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ORIGINAL ARTICLE

Isolation and molecular characterization of nephropathic infectious bronchitis virus isolates of Gujarat state, India

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Abstract Infectious bronchitis (IB) is a common, highly contagious, acute, and economically important viral disease of chickens caused by Infectious bronchitis virus (IBV, sp. *Avian coronavirus*). Five pooled tissue suspensions of 50 layer birds and one reference Massachusetts vaccine strain were inoculated into specific pathogen free (SPF) chicken egg for isolation of IBV. Reverse-transcription polymerase chain reaction (RT-PCR) was carried out using post inoculated allontoic fluid to amplify the spike (S) glycoprotein of S1 subunit of IBV. All the eggs inoculated with five pooled tissue samples and vaccine sample showed dwarfing and curling of SPF embryos indicative of IBV. All the five samples and the vaccine sample produced the expected amplicons of 466 bp by RT-PCR. The sequencing of five isolates revealed that all the five sequences were 99.09–100 % similar among themselves and showed 99.10–100 % nucleotide identity with the vaccine strain. On multiple sequence alignment it was found that our isolates were more similar at S1 subunit nucleotide level with the reference Ma5 and H120 vaccine strains than the reference Mass41 strain. The sequences of Anand isolates revealed further genetic changes in the circulating IBV in comparison to previous isolate of Gujarat as well as higher differences with the strains isolated in other states showing substantial changes at genetic level in Indian IBV isolates, which may partially explain the increasing incidences of IB in the country in spite of the vaccination.

Keywords Infectious bronchitis virus · S1 subunit · RT-PCR · Sequencing

Introduction

Infectious bronchitis is a highly contagious disease of respiratory and urogenital tract of chickens which causes major economic losses to the global poultry industry. Infectious bronchitis virus (IBV, sp. *Avian coronavirus*) belongs to the order *Nidovirales*, family *Coronaviridae*, and genus *Gammacoronavirus.* In India, vaccination against IB is carried out using Massachusetts 41 (Ma41) strain. Different IBV variants have emerged causing nephropathogenic and reproductive problems which infected the vaccinated flocks and hence it demands a dramatic change in vaccination programmes. IB is known to be one of the major contagious diseases of the respiratory and urogenital tract of chickens [7, 13]. IBV is a very dynamic and evolving virus, causing major economic losses to the global poultry industry [1].

One or more serotypes can be endemic in regions with intensive poultry production [4]. IBV was first recognized as avian respiratory pathogen in 1930, after that many IBV vaccines were introduced to tackle this problem {H52, H120, M41, 4/91(793/B) and other strains}. Recently, different IBV variants have emerged causing nephropathogenic and reproductive problems which demands a dramatic change in vaccination programmes [17]. IB vaccine has been produced

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by passage of the virus in embryonated eggs. The main barriers to producing recombinant vaccines against IBV appear to be reproduction of the conformationally dependent epitopes on the spike glycoprotein that induces neutralizing antibodies and a protective local immune response in the upper respiratory tract of the bird [8].

In India, nephropathic form of IB usually goes un-noticed because it is confused with metabolic visceral gout. Some IBV strains are intrinsically nephropathogenic i.e. they reproducibly cause nephritis when inoculated experimentally into SPF chickens, causing mortality [14]. IBV infects mainly the lower nephron down to the collecting duct epithelial cells [6].

The spike (S) glycoprotein gene is composed of S1 and S2 subunits and it is the most variable gene in the IBV genome. Spike subunit 1 (S1) is about 1644 nucleotide (nt) in length. S1 subunit is an ideal target to type strain by RT-PCR and sequencing because of its high variability which can differ from 20 to 25 % and even up to 50 % in amino acid sequence among other serotype of IBV [5].

In spite of vaccination to the poultry, there was a sporadic outbreak of IB in the Gujarat state, India. Thus, the present study was undertaken to isolate, identify and characterize the nephropathogenic strains of IB causing severe mortality in the poultry farm of Anand, Gujarat state, India.

