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Original Article

Emergence of variant avian infectious bronchitis virus in India

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Abstract

Background: Infectious bronchitis virus (IBV) is the etiological agent of an acute and highly contagious disease. Infectious bronchitis (IB) affects chicken of all ages and poses major economic loses to the poultry industry worldwide. The continuous evolution of the spike protein (S1) of IBV is responsible for the prevalence of many serotypes/genotypes around the world. Multiple lineages of IBV strains have been detected in chicken flocks in India since 2003. **Aims:** To detect IBV genotypes prevalent in India. **Methods:** Organ samples from 20 IBV-positive flocks with variable clinical signs were used for the amplification of the *S1* gene of IBV by reverse transcriptase-polymerase chain reaction (RT-PCR). **Results:** Positive PCR amplicons were sequenced. Sequence analysis showed that 14 field isolates belonged to the GI-1 genetic lineage (Mass 41 serotype), two field isolates belonged to the GI-13 (UK 4/91 variant IBV strain), one field isolate grouped with GIII, GV, and GVI genetic lineage and three belonged to a variant genotype unique to India (GI-24). Phylogenetic analysis also showed a similar type of grouping within the field isolates. Among the fourteen GI-1 isolates, 12 were isolated between 2003 and 2006 and only two were isolated between 2009 and 2011. The two field isolates belonging to GI-13 were isolated in 2007, another one belonging to GIII, GV, and GVI was isolated in 2010 and three field isolates were not close to any reference IBV sequences isolated in 2006 (IND-TN-168-06), 2010 (IND-TN-280-10) and 2011 (IND-TN-290-11). **Conclusion:** A unique variant of IBV is emerging in India (GI-24). Our findings will have important implications for future vaccine intervention.

Key words: Infectious bronchitis virus, Phylogeny, Sequence, *Spike protein 1* gene, Variant infectious bronchitis virus

Introduction

Infectious bronchitis (IB) is one of the most common diseases of chicken and difficult to control. It is caused by infectious bronchitis virus (IBV) which belongs to family Coronaviridae, genus Gamacoronavirus (Cavanagh and Naqi, 2003; International Committee on Toxonomy of Viruses). Infectious bronchitis virus is primarily a respiratory pathogen but it can also replicate in tissues such as the kidney, gonads and alimentary tract and is characterized by respiratory signs, nephritis and reduced egg production. The infectious bronchitis virus can spread through air or by mechanical transmission between birds, houses and farms. Infectious bronchitis virus exists in multiple serotypes due to frequent point mutations and recombinations (Cavanagh *et al*., 1992; Cavanagh *et al*., 1997). Immunizing chicken with one serotype has shown to induce partial or no protection against other serotypes. Hence, genotyping field IBV isolates is an important process to help recognize the circulating IBV genotype and evaluate current vaccination programs.

The genome of IBV consists of a single-stranded positive sense RNA of 27.6 kb (Boursnell *et al*., 1987) that codes for three major structural proteins, the spike (S), membrane (M), and the nucleocapsid (N) protein. The spike protein (S1) is post - translationally cleaved into two subunits, S1 and S2 (Cavanagh, 1983). Spike protein glycoprotein is responsible for generating neutralizing and serotype-specific antibodies (Koch *et al*., 1990; Kant *et al*., 1992).

Infectious bronchitis virus *S1* gene sequences comprise of three hypervariable (HVR) regions, HVR I (amino acid residues 38-67), HVR II (amino acid residues 97-141), and HVR III (amino acid residues 274-387). It has been reported that the sequences of HVRs of the *S1* gene correlate well with virus neutralization tests and can be used for genotyping IBV isolates (Kusters *et al*., 1989; Moore *et al*., 1997; Lee *et al*., 2003). Therefore, genotyping IBV field isolates based on HVRs of the *S1* gene would be a useful tool in determining the epidemiology of field IBV and thus predicting the efficacy of vaccines against field isolates.

