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Molecular epidemiology of aMPV-B in Europe

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Summary

In the present study one hundred and sixteen partial G gene sequences of Avian metapneumovirus (aMPV) subtype B, obtained during routine diagnostics in different European Countries in the last few years (2014-2019), were analysed by sequence and phylogenetic analyses in order to draw an updated picture of the molecular characteristics of circulating strains. Nucleotide sequences were compared with other sequences of European and non-European aMPV-Bs collected prior to that period or retrieved from GenBank. Phylogenetic relationships among the aMPV-B strains, reconstructed using the Maximum Likelihood method implemented in MEGA X, demonstrated that aMPV-B has evolved in Europe from its first appearance, frequently displaying a clear relation with the geographic area of detection. The 40% of aMPV-B viruses analysed were classified as vaccine-derived strains, being phylogenetically related, and showing high nucleotide identity with live commercial vaccine strains licensed in Europe. The remaining 60% were classified as field strains since they clustered separately and showed a low nucleotide identity with vaccines and vaccine-derived strains. The phylogenetic tree showed that the virus has continued to evolve from its first appearance in the ’80s since more recently detected strains belonged to clades phylogenetically distant from the older strains. Unlike vaccine-derived strains, field strains tended to cluster according to their geographic origin and irrespective of the host species where the viruses had been detected. In conclusion, the molecular characterization of aMPV-B and the differentiation between vaccines and field strains through G gene sequence analysis can be a useful tool towards correct diagnosis and should be routinely applied in order to better address the control strategies.

***Key words*: Avian metapneumovirus, subtype B, Europe, molecular characterization**

Introduction

Avian metapneumovirus (aMPV) infections in Europe have been an issue since its first appearance both in turkeys, chickens and in other minor species (Toquin, Bayon-Auboyer, Eterradossi, & Jestin, 1999; Catelli et al., 2001; Cecchinato, Ferreira, Munir, & Catelli, 2017; Cecchinato et al., 2018), causing economic losses mainly due to respiratory or reproductive problems (Cecchinato et al., 2012), often exacerbated by secondary bacterial infections (Giovanardi et al., 2014).

First aMPV isolations in Europe date back to the second half of the 1980s, when Turkey Rhinotracheitis (TRT) outbreaks appeared in the United Kingdom (McDougall and Cook, 1986) and France (Giraud, Bennejean, Guittet, & Toquin, 1986). As the virus was spreading all over Europe and the clinical problems in poultry farms became increasingly serious, during the early 1990s (Cook et al. 1989a e 1989b) live attenuated vaccines were developed and became commercially available. By analysing strains circulating in Europe, Juhasz and Easton (1994) reported differences in the G gene between aMPV isolates and proposed the classification into A and B subtypes and confirmed the co-circulation of both subtypes. After this initial study, and due to the increasing use of sequence analysis, two further subtypes, named C and D, were identified in France (Bayon-Auboyer, Arnauld, Toquin, & Eterradossi, 2000; Toquin et al., 2006).

Despite these reports, molecular data on aMPV strains circulating in Europe are still poor and scattered (Catelli et al., 2004; Cecchinato et al., 2013a; Listorti et al., 2014; Franzo et al., 2017; Tucciarone et al., 2017 a 2018; Ball, Forrester, & Ganapathy, 2018; Andreopoulou et al., 2019), as most of them originate from few countries. In the last decades, subtype B has been generally the most frequently encountered subtype in Europe, although subtype A has been sporadically reported (Lupini et al., 2011).

In order to update the epidemiological picture of circulating strains, the present study was designed to molecularly characterize, by partial G gene sequencing, aMPV subtype B strains detected in Europe in the last years.

Material and Methods

Sample collection

The survey covered the time period from 2014 to 2019 and targeted aMPV subtype B detected during routine diagnostic activity performed in Italy by the Universities of Bologna and Padua or in Spain by the Centre de Sanitat Avícola de Catalunya i Aragó (CESAC).

