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**Molecular characterization of whole genome sequence of infectious
bronchitis virus 624I genotype confirms the close relationship with Q1
genotype**

Running Title: Correlation between IBV genotypes 624I and Q1

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Abstract

Infectious Bronchitis virus (IBV) genotype Q1 was detected for the first time in China in 1996, and then spread worldwide. The first report of Q1 genotype in Italy occurred in 2011 and a deep molecular investigation of a Q1 isolated in Italy in 2013 has led to speculation regarding the origin of this genotype. Phylogenetic analysis of the S1 sequence of a Q1 Italian strain revealed a close relationship with sequences of the 624I strains circulating in Italy in the early 1990s and this led to the idea that 624I was an ancestor of the Q1 genotype. Despite the fact that most heterogeneity of IBVs occurs in the S1 gene, the sequence analysis of this gene alone was not sufficient to confirm or deny this hypothesis. In the present study, an Italian 624I (gammaCoV/AvCov/Ck/Italy/IP14425/96) was fully sequenced for the first time and compared to all available complete Q1 genome sequences. This analysis confirmed the genetic correlation between GammaCoV/AvCov/Ck/Italy/IP14425/96 and Q1 strains, suggesting a common origin between 624I and Q1 genotypes.

65 **Introduction**

66

67 Infectious bronchitis (IB) is an avian disease distributed worldwide that represents one of the
68 most persistent health problems of the commercial poultry industry (Cook et al., 2012, de Wit
69 et al., 2011). It is caused by a Gammacoronavirus called Infectious Bronchitis Virus (IBV) and
70 has a positive sense single stranded 27.6kb RNA genome (Jackwood and de Wit, 2013). The
71 IBV genome can evolve rapidly by mutation and recombination events, resulting in the
72 emergence of new IBV variants which sometimes confer minimal or negligible cross
73 protection (de Wit et al., 2011). The majority of such variants causes a transitory problem,
74 which then disappear or remain confined into a specific geographical region. However a few
75 variants can persist and spread to new areas where they continue to cause disease (de Wit et
76 al., 2011; Jackwood 2012).

77 Recently, genotypes of Asian origin have spread to cause worldwide disease and major
78 economic losses (de Wit et al., 2011). IBV Q1 is one such genotype. It was first detected in
79 China in 1996 (Yu et al., 2001) and then reported in Asia, Middle East, Europe and South
80 America (Ababneh et al., 2012, Huang et al., 2004, Jackwood, 2012, Marandino et al., 2015,
81 Rimondi et al., 2009). In Italy the Q1 genotype was reported for the first time in 2011 after
82 causing an outbreak of disease associated with respiratory signs, increased mortality, kidney
83 lesions and proventriculitis (Toffan et al., 2013). Since then, the genotype has been
84 continuously detected in Italy (Massi et al., 2015). Phylogenetic analysis performed using full
85 or partial S1 sequences showed a high identity (>99%) with Chinese Q1 isolates (Franzo et al.,
86 2015, Massi et al., 2015, Toffan et al., 2013). At a similar period, a high identity (94,1%)
87 between those strains and strains belonging to the 624I genotype was observed (Franzo et al.,
88 2015, Massi et al., 2015), such that the recently proposed new IBV nomenclature based on the
89 S1 sequence placed them in the same lineage (GI-16) (Valastro et al., 2016).

90 IBV 624I had been reported for the first time in Italy in 1993 (Capua et al., 1994, Capua et al.,
91 1999), producing disease associated with kidney lesions and drop in egg production in
92 breeders and layers (Capua et al., 1996). This genotype continued to be detected in Italy until
93 2004 and then reappeared from 2010 when it was again detected in a few broiler farms
94 affected by respiratory disease located in different areas of Italy. In the following years the
95 number of detections increased (Massi, 2013) but since 2013 the 624I genotype has not been
96 further detected (Massi et al., 2015).