In India, until recently, the most common form of IBV was the respiratory form of Mass 41 (Sukumar and Prabakar, 1993; Kumanan *et al*., 2003; Sureshkumar *et*

al., 2007). However, Elankumaran *et al*. (1999) reported serological evidence for the presence of the IBV variant 793/B, but failed to isolate the variant virus. Bayry *et al*. (2005) reported the presence of a single isolate of nephropathogenic IBV (PDRC/Pune/Ind/1/00) in India, isolated in 1999. Subsequently, Gaba *et al*. (2010) and Sumi *et al*. (2012) carried out virus isolation and genotyping and reported the presence of a single isolate of 793/B, a variant IBV. Recent studies showed that the field isolates belonged to the Mass 41 genotype (Patel *et al*., 2015; Parveen *et al*., 2017; Jakhesara *et al*., 2018). In India, in general, layer birds and broiler breeder birds are vaccinated with H120 vaccines during the 1st week, live attenuated vaccine (H120) in their 12th week, and IB killed (Mass 41) during their laying period. Commercial broiler birds are given a live attenuated vaccine (H120) during the first week of age. Despite the use of H120, IB problems are commonly found in vaccinated chickens. However, IBV genotyping studies using a large number of samples collected from chickens with different clinical manifestations and different geographical locations have not been carried out in India. This study highlights the results of IBV genotyping performed in India on samples collected during a 9-year period, from 2003-2011. Other Indian IBV S1 sequences available in the GenBank since 2011 were also analyzed in order to discover the molecular nature of the IBV prevalent in India.

Materials and Methods

Samples

Three hundred and eleven samples of trachea and kidneys of chickens from three major poultry housing states of India, Tamil Nadu, Andhra Pradesh and Karnataka, showing signs suggestive of IB were collected between 2003 and 2011 and used for this study. Among the 311 samples, 246 samples were kidney samples and 65 were tracheal samples. Most of the samples were from Tamil Nadu [181 - Kidneys (156) and Trachea (25)], followed by Andhra Pradesh [74 - Kidneys (52) and Trachea (22)] and Karnataka [56 - Kidneys (38) and Trachea (18)]. These samples were from vaccinated broiler birds (105 flocks), layers (180 flocks) and breeders (5 flocks). Details of 20 IBV positive samples are given in Table 1.

Sample preparation and isolation of IBV

Trachea and kidneys were homogenized using sterile Mortar and Pestle with liquid nitrogen and prepared in a 20% suspension in nuclease free phosphate buffered saline (PBS) (pH = 7.2) with antibiotics (10,000 IU/penicillin, 10,000 mg/ml streptomycin). Sample suspensions were centrifuged at 12,000 rpm (16128 × g) for 10 min at 4°C and the supernatant was filtered through 0.22 µm sterile syringe filters. Infectious bronchitis virus was isolated from these samples by inoculating into the allantoic cavity of 9-10 day-old specific pathogen free embryonated chicken eggs (SPF-ECE). After 5 to 6 passages in ECE, allantoic fluids were used to extract RNA to determine the presence of IBV by reverse transcriptase-polymerase chain reaction (RT-PCR). Allantoic fluids were confirmed negative for Newcastle disease virus (NDV) by RT-PCR and infectious laryngotracheitis virus by PCR.

RNA isolation and RT-PCR assay

Allantoic fluids of each sample were used to extract Total RNA using Trizol LS reagent (Invitrogen, USA) as per manufacturer's instructions. RNA was converted into complementary DNA (cDNA) using a Revert-Aid cDNA synthesis kit (Fermentas, USA) with random hexa primers. The synthesized cDNA was used to amplify the HVR regions of *S1* gene by RT-PCR using the forward primer 5´- TGG TTG GCA TTT ACA CGG GG-3´ (Wang and Tsai, 1996) and reverse primer 5´ -CTC GAA TTC C NGT RTT TRA YTG RCA-3´ (Keeler *et al*.,