Samples originated from different European countries and were usually collected during outbreaks of respiratory disorders referable to aMPV infections in turkey, chicken or guinea fowl flocks. Sampling was also performed for epidemiological purposes in absence of clear clinical signs.

As a rule, samples consisted of pools of 10 rhino-pharyngeal swabs per flock as previously suggested (Catelli et al., 2004).

RNA extraction and PCR analysis

RNA was extracted from each pool of swabs and subjected to a nested RT-PCR targeting the G gene (Cavanagh, Mawditt, Britton, & Naylor, 1999, slightly modified) which allows the simultaneous detection and differentiation of subtypes A and B. The reverse transcription (RT) reaction was primed using oligonucleotide G6- (5'- CTGACAAATTGGTCCTGATT- 3'). Subsequently the same oligonucleotide was used together with oligonucleotide Gstart+ (5'- CAAGTATCCAGATGGGGTC- 3') for the first PCR round. The obtained PCR product was then subjected to a second PCR round with oligonucleotide G5- (5'- CAAAGAGCCAATAAGCCCA- 3') in conjunction with oligonucleotides G8+A (5'-CACTCACTGTTAGCGTCATA-3') and G9+B (5'-TAGTCCTCAAGCAAGTCCTC-3'). The predicted amplicon length is of 268 bp for subtype A and 361 bp for subtype B. Depending on the laboratory, this step was preceded by a multiplex real-time RT-PCR (qRT-PCR) screening test targeting the SH gene (Cecchinato et al., 2013b) and only positive samples were subsequently amplified by RT-nested PCR.

Partial G gene sequencing

The partial G gene amplicons (361 bp-long) obtained were purified using ExoSAP-IT™ *Express* PCR Product Cleanup (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions and sequenced in both directions using RT-nested PCR primers G5- (5′-CAAAGAGCCAATAAGCCCA-3′) and G9+B (5′-TAGTCCTCAAGCAAGTCCTC-3′), by a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands). The obtained sequences were named using the following nomenclature: aMPV/B/Country of origin/Host species (Turkey: Ty, Chicken: Ck or Guinea fowl)/sample ID number/year of detection.

Sequence and phylogenetic analysis

Nucleotide sequences were edited and assembled using BioEdit software, then, using Clustal W, aligned against and compared with G gene sequences of:

- The most commonly used subtype B vaccines (i.e. aMPV/B vaccine - strain VCO3; aMPV/B vaccine - Strain 11/94; aMPV/B vaccine - strain 1062 and aMPV/B vaccine - strain PL21)
- 82 already available European aMPV B strains (Table S1)
- 59 non-European aMPV-B strains, retrieved from *GenBank* (Table S2).

Phylogenetic relationships among the aMPV-B strains were reconstructed using the Neighbor-Joining method and Kimura 2-parameter model implemented in MEGA X (Kumar et al., 2018). The substitution model was selected based on Bayesian information criterion (BIC), calculated using the same software. The branch support was calculated by performing 1000 bootstrap replicates; only branches supported by bootstrap values ≥ 70% were considered reliable. Complete deletion option was selected before the analysis begins, to remove sites containing missing data or alignment gaps.

Within-group mean pairwise genetic p-distance was estimated using MEGA X in order to evaluate the genetic heterogeneity of aMPV population. Overall nucleotide similarity was further estimated by calculating the arithmetic mean of the sequence identity values, obtained using the Sequence Identity Matrix tool on BioEdit software, between field strains or vaccine strains belonging to the same phylogenetic cluster.

Results

116 samples tested aMPV positive, all belonging to subtype B (n. 27 from France, n. 18 from Italy, n. 27 from Romania, n. 36 from Spain and n. 8 from the United Kingdom) and 116 partial G gene sequences were obtained. Sequence data have been submitted to the GenBank database under accession numbers MT432826-MT432923 and MT436220-MT436237 (Tables S3 to S7).

Phylogenetic analysis was carried out by reconstructing both a comprehensive tree (Figure 1) and five Country- specific trees (Figures S1 to S5). Furthermore, a phylogenetic tree including selected European and non-European aMPV-B sequences was generated (Figure 2). The number of nucleotide positions included in the final datasets subjected to phylogenetic analysis is specified in the legends of the figures.

