histopathological studies

A portion of each sample (approximately 0.5 cm^2) was placed in 10% formalin solution, embedded in paraffin for 24 h and stained with hematoxylin and eosin for microscopic examination following the protocols described by Kiernan (2008).

2.4. Isolation of viral DNA

For the extraction of total DNA from the chicken organs for real-time PCR assays, a fresh sample of 0.2–0.4-g was collected, 2 mL of phosphate-buffered saline (PBS) was added, and the mixture was macerated using a rotor homogenizer. The macerated samples were frozen and thawed 3 times to release the virus, and the cellular remains were eliminated by centrifugation at 3000*g* by 15 min. Total DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen, Mexico City, Mexico).

2.5. Real-time PCR

Quantification of FAdV-11 genome copies in each analyzed organ was performed by real-time PCR using a LightCycler 2.0 (Roche Applied Science) thermocycler. The 25 µl reaction mixture was prepared with 50–200 ng of purified DNA from each organ, 0.7 µM of each primer (52K-fw and 52K-rv) (Günes et al., 2012) and HotStart-IT SYBR Green qPCR mixture (Affymetryx, USA). The amplification conditions were as follows: 95 °C for 5 min, and 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 10 s and a last melting step between 60 and 95 °C for confirmation of PCR product Tm, as previously reported (Günes et al., 2012). Data analysis was performed using the LightCycler 4.1 software package (Roche Applied Science), automatically adjusting the threshold value. The standard curve was performed as previously reported (Günes et al., 2012).

2.6. Genome sequencing

The viral DNA of FAdV-11 strain MX95 for sequencing was purified from the supernatant of a primary culture of hepatocytes infected with the same virus as we previously reported (Vera-Hernández et al., 2016). The whole-genome sequencing of FAdV-11 was performed using Next-Generation Sequencing on an Illumina Hiseq 2500 system with paired reads of 150 bp in length, with an average of 1.5×10^{8} reads.

2.7. Genome assembly and analysis

The whole adenovirus genome was assembled using DNASTAR's Lasergene Genomics Suite software package to perform de novo assembly and guided assemblies using the genome of the FAdV-4 strain SHP95 (KP295475) and FAdV-11 strain HBQ12 (KM096545) as references. The repeated sequences present in the genome were identified using the Tandem Repeats Finder software package (Benson, 1999). The percentage of identity for every gene was calculated using Genius software (Biomatters Ltd).

3. Results

3.1. Virus isolation

Concentrated extracts obtained from liver samples from apparently healthy chickens caused a cytopathic effect in LMH monolayers. The presence of FAdV was confirmed by real-time PCR using universal oligonucleotides (Günes et al., 2012) for detection of all species of FAdVs. The supernatant of the LMH monolayers infected was collected, and 5 mL of virus suspension was concentrated at 10% from the original volume using Amycon ultrafilters with pore size of 100 kDa (Millipore, USA). Using this virus suspension, serial dilutions of 10^{-3} to 10^{-7} were performed and plated on monolayers of LMH cells with agar medium. Dilution at 10^{-3} gave lysates with no or low separation between viral plaques, while dilutions at 10^{-4} and 10^{-5} produced separate plaque formation. No apparent plaque formation was detected in plates with dilutions at 10^{-6} and 10^{-7}. From the 10^{-5} dilution, individual plaques were collected and used to infect plates with fresh