97 A recent retrospective study carried out on 123 IBV strains isolated in Italy between 1963 and
98 1989 revealed that 624I genotype had not only circulated long before its first reporting in
99 1993, but that in fact it has been one of the major IBV genotypes circulating in the Country at
100 that time (Taddei et al., 2012). Evidence of the presence of this genotype have also been found
101 in Slovenia, where several 624I strains were isolated between 1991 and 1999 (Krapez et al.,
102 2010), in Poland and South Africa (Capua et al., 1999) and eventually in Russia where 624I
103 genotype was reported in 2002 (Bochkov et al., 2006).
104 The high identity observed between Q1 and 624I genotypes raise questions regarding their
105 possible related origins. IBV 624I has been hypothesized to be an ancestor of the Q1 genotype
106 (Franzo et al., 2015, Massi, 2013), but unfortunately the unavailability of any 624I full genome
107 sequence didn't allow such final conclusions to be drawn.
108 In the present study an IBV 624I was fully sequenced and phylogenetic analysis performed
109 both using a dataset based on available IBV full-length genome sequences and, due to the
110 larger number of published sequences, a dataset based on full S1 gene. In addition,
111 recombination analysis was carried out using the complete IBV 624I and Q1 strains.

112
113

114 **Materials and Methods**

115

116 **Virus**

117 IBV 624I strain was isolated in 1996 during a disease outbreak in chicken farms located in
118 Northern Italy. The virus was isolated in specific pathogen free (SPF) chicken eggs and the 3th
119 passage was propagated in SPF chicken embryo tracheal organ cultures (TOC). After isolation
120 the virus underwent serological analysis, resulting as belonging to the 624I serotype. In this
121 study this virus is named gammaCoV/AvCov/Ck/Italy/IP14425/96.

122

123 **RNA extraction, RT-PCR and sequencing**

124 The RNA was extracted from the supernatant of infected TOC using Qiamp viral RNA mini kit
125 (Qiagen, Hilden, Germany) following the manufacture's protocol. Viral RNA was firstly retro-
126 transcribed using Super Script III enzyme (Invitrogen, Carlsbad, USA) then amplified using
127 Ranger enzyme (Bioline, London, UK) according to the manufacturer's instructions. Retro-
128 transcription, amplification and sequencing were carried out using primers previously
129 designed for IBV full genome sequencing (Franzo et al., 2015, Listorti et al., 2017). Where
130 primers did not work due to sequence differences, new primers were designed based on the

131 newly determined sequences flanking those genome regions (Table 1). Sequencing was
132 performed by Source BioScience (Nottingham, UK). Each genome fragment was sequenced
133 twice. Where gammaCoV/AvCov/Ck/Italy/IP14425/96a sequence differed from those of Q1
134 strains, the locations were sequenced again, starting with a new retro-transcription of the
135 region.

136

137

138 **RT-PCR of the 3' END of genome**

139 3' end of the genome was determined using a 3'RACE protocol previously described (Laconi et
140 al., 2016). Briefly, RT was performed with a primer containing 20 Ts followed by an adaptor
141 sequence at its 5' terminus. This was amplified by PCR using 2 primers, one within the end of
142 the genome and one matching the adaptor (Table 1). These PCR products were sequenced
143 towards the polyA tail.

144

145 **Sequences analysis and comparison**

146 Chromatograms were analysed using the program Chromas
147 (<http://technelysium.com.au/wp/chromas/>) and sequences aligned using BioEdit
148 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) against the genome of IBV strains
149 gammaCoV/Ck/Italy/I2022/13 (KP780179) and CK/CH/LDL/97I (JX195177). Open
150 Reading Frame (ORF) prediction was carried out using ORFfinder program
151 (<https://www.ncbi.nlm.nih.gov/orffinder/>).

152

153 **Complete genome sequences analysis**

154 A data set containing 313 complete genome sequences (nt) of IBV was downloaded from ViPr,
155 an open source bioinformatics database and analysis resource for virology research. To
156 minimize the computational load, cd-hit-est test of the CD-HIT Suite (Li and Godzik, 2006)
157 was used to cluster sequences that shared over 98% identity and a prototype sequence within
158 each cluster was selected. After clustering, 187 representative sequences remained.
159 Sequences were aligned using ClustaW and phylogenetic analysis was carried out with
160 MEGA7 software (Kumar et al., 2016) using Maximum Likelihood method with Tamura-Nei
161 substitution model and 1,000 bootstrap replicates to assess the robustness of the branches.

