Molecular epidemiology of infectious bronchitis virus and avian metapneumovirus in Greece

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ABSTRACT Respiratory diseases like infectious bronchitis virus (IBV) and avian metapneumovirus (aMPV) have been held accountable for major losses for poultry production. Nevertheless, scarce information was present dealing with the prevalence and molecular epidemiology of these infections in Greece and the efficacy of currently applied control strategies. To fill this gap, a specific epidemiological study was designed. A total of 106 broiler and layer farms, including 10 backyard and 96 commercial flocks, were sampled between March 2016 and May 2017, and the obtained tracheal swabs were tested for IBV and aMPV using RT-PCR based techniques followed by sequencing. For each farm, data regarding production type, flock features, clinical signs, and vaccination program were also recorded. Different associations between vaccination protocol, production type, animal category, birds density, age, presence of clinical signs, and IBV and/or aMPV infection were tested. Both IBV and aMPV field strain prevalence were proven high, approximately 20 and 30%, respectively, being the GI-19 lineage (14 out of 19; 73.6%) and B subtype (30 out of 30; 100%), the most commonly detected IBV and aMPV genetic types. Infection with IBV field strains was significantly associated with clinical sign presence (odds ratio = 8.55 [95CI = 2.17–42.90]). Remarkably, only the vaccination protocol involving a double vaccination at 1 D of age was proven protective against IBV-induced symptomatology, with the odds of developing disease being 4.14 [95CI = 1.34–14.51] times lower.

No association was demonstrated between aMPV infection and clinical outbreaks or between aMPV and IBV detection, suggesting the marginal role of the former pathogen in poultry farming.

Globally, the present study provides the first detailed investigation of the epidemiological scenario of 2 viruses traditionally considered of pivotal relevance in poultry farming and demonstrates that remarkable benefits could be obtained with just minor adjustments in vaccination protocols.

Key words: infectious bronchitis virus, avian metapneumovirus, Greece, epidemiology, vaccination

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INTRODUCTION

Since 2009, Greek economy has undergone a remarkable crisis, leading to a decrease in per-capita gross domestic product of approximately 40% in the following 7 years (http://www.worldbank.org/). Repercussions in primary needs, like food access, have also been noted, with a relevant decline in the supply of animal origin protein (g/capita/day) (http://www.fao.org/faostat/). Traditionally, poultry meat and eggs have been considered cheap sources of high biological value proteins. Nevertheless, although the poultry production remained substantially stable over time, production price index of chicken meat increased by about 25% between 2009 and 2014 with relevant consequences on prices for the final customers and their alimentary habits (http://www.fao.org/faostat/). As easily predictable, this dramatic situation went with the bankruptcy of over 60,000 poultry farms in the last decade (http://ec.europa.eu/eurostat), especially the small-medium sized ones.

A multitude of economic and political factors have contributed to the present scenario, including a drop in agricultural investment from 3.8 billion in 2008 to 1.4 billion euros in 2012 (http://www.fao.org/faostat/). Based on these facts, a short-term massive modernization of the productive farming, leading to productive cost reduction, can hardly be imagined. Nevertheless, an improvement in farm management could surely

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provide immediate and relevant advantages for poultry production and therefore for Greek citizens.

Viral respiratory diseases represent a major threat for poultry farming because of direct losses, costs, and constraints associated to their control.

Among respiratory infections, infectious bronchitis virus (**IBV**) has established itself as one of the most widespread and damaging poultry diseases, at least in Europe (Jackwood et al., 2012). As the other single-stranded RNA viruses, IBV is featured by a high substitution and recombination rate (Duffy et al., 2008; Simon-Loriere and Holmes, 2011), which has led to the emergence of several genotypes and lineages over time (Valastro et al., 2016). Additionally, the wide commercial network among countries has been favoring the spreading of several livestock diseases all over the world (Franzo et al., 2016a, 2017a, 2018), enhancing the likelihood of introducing new strains with different immunological features in naive animal populations. This high genetic and antigenic heterogeneity has severely hindered the implementation of effective control measures, which currently rely mainly on vaccination application (de Wit et al., 2011). In fact, a limited cross-protection has been demonstrated especially between different genotypes, although the genetic distance is a poor proxy of immunological features (de Wit et al., 2011). Besides the development and administration of homologous vaccines, the immunization using multiple vaccines, based on different genotypes, has been proven beneficial, broadening the protection spectrum (Cook et al., 1999). However, other practical managerial factors like vaccine application route, dose, timing, and housing conditions can substantially reduce the vaccination efficacy (de Wit et al., 2010).