Materials and method

Sample

Total 50 dead layer birds (25–35 weeks old) were examined by necropsy during April 2013 to May 2013 for suspected IBV infection showing gross lesions like swollen and pale kidneys with distended tubules, distended ureters with urate deposition at Department of Pathology, College of Veterinary Science & Animal Husbandry, AAU, Anand. All the birds were vaccinated with IB vaccine which contains Massachusetts type strain. Tissues (trachea, kidney and lung) from ten birds were pooled and processed as single sample. Total five pooled samples were prepared and labeled as ANDGUJIBV1, ANDGUJIBV2, ANDGUJIBV3, ANDGUJIBV4 and ANDGUJIBV5. All the five pooled samples were filtered using 0.45 µm syringe milipore membrane filter. Filtered suspensions were stored at −20 °C and used for inoculation in SPF embryonated eggs.

Reference strain of IBV

Avian infectious bronchitis live vaccine Massachusetts type strain obtained from the Ventri Biologicals, Vaccine Division, Venketshwara Hatcheries Pvt. Ltd., Pune, was used and labeled ANDGUJIBV-vac as the reference virus.

Virus isolation

All the five pooled tissue homogenates were inoculated in 200 µl volumes into the allantoic cavity of embryonated SPF eggs of 10 days old and then incubated at 37 °C. IB vaccine virus strain and PBS were also inoculated in 200 µl volume as positive and negative controls respectively. Allantoic fluids were harvested at 96 h post inoculation. Three successive blind serial passages were performed. The allantoic fluids were harvested and stored at −20 °C.

Detection of IBV using one step RT-PCR

The RNA for RT-PCR was extracted from the allantoic fluid using QiaAmp viral RNA mini kit (Quagen, Germany, cat no 52904, Lot no 142358315) according to the manufacturer's instructions. RT-PCR was carried out using primer sets XCE2-F CACTGGTAATTTTTCAGATGG and XCE2-R CCTCTATAAACACCCTTGCA targeting the spike glycoprotein of S1 subunit of IBV [2]. RT-PCR was performed using Qiagen one step RT-PCR Kit (Qiagen, Germany, cat no. 210210, Lot no 145017312). The RT-PCR reaction was carried out in 200 µl PCR tube containing 25 µl reaction mixture comprises of 5 µl Qiagen OneStep RT-PCR Buffer (5×), 1 µl of dNTP mix (10mMol), 1.5 µl of each primer (15 pmol), 1 µl of Qiagen OneStep RT-PCR enzyme mix, 10 µl of RNA template (30 ng/ul) and 5 µl of RNAse free water. The RT-PCR reactions were performed in thermocycler (Veriti Thermal Cycler, Applied Bioscience, USA) with following cycles; reverse transcription 50 °C for 30 min, initial denaturation of 94 °C for 10 min followed by 30 cycles of denaturation, annealing and extension at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 1 min, respectively, and the final extension was carried out at 72 °C for 10 min.

To confirm the targeted PCR amplification, 5 µl of the PCR products from each tube was mixed with 1 µl of 6X gel loading buffer and electrophoresed along with 100 bp DNA molecular weight marker (GeneRuler, MBI Fermentas) on 2.0 % agarose gel containing ethidium bromide (at the rate of 0.5 µg/ml) at constant 80 V for 30 min in 0.5× TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

