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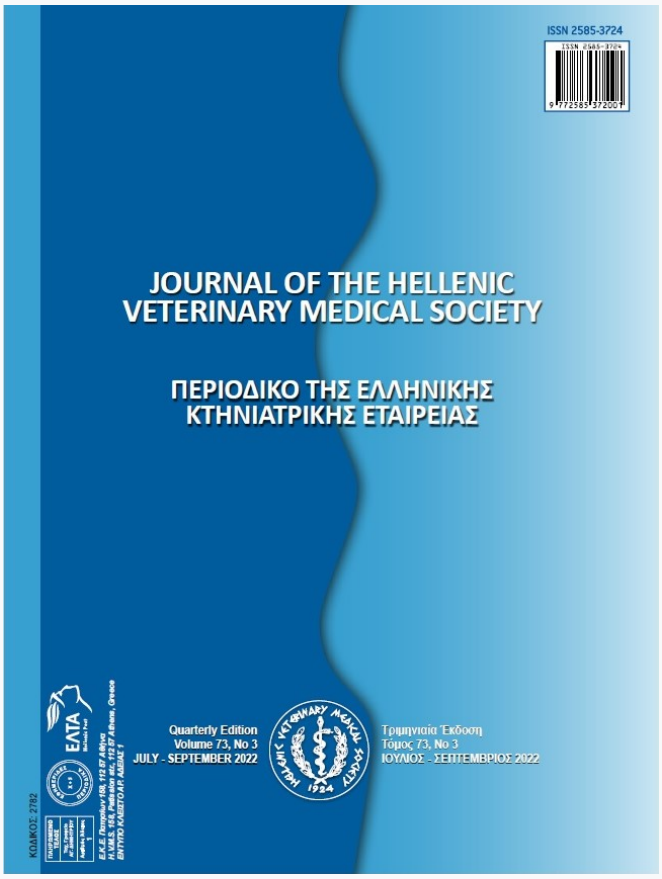

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Research article
Ερευνητικό άρθρο

Characterization of fowl aviadenovirus species in backyard flocks in Turkey

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ABSTRACT: Fowl adenoviruses (FAdVs) have a worldwide distribution in poultry at all ages and the incidence of the disease has been in an increasing trend in the last decade. Most of the studies had revealed the status quo of disease in commercial chickens, especially in broilers; however, there was not sufficient data to estimate in backyards. Thus, the primary goal of this study was to investigate the presence of infections in backyard poultry operations. For this purpose, 389 internal organ samples were collected from 56 flocks from clinically suspected backyard chickens at 10 to 35-week-age. Samples were tested by the PCR method targeting the nucleotide sequences of hexon genes (A-D). Amplified genes were sequenced by the Sanger sequencing method and the elicited data were further used for the phylogenetic analyses. Of the 389 samples, 23.9% were found to be FAdV positive which was equal to 17 out of 56 flocks sampled for this study. This is the first study being carried out in backyard operations and two species (Fowl adenovirus D and Fowl adenovirus C) and four serotypes (FAdV-8a, FAdV-3, FAdV-4, and FAdV-10) have been discovered for the first time in Turkey. As a result, the outcomes of this study provide a better understanding of the epidemiology of FAdV in Turkey; hence, will aid in creating a robust control and prevention strategy.

***Keywords*:** FAdV; serotypes; backyard; PCR;phylogenetic tree

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INTRODUCTION

Fowl adenoviruses are well-recognized etiologic agents of poultry birds, frequently contributing to clinical infections with the coexistence of other microbes. On the other hand, some certain species do cause severe disease, such are known as turkey haemorrhagic enteritis in Turkeys, quail bronchitis or egg drop syndrome. Although the Adenoviridae family comprises six genera in total, Fowl adenoviruses distribute into only three genera (*Atadenovirus*, *Aviadenovirus*, *Siadenovirus*), which consist of 8 species (A-E) and 11 serotypes (Benkő et al., 2022). A typical adenovirus virion composes of a 43-45 kbp-length double-stranded DNA, being surrounded by icosahedral symmetric, non-enveloped icosahedral capsid. Virions are mainly structured by three different polypeptides, often referred to as major capsid proteins (MCPs), which are hexon, penton base, and fiber proteins (Reddy and Nemerow 2014).