162

163 **S1 gene sequences analysis**

164 A data set containing all available complete or nearly complete S1 gene sequences (at least
165 1000bp) was downloaded from ViPr. After clustering, 320 sequences remained. The
166 sequences were aligned using ClustalW method, and a phylogenetic tree was constructed
167 using the Maximum Likelihood method with Kimura 2 parameter substitution model and
168 1,000 bootstrap replicates to assign confidence level to the branches in MEGA 7 software.
169 Based on these results a subset of sequences clustering with
170 gammaCoV/AvCov/Ck/Italy/IP14425/96 was selected and expanded to its original number
171 of taxa. Sequences belonging to this dataset were aligned with ClustalW method, and a
172 phylogenetic tree was reconstructed using the parameters previously described.

173

174 **Recombination event analysis**

175 Presence of past recombination events for strain gammaCoV/AvCov/Ck/Italy/IP14425/96
176 was evaluated using RDP4 software (<http://web.cbio.uct.ac.za/~darren/rdp.html>)(Martin et
177 al., 2017). Occurrence of possible recombination events was also evaluated for the available
178 Q1 full genome sequences: gammaCoV/Ck/Italy/I2022/13 (KP780179), an Italian isolate
179 from 2013 and CK/CH/LDL/97I (JX195177), a Chinese isolate from 1997. The Kimura 2
180 parameter substitution model with a window size of 200 nucleotides and a step size of 20
181 nucleotides was used to calculate the pairwise percentage of identity between
182 gammaCoV/AvCov/Ck/Italy/ IP14425/96, the Q1 strains and 13 complete genome sequences
183 of relevant strains, selected on the previous phylogenetic analysis. Phylogenetic analysis was
184 performed for those genome portions where a sharp change in percentage of identity strongly
185 suggested recombination events using a dataset including all the sequences available for the
186 given regions.

187

188 **Results**

189

190 **Genome organization of strain gammaCoV/AvCov/Ck/Italy/ IP14425/96**

191 A consensus sequence of 27.573bp was obtained (minimum coverage 2X), with the 5' UTR
192 incomplete by approximately 100nt. The ORF analysis predicted 13 ORFs and revealed the
193 following genome organization: 5'UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-3'UTR (Table
194 2). The same genome organisation was observed for viruses gammaCoV/Ck/Italy/I2022/13,
195 CK/CH/LDL/97I and UY/09/CA/01, all belonging to the Q1 genotype (Table 2).

196

197 **Accession number**

198 Sequence of the IBV strain gammaCoV/AvCov/Ck/Italy/ IP14425/96 was submitted to the
199 GenBank database and the following accession number was assigned: MG021194.

201 **Phylogenetic analysis of full genomes of IBV strains**

202 The phylogenetic analysis of the 187 representative full IBV genome sequences demonstrated
203 that gammaCoV/AvCov/Ck/Italy/IP14425/96 clustered together with Q1 strains
204 gammaCoV/Ck/Italy/I2022/13, CK/CH/LDL/97I and UY/09/CA/01, occupying a basal
205 position in the specific cluster (Figure 1). In the same clade the Uruguayan strain
206 UY/11/CA/18 (MF421320), was also present, previously ascribed to the SAI genotype
207 (Lineage G-11) (Figure 1) (Marandino et al., 2015).

208 GammaCoV/AvCov/Ck/Italy/IP14425/96 624I strain showed the highest sequence identity
209 with the Italian Q1 strain gammaCoV/Ck/Italy/I2022/13 (*p*-distance 0.054), while the
210 percentage of identity slightly decreased when the virus was compared to the Chinese (*p*-
211 distance 0.058) and the Uruguayan (*p*-distance 0.062) Q1 strains.

213 **Phylogenetic analysis of full S1 sequences**

214 Phylogenetic analysis using a dataset characterized by 320 representative full S1 sequences
215 showed that gammaCoV/AvCov/Ck/Italy/IP14425/96 clustered together with strains
216 previously identified as 624/I genotype isolated in Italy in the late '80s and early '90s and
217 strains belonging to Q1 genotype, isolated in Italy, in China, in Taiwan and South America
218 (Figure 2A). The highest sequence identity was observed with strain 624I/94/JQ901492.1 (*p*-
219 distance = 0.036) while the identity was lower when compared to Q1 strains (data not
220 shown).