Sequencing and sequence analysis

Five pooled samples along with vaccine positive control, each containing 50 µl of PCR product at a concentration of 200 ng/µl was out sourced to the Xcelris Labs Limited, Ahmedabad, Gujarat for direct sequencing by Sanger sequencing method. Sequences were aligned with each other

using seqScape v 5.2.0 sequence analysis software. The sequence similarity search with the DNA public Database available at GeneBank, maintained by NCBI, was carried out with the help of Basic Local Alignment Search Tools-BLAST (http://www.ncbi.nlm.nih.gov/blast). For further comparison, nucleotide sequences of three reference vaccine virus strains (Ma5, H120 and Mass41, with Accession Nos. AY561713, KF188436 and AY851295 respectively) were retrieved from the GeneBank database. Sequences were compiled and analyzed using BioEdit software [10]. All the nucleotide sequences were aligned for multiple sequence analysis using Clustal W program. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 6 [18]. For the comparative sequence analysis and phylogenetic studies, Anand isolates and vaccine virus were compared with sequences of spike glycoprotein of S1 subunit of IBV available in the GeneBank of NCBI. A total of 16 strains/isolates with their gene accession numbers were used for sequence analysis and phylogenetic study (Table 1).

Results

Virus isolation

Five pooled samples, one vaccine virus sample and one negative control (PBS solution) were inoculated in seven different SPF embryonated eggs. During first 24 h of inoculation, egg embryos were alive, however the embryos died after 72–96 h post inoculation after the third passage, except in the negative control. All the five pooled field samples and the vaccine virus showed gross signs in embryos i.e., dwarfing and curling, which was absent in the negative control (Fig. 1).

Detection by RT-PCR

All the five field samples and the vaccine virus produced the expected amplicons of 466 bp by RT-PCR, whereas no amplification was observed in negative control (Fig. 2).

Sequencing of IBV

Length of the nucleotide sequences for ANDGUJIBV1, ANDGUJIBV2, ANDGUJIBV3, ANDGUJIBV4, ANDG UJIBV5, and ANDGUJIBV-vac were 444, 440, 447, 439, 441, and 442 bp respectively. Five nucleotide sequences of spike glycoprotein of S1 subunit fragment of Anand IBV isolates and that of the vaccine were submitted to GenBank of NCBI database. The accession number was designated as KJ577258 (ANDGUJIBV1); KJ577259 (ANDGUJI BV2); KJ577260 (ANDGUJIBV3); KJ577261 (ANDGU JIBV4); KJ577262 (ANDGUJIBV5) and KJ577263 (ANDGUJIBV-vac).

Phylogenetic analysis of partial S1 subunit

Phylogenetic analysis was performed with the reported sequences of nine Indian IBV isolates and seven foreign isolates available in GenBank which revealed five distinct groupings (clusters) with Indian and abroad isolates appearing to be on the same phylogenetic scale with that of Anand IBV isolates. Cluster I consisted of all five Anand isolates and vaccine virus. Cluster II was made up of Andhra Pradesh (M41), Andhra-Pradesh (H120), Europe (Ma5) and USA (Mass41). Cluster III was made up of isolates from Gujarat (hbl 15/09), Tamilnadu (India/NMK/72/IVRI/10), UK (4/91 pathogenic), China (CK/CH/Chongqing/0909) and Australia (Australia-T). Cluster IV

Table 1 List of published IBV sequences and their accession numbers used for phylogenetic analysis

Sr. no.	Geographical origin and year	GenBank accession no.	Isolate/strain
1	Gujarat (2009)	AB538873	hbl 15/09
2	Maharashtra (2004)	AY091551	PDRC/Pune/India/9/99
3	Uttar Pradesh (2010)	HQ291840	India-744-AD-04
4	Tamilnadu (2010)	HM748585	India/NMK/72/IVRI/10
5	Andhra Pradesh (2009)	GQ219712	M41
6	Andhra Pradesh (2013)	KF188436	H120
7	Orissa (2010)	GU967409	IBS1_ORS_08_9
8	Punjab (2010)	GU967396	IBS1_PUB_08_25
9	Assam (2010)	GU967391	IBS1_ASM_08_12
10	UK (2001)	AF093794	4-91 pathogenic(793/B) serotype)
11	USA (2001)	AF363590	VA/7996/99(Arkansas)
12	Europe (2004)	AY561713	Ma5 (Mass)
13	USA (2001)	AF363591	MS/8306/99 (Connecticut)
14	Australia (2004)	AY775779	Australia-T
15	China (2011)	GU938414	CK/CH/Chongqing/0909
16	USA (2006)	AY851295	Mass41

