



Infectious Bronchitis Virus Detection and Isolation from Broiler Chicken

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ABSTRACT

Infectious bronchitis virus was detected in tissue samples of 16.39% (n=10) non-vaccinated commercial broiler flocks showing respiratory and or nephrotic lesions by real time RT-PCR and RT-PCR amplification of 5'-UTR and N gene. Tissue/swab suspensions from all 10 flocks were inoculated in 9-11 day old SPF embryonated chicken eggs by allantoic route. Nephropathogenic IBV was isolated from samples of 2 farms from Vijaywada region of Andhra Pradesh. Necropsy of birds from above two farms revealed lesions of swollen and mottled kidneys in many birds and visceral gout in few birds. Histopathological examination confirmed nephropathogenicity of the isolates. S1 gene amplification from two IBV isolates viz. BVC/2017/VSD/IBV/South/1 and BVC/2017/VSD/South/2 and subsequent sequencing revealed 92.7 to 96% amino acid sequence identity with the sequences of Indian nephropathogenic isolates published earlier. Amino acid sequences of two isolates shared 73.8%, 75.3% and 72.1% identity with M41, H120 and 4-91 type as respectively, which indicated substantial genotypic divergence from M41 and H120 (major respiratory and vaccine strain) and 4-91 strain. Of the samples from 8 flocks with respiratory lesions (positive for IBV by RT-PCR however, negative on isolation); 6 were found positive for *Mycoplasma gallisepticum* and or *Avian influenza* (H9) by PCR and RT-PCR test respectively.

Keywords: Broiler chicken, IBV, RT-PCR, nephropathogenic, virus isolation, SPF eggs

Poultry industry is one of the fastest growing sectors of Indian agriculture, with annual growth rates of 5.57 and 11.44% in egg and broiler production respectively. It accounts for about 1% of India's gross domestic product (GDP) and 11.70% of the GDP from the livestock sector. Presently, this sector is facing challenges on several fronts. In recent years, it is observed that, respiratory disease complex is becoming one of the major challenges amongst broilers and mortality rate has increased above normal, primarily because of respiratory disease complex and metabolic disorders. Infectious bronchitis (IB) is one of the important etiologies causing respiratory disease complex. Also, certain strains of IB are reported to cause pathology in reproductive system, kidneys, muscles etc.

IBV is highly mutagenic and is continuously evolving. Since, its first isolation in 1930s, over 50 serotypes or variants have been reported worldwide (Cavanagh and

Naqi, 2003). There is very little or no cross protection between different serotypes. S1 glycoprotein of the virus comprises of two glycopolypeptides viz. S1 and S2. S1 is considered to be most important in terms of variability (Kusters *et al.*, 1989) and neutralizing antibodies are mainly directed against it.

Outbreaks of infectious bronchitis involving nephropathogenic strains are being reported more commonly as compared to respiratory form in India. In India vaccine prepared from Massachusetts strain M41 and H120 is being used for prevention of infectious bronchitis. These vaccines show very little or no cross protection for different serotypes circulating in the field. Considering these facts, the study was planned to detect infectious bronchitis in broiler chickens with respiratory/nephrotic signs or lesions by using RT-PCR and isolation

in SPF eggs. S1 gene characterization was done to classify genotype of isolates obtained in the study.

MATERIALS AND METHODS

Sample collection

Samples were collected from 61 commercial broiler chicken farms located in different parts of Maharashtra and adjoining states. Information on parameters such as age, morbidity and mortality, clinical signs, history of IBV vaccination, etc. was recorded.

Trachea, lungs and kidney tissues were collected aseptically using sterile set of equipments into sterile container containing phosphate buffered saline (PBS) and on ice from commercial broiler chicken died with history of respiratory/nephritic signs and lesions for molecular detection of infectious bronchitis virus and its isolation in specific pathogen free (SPF) embryonated chicken eggs during necropsy.

Gross and microscopic examination

Necropsy examination was done as per standard procedure. The gross lesions were noted thoroughly and tissues viz. trachea, lungs and kidneys were collected in 10 % neutral buffered formalin for histopathological examination. The tissues were processed by routine paraffin embedding technique and sections were stained with Hematoxyline and Eosin stain as per method described by Luna, (1968).

