

Genotypic characterization of infectious bronchitis viruses from India

K Suresh Kumar, G Dhinakar Raj*, A Raja and P Ramadass

Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University
Chennai 600 007, India

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Infectious bronchitis (IB) is an acute, highly contagious and economically important disease of chickens. In India, IB vaccinations are carried out using vaccines belonging only to the Massachusetts 41 (Ma41) serotype. However, the vaccine strain used may not always be protective and it becomes necessary to continuously monitor the viruses causing disease. There has been anecdotal evidence of IB outbreaks even in vaccinated flocks in India. Tissue suspensions were inoculated intra-allantoically into 11-day-old embryonated chicken eggs. The allanotic fluids collected at 48 h post-inoculation (PI) were used in reverse transcription-polymerase chain reaction (RT-PCR) to amplify a part of S1 gene. The partial S1 gene products obtained were sequenced. All the seven isolates had sequences with 94.8-98.8% homology with the vaccine strain, H120. The cross-neutralization tests were used to identify the serotype of the field isolates. All the four isolates showed greater than 50% antigenic relatedness value indicating their classification in Massachusetts's serogroup. The commercial vaccines conferred protection to the viruses isolated.

Keywords: infectious bronchitis virus, chickens, genotyping, antigenic relatedness, protectotyping

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Introduction

Infectious bronchitis (IB) is an acute, highly contagious disease of chickens characterized by tracheal rales, coughing, sneezing along with excess accumulation of mucus in bronchi. Other disease manifestations such as decline in egg production and quality, kidney damage, enteritis and even pectoral myopathy have also been observed¹. IB is of economic importance because it causes decrease in weight gain, feed efficiency, egg production and quality². It is caused by a virus belonging to the genus Coronavirus of the Family *Coronaviridae*. IB virus (IBV) is not a single homogenous type but occurs in different serotypes. Variant serotypes continue to be associated with outbreaks of disease in many countries³⁻⁵. In India, IB vaccinations are carried out using vaccines belonging only to the Massachusetts 41 (Ma41) serotype. However, the vaccine strain used may not be always be protective and it becomes necessary to continuously monitor the viruses causing disease. There has been no extensive genomic characterization of IB viruses circulating in India although there has been serological evidence of the

presence of an IBV variant, 793/B antibodies⁶. There has been anecdotal evidence of IB outbreaks even in vaccinated flocks in India that prompted us to study the nature of IBV involved in field outbreaks. The virus isolates were characterized genotypically, serotypically and protectotypically.

Materials and Methods

Virus Isolation

Trachea, proventriculus, kidney, ileo-caecal junction or oviduct samples were collected from IB suspected birds or received from clinicians, from different parts of India. Pooled 20% tissue suspensions were inoculated intra-allantoically into 11-day-old embryonated chicken eggs (ECE).

Diagnostic RT-PCR

The allanotic fluids of ECR were collected at 48 h post-inoculation (PI) and used in reverse transcription-polymerase chain reaction (RT-PCR) initially for a portion of the nucleoprotein (N) gene using primers and cycling conditions described earlier⁷. Briefly, total RNA extracted by solution D method from the infected allantoic fluids was reverse transcribed in to cDNA using a cDNA synthesis kit (MBI Fermentas, USA). The cDNA was used with 'N' gene specific primers for amplification of external

*Author for correspondence:

Tel: 91-442-25381506 extn ABT; Fax: 91-442-25389445

E-mail: dhinakarraj@yahoo.com

product (402 bp) with the following cycling conditions: initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 45 sec; 60°C for 1 min; 72°C for 2 min, followed by a final extension of 72°C for 7 min. A nested reaction that amplified a 380 bp product was used using 1-2 µL of the first round reaction and the same reaction conditions with an internal set of primers. A total of 47 samples were screened using these primers.

Phylogenic RT-PCR

All the samples positive by N gene nested RT-PCR were passaged in ECE thrice in quick succession and the allantoic fluids were concentrated by centrifugation at 35,000 rpm for 2.5 h (Beckmann, 70Ti rotor). RNA was extracted from the pellet and RT-PCR conducted for a portion of the spike protein (S₁) gene using primers and cycling condition described earlier⁸. The sensitivity of amplification of the N and S₁ genes was estimated by carrying out RT-PCR from dilutions 10⁰ EID₅₀ to 10⁶ EID₅₀⁹.