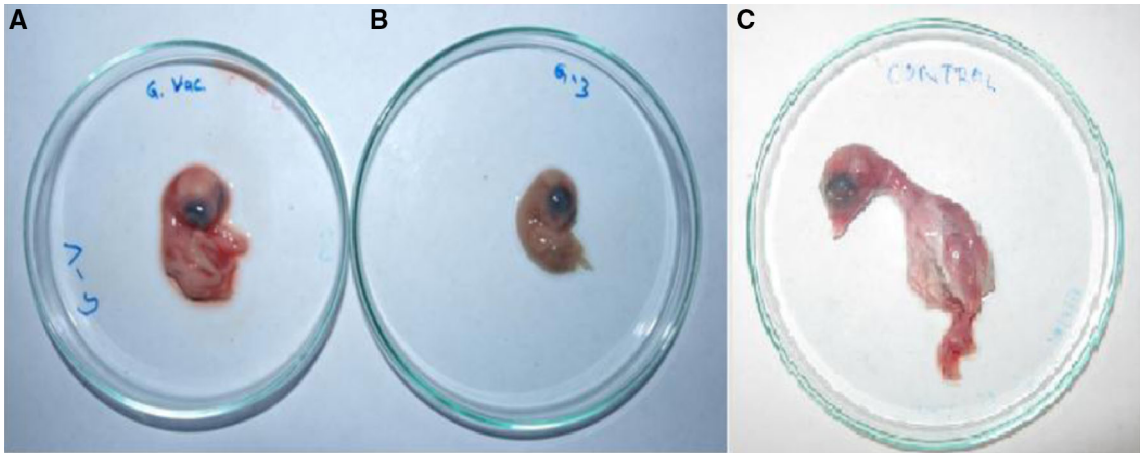

Fig. 1 Embryo showing dwarfing and curling of SPF egg embryos suggestive of IBV. **A** Vaccine virus, **B** suspected tissue sample; **C** negative control (PBS solution)

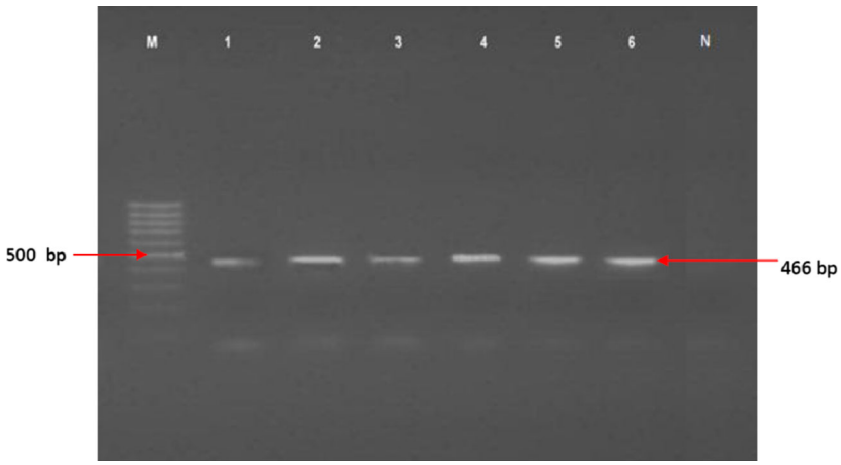

Fig. 2 Agarose gel electrophoresis of IBV S1 gene specific RT-PCR products of 466 bp. *Lane -M* ladder-Molecular weight marker (100 bp), *1–5* Field Isolates; *6* Positive control; *N* Negative control