Sample preparation and RNA extraction

Tissue suspensions/inocula of 10 % weight/volume were prepared in sterile phosphate buffered saline solution (pH 7.2) by trituration and freeze-thaw cycles followed by centrifugation at 1600 × g for 30 min at 4°C and filtration through 0.20 µm filters. RNA from tissue suspension was extracted using TRIZOL[®] reagent (Ambion LifetechnologiesTM, USA) as per method described by Chomczynski (1993) with minor modifications in the procedure. The concentration of the RNA samples was obtained by using spectrophotometer (Multiskan Go, Thermo Scientific, USA). RNA was extracted from pooled tissue samples of 61 commercial broiler chicken farms and then subjected to RT-PCR detection of IBV.

Real time reverse transcriptase PCR detection of 5'untranslated region of IBV

Real-time RT-PCR assay was performed to detect 5'-UTR region of IBV genome. A forward primer IBV5'GU391 (5' GCTTTTGAGCCTAGCGTT 3') and a reverse primer IBV5'GL533 (5'GCCAATGTTGTC ACTGTCTATTG 3') and a Taqman[®] dual-labeled probe IBV5'G probe (5'-FAM- CACCACCAGAACCT GTCACCTC-BHQ1-3') located at nucleotide positions 494–473 of the IBV M41 strain genome sequence designed by Callison *et al.* (2006) were used in this study. The primers and probe were synthesized by Eurofins Genomics India Pvt. Ltd., India. The real time RT-PCR reactions were set up in duplicate for each sample in a total 20 µl volume using Step One Real Time PCR System (Applied Biosystems, LifetechnologiesTM, USA) and contained following components.

Components	Quantity (µl)
4 × TaqMan [®] Fast Virus 1-Step Master Mix Make- Applied Biosystem, Lifetechnologies TM , USA Catalogue No. 4444432	10
Forward Primer (500 nmol)	1.0
Reverse Primer (500 nmol)	1.0
Probe (250 nmol)	1.0
RT- PCR grade Water	5.0
Template	2
Total Volume	20

Following thermal cycling conditions were followed to amplify the desired segment.

Step	Stage	No. of Cycles	Temperature	Time
Reverse transcription	1	1	50° C	5 minutes
RT inactivation/ initial denaturation	2	1	95°C	20 Seconds
Amplification	3	40	95°C 60°C	3 Seconds 30 Seconds

In Real Time RT-PCR assay, the threshold was calculated automatically by the machine. Results were analysed in comparison with ct values of positive and negative control.

Reverse transcriptase polymerase chain reaction (RT-PCR) to detect N gene of IBV

The RT-PCR was performed to amplify N gene of IBV using gene specific primers as described by Handberg *et al.*, (1999). The RT-PCR was carried out by use of a program including both the RT reaction and PCR. The reaction was carried out in total 20 µl volume and consisted 10 µl of 2X Master Mix, 0.1 µl of Reverse Transcriptase enzyme, 0.5 each of forward and reverse primers, 3.0 µl template and 5.9 µl of NFW. The thermal cycling conditions consisted of cycle 1, 30 min at 48°C (RT reaction); cycle 2, 94°C for 120 s (Initial denaturation); cycle 3 to 41, 95°C for 30 s (denaturation), 52°C for 30 s (annealing) and 72°C for 40 s (extension); cycle 42, 72°C for 5 min (final extension). To confirm the targeted PCR amplification, 5 µl of PCR product was mixed with 1 µl of 6X gel loading buffer and electrophoresed on 2.0 per cent agarose gel along with 100 bp DNA ladder (Thermo Fisher Scientific, USA). The amplified product of 453 bp size was visualized as a single compact band of expected size under UV light and documented by gel documentation system (UVIDOC HD6 Touch, England, UK).

Detection of nucleoprotein gene (NP) of influenza A and H9 gene of avian influenza virus by reverse transcriptase PCR

Viral RNA was extracted from tissue suspension was used to amplify NP gene of influenza A and H9 gene of avian influenza virus as per method described by Lee *et al.* (2001) and Xie *et al.* (2006) respectively.

