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SHORT COMMUNICATION

Genotyping and phylogenetic analysis of infectious bronchitis virus isolated from broiler chickens in Kashmir

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Abstract Infectious bronchitis virus (IBV) is responsible for significant economic losses to the poultry industry across the world. The enormous genetic diversity of IBV poses difficulty in diagnosing and controlling the virus. To understand the nature of IBV prevalent in the Kashmir Himalayas, we characterized two field strains, isolated from non-immunized broiler chickens, by sequence and phylogenetic analysis of S1 subunit of the spike glycoprotein. The analysis revealed both the isolates are identical to each other, with nucleotide and amino acid sequence identities of 99.4% and 98.4%, respectively. They exhibit variable sequence divergence in the S1 gene to that of the reference serotypes. Both are of "Massachusetts type" belonging to GI-1 lineage of the IBV génotype. The phylogenetic analysis revealed both of the isolates clustered into the same branch as that of many IBV strains recently reported from China and Iran. Likely, these regionally-related isolates represent revertant vaccine strains which may have been disseminated across the region by wild migratory birds. This study provides the first report of molecular evidence and phylogenetic characterization of the IBV from the Kashmir Himalayas and implicate the possible role of wild migratory birds in the spread of IBV in the region.

Keywords Infectious bronchitis virus (IBV) · Spike protein · Broiler chickens · Phylogenetics · Kashmir Himalayas

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Infectious bronchitis virus (IBV) belongs to the genus gamma-coronavirus in the family *Coronaviridae* order *Nidovirales* and represents *Avian Coronavirus* (ACoVs). Its virion is enveloped, spherical, about 120 nm in diameter, and is surrounded by characteristic spike-shaped glycoprotein. The genome is monopartite, linear ss (+) RNA of approximately 27.6 kbp in size encoding four structural protein designated S (spike), M (membrane), E (envelope) and N (nucleoprotein) [3]. Additionally, two accessory genes have been described that code for non-structural proteins 3a/3b and 5a/5b [4].

IBV, like most other RNA viruses, shows a huge genetic variation due to lack of proofreading activity by viral RNA-dependent RNA polymerase. This genetic heterogeneity allows IBV to quickly adapt to selection pressures from host immune responses leading to the emergence of new variants. Further, genetic recombinations can occur between two different IBV strains at certain "hot spot" regions of the genome resulting in distinct variants. The enormous diversity of IBV has led to its classification into serotypes, protectotypes, and genotypes. Currently genotyping, which is based on the sequence similarity between S1 subunit of the spike glycoprotein, is by far the most used system. The S1 subunit forms the globular head of the spike and mediates receptor binding during the virus entry into the host cell. S1 is the major determinant of protective immunity and carry most of the virus-neutralizing epitopes

Table 1 Pairwise nucleotide and amino acid divergence (π*) between S1 gene of IBV/JKGBL strains and major serotypes of IBV

	JKGBL01	JKGBL02	MASS	Conn	Florida	Gray	JMK	Holte	Arkansas
JKGBL01		0.006	0.026	0.052	0.058	0.200	0.204	0.204	0.245
JKGBL02	0.016		0.026	0.053	0.058	0.200	0.203	0.206	0.243
MASS	0.049	0.045		0.041	0.046	0.199	0.201	0.200	0.240
Conn	0.095	0.095	0.090		0.011	0.205	0.206	0.208	0.243
Florida	0.103	0.103	0.099	0.028		0.209	0.209	0.209	0.244
Gray	0.236	0.236	0.236	0.236	0.241		0.010	0.161	0.197
JMK	0.246	0.246	0.241	0.239	0.244	0.016		0.160	0.192
Holte	0.259	0.262	0.259	0.254	0.254	0.204	0.199		0.218
Arkansas	0.272	0.267	0.272	0.262	0.267	0.211	0.196	0.249	

Nucleotide Divergence (upper triangle); Aminoacid Divergence (lower triangle)

* The value indicates the number of nucleotide or amino-acid substitutions per site between the sequences

including serotype-specific epitopes. In fact, most of the variations in the virus have been mapped to three major hypervariable regions (HVRs) located within the N-terminal region of the S1protien. These HVRs lies between the amino acid residues 56–69 (HVR-1), 117–131 (HVR-2), and 274–387 (HVR-3) [11].