Despite these limitations, a properly targeted and performed vaccination has been proven to effectively control the emergence of clinical signs and diminish the viral circulation, both in experimental and field conditions (Terregino et al., 2008; Geerligs et al., 2011; Feng et al., 2015; Franzo et al., 2016b). The noteworthy economic impact and need for properly targeted vaccination plans would impose a good and updated knowledge of the local epidemiological scenario, allowing to select the most effective control strategies and minimize the cost–benefit ratio.

Nevertheless, this information is unforgivably lacking in most counties and, especially in Greece, limited data are available about IBV molecular epidemiology (Koutoulis and Nikolaos, 2013), leading to the selection of vaccination protocols based on habits and personal opinions rather than on evidence-driven criteria.

Based on these premises, the primary objective of this study was to obtain a representative picture of IBV epidemiology in broiler and layer farms in Greece, monitoring the efficacy of currently implemented control measures and favoring their future improvement.

Recently, some studies have suggested the increasing role of avian metapneumovirus (**aMPV**) in respiratory outbreaks not only in turkey but also in broiler farms, potentially as a primary pathogen (Tucciarone et al., 2018b). Although aMPV presence was already reported in Greece (Tucciarone et al., 2017), no accurate information is available about its distribution and clinical relevance. Consequently, the prevalence and impact of aMPV on Greek poultry farming has been investigated as a secondary objective of the present study.

MATERIAL AND METHODS

Study Design

According to the primary objective of this study, a minimum of 100 farms had to be included in order to estimate the prevalence of IBV in Greek flocks with a confidence level of 95% and a precision of ±10%, assuming an expected prevalence of 50% as a worst-case scenario. The sampling was stratified according to the number of farms in 3 major Greek regions. Particularly, the geographical allocation of broiler farms in Greece is concentrated in Epirus (in North-Western Greece), Central Macedonia, and Central Greece, where 41.8, 19.4, and 29.6% of poultry meat is annually produced, respectively (Hellenic Ministry of Rural Development and Foodata, collected on December 2016). Efforts were also made to sample both commercial and backyard farms as well as broiler and layer flocks proportionately to their frequency.

Farms were randomly selected from the Ministry of Agriculture register using the above-mentioned criteria. For each farm, 10 tracheal swabs were collected from randomly selected animals and air-dried. Whenever possible, the following data were collected: sampling date, farm geolocalization, animal category (broilers or layers), production and housing system (backyard, commercial or organic, i.e., reared according to Council Regulation 834/2007 and Commission regulation 889/2008), age of poultry, population size, vaccination protocols for aMPV and IBV. Farms were inspected by veterinarians involved in the research project and, in case of clinical signs presence, they were recorded for further evaluation. All samples were delivered to the Veterinary Infectious Disease laboratory (Department of Animal Medicine, Production and Health, Padua University, Italy).

IBV and aMPV Detection

Swab pools were eluted in 2 mL of PBS and widely vortexed. Approximately 200 μL of the obtained solution was extracted using the High Pure Viral RNA Kit (Roche, Basel, Switzerland), following the manufacturer's instructions.

IBV diagnosis was performed by RT-PCR using the SuperScript III One-Step RT-PCR System with PlatinumTaqDNA Polymerase kit (Thermo Fisher, Waltham, MA) with the primer pairs XCE-1 and XCE-2 described by Cavanagh et al. (1999), and

obtained amplicons were sequenced using the same primers.

aMPV detection and subtyping were performed using the real-time RT-PCR described by Franzo et al. (2014). Partial G gene sequences were obtained from positive samples by RT-PCR performed using the Gstart+ and G6- primers described by Cecchinato et al. (2010). Real-time RT-PCR and RT-PCR were performed using the SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher) and the SuperScript III One-Step RT-PCR System with Platinum-TaqDNA Polymerase kit (Thermo Fisher), respectively. RT-PCR positive samples were Sanger sequenced. IBV and aMPV sequences obtained in this study and related metadata were submitted to the GenBank database under the accession numbers M869172-MG869260 and MG869147-MG869171, respectively.

Phylogenetic Analysis

IBV strain genotyping was performed by comparing detected strains with the reference dataset proposed by Valastro et al. (2016). Briefly, sequences were aligned using MAFFT (Standley, 2013) and recombination analysis was performed using RDP4 (Martin et al., 2015); settings for each method were adjusted following the recommendations of the RDP4 manual, considering the database features. After removal of recombinant sequences, a phylogenetic analysis was performed using the maximum likelihood **(ML)** method implemented in PhyML (Guindon et al., 2010), choosing as tree rearrangement strategy a combination of nearest neighbor interchange and subtree pruning and regrafting. The evolutionary model was selected based on the Bayesian Information Criteria calculated using JmodelTest (Darriba et al., 2012). The reliability of each clade was evaluated using the Shimodaira-Hasegawa-Approximate likelihood-ratio test implemented in PhyML (Anisimova and Gascuel, 2006).