Table 1: Details of IBV field isolates used in this study

S. No.	Year of isolation	Isolate No.	Type of bird	Age in weeks	Type of sample collected	Clinical signs	Accession number
1	2003	Ind/113/03	CB	40 wks	Trachea	Respiratory distress	JX966392
2	2003	Ind/114/03	CB	40 wks	Trachea	Respiratory distress	EF165593
3	2003	Ind/TN/04/03	CB	4 wks	Kidney	Nephritis, pale kidneys	EF165596
4	2003	Ind/TN/20/03	CL	36 wks	Kidney	Pale and Enlarged kidneys	EF165597
5	2003	Ind/TN/92/03	CL	26 wks	Kidney	Nephritis	EF165598
6	2003	Ind/TN/95/03	CL	31 wks	Trachea	Tracheal congestion, respiratory distress	EF165599
7	2003	Ind/TN/97/03	CL	42 wks	Trachea and cloacal swabs	Drop in egg production	EF165600
8	2003	Ind/TN/98/03	CL	36 wks	Kidneys	Pale kidneys with urate deposits	EF165601
9	2005	Ind/AP/151/05	BB	3 wks	Kidneys	Pale kidneys	JX966393
10	2005	Ind/KA/152/05	BB	3 wks	Kidneys	Nephritis, gout	EF165595
11	2006	Ind/TN/162/06	CL	3 wks	Kidneys	Nephritis	JX966394
12	2006	Ind/TN/163/06	CL	2 wks	Kidneys	Pale kidneys	JX966395
13	2006	Ind/TN/168/06	CL	3 wks	Kidneys	Pale kidneys	JX966396
14	2007	Ind/TN/174/07	CL	14 wks	Kidneys	Gout	JX966397
15	2007	Ind/TN/175/07	CL	25 wks	Kidneys	Gout	JX966398
16	2009	Ind/TN/183/09	CL	32 wks	Trachea	Respiratory signs	JX966399
17	2009	Ind/TN/270/09	CL	28 wks	Trachea	Respiratory signs	JX966400
18	2010	Ind/TN/280/10	CL	3 wks	Kidneys	Nephritis	JX966401
19	2010	Ind/TN/284/10	CL	3 wks	Kidneys	Nephritis	JX966402
20	2011	Ind/TN/290/11	CL	3 wks	Kidneys	Nephritis	JX966403

CL: Commercial layers, CB: Commercial broilers, and BB: Broiler breeders

1998) which covers HVR regions I and II from nucleotide position 114 to 708 (Beaudette strain Accession number M95169). Reverse transcriptase-polymerase chain reaction was carried out in Eppendorf Thermal cycler using AmpliTaq Gold fast PCR master mix (ThermoFisher, USA) with the following cycling conditions: initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s; annealing at 54°C for 30 s and extension at 72°C for 45 s and a final extension of 72°C for 10 min. The amplified RT-PCR products (600 bp) were analyzed by electrophoresis using 2% agarose gel and visualized using BioRad gel documentation system.

Sequencing of *S1* gene of IBV

Reverse transcriptase-polymerase chain reaction products were purified using AuPreP gel extraction kit (M/s. Life Technologies, USA) as per the manufacturer's instruction. Sequencing of the PCR products with forward and reverse primers was done in the Center for Genomic Services (TCGS), New Delhi, India using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

S1 gene sequence analysis

The sequence data were aligned by MegAlign program (DNASTAR) using the Clustal W multiple sequence alignment algorithm. *S1* gene sequences of major variant IBVs circulating in the world and other major vaccine strains were included in the multiple sequence alignment (Table 2), and nucleotide and amino acid similarities were determined using DNASTAR. Multiple sequence alignment of *S1* gene sequences was also performed using the ClustalX program to construct a phylogenetic tree using the neighbor-joining (NJ) approach with bootstrap values of 1000 replicates and a p-distance model using, MEGA 7.0.

Accession numbers of nucleotide sequences

The nucleotide sequence data from the HVR region of 20 IBV isolates reported here were submitted to GenBank, and were assigned the accession numbers shown in Table 1.