Detection of 16S rRNA gene of *Mycoplasma gallisepticum* by PCR

The DNA from tissue samples/swabs was extracted by using TRIZOL[®] reagent (Ambion Lifetechnologies[™], USA) as described by Chomczynski, (1993). The PCR reaction was performed using primers and method (with minor modification) described by Lauerman, (1998) and Marois *et al.* (2000) to amplify 16S rRNA gene to yield 186bp product size.

Virus isolation

0.2 µl of inocula or tissue suspensions (10% w/v) were inoculated into the allantoic cavity of 9-day-old SPF

embryonated chicken. Eggs were candled daily for 6 days. Allantoic fluid was collected 48 hr post inoculation and used for further passage in 9-day-old SPF embryonated chicken eggs. Eight passages were given for each sample. Allantoic fluids collected were tested for IBV by RT-PCR. The effects on embryos were studied and recorded in each passage.

S1 gene amplification and sequencing

Genomic RNA of IBV two isolates (BVC/2017/VSD/ South India/1 and 2) was extracted from allantoic fluid by Trizol method. IBV specific universal primers, SIUni2+:5'-CCCAATTTGAAAACCTGAACA-3' and SIUni1:5'-CCTACTAATTTACCACCAGA-3' described by Adzhar *et al.* (1996) was used for molecular characterization of S1 gene by RT-PCR. Amplicons (1600 bp) were gel purified using PureLink[™] Quick Gel extraction kit (Invitrogen, Carlsbad, CA). The purified PCR products were sequenced in both directions using BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Nucleotide sequences were subjected to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/>) to confirm that the sequences represented IBV. The sequences were compiled, aligned and phylogenetic trees of the nucleic acid and putative amino acid sequences was constructed using neighbor-joining method using MEGA 6 (Tamura *et al.*, 2012).

RESULTS AND DISCUSSION

Detection of 5'-UTR region and N gene of IB virus genome by RT-PCR

RNA extracted from tissue samples of all 61 broiler farms was used to amplify 5'- UTR of IBV genome using Real Time RT-PCR. Results of amplification were analyzed in comparison with cycle threshold values of positive and negative control. Out of 61 commercial broiler farms, samples from 10 farms (16.39%) showed amplification of 5'-UTR region of IBV genome (Fig. 1). Subsequently, RNA from all samples was also subjected to RT-PCR detection of N gene of IBV. All the 10 samples which tested positive for amplification of 5'-UTR region of IBV genome also showed amplification of N gene of IBV (Fig. 2). Kumar *et al.* (2007) reported 21.28 % samples positive for IBV isolation in SPF embryonated chicken egg and

RT-PCR targeting N gene of IBV. Ducatez *et al.* (2009) reported 26% chickens positive for IBV by RT-PCR. Similarly, Gola *et al.* (2017) diagnosed IB in 9 broiler flocks during August 2011 to December, 2012. 16.39% prevalence in unvaccinated and diseased broiler flocks recorded in this study can be regarded as high.

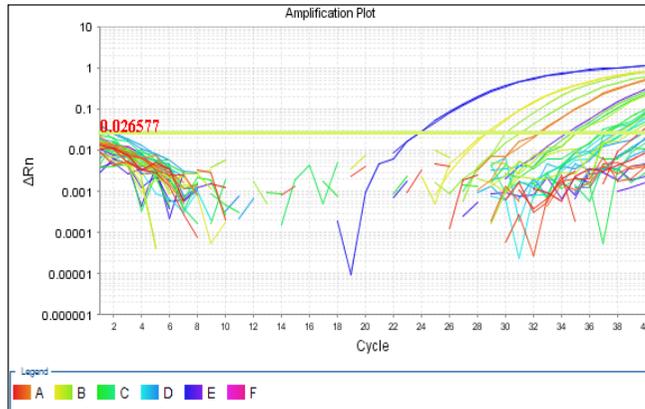


Fig. 1: Amplification plot for Real time RT-PCR for 5'-UTR gene of for IB positive sample

