Phylogenetic and phylodynamic analyses of subtype-B metapneumovirus from chickens in Tunisia

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ABSTRACT Swollen Head Syndrome (**SHS**) is an economically important viral disease of chickens caused by avian metapneumovirus (**aMPV**). The virus comprises 6 different subtypes (A,B,C,D, New-1 and New-2). To date, no information was available on the presence of the virus in Tunisian poultry.

The present work aims to detect the presence of (aMPV) in broiler chicken in Tunisia, then to characterise the isolates in order to determine their subtype and to estimate their geographic origin of introduction.

A total of 289 samples were collected, aMPV detection was detected by real time RT-PCR and molecular characterization was warried out by Sanger sequencing on the glycoprotein (**G**) gene. Phylogenetic analysis was carried out using Beast 2 software.

Out of the 289 samples, 21 were revealed positive to aMPV. Only 2 isolates have been confirmed by sequencing analysis ; one isolate sampled in 2015 and another in 2019. Based on the partial G gene sequence, analysis of these 2 Tunisian isolates showed that they belong to subtype B. The isolate sampled in 2015, appeared to be phylogenetically related to derived vaccine strain. However, the one sampled in 2019 appeared to be a field strain. Phylodynamic analysis provided evidence that this field strain derived from a Spanish strain and probably the virus has been introduced from Spain to North Africa back in 2016.

This study is the first that highlighted the circulation of (aMPV) in Tunisia. It is possible that aMPV has been circulating in Tunisia and neighboring countries without being detected. Also, multiple strains could be present and therefore multiple introductions have happened. Through this study, we shed the light on the importance of reinforcing farms biosecurity as well as virological surveillance.

Key words: avian metapneumovirus, subtype B, phylogenetic, phylodynamic

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INTRODUCTION

Avian metapneumovirus (**aMPV**) is a member of the Pneumoviridae family, genus Metapneumovirus, that causes severe respiratory disorders in infected chickens (Afonso et al., 2016). Indeed, the virus causes immunosuppression, expressed by lower immunity level of the respiratory system and predisposes birds to other viral infections (infectious bronchitis) and bacterial (*Escherichia coli, Ornithobacterium*) pathogens. aMPV was first described as a Turkey Rhinotracheitis Virus (**TRTv**), affecting mainly turkey (Buys and Du Preez, 1980). In 1984, the disease appeared in chickens, and named swollen head syndrome (**SHS**) by Thomson and Morley. All aMPV strains have been classified under 4 subtypes designated A, B, C, and D showing major differences not only in the genome but also in their biological features like antigenicity (Juhasz and Easton, 1994). Recently, 2 novel subtypes were detected in monk parakeet and in gull (Canuti et al., 2019; Retallack et al., 2019) and not yet classified. The 2 subtypes A and B are described as largely dominant in Europe; the subtype C (Colorado) seems to be the subtype isolated in the United States

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and has not been reported in any other parts of the world until the yr 2013 where it was detected in China, with antigenic and biological characteristics slightly different from other subtypes (Lee et al., 2007; Wei et al., 2013). Concerning the subtype D, it has only been reported in France (Bäyon-Auboyer et al., 2000). This classification is based on the aMPV G protein known to be the major viral attachment protein that exhibits extensive genetic and antigenic variations (Juhasz and Easton, 1994; Govindarajan et al., 2010). This structural glycoprotein plays an important role in the virus pathogenicity and the disease protective immunity (Govindarajan et al., 2010, Yu et al., 2010). For this reason and because of the high level of genetic variations of the virus, analysis of the G gene to determine its molecular relationship and differentiate between field and derived vaccine strains is very important.

In this study, detection of circulating aMPV strains and analysis of their G genes were carried out for clinical cases in commercial and farm chickens infected with Swollen Head Syndrome disease. Therefore, isolation of aMPV and sequence analysis of G gene may help better knowledge of the molecular characters of field aMPV in Tunisia to adapt the correct vaccine, giving that the efficacy of a protective vaccine strain is affected by mutations altering the balance of G protein expression (Naylor et al., 2007).