Regardless of the Country of origin, aMPV strains detected from 2001 to 2019, herein referred to as "recent strains", were distinguished from those clustering with vaccines and field strains detected prior to the 1990s, referred to as "older strains" (from which the vaccines were established by attenuation) and those forming independent clades.

As a rule, aMPV strains were referred to as "vaccine-derived strains" if the maximum nucleotide sequence identity with a reference vaccine strain was greater than or equal to 99 % (2 or less nucleotide differences) and if they fell into the same phylogenetic cluster. The remaining strains were classified as "field strains".

142 On the above basis, 70 out of 116 aMPV-B strains were labelled as field strains (60%); the remaining
143 46 strains (40%) were identified as vaccine-derived strains.

144 A certain degree of geographic clustering was observed among Romanian, Spanish, French and
145 Italian field strains (Figure 1). Some Italian strains also clustered together with some recent Greek
146 strains.

147 French aMPV field strains (Figure S1) were divided in two clusters. The largest cluster displayed a
148 within-group mean p-distance of 0.003 (overall nucleotide similarity of 99.7%) and included chicken,
149 turkey and guinea fowl strains detected from 2017 to 2019, while the smallest had a within-group
150 mean p-distance of 0.000 (overall nucleotide similarity of 100%) and contains turkey strains detected
151 in 2018. Vaccine-derived strains clustered respectively with vaccine strain PL21, 11/94 or VCO3 and
152 were mostly detected in homologous-vaccinated turkeys or chickens, but also in unvaccinated guinea
153 fowls. The cluster containing vaccines, vaccine-derived strains and older French strains displayed a
154 within-group mean p-distance of 0.006 (overall nucleotide similarity of 99.4%)

155 Italian field strains were grouped in two main clusters (Figure S2): the larger one was composed of
156 several sub-clusters including field strains detected from 2010 to 2019 in turkey, chicken, and guinea
157 fowl flocks and showed a within-group mean p-distance of 0.010 (overall nucleotide similarity of
158 99.0%). The smaller one included field strains detected from 2001 to 2009 and in 2016 in turkeys and
159 chickens, with a within-group mean p-distance of 0.004 (overall nucleotide similarity of 99.6%).
160 Vaccine-derived strains clustering with aMPV-B vaccine strains VCO3 or 1062 were detected from
161 vaccinated turkeys. The cluster containing vaccines, vaccine-derived strains and older Italian strains
162 displayed a within-group mean p-distance of 0.007 (overall nucleotide similarity of 99.3%).

163 Romanian aMPV-B field strains (Figure S3), detected from turkeys or chickens, fell within a single
164 phylogenetic group showing a within-group mean p-distance of 0.001 (overall nucleotide similarity
165 of 99.9%). The vaccine-derived strains, all detected in vaccinated turkey flocks, clustered with

166 vaccine strains PL21, 11/94 or VCO3. The cluster containing vaccines and vaccine-derived strains
167 displayed a within-group mean p-distance of 0.007 (overall nucleotide similarity of 99.3%).

168 In the Spanish tree (Figure S4) field strains fell within two main clusters: a larger one (within-group
169 mean p-distance: 0.006 and overall nucleotide similarity of 99.4%) containing strains detected from
170 2014 to 2017; a smaller one (within-group mean p-distance: 0.003 and overall nucleotide similarity
171 of 99.7%) containing viruses detected from 2014 to 2015. Spanish vaccine-derived strains clustered
172 with vaccine strain 11/94 or PL21. The cluster containing vaccines, vaccine-derived strains and older
173 strains displayed a within-group mean p-distance of 0.008 (overall nucleotide similarity of 99.2%).

174 The British phylogenetic tree (Figure S5) exclusively displayed vaccine-derived strains, clustering
175 with vaccine strain PL21, 11/94 or VCO3 (within-group mean p-distance: 0.007 and overall
176 nucleotide similarity of 99.3%).