The partial S₁ gene products obtained were sequenced using an automated sequencer (ABI Prism). A commercial vaccine virus (H120) that is commonly used in the field was also sequenced. The nucleotide sequences were aligned using the MegAlign programme of the LaserGene software (DNASTAR INC., USA) and subjected to phylogenetic analysis. For comparison, published sequences of IB viruses available in GenBank and the vaccine virus sequenced were included.

Serotyping of Field Isolates

The cross-neutralization tests used to characterize the field isolates were as described in the OIE manual¹⁰. Four field isolates (008, 177, S3, U4) and a vaccine strain H120 and their respective sera were used. Hyperimmune sera were raised in chicks as described previously¹¹. Each virus was reacted with its homologous serum and also with panels of serially diluted four other heterologous sera. The end points of the homologous and heterologous neutralization titres were determined as the dilution, which showed no signs of dwarfing (complete neutralization) after incubation of inoculated eggs for five days. Each time a control with 10^{2.0} EID₅₀ of the respective virus (without antisera) was inoculated into 4 eggs to determine the ability of the diluted virus to cause dwarfing. The antigenic relatedness values (R) were calculated by using homologous and heterologous

titres¹². Two viruses having an 'R' value greater than 50% were considered to be related¹³, whereas those with values less than 50% were considered unrelated.

Protectotyping of a Field Isolate

Two groups of six chicks each were inoculated with H120 or Massachusetts serotype (Ma5) vaccines at day old at the recommended vaccine dose of 10^{3.0} EID₅₀ per bird oculo nasally. One group (of six chicks) was left unvaccinated to serve as challenge controls. At 3 weeks of age all the three groups were challenged with a representative field isolate 008 (10^{3.0} EID₅₀) oculo nasally. Four days post-challenge the birds were killed humanely and assessed for protection ensured by the vaccines against the field virus. In each group, trachea from 3 birds was used to assess ciliostasis¹⁴. An individual chick was recorded as protected against challenge if the ciliostasis score for that trachea was less than 20. For each group, a 'protection score' was calculated by the formula:

$$1 - \frac{\text{Mean ciliostasis score of vaccinated/infected group}}{\text{Mean ciliostasis score of challenge controls}} \times 100$$

The maximum obtainable protection score is 100. The higher the score reaching towards 100, higher the level of protection provided by that vaccine. Tracheas from the remaining three birds from each group were used for virus isolation in ECE.

Results

Diagnostic and Phylogenic RT-PCR

A total of 47 samples were screened using primers specific to amplify the part of N-gene of IBV. Ten samples were found positive by N gene nested PCR and all were amplified using S₁ gene primers after consecutive passages and concentration. The sensitivity of the N-gene RT-PCR was found to be 10^{3.0} EID₅₀ while that of the nested PCR was 10⁰ EID₅₀ and that of the S gene PCR was 10^{4.0} EID₅₀ (Fig. 1). Seven isolates and one vaccine virus belonging to H120 strain were used in sequence analysis. The sequence data of other 3 isolates were not of good quality to be used for analysis. The nucleotide sequence of the H120 vaccine virus sequenced in this study was 100% similar to the already available sequence for that virus in GenBank (Accession No. M21970). The other 7 sequences had nucleotide percent similarity with H120 strain ranging from 94.8 to 99.8%. The phylogenetic tree constructed based on aligned sequence data is shown in Fig. 2.

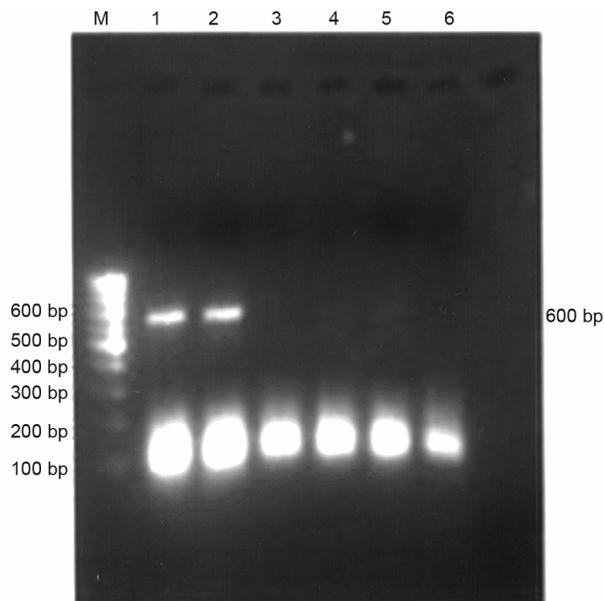


Fig. 1—Sensitivity of S1 gene PCR: Lanes 1-5, 10^5 to 10^1 EID₅₀; Lane 6, Negative control; M, 100 bp Marker lane