Our study was aimed at assessing comprehensive molecular characterization of a currently circulating aMPV strains in Tunisia in order to provide updated insights into the characteristics and the global spreading of the aMPV isolates, using a phylodynamic approach. The objective was oriented towards studying their origin whether they are field or vaccine derived strains. as well as identifying the origin of introduction of the aMPV strains to Tunisia. Our data might help understanding aMPV dissemination and providing a basis for animal health strategies aimed at controlling and preventing aMPV infection.

MATERIALS AND METHODS

Samples

A total of 289 suspected samples were collected by veterinarians, during the yr 2015−2019, from commercial and traditional broilers and broiler breeder flocks in all regions of the country where diseased chickens are declared, showing the following clinical signs such as couching, ocular and nasal discharge and swelling of sinuses. The broiler chickens were aged 4 to 6 wk while the breeders were 13 to 15 wk of age. The Broiler breeder chickens were vaccinated at 10 wk with a commercial aMPV vaccine. The broiler chickens were not vaccinated against aMPV. Nasal turbinate samples, Orophrayngeal and tracheal swabs and internal organs, including lungs and trachea were collected from necropsied dead animals sent to the Laboratory of Epidemiology and Veterinary Microbiology then conserved frozen at −80°C until viral RNA extraction. Orophrayngeal and tracheal swabs were resuspended in minimum essential medium with gentamicin, clarified by centrifugation at 8,000 rpm for 5 min and used immediately for RNA extraction and aMPV isolation. The aMPV subtype B vaccine strain was used as a positive control for RT-PCR.

RNA Extraction, Detection, and Molecular Subtyping of aMPV

RNA extraction was performed using Pure Link RNA Mini kit (Thermo Fisher Scientific, Carlsbad, CA), according to the manufacturer's instructions. For detection of aMPV, SYBR Green I real time RT-PCR was carried out to screen all the 289 isolates by amplifying a highly conserved fragment of sequence in the N open reading frame (Lemaitre et al., 2018). For the revealed positive ones, G and M genes were amplified by conventional RT-PCR for typing using easyScript One-step RT-PCR Super Mix (TransGen Biotech, Beijing, China) and primers as described in Table 1. The cycling conditions for conventional RT-PCR were set as follows: one cycle of 45°C for 20 min, 94°C for 5 min and 37 cycles of 94°C for 30 s, 54/54/51 and 57°C for A, B, C, and D (aMPV) type, 72°C for 1 min for A, B, C, and 2 min for D (aMPV) type, following by a final extention in 72°C. The PCR products were separated on 2% agarose gel and identified by ethidium bromide. RNA was tested for avian influenza virus, infectious bronchitis virus, and Newcastle disease virus by real time RT-PCR.

Table 1. Oligonucleotide primers used for detection and typing of avian metapneumovirus.

Gene	Primer names	5′-3′ nt sequence	aMPV type	Band size (bp)	References
N	Pan MPV/N1fwd	CTGTTTGTGAACATTTTYATGCA	All	107	(Lemaitre et al., 2018)
	Pan MPV/N1 AMPVD fwd	CTGGTTGTGAACATATTCATGCA			
	Pan MPV/N1Rev	ACAGAGACATGGCCTAACATDAT			
G	Ga*	CCG GGA CAA GTA TCT CTA TGG	A	504	(Bäyon-Auboyer et al., 1999)
	G2-	CCA CAC TTG AAA GAT CTA CCC			
G	Ga*		B	312	
	G12-	CAG TCG CCT GTA ATC TTC TAG GG			
M	C1	GATGACTACAGCAAACTAGAG	C	468	(Shin et al., 2000)
	C2	CTTCAGGACATATCTCGTAC			
G	G+50	GCGATGCCCAGTTAATGAA	D	956	(Bäyon-Auboyer et al., 2000)
	G-1005	CCCCTTACAAACACTGTTC			

Isolation of aMPV

Samples confirmed to be aMPV positive by real time PCR were inoculated on primary tracheal culture layers for 4 blind passages then used to infect Vero cells until cytopathic effect (**CPE**) revealed (Rivera-Benitez et al., 2014).

