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BIOTECHNOLOGY

Phylogenetic analysis of S1 gene of infectious bronchitis virus reveals emergence of new genotype

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Abstract

In India the most common vaccine strain against infectious bronchitis (IB) virus (IBV) is Mass strain (M41). Most of the organized and unorganized poultry farms use Mass strain for vaccination of parent stock. But even after taking all precautions the incidences of IB outbreak are common in poultry population. IBV, a major pathogen of poultry flocks, circulates in the form of several genotypes and serotypes. Only a few amino acid changes in the S1 subunit of wild type proteins may results in mutants unaffected by current vaccine. In the year 2008 one strains of IBV was isolated from vaccinated chicken flocks. The results from sequencing of S1 gene showed that this strain was distinct from classic IBV strains of H120, M41 etc. Compared to H120 and M41 vaccine strain, point mutation occurred at many positions in the S1 protein of this field strain. The homology of the nucleotide and amino acid sequences of the S1 gene of this isolate was 79.0%-99.6% and 74.5%-98.8%, respectively with relation to major vaccine strains used worldwide. The results from this study indicate that different IBV strains cocirculate in the chicken population in India.

Highlights

- Sequencing of S1 gene of Indian field isolate represents a unique sub-genotype.
- These findings indicated that several other unknown genotypes may be circulated in field condition.
- Results validate the need of permanent monitoring of circulating strains to make effective vaccination strategies.

Keywords: Infectious bronchitis, IBV, spike protein, S1 gene, phylogenetic analysis

Infectious Bronchitis (IB) is a highly contagious disease of chickens caused by infectious bronchitis virus (IBV), which is a member of the Coronaviridea family and contains a single stranded, positive sense RNA genome of about 27.6 kb. The genome of IBV encodes 3 major structural proteins: the spike glycoprotein (S), the membrane glycoprotein (M) and the phosphorylated nucleocapsid protein (N) (Cavanagh and Naqi 2003). Clinically, the disease causes respiratory distress, drop in egg production and quality in layers. Some strains of IBV are associated with nephritis (Jackwood 2012, Cavanagh 2005, Cavanagh 2003).

Especially S1 defines the serotype of the virus; even single base mutation can apparently play a critical role in the generation of serologically distinct but, genetically closely related strains. The molecular characterization of IBV is based mainly on analysis of the S1 gene (Cavanagh 2001). The S protein is very diverse in terms of both nucleotide sequence and deduced primary protein structure, especially in the upstream part of S1. This region contains hypervariable regions (HVRs) distributed along the less variable and more conserved sequences of this protein (Cavanagh 1995, Kusters *et al.* 1989). The S1 sub-unit of spike protein mediates virion attachment to IBV host cells and is a major target of neutralizing

antibodies in chickens. Evolution of new genotype is primarily associated with the alteration in S1 protein sequence (Kant *et al.* 1992, Cavanagh *et al.* 1988). Therefore characterization of IBV is mainly based on the analysis of the variable S1 gene or the expressed S1 protein (Fellahi *et al.* 2015, Lee *et al.* 2003). Several IBV variants are distributed globally. More than 20 IBV serotypes are differentiated worldwide that evolved from genomic insertions, deletions, substitutions and/or RNA recombinations of S1 gene (Alvarado *et al.* 2005, Gelb *et al.* 1991). This large diversity is the actual cause of vaccine failure or partial efficacy of vaccine and hence new outbreaks reported regularly (Cavanagh 2003).

All above facts make the S1 gene most suitable candidate for viral characterization, serotyping, immunological studies, host-virus interaction studies etc. Therefore in this experiment the S1 gene of isolate was sequenced and is characterized to identify its relationship to reference IBV strains by nucleotide sequence analysis.

Methodology

Collection of field sample

Clinical samples of poultry collected from the field showing post mortem lesions of visceral gout, bronchitis and nephritis. IBV isolate used in the present study were collected from the field outbreak at adjoining area of Jabalpur, M.P. Isolation of the virus from the field sample was done by the method of Zhou *et al*. (2004). Characterization of virus was done by CAM inoculation in 9-11 days old embryonated chicken eggs which results in teratogenic changes like curling and dwarfing of embryo.