The members of the *aviadenovirus* genus present worldwide distribution. Fowl aviadenovirus D (serotype 2 and 11) and E (serotype 8a and 8b) are known to be closely associated with the inclusion body hepatitis (IBH) (Schachner et al., 2018). Since the first observation in the United States in 1963, IBH outbreaks had occurred periodically in different countries such as Mexico, 1975; Pakistan, 1987; Iraq, 1991 (Helmboldt and Frazier 1963; Anjum et al., 1989; Abdul-Aziz and Al-Attar 1991; Antillón and Lucio 2016). Similarly, IBH disease cases have been reported from Minor Asian territories and surrounding countries such as Greece, Saudi Arabia, Iran recently (Khodakaram-Tafti et al., 2016; Mohamed et al., 2018; Cizmecigil et al., 2020; Franzo et al., 2020). In addition, 8a and 8b serotypes can be detected from adenoviral gizzard erosion (AGE) cases, although the causative agent is generally accepted as Fowl aviadenovirus D (FAdV-1) (Okuda et al., 2004; Mase and Nakamura 2014). Hepatitis hydropericardium syndrome (HSS) is considered to be a more severe form of IBH possessing a higher mortality rate and incidence in poultry birds (Fitzgerald et al., 2020). Numerous studies have pointed out the fowl adenovirus type C (FAdV-4) attributes to HSS (Hess et al., 1999; Li et al., 2016, Vera-Hernández et al., 2016).

The economic consequences of fowl adenovirus infection in poultry have not been undertaken extensively and yet, some adenovirus species have remained unidentified since they are clinically neglectable (MacLachlan and Dubovi 2017). IBH and HHS can lead to significant losses in younger chicks (3 to 6 weeks), while AGE appears to be more independent from age determinants (Schachner et al., 2018). The mortality rate of the IBH can be between 5% to 30% in broilers, whereas rates can dramatically reach up to 80% death rate in HSS cases (McFerran and Smyth 2000). Fowl adenoviruses can spread via both horizontal and vertical routes, which enable viruses to survive in nature (Niczyporuk 2018) Furthermore, recent findings revealed that intertypic recombination mechanisms contributed to the molecular evolution of viruses and the emergence of hybrid serotypes (Schachner et al., 2019). In this context, backyard farming activities play a crucial role in the virus spillover events thereby assisting the circulation and possibly the evolution of viruses (Ayala et al., 2020). Considering the scarcity of literature reporting the incidence of adenoviruses in backyard flocks, we sought to investigate and characterize fowl adenoviruses using molecular detection methods in backyard poultry birds in Turkey.

MATERIALS AND METHODS

Sampling and nested PCR assay

A total of 389 samples were collected in Malatya, Elazig, Bingol, and Diyarbakir from 56 flocks between February 2018 and September 2018. Veterinary clinicians were asked to report and take internal organ samples from suspicious backyard chickens whose ages were between 10 and 35 weeks. Internal organ samples were homogenized and diluted 1:10 with 1 M phosphate-buffered saline. After the centrifugation (10 min at 3500 rpm; at +4°C) supernatants were submitted to a nucleic acid extraction procedure using a GF-1 Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions. Eluted nucleic acids were stored at −80ºC until use.

The primer pairs targeting the immunogenic determinant loop 1 (L1) region of the hexon gene were utilized for the nested PCR method as previously reported (Meulemans et al. 2001). The Hexon A (CAARTTCAGRCAGACGGT)/Hexon B (TAGTGATGMCGSGACATCAT) set, and Hexon C (SKCSACYTAYTTCGACAT)/Hexon D (TTRTCWCKRAADCCGATGTA) set would amplify 897 bp and 580 bp partial hexon gene, respectively. PCR mixtures were prepared in 25-µL volumes containing 12.5 µL of Quick-Load® Taq 2X Master Mix (NEB, USA), 200 nM of sense/antisense primers, and ~ 300 ng template DNA. A 1 µl aliquot from the Hexon A/B PCR

product in the first round was submitted to the Hexon C/D PCR, and the second-round PCR was conducted with the same conditions. PCRs were set up with an initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 94°C for 1 min; annealing at 60°C for 1 min and extension at 72°C for 90 sec; a final extension step at 72°C for 10 min. The final amplicons were separated by electrophoresis in 1.5% agarose gels stained with ethidium bromide (0.5 μg/mL) and DNA bands were determined under UV light.