A phylogeographic analysis was performed on the field QX genotype strains (GI-19 lineage) as described by Franzo et al. (2017a) to evaluate the introduction routes of this genotype in Greece. For this purpose, an updated version of the sequence dataset described in Franzo et al. (2017a) was selected.

A fully comparable approach was used to perform the phylogenetic analysis of aMPV strain. A slightly modified version of the sequence dataset described in Tucciarone et al. (2018b) was selected. The phylogeographic analysis was not carried out on aMPV due to the limited number of freely available aMPV sequences annotated with adequate metadata.

Statistical Analysis

Different associations between vaccination protocol, production type, animal category, bird density, age, presence of clinical signs, and IBV and/or aMPV infection were tested. To allow a robust statistical analysis, sparse data were aggregated in a limited number of categories. Particularly, the different vaccination strategies were summarized as "vaccination: yes/no," and 2 new variables were created to deal with clinical signs. The first one simply reported the presence or absence of symptoms, while the second classified them in "none," "respiratory," "enteric," "reproductive," and "others." A further variable was defined to differentiate IBV vaccine and field strains. The classification in 1 of the 2 bins was based on phylogenetic analysis and genetic distance evaluation, by comparing each strain with the known used vaccines. Strains part of the same lineage and with a percentage of identity higher than 99% compared to the implemented live-attenuated vaccines were included in the category "vaccine." The remaining strains were classified as "field strain."

The presence of statistical differences between groups was evaluated using the chi-square or Fisher's exact tests when dealing with categorical variables, whereas the Student's t and Mann-Whitney tests, depending on the data distribution, were used for continuous variables. When more than 2 groups were involved in the comparison, ANOVA or Kruskal–Wallis tests, followed by post hoc tests (Student t or Mann-Whitney tests) with Bonferroni correction, were performed.

Logistic regression was performed to assess the relevance of different variables in clinical signs emergence and calculate the respective odds ratio. The statistical significance level was set to P-value < 0.05.

Farm level prevalence and 95% CI, calculated using the asymptotic (Wald) method based on a normal approximation, were obtained using the *prevalence* package (Devleesschauwer et al., 2014) in R. The estimation of individual level true prevalence and 95 high posterior density **(95HPD)** from apparent prevalence obtained by testing pooled samples was performed by means of a Markov Chain Monte Carlo sampling approach, within the Bayesian framework implemented in the *truePrevPools* function of the same R package (Devleesschauwer et al., 2014). Test sensitivity and specificity priori were defined as a uniform distribution ranging between 80–100% and 90–100%, respectively. These informed priori were chosen based on expert's opinion and participation to different proficiency tests. The priori of the true prevalence was set to an uninformative beta ($\alpha = 1$, ß $= 1$) distribution.

RESULTS

Background

A total of 106 farms, 10 backyard (i.e., less than 50 animal, used for self-consumption), and 96 commercial flocks (more than 1000 animals), were included in the study between March 2016 and May 2017. Among those, 78 were broiler farms, whereas 28 were layers farms. The geographical farm distribution is reported in Figure 1. IBV vaccination was applied in 72 farms,