The domestic poultry is considered the primary host of IBV affecting all ages. The virus also has been isolated from other species such as pheasants, quail, patridge, geese, pigeon, guinea fowl, duck and peafowl. IBV or IBV-like coronaviruses has also been detected in wild aquatic birds [7, 12, 15], implicating their role in the epidemiology of the IBV. Nevertheless, IBV has a major economical impact on commercial poultry chickens causing significant mortality, morbidity and production losses. The initial target of IBV is the epithelial cells of the respiratory tract, which is often followed by secondary infection of *Escherichia coli*, accentuating respiratory signs. Respiratory disease is most frequently observed syndrome caused by IBV in broiler chickens at 2–8 weeks of age [8]. The variations in S1 subunit can advance tissue tropism of IBV to several non-respiratory tissues, such as the gastrointestinal tract, the kidney, and the oviduct. Infection of the enteric tissues is usually not associated with clinical signs, while replication in the kidney might cause nephropathy with high mortality (up to 60%) in young chicks [2]. Oviductal infection can cause permanent damage in immature birds, and, in hens can lead to a reduction or cessation of egg-laying, production of thin-walled and misshapen shells with a loss of shell pigmentation [8].

In the present study, we report the genotypic and phylogenetic characterization of two virulent IBV strains obtained recently from outbreaks among broiler chickens in the Kashmir Himalayas (34.0333°N 74.6667°E). Currently, there is no information regarding the status of IBV among poultry in Kashmir, its epidemiology, and economic impact. Also, the IBV variants prevalent in the poultry has not been genetically characterized, which is important before contemplating the use of a vaccine against this disease.

A total of twenty-five suspected IBV cases were brought to the Division of Pathology for diagnosis between February and July 2014. The samples consisting of oral swabs from the live birds, and the trachea, kidneys and lungs from the dead ones, were collected. Total RNA was extracted from the samples using Trizol reagent (Sigma, San Diego, USA) according to the manufacturer's instructions. The RNA was converted into cDNA using Revert Aid cDNA synthesis kit (Thermofisher, USA) and screened for the presence of the IBV genome by RT-PCR using degenerate primers (5′-TGAAAACTGAACAAAAGA-3′/5′-CNGTRTTRTAYTGRCA-3′ [1]) targeting S1 subunit of the spike glycoprotein gene. Two of the twenty-five cases were detected positive for IBV in the RT-PCR. Both the cases were broiler chickens from two different poultry farms in the Ganderbal district of the Kashmir valley. In both the farms, live birds showed respiratory signs including sneezing, tracheal rales, and nasal discharge, whereas the dead birds showed respiratory tract involvement with slight hemorrhage or serous catarrhal exudates in the trachea, nasal passages, and sinuses. The birds at both the farms were 5 weeks of age and had been vaccinated against Newcastle disease and infectious bursal disease, but not against IBV.

To obtain the full-length S1 gene sequence, the samples from the two cases were subjected to RT-PCR using specific primer pair (5′-CCCAATTTGAAAACTGAACA-3′/5′-CCTACTAATTTACCACCAGA-3′ [6]). The DNA fragments of the expected size (~ 1600 bp) were amplified in the samples corresponding to the full-length S1 gene fragment (Supplementary Fig. 1). The amplicons were cloned into pGMET vector and sequenced on Applied

