

Molecular epidemiology of fowl adenoviruses in Greece

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ABSTRACT Outbreaks of inclusion body hepatitis (IBH) and adenoviral gizzard erosion have been anecdotally reported in Greece since approximately 2011. However, a relevant increase in clinical outbreaks compatible with IBH has been described since 2014. Unfortunately, with limited exceptions, only serological assays were performed, and involved strains were not properly characterized. In the present study, 35 outbreaks were investigated in the period between July 2017 and February 2018 in Greece. In addition to clinical and histopathological diagnosis, fowl adenovirus (FAdV) presence was investigated by PCR and sequencing. Thirty-four out of 35 samples tested FAdV positive. Twenty-nine (85.29%) and 5 (14.71%) strains were classified as FAdV-E and FAdV-D, respectively. Fowl adenovirus-E strains were genetically homogeneous and formed an independent cluster of Greek-only sequences,

including the sole previously available sequence, suggesting the prolonged circulation of this species in Greece. On the contrary, FAdV-D strains were more heterogeneous and closely related to strains sampled in other European countries, testifying the occurrence of multiple introduction events. The evaluation of phylogenetic relationships, geographic clustering, age of infection, and origin of the broiler breeder flocks suggests that both vertical and horizontal transmission are important in FAdV epidemiology in Greece and highlights the limited efficacy of currently implemented control measures. Of note, a significantly higher mortality was observed in precociously infected flocks, likely because of the higher susceptibility of younger animals. This evidence stresses the need of preventing vertical and/or early infection to limit the economic impact of adenovirus-induced diseases.

Key words: fowl adenovirus, Greece, epidemiology, species, sequencing

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INTRODUCTION

Fowl adenoviruses (FAdV) belong to the genus *Aviadenovirus* within the family *Adenoviridae*. They are classified into 5 different species (FAdV-A to FAdV-E) because of their molecular structure and into 12 serotypes (FAdV-1 to -8a and -8b to -11), as a result of cross-neutralization tests (King et al., 2011; <http://ictv.global/report/>).

Fowl adenoviruses have been isolated from cases of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome, and adenoviral gizzard erosion (Hess, 2013).

For more than 10 yr, FAdV have been described worldwide, highlighting their widespread circulation in chicken flocks. Although, the presence of immunosuppressive factors was initially considered of great relevance in the pathogenesis of IBH, in recent years, a rising interest toward the investigation of FAdV as primary etiologic agents emerged (Hess, 2017).

Although an increase in FAdV-associated outbreaks frequency has been documented in many countries all over the world, the reported cases are still an underrepresentation of the actual epidemiological situation (Schachner et al., 2018).