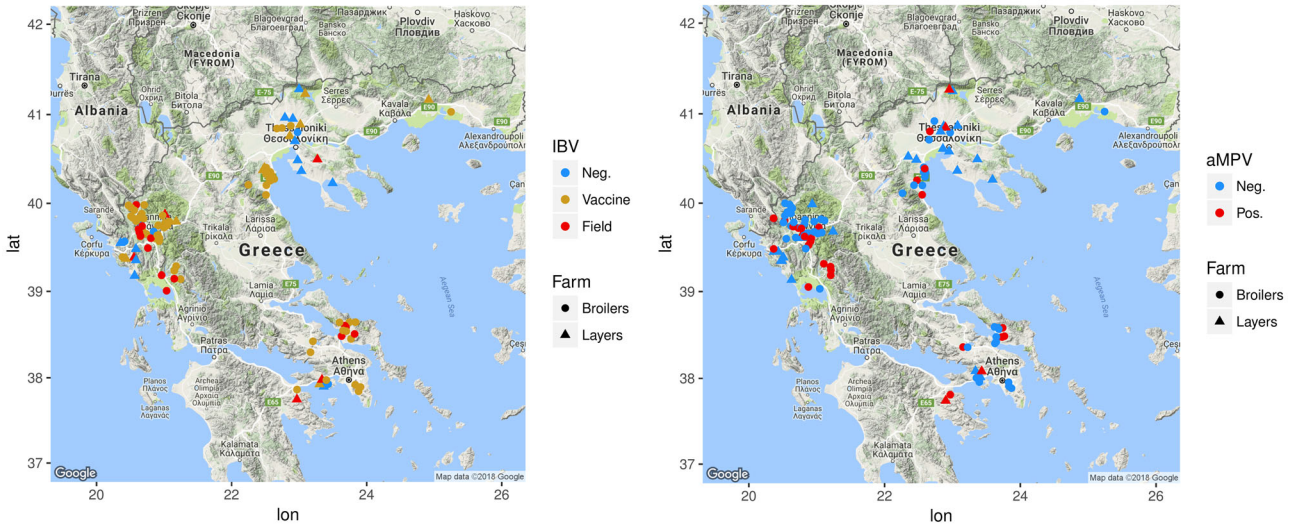

Figure 1. Maps reporting the geolocalization of broiler (full circle) and layer (full triangle) tested farms. Flocks have been color coded based on IBV (left) and aMPV (right) positivity. For IBV only, the distinction between field and vaccine-derived strains is also reported. A slight jittering has been applied to farm location to avoid point overlapping.

whereas only 5 flocks were vaccinated against aMPV. The most commonly implemented IBV vaccination involved a combination of Mass (H120 strain) and 793B (1/96 strain)-based vaccines. A lower number of farms used Mass alone (n = 10) or in combination with 4/91 strain (n = 4) or QX genotype (n = 3)-based vaccines. A total of 43 flocks received a double vaccination at hatchery, 25 a delayed booster, and 4 a single vaccination.

Clinical signs were observed in 33 farms, including outbreaks of respiratory (n = 17), reproductive (n = 2), and enteric (n = 2) signs. Twelve clinical episodes were not classified in any of the previous categories. No significant differences were observed neither between commercial and backyard flock nor between broilers and layers in terms of clinical signs occurrence.

IBV Epidemiology

Of 106, 89 farms were IBV positive (i.e., farm level prevalence = 83.96% [95CI = 76.98% to 90.95%] and estimated individual level prevalence = 13.7% [95HPD = 7.7% to 19.7%]). Sequencing and phylogenetic analysis (Figure 2) allowed to demonstrate the circulation of 4 genotypes characterized by different prevalence: 69 (65.09%) 793B, 14 (13.20%) QX, 4 (3.77%) Mass, and 1 (0.94%) Ark strains. All Ark and Mass strains were classified as vaccines, as well as 65 out of 69 793B-like strains (i.e., 53 1/96 and 12 4/91 strains). Among the 793B-like strains, 4 were distantly related to currently used vaccines and were thus classified as field strains. All QX strains were classified as field strains.

Additionally, 1 recombinant strain (12|Greece|Ioannina|07–07-2016) (0.94%) was identified. The 2 parental strains, although not identical, showed a close similarity with QX (region 1–177) and 793B (region 178–375) vaccines.

Consequently, a total of 19 strains (14 QX, 4 793B, and 1 recombinant) were considered field strains, corresponding to a farm level prevalence of 17.97% (95CI = 10.62% to 25.22%) and an estimated individual level prevalence of 1.90% (95HPD = 0.10% to 4.30%).

Overt clinical signs were observed in 12 out of 34 (35.29%) unvaccinated and 21 out of 72 (29.16%) vaccinated farms, respectively. However, the difference was not statistically significant ($P = 0.524$). An even lower association was evidenced when only healthy animals and those with respiratory signs were included in the study ($P = 1$). Additionally, no statistical difference was evidenced in IBV field strain frequency between vaccinated (14 out of 67; 20.89%) and unvaccinated (5 out of 22; 22.73%) IBV-positive flocks.

To evaluate the effect of vaccination timing, another variable was defined differentiating the combined vaccination at 1D of life from other vaccination strategies (i.e., not-vaccinated, combined vaccination at 1 D of age, other vaccination schedules). No association between administration timing and frequency of field strain detections was proven. However, the odds of developing disease were 4.14 times lower (95CI = 1.34 to 14.51; P-value = 0.017) when both vaccines were simultaneously administered compared to unvaccinated flocks. No significant difference was demonstrated between farms using other vaccination protocols and unvaccinated ones; odds ratio = 2.26 (95CI = 0.83–6.37; P-value = 0.12) (Supplementary Figure S1).

The presence of clinical signs was significantly higher ($P < 0.001$) in farms where IBV field strains were detected. Particularly, the odds of developing clinical signs were 8.55 (95CI = 2.17 to 42.90) times higher in flocks where field strains were detected. No particular geographical (Figure 1) or temporal (Supplementary Figure S2) pattern was observed. No association was

