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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.

Introduction

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

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

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

A recent retrospective study carried out on 123 IBV strains isolated in Italy between 1963 and 1989 revealed that 624I genotype had not only circulated long before its first reporting in 1993, but that in fact it has been one of the major IBV genotypes circulating in the Country at that time (Taddei et al., 2012). Evidence of the presence of this genotype have also been found in Slovenia, where several 624I strains were isolated between 1991 and 1999 (Krapez et al., 2010), in Poland and South Africa (Capua et al., 1999) and eventually in Russia where 624I genotype was reported in 2002 (Bochkov et al., 2006).

The high identity observed between Q1 and 624I genotypes raise questions regarding their possible related origins. IBV 624I has been hypothesized to be an ancestor of the Q1 genotype (Franzo et al., 2015, Massi, 2013), but unfortunately the unavailability of any 624I full genome sequence didn't allow such final conclusions to be drawn.

In the present study an IBV 624I was fully sequenced and phylogenetic analysis performed both using a dataset based on available IBV full-length genome sequences and, due to the larger number of published sequences, a dataset based on full S1 gene. In addition, recombination analysis was carried out using the complete IBV 624I and Q1 strains.

Materials and Methods

Virus

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

RNA extraction, RT-PCR and sequencing

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

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

RT-PCR of the 3' END of genome

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

Sequences analysis and comparison