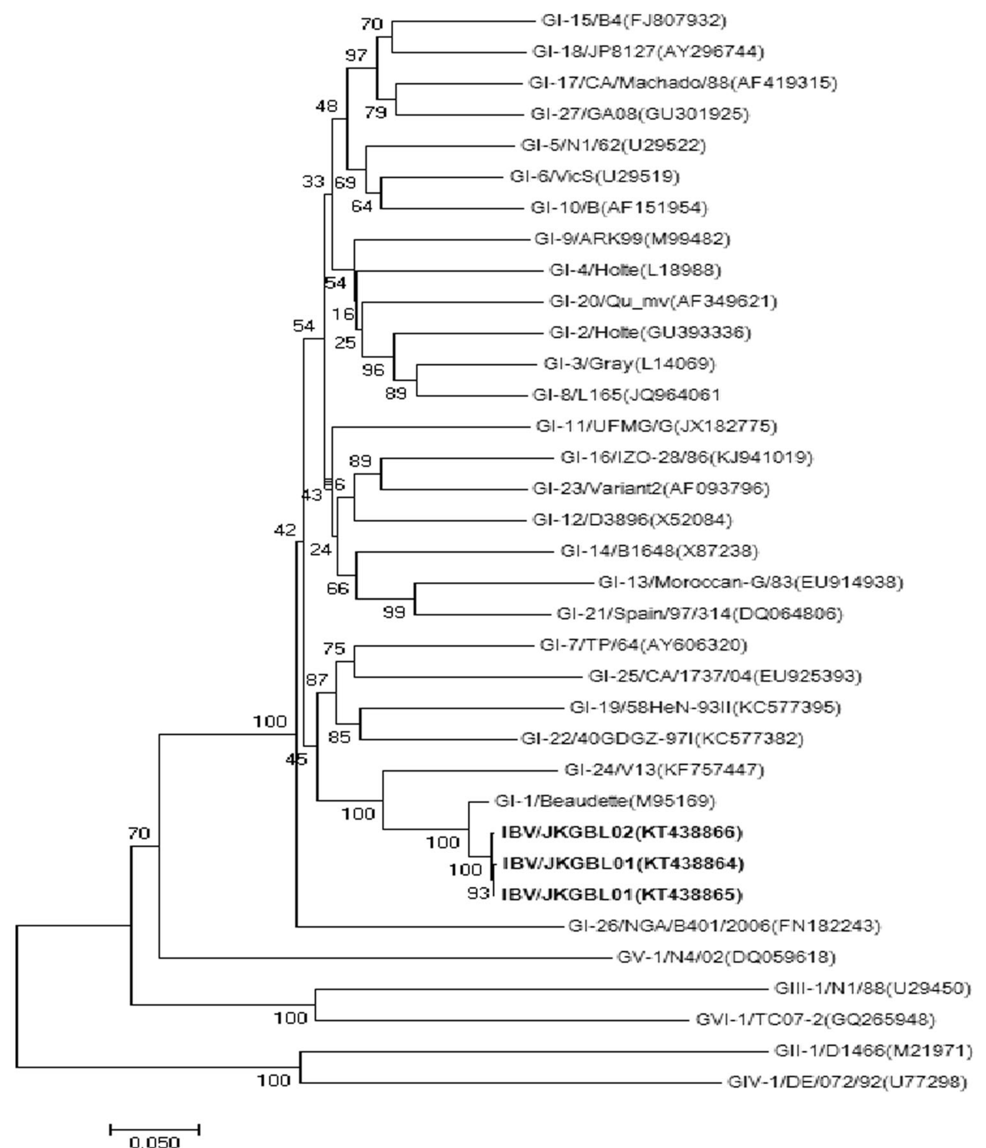

Fig. 1 Phylogenetic tree showing a relationship of IBV/JKGBL strains with the reference genotype strains. The tree analysis was obtained from the whole S1 sequences (1593 bp) of the two IBV/JKGBL strains along with the 32 prototype strains retrieved from the GenBank. Both the JKGBL01 and JKGBL02 clustered with sequence belonging to the Beaudette (accession number M95169) representing genotype I lineage 1 (GI-1). The sequences were aligned with Clustal W and the tree was build using MEGA7 software. The prototype strains are designated by the genotypes and lineages followed by the strain name and GenBank accession number in parenthesis as described by Valstro et al. [16]. The isolates sequenced in this study are highlighted in bold. The scale of the bar represents genetic divergence i.e. the number of nucleotide substitutions per site

Biosystems 3730xl DNA Analyzer at the Center for Cellular and Molecular Biology (CCMB), Hyderabad, India. The S1 gene sequences were submitted to GenBank and assigned the accession numbers KT438864 and KT4388645 for gammaCoV/chicken/IBV/Jammu and Kashmir/Ganderbal/2014/01 (IBV/JKGBL01) and KT438866 for gammaCoV/chicken/IBV/Jammu and Kashmir/Ganderbal/2014/02 (IBV/JKGBL02). The two strains, IBV/JKGBL01 and IBV/JKGBL02, were identical up to 99.4% (Divergence, $\pi = 0.006$) at the nucleotide level to each other. There were nucleotide substitutions at 8 positions that all led to the amino-acid changes in the translated S1 protein; i.e. the changes were non-synonymous. The predicted amino-acid differences between the two strains was 98.4% ($\pi = 0.016$) and these changes were both mapped within and outside of the 3 HVRs reported in the S1 protein (Supplementary Table 1).

