Virus Research 210 (2015) 77–80

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

# Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013

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ARTICLE INFO

*Article history:*
Received 13 May 2015
Received in revised form 24 June 2015
Accepted 8 July 2015
Available online 17 July 2015

*Keywords:*
IBV
Genotype
Phylogenesis
Recombination
Complete genome

ABSTRACT

Since 1996 a new Infectious Bronchitis virus (IBV) genotype, referred to as Q1, circulated in China and was reported for the first time in Italy in 2011, associated with an increase of mortality, kidney lesions and proventriculitis. During northern Italian outbreak of respiratory disease in a broiler flock in 2013, an IBV strain was detected by RT-PCR and characterized as Q1-like based on partial S1 sequence. The virus was isolated and named γCoV/Ck/Italy/I2022/13. All coding regions of the isolate were sequenced and compared with 130 complete genome sequences of IBV and TCoV, downloaded from ViPR. This showed the highest identity with a Chinese strain CK/CH/LDL/97I (*p*-distance = 0.044). To identify potential recombination events a complete genome SimPlot analysis was carried out which revealed the presence of possible multiple recombination events, but the minor parent strains remained unknown. A phylogenetic analysis of the complete S1 gene was performed using all complete S1 sequences available on ViPR and showed the isolate clustered with an Q1-like strain isolated in Italy in 2011 (*p*-distance = 0.004) and a group of Chinese Q1-like strains isolated from the mid 90's (*p*-distance equal or higher than 0.001). It could be hypothesized that the isolate descended from the Italian 2011 Q1-like strain or was the result of a separate introduction from China through commercial trade or migratory birds; but the data currently available does not distinguish between these possibilities.



*Infectious bronchitis virus* (IBV), belonging to family *Coronaviridae*, genus *Coronavirus*, is a positive-sense, single-stranded RNA virus of about 27.6 kb (5-UTR-1a/1ab-S-3a-3b-E-M-5a-5b-N-3 UTR) causing major economic losses in the poultry industry (Jackwood and de Wit, 2013). Currently, control strategies are mainly based on widespread use of vaccination. Nevertheless, vaccine-induced immunity is generally poorly protective between strains due to limited cross protection afforded between strains (Cook et al., 2012; Sjaak de Wit et al., 2011) and this is currently considered to result from antigenic diversity due to S1 protein variability. The viral genome can mutate rapidly through substitutions, insertions, deletions and recombinations and this results in the emergence of a large number of IBV variants, characterized by little or negligible cross protection (Jackwood et al., 2012; Thor et al., 2011). In recent times, different genotypes of apparent Asian origin, have spread to other countries and continents, sometimes with economic consequences (Sjaak de Wit et al., 2011). Different strains closely related to the proposed Q1 genotype, isolated for the first time in the mid 90's (Yu et al., 2001a,b), have more recently been described in Italy (Franzo et al., 2014; Toffan et al., 2011, 2013) in association with respiratory disease. However, little sequence information is available about this genotype despite its presence in Asia, Middle East, Europe and South America (Ababneh et al., 2012; Alvarado, 2012; de Wit et al., 2012; Huang et al., 2004; Jackwood, 2012a,b; Rimondi et al., 2009; Sesti et al., 2014a,b). This is the first report of a full genome sequence of a Q1 like isolate together with its comparison to currently available full length IBV genomes on world databases. Additionally a comparison with a broader S1 protein database, typically used for classification purposes, was performed.

To this end, 10 swabs were collected (May 2013) from 35 days old chickens showing respiratory signs, raised in a commercial broiler farm located in Northern Italy. Virus was isolated in chicken embryo tracheal organ cultures (TOC) (Cook et al., 1976). Ciliostasis observed 3 days after inoculation was taken as the indicator

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http://dx.doi.org/10.1016/j.virusres.2015.07.008
0168-1702/

of the presence of the virus. This was confirmed when RNA was extracted from the TOC medium and an IBV specific RT-Nested PCR was performed (Cavanagh et al., 1999). Virus recovered from the third passage was named γCoV/Ck/Italy/I2022/13 (proposed classification Ducatez, 2014) and used for the following steps. A two-step RT-PCR protocol was developed to amplify the full genome of IBV through several overlapping amplicons. Different sets of primers were designed using Primer3 on the basis of the sequence already published. Reverse transcription was performed with the commercial kit Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific, Carlsbad, CA) while PCR was performed using the Phusion Hot Start II High-Fidelity DNA Polymerase kit (ThermoFisher Scientific, Carlsbad, CA). Both RT and PCR phases were thoroughly optimized with respect to primers (available on request), thermal profiles and reagents, so as to achieve an acceptable final yield in absence of non-specific products. The final protocol included the following steps.: 2 μL of RNA were added to a pre-mix comprising dNTP mix (final concentration 0,5 mM), 15 pmol of primer and water up to a volume of 14,5 μL. After a denaturation step at 65 °C for 5 min the pre-mix was added with 5X RT Buffer, 20 U of Thermo Scientific RiboLock RNase Inhibitor, 20 U Maxima H Minus ReverseTranscriptase. Water was added to reach final volume of 20 μL. Reverse transcription was performed at 50 °C for 90 min and terminated through a step at 85 °C for 5 min. Several PCRs were optimized to cover all coding regions of IBV genome using overlapping amplicons of approximatively 2 kbp. Four μL of cDNA were added to a standard mix including 5X Phusion HF buffer, 200 μM of each dNTP, 0,5 μM of forward and reverse primers, and 0,02 U/μL of Phusion Hot Start II DNA polymerase. Nanopure water was added to a final volume of 50 μL Sequencing was performed at Macrogen (Macrogen Europe). After the initial activation at 98 °C for 30 s, 40 cycles were performed at 98 °C for 10 s, 60 °C for 20s and 72 °C for 150 s. Each amplicon was sequenced using 4 primers including those used for PCR plus two additional internal primers. The list of primer used for reverse transcription, PCR and sequencing is available as Supplementary material 1.