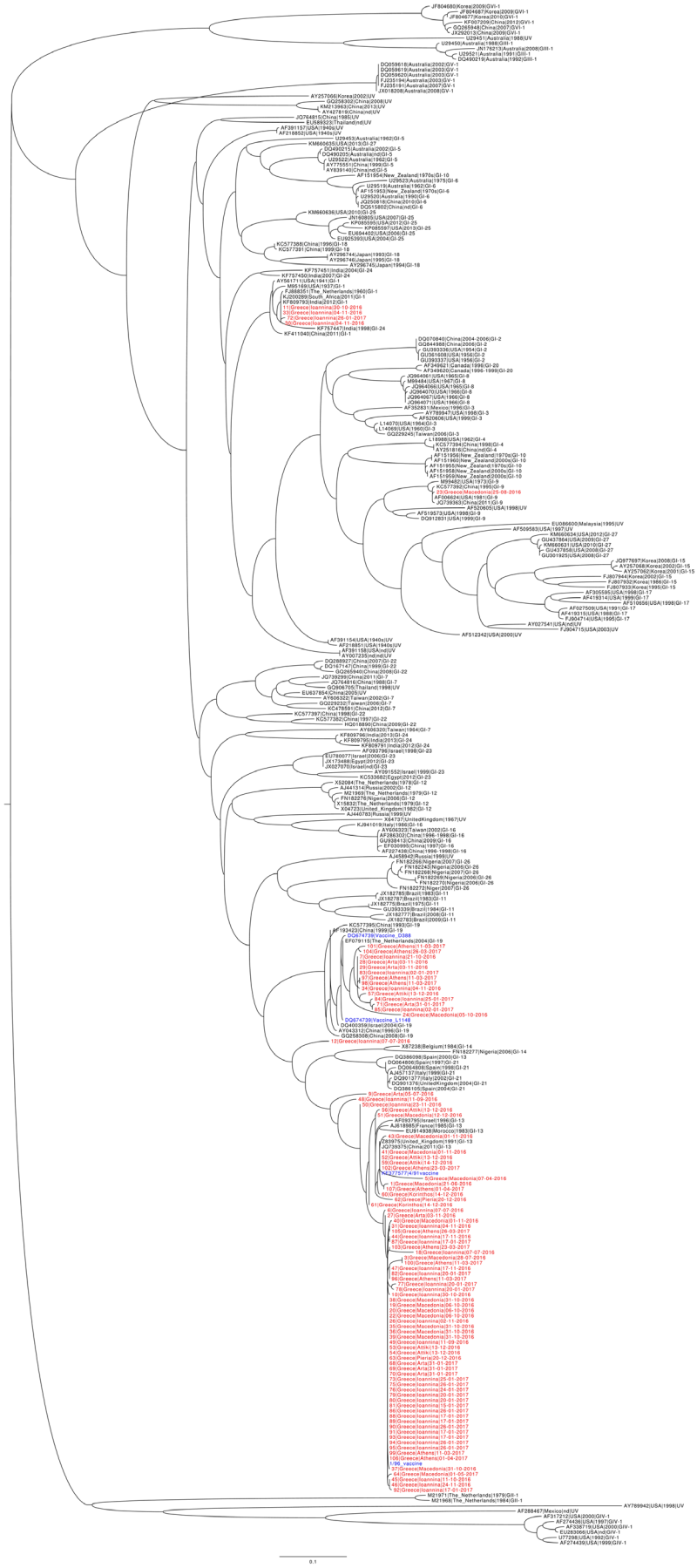

Figure 2. Maximum-likelihood phylogenetic tree comparing the IBV strains obtained in the present study (highlighted in red) with the reference set proposed by Valastro et al. (2016). Reference vaccine strains are reported in blue.