Sequencing and Phylogenetic analysis

Five DNA amplicons were randomly selected for further investigation. For this purpose, amplicons with the expected size were separated from the gel by scalpel and purified using GF-1 AmbiClean Kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions. Purified amplicons were then bidirectionally sequenced using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The quality of each sequencing raw data was evaluated using Geneious Prime® 2022.0.1 software (Kearse et al., 2012) and chromatograms with discernible peaks were discarded. The good-quality reads for each sample were aligned with each other to obtain final sequencing data, which were deposited to the GenBank with accession numbers as follows: MN717240, MN717241, MN717242, MN717244, MN717245 (Table 1).

Table 1: Species and serotypes of strain detected in the present study

Strain name	Species	Serotype	Accession No
FAdV11/TUR/TypeE	E	8b	MN717240
FAdV19/TUR/TypeE	E	8a	MN717241
FAdV33/TUR/TypeD	D	3	MN717242
FAdV61/TUR/TypeC	C	4	MN717244
FAdV70/TUR/TypeC	C	10	MN717245

Sequencing data were initially subjected to multiple sequence analysis (MSA). Briefly, 549-bp partial hexon gene sequence data were aligned with other publicly available FAdV sequences in the National Centre for Biotechnology Information (NCBI) using the MUSCLE algorithm (Edgar 2004). In order to simplify analyses, sequence outputs obtained from this study were queried in the BLASTn database to include closely related strains only. Phylogenetic analysis was further conducted using the PHYML algorithm (Guindon et al., 2010). For this purpose, the best-fit model was selected using MEGA X (Stecher et al., 2020) and the lowest BIC score presenting model (TN93+G+I) was chosen for generating ML tree, which was bootstrapped 100 times (Tamura and Nei 1993).

RESULTS

PCR Detection of Field Samples

Of the 389 samples, 23.9% were found to be FAdV positive (Table 2). The virus was identified from 30.4% (17/56) of the flocks subjected to this study, whereas the positivity was 18.8% in Malatya,15.8% in Elazig, 60% in Bingol, and 45.5% in Diyarbakir on a provincial basis. In addition, fowl adenovirus presence was detected at least three flocks in each province.

Table 2: Distribution of positive samples and flocks

Provinces	No of positive flocks/ no of sampled flocks (proportion)	No of positive samples/ no of total samples (proportion)
Malatya	3/16 (18.8%)	23/106 (21.7%)
Elazig	3/19 (15.8%)	27/128 (21.1%)
Bingol	6/10 (60%)	21/73 (28.8%)
Diyarbakir	5/11 (45.5%)	22/82 (26.8%)
Total	17/56 (30.4%)	93/389 (23.9%)

Multiple Sequencing Analysis (MSA)

Multiple sequence analysis (MSA) based on 549-bp partial hexon gene sequences revealed the high variation between Turkish isolates ranging between 54.86 – 95.63%. The FAdV61/TUR/TypeC and FAdV70/TUR/TypeC showed the highest nucleotide identity (95.63%); however, restriction enzyme profile was not conserved among these two samples. Despite the high identity, only FAdV70/TUR/TypeC carried the *Mlu*I restriction motif (ACG CGT) between 511th and 516th positions. Similarly, both MluI and *BsiW*I (CGTACG) restriction motifs were detected in FAdV19/TUR/TypeE between 322-327 and 341-346 positions, respectively, whereas none of them was available in the FAdV11/TUR/TypeE genome because of the transversion substitution events. These two strains presented significantly high identity (78.69%). Notably, the *BsiW*I motif also existed in the FAdV33/TUR/TypeD sequence, but in a different position (between 35 and 40).

MSA was further applied to the predicted hexon protein sequence of partially obtained nucleotide sequencing data. Briefly, codons were converted to the

183 aa-length deduced polypeptide sequence and compared to each other. The predicted aa sequences had a high level of variation in sequences, which was between 18.58-93.44%. The FAdV61/TUR/TypeC and FAdV70/TUR/TypeC showed the highest similarity count (171 out of 183 aa) whilst FAdV33/TUR/TypeD significantly differed from the rest of the strains (34-45 out of 183 aa). FAdV11/TUR/TypeE was further compared with other Turkish strains since it exhibited high homology within the species. MSA demonstrated that FAdV11/TUR/TypeE exhibited 96.36-98.36% nucleotide identity and had the highest identity to strains TR/BVKE/R/B-8 (MK937071) and TR/BVKE/R/Y (MK937076). Notably, the only alanine-glycine mutation in the 79th residue occurred in Turkish isolates and this was commonly shared with Iranian and Malaysian strains.