177 The tree reconstructed with representative aMPV-B sequences from all over the world (Figure 2)
178 confirmed the Country-specific clustering trend seen for the European strains. Well-defined clusters
179 were identified for field strains circulating in Brazil, Iran, Israel and Turkey. Vaccines, vaccine-
180 derived and older strains clustered together and were showed in the figure compressed in two
181 subtrees. Within-group mean p-distance and overall nucleotide similarity of the cluster containing
182 vaccine PL21 were respectively 0.007 of 99.3%,

183

184 **Discussion**

185 In the present study, one hundred and sixteen aMPV-B partial G gene sequences, obtained during
186 routine diagnostics in different European Countries in the last few years, were analysed by sequence
187 and phylogenetic analyses in order to molecularly characterize them.
188 The G gene, which harbours mutations at variable positions between aMPV-B strains, was
189 conveniently amplified by the routine diagnostic RT-PCR protocol employed in the study, and its

190 sequencing proved useful for epidemiological purposes. Furthermore, its variability can give an
191 indication of whether vaccine or field strains are present, making the differentiation relatively easy
192 and cheap to perform.
193 Live attenuated aMPV vaccines are widely administered to prevent disease in turkeys and chickens,
194 and the recovery of vaccine-derived strains is not unusual both in vaccinated or in unvaccinated
195 flocks (Banet-Noach et al., 2009; Lupini et al., 2011; Chacon et al., 2011; Cecchinato et al., 2013a;
196 Listorti et al., 2014; Arafa et al., 2015; Bayraktar et al., 2019; Andreopoulou et al., 2019).
197 The 40% of the aMPV-B strains detected in the present study were classified as vaccine-derived
198 strains, being phylogenetically related and showing high nucleotide identity with live commercial
199 vaccine strains licensed in Europe. As expected, vaccine-derived strains formed separate clusters
200 depending on the vaccine strain of origin and were detected in all tested European countries.
201 A large part of the vaccine-derived strains analysed in the present study was detected in
202 homologous-vaccinated birds, from approximately two to four weeks after vaccination, and only
203 occasionally in unvaccinated birds. Reversion to virulence of aMPV subtype A or B has been
204 previously demonstrated (Catelli et al., 2006; Brown et al., 2011, Cecchinato et al., 2014).
205 Therefore, the detection of vaccine-derived strains closely related to the applied vaccine,
206 concurrently with respiratory signs, could be reliably linked to the vaccine reversion to virulence.
207 The detection of the strain aMPV/B/France/GuineaFowl/1060/18 in unvaccinated guinea fowls is
208 noteworthy, since its partial G gene sequence shared 100% nucleotide identity with the vaccine
209 strain 11/94. It could be speculated that the vaccine virus could had been introduced as a
210 contaminant by personnel, fomites, vehicles movement or airborne from neighbouring premises.
211 The field veterinarian reported the presence of a turkey farm at approximately 500 meters from the
212 guinea fowl one. Vaccinal strain 11/94 was applied in the turkey flock and the use of the litter from
213 the turkey farm for fertilization of the surrounding crops was reported. The ability of vaccine-
214 derived aMPVs to spread beyond the administration site is well known and it has been proven by

Lupini et al. (2011) following a turkey rhinotracheitis outbreak caused by aMPV subtype A in unvaccinated turkeys.

The remaining 60% aMPV-B viruses analysed in the present study were classified as field strains since they clustered separately and showed a low nucleotide identity with vaccines and vaccine-derived strains.

The phylogenetic tree reconstructed with European sequences showed that the virus has continued to evolve from its first appearance in the ’80s. In fact, more recently detected field strains belonged to clades phylogenetically distant from the older field strains, confirming the previously-reported aMPV tendency to evolve over time (Cecchinato et al., 2010).

Unlike vaccine-derived strains, field strains tended to cluster according to their geographic origin, with few exceptions. Distinct clusters were observed for French, Italian, Romanian and Spanish strains, yet some Italian and Greek field isolates clustered closely together, indicating a potential transmission route between these two countries (Tucciarone et al., 2017; Andreopoulou et al., 2019).