Sequencing of the G Gene

The RT-PCR products, corresponding to the predicted size, were sequenced by Sanger dideoxy sequencing method. Shrimp alkaline phosphatase and exonuclease I were used to remove the excess dNTPs and primers as follows: add 0.5 μL exonuclease I (20 U/μL) and 0.5 μL shrimp alkaline phosphatase (1 U/μL) to each PCR sample (5 μL) in a final volume reaction of 10 μL and incubate in thermal cycler at 37°C for 15 min then in 80°C for 15 min to inactivate the enzymes. Purified DNA was then sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit that contains thermally stable AmpliTaq DNA polymerase, modified dNTPs, and a set of dye terminators labeled with high-senility dyes. The sequencing reaction was prepared as follows: 10 μL PCR product of each gene, 2 μL BigDye sequencing mix, 4 μL Buffer 5X, 1 μL of specific primer (10 μM), and 4 μL RNase-free H2O. The sequencing program consisted of a predenaturation step at 96°C for 3 min followed by 25 cycles of 10s denaturation at 96°C, 5 s annealing at 50°C, and 150 s elongation at 60°C. Overlapping DNA fragments were sequenced in both directions from 2 independent PCRs to generate a consensus sequence for each isolate. The finished sequencing products were washed with 50 μL of 80% ethanol, centrifuged at 13,000 rpm for 15 min, and dried at room temperature. The DNA was then resuspended in 20 μL RNase-free water. Sequencing reactions were run and analyzed in an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA).

Phylogenetic Analysis

1. After sequencing, the obtained nucleotidic and amino acid sequences of G gene of the aMPV isolates were analyzed by BioEdit (version 7.2.5.0), and the sequence similarity was checked against sequences deposited in GenBank using a BLAST search at the NCBI site (http://www.ncbi.nlm.nih.gov). The sequences were aligned using MAFFT online software (Katoh et al., 2019). Phylogenetic trees were constructed using maximum likelihood method. Substitution model was selected according to the lowest Bayesian Information Criterion (**BIC**) calculated using Model Selection tool on MEGAX software (Kumar et al., 2018). The best model selected was General Time Reversible (**GTR**) model. The analysis was set to run at 1,000 bootstrap replicates to assign confidence levels to the branches tree.

The partial deletion option was selected at a 95% threshold for sites with missing data or gaps in alignment. The overall nucleotide similarity was estimated by calculating the arithmetic means of the sequence identity values, obtained using the Sequence Identity Matrix tool on the BioEdit software. The resulting TN1015/17 and TN1000/19 sequences of the G gene were submitted to GenBank under the accession numbers MH411239 and MZ229769, respectively. Reference nucleotide sequences of aMPV were extracted from the GenBank database. Vaccine sequences were kindly provided by Dr Caterina Lupini of the Department of Veterinary Medical Sciences, University of Bologna, Italy.

Analysis of Recombination Events

Recombination Detection Program 4 (**RDP** v.4.97) software was used to detect potential recombination sites in G gene of metapneumovirus sequence using default settings. Only recombination events supported by no fewer than 5 independent methods will be regarded as positive and eliminated for the phylodynamic analyses.