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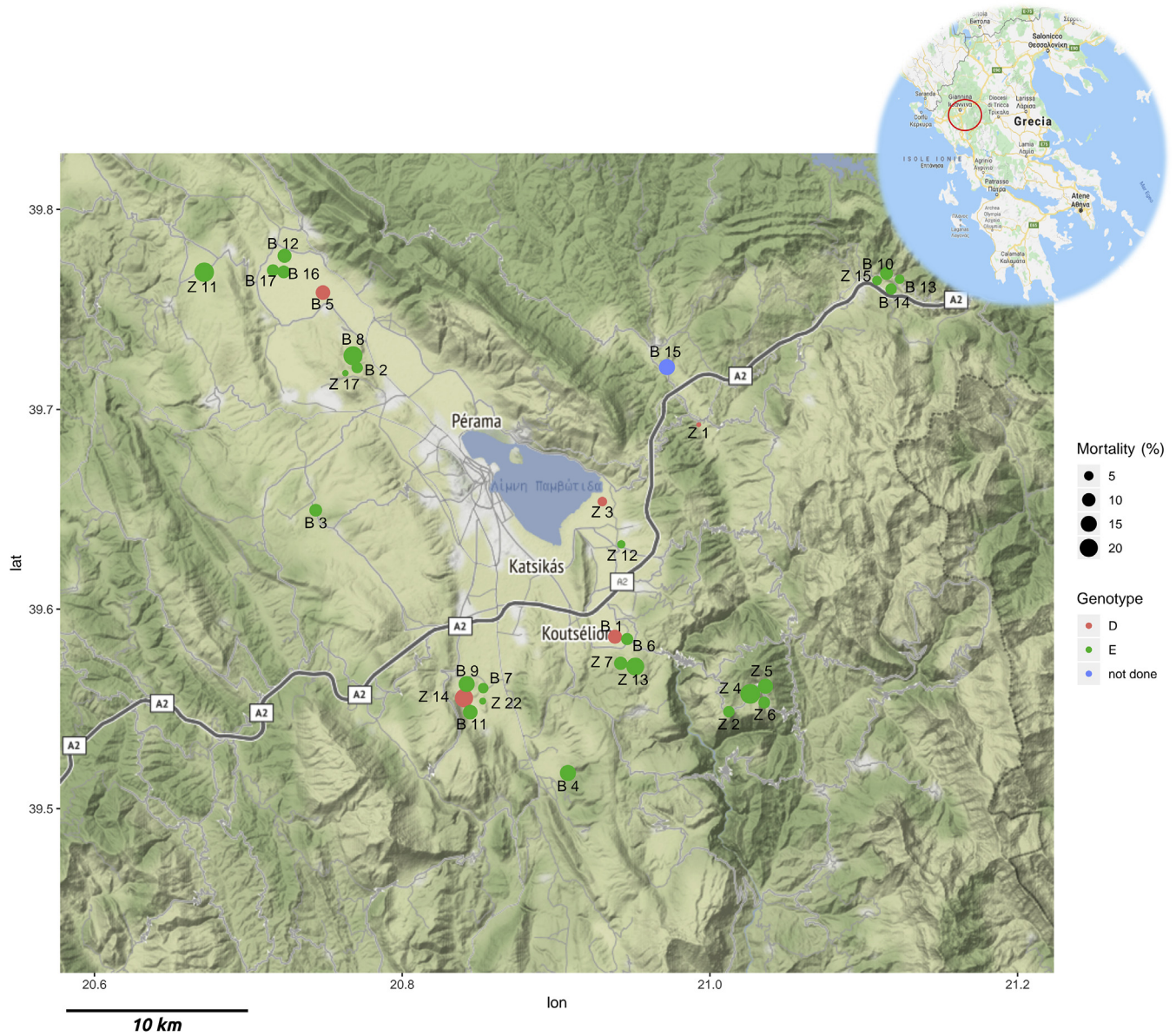


Figure 1. Maps reporting the geolocalization of the tested broiler farms (full circles). Flocks have been color-coded based on the detected FAdV species. The diameter of the circle is proportional to the observed mortality (in percentage). A slight jittering has been applied to farm location to avoid point overlap. The area investigated in the present study is reported in the top-right insert. Abbreviation: FAdV, fowl adenovirus.

The higher incidence of FAdV has been speculated to be linked to the efficacy of the elevated standards of biosecurity in preventing FAdV natural infection in breeder stocks, which causes a lack of immunity in parental birds and therefore the absence of maternal-derived immunity in the progeny (Schachner et al., 2018).

In Greece, an increased number of IBH outbreaks were recorded since 2014, when a sudden rise in mortality rates of broiler flocks was reported. In some affected flocks, birds suddenly died with no evident clinical signs. However, in many flocks, birds exhibited signs and lesions suggestive of IBH infection. The *postmortem* examination revealed enlarged, pale yellow-colored, friable livers with scattered hemorrhages and necrosis. The kidneys were swollen, enlarged, pale, and mottled with multiple hemorrhages as well.

Furthermore, anemia and icterus of the skin were observed in affected birds (Prentza and Koutoulis, 2017). Additionally, Koutoulis (2015) reported FAdV-A cases in broiler breeders and their progeny, which manifested major heterogeneity in flock size and ulcers in gizzard. Anecdotally, many problems of IBH and adenoviral gizzard erosion have been informally reported since 2011. However, the diagnosis is still essentially performed by ELISA methods, resulting in elevated antibody titers of FAdV without a further characterization of the involved species.

The aim of our study was to investigate the clinical and molecular epidemiology of FAdV in Greece during massive hepatitis outbreaks occurred between 2017 and 2018 and try to find the likely source of FAdV-induced disease outbreaks.

Table 1. Summary table reporting the metadata associated to the considered samples.