The molecular epidemiology of aMPV within each country was analysed in detail reconstructing country-related phylogenetic trees. Heterogeneous field strain populations seemed to co-exist within single European countries, with the only exception of Romania, where all identified strains were part of just one clade. This last finding could be explained by keeping into account that all the processed samples came from different sites within the same company in which viral circulation could be compartmentalized (Franzo et al., 2020).

French, Italian, and Spanish trees showed a rather heterogeneous field strain population, as the strains fell into more than one cluster, divided in several sub-clusters. The heterogeneity was further deduced from the within-group mean p-distance values. The highest value, indicating the highest heterogeneity of nucleotide sequence, was observed for Italian field strains, presumably because of

239 the larger number of sequences available over a broader time period and coming from different
240 commercial poultry companies.

241 Furthermore, time-related clustering was visible for Italian and Spanish field strains, as strains
242 detected in the last few years tend to form separate clusters or sub-clusters.

243 As a common finding, recent field aMPV-B strains, belonging to different and distant phylogenetic
244 clades, were detected in the presence of respiratory signs in vaccinated flocks. Due to the G gene
245 variability observed in recent aMPV-B strains, and knowing that the encoded surface G
246 glycoprotein is a key antigen for vaccine-induced immune protection (Naylor et al., 2007) and can
247 evolve in order to avoid vaccine-induced immunity (Catelli et al., 2010; Cecchinato et al., 2010),
248 re-assessment of protection conferred by commercially available vaccines against currently
249 circulating aMPV field strains might be necessary to improve disease control strategies.

250 The obtained phylogenetic data revealed that aMPV-B strains clustered together irrespective of the
251 host species where the viruses had been detected. A recent experimental challenge study showed
252 that both chickens and turkeys are susceptible to aMPV-B infection with the same virus isolate
253 (Brown et al., 2019). Moreover Cecchinato et al. (2018) reported an outbreak of respiratory disease
254 in guinea fowls caused by aMPV-B strain identical to the ones circulating in the surrounding turkey
255 flocks. Therefore, no evidences are currently available to support a host-specific adaptation of
256 aMPV variants.

257 As a final remark, the tree reconstructed on European and non-European strains further confirmed
258 the geographic clustering of field strains. As a rule, field strains grouped together for country
259 location and time period, suggesting a local evolution tendency of the virus that might have taken
260 place after a single introduction event. The majority of recently detected European field strains were
261 located in a big subtree indicating a certain genetic similarity and supporting the wide circulation of
262 a quite homogeneous aMPV subtype B clade in European countries, with the only exception of two
263 clusters of phylogenetically-distant French and Spanish strains.

In conclusion, the molecular characterization of aMPV subtype B and the differentiation between vaccines and field strains through G gene sequence analysis can be a useful tool towards a correct diagnosis and should be routinely applied in order to better address the control strategies. In this respect, current vaccine research is focused on reducing the issues connected to live attenuated vaccine reversion to virulence or the selection of potentially virulent subpopulations (Franzo et al., 2015). Therefore, considering the associated risks, further efforts should be directed at improving administration and biosecurity measures, in order to reduce their prolonged circulation and spreading.

Several research groups have attempted to develop efficacious, more stable and safer next-generation vaccines but, despite this effort, conventional live attenuated vaccines still provide the greatest protection after homologous challenge (Qingzhong et al., 1994; Tarpey et al., 2001; Kapczynski and Sellers, 2003; Kapczynski, 2004; Chary, Njenga, & Sharma, 2005; Yu et al., 2013; Hu, Roth, Zsak, & Yu, 2017).

Promising reverse genetics systems for aMPV have been developed in recent years for A, B and C subtypes (Naylor et al., 2004; Yu et al., 2010; Laconi et al., 2016) other than being an exceptional tool for the study of the virus properties (Brown et al., 2011), can be used for the development of rationally modified aMPV vaccines (Naylor, Lupini, & Brown, 2010) or recombinant vaccines (Falchieri et al., 2013) expressing foreign genes.

Conflict of Interest Statement

The authors declare no conflict of interest.

Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the original research data of this

289 article was obtained from oro-pharyngeal swabs collected for routine diagnostics purpose by field
290 veterinarians.
291
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Figure legends

Figure 1. Phylogenetic tree based on G gene nucleotide sequences of 60 selected European aMPV-B strains detected in the present study (identical sequences within a country were removed), including the most commonly used subtype B vaccines and European aMPV-B strains previously obtained or retrieved from GenBank. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. All nucleotide positions containing gaps and missing data were eliminated, there were a total of 246 positions in the final dataset. The two subtrees containing vaccines and vaccine-derived strains have been compressed. Field aMPV-B strains clusters are included in square brackets and coloured by country of origin. Only bootstrap values ≥ 70 are reported.

Figure 2. Phylogenetic tree based on G gene nucleotide sequences of selected European and non-European aMPV-B strains, previously obtained or retrieved from GenBank. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. All nucleotide positions containing gaps and missing data were eliminated, there were a total of 171 positions in the final dataset. The two subtrees containing vaccines and vaccine-derived strains have been compressed. Field aMPV-B strains clusters are coloured by country of origin. The country of origin was added in brackets next to the strain name when it was not clearly specified in the very name. Only bootstrap values ≥ 70 are reported.

Supplementary figure legends

Figure S1. Phylogenetic tree based on G gene nucleotide sequences of 27 French aMPV-B strains detected in the present study, including the most commonly used subtype B vaccines (marked with a green triangle) and 2 French aMPV-B strains previously obtained. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. All nucleotide positions containing gaps

494 and missing data were eliminated, there were a total of 253 positions in the final dataset. Field
495 aMPV-B strains clusters are included in square brackets. Only bootstrap values ≥ 70 are reported.

496

497 **Figure S2.** Phylogenetic tree based on G gene nucleotide sequences of 18 Italian aMPV-B strains
498 detected in the present study, including the most commonly used subtype B vaccines (marked with
499 a green triangle) and 42 Italian aMPV -B strains, previously obtained or retrieved from GenBank.
500 The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. All
501 nucleotide positions containing gaps and missing data were eliminated, there were a total of 252
502 positions in the final dataset. Field aMPV-B strains clusters are included in square brackets. Only
503 bootstrap values ≥ 70 are reported.