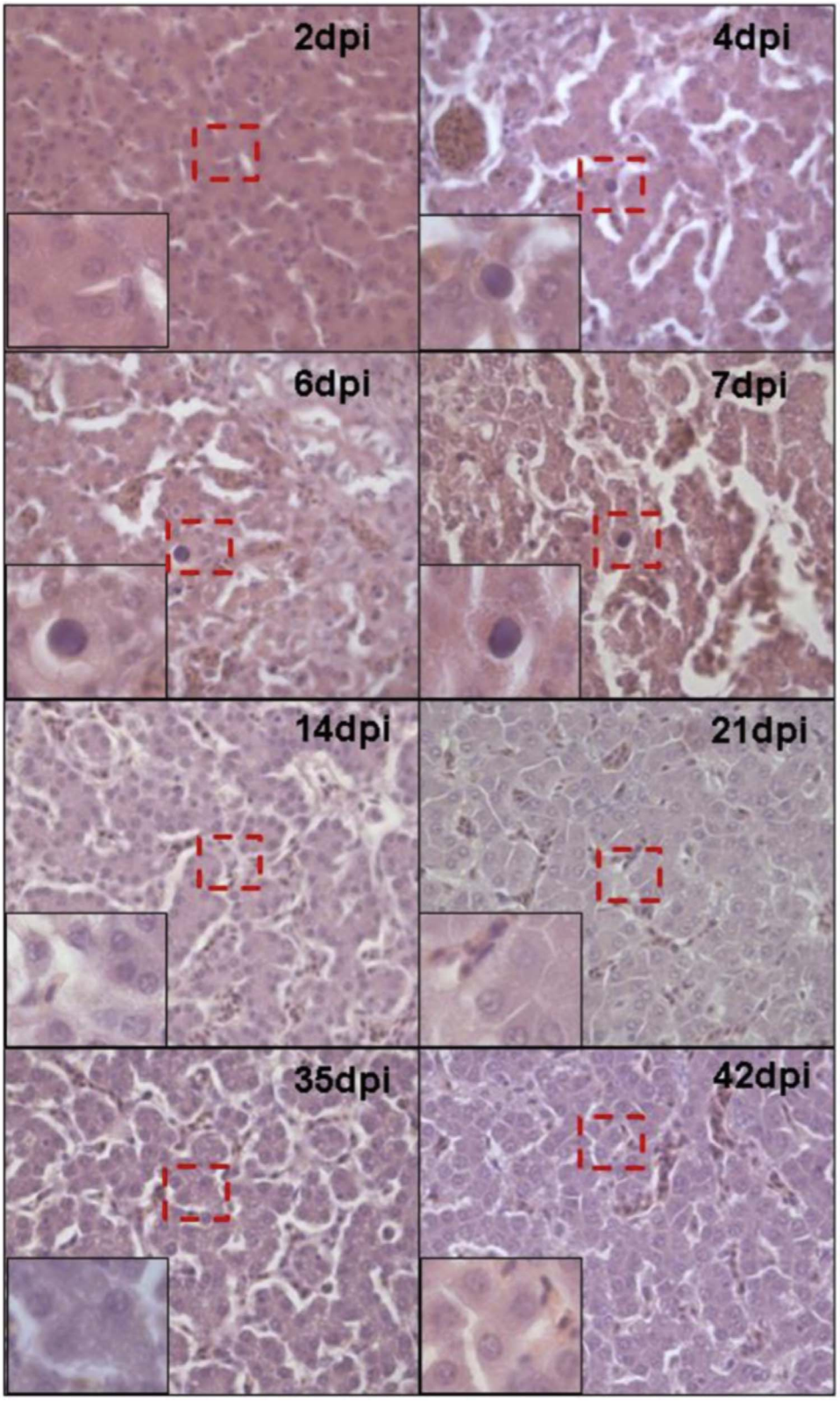

Fig. 1. Histological changes in the livers of birds infected with SPH95. Inclusion bodies were observed at day 5 and 7 post-infection (arrows). However, at 2, 3 and 4 weeks post-infection, there were not obvious inclusion bodies in the liver (all images were taken at 40× and close up at 100× of magnification).