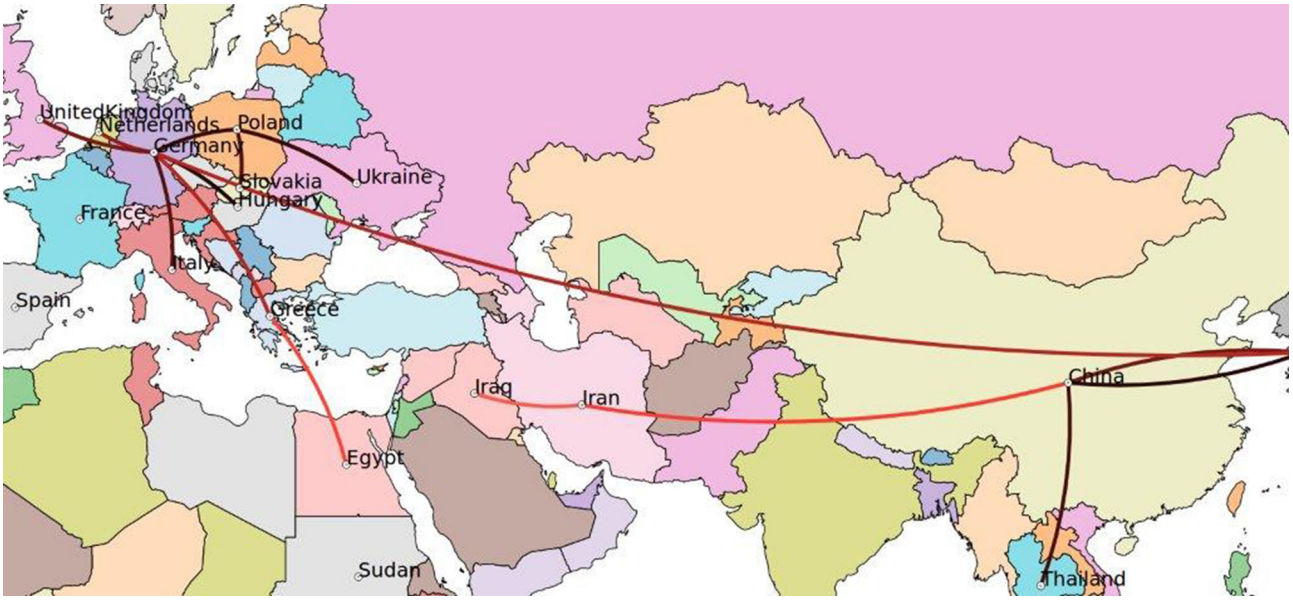

Figure 3. Well-supported migration paths (i.e., BF>20) among countries are depicted. The line color is proportional to migration event age, being the more ancient ones coded in black and the more recent ones in red. The location of each country has been matched with its centroid.

found between animal age and IBV infection status (i.e., negative, field, or vaccine strain). However, the average bird population was higher in field strain positive broiler farms ($P = 0.044$).

The results of phylogeographic analysis globally mirrored those reported by Franzo et al. (2017a). However, a highly supported migration rate was identified from Germany to Greece (Bayesian factor = 156.8) and from Greece to Egypt (Bayesian factor = 265.3) (Figure 3, Supplementary Figure S3 and Supplementary animation). According to most recent common ancestor estimation, QX genotype introduction in Greece could have occurred approximately in 2009, while the exportation to Egypt was estimated 4 yr later.

Another potential linkage, even if devoid of adequate support, was evidenced between Greece and Hungary (Supplementary Figure S3).

aMPV Epidemiology

Of 106, 32 farms were positive to aMPV, corresponding to a farm level prevalence of 30.18% (95CI = 21.45% to 38.92%) and an estimated individual level prevalence of 2.8% (95HPD = 0.1% to 6%). All strains were part of the same aMPV subtype B cluster, which also included sequences from strains collected in Italy and Romania (Figure 4).

The only 2 exceptions were the strains 101 and 102, which clustered with NEMOVAC® (Merial) and Nobilis Rhino CV (MSD) vaccine strains, respectively. None of these 2 farms had been vaccinated against aMPV. Similarly to IBV, no particular geographical or temporal patterns were observed (Figure 1 and Supplementary Figure S4).

Of 32, 10 (31.25%) infected flocks showed clinical signs, a fully comparable percentage to uninfected ones (i.e., 23 of 74; 31.08%). Therefore, no association was demonstrated between aMPV and presence of clinical signs. The same results were obtained considering only healthy animals and those with respiratory signs. Similarly, no association was proven between aMPV and IBV infection ($P = 0.09$). Nevertheless, IBV field strain infected farms displayed a slightly higher frequency of aMPV infection compared to expectations, under the null hypothesis of no interaction between the 2 pathogens. Similarly, aMPV was underrepresented in IBV-negative farms (Supplementary Figure S5).

No association was found between aMPV infection and animal population size. However, a significant age difference ($P = 0.047$) was observed in broilers, whose age was lower in aMPV-positive flocks (mean = 38.8 D) than in negative ones (mean = 49.32 D). Finally, 2 out of 5 vaccinated flocks were infected with field aMPV field strains and one of those developed clinical signs. However, the small sample size of vaccinated animals impeded any reliable analysis.

DISCUSSION

Infectious bronchitis is a major threat for intensively raised poultry, causing direct losses due to animal mortality, reduced daily weight gain, and decreased egg production and quality (Cavanagh, 2007).

Additionally, the increased susceptibility to secondary infections, besides the additional direct costs, typically requires further actions that weight on the farm balance and are often discouraged by current European Union policies, like antimicrobial administration.