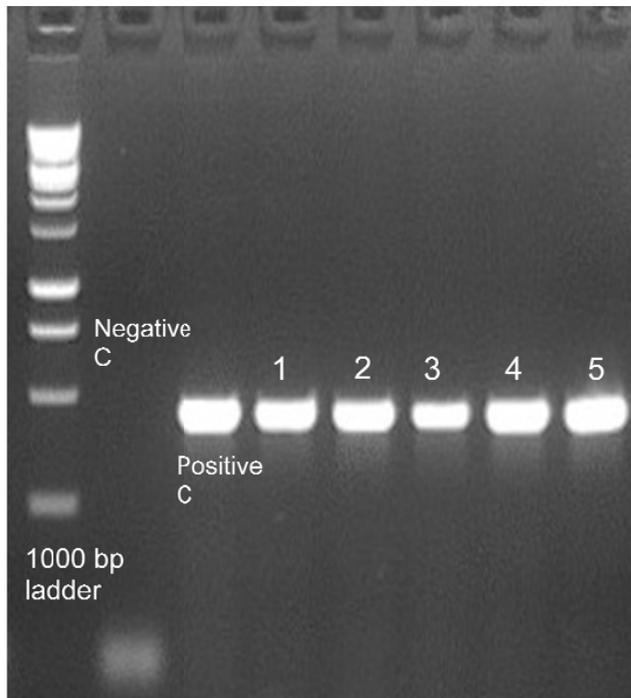


Fig. 2: Picture showing RT-PCR amplification of N gene (453 bp product) of IBV of 5 farms designated as 1,2,3,4 and 5. Well No. 1 from left beside 1000 bp ladder is negative control and positive C is positive control

Virus isolation

Samples from two broiler farms from Vijaywada region of Andhra Pradesh caused typical curling, dwarfing of embryos (button like embryo), subcutaneous hemorrhages and poor feathering out of total 10 samples inoculated in SPF embryonated eggs after seventh and eighth passage (Fig. 3). Similar type of changes (curling and dwarfing) in embryos were reported by Bayry *et al.* (2004); Xie *et al.* (2011); Selim *et al.* (2012) and Balasubramaniam *et al.* (2013), when IBV positive samples were inoculated in 9-11 day old SPF embryonated chicken eggs.



Fig. 3: Shows curling and dwarfing of embryos in IB positive cases

Singh *et al.* (2009); Balasubramaniam *et al.* (2013); Patel *et al.* (2015) and several other authors have recorded curling and dwarfing of embryos at third to fifth passage level in 9-11 day old SPF embryonated eggs.

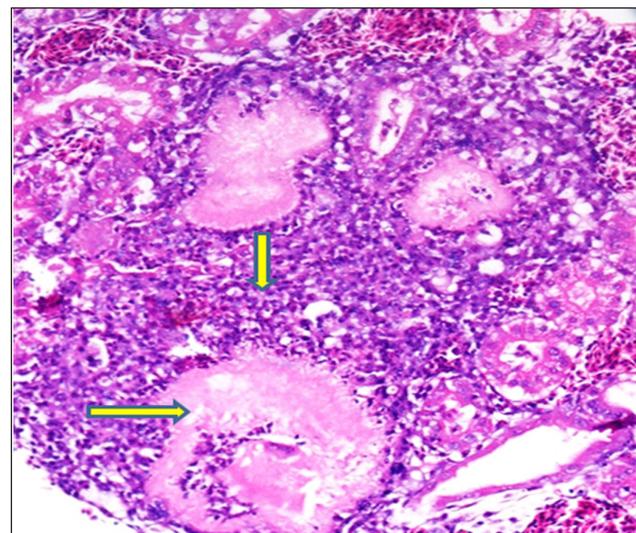


Fig. 4: Section of kidneys showing urate deposits (horizontal arrow) surrounded by mononuclear infiltrate (Vertical arrow) 200x H&E

However, in the present investigation curling and dwarfing in embryos was noted after seventh and eighth passage for both the isolates. Sreevastava, (2008) reported curling and dwarfing of embryos in very few isolates at third passage and further they stated that curling and dwarfing is more consistent at higher passage levels. Also, Dinakar *et al.* (2010) has clearly mentioned that, only egg adopted strains of IBV induce curling and dwarfing at third passage and field isolates do not do so until after several passages. Results of the present study also indicate that, field isolates may not induce typical curling and dwarfing of embryos after third passage in all cases and may need further passages to induce said changes upon inoculation. Hence, while isolating IBV in SPF embryonated chicken eggs several passages may be upto 7 or more be attempted before concluding results of isolation of IBV from field cases. The kidneys of embryos from above two cases revealed typical mottling due to deposition of urate crystals. Bayry *et al.* (2004) reported uric acid deposition in the kidneys of embryos inoculated with nephropathogenic IBV. Sreevastava, (2008) and OIE manual (2013) also reported similar changes in embryos upon inoculation of nephropathogenic strains of IBV. All above findings indicated that the two isolates (BVC/2017/VSD/IBV/South /1 and BVC/2017/VSD/IBV/South /2) obtained the present study were of nephropathogenic nature.