Phylogeographic Analysis

Only non derived strains were included in the phylogeographic analysis. Metadata corresponding to the country of origin and the year of sampling were considered in the analysis, using Beast 2.5 software (Bouckaert et al., 2019). Substitution model averaging was estimated using boMdelTest package on Beast 2.5 (Bouckaert and Drummond, 2017). Phylogeographic analysis was carried out considering country of origin as a discrete trait and year of sampling as sampling date. GTR was used as substitution model. Uncorrelated relaxed lognormal clock was used as a molecular clock model (Drummond et al., 2006). Bayesian skyline was used as population model for this analysis (Drummond et al., 2005). The analysis was set to run for 30 million iterations. Tracer software was used to assess for Effective Sampling Size (**ESS**). Maximum credibility tree was generated and annotated using Tree Annotator. The resulting tree was edited and visualized using ggmap (Kahle and Wickham, 2013) and ggplot2 (Wickham, 2016) packages on R 3.5 Software (R Foundation for Statistical Computing, Vienna, Austria);

All results were rendered to KML format using SpreadD3 software and visualized on Google Earth (Bielejec et al., 2016).

RESULTS

Detection and Isolation of aMPV

Twenty one samples out of 166 broiler and 123 breeder chickens, corresponding to 7.69%, were detected as positive for aMPV, by real time RT-PCR. Among the 21 positive samples, 12 were from broilers and 9 from breeder chickens. All detected aMPV were from

commercial flocks, no aMPV has been detected in the 16 traditional flocks. Ten among the 12 broiler samples and 7 among the 9 broiler breeder chickens were also screened positive for co-infection with infectious bronchitis virus and were discarded for aMPV isolate characterization. Only the 2 remaining broiler breeder samples whose are (TN1015/17 and TN1000/19), induced a cytopathic effect after 4 blind passages in primary trachea cultures followed by 4 blind passages in Vero cells. Supernatants from the fifth Vero culture passages were used for RNA extraction followed by conventional RT-PCR to identify the aMPV subtype. A product size corresponding to approximately 312 bp indicated that the viral genome corresponds to the subtype B strain which was isolated from 2 different regions in the eastern and the southern Tunisia.

Sequence Identities and Phylogenetic Analysis

The partial G gene sequences of TN1015/17 and TN1000/19 isolates were 312 nucleotides in length. Analysis of their nucleotide sequences, showed 68 to 100% nucleotide sequence similarity with aMPV subtype B sequences; 46 to 50% with aMPV subtype A sequences; 44 to 46% with aMPV subtype D sequences, 31 to 46% with aMPV subtype C sequences, 31 to 38% with the new subtype isolated in New Foundland in Canada and 36 to 37% with the second new subtype isolated in United States. The partial G sequences of both isolates were compared to that of other strains from different countries and different subtypes retrieved from GenBank. According to the database, the highest percentage identity of 100% was observed for TN1015/17 Tunisian strain with that of an aMPV-B isolated in Russia (JN651915), and 99% with the Algerian and the Nigerian strains (KP892758 and AM490058), already classified as derived vaccine strains. For the second isolate, TN1000/19, the analysis has revealed 100% phylogenetic similarity with the Spanish strain (MT432857) which is a field strain. The tree constructed based on the alignments of sequence data sets of the G gene, showed 6 main clusters. The upper end of the tree contained the subtype B while the lower end contained subtype C metapneumovirus. The subtypes D, A, and the 2 new metapneumovirus were grouped in the middle of the tree. Both Tunisian TN1015/17 and TN1009/19 isolates were clustred with subtype B (Figure 1). In the cluster forming subtype B metapneumovirus, the Tunisian isolate TN1015/17 was clustered with the derived vaccine strains; however, the TN1000/19 isolate was clustred with field strains (Figure 2).