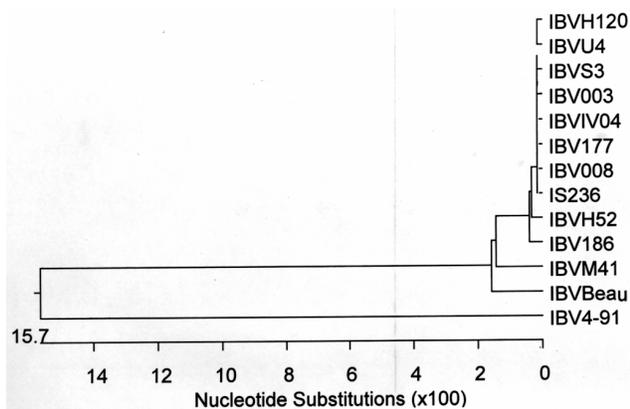


Fig. 2—Phylogenetic tree based on nucleotide sequence alignment of the S1 gene variable region of different IBV isolates. (Branched distance correspond to sequence divergence).

Serotyping

In the cross-neutralization tests, the end point titres of all the 4 isolates tested had less than 2-fold differences with the H120 vaccine sera titres (Table 1). All the four isolates showed greater than 50% antigenic relatedness value indicating their classification in Massachusetts's serogroup.

Protectotyping

Since all the isolates tested belonged to the same serogroup Mass 41, one representative isolate (008) was selected for use in the *in vivo* protection experiment. The ciliostasis score of the unvaccinated, challenged

Table 1—Antigenic relatedness (R) values of IBV isolates

Isolates	Relatedness (%)				
	H120	008	177	S3	U4
H120	100				
008	70.7	100			
177	50	70.7	100		
S3	50	50	50	100	
U4	50	70.7	70.7	50	100

group was 22 out of maximum score of 40 obtainable. However, the vaccinated challenged birds had a score of 4.6. The protection scores for both the H120 and Ma5 vaccinated groups were 79%. The tracheal homogenates of the unvaccinated challenged chickens were all positive for virus while those of chickens vaccinated with IBV vaccines were negative.

Discussion

In India, Newcastle disease (ND) has always been the most prevalent respiratory pathogen in chickens. The incidence and isolation of IBV has been rare, probably due to the ubiquitous presence of ND viruses that masked IBV infections. Now extensive vaccination with several types of ND vaccines is routinely being used, bringing the disease largely under control. In this context, IB has started causing great economic losses. In this study, no distinct variant genotype of IBV could be detected. All the seven isolates had sequences of 94.8-98.8% homology with the vaccine strain, H120. The least homology (high percent divergence) was with the isolate IBV 186 (94.8%). All the isolates sequenced had 1-4 nucleotide differences from that of the H120 vaccine virus. Thus it appears that the vaccine viruses are acquiring point mutations and insertions during its spread in the field. Although speculative, it is possible that in due course, the accumulation of these mutations may lead to viruses that may escape protection by existing vaccines¹⁵. Serotype analysis by cross-neutralization tests also confirmed them as Mass serotype. The commercial vaccines conferred protection to the viruses isolated. Mass serotype based vaccines are used worldwide, though they are protective against the homologous serotype they spread in field^{7,15}, creating a confusing epizootiological problem. The exact discrimination of wild type and Mass vaccine strains is very difficult.

The sequencing results demonstrated the co-circulation of vaccine and wild type infectious bronchitis viruses belonging to Mass 41 serogroup and are further justification for continuous monitoring of circulating strains in order to rationally modify

vaccination strategies to make them appropriate to the field situation. However, there was no evidence of any variant viruses present at least among the few IBV sequenced in this study.

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