Results

Isolation and identification of IBV

Two hundred and ninety flocks of poultry suspected for IBV were tested for the presence of IBV by RT-PCR from 2003 to 2011. Most of these cases were from commercial layer birds with a history of nephritis and very few with respiratory problems. The epidemiological

Table 2: Reference sequences used in this study

Strain	GenBank accession number	Strain	GenBank accession number
Australia	AY775779	IND-HBL1310	AB861538
Australia-VicS	KF931628	IND-IB257	KF809781
Belgium-B1648	KR231009	IND-IB422	KF809791
China-AH3	JQ900137	IND-MZ1	KX832218
China-HC1312	KX107748	IND-PDRC	AY091551
China-JL1312	KX107768	IND-IB236	KF809778
China-LX4	AY189157	IND-IB238	KF809779
China-NTP	KX107792	IND-IBV470	KF809795
China-NX1401	KX107803	IND-MZ2	KX832219
China-SCMS4	JF951377	IND-MZ3	KX832220
China-SCZJ2	KX721498	IND-21-B-2008	KF360981
Egypt-F03	DQ487085	IND-V24	KF757450
Europe-H120	KR605489	IND-V39	KF757456
Israel-Variant	EU780077	IND-23-B-2008	KF360983
Italy-IT02	AJ457137	IND-IBV422	KF809791
Europe-D274	X15832	IND-415	KF809790
Pak	KX013102	IND-IB-MZ-1	KX832218
Taiwan	DQ646405	IND-IBV238	KF809779
UK-4-91	JN192154	IND-IBV236	KF809778
USA-Ark99	L10384	IND-IB-MZ-3	KX832220
USA-Cal99	AY942738	IND-IB-MZ-2	KX832219
USA-Connecticut	L18990	IND-HBL-IB13	AB861538
USA-M41	AY561711	IND-IBV257	KF809781
IND-IB-MZ6	KX832223	IND-IBV506	KF809796
IND-V17	KF757449	IND-V57	KF757464
IND-V6	KF757440	IND-IBV398	KF809789
IND-V7	KF757441	IND-IBV270	KF809783
IND-V62	KF757469	IND-GE1	KF757465
IND-V65	KF757472	IND-GE2	KF757466
IND-V64	KF757471	IND-IB-MZ5	KX832222
IND-V63	KF757470	IND-IB-MZ4	KX832221
IND-V28	KF757454	IND-HBL-13	AB86260
IND-V27	KF757453		

information regarding the field isolates used in this study is presented in Table 1. All layer birds and broiler breeder birds were vaccinated with two doses of live attenuated Mass 41 vaccine with or without Newcastle disease (ND) vaccine. Commercial broiler birds received a single vaccine. Successful isolation of IBV required at least 5 passages. The embryonic lesions suggestive of IBV such as dwarfing and curling of toes were not observed for all the isolates. Regardless of embryonic lesions, allontoic fluids from all the isolates were tested for IBV by RT-PCR.

Sequence analysis of *S1* gene of IBV

Of the 311 samples analyzed, 20 were positive (6.4%) by RT-PCR and all the RT-PCR products were sequenced. Sequences of 20 field isolates were compared with the reference *S1* gene sequences from GenBank (Table 2). Infectious bronchitis virus sequence genetic lineage for field isolates were assigned as suggested by Valastro *et al*. (2016). Nucleotide sequence and amino acid sequence similarities of field isolates are given with the reference sequences in Table 3. Sequence analysis showed that 14 isolates belonged to the GI-1 lineage (Mass 41 genotype). The nucleotide sequence identity percentage of field isolates was between 85.0% to 96.2%, for the Mass 41 reference sequence (AY561711). For the H120 vaccine, these 14 field isolates had nucleotide sequence identities between 85.8% to 100%. The field isolate, IND-TN-183-09 had 100% nucleotide and amino acid identities with the H120 vaccine sequence. Two field isolates, IND-TN-174-07 and IND-TND-175-07 had 95.6% and 97.4% nucleotide sequence identities with the UK 4/91 variant IBV strain (GI-13 lineage) and IND-TN-284-10 had a 92% nucleotide sequence identity with the Indian nephropathogenic IBV (AY091151). Three field isolates, IND-TN-168-06, IND-TN-270-09 and IND-TN-290-11 had nucleotide sequence identities from 65.7% to 77.8% with Mass 41 genotype (AY561711) and nucleotide sequence identities of 70.9% to 79.6% with the H120 vaccine. The analysis of IBV genetic lineage and year of isolation showed that between 2003 and 2006, 12 out of 14 GI-1s were detected, whereas all six variant IBVs were detected from 2006 to 2011 (Table 4) (Fig. 1).