Phylogenetic Analysis

Phylogenetic analysis was performed using selected strains from available data in the NCBI database (Figure 1). The phylogenetic tree demonstrated that FAdV61/TUR/TypeC and FadV70/TUR/TypeC clustered with the type C fowl adenoviruses; however, they fell into different branches. FAdV61/TUR/TypeC clustered with serotype 4 strains, while FAdV70/TUR/TypeC was with the serotype 10 strains. FAdV61/TUR/TypeC showed the highest identity (99.62%) to Italian field isolates (isolate 5997 and isolate 3890) and an Indian isolate (isolate B1-7; %99.44). Furthermore, FAdV70/TUR/TypeC was identical to the 23548/s/2010 Debrecen strain. In the phylogenetic tree, FAdV61/TUR/TypeC and FAdV70/TUR/TypeC clustered with the corresponding isolates with good bootstrap value, 94% and 75%, respectively (Figure 1).

Type E aviadenoviruses separated into multiple branches depending on their serotypes in the phylogenetic tree and FAdV19/TUR/TypeE and FAdV11/TUR/TypeE were clustered with the serotype 8a and 8b strains, respectively. FAdV19/TUR/TypeE presented 98.36% nucleotide identity to isolate 09-8990

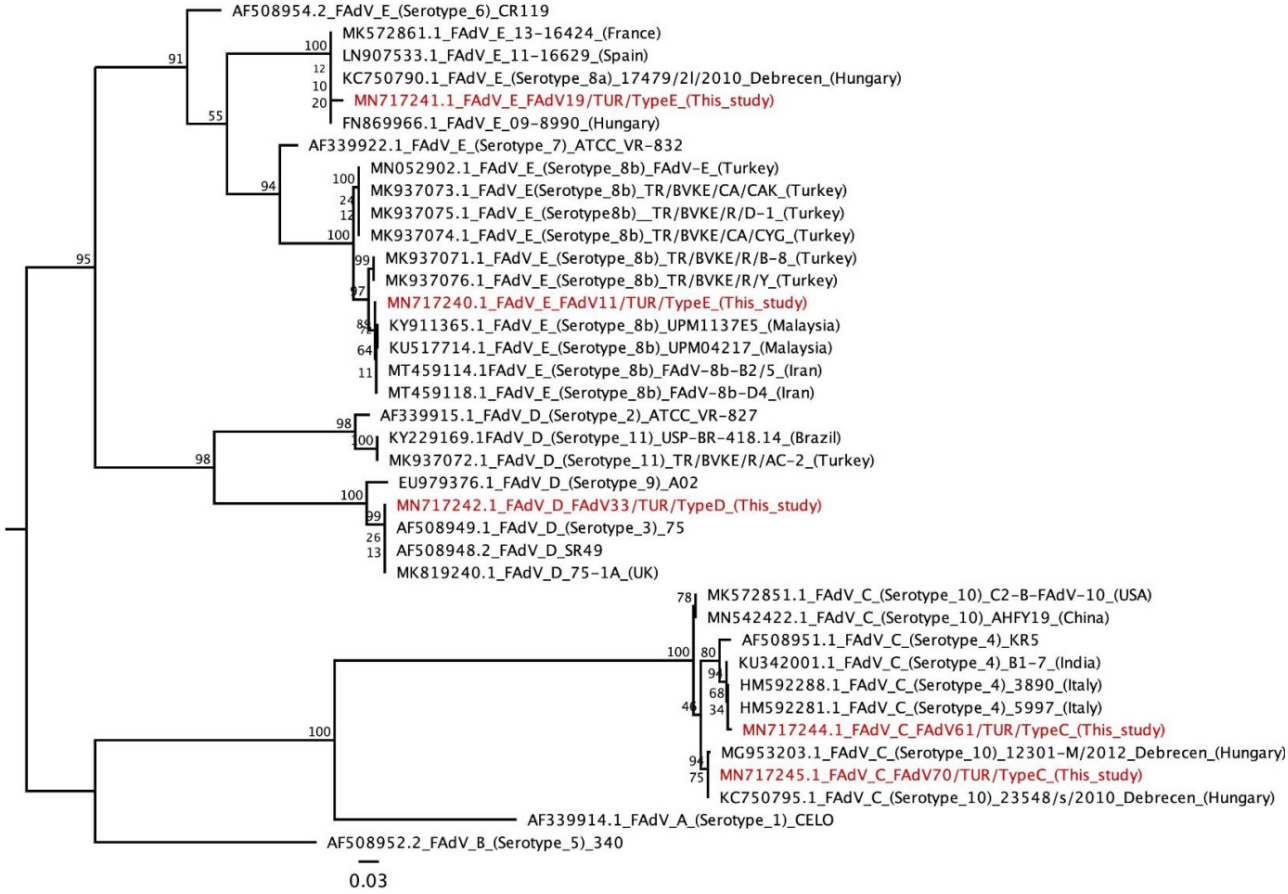

Figure 1: Phylogenetic tree showing the relationship of Turkish strains (red color) with other fowl adenovirus strains. The maximum likelihood (ML) tree was constructed using TN93 + G +I model in the PHYML algorithm. Bootstrap values supporting the branch points are expressedas the percentage of 100 replicates

(FN869966.1), strain 17479/2l/2010 (KC750790.1), isolate 11-16629 (LN907533), and strain 13-16424 (MK572861) and grouped into a single clade with 100% bootstrap value. On the other hand, FAdV11/TUR/TypeE participated in a clade which included Malaysian strains (strain UPM1137E5, KY911365; isolate UPM04217, KU517714) and Iranian strains (isolate FAdV-8b-B2/5, MT459114; FAdV-8b-D4, MT459118). Notably, previously reported Turkish strains separated from FAdV11/TUR/TypeE with good bootstrap value (89%). In addition, The highly varied FAdV33/TUR/TypeD strain was identical to a group D Aviadenoviruses (strain 75, AF508949; strain SR49, AF508948; strain 75-1A, MK819240), and therefore, they branched together in a clade with high bootstrap value (99%).

DISCUSSION

The majority of the studies on the fowl adenoviruses have been conducted in the chickens raising in the professional types of keeping whereas the infections in the farmyard flocks have been neglected so far. Backyard poultry farming has been in an increasing trend in the rural areas of Turkey, with lacking proper biosecurity measurements (Özdemir 2020). This omission frequently leads to the reciprocal transmission of pathogens between local poultry farming areas and wildlife which may have very serious consequences in the industry (Gilchrist 2005; Muzaffar and Takekawa 2010). Thus, the primary goal of this study was to provide information about the molecular characteristics and possible variations of the FAdV species in backyard flocks in Turkey.