Characterization of Residues Associated With TN1000/19 Tunisian Isolate

The nucleotide sequences of the different strains were aligned and the multiple alignments revealed point mutations common to the Spanish and the Tunisian isolates. Indeed, 2 transition-type substitutions, A> G and G> A, at positions 204 and 277, respectively, marked these isolates. An exhaustive comparison of the nucleotide sequences of the G gene of the Tunisian isolates with all strains in GenBank, showed that these are unique and characteristic mutations of both the Tunisian and the Spanish isolates, thus forming a signature specific to them. In order to look for a possible change in the structure of the G protein, the nucleotide sequences were converted into proteins to analyze the amino acid sequences. Alignment of the amino acid sequences revealed that the amino acid sequences obtained from both mutations of A> G at position 204 and that of G> A at position 277, indicated that the first mutation was silent, thus keeping the amino acid proline at position 68, while the second mutation was at the origin of the substitution of the amino acid glycine (G) by argine (R).

Phylogeographic Analysis

A phylogeography study carried out, using Beast 2.5 Software, resulted in the Maximum clade credibility tree shown in Figure 3. The Overall tree structure places the isolates originating from one location together in the same branch of the tree. Thus, the isolates originating from Brazil clearly diverged from the root of all other isolates. Similarly, the Iranian isolates diverged from all other European branches. Although European isolates didn't form a separate cluster. The emergence of a separate branch for each of the following countries: France, Romania, Spain, and Italy may be clearly noticed. However, Greek isolates clustered within the Italian branch. Interestingly, the Tunisian isolate TN1000/19 clustered within the Spanish isolate branch. The Time to Common Ancestor for the Tunisian and the Spanish isolates is estimated around the yr 2016 (95% HD 2015−2017).

DISCUSSION

Swollen Head Syndrome is a disease of chickens, distributed worldwide and having serious economic consequences. Isolation of aMPV is extremely difficult because excretion of the virus from the tissue happens to be for a very short time after infection. Consequently, it is hard to establish the time of onset of infection and the best time to sample collection in the field. Furthermore, isolation and identification of the virus by RT-PCR became very difficult after the fourth day of virus infection (Cook et al., 2001). For this reason, we were able to only isolate, characterize and sequence the partial G gene of the 2 aMPV reported isolates from commercial broiler breeder flocks in eastern and southern regions of Tunisia during 2015 to 2019. This is the first time the aMPV has been isolated, in Tunisia, and the only subtype B virus identified. Actually, aMPV has commonly been suspected in our country, as co-infecting agent during clinical manifestations without a precise knowledge of its possible spreading. It could be also possible that

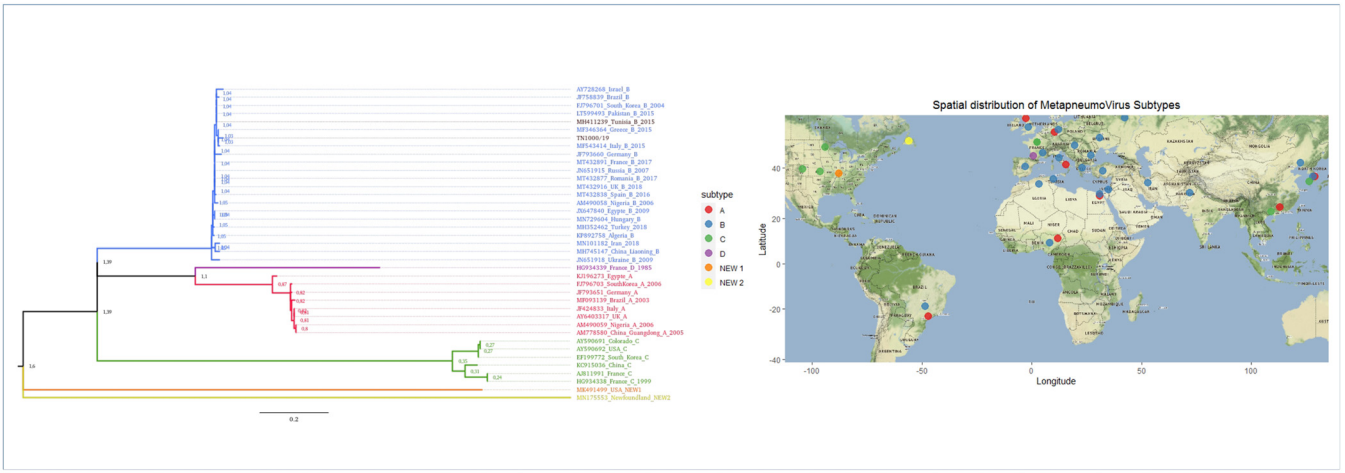

Figure 1. The geographical distribution of the different subtypes detected up to 2021, including the Tunisian isolates.

