Veterinary Microbiology 167 (2013) 357–363

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Molecular typing of fowl adenoviruses, isolated in Hungary recently, reveals high diversity

Győző L. Kaján [a,*], Sándor Kecskeméti [b], Balázs Harrach [a], Mária Benkő [a]

[a] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, PO Box 18, H-1581 Budapest, Hungary

[b] Immunological, Virological and TSE Laboratory, Veterinary Diagnostic Directorate, National Food Chain Safety Office, Bornemissza u. 3-7, H-4031 Debrecen, Hungary

ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 10 September 2013
Accepted 17 September 2013

Keywords:
Emerging pathogen
Fowl adenovirus
Genotyping
Gizzard erosion
Inclusion body hepatitis
Taxonomy

ABSTRACT

Molecular typing of 26 recent fowl adenovirus (FAdV) isolates obtained in Eastern Hungary between 2006 and 2011 was performed. The viruses were isolated on primary chicken embryo liver cell cultures from carcasses of chickens, sent for diagnostic investigation. The isolates were subjected to a PCR to amplify a fragment from the hexon gene. Sequence analysis of the amplicons revealed the presence of all the five FAdV species in the country, among them FAdV-B, which has been barely found in Hungary before. The three strains classified as FAdV-B might represent a novel type within this species, as their nucleotide sequence identity to strain 340 (type FAdV-5) is below the commonly accepted intratype limit. Most of the strains (63%) were classified into species FAdV-E (12 strains) and FAdV-D (7 strains) in the study. Four of the isolates proved to be the mixture of two adenovirus strains belonging to two different FAdV types, in three from these four cases, these two types even belonged to two different species. Analysing the pathological findings bolsters certain established connections between some FAdV types and disease conditions. Gizzard erosion was found in connection with FAdV-1 strains only, and inclusion body hepatitis with FAdV-D and -E strains.

1. Introduction

Adenoviruses are non-enveloped double stranded DNA-viruses. Currently, the family *Adenoviridae* is divided into five officially accepted genera (Harrach et al., 2011). Chickens can be infected by fowl adenoviruses (FAdVs), belonging to the genus *Aviadenovirus* (Harrach and Kaján, 2011), but also by the egg drop syndrome virus (duck adenovirus 1, genus *Atadenovirus*) (Both, 2012; Harrach et al., 1997) and by the turkey hemorrhagic enteritis virus (turkey adenovirus 3, genus *Siadenovirus*) (Davison and Harrach, 2011). FAdVs were classified into 12 serotypes: FAdV-1–8a and -8b–11 (McFerran et al., 1972; McFerran and Connor, 1977; McFerran and Smyth, 2000), and these types into five groups based on restriction fragment length polymorphism (Zsák and Kisary, 1984). These groups (FAdV-A–E) were retained when the adenovirus species were established (Harrach et al., 2011) supported by phylogenetic analyses based on hexon sequences (Marek et al., 2010a; Meulemans et al., 2004; Raue and Hess, 1998).

FAdVs can be transmitted vertically and horizontally by fecal route. Persistent infection often occurs and can lead to virus shedding in immunocompromised birds. Generally more than one adenovirus type can be isolated from diseased flocks, especially if they were established from several parent flocks. The most significant diseases associated with FAdVs in chicken are the inclusion body hepatitis, the hydropericardium syndrome and the gizzard erosion (Adair and Fitzgerald, 2008; Smyth and McNulty, 2007).

* Corresponding author. Tel.: +36 1 4674091; fax: +36 1 4674076.
E-mail address: kajan.gyozo@agrar.mta.hu (G.L. Kaján).

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.09.025

Profound investigation revealed that gizzard erosion is associated with pathogenic strains of FAdV-1 (Kecskeméti et al., 2012; Ono et al., 2001), the only type constituting species FAdV-A. The sequence of the long fiber (fiber-1) is assumed to be a marker of pathogenicity (Okuda et al., 2006), however European pathogenic strains could not been distinguished from apathogenic reference strains based on this method (Domanska-Blicharz et al., 2011; Marek et al., 2010b).