221 The subtree obtained with the expanded dataset shows 3 clades, of which one contained all
222 624/I strains and occupied a basal position with respect to the others. In the remaining two
223 clades, Q1 Italian, Chinese and some of the Taiwanese strains, cluster together, while the
224 remaining Q1 Taiwan strains and all South American Q1 strains form a distinctive
225 phylogenetic group (Figure 2B).

227 **Recombination analysis**

228 Recombination analysis was performed to assess the possible recombinant nature of the 624I
229 strain gammaCoV/AvCov/Ck/Italy/IP14425/96 and of the Q1 strains
230 gammaCoV/Ck/Italy/I2022/13 and CK/CH/LDL/97I.

Possible recombination events were identified in the 1a gene sequence of the strain CK/CH/LDL/97I with a H120 vaccine strain (FJ888351) (Figure 3A). Neither gammaCoV/Ck/Italy/I2022/13 nor gammaCoV/AvCov/Ck/Italy/IP14425/96 Q1 strains showed a similar recombination event in the 1a gene (Figures 3B and 3C).

A phylogenetic analysis was performed considering only the 1a gene, revealing that CK/CH/LDL/97I clustered with H120 and Mass strains, while gammaCoV/AvCov/Ck/Italy/IP14425/96 and gammaCoV/Ck/Italy/I2022/13 form a distinctive clade together with a QX-like Italian strain (ITA/90254/2005 - FN430414) (Figure 4).

Discussion

The genome of the IBV strain gammaCoV/AvCov/Ck/Italy/IP14425/96 isolated in Italy in 1996 was fully sequenced and this represents the first report of a full genome sequencing of a virus belonging to the 624I genotype. The isolate shows a genome organisation slightly different when compared to the genome organization of most IBVs previously reported (5'UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3'UTR) (Cavanagh, 2005), since ORF analysis showed the presence of accessory genes 4b, 4c and 6b already reported for TCoV and other IBVs (Abolnik, 2015, Hewson et al., 2011). It is not clear whether the scarcity of reports of presence of the accessory genes 4b, 4c and 6b in IBVs is due to their absence in some genomes; or whether it depends on algorithms and software used by other authors for those ORF's detections. A recent ORF analysis of the genome of the Q1 strain gammaCoV/Ck/Italy/I2022/13, (Marandino et al., 2017) didn't support the presence of ORF 6b in contrast to the results presented here. On the contrary, a recent study confirmed the expression of the 4b protein after M41 IBV infection *in vitro* (Bentley et al., 2013). IBV accessory genes 3a, 3b, 5a and 5b are known to be not necessary for viral replication, but several studies demonstrated their involvement in the pathogenicity of the virus (Kint et al., 2015a, Kint et al., 2015b, Kint et al., 2016, van Beurden et al., 2017). A similar function might be hypothesised also for genes 4b, 4c and 6b, especially in the light that the 4b homologous gene in the MERS-CoV has been reported as an antagonist of type I interferon response (Yang et al., 2013) and that the 6b homolog in SARS-CoV was shown to be able to induce apoptosis (Ye et al., 2008). More studies need to be done to improve the knowledge on these 3 accessory genes, in particular whether they are peculiar of certain genotypes and whether their expression influences the pathogenicity or the tropism of the virus.

Phylogenetic analyses performed using two different datasets, one built with IBV complete genome sequences and one built with IBV complete or nearly complete S1 gene sequences,

showed that 624I and Q1 genotypes clustered together. Our findings, strongly suggest a common origin between the two genotypes. The basal location of the 624I strain in both the phylogenetic trees, coupled with the epidemiological data available, suggests that this genotype might have played a role in the emergence of Chinese Q1. This model requires long-distance intercontinental dispersion of the 624I genotype and this possibility is supported by its ability to circulate for extended periods within the same country (Taddei et al., 2012) and beyond a geographical area (Capua et al., 1999, Krapez et al., 2010). Unfortunately there's no comprehensive model explaining the intercontinental dispersal of the 624I genotype. Some hypothesis can be proposed, such as migratory birds, illegal trading and poultry movement. Albeit speculative, these hypotheses seem to be supported by the detection of 624I genotype in Russia, which represents an intermediate position between Europe and the Far East (Bochkov et al., 2006). Further investigations are needed since, despite the IBV worldwide dissemination has been observed and accepted for other genotypes (Franzo et al., 2017), the mechanism behind this evidence is not fully understood.