Phylogenetic tree analysis of 20 field isolates showed that 14 field isolates, IND-113-03, IND-114-03, IND-TN-04-03, IND-TN-20-03, IND-TN-92-03, IND-TN-95-03, IND-TN-97-03, IND-TN-98-03, IND-AP-151-05, IND-KA-152-05, IND-TN-162-06, IND-TN-163-06, IND-TN-270-09, IND-TN-183-09 belonged to GI-1 genetic lineage (Mass 41 genotype), two field isolates, IND-TN-174-07 and IND-TN-175-07 belonged to GI-13 genetic lineage (UK 4/91 genotype), one field isolate, IND-284-10 belonged to GIII-1, GV-1, and GVI-1 genetic lineage (Indian nephropathogenic IBV) and three field isolates, IND-TN-168-06, IND-TN-280-10, IND-TN-290-11 belonged to GI-24 lineage (Fig. 2). A phylogenetic tree was also constructed using the *S1* gene sequences of IBV isolates from India, isolated after 2011. Most of these isolates belonged to the GI-24 genetic lineage and indicated an emergence of a unique variant IBV in India (GI-24) (Fig. 2).

Table 3: Nucleotide and amino acid identity percentage of *S1* gene sequences of Indian isolates with the reference IBV strains. First line of each row represents nucleotide identities and second line represents amino acid identities of the field isolate with the corresponding reference sequence