Actually, the present study demonstrates a high IBV prevalence (i.e., about 85% and 15% at farm and individual level, respectively). However, most of these

Figure 4. Maximum-likelihood phylogenetic tree comparing the aMPV strains obtained in the present study (highlighted in red) with a set of reference sequences.

detections are, with all likelihood, ascribable to the sampling of vaccine-derived strains. These results are not surprising since the within-farm prolonged circulation of live-attenuated vaccines was already proven by other authors (Cavanagh et al., 1999; Tucciarone et al., 2018a). Vaccine-like strains were detected in unvaccinated farms or in flocks using other commercial vaccines, further confirming their ability to effectively spread among farms, affecting the epidemiological scenario and complicating its interpretation (Franzo et al., 2014). Remarkably, a recombinant virus originating from parental strains closely related to QX (GI-19) and 793B (GI-13) vaccines has been identified in a farm where no vaccination scheme against IBV

was in place. The widespread vaccination application creates favorable conditions for co-infection, and thus for recombination to occur, particularly when partial vaccination coverage is attained (Moreno et al., 2017). This risk cannot be underestimated since a high frequency of recombination between vaccine and field strains has frequently been reported (Cavanagh et al., 1992; McKinley et al., 2011; Jackwood et al., 2012), and high fitness variants can effectively emerge and spread through this evolutionary mechanism (Moreno et al., 2017). Nevertheless, in this particular case, the detected recombinant more likely represents a transient variant exhibiting a limited fitness, since it has been identified only once and in a limited geographic area.

The economic costs, the effect on IBV epidemiology, and associated risks suggest that IBV vaccination should be performed only when necessary, that is: (1) if the pathogen is actually circulating and causing relevant damages; (2) if the applied vaccination strategies are proven to be effective in controlling the disease.

The present study demonstrates the wide circulation of IBV field strains in Greece, particularly belonging to the GI-19 lineage. The introduction of this genotype was traced back to 2009, likely by means of a single introduction event from Germany. Remarkably, this result fully coincides with the first reported detection of the QX genotype in Greece (Koutoulis et al., 2013), strengthening the reliability of the performed analysis. However, it must be stressed that the limited availability of properly annotated IBV sequences could have prevented the identification of the actual introduction source. Therefore, the need for more intensive collaborative efforts and molecular biology data sharing, especially within European Union borders, is stressed once more. Since its introduction, QX genotype has spread in the country, justifying the widespread detection in all the sampled Greek regions (Figure 1).

As expected, field strains are more commonly detected in the presence of clinical signs compared to what expected by chance, demonstrating IBV primary pathogenic role, especially in high densely populated farms. At first glance, no differences were evidenced in field strain or clinical sign frequency between vaccinated and unvaccinated flocks. These data are quite surprising since most of vaccination schedules in Greece were based on the "protectotype" concept (Cook et al., 1999) or, less frequently, on the use of the homologous vaccine, 2 approaches that have been proven valuable in controlling the emergence of overt clinical signs in experimental and field conditions (Terregino et al., 2008; Geerligs et al., 2011; Feng et al., 2015; Franzo et al., 2016b). Therefore, variables related to animal or vaccination management are probably affecting the vaccination efficacy (de Wit et al., 2010). Additionally, other factors, like general animal health and not investigated co-infections, could have negatively affected the immunization. Interestingly, while Franzo et al. (2016b) demonstrated the efficacy of both Mass+793B and Mass+QX-based vaccination in reducing viral circulation and clinical outbreaks compared to Mass vaccination alone, the most relevant benefits of double vaccination administration were observed when both vaccines were provided at 1 D of life, mainly at hatchery (Franzo et al., 2016b). A comparable scenario was also demonstrated in the present study. Despite the fact that no association between administration timing and frequency of field strain detection was demonstrated, the flocks where both vaccines (Mass+793B or QX) were provided at 1 day of life displayed 4.14 (95CI 1.34 to 14.51) times lower odds of developing disease compared to not vaccinated farms. On the other hand, other vaccination strategies did not provide any protective effect compared to no vaccination at all. On the contrary, the increased odds (i.e., 2.26) of developing clinical signs in these flocks, although not significant from a statistical perspective, could be attributable to vaccine reversion to virulence or to the respiratory epithelium damages due to vaccine replication, which can be more marked when partial animal coverage allows for prolonged rolling reactions (McKinley et al., 2008; Cook et al., 2012).

Remarkably, both field and vaccine-derived strains were detected in diseased farms using single or delayed vaccination (data not shown).

Consequently, a combined vaccination can be more effective in avoiding the emergence of clinical signs, even if it is not effective in preventing viral infection. These results are in contrast with the traditional belief that a delayed administration of the second vaccine dose should be beneficial because of the recovery time needed by the tracheal epithelium after the first vaccination and the booster effect provided by the second vaccination (Winterfield and Fadly, 1972; Di Matteo et al., 2000). Nevertheless, the absent or negligible competition between co-administered vaccines was proven in the field (Tucciarone et al., 2018a), and the effectiveness of this strategy was already proven experimentally (Cook et al., 1999) and through epidemiological studies (de Wit et al., 2010), although conflicting evidences were reported between broiler and layer flocks (de Wit et al., 2010). While dedicated studies will be necessary to demonstrate the biological mechanisms behind these empirical evidences, the higher animal coverage and the greater control on vaccination administration conditions when vaccines are provided at hatchery can probably balance and outperform the replicative and immunological advantages expected when the second vaccination is postponed.