included isolates from Orissa (IBS1_ORS_08_9), Assam (IBS1_ASM_08_12) and Punjab (IBS1_PUB_08_25). Cluster V consisted of Maharashtra (PDRC/Pune/India/9/99) and USA strains (VA/7996/99-Arkansas, MS/8306/99- Connecticut). Uttar Pradesh (India-744-AD-04) isolate remained out grouped from other five clusters. Present study shows that Anand isolates were 99.32–99.55 % similar with the strains M41 and H120 and 62.61–63.18 % similar with PDRC/Pune/Ind/1/00 isolate, while least percent nucleotide identity was found with the strains MS/8306/99 (Connecticut) and VA7996/99 (Arkansas) which was 53.43 and 55.71 % respectively. This indicates that Anand isolates were of Massachusetts type (Fig. 3).

Discussion

IB is one of the most economically significant diseases of the intensive poultry industry in the world. Vaccination programmes rely mainly on the use of the Massachusetts strain, which is the most commonly used IBV vaccine strain in India; however, presence of the disease in vaccinated chickens is commonly observed, causing a major economic impact all over the world [12]. There is no detailed information available on the genetic makeup of any of the IBV strains used in the commercial vaccines. Since the IBV vaccines or the vaccine strains used in Indian poultry population are usually imported from abroad, it is always advisable to use an indigenous IBV vaccine strain as the immuno-prophylactic agent against prevailing field strains.

Isolation of IBV by passaging the tissue samples in embryonated SPF eggs is a simple method. Dwarfing and curling signs were suggestive of presence of IBV [3, 16]. RT-PCR technique is increasingly being used to identify the spike glycoprotein genotype of IBV field strains and found to be very specific and sensitive for the detection of IBV [9, 11, 15, 16].

The sequencing of five field samples (Anand isolates) revealed that all the five sequences were 99.09–100 %

similar among themselves. Sequences of five samples compared with the vaccine sample, indicated 99.10–100 % similarity. The sequence alignment of Ma5, H120 and Mass41 reference strains with Anand isolates exhibited 97.75–99.55 % identity. The sequence alignment of Anand isolates with two references (Ma5 and H120) vaccine strains revealed 99.32–99.55 % identity and with Mass41 strain, lower identity of 97.75–97.96 % (Table 2).

The deduced amino acid sequences of Anand isolates and vaccine virus were also aligned using the ClustalW programme. The amino acid sequences of the Anand isolates and vaccine virus showed no variation. Multiple amino acid sequence alignment revealed that all the strains under comparison had similar amino acid sequences except the reference vaccine virus strain Mass41, which showed I (isoleucine) and S (serine) at position 246 and 379, while all the other strains including Anand isolates showed T (threonine) and L (leucine) at the same positions. This again substantiates the earlier observation that our isolates were more similar to the reference vaccine strains Ma5 and H120 than Mass41 strain. The nucleotide/aminoacid differences between IBV Anand isolates and other reported sequences were based on the Clustal Alignment of the Nucleotide sequences of S1 subunit fragment of Anand isolates, vaccine strain and reference strains as mentioned in Table 3. The three reference vaccine strains i.e. Ma 5, H120 and Mass41 showed SNVs at positions 708, 1143 and 1146 with 'C', 'T' and 'A' respectively, while the Anand isolates including the vaccine strain showed 'T', 'C' and 'G' at the same positions. Mass41 (AY851295) showed additional seven SNVs at positions 737, 846, 861, 1017, 1104, 1119 and 1136 with T, T, T, C, T, T, and C nucleotide respectively, while all other strains showed C, A, C, T, C, C and T at the respective positions. Thus, Mass41 strain showed maximum variation of ten nucleotides at different positions, which also showed the least percent nucleotide identity with our isolates. This indicates that our isolates were more similar at S1 subunit nucleotide level with the reference Ma5 and H120 vaccine strains than the reference Mass41 strain (Table 3).