Reference virus/Field virus	IND-113-03	IND-114-03	IND-TN-04-03	IND-TN-20-03	IND-TN-92-03	IND-TN-95-03	IND-TN-97-03	IND-TN-98-03	IND-AP-151-05	IND-KA-152-05	IND-TN-162-06	IND-TN-163-06	IND-TN-168-06	IND-TN-174-07	IND-TN-175-07	IND-TN-183-09	IND-TN-270-09	IND-TN-280-10	IND-TN-284-10	IND-TN-290-11
Aus-Vic	68.3	67.9	67.8	67.6	68.8	67.4	68.5	68.5	68.1	68.1	61.5	67.9	76.9	36.3	37.0	71.1	76.4	42.5	72.5	71.6
KF931628	60.3	59.6	53.4	58.6	59.3	56.9	59.6	59.6	57.6	57.6	62.5	60.0	68.1	20.5	19.2	63.0	54.5	30.1	59.1	58.3
Belg-B1648	36.8	37.7	34.6	35.3	37.3	36.6	37.7	37.7	39.1	39.1	36.3	37.6	34.8	84.8	85.1	36.7	37.8	42.9	70.5	69.9
KR231009	21.3	22.0	20.0	21.3	22.6	21.7	22.0	22.0	23.0	23.0	22.7	21.0	21.6	83.8	83.9	21.3	27.8	30.1	57.6	58.1
China-LX4	73.2	73.9	67.8	72.4	74.2	72.8	74.5	74.5	74.1	74.1	66.8	72.2	71.6	38.6	38.9	75.3	77.0	41.6	74.5	69.0
AY189157	63.8	64.9	58.6	63.8	64.4	62.1	64.9	64.9	64.4	64.4	65.0	67.3	61.1	20.5	16.4	67.1	64.6	28.8	66.7	55.6
Europe-D274	70.0	69.6	66.1	68.1	69.9	70.7	70.2	70.2	70.3	70.3	63.7	68.5	72.1	38.3	37.7	73.2	76.1	46.1	74.0	70.0
X15832	58.6	57.9	55.2	56.9	57.6	55.2	57.9	57.9	59.3	59.3	62.5	60.0	62.5	17.8	23.3	64.4	64.6	27.4	69.5	60.0
Europe-H120	**96.2**	**96.2**	**91.3**	**93.5**	**96.8**	**95.7**	**96.7**	**96.7**	**97.3**	**97.3**	**96.3**	**96.3**	79.6	36.3	36.9	**100**	**98.3**	43.4	74.0	73.5
KR605489	**91.4**	**89.5**	**84.5**	**87.9**	**91.5**	**86.2**	**89.5**	**89.5**	**93.2**	**93.2**	**92.5**	**92.7**	75.0	19.2	19.2	**100**	**88.3**	37.0	71.0	66.7
IND-PDRC	74.0	73.3	71.3	73.9	75.9	75.0	76.1	76.1	76.3	76.3	41.8	76.8	39.3	38.2	38.6	79.5	74.4	47.9	**92.0**	34.7
AY091151	66.1	63.8	62.1	63.8	66.1	61.0	63.8	63.8	66.1	66.1	45.2	66.1	12.5	20.5	21.9	75.3	62.0	43.8	**84.8**	16.4
Israel-Variant	71.3	69.3	62.9	72.7	71.8	71.0	71.6	71.6	71.8	71.8	74.7	72.1	36.1	33.6	34.9	74.0	79.9	42.9	73.0	58.1
EU780077	62.1	61.4	58.6	65.3	62.7	60.3	61.4	61.4	62.7	62.7	70.0	63.6	18.9	20.5	21.9	65.8	63.3	31.5	65.2	56.9
Italy-IT02	68.4	66.5	63.5	70.5	72.7	68.8	68.8	68.8	75.7	75.7	71.9	69.7	36.6	85.2	86.0	70.8	72.8	44.3	69.7	62.9
AJ457137	62.1	61.4	58.6	63.9	68.1	60.3	61.4	61.4	62.7	62.7	70.0	63.6	16.2	86.6	87.4	64.4	60.8	30.1	63.6	61.1
Taiwan	76.0	76.6	72.1	74.1	76.3	75.5	77.2	77.2	76.8	76.8	68.6	74.3	76.9	35.8	36.6	77.5	76.1	46.6	73.5	71.2
DQ646405	73.7	73.2	64.9	76.4	71.8	70.2	75.0	75.0	74.1	74.1	43.1	75.9	73.2	19.4	19.2	76.4	64.6	23.0	68.2	64.8
UK-4-91	36.8	37.7	34.6	35.3	37.3	36.6	37.7	37.7	38.6	38.6	35.8	35.5	37.5	**95.6**	**97.4**	36.3	78.4	37.7	74.0	59.5
JN192154	21.3	20.3	18.3	19.7	22.6	20.0	20.3	20.3	19.7	19.7	22.7	17.7	23.0	**93.0**	**94.4**	18.7	64.6	27.4	66.7	50.5
USA-Ark99	63.9	64.1	60.7	65.7	65.1	63.6	64.7	64.7	66.5	66.5	68.7	64.2	70.2	34.5	34.8	69.6	71.1	42.0	68.0	66.1
L10384	58.6	56.1	53.4	55.2	59.3	53.4	56.1	56.1	59.3	59.3	60.0	58.2	65.3	21.9	21.9	64.4	57.0	28.8	57.6	58.3
USA-Cal99	66.7	66.8	62.8	65.4	67.7	66.3	67.4	67.4	69.7	69.7	71.4	68.4	72.0	35.0	36.6	71.4	68.9	42.5	70.0	70.3
AY942738	62.1	61.4	58.6	60.3	62.7	58.6	61.4	61.4	62.7	62.7	62.5	63.6	63.9	20.5	21.9	67.1	58.2	27.4	60.6	56.9
USA-M41	**96.2**	**95.7**	**90.7**	**94.1**	**96.2**	**96.2**	**96.2**	**96.2**	**95.7**	**95.7**	**95.9**	**95.2**	77.8	34.6	38.6	**95.6**	**95.3**	43.8	71.5	69.3
AY561711	**89.7**	**87.7**	**82.8**	**86.2**	**88.1**	**84.5**	**87.7**	**87.7**	**89.8**	**89.8**	**92.5**	**90.9**	68.1	19.2	17.8	**91.8**	**81.8**	13.7	60.6	59.7

Table 4: Genotyping of Indian isolates based on *S1* gene sequences

Genotype	Year of isolation	Isolates
Mass 41 IBV	2003	IND-113-03
	2003	IND-114-03
	2003	IND-TN-04-03
	2003	IND-TN-20-03
	2003	IND-TN-92-03
	2003	IND-TN-95-03
	2003	IND-TN-97-03
	2003	IND-TN-98-03
	2005	IND-AP-151-05
	2005	IND-KA-152-05
	2006	IND-TN-162-06
	2006	IND-TN-163-06
	2009	IND-TN-270-09
	2009	IND-TN-183-09
UK 4/91 IBV	2007	IND-TN-174-07
	2007	IND-TN-175-07
Indian nephropathogenic IBV	2010	IND-TN-284-10
Variant IBV	2006	IND-TN-168-06
	2010	IND-TN-280-10
	2011	IND-TN-290-11