The recombination analysis showed that the Chinese strain CK/CH/LDL/97I underwent recombination with a H120 vaccine strain, which has been previously demonstrated to be involved in recombination events leading to reversion to virulence and the emerge of new genotypes in China (Zhang et al., 2010). The identification of such a recombination event within the 1a gene might explain the relatively high genetic diversity between 624I strain gammaCoV/AvCov/Ck/Italy/IP14425/96 and Q1 Chinese strain CK/CH/LDL/97I, two viruses isolated only one year apart.

The absence of such recombination in the genome of Q1 Italian strain gammaCoV/Ck/Italy/I2022/13 suggests that not all Q1 strains emerged as a result of a recombination event with a H120 strain. The absence of a recombination event in the 1a gene of Q1 Italian strain gammaCoV/Ck/Italy/I2022/13 might indicate that Chinese and Italian Q1 strains are the result of independent evolutions from the 624I genotype. However, the huge differences in field conditions and therefore genetic pressures between the two countries, together with the phylogenetic results based on S1 gene sequences in which Q1 Italian strains cluster together with Chinese and Taiwanese strains, make this hypothesis highly unlikely.

Taken as a whole, the data presented in this study suggest the 624I genotype to be the ancestor of Q1.

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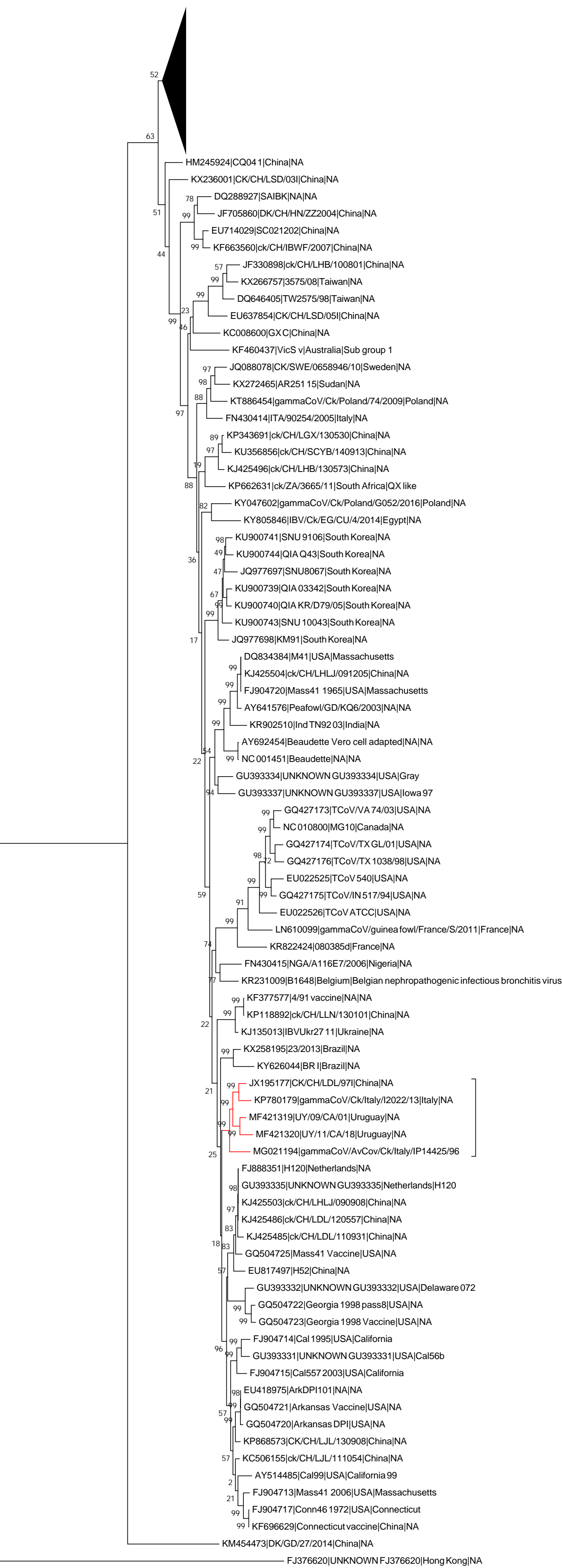
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