Isolation of viral RNA and cDNA synthesis

Total RNA was isolated from the isolate by Tri-Reagent (Sigma) method as per the manufacturer's protocol. The RNA extracted was immediately used for cDNA synthesis by using reverse transcriptase (Fermentas).

PCR amplification and Cloning of S1 gene

S1 gene specific primers were designed by DNA Star Lasergene software (Germany). The complete S1 gene was amplified with these specific primers (IBS1F1- TGAAAACTGAACAAAAGACA and IBS1R1- CATAACTAACATAAGGGCAA). The gene was amplified at 48ºC annealing temperature in 25µl reaction volume with 1mM $MgCl_2$ concentration. The amplified S1 gene fragment was confirmed by nested PCR using internal primers of the hyper variable region-1 and 2 (HVR12F- TGCAGGCTCTTCATCTG and HVR12R-TAGGTGATCCATCACAC).

Sequencing of S1 gene

The sequencing of S1 gene was done thrice by sequencing of PCR amplified product using IBS1F1, IBS1R1 and HVR12F primers. The purified PCR product (amplified S1 gene) used as template for the sequencing reaction. Sequencing was done in automatic 310 Genetis Analyzer (ABI, USA). All the generated sequences were aligned to generate the complete stretch of S1 gene fragment. Several other important vaccine strains were used to compare the generated sequence to identify the homology (Table 1).

Table 1: The reference vaccine strains of IBV used to compare the S1 gene sequence

Sl. No.	Accession no.	Strain	Country
1	L18990	Cann	—
2	M21970	H120	—
3	AY839140	JAAS	China
4	AY839144	Jilin	China
5	AY856348	IBN	China
6	AF169859	Ark	USA
7	DQ834384	M41	USA
8	AY514485	Cal99	USA
9	AY692454	Beau	USA
10	NC_001451	Beau	UK

Results and Discussion

Emergence of new genotypes of IBV becomes a major issue of vaccine failure. Studies of genetic diversity become a popular tool to identify the variations and its outcomes (Fellahi *et al.* 2015, Ma *et al.* 2012, Yan *et al.* 2011, Bochkoy *et al.* 2007). The spike glycoprotein of IBV is post-translationally cleaved into two subunits, S1 and S2 (Cavanagh and Naqi 2003). The S1 protein forms the N-terminal portion of peplomer and contains antigenic epitopes mainly within three hyper variable regions (HVR) (Moore *et al.* 1997, Cavanagh *et al.* 1988). Variation

in S1 sequence, has been used for differentiate the IBV serotypes (Abdel-Moneim *et al.* 2006, Kingham *et al.* 2000, Kwon *et al.* 1993). The generation of genetic variant is thought to be resulted from few amino acid changes in spike glycoprotein of IBV (Cavanagh *et al.* 1992, Kant *et al.* 1992).

The S1 gene sequence of the IBV field isolate was partially sequenced in the present study. The generated nucleotide sequence and deduced amino acid sequence (Fig. 1 and 2) of the isolate was compared with the reference strain sequences retrieved from GenBank from different region of the world.

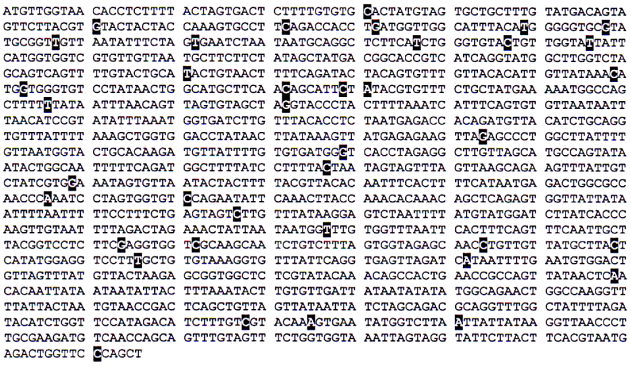

Fig. 1: Complete nucleotide sequence of S1 gene of field isolate

*Nucleotides in black background are the point mutation with respect to M41 reference strain