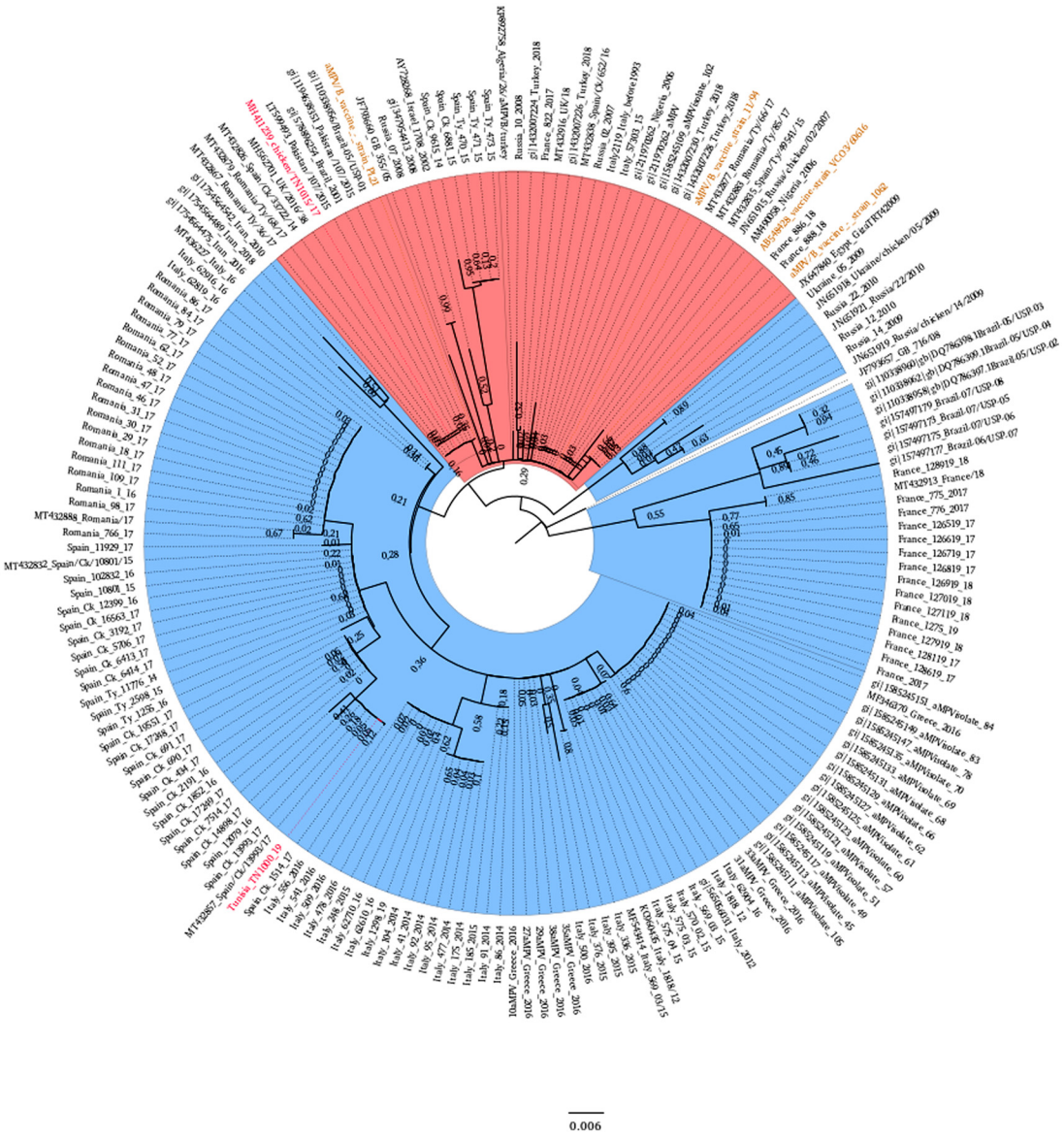

Figure 2. Maximum likelihood phylogenetic tree of partial G gene of (aMPV) subtype B. Worldwide selected sequences were based on countries of sampling information. The analysis involved 182 sequences of G gene. Red colored clades correspond to vaccine derived strains while blue colored clades corresponds to Tunisian field strains TN1015/17 and TN1000/19, isolated in 2017 and 2019. The tips of