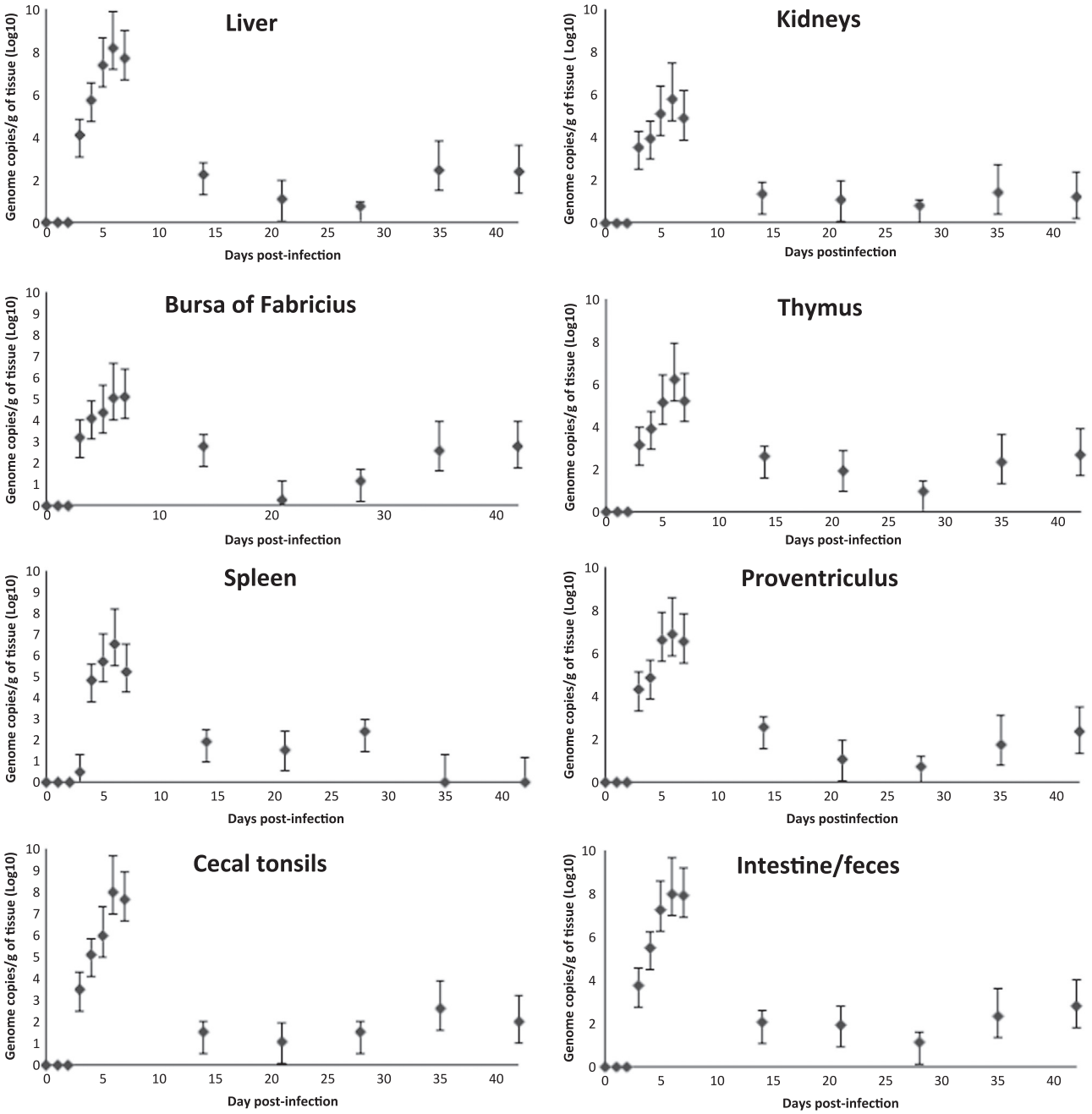

Fig. 2. Tissue tropism and genome copies of SPH95 in different organs from day 1 until day 28 post-infection. Three organs of the different birds were analyzed every day until day 7. Subsequently, viral genome quantification was performed at days 14, 21, 28, 35 and 42. Viral genome copies are expressed as the base-10 logarithm of the number genome copies. The mean (small rhombuses) and standard deviation (vertical lines) are provided for virus genome copies.

LMH cells to propagate the virus.