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

Complete genome sequences analysis

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

S1 gene sequences analysis

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

Recombination event analysis

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

Results

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

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

Accession number

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

Phylogenetic analysis of full genomes of IBV strains

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

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

Phylogenetic analysis of full S1 sequences

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

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

Recombination analysis

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

231 Possible recombination events were identified in the 1a gene sequence of the strain
232 CK/CH/LDL/97I with a H120 vaccine strain (FJ888351) (Figure 3A). Nor
233 gammaCoV/Ck/Italy/I2022/13 neither gammaCoV/AvCov/Ck/Italy/IP14425/96 Q1 strains
234 showed a similar recombination event in the 1a gene (Figures 3B and 3C).
235 A phylogenetic analysis was performed considering only the 1a gene, revealing that
236 CK/CH/LDL/97I clustered with H120 and Mass strains, while gammaCoV/AvCov/Ck/Italy/
237 IP14425/96 and gammaCoV/Ck/Italy/I2022/13 form a distinctive clade together with a QX-
238 like Italian strain (ITA/90254/2005 - FN430414) (Figure 4).
239
240 **Discussion**
241 The genome of the IBV strain gammaCoV/AvCov/Ck/Italy/IP14425/96 isolated in Italy in
242 1996 was fully sequenced and this represents the first report of a full genome sequencing of a
243 virus belonging to the 624I genotype. The isolate shows a genome organisation slightly
244 different when compared to the genome organization of most IBVs previously reported