Fig. 2: Alignment of partial amino acid S1 gene sequence of field isolate with the reference strains (M41 and H120) sequence

‡ - indicate variation in amino acid with reference strain

* - indicate identical amino acid

This molecular study has revealed that a new genotype can emerge as a result of only a few changes in the amino acid composition in the S1 subunit of the spike protein of virus (Cavanagh *et al.* 1992). Alignment of the S1 gene sequence of the isolate with various vaccine strains revealed high homology with H120 and M41 strains. In present study although the sequence of isolate showed 97-99% similarity with other vaccine strains (M41 and H120), there were 38 replacement in nucleotide sequence and 24 predicted amino acid variations. The hypothesis is that these variations may play role on mutation in vaccine virus and may be the reason for vaccine failure, as even point mutation can generate antigenic variation (Jia *et al.* 1995). Nucleotide sequence of 1556 bp was aligned with published vaccine strain sequences. The nucleotide sequence showed 99.6% homology with H120 strain indicative of common origin of both strains. Surprisingly showed less similarities (97.3%) with M41 strain, although it is the commercial vaccine strain in India (Table 2). Generated gene sequence of the field isolate showed 38 point mutation at different positions throughout the sequence as comparison to M41 (Fig. 1).

Table 2: Percent identity and divergence of nucleotide sequences of field isolated S1 gene from different reference strains of IBV

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11		
1		97.5	99.6	99.5	97.2	97.4	94.6	79.0	80.3	82.1	80.7	1	S1 gene sequence
2	2.6		97.8	97.6	97.9	98.2	95.5	79.5	80.5	82.8	81.2	2	DQ834384 M41 USA
3	0.5	2.3		99.7	97.4	97.6	94.9	79.4	80.4	82.4	81.0	3	M21970 H120
4	0.5	2.4	0.3		97.3	97.6	94.8	79.3	80.3	82.4	80.9	4	AY856348 IBN China
5	2.9	2.1	2.7	2.8		99.5	94.6	79.3	80.7	83.1	80.9	5	NC_001451 Beau UK
6	2.6	1.8	2.4	2.5	0.5		94.9	79.4	80.7	83.1	81.1	6	AY692454 Beau USA
7	5.6	4.5	5.3	5.5	5.4	5.2		78.7	79.9	82.3	80.5	7	L18990 Cann
8	25.2	24.2	24.7	24.9	24.3	24.3	25.5		85.4	80.4	96.1	8	AF169859 Ark USA
9	24.0	23.9	23.9	23.8	23.2	23.3	24.4	16.0		79.8	86.4	9	AY514485 Cal99 USA
10	20.6	20.0	20.3	20.4	19.9	19.7	20.8	23.4	23.2		80.7	10	AY839140 JAAS China
11	23.1	22.2	22.6	22.8	22.5	22.2	23.3	4.0	14.6	22.9		11	AY839144 Jilin China
	1	2	3	4	5	6	7	8	9	10	11		

Divergence

The deduced amino acid sequence showed 24 amino acids variation compared to the M41 strain. The amino acid sequence showed 95.4% identity with M41 strain, while 98.8% with H120 strain of IBV (Table 3). The major difference seen at the position 128 to 131 where Lysine, Asparagine, phenylalanine and Leucine of M41 strain were replaced by Glutamine, Histidine, Serine and Isoleucine in our sequence. The notable thing was that here Phenylalanine replaced by Serine, means hydrophobic amino acid is replaced by polar amino acid (Fig. 2). This identified variation lies

between 128 to 131 residues of deduced amino acid sequence. This sequence region correspond to known HVR2 (117 -131 residues) of IBV-S1 gene. It was well documented that HVR1 and HVR2 contain sequences that have been associated with specific IBV serotypes (Cavanagh *et al.* 1988, Kusters *et al.* 1989) and serotype specific neutralizing epitopes (Kant *et al.* 1992).