3.2. Clinicopathological characterization of FAdV-11 MX95 in SPF chickens

The pathogenicity of FAdV-11 MX95 was determined in specific-pathogen-free chickens (at 1 day of age) with 1×10^6 TCID. Under the conditions described above, no mortality or sign of illness was observed in chickens during the 52 days of the trial. At the necropsy, the birds showed a few petechiae and slight icterus in the liver between days 5 and 7 post-infection, but no obvious pathological lesions were observed in other organs (Supplementary material 1).

The histopathological analysis of birds infected with FAdV-11 MX95 of the liver showed the presence of inclusion bodies between days 4 and 7; however, inclusion bodies were not detected at day 14 post-infection or after (Fig. 1). No lesions were detected by histopathology in other organs of the infected birds (Supplementary material 2).

3.3. Distribution of FAdV-11 in organs

Samples from the liver, thymus, spleen, bursa of Fabricius, intestine, cecal tonsils, kidneys and proventriculus were collected from sacrificed infected birds. Real-time PCR was performed to quantify the viral genome copies in each organ (Fig. 2). Genome copies were detected at day 3 post-infection, and the highest number of genome copies was reached in all organs at day 6 post-infection. At day 7 post infection and after, the genome copy number decreased; but the virus could be detected until day 42 of the trial with exception of the spleen samples.

Table 1
The positions of ORFs found in the genome of FAdV-4 SHP95 that encode potential proteins.

Gene	Strand	Location	No. of bp	No. of aa
orf 0	r	440–808	369	122
dUTPase	r	847–1338	492	163
orf 8	l	1494–1736	243	80
orf 1B	r	1500–1730	231	76
orf 1C	f	1678–1878	201	66
orf 2	r	1952–2755	804	267
orf 7	l	2347–2667	321	106
orf 24	l	2838–3413	576	191
orf 14	l	3537–4052	516	171
orf 13	l	4263–5060	798	265
orf 12	l	5245–5844	600	199
IVa2	l	6131–7336	1206	401
DNA pol	l	7333– 11,253	3921	1306
pTP	l	11,250–13,106	1857	618
52K	r	13,247–14,455	1209	402
pIIIa	r	14,442–16,217	1776	591
Penton base	r	16,223–17,935	1713	570
pVII	r	17,976–18,212	237	78
pX	r	18,451–19,050	600	199
pVI	r	19,178–19,864	687	228
hexon	r	19,977–22,829	2853	950
protease	r	22,843–23,460	618	205
DBP	l	23,576–24,889	1314	437
100K	r	25,410–28,583	3174	1057
22K	r	28,252–28,809	558	185
33K	r	28,829–29,173	345	114
pVIII	r	29,213–29,938	726	241
fiber	r	30,189–31,907	1719	572
orf 22	l	31,967–32,539	573	190
orf 20	l	33,023–33,871	849	282
orf 19	l	34,256–36,478	2223	740
gam1	r	37,938–38,771	834	277
orf 17	l	39,866–40,366	501	166
orf 11	r	40,278–40,646	369	122
orf 11B	r	40,730–41,122	393	130
orf 23	l	41,924–42,985	1062	353

r=rightward transcribed strand.
l=leftward transcribed strand.

3.4. Genome characteristics

The virus genome of FAdV-11 MX95 is available in the GenBank database under accession number KU746335. The genome size is 44, 326 bp with a G+C percentage of 53.68%, and it codes for 36 putative open reading frames (ORFs) (Table 1). The genes involved in the infection and replication process are located in the central region of the genome; whereas genes that are not conserved within the *Adenoviridae* family are grouped at both ends (Marek et al., 2012; Griffin and Nagy, 2011).

The genome of MX95 has 99.3% similarity with FAdV-11 strain HBQ12 (KM096545) (Zhao et al., 2015), 93.3% similarity with FAdV-9 strain A-2A (AF083975) (Ojkic et al., 2002), and 90.8% similarity with FAdV-2 partial genome (EF458160 and DQ208708) (Corredor et al., 2006, 2008); all these strains belong to species D (to date, there are no reports of genome sequences available on the GenBank database for FAdV-3).