Genotyping of IBV based on S1-gene sequence strongly correlates to their serotype and is used more frequently for its convenience as compared to the serotyping. However, in view of inconsistencies reported in genetic typing based on partial S1 gene sequences [9], we analyzed the two strains based on the whole S1 gene sequence. The S1 sequence comparison with the standard seven serotypes of IBV revealed that the IBV/JKGBL strains show the least divergence in amino acid and nucleotide sequence with the Mass serotype and maximum divergence with the Arkansas serotype (Table 1). Mass serotype is usually reported to cause respiratory signs [5] and we also observed both the JKGBL strains were mainly associated with the respiratory disease in the chickens.

Recently, a new coherent classification scheme for IBV strains based on the S1gene phylogeny has been proposed [16]. Thus, we retrieved the complete S1 gene of 32

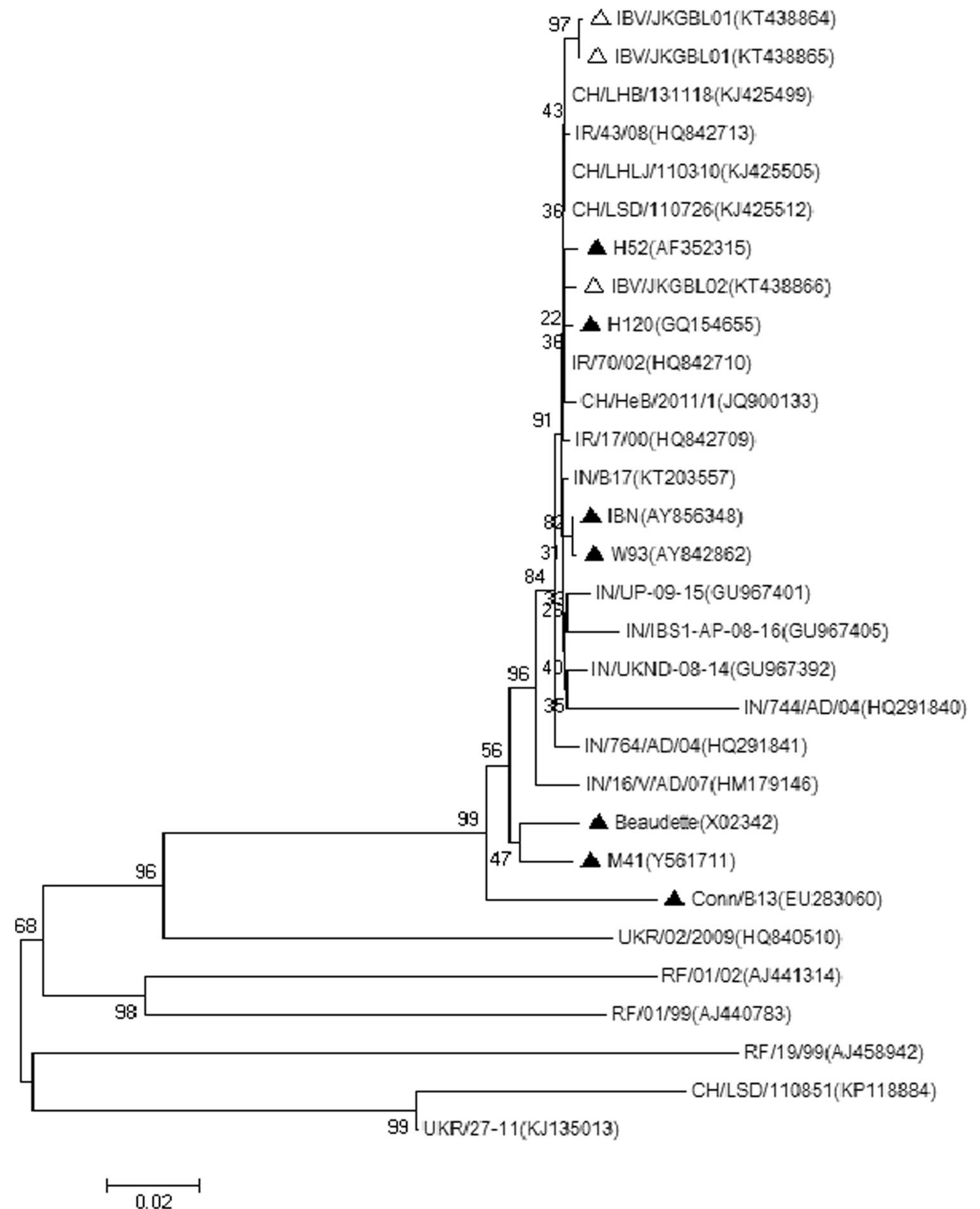

Fig. 2 Phylogenetic tree showing relationship of IBV/JKGBL strains with the regional and vaccine strains. The tree shows the relationships between the S1 gene sequence of two IBV/JKGBL (Δ) strains and the IBV strains from Asian–Indian migratory bird flyway including India (IN), Iran (IR), China (CH), Russian Federation (RF) and Ukraine (UKR) and the vaccine strains (shaded triangle) used in the region. The IBV/JKGBL strains clustered with the strains recently reported from China and Iran. The IBV/JKGBL strains show maximum identity with H120 and H52 strains, both of which are the 'Massachusetts type' vaccine strains. The tree was constructed by the neighbor-joining method with 1000 bootstrap replicates in MEGA7 software. The regional strains were designated by the country of origin, and the strain name as in Genbank and acession number in paranthesis. The vaccine strains were designated by the strain name and GenBank accession number in paranthesis. The scale of the bar denotes the number of nucleotode subustutions per site

prototype strains and subjected them to analysis along with the S1 gene sequences of two IBV/JKGBL strains using the Neighbor-Joining method [13] in the MEGA7 software suite. The analysis revealed that both of the strains belong to Genotype I lineage 1 (GI-1) of IBV (Fig. 1). The GI-1 lineage represents most widely distributed genotype throughout the world, more likely due to the extensive use of homologous vaccine strains. As the IBV vaccines are live attenuated, there are chances of pathogenic reversion due to back passaging if the vaccines are not applied properly. Although both the JKGBL strains originated from non-vaccinated broiler flocks, there are chances that they are revertant strains which may have originally evolved from the live attenuated vaccine strains used in the region.