The nucleotide sequences of IBV S1 gene normally differ by 20-25% among serotypes, while amino acid sequences vary as little as 2% (Cavanagh *et al.* 1992). The variation of four amino acids in HVR2 of S1 subunit possibly has some significant role (i.e. Viral tropism, neutralization ability etc.) (Wang and Huang 2000, Cavanagh *et al.* 1997). Further studies are needed to determine the role of the substitution in S glycoprotein (Moore *et al.* 1997).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	
1		95.4	98.8	98.6	95.4	95.8	89.9	74.5	76.4	80.9	76.3	1
2	4.8		96.4	96.2	95.0	95.6	90.5	74.9	76.8	81.2	76.8	2
3	1.2	3.7		99.4	96.1	96.5	90.9	75.2	77.2	81.4	77.2	3
4	1.4	3.9	0.6		95.9	96.4	90.5	75.2	77.4	81.4	77.2	4
5	4.8	5.2	4.0	4.2		98.8	89.1	76.0	78.3	81.8	77.9	5
6	4.4	4.6	3.5	3.7	1.2		89.5	75.6	77.9	81.6	77.7	6
7	10.7	10.0	9.6	10.0	11.4	11.1		75.3	75.9	81.3	76.7	7
8	31.0	30.5	29.9	29.9	28.8	29.4	30.1		82.3	79.3	93.9	8
9	27.8	27.3	26.7	26.5	25.1	25.7	28.0	19.7		80.7	84.3	9
10	21.9	21.5	21.2	21.2	20.6	20.9	21.3	24.6	21.9		80.9	10
11	28.6	27.8	27.3	27.3	26.2	26.5	28.0	6.4	17.2	22.1		11
	1	2	3	4	5	6	7	8	9	10	11	

1	S1 gene sequence
2	DQ834384 M41 USA
3	M21970 H120
4	AY856348 IBN China
5	NC_001451 Beau UK
6	AY692454 Beau USA
7	L18990 Cann
8	AF169859 Ark USA
9	AY514485 Cal99 USA
10	AY839140 JAAS China
11	AY839144 Jilin China

Table 3: Percent identity and divergence of amino acid sequences of field isolated S1 gene from different reference strains of IBV

The new novel genotype of IBV are emerging continue. Widespread uses of various vaccines made from heterotypic IBVs are probably the major cause. Recombination as a consequence of mixed infection, play important role in the emergence of such novel genetic variant (Fellahi *et al.* 2015, Dolz *et al.* 2006, Lai and Cavanagh 1997). Phylogenetic analysis is a most preferred method to identify the origin of new stains.

Hence the phylogenic tree was constructed from the nucleotide sequences of isolate and reference strains. The present isolate forms a clear common branch with the H120 strain (Fig. 3). The sequence analysis of the partial S1 gene demonstrate that this Indian isolate represent a unique sub-genotype compared to other reference strains of various countries.

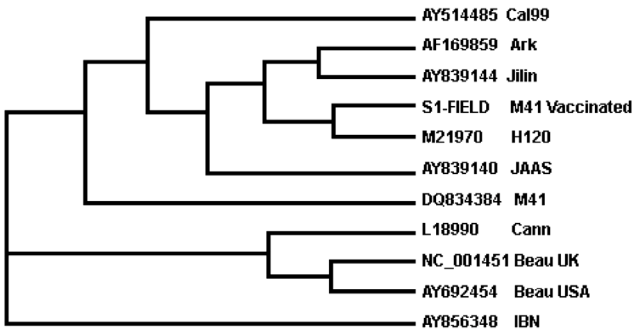

Fig. 3: Phylogenetic relationship of obtained field isolate and selected reference strains based on partial nucleotide sequence of S1 gene

Conclusion

This molecular study has revealed that a new genotype can emerge as a result of only few changes in the amino acid composition in the S1 subunit of the virus. The variant presented specific nucleotide and amino acid sequence variation in the S1 gene in comparison to the M41 mass vaccine strain that might be associated with the occurrence of clinical disease in vaccinated flocks. Vaccination by M41 mass strain did not provide satisfactory protection against challenges with this newly recovered genotype. Evaluation of cross protective capability of IBV vaccine(s) and newly recovered field isolates should be performed regularly to ensure optimal control of IBV. Our sequencing results demonstrate a co-circulation of wild-type IBV in chicken. These results justify the permanent monitoring of circulating strains in order to modify the vaccination strategies regularly.

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