The inverted terminal repeats (ITRs) located at both ends of the genome are 72 bp in length. Repetitive sequences were calculated with Tandem Repeats Finder software; to avoid confusion with gene terminator sequences, we considered a sequence repetitive when the copy number of the sequence was more than 3. Under these criteria, the genome contains five regions of repeated sequences (TRs), the characteristics of which are described in Table 2.

Besides the differences in some nucleotide in orf (Fig. 3, Supplementary material 4), the biggest differences are in the repeat regions (TR) were located, particularly in TR1 and TR2. The TR1 of FAdV-11 HBQ12 is larger in 13 bp, representing less than 0.5 repetitions regarding FAdV-11 MX95-S11. Furthermore, the region TR2 of FAdV-11 MX95 is larger in 270 bp, which is equivalent to two additional copies regarding TR2 of FAdV-11HBQ12. The role in the virus replication cycle of TR sequences has not been determined, but some authors hypothesize that these sequences may regulate the expression of viral genes (Gruss et al., 1981).

Table 2
The positions of repetitive sequences in the genome of FAdV-11 MX95.

Repetitive sequence	Location	Period size	Copy number	%GC
ITR[a]	1–72, 44,275–44,326	72	2	53
TR-A	28,428–28,459	3	10.7	65
TR-B	37,004–37,101	20	4.8	27
TR-1	37,194–37,307	20	5.6	32
TR-2	38,833–39,833	135	7.4	74.5
TR-3	41,872–41,914	14	2.9	26

All of the TR regions were named based on the similitude at the position of the repetitive sequence according to FAdV-4 ON1 GenBank access GU188428 (Griffin and Nagy, 2011), and FAdV-10 C-2B Genbank access EF458162 and DQ208710 (Corredor at al., 2006, 2008).
[a] Inverted terminal repeats are considered repetitive sequences because are redundant elements at both ends of the DNA.

3.5. Fiber protein

The MX95 Fiber protein has 572 amino acid residues as in other FAdV-11. At the extreme -NH_2 end of the protein, we identified the basic residue-rich sequences RKRP (residues 17–20) and VYPF (residues 53–56), which are hypothetically involved in nuclear localization and interaction with the Penton base, respectively (Grgic et al., 2011). The percentage similarity of Fiber protein with FAdV-11 strain HBQ12 (KM096545) is 100%, and it is 99.8% with isolate 05-50052-2924-1 (JQ034217). In an amino acid residue alignment we do not see differences in specific amino acids associated with HHS among the serotype 4 strains (Pallister et al., 1996; Marek et al., 2012). However, it should be mentioned that virulence markers have not been reported in the amino acid sequence of the Fiber protein in the FAdV-11 causing IBH (Grgic et al., 2014).

4. Discussion

FAdVs are present in chicken farms worldwide and cause two main illnesses: inclusion body hepatitis and hydropericardium hepatitis syndrome (Ganesh and Raghavan, 2000; Asthana et al., ,2013; Zhao et al., 2015).

FAdV-11 MX95 was isolated from a healthy farm; however, from liver samples it was possible isolate a fowl adenovirus identified as FAdV-D serotype 11 according to the Hexon sequence (data not shown).

The clinicopathological characterization of the virus in SPF chickens showed that it is a low pathogenicity virus that does not cause mortality, not even clinical signs of the illness. Nevertheless, necropsy of the birds revealed that the virus produces some damage in the liver characterized by slight yellowing. The histopathology of the studied livers showed inclusion bodies between days 4 and 7, similar to what occurs with other viruses. (Chandra et al., 2000; Mase et al., 2012). It should be noted that no damage was observed in other organs of the birds in the necropsy or in the histopathology (Supplementary materials 1 and 2). These analyses show that FAdV-11 MX95 is a low pathogenicity virus and is not a primary mortality-causing agent.