Blast search of the S1 sequences of the two IBV/JKGBL strains showed their maximum identity with the IBV strains recently reported from China. Thus, we retrieved complete S1 sequences of the IBV strains reported from surrounding countries including China, India, Iran, Ukraine and Russian Federation, and subjected them to the phylogenetic analysis along with our strains. The analysis also included the common vaccine strains used in the region. The analysis revealed that the JKGBL01 and JKGBL02 strains are more closely related to the IBV strains reported from China and Iran (Fig. 2). The analysis also revealed that the two strains were more closely related to the "Mass type" vaccine strains, in particular H120 and H52. Since there is the least possibility of domestic birds moving between Kashmir

valley, China, and Iran due to the presence of long stretch of Himalayan Mountains and non-existent trade links, we hypothesize the role of wild migratory birds in the dissemination of the IBV strain across the region. The Kashmir valley forms a part of the Asian-migratory flyway with major birding sites at Hygam, Hokersar, Shalibug and Mirgund wetland reservoirs. The major migratory birds prevalent in Kashmir include Brahminy Duck, Tufted Duck, Gadwall, Garganey, Greylag Goose, Mallard, Common Merganser, Northern Pintail, Common Pochard, Ferruginous Pochard, Red-Crested Pochard, Ruddy Shelduck, Northern Shoveler, Common Teal, and Eurasian Wigeon. These migratory birds usually arrive in Kashmir each year during September–October from Siberia, Central Asia, China and Indian sub-continent and leave back during March–April. The role of migratory birds in the dissemination of IBV has been highlighted in a study by Huges et al. [7] wherein they show avian coronavirus isolated from healthy migratory birds in England have a high degree of genetic identity with the IBV H120 vaccine strain. Similarly, coronaviruses sharing close proximity to the IBV H120 vaccine strain have also been detected in healthy, unvaccinated, domestic peafowl and as well as wild peafowl in China [10, 14]. In Kashmir, live and inactivated Mass type vaccines based on H120 strains are extensively used by poultry farmers for the control of IBV. The two JKGBL strains showed close proximity to the IBV H120 vaccine strain and therefore may represent a revertant attenuated vaccine strains that may have arisen as a result of the widespread use of IBV vaccines in the local poultry populations and have been further disseminated across the region by the wild migratory birds. To better understand the potential role of the wild migratory birds in the transmission, dissemination, and evolution of IBV strains, the surveillance of coronaviruses in the wild migratory birds across the region is warranted.

Altogether, the present study provides first molecular evidence of the Mass type IBV strains circulating among the poultry population in the Kashmir Himalayas, that share a genetic proximity to the recently isolated Chinese and Iranian strains. Most likely the strains may be revertant vaccine strains that may have arisen in the local poultry population, and may have been disseminated across the region by wild migratory birds. In the future, the role of these migratory birds in the evolution and transmission of IBV, needs a thorough investigation.

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