the Tunisian strains are colored in red. Vaccine strains tips are colored in orange.

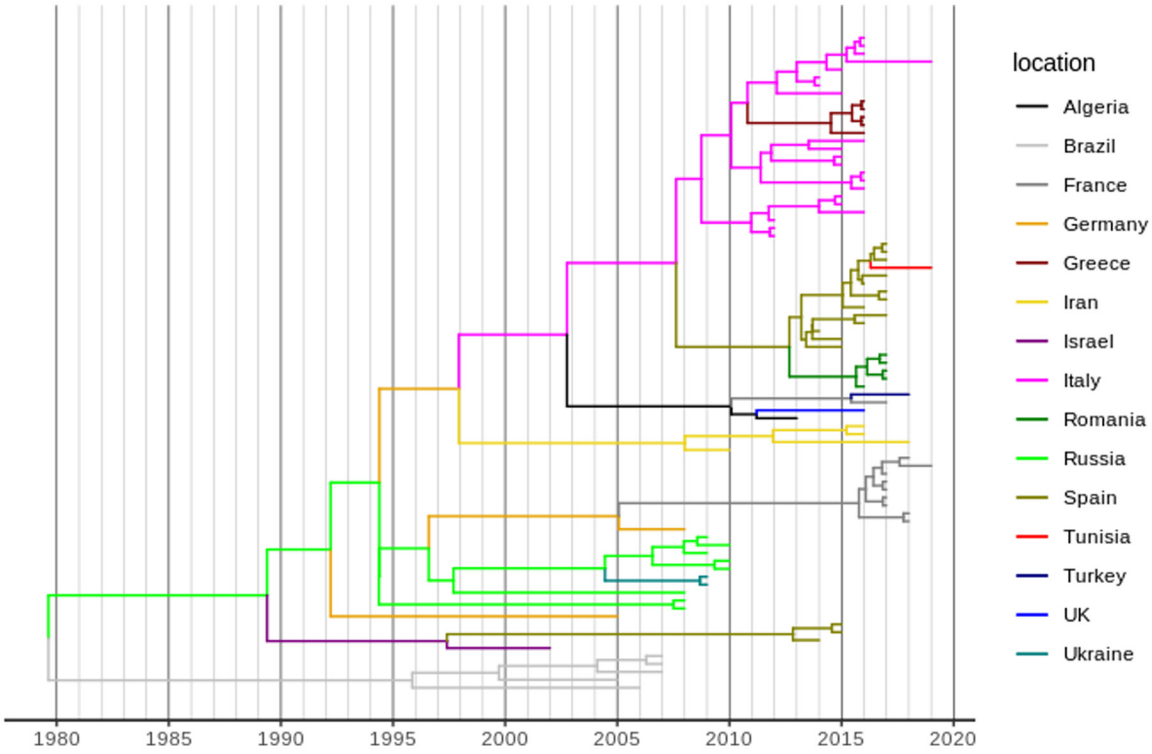

Figure 3. Time scale phylogenetic tree of aMPV subtype B isolates performed using G gene sequence data. Reconstruction of ancestral locations was performed using Beast 2.5. Branches of the tree are colored according to locations.

aMPV subtype B had been circulating in Tunisia without ever being detected.