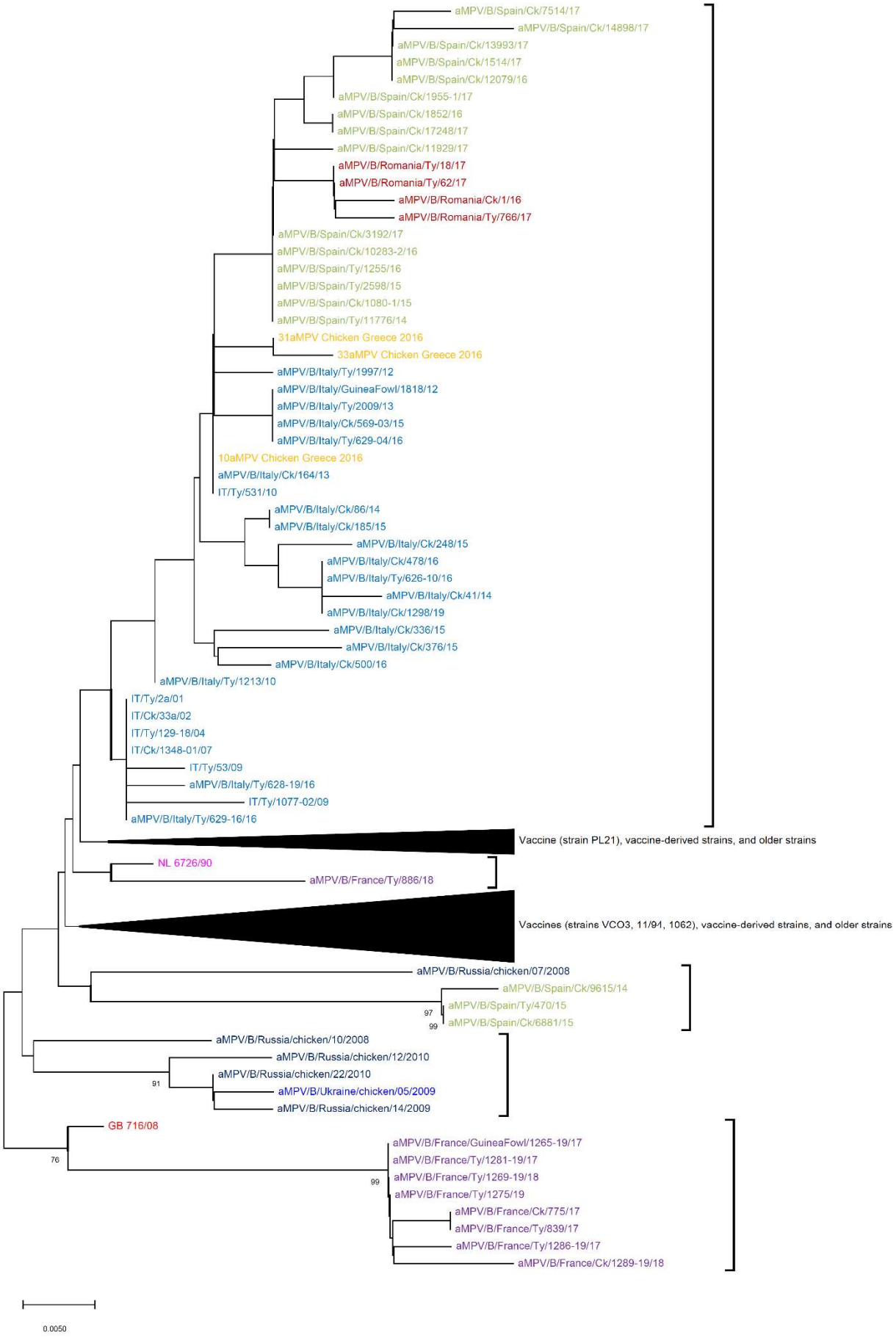
504

505 **Figure S3.** Phylogenetic tree based on G gene nucleotide sequences of 27 Romanian aMPV-B
506 strains detected in the present study and the most commonly used subtype B vaccines (marked with
507 a green triangle). The evolutionary history was inferred using the Neighbor-Joining method in
508 MEGA X. All nucleotide positions containing gaps and missing data were eliminated, there were a
509 total of 282 positions in the final dataset. Field aMPV-B strains clusters are included in square
510 brackets. Only bootstrap values ≥ 70 are reported.

511

512 **Figure S4.** Phylogenetic tree based on G gene nucleotide sequences of 36 Spanish aMPV-B strains
513 detected in the present study and the most commonly used subtype B vaccines (marked with a green
514 triangle). The evolutionary history was inferred using the Neighbor-Joining method in MEGA X.
515 All nucleotide positions containing gaps and missing data were eliminated, there were a total of 303
516 positions in the final dataset. Field aMPV-B strains clusters are included in square brackets. Only
517 bootstrap values ≥ 70 are reported.