Various methods have been proposed to detect FAdV from samples including electron microscopy, ELISA or IFA (Hess 2000). The conventional PCR and restriction fragment length polymorphism (RFLP) together are frequently applied for the determination of serotypes (Zsák and Kisary 1984; Okuda et al., 2006). In this study, nested PCR and sequencing were initially utilized as previously described (Meulemans et al., 2001), then restriction maps were determined regarding *Mlu*I and *BsiW*I enzymes by *in silico* method. Unfortunately, PCR products obtained from the first round of nested PCR were below the detectable limit under the UV light; hence, data elicited from the second round of PCRs were taken into consideration only. Results demonstrated the strains in the same serotype (FAdV11/TUR/TypeE and FAdV19/TUR/TypeE) can present different restriction motif patterns because of point mutations. The strong negative selection pressure exists on the structural gene of adenoviruses (Cheng et al., 2018) therefore, we conjectured that the evolutionary mechanisms might disrupt the restriction enzyme motifs thereby nullifying the validity of RFLP analysis.

Despite having worldwide distribution, the incidence of fowl adenoviruses directly related to genera: Fowl adenovirus D and E genera occur the most common with the percentage of 34% and 50%, respectively. In contrast, fowl adenovirus C has rather a minuscule part of the overall distribution with only two per cent (Kiss et al., 2021). Two recent studies carried out in Turkey have pointed out the occurrence of FAdVs in commercial broilers; however, fowl adenovirus prevalence remained unknown because of the low number of samples (Cizmecigil et al., 2020; Şahindokuyucu et al., 2020). The CELO and EDS'76 were comprehensively investigated in the serum samples obtained from 107 commercial farms using AGID and HI methods, and the positivity rates were reported at 8.4 and 17.7%, respectively (Sayim et al., 1988). In this study, we collected samples from backyard flocks in non-urbanized areas in the Eastern provinces of Turkey and determined a 23.9% positivity rate of the overall samples using nested PCR. This rate seems to vary through the provinces between 21.1% and 28.8%.