Based on this evidence, an improvement on IBV control is potentially easily achievable in Greece and could be achieved simply by acting on a more conscientious management of already applied vaccination strategies, without requiring the implementation of other expensive control strategies or structural interventions.

The analysis of aMPV epidemiology provided a comparably interesting scenario, demonstrating the high

prevalence (about 30 and 3% at farm and individual level, respectively) of this virus in Greek poultry farming, an evidence already reported in Italy (Tucciarone et al., 2018b). However, some major differences distinguish the 2 countries. Turkey farming has a marginal role in Greece compared to Italy (http://www.fao.org/faostat/), and therefore a stable aMPV circulation in poultry farms rather than frequent spill-over from turkey ones is likely.

Similarly to Italy (Tucciarone et al., 2018b), approximately 30% of Greek flocks reporting clinical signs were aMPV positive. Nevertheless, in this case the study design allowed to demonstrate the absence of any statistical association between aMPV infection and symptomatology development, which suggests the marginal role of this pathogen, at least in Greece. Comparable results were obtained when only broiler farms were considered (data not shown), like in the Italian study (Tucciarone et al., 2018b). Since the aMPV frequency did not differ between diseased and healthy flocks, its detection as an incidental finding in the presence of other primary etiological agents is more likely.

aMPV and IBV co-infection was common, being approximately 68% of aMPV-positive samples detected in presence of IBV vaccines and approximately 25% in presence of field strains, which is in sharp contrast with the Italian situation, where only approximately 4% of aMPV-positive samples were co-infected with IBV field strains. Nevertheless, no interaction was proven between aMPV and IBV neither in terms of infection frequency nor in the emergence of clinical signs. Different factors could explain the reported differences. At first, Italian and Greek aMPV strains could differ in their biological features. However, all these strains seem to be genetically related, at least in the considered genome region (Figure 4), lessening this hypothesis. It must be stressed that IBV has been effectively controlled in Italy, an evidence that can easily justify the lower co-infection frequency. Additionally, it has been suggested that the successful IBV vaccination could have altered the competitive equilibrium between IBV and aMPV, favoring the latter (Tucciarone et al., 2018b). However, the lack of any significant interaction between aMPV and IBV herein demonstrated seems to contradict this hypothesis. Another suggestive hypothesis is the presence of different poultry genetic lines that could exhibit a differential susceptibility to aMPV.

More probably, a combination of different factors including viral strain and host biological features, poultry management, and different study design determines the different scenarios observed in Italy and Greece.

As a final remark, with the exception of 2 vaccine-derived viruses, all identified Greek aMPV strains appeared to be part of just one clade, which suggest a single (or a limited number) introduction event followed by local spreading (Figures 1 and 4), similarly to what evidenced for IBV GI-19 lineage. Additionally, the close genetic similarity with recently detected strains (Franzo et al., 2017b; Tucciarone et al., 2018b) supports the wide circulation of a quite homogeneous aMPV subtype B clade in several European countries. However, the lack of adequate aMPV molecular epidemiology data impedes any reliable conclusion and requires further, more extensive, studies.

CONCLUSIONS

The present study results provide a robust depiction of IBV and aMPV epidemiology in Greece, investigating the potential introduction routes in this country and evaluating their prevalence, distribution, clinical relevance, and genetic features. This information, coupled with the evaluation of currently applied control measure efficacy, could be used to guide the sanitary policies, not only in Greece but on a broader scale too, allowing an appropriate targeting and optimization of future investments and minimizing disease associated costs.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Supplementary Figure S1. Mosaic plot depicting the relationship between clinical sign presence and applied vaccination protocol. The area of each cell is proportional to the count size. Cells have been color coded and lines dotted based on standardized residuals of the corresponding in chi-square test (a standardized residual greater than 2 or lower than –2 is indicative of statistical significance). The number of samples has been indicated within each cell.

Supplementary Figure S2. Timeline reporting the collection date of each sample. Results of IBV diagnosis and differentiation between field and vaccine-derived strains are also reported.

Supplementary Figure S3. Maximum clade credibility phylogenetic tree (obtained through BEAST analysis) based on non-recombinant GI-19 sequences. Branches have been color coded based on the location with the highest estimated posterior probability.

Supplementary Figure S4. Timeline reporting the collection date of each sample. Results of aMPV diagnosis are also reported.

Supplementary Figure S5. Mosaic plot depicting the relationship between IBV and aMPV positive flocks. The area of each cell is proportional to the count size. Cells have been color coded and lines dotted based on standardized residuals of the corresponding in chi-square test (a standardized residual greater than 2 or lower than –2 is indicative of statistical significance). The number of samples has been indicated within each cell.

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