Traditionally, serological methods (i.e. virus neutralization) were used for the typing of FAdV isolates (McFerran et al., 1972; McFerran and Connor, 1977). With the increased number of serotypes the availability of a full panel of reference FAdV strains and antisera became restricted to a couple of laboratories. Molecular methods, i.e. PCR and sequencing, might circumvent this difficulty by comparing the results to deposited gene sequences. Several PCR methods have been published for the diagnostics of adenoviruses infecting poultry, some of these target the gene of the major capsid protein, the hexon (Mase et al., 2009; Meulemans et al., 2004; Raue et al., 2005; Raue and Hess, 1998), some the gene of the viral DNA polymerase (Hanson et al., 2006; Kaján et al., 2011; Wellehan et al., 2004). More sophisticated techniques have also been developed, using real-time PCR (Günes et al., 2012) and the high-resolution melting-curve analysis of the products (Marek et al., 2010a; Steer et al., 2009), as well as pyrosequencing (Pizzuto et al., 2010) or loop-mediated isothermal amplification (Xie et al., 2011).

In the majority of the recent surveys, novel FAdV isolates have been studied by molecular methods then classified into the existing serotypes (Choi et al., 2012; Lim et al., 2011; Mase et al., 2012; Ojkić et al., 2008b; Steer et al., 2011). We also performed our screening this way. Obviously, the type identity would require serologic confirmations, especially when a seemingly novel FAdV type is found/discovered. A fully molecular typing scheme to determine FAdV "genotypes", as proposed by Marek et al. (2010a), would be more straightforward, but ideally a universal system suitable for the typing and naming members of every group of the *Adenoviridae* family, including the most numerous human adenoviruses, should be established. To this end, comprehensive studies comparing the results of serological and molecular tests would be essential. With the increasingly easier and cheaper availability of whole genome sequencing possibilities, an appropriate algorithm could be established in the near future.

Although FAdVs are commonly isolated from chickens, recent publications on the prevailing different types in different countries of Europe are scarce. The present paper describes the molecular typing of 26 FAdV isolates obtained in Hungary between 2006 and 2011.

2. Materials and methods

2.1. Origin of the strains

The FAdV strains, described in this paper, were isolated from dead chicken originating from Eastern Hungarian broiler and layer pullet flocks with various clinical signs. The carcasses were sent to the Veterinary Diagnostic Directorate of the National Food Chain Safety Office, Debrecen, for diagnostic investigation. For virus isolation attempts, a 10% suspension was prepared from a pool of organs, including liver, heart, guts and sometimes tendons or the mucosa of the gizzard if these showed pathological alterations, in Eagle's Minimum Essential Medium (Sigma–Aldrich) containing penicillin (100 IU ml^{-1}) and streptomicin (0.1 mg ml^{-1}). After a low-speed centrifugation, freshly prepared primary chicken embryo liver cell cultures in test tubes were inoculated with the supernatant. The inoculated and control cell cultures were examined daily for the presence of cytopathic effects. After 4–6 days the supernatant of cultures without cytopathic effects were passaged blindly three times. When maximal cytopathic effect was observed, tissue cultures were frozen. The cells and the supernatant were mixed and subjected to two additional cycles of freezing and thawing. The mixtures were lyophilized in 0.5 ml aliquots.

2.2. PCR and sequencing

The lyophilized virus stocks were resolved in 1 ml sterile ultrapure water, then hundred fold dilutions were prepared also in sterile ultrapure water. Without further treatment, the diluted virus suspensions were subjected to a nested PCR published by Meulemans et al. (2004) with slight modifications. This method amplifies the immunogenic determinant loop 1 (L1) region of the hexon gene. The reaction volume was 50 μl consisting of 38.75 μl sterile ultrapure water, 5 μl of 10X DreamTaq Green Buffer, 2 μl of $MgCl_2$ solution (25 mM), 1 μl of deoxyribonucleotide mixture (10 mM), 1 μl of each primer (125 mM), 0.25 μl DreamTaq DNA Polymerase enzyme (Thermo Fisher Scientific) and 1 μl target solution for both rounds of the nested PCR. The thermal profile of the first reaction consisted of an initial denaturation step (at 94 °C for 5 min) followed by 35 cycles with denaturation (at 94 °C for 30 s), annealing (at 60 °C for 30 s) and elongation (at 72 °C for 45 s) steps. At the end of the program, the final elongation (synthesis) at 72 °C was allowed to proceed for 5 min. The annealing temperature of the second reaction was 55 °C, otherwise the same conditions were applied as in the first round. The PCR products were analyzed by agarose gel electrophoresis. The DNA fragments were purified directly from the reaction mixture with the use of the GeneJet PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

The sequence of the purified PCR products was determined on both DNA strands using the appropriate inner primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies) following the manufacturer's instructions. The electrophoresis was performed by a commercial supplier on an ABI PRISM 3100 Genetic Analyzer.

2.3. Molecular cloning of mixed isolates

Isolates, producing clean, easy-to-read sequence data, were assumed to contain only one FAdV strain. However, if the sequence trace file indicated that the PCR products