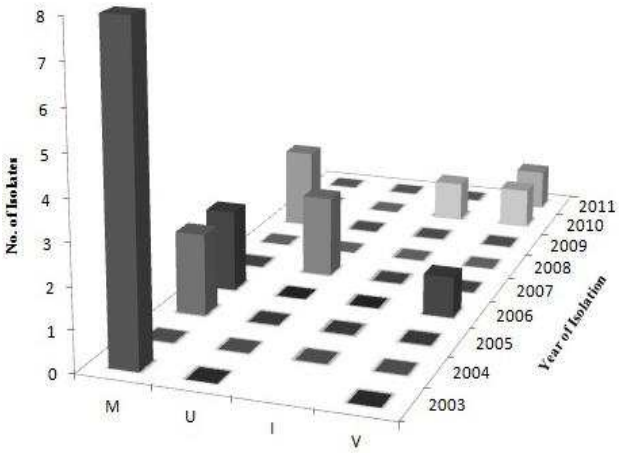

Fig. 1: Distribution of IBV genotypes during the period of 2003-2011. M: Mass 41 IBV, U: UK 4/91 IBV, I: Indian nephropathogenic IBV, and V: Variant IBV

Discussion

In this report, we analyzed 20 field isolates of IBV from India from 2003 to 2011 using RT-PCR and sequencing HVR I and HVR II of the *S1* gene. Based on the following observations from the present study, from the 20 isolates studied, 3 can be considered as new IBV genotypes. First, nucleotide sequence analysis of HVR regions of *S1* gene sequences of field isolates with reference strains showed that IND-TN-168-06, IND-TN-280-10, and IND-TN-290-11 isolates did not belong to the variants reported previously (Bande *et al*., 2017) but rather belonged to the GI-24 genetic lineage (Valastro *et al*., 2016), which is unique to India. Second, phylogenetic analysis confirmed that these three isolates did not group with the vaccine sequences or variant IBVs. Third, subsequent reports of GenBank nucleotide sequences of IBV from India clearly showed an emergence of a unique IBV variant in India (GI-24).