However the virus is capable of replicating in SPF birds and remaining in many organs including lymphoid organs (bursa of Fabricius, thymus, cecal tonsils and spleen) (Fig. 2). Additionally,

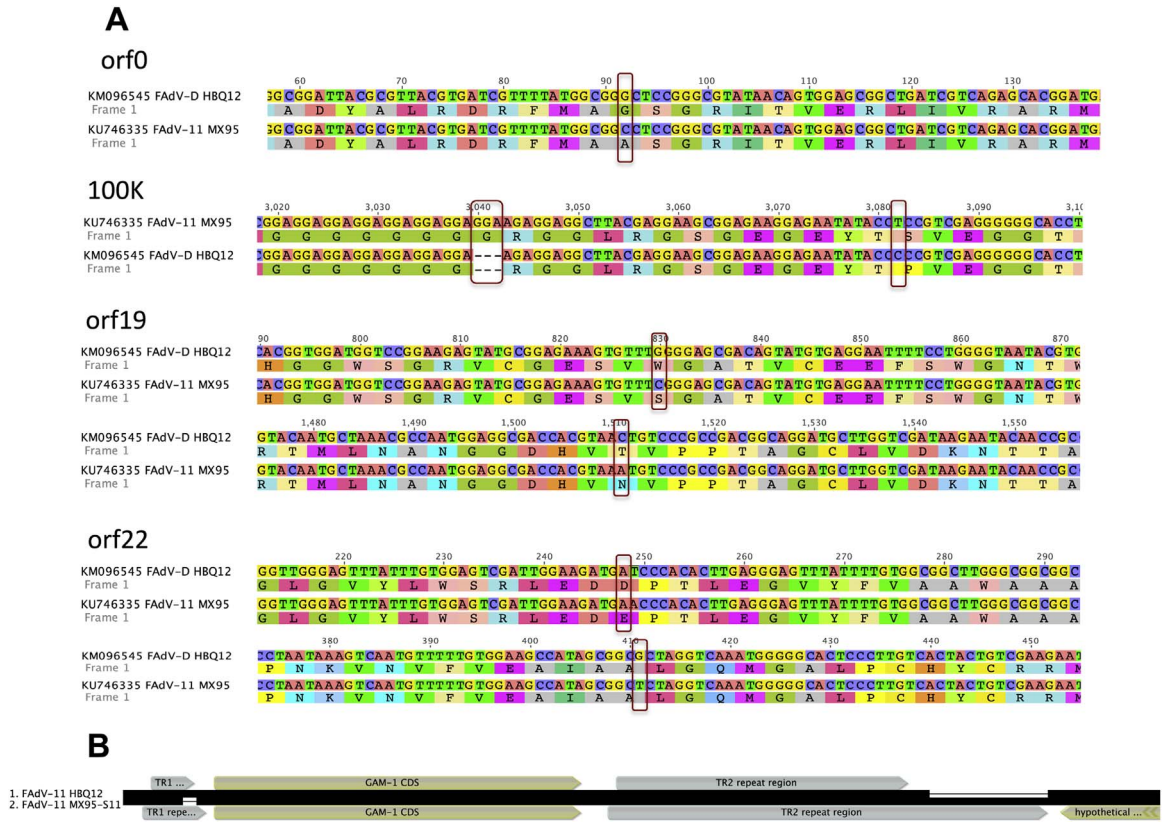

Fig. 3. A. Punctual nucleotide differences in a segment of a pairwise partial alignment of orf0, 100K, orf19 and orf22. In orf0, HBQ12 and Mx95 strains have a single nucleotide difference with change of amino acid G→A. In 100K a triplet insertion in Mx95 and a single nucleotide change in position 3082. In orf19, there are two not silent mutations in nucleotide 830 and nucleotide 1511. Orf22 have two nucleotide changes, the differences in nucleotide 248 generate a substitution of amino acid D→E; but the changes in nucleotide 411 generate a silent mutation. **B.** Size differences in TR1 and TR2 regions.

there is a relationship between the appearance of inclusion bodies and the number of genome copies detected by real-time PCR. With the exception of the spleen, it was possible to detect genomic FAdV DNA by real-time PCR throughout the experiment.