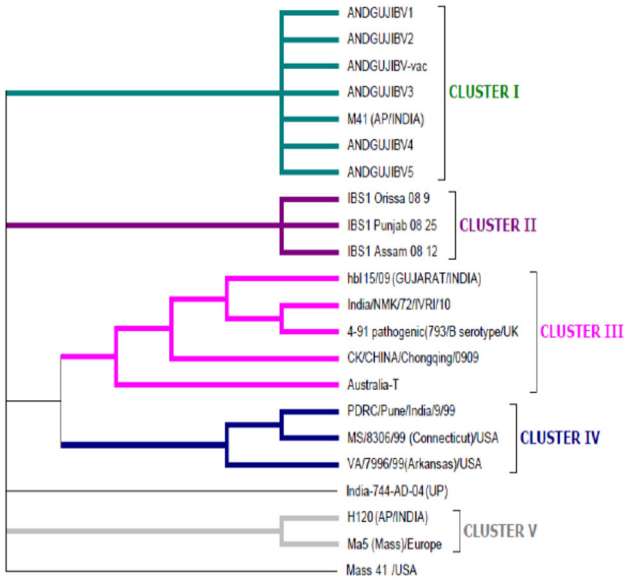

Fig. 3 Unrooted Phylogenetic tree showing relationship between Anand IBV isolates and other sequences of IBV based on nucleotide sequences of S1 region of IBV

Phylogenetic analysis revealed that the Anand isolates and the reference M41 (Massachusetts type) strain grouped into the same cluster I and thus showed higher genetic similarity. However, the Anand isolates were of genetic distance with the Arkansas and Connecticut reference strains, which grouped into cluster IV. Nephropathogenic reference isolates also formed well defined separate clusters II and III with nucleotide identity of 63.27–69.59 and 77.59–87.05 % respectively with Anand isolates. Grouping of Anand isolates in a separate cluster I at a genetic distance with the nephropathogenic strains further indicate the fast emergence of variants in IBV. The Anand isolates showed similarities in the range of 77.59–78.08 % with earlier reported isolate of Gujarat and 78.64–79.19 % with the Tamil Nadu (India/NMK/72/IVRI/10) isolate. The sequence of Gujarat IBV isolate was reported in the year 2009 and the lower similarity as observed in the current

Table 2 Percentage nucleotide and amino acid identity amongst the IBV isolates/strains under comparison

IBV isolates/strains	Percentage nucleotide identity (% nt), nucleotide differences (d), percentage amino acid identity (% A) and amino acid differences (D) between IBV Anand isolates with vaccine and reference strains			
	% nt	d	% A	D
Vaccine	100	0	100	0
Reference Ma5 (Mass)	99.32–99.55	3	100	0
Reference H120	99.32–99.55	3	100	0
Reference Mass41	97.75–97.96	10	98	2

Table 3 Nucleotide and amino acid changes in spike glycoprotein of S1 subunit of Anand isolates, vaccine sample and reference strains of IBV

Position	Anand isolates	Vaccine sample	Ma5	H120	Mass41
Nucleotides					
708	T	T	C	C	C
737	C	C	C	C	T
846	A	A	A	A	T
861	C	C	C	C	T
1017	T	T	T	T	C
1104	C	C	C	C	T
1119	C	C	C	C	T
1136	T	T	T	T	C
1143	C	C	T	T	T
1146	G	G	A	A	A
Amino acids					
246	T	T	T	T	I
379	L	L	L	L	S

study indicates further genetic changes in the circulating IBV in Gujarat, which may partially explain the increasing incidences of IB in the country in spite of the vaccination programme.

Unifying the overall findings, the present study has given an insight into the ever-changing genetic makeup of IBV, and probably addresses the IBV vaccine failures in field conditions. The results of present study revealed that different strain variants of IBV may be circulating among chicken populations in India and recent outbreaks of nephropathogenic IB were due to this reason. The finding of IBV strains in field samples and its crucial genetic distance with other reported IBV isolates demands a continuous scientific effort in the country to monitor and characterize the circulating IBV so as to get rid off this important disease menace faced by poultry population.

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