**Table 1**
Gene and protein lengths of strain γCoV/Ck/Italy/I2022/13.

| Gene | 1ab | Spike | 3a | 3b | 3c | M | 5a | 5b | N |
|---|---|---|---|---|---|---|---|---|---|
| Length nt | 19893 | 3501 | 174 | 195 | 309 | 681 | 198 | 249 | 1230 |
| Length aa | 6631 | 1167 | 58 | 65 | 103 | 227 | 66 | 83 | 410 |

All chromatograms were visually inspected using FinchTV (http://www.geospiza.com/Products/finchtv.shtml) and sequences were trimmed to remove primer contamination and bases with a phred score lower that 20 using Geneious 8.0.5 (http://www.geneious.com/). Sequences were aligned to a reference IBV-QX genotype sequence (Acc.Num. JQ088078) and consensus sequences was generated using the same software. Complete genome sequences including IBV and TCoV (130) were downloaded from ViPR (Pickett et al., 2012) and aligned using MAFFT version 7(Katoh and Standley, 2013). The substitution model was selected on the basis of Bayesian information criterion (BIC) calculated using Jmodeltest 2.1.6 (Darriba et al., 2012). A complete genome phylogenetic tree was reconstructed using the MaximumLikelihood (ML) method implemented in PhyML 3.0 (Guindon et al., 2010). A combination of Nearest neighbor interchange (NNI) and sub-tree pruning and regrafting (SPR) were selected as the tree rearrangement strategy. To evaluate robustness of the monophyly of the taxa subsets, a fast non-parametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), developed and implemented in the PhyML 3.0 (Anisimova et al., 2011), was used.

A NeighborNet network was reconstructed using SplitsTree4 v4.12.3 (Huson and Bryant, 2010). Phylogenetic network was used to display the incompatibilities and ambiguous phylogenetic signals within datasets and provided a preliminary overview on the extent of recombination phenomenon. Presence of recombination within dataset was also tested using the Phi test implemented in the same software. Possible recombinant nature of the isolated strain was also evaluated using SimPlot. Briefly, a sliding window of 300 nt

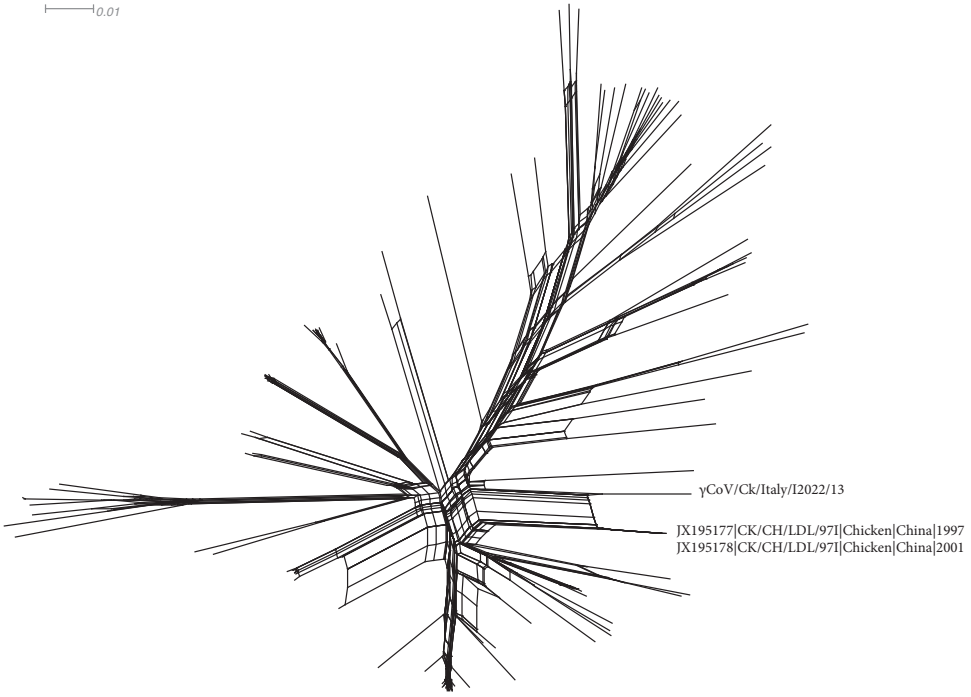


**Fig. 1.** Phylogenetic network based on NeighborNet method including 130 complete IBV and TCoV genomes. For easiness of representation, only strain reported in the present study and closely related strains (i.e., JX195177 and JX195178) are labelled.