

Original Research Article

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Detection and Characterization of Infectious Bronchitis Virus in Desi Birds by Molecular Assay

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ABSTRACT

Keywords

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Infectious Bronchitis (IB) is highly contagious and acute, with economically importance disease of poultry. 185 samples including tracheal swab and faecal swab were collected from apparently healthy and infected birds with respiratory disorders. An attempt made to isolate the virus through inoculation of specific pathogen free embryonated egg (SPFEE) as cultural medium. The embryo showing dwarfism were identified and subjected to molecular diagnosis. Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify N-Protein and sequenced. Factors responsible for prevalence of IB in desi chicken were studied. Out of 185 samples 7 samples showed dwarfism in SPFEE. 4 out of 7 samples, found positive for IBV by RT-PCR.

Introduction

Poultry are kept in most areas of the world and it provides an acceptable form of animal protein to most people throughout the world. During the last decade, many developing countries have adopted intensive poultry production in order to meet the demand for this form of animal protein. Infectious bronchitis virus (IBV) is a major cause of disease in domestic fowl and causes an acute, highly contagious disease of the respiration and sometimes urogenital tracts (King and Cavanagh, 1991). Morbidity is usually high in all ages of chickens, drop in egg production, affect internal quality to eggs and high mortality (10%-30%) occurs in chickens less

than 6 week old (Wang *et al.*, 1997). Infected chicks are the major source of virus excretion in the environment. Contaminated equipment and material are a potential source for indirect transmission over large distances. Clinical signs will develop in contact chicks within 36 hours and in nearby sheds within one to two days. Infection is resolved within fourteen days with a rise in antibody titres. In a small number of chicks, latent infection is established with subsequent erratic shedding of virus for a prolonged period of time via both faeces and aerosol. Movement of live birds should be considered as a potential source for the introduction of IBV. Isolation

and identification of IBV is needed for positive diagnosis. The preferred method of isolation is to passage a sample in SPFEE (FAO, 2010). Molecular techniques such as RT-PCR, sequencing and real time PCR, have been used for rapid and sensitive detection of IBV viral genome either in allantoic fluids from embryonated chicken eggs (ECE) inoculated with field samples or directly from the tissue samples. Several authors used different targets like the nucleoprotein gene (N-gene) (Suresh Kumar *et al.*, 2007.), Spike protein 2 (S2) (Kumanan, 2003), Spikeprotein 1(S1) (Okino *et al.*, 2005), M (Williams *et al.*, 1993) to diagnose IB by RT-PCR.

However, those techniques detect only one specific pathogen at a time. The objectives of the present study were to develop RT-PCR for detection of the N glycoprotein genes of IBV field isolates and to sequence N glycoprotein genes to study genetic variation and relationships of field strain with other IBV vaccine strains. Though no disease was witnessed at the time of sampling, identified viruses including variant IBV strains, may still pose a threat for both backyard poultry and commercial poultry.

Materials and Methods

Study area

The study was conducted in organised and unorganised backyard poultry in Namakkal district, Tamil Nadu, India during October, 2016 to May, 2017.

Sample collection

A total of 27 tracheal swab and 31 rectal swab were collected from apparently healthy birds from 4 taluks of Namakkal district and were transported in 1% PBS solution to the laboratory for further processing.

Reference virus (Vaccine virus- positive control)

Vaccine virus (Massachusetts H120 strains) received from commercial market and is used for standardizing the RT-PCR protocol (Table 1).

Virus isolation (Samples)

The collected swabs were inoculated intra allantoically into 10 day old SPFEE.

Collection and storage of allantoic fluid from SPFEE showing dwarfism were done properly.

Extraction of viral RNA

Viral RNAs were extracted using Trizol method (Okino *et al.*, 2005). 750µl of Trizol LS (Ambion) is added to 250µl of sample (gently vortex), incubate at 37⁰ C for 5 min, add 200 µl of chloroform, shake vigorously with hand for 15 sec, incubate at 37⁰ C for 5 min, centrifuge at 13000 rpm for 15min at 4⁰ C, take the aqueous phase still some good amount left, precipitate the RNA by 0.5 ml Isopropanol (Emparta) ice cooled, incubate at -20⁰ C for 20min, centrifuge at 13000rpm for 20min at 4⁰ C, remove the supernatant and add 1ml of 70% Ethanol (Jebsen and Jessen GmbH and Co., Germany) and vortex, centrifuge at 12000 rpm for 5min at 4⁰ C, remove the supernatant and air dry the pellet, add 20 µl of nucleus free water (NFW) to the pellet, pipette it gently and thoroughly on the sides of the tube, keep the samples in the water bath at 60⁰ C for 10min.