Sample	Mortality (%)	Clinical sign onset	Species	Breeder origin
Z 1	3%	18	D	4; 34
Z 2	6.97%	20	E	34; 13
Z 3	5.1%	16	D	4
Z 4	24.3%	20	E	25; 4
Z 5	13%	20	E	34; 21; 5; 10; 18; 2
Z 6	7.5%	27	E	26; 7; 17
Z 7	9.75%	25	E	7; 26
Z 8	3.98%	30	E	16
Z 9	7.4%	29	E	16; 19; 4
Z 10	15.45%	16	E	32; 1
Z 11	23.49%	8	E	31; 3
Z 12	4.1%	27	E	25; 5
Z 13	19.3%	18	E	32
Z 14	20.2%	21	D	30; 32
Z 15	5.15%	20	E	33; 12
Z 16	7.1%	15	E	33
Z 17	3.2%	21	E	14; 25
Z 22	3.39%	19	E	30
B 1	11%	16	D	4; 22
B 2	7%	28	E	3
B 3	9%	17	E	33; 3
B 4	15%	16	E	1
B 5	12%	15	D	33; 1
B 6	8%	14	E	23
B 7	6%	23	E	32
B 8	22%	16	E	31
B 9	15%	16	E	31
B 10	9%	23	E	25
B 11	12%	16	E	1
B 12	11%	20	E	31
B 13	7%	16	E	36
B 14	5%	22	E	31
B 15	15%	16	Negative	23
B 16	8%	23	E	30
B 17	9%	25	E	23; 12
Z18		Embryos		32
Z19		Embryos		1
Z20		Embryos		33
Z21		Embryos		23

Abbreviation: FAdV, fowl adenovirus.

Mortality percentage, animal age at disease onset, detected FAdV species, and breeder flock source (coded by numbers) have been reported.

MATERIALS AND METHODS

Samples

Liver samples were collected from 35 affected broiler flocks of different size, located in a limited geographic region of Greece (Figure 1). Flocks were included in the study because of an increase in mortality rate in presence of clinical signs and lesions compatible with FAdV infection.

Specimens from liver tissue with necrotic focal lesions were collected from freshly dead birds and put into 10% neutral formalin solution for histopathological investigation, whereas the rest of the liver was stored at -20°C until processing.

To investigate the occurrence of vertical transmission of FAdV, dead-in-shell chicks from the suspected broiler breeder farms (supplying the affected broiler flocks and experiencing a decrease in reproductive performance) were retrieved from the eggs at the lowest point of hatchability. Embryos were examined, and liver samples were collected. Organ samples were pooled and frozen at -20°C until DNA extraction.

Moreover, for all sampled flocks, the origin breeder flock was traced back, and the presence of clinical signs or lower productive performances was recorded (Table 1). All considered analyses and medical procedures were performed in the context of routine diagnostic and clinical activity, and no experimental treatments or additional assays were implemented during the study.

Histopathology

Tissues were fixed in 10% formalin, processed by conventional methods and embedded in paraffin wax. Sections were cut at $4\ \mu\text{m}$, mounted on glass slides, and stained by hematoxylin and eosin according to standard procedures.

Molecular Biology

Pieces of liver tissue were collected, added with PBS in a ratio 1:10 (g/mL), and mechanically homogenized. DNA was extracted from 200 μL of homogenate using

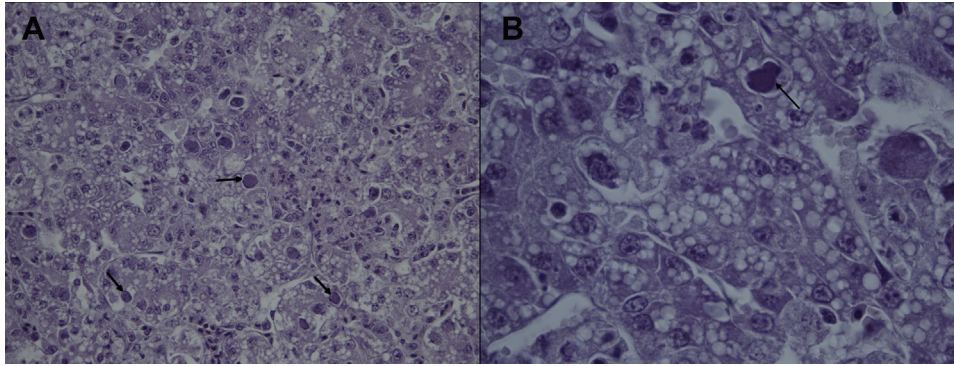


Figure 2. Hepatic degeneration in presence of eosinophilic intranuclear inclusion body (arrows) in hepatocytes (H&E; 400X [A] and 1000X [B]).

the DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) kit according to manufacturer instructions. Amplification of the partial Hexon gene (1,355 bp) was attempted on all samples with the primer pair HexonA (5'-CAARTTCAGRCAGACGGT-3') -H2 (5'-AAGG-GATTGACGTTGTCCA-3') using the PlatinumTaq DNA Polymerase kit. Five μ L of extracted DNA were added to a standard mix composed of 1X PCR buffer, 1.5 mmol $MgCl_2$, 0.2 μ mol of each dNTP, 0.5 of each primer, and 2 U of PlatinumTaq DNA Polymerase. Molecular biology grade water was added up to the final volume of 25 μ L. The following thermal protocol was selected: 94°C for 2 min, followed by 45 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 50 s. A final extension phase at 72°C for 5 min was also performed. Presence and specificity of PCR products were assessed by 2% SYBR Safe (Invitrogen) stained agarose gel electrophoresis.

Positive samples were Sanger sequenced at Macrogen Spain (Madrid, Spain) in both strands using the same PCR primers. Chromatograms were evaluated with FinchTV (<http://www.geospiza.com>), and consensus sequences were obtained using ChromasPro (Version 2.0.0, Technelysium Pty Ltd.).

Sequence Analysis

The hexon gene sequencing was selected because it represents a variable and phylogenetically informative region of the viral genome, commonly used to allocate isolates to individual species and characterize the epidemiological links (Ganesh et al., 2001; Meulemans et al., 2004; Marek et al., 2010).

Obtained sequences were preliminary used for species identification using BLAST (Madden, 2013). Thereafter, a multiple alignment of obtained sequences was performed. To account for the coding nature of the considered region, the alignment was performed at amino-acid level and then back translated to nucleotide sequences using MAFFT (Standley, 2013) method implemented in TranslatorX. Pairwise genetic distance was calculated using MEGA X (Kumar et al., 2018), whereas a maximum likelihood phylogenetic tree was reconstructed using IQ-Tree (Trifinopoulos et al., 2016) selecting as substitution model the one with the

lowest Bayesian information criterion, calculated using JmodelTest (Darrriba et al., 2012). Phylogenetic tree reliability was evaluated performing 10,000 ultrafast bootstrap replicates.

To evaluate the relationship between Greek samples and worldwide sampled ones, a full collection of Hexon gene sequences was downloaded from GenBank (accessed 25/05/2019) and aligned to the sequences obtained in the present study. Nevertheless, considering the different location and length of publicly available sequences, short (i.e., less than 600 bp) and/or poorly aligned ones or those with unknown bases or frameshift mutations were excluded from further analysis to achieve a compromise between sequence number and alignment length. Sequence names were annotated with the corresponding host, country, and collection date, when available. The phylogenetic tree was reconstructed using the previously described approach.

Statistical Analysis

Data management and statistical analyses were performed using the R package and related dependencies. The correlation between genetic and geographic distances was assessed using Mantel test implemented in *ecodist* library (Goslee et al., 2007). More in detail, the genetic matrix distance was calculated using *ape* library (Paradis et al., 2014), whereas the among farm geographic matrix distance was obtained using *geosphere* library (Hijmans et al., 2017). The difference in mortality between FadV species was analyzed using Mann-Whitney test. The relationship between clinical sign onset and mortality was assessed by fitting a linear model. Statistical significance level was set to P -value <0.05 for all analyses.

RESULTS

Greece FAdV Epidemiology and Pathology

Thirty-five flocks were affected by clinical outbreaks compatible with IBH, between July 2017 and February 2018, in the Regional unit of Ioannina.