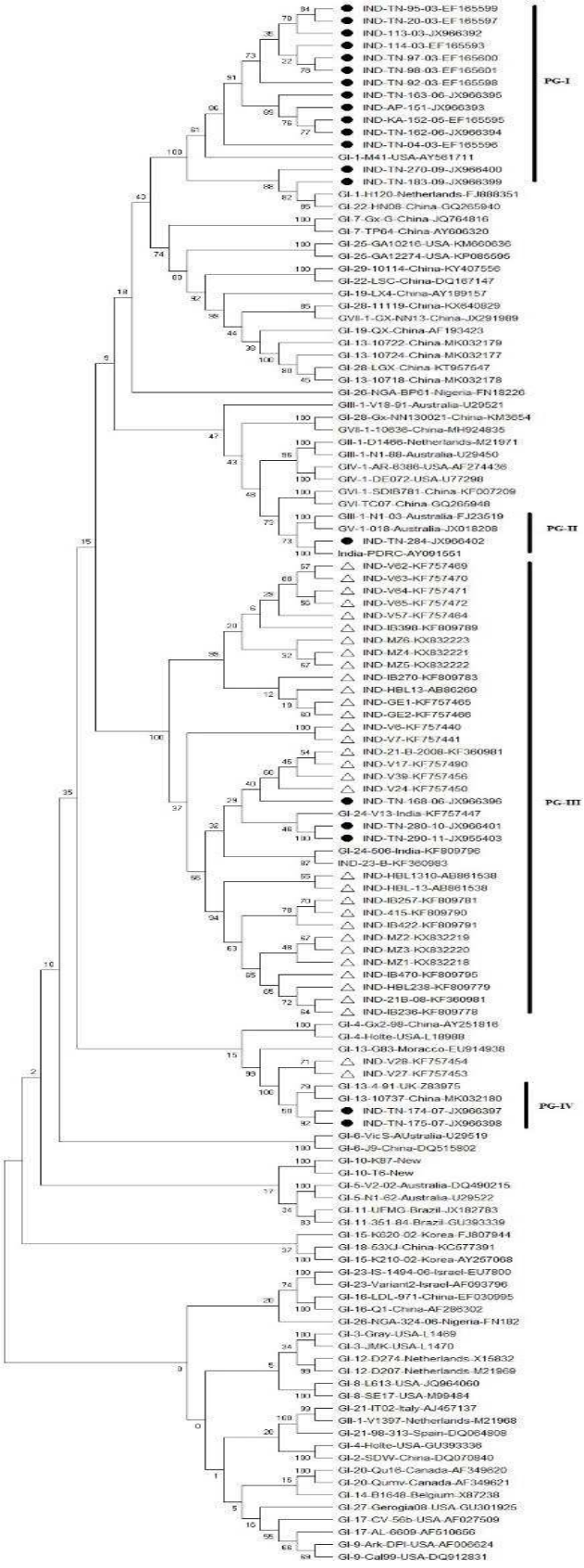

Fig. 2: Phylogenetic tree constructed by the neighbor-joining method with 1000 boot strap values (Mega 7.0) for the field isolates collected during the period of 2003-2011 (•) and for the field isolates from India available in GenBank during the period of 2011-2016 (Δ)

For majority of poultry flocks in India, IB control strategy is based mainly on the use of attenuated live Mass 41 strain, H120 vaccine. However, despite extensive vaccination, the number of IBV-suspected cases was large. Most gout cases, even in chickens less than a week old were suspected to be IB; however,, such cases were negative upon analysis, indicating other causes (Sályi, 1999) for these birds.

To date, only stray reports on the presence of IBV variants are seen in the Indian literature (Bayry *et al*., 2005; Gaba *et al*., 2010; Sumi *et al*., 2012). Although the number of genotyped samples is few, a clear pattern seems to be emerging. IBV in the early years of 2003-2006 was predominantly Mass 41 type. Earlier reports of IBV isolations in these years also revealed only the Mass type (Kumanan *et al*., 2003; Suresh Kumar *et al*., 2005). As seen in this study (n=7) and reports by Gaba *et al*. (2010) (n=1) and Sumi *et al*. (2012) (n=2), variant viruses seem to be emerging in India since 2006.

These observations confirm that one or more genotypes of variant IBV are circulating in Indian farms and are more likely to diversify further. This could likely result in vaccination failures if only Mass type vaccines are used. The emergence of new IBV genotypes or serotypes (Ducatez *et al*., 2009; Lim *et al*., 2011; Mahmooda *et al*., 2011) along with the poor cross-protection observed among IBV serotypes have complicated IB control programs (Cavanagh, 2007). Moreover, the prevalence of IBV in Asian countries shows the presence of genotypes like QX, LX4, Mass 41, 4/91, IS1494, D274 and some local and regional IBV genotypes (Bande *et al*., 2017; Lin and Chen, 2017; Sadri *et al*., 2018).

The IBV isolates used in this study were isolated from the major poultry rearing areas of India where outbreaks of IBV have been increasing annually. Our results support the idea that the vaccine presently in use is not protecting the birds against these field variant IBVs. Since the S1 of IBV is related to host range and cell tropism (Kuo *et al*., 2000; Casais *et al*., 2003), alterations in the pathogenic and antigenic characteristics of IBV are expected (Wang *et al*., 1994). Moreover, the use of live attenuated vaccines could produce new variant IBVs by recombining with field isolates resulting from the spread of vaccine strains (Farsang *et al*., 2002; Chen *et al*., 2009).

The present study indicates an emergence of a unique variant IBV in India (GI-24). Moreover, the observations indicate that these variant IBVs might increase the genetic diversity of IBV and hamper IB control in India. Hence a continuous IBV isolation and genotyping program is recommended, not only for the study of virus evolution but also for effective modifications of vaccination programs (Cavanagh *et al*., 2005). It may also be necessary to ponder the need for inclusion of new variant IBV strains for vaccine preparation to ensure a broader spectrum of protection against IBV infection.

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