Oligonucleotide primers

Primers were chosen based on the cDNA sequences of genomic segments NP gene of IBV. A set of primers were used to amplify NP gene at 410bp. Primer sequences were as follows

cDNA synthesis

RT-PCR reactions were performed according to procedures provided by Qiagen One Step RT-PCR kit (Germany). Briefly, the RT-PCR reactions were performed in 25µl containing 5 µl RNA template, 5 µl PCR Buffer mix, 1 µl PCR enzyme mix, 1 µl dNTP mix. 1 µl FP, 1 µl RP, 11 µl NFW. A slight modifications done to RT- PCR procedure given by Liu *et al.*, (2003). Reverse Transcription was carried out at 50⁰ C for 30min. PCR reactions include initial denaturation for 95⁰ C for 3min, followed by 35 cycles of denaturation for 45 seconds at 94⁰ C, annealing for 30 sec at 56⁰ C, and extension for 30 sec at 72⁰ C and a final extension cycle at 72⁰ C for 7 min with holding temperature of 4⁰ C.

Analysis of amplified products

After completion of PCR, 5 ml of reaction mixture was loaded onto 1.2% agarose gels for 40min at 80V in 1% TAE buffer (4.84gm of Tris Base, 1.142ml of glacial acetic acid, 2ml of EDTA, pH 8.3), containing 2µl ethidium bromide(Bio basic, Canada), for electrophoresis and subsequent visualization by UV transillumination. DNA ladder of 1 kb (Genedirex) was electrophoresed as size marker for determination of the length of the amplified fragments.

Results and Discussion

Isolation of IBV

The samples were collected from apparently healthy and clinically affected birds from four

different blocks in Namakkal district. The primary symptoms observed in live birds was dyspnoea which was frequently associated with other symptoms, including apathy, diarrhoea, asthenia, locomotion ataxia, or general paralysis. Samples were processed in laboratory of Department of Veterinary Preventive Medicine, VC&RI and Namakkal. Suspected samples were inoculated in 10 day old SPFEE. Observed every 24hrs once. Death of the embryo found in 4-5th day. The embryo showing dwarfism in SPFEE, Allantoic fluid of that SPFEE were collected aseptically.

Identification of IBV

RNA extracted by Trizol method, cDNA synthesis done by Qiagen one step RT-PCR kit. The RNA from reference and recent isolates was reverse-transcribed to cDNA and amplified. Examination of the amplified PCR products following electrophoresis in 1.2% agarose gels indicated that all amplified cDNA showed almost identical mobilities. Amplification of the NP genes of IBV by RT-PCR using primer pairs 5'-3' and 3'-5' generated a specific DNA band of 410 bp, respectively (Fig. 1), suggesting that no apparent deletions or insertions in the tested regions of NP genes of IBV. The negative controls produced no PCR products.

