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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

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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

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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