245 (5'UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3'UTR) (Cavanagh, 2005), since ORF analysis showed the
246 presence of accessory genes 4b, 4c and 6b already reported for TCoV and other IBVs (Abolnik,
247 2015, Hewson et al., 2011). It is not clear whether the scarcity of reports of presence of the
248 accessory genes 4b, 4c and 6b in IBVs is due to their absence in some genomes; or whether it
249 depends on algorithms and software used by other authors for those ORF's detections. A
250 recent ORF analysis of the genome of the Q1 strain gammaCoV/Ck/Italy/I2022/13,
251 (Marandino et al., 2017) didn't support the presence of ORF 6b in contrast to the results
252 presented here. On the contrary, a recent study confirmed the expression of the 4b protein
253 after M41 IBV infection *in vitro* (Bentley et al., 2013). IBV accessory genes 3a, 3b, 5a and 5b
254 are known to be not necessary for viral replication, but several studies demonstrated their
255 involvement in the pathogenicity of the virus (Kint et al., 2015a, Kint et al., 2015b, Kint et al.,
256 2016, van Beurden et al., 2017). A similar function might be hypothesised also for genes 4b, 4c
257 and 6b, especially in the light that the 4b homologous gene in the MERS-CoV has been
258 reported as an antagonist of type I interferon response (Yang et al., 2013) and that the 6b
259 homolog in SARS-CoV was shown to be able to induce apoptosis (Ye et al., 2008). More studies
260 need to be done to improve the knowledge on these 3 accessory genes, in particular whether
261 they are peculiar of certain genotypes and whether their expression influences the
262 pathogenicity or the tropism of the virus.
263 Phylogenetic analyses performed using two different datasets, one built with IBV complete
264 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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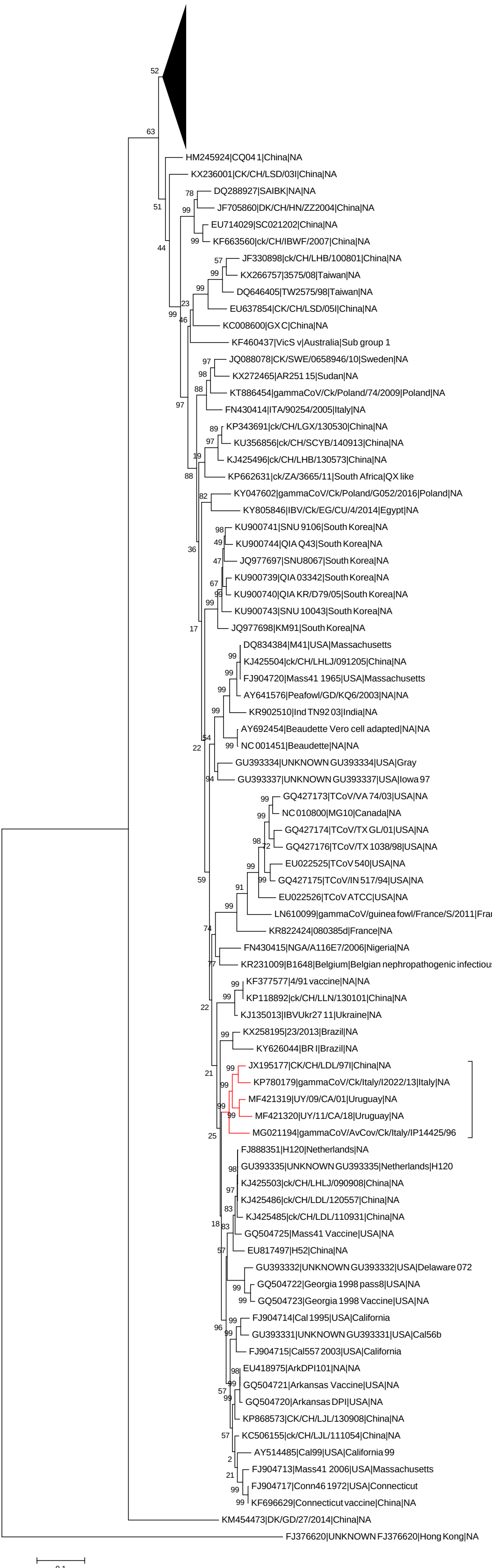
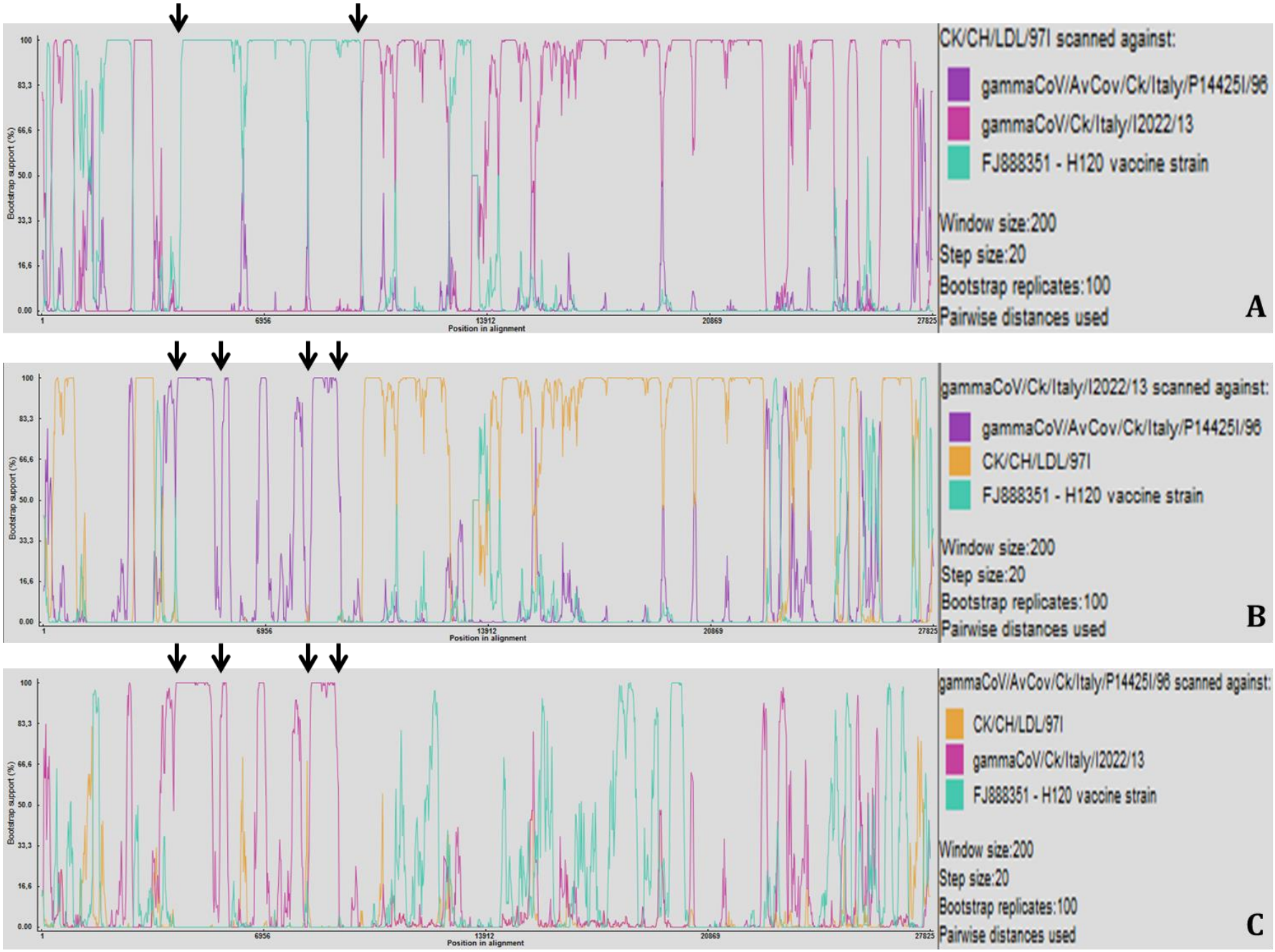