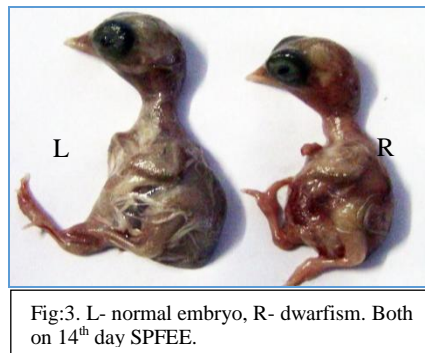
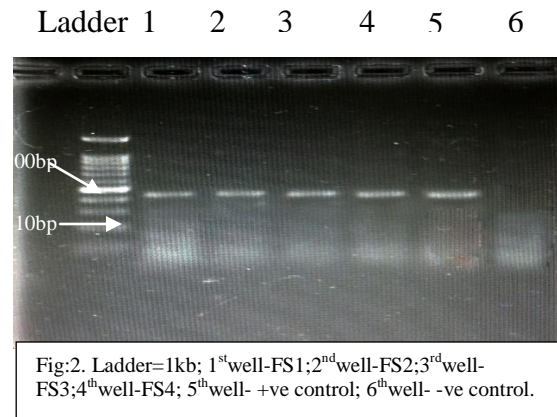
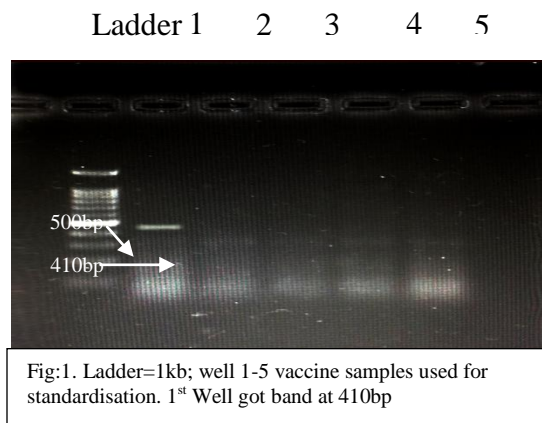
Seven IB-suspected field samples were subjected to RT-PCR assays. Results revealed that 410bp in length were amplified from four samples (Fig. 2). Results revealed that RT-PCR provided a sensitive approach for diagnosis of IBV infections (Table 2).

Table.1 Details of PCR primers

Primer	Primer direction	Sequences (5'-3')	Gene location	Product size
		AAT TTT GGT GATGAC AAG		
Forward	Sense	ATG	N-glycoprotein	413bp
Reverse	Anti-sense	CAT TGT TCC TCT CCT CAT CTG.	N-glycoprotein	413bp

Table.2 Details of field sample positive for IBV by egg inoculation and RT-PCR

S.no	Place	Type of bird	Age of the bird	Samples collected	SPFEE inoculation	RT-PCR
1	Thiruchengode	DB	20W	Tracheal swab	+	+
2	Ilupali	DB	20W	Tracheal swab	+	+
3	Sendhamangalam	DB	25W	Tracheal swab	+	-
4	Valaiyapatti	DB	8W	Tracheal swab	+	+
5	Kabilarmalai	DB	18W	Tracheal swab	+	-
6	Aandalurgate	DB	10W	Tracheal swab	+	+
7	Anagur	DB	42W	Tracheal swab	+	-



Out of 185 samples, 7 samples (3.7 %) showed dwarfism in SPFEE (Fig. 3). Out of 7 samples, 4 samples (57.14%) showed sensitivity for RT-PCR (Fig. 2). Remaining 3 samples were not shown any bands. Of the total number of samples tested for IBV from both sites (n = 185), 4 were positive from in and around Namakkal, resulting in an overall prevalence of 2.16% (4/185). All of the samples that were positive for IBV were from domestic chickens. According to the collected information, few birds were vaccinated against Newcastle disease

virus (NDV), and fowl pox virus (FPV). The others were not vaccinated. There was no report of outbreak in our study areas. Desi birds near to commercial chicken farms shows presence of IBV in their flock.

This study is to identify the circulating IBV genotypes in backyard poultry of Namakkal. For this study, an epidemiologically representative number of samples were collected from four taluks of Namakkal and brought to the laboratory of the Department of

Veterinary Preventive Medicine. Isolation done for IBV field isolates and allowed for RNA extraction once back in the laboratory, proving useful for analysing field isolates away from the point of sampling (Awad *et al.*, 2014; Moscoso *et al.*, 2005). Using RT-PCR we identified the prevalence of viruses within backyard flocks in relation to location and poultry species. Four samples were identified as IBV positive, with an overall prevalence of 2.16%. This finding is lower than previously reported in commercial farms within Namakkal district (Chandrasekar *et al.*, 2015). Age group of 8w-42w showing positive cases. However both studies sampled chickens within a higher density environment, which may have contributed to the higher prevalence rate compared with the backyard flocks. All the 4 positive samples were from non-immunized against IBV. Alert signs in the flocks were not much peculiar with morbidity, mortality, or decreased egg production. The prevalence is relatively normal in Namakkal backyard population. IBV is one of the most dangerous respiratory viruses in backyard poultry. There was no geographical significance was observed for IBV.

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