The FAdV-C has been remarked as a causative agent of HSS and the novel variants of FAdV-C have emerged in multiple outbreaks since 2015 (Ye et al., 2016). Furthermore, FAdV-C serotype 4 was identified with hydropericardium syndrome (HPS) cases in China (Li et al., 2018). Recently, FAdV-C serotype 4 was found in both backyard flocks in California (Mete et al., 2021). Hypervirulent strains of serotype 4 can maintain subclinical infection in ducks and contribute to virus shedding (Pan et al., 2017). In this study, FAdV-C serotype 4 (FAdV61/TUR/TypeC) showed the highest identity to Italian field isolates (isolate 5997 and isolate 3890) previously identified by the pyrosequencing method and an Indian isolate (isolate B1-7), whereas FAdV-C serotype 11 (FAdV70/TUR/TypeC) was identical to the 23548/s/2010 Debrecen strain, which was isolated from clinically infected juvenile layers (Pizzuto et al., 2010; Kaján et al., 2013). Taken together, FAdV-C serotypes might exist in both commercial and backyard poultry regardless of clinical manifestation of birds and might contribute to multiple syndromes such as HSS and HPS in Turkey. Additionally, we have detected FAdV-C sero-

type 4 (FAdV61/TUR/TypeC) and 11 (FAdV70/TUR/TypeC) for the first time in the backyard poultry operations in Turkey. Nonetheless, further information is needed to elucidate potential FAdV transmission between the backyard and commercial flocks.

FAdV-D and FAdV-E species together are frequently isolated from inclusion body hepatitis (IBH) cases. For example, a detailed study exhibited that FAdV-D serotype 11 is responsible for up to 33 per cent of the IBH that occurred in Spain in the last decades, while FAdV-E serotype 8a and 8b were 64.7% (Bertran et al., 2021). Recently, the presence of FAdV-D serotype 11 and FadV-E 8a and 8b has been documented in Turkey (Cizmecigil et al., 2020; Şahindokuyucu et al., 2020). In this study, FAdV33/TUR/TypeD (MN717242) strain was identical to the European-originated representative strain (SR49, AF508948) defined as "serotype 3" (Meulemans et al., 2004). Strict genetic conservation and cross-reaction rates were found within the members of the serotype 3; therefore, more varied genetic regions (i.e. ORF19) could be considered to differentiate these strains from each other (Schachner et al., 2019). We also detected another FADV-E serotype 8b (FAdV11/TUR/TypeE, MN717240) which carried unique mutations discriminating from previously reported Turkish 8b strains and amino acid similarities with Iranian isolates in several positions. These results raised the question of whether recombination events exist or not; thus, whole-genome sequencing and cross-reaction tests must be availed of to characterize fowl adenoviruses in Turkey (Schachner et al., 2019). Finally, we exhibited the FAdV-E serotype 8a in backyard poultry chicks (FAdV19/TUR/TypeE, MN717241) which presented close phylogenetic relation with various strains isolated from Hungary, France and Spain (Kaján et al., 2013; Schachner et al., 2019).

We are aware that there are several limitations to this study. First, internal organs from clinically infected chickens were not subjected to the histopathological examination for more accurate IBH, HSS or AGE diagnoses. Second, only a few samples were sequenced (5 out of 93), whereas the rest of the strains remained unknown. Finally, this study concentrated on the fowl adenovirus infections only, even though concurrent diseases have been reported (Toro et al., 2000; Mei et al., 2020). It is also important to note that simultaneous infections with multiple strains of fowl adenovirus can enhance the viral replication capacity of strains and increase the viral load (Liu et al., 2021). Notwithstanding, the outcomes of this study may provide valuable information for the further investigation of the fowl adenoviruses in the same geography.

CONCLUSIONS

In this study, we contributed to an expanding understanding of molecular characteristics of fowl adenovirus strains in the backyard flocks which might become a reservoir for the transmission of some diseases in wildlife. In our understanding, large-scale national studies should be conducted in different avian species, not only commercial and backyard chickens, but also turkeys, geese, ducks, raptors and migrating birds to understand the epidemiology of fowl adenoviruses and gain sufficient information about the species and serotypes. We reckon that these data would be helpful to conceive rational control and prevention strategies against adenoviral diseases in poultry.

CONFLICT OF INTEREST

None of the authors declare a conflict of interest.

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