HM245924|CQ04 1|China|NA
KX236001|CK/CH/LSD/03I|China|NA
DQ288927|SAIBK|NA|NA
JF705860|DK/CH/HN/ZZ2004|China|NA
EU714029|SC021202|China|NA
KF663560|ck/CH/IBWF/2007|China|NA
JF330898|ck/CH/LHB/100801|China|NA
KX266757|3575/08|Taiwan|NA
DQ646405|TW2575/98|Taiwan|NA
EU637854|CK/CH/LSD/05I|China|NA
KC008600|GX C|China|NA
KF460437|VicS v|Australia|Sub group 1
JQ088078|CK/SWE/0658946/10|Sweden|NA
KX272465|AR251 15|Sudan|NA
KT886454|gammaCoV/Ck/Poland/74/2009|Poland|NA
FN430414|ITA/90254/2005|Italy|NA
KP343691|ck/CH/LGX/130530|China|NA
KU356856|ck/CH/SCYB/140913|China|NA
KJ425496|ck/CH/LHB/130573|China|NA
KP662631|ck/ZA/3665/11|South Africa|QX like
KY047602|gammaCoV/Ck/Poland/G052/2016|Poland|NA
KY805846|IBV/Ck/EG/CU/4/2014|Egypt|NA
KU900741|SNU 9106|South Korea|NA
KU900744|QIA Q43|South Korea|NA
JQ977697|SNU8067|South Korea|NA
KU900739|QIA 03342|South Korea|NA
KU900740|QIA KR/D79/05|South Korea|NA
KU900743|SNU 10043|South Korea|NA
JQ977698|KM91|South Korea|NA
DQ834384|M41|USA|Massachusetts
KJ425504|ck/CH/LHLJ/091205|China|NA
FJ904720|Mass41 1965|USA|Massachusetts
AY641576|Peafowl/GD/KQ6/2003|NA|NA
KR902510|Ind TN92 03|India|NA
AY692454|Beaudette Vero cell adapted|NA|NA
NC 001451|Beaudette|NA|NA
GU393334|UNKNOWN GU393334|USA|Gray
GU393337|UNKNOWN GU393337|USA|Iowa 97
GQ427173|TCoV/VA 74/03|USA|NA
NC 010800|MG10|Canada|NA
GQ427174|TCoV/TX GL/01|USA|NA
GQ427176|TCoV/TX 1038/98|USA|NA
EU022525|TCoV 540|USA|NA
GQ427175|TCoV/IN 517/94|USA|NA
EU022526|TCoV ATCC|USA|NA
LN610099|gammaCoV/guinea fowl/France/S/2011|France|NA
KR822424|080385d|France|NA
FN430415|NGA/A116E7/2006|Nigeria|NA
KR231009|B1648|Belgium|Belgian nephropathogenic infectious bronchitis virus
KF377577|4/91 vaccine|NA|NA
KP118892|ck/CH/LLN/130101|China|NA
KJ135013|IBVUkr27 11|Ukraine|NA
KX258195|23/2013|Brazil|NA
KY626044|BR I|Brazil|NA
JX195177|CK/CH/LDL/97I|China|NA
KP780179|gammaCoV/Ck/Italy/I2022/13|Italy|NA
MF421319|UY/09/CA/01|Uruguay|NA
MF421320|UY/11/CA/18|Uruguay|NA
MG021194|gammaCoV/AvCov/Ck/Italy/IP14425/96
FJ888351|H120|Netherlands|NA
GU393335|UNKNOWN GU393335|Netherlands|H120
KJ425503|ck/CH/LHLJ/090908|China|NA
KJ425486|ck/CH/LDL/120557|China|NA
KJ425485|ck/CH/LDL/110931|China|NA
GQ504725|Mass41 Vaccine|USA|NA
EU817497|H52|China|NA
GU393332|UNKNOWN GU393332|USA|Delaware 072
GQ504722|Georgia 1998 pass8|USA|NA
GQ504723|Georgia 1998 Vaccine|USA|NA
FJ904714|Cal 1995|USA|California
GU393331|UNKNOWN GU393331|USA|Cal56b
FJ904715|Cal557 2003|USA|California
EU418975|ArkDPI101|NA|NA
GQ504721|Arkansas Vaccine|USA|NA
GQ504720|Arkansas DPI|USA|NA
KP868573|CK/CH/LJL/130908|China|NA
KC506155|ck/CH/LJL/111054|China|NA
AY514485|Cal99|USA|California 99
FJ904713|Mass41 2006|USA|Massachusetts
FJ904717|Conn46 1972|USA|Connecticut
KF696629|Connecticut vaccine|China|NA
KM454473|DK/GD/27/2014|China|NA
FJ376620|UNKNOWN FJ376620|Hong Kong|NA
0,1


Bootstrap support (%)
100
83,3
66,6
50.0
33,3
16,6
0.00
1
6956
13912
20869
27825
Position in alignment
CK/CH/LDL/97I scanned against:
gammaCoV/AvCov/Ck/Italy/P14425I/96
gammaCoV/Ck/Italy/I2022/13
FJ888351 - H120 vaccine strain
Window size:200
Step size:20
Bootstrap replicates:100
Pairwise distances used
A
gammaCoV/Ck/Italy/I2022/13 scanned against:
gammaCoV/AvCov/Ck/Italy/P14425I/96
CK/CH/LDL/97I
FJ888351 - H120 vaccine strain
Window size:200
Step size:20
Bootstrap replicates:100
Pairwise distances used
B
gammaCoV/AvCov/Ck/Italy/P14425I/96 scanned against:
CK/CH/LDL/97I
gammaCoV/Ck/Italy/I2022/13
FJ888351 - H120 vaccine strain
Window size:200
Step size:20
Bootstrap replicates:100
Pairwise distances used
C