S1 gene amplification and sequence analysis

S1 gene was successfully amplified using IBV specific universal primers (Fig. 5) for both the isolates. The amplified products were visualized as a single compact band of expected 1600 bp size under UV light and documented by gel documentation system. The nucleotide and amino acid identities of S1 gene sequences of both isolates obtained in this study shared 92.7 to 96% 73.8%, 75.3% and 72.1% identity with other Indian IBV nephropathogenic strains, M41, H120 and 4/91 type, respectively which indicate substantial genotypic divergence from M41 and H120 (major respiratory and vaccine strain) and 4/91 strain. The only published data on the circulation of local Indian IBV variant was that of Bayry *et al.* (2005) who described the emergence in India of a unique nephropathogenic IBV. S1 gene sequence analysis of the two isolates in this study revealed high degree of similarity with reported nephropathogenic IBV isolates. In India chickens are

vaccinated against only Massachusetts M41 or H120 strains. It can be concluded that, strains other than vaccine are also circulating in the amongst chicken.

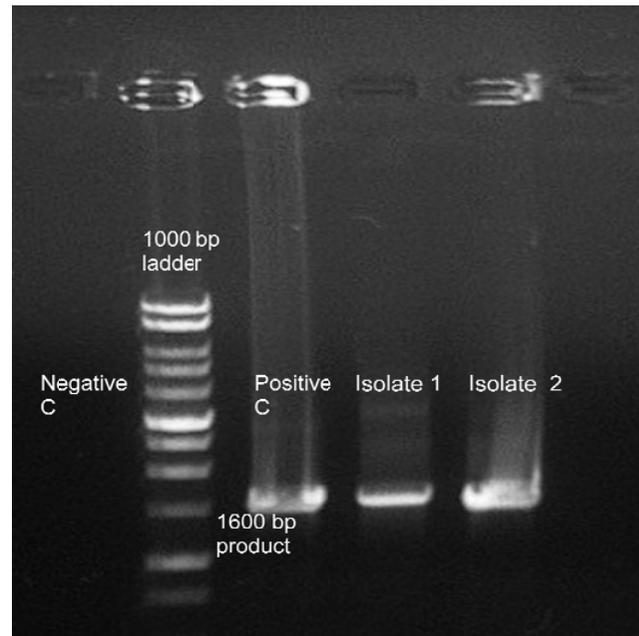


Fig. 5: Picture depicting results of PCR for amplification of S1 gene for isolates BVC/2017/VSD/IBV/south/1 and 2

Clinical picture, gross and microscopic Lesions

The clinical signs noted in birds of two farms showed isolation of IBV were depression, ruffled feathers, increased water consumption and vent pasting in few birds. Cumming, (1963) and Lee *et al.* (2004) also reported similar signs in nephropathogenic form. The mortality noted was 5 and 7% for two farms. Gaba *et al.* (2010) observed mortalities upto 30% in nephropathogenic form of IB. Cavanagh and Gelb (2008) reported 1% weekly mortality in cases involving nephritis. Meulemans, (1987) quoted that, 25% mortality is common in younger chicks affected with IB nephritis. There exists great variation in mortalities caused by nephropathogenic IBV which we think should be interpreted considering age of the affected chicken, virulence of the virus and secondary complications.

Necropsy of dead birds at both these farms revealed swollen and mottled kidneys in few birds without lesions of visceral gout and in few birds lesions of visceral gout.

Cumming, (1963) also described swollen kidneys with pale tubules and distension of ureters as principal lesions in case of nephropathic form. Visceral gout has been described by several authors in nephropathic form (Bayry *et al.*, 2004 and Gaba *et al.*, 2010).