The two virus isolates considered in this work belong to the subtype B of the aMPV. The subtype B has been described in multiple countries worldwide, especially in Europe and the Mediterreanean region (Andreopoulou et al., 2019; Franzo et al., 2020); although, strains of the subtype B have different evolutionnary origins. Some isolates have been proven to be vaccine related and their apparition was due to a reversion of virulence (Mescolini et al., 2021).

The first isolate TN1015/17, an aMPV-B detected in 2017, was classified as a vaccine-derived strain, since it has shown high nucleotide identity and was phylogenitically related to live vaccine strain (PL21). The second isolate TN1000/19, aMPV-B detected in 2019, was classified as a field strain showing 100% nucleotide sequence similarity with the Spain strains.

To investigate the history of aMPV subtype B transmission in Tunisia, we performed fine scale phylogenetic analyses, based on informative partial G gene sequencing data. Our results showed that the TN1000/19 strain is likely to have originated in Spain. A phylodynamic study revealed that the estimated date of divergence time for the isolate has happened around the year 2016.

The Maximum likelihood tree performed in order to analyze the clade appartenance of the Tunisian isolates is comparable to phylogenetic tree performed in previous studies where vaccine-derived strains clustered serapately from field strains (Mescolini et al., 2021). The isolate TN1015/17, sampled in 2017 in Tunisia, clustered with high support with vaccine derived strains. Whereas isolate TN1000/19 sampled in 2019 in Tunisia clustered with field strains of European origin. Thus, Metapneumoviruses circulating among poultry in Tunisia are diverse and have different origins.

Phygeographic analysis was undertaken based on year of sampling as well as country of origin, as it has been done in previous studies (Franzo et al., 2020). Uncorrelated lognormal model was used as molecular clock model to allow for different rates among branches (Drummond et al., 2006). Overall, the tree structure is comparable to that of previous studies where isolates tend to cluster according to their country of origin (Franzo et al., 2020). The Tunisian isolate TN1000/19 clustered within the branch of isolates of Spanish origin. It is very likely that this Tunisian isolate was introduced from Spain around 3 yr before its isolation from the field; as tMRCA is estimated at yr 2016 (95% HD 2015 −2017).

The hypothesis of virus transmission through the networks of migratory birds between Africa and Europe can be incriminated. A second hypothesis can also be considered: given that this strain has the highest percentage of identity of its G gene with the Spanish aMPV-B strain; trade between countries of North Africa and Europe may be suspected of being involved in the spread of the virus. However, due to the limited number of studies and the lack of aMPV sequences available in public databases from North African countries, including Tunisia, Morocco, Libya, and Algeria, further studies are needed to fully explain the epidemiological models. It is also possible that aMPV subtype B has already been circulating in these countries without ever

being detected, due to the lack of diagnostic laboratoties and the difficulties of metapneumoviruses isolation. Our phylodynamic analyses are in accordance with the results reported in the recently published study (Franzo et al., 2020), in which sequences belonging to other continents like South America, North Africa, and Asia were included in addition to Europe.

Our study shed the light on the existence of this virus responsible of a major disease of poultry and hilighted its multiple origins. This suggests that basis for aMPV control in Tunisia needs to consider reinforcement of biosecurity measures in the farms and intensive virological surveillance including sequencing of field isolates and differentiate them from vaccine derived strains. Further studies are needed for better understanding of aMPV-B spread in Tunisia with a larger number of samples.

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DISCLOSURES

The authors declare no conflict of interest. The funders had no influence on the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to publish the results.

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