All flocks were sampled and tested (Figure 1). Of those, 3 farms were sampled twice (i.e., samples Z7 and

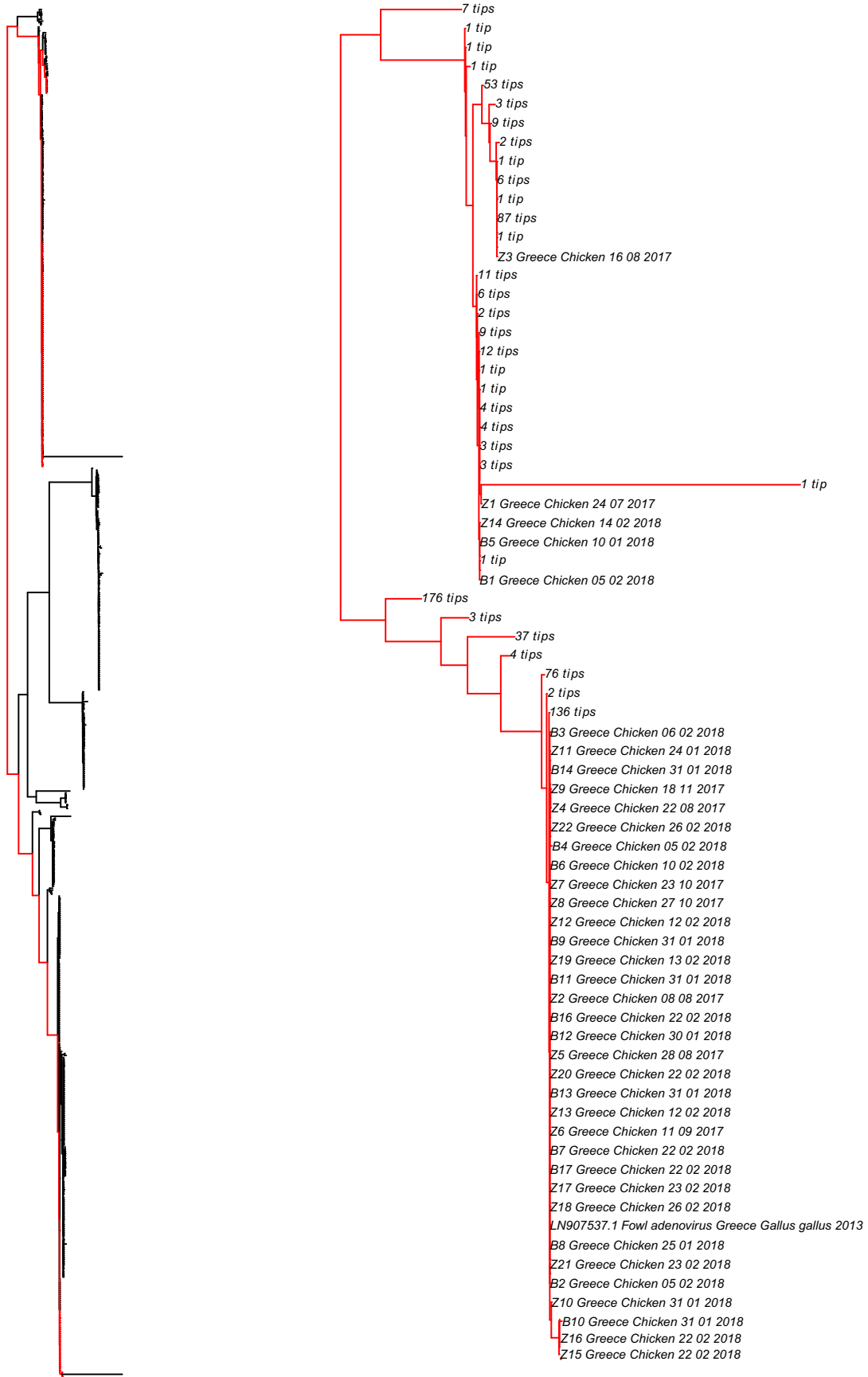


Figure 3. Maximum likelihood phylogenetic tree based on the international data set. Branches leading to Greek sequences are highlighted in red and magnified in the right insert. For representation easiness, only Greek sequences labels are reported while the number of hidden strains is indicated. The complete tree is reported as [Supplementary Figure 1](#).

Z13, Z9 and Z10, and B16 and B17 originated from the same farms, respectively). All birds belonged to the same breed (Ross 308).