On the other side, in 2015 the genome sequence was published for a virulent FAdV-11, which is capable of inducing HHS (Zhao et al., 2015). To our knowledge, this is the first report of a FAdV-11 capable of inducing HHS. When we compare the genome of FAdV-11 strain HBQ12 (Zhao et al., 2015) to that of MX95, we find a high percentage of similarity (99.3%). In a comparative analysis of the two strains at each one of the orfs, we showed that in 32 of 36 orfs there is 100% similarity (with the exception of orf0, 100K, orf19 and orf22 with 99.8, 99.8, 99.5 and 99.4% similarity, respectively) (Fig. 3; Supplementary material 4). Additionally, we notice that the most of the observed differences in the genome are in noncoding regions. Even though the pathogenic differences between MX95 and HBQ12 are significant, the differences in their genome sequences are very few; and this distinctiveness is not clearly attributed to a specific gene.

It has been hypothesized that TR regions may have a role in regulating transcription or translation of some genes (Gruss et al., 1981; Chinen et al., 1996; Kawakami and Watanabe, 2003). In humans, it has been proved that thymidylate synthase gene (TS) is regulated by a region TR at the 5 'end of the gene. On TS Protein exists a direct relationship between the number of repetitions of TR sequence on the 5′ end and translation; that is, a highest number of periods increase the quantity of TS protein (Kawakami and Watanabe, 2003). In this sense, there are not significant differences on the number of repetitions between HBQ12 and MX95 strains. Furthermore, Chinen and co-workers determined at 1996 that a high number of repetitions of TR at the 3′ end of the gene SEC14L have a negative effect on the translation (Chinen et al., 1996).

Additionally, it is interesting that these regions TR are at both ends of gam-1 (on FAdV-11 HBQ12 and MX95 and all the serotypes of FAdVs). In fact, TR1 is at the 5 'end and TR2 at the 3′ end of *gam*-1 (Fig. 3B). As previously demonstrated, GAM-1 is a protein with antiapoptotic cell survival activity that enhances late in infection and facilitate spread of virus on the host tissue (Chiocca et al., 1997).

If the effect of the number of repetitions of TR has a similar effect on birds than described in humans, the fact that TR2 has a greater number of repetitions decrease the amount of GAM-1, which would help increase infection of the organs delaying apoptosis; this could be the reason on differences in virulence between FAdVs. However, this hypothesis should be demonstrated experimentally.

It should be mentioned that in FAdV-4, the genetic differences between pathogenic FAdV-4 (causing HHS) (Vera-Hernández et al., 2016) and non-pathogenic strains (Marek et al., 2012; Griffin and Nagy, 2011) total 1.9%.

With respect to genes that could be involved in virulence (particularly in HHS), several studies have suggested that the fiber protein could be a pathogenicity determinant for avian Adenovirus (Pallister et al., 1996; Marek et al., 2012). This hypothesis is based on amino acid sequences of pathogenic and non-pathogenic strains showing amino acid substitution (G→D at position 219 in strain KR5) changes when pathogenic and non-pathogenic strains are compared (Marek el at., 2012). In this study, we do not detect any differences between the amino acids of Fiber proteins from MX95 and HBQ12. These facts suggest that the difference in virulence between the two strains is not associated with Fiber protein in FAdV-11. Finally, it must be considered that some studies have shown that non-serotype 4 strains may induce HHS in immunosuppressed birds (Nakamura et al., 2003); similarly, the association of FAVs with chicken anemia virus may enhance HHS (Toro et al., 2000).

In this report, we present the genome and pathological characterization of a low pathogenicity strain of serotype 11 of fFowl Adenovirus species D. Currently, a lower number of FAdV genomes are available in the GenBank database, and most of the orfs are not characterized. It is necessary to continue with studies on protein function characterization and on the genetic determinants of virulence.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2016.11.006.

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