Figure S5. Phylogenetic tree based on G gene nucleotide sequences of 8 British aMPV-B strains detected in the present study, including the most commonly used subtype B vaccines (marked with a green triangle) and 10 British aMPV -B strains, previously obtained or retrieved from GenBank. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. All nucleotide positions containing gaps and missing data were eliminated, there were a total of 302 positions in the final dataset. Only bootstrap values ≥ 70 are reported.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IT/Ty/531/10
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IT/Ty/129-18/04
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Vaccine (strain PL21), vaccine-derived strains, and older strains
NL 6726/90
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Vaccines (strains VCO3, 11/91, 1062), vaccine-derived strains, and older strains
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97
99
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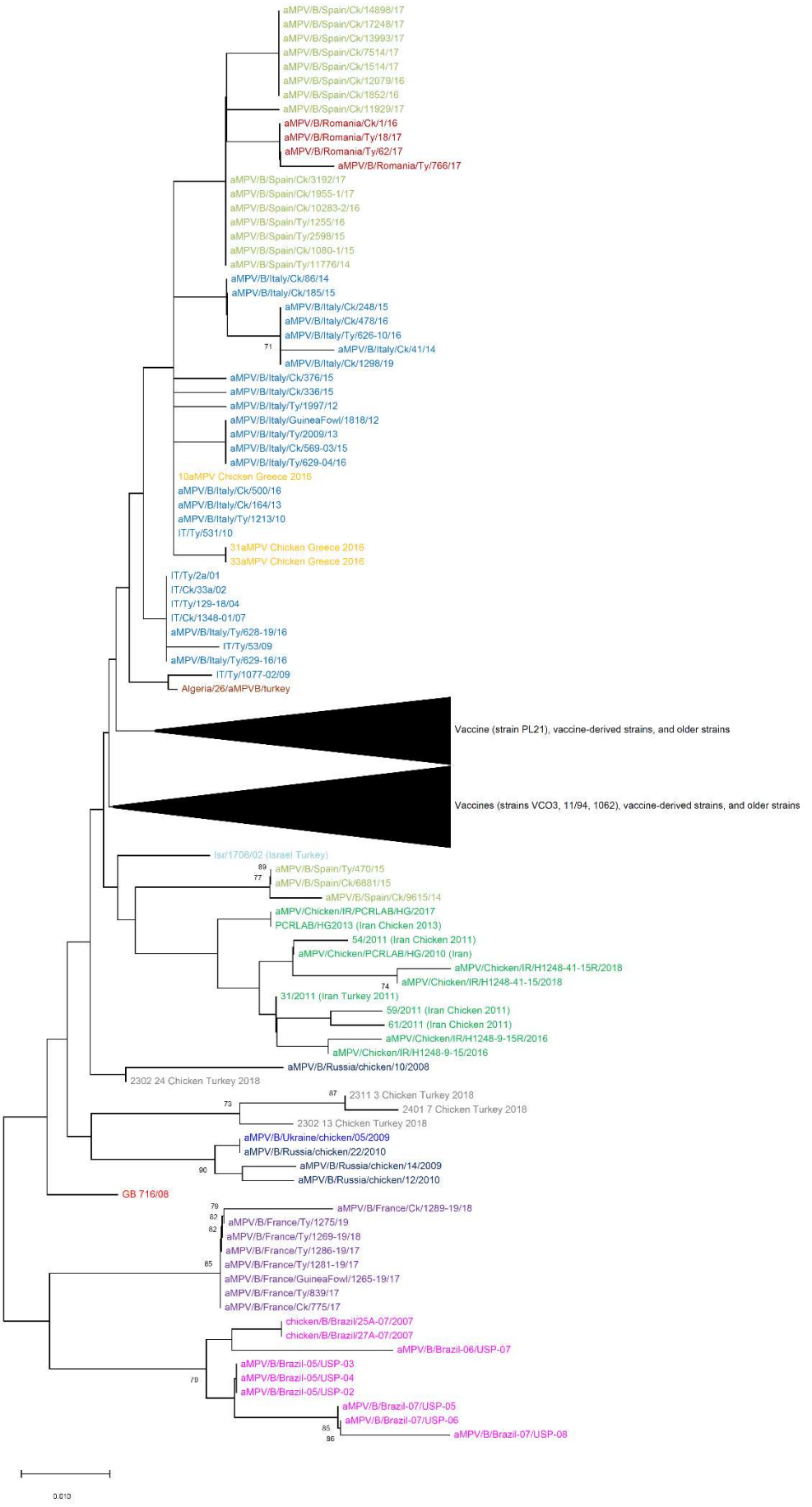
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IT/Ty/1077-02/09
Algeria/26/aMPVB/turkey
Vaccine (strain PL21), vaccine-derived strains, and older strains
Vaccines (strains VCO3, 11/94, 1062), vaccine-derived strains, and older strains
Isr/1708/02 (Israel Turkey)
89
77
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