Some of the affected broiler flocks originated from the same broiler breeder farm (Table 1). Of those, some experienced a moderate drop in the number of hatched chicks between 39 and 49 wk of life, whereas egg production remained normal. No further clinical signs were observed in the breeder farms.

The outbreaks of IBH in broilers were characterized by mortality starting as early as 8 d of age. In most cases, an increase in mortality was detected at around 18 d of age, peaking within 4 to 6 d and ceasing within 9 to 12 d. Overall, the mortality ranged from 3 to 24.3%. Higher mortality was observed in flocks experiencing early disease onset (less than 3 wk) ($12.465 \pm 5.912\%$) compared with delayed infection ($8.628 \pm 5.505\%$) ($W = 198.5$, P -value = 0.05). Animals displayed lethargy, huddling with ruffled feathers, inappetence, and yellowish mucoid droppings.

At necropsy, livers were enlarged, pale, and friable, sometimes with necrotic foci and hemorrhages. Kidneys were enlarged and mottled with multiple hemorrhages. Icterus of the skin and subcutaneous fat were also observed.

The most characteristic histological lesions of all analyzed samples were eosinophilic intranuclear inclusion bodies in the hepatocytes (Figure 2). In addition, fatty degeneration and necrosis were observed.

FAdV Molecular Characterization

Thirty-four out of 35 flocks tested FAdV positive and were molecularly characterized. Twenty-nine (85.29%) and 5 (14.71%) strains were classified as FAdV-E and FAdV-D, respectively. When farms were sampled several times, the same viral species (i.e., FAdV-E) was consistently detected. All liver samples ($n = 4$) collected from embryos tested positive to FAdV-E.

More in detail, the FAdV-E strains detected in the present study were genetically homogeneous in the considered hexon gene region (final alignment length=1,245 bp): mean p-distance = 0.002, range: 0-0.018. Particularly, one major clade of identical sequences comprising most of the strains originating from broiler flocks and all the embryo-derived ones was identified (Figure 3). The strains Z7-Z13 and B16-B17, longitudinally collected from 2 distinct farms, were also part of this clade. However, samples Z9 and Z10, also collected from the same farm almost 2 mo apart, showed different sequences (p-distance = 0.002).

Although FAdV-D strains appeared more heterogeneous (mean p-distance = 0.019, range: 0-0.045), strains Z14, B1, and B5 were closely related from a genetic perspective (Figure 3). Nevertheless, the farms of origin were geographically distant, and the animals had different breeder sources (Figure 1 and Table 1).

When the geographical pattern was evaluated, a certain tendency toward outbreak clustering was observed (Figure 1). Although FAdV-D and FAdV-E

were never detected in the same farm, neighboring flocks were infected with different FAdV. Accordingly, no statistical association was found between genetic and geographic distance (P -value = 0.09).

Average mortality was similar between flocks infected with FAdV-D (average mortality: 10.26 ± 6.74003) and FAdV-E (average mortality: 10.24414 ± 5.958495) ($W = 71$, P -value = 0.9612).

A significant negative relationship was found between clinical sign onset and mortality for FAdV-E ($b = -0.628$, P -value = 0.002) but not for FAdV-D ($b = 1.467$, P -value = 0.370).

International Data set

To evaluate the relationships between Greek and worldwide FAdV sequences, a final data set of 705 sequences (alignment length = 764 bp) was prepared. A monophyletic cluster with high bootstrap support (i.e., 99%) including all and only Greek FAdV-E sequences was identified. Remarkably, a sequence sampled in the same country in 2013 was also part of this cluster (Figure 3 and Supplementary Figure 1).

On the contrary, FAdV-D were classified in 3 different clusters and were more closely related to strains previously sampled in European countries such as Germany, Hungary, Poland, Sweden, and United Kingdom (Supplementary Figure 1).

DISCUSSION

An increase in the number of IBH clinical outbreaks has been reported since 2014. However, although the clinical signs and lesions were suggestive of FAdV infection, a proper molecular detection and characterization of the involved strains was not performed. The present study allowed to confirm the high frequency of FAdV infection in the tested farms (97,14%). While this value cannot be representative of the actual prevalence, it suggests the high accuracy of clinical diagnosis, supporting the reliability of the previous anecdotal reports. The detected strains belonged to FAdV-D and FAdV-E, confirming the pivotal role of these species in IBH (Kichou et al., 2020). A higher FAdV-E frequency was detected. Particularly, FAdV-E strains were part of a homogeneous clade composed by Greek-only sequences, including those obtained in the present study and a previously published one, sampled in 2013. This suggests the persistent circulation of FAdV-E in Greece and its role as a major species. How it was able to persist and circulate among farms is hard to be established. Both vertical and horizontal transmission are involved in FAdV epidemiology (Grgić et al., 2006; Sharma and Ahmed, 2018). In this case also, several evidences pose in favor of the vertical transmission role. All strains sequenced from the embryos were part of the main FAdV-E clade and therefore identical to those detected in affected broiler farms, suggesting breeders were likely the infection source. Seventeen farms demonstrated an early (21 d) clinical sign onset, more compatible with a

vertical infection rather than an introduction from external sources. A precocious infection because of poor cleaning and disinfection and/or depopulation could also be plausible. However, 7 out of 17 farms with early mortality received pullets from breeders whose embryos were FAdV-E infected. Thus, at least in some instances, the vertical transmission can be definitely stated. On the other hand, it must be stressed that the only negative flock received animals from infected breeders as well. A previously reported intermittent viral excretion could explain this apparently contradictory scenario (Grgić *et al.*, 2006; Fitzgerald, 2017).

A significant association was found between disease emergence age and mortality for FAdV-E only. Because of the substantial genetic identity of the considered strains, a higher susceptibility of younger animals, rather than virulence differences, is likely in place. In Greece, breeders are not vaccinated against FAdV; therefore, animals can be expected to be fully susceptible since the first days of life. These evidences stress the need of effectively preventing vertical or early infections due to within-farm viral persistence, to limit and control disease-related costs. A similar association was observed for FAdV-D outbreaks; however, their low number decreases the statistical power and prevents any reliable conclusion.

Although vertical transmission could be advocated in several outbreaks, horizontal one is also likely involved in the Greek epidemiological scenario. The late outbreaks emergence in different flocks barely fits with a precocious, vertical infection. Remarkably, other studies have reported a similar picture, with clinical cases of FAdV infections occurring at various ages and therefore suggestive of both horizontal and vertical transmission (Niu *et al.*, 2016, 2018; Chen *et al.*, 2019). Moreover, the link between the infected broilers and breeder source was often unclear and hardly compatible with this transmission path. In fact, the presence of different strains in the same flock at different time points highlights that, at least in some instances, a new virus was introduced at later stages of the cycle. These evidences, coupled with the absence of a clear geographic pattern, support the limited efficacy of the implemented biosecurity measures, which allowed the wide circulation of FAdV-E and introduction within farm, likely mediated by both short range-direct contacts (i.e., local farm clusters) and long range-indirect ones. Finally, the contamination of eggs belonging to different batches at hatchery cannot be excluded.

FAdV-D analysis strengthens and expands the above-mentioned scenario, whereas the 3 identical FAdV-D strains were identified in geographically distant farms, who received pullets from different breeder flocks, implying indirect long-distance contacts.

Additionally, the relevant genetic distance compared with other Greek FAdV-D strains and their distribution in the phylogenetic tree pose in favor of multiple introduction events from foreign countries. Particularly, strain Z3 was closely related with others collected in Central Europe, including Germany, whose role in the exportation of infectious bronchitis virus strains in Greece was already suggested (Andreopoulou *et al.*,

2019). Because the linkage was ascribed to the importation of German breeders, a mixed scenario featured by horizontal transmission between countries followed by vertical transmission of the newly introduced strains can be hypothesized.

Overall, the present study demonstrates a wider than expected FAdV circulation (particularly FAdV-D and -E) and the limited effectiveness of applied control measures both in preventing new strain importation from foreign countries and their within-country circulation. Although more extensive studies would be of benefit to understand the actual risk factors of FAdV introduction and clinical sign emergence, both horizontal and vertical transmission seem to contribute to FAdV epidemiology in Greece. The characterization of the involved species and the knowledge of their epidemiology could contribute to the planning of more effective control strategies and introduction of protective vaccines, if necessary.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.07.019>.

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