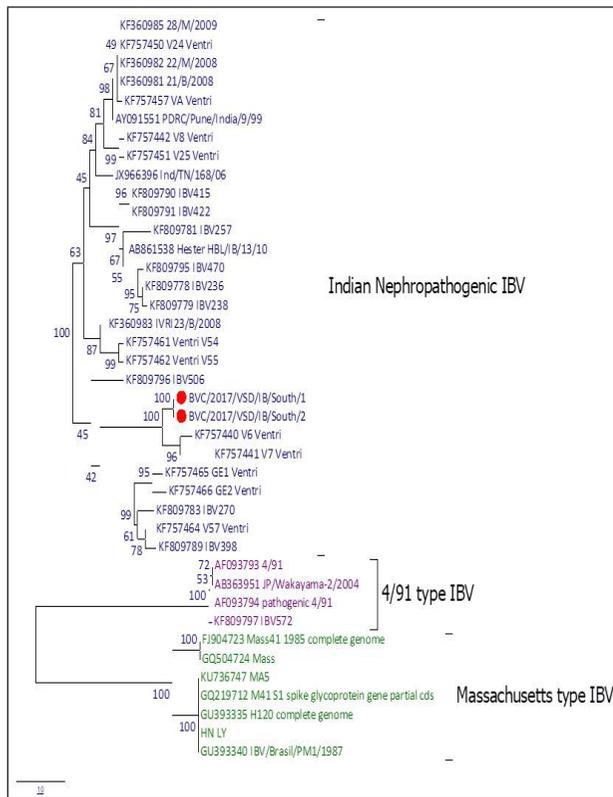


Fig. 6: Phylogenetic tree of S1 genes sequences for two isolates with the Indian Nephropathogenic isolates and the 4/91 and Massachusetts type reference strains

Trachea showed mild catarrhal inflammation only in few birds. Lungs, air sacs, spleen, gizzard, proventriculus, bursa of Fabricius did not revealed appreciable gross lesions. Histopathologically, there were focal areas of necrosis and multifocal areas of tubular degeneration in the kidneys. Kidneys also revealed focal deposition of urate crystals in tubules and glomeruli surrounded by zone of inflammatory cell infiltration predominantly mononuclear cells i.e. tophi (Fig. 4). Focally, the interstitium showed minimal to mild infiltration of inflammatory cells (predominantly mononuclear cells) and widening of interstitium. Albassam *et al.* (1986) described granular degeneration, vacuolation and desquamation of the tubular epithelium with massive infiltration of heterophils in the interstitium

in acute stages and interstitial lymphocytic nephritis in chronic stage of diseases as important microscopic lesions). Histopathologically, liver and heart showed urate deposits in capsule and pericardium respectively in few birds. Visceral gout has been described by several authors in nephropathic form (Bayry *et al.*, 2004 and Gaba *et al.*, 2010). Trachea revealed mild hyperplasia of goblet cells and congestion of vessels.

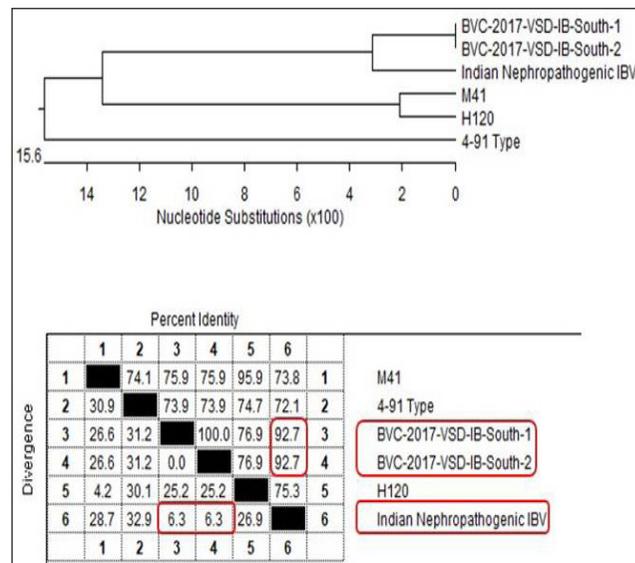


Fig. 7: Percent S1 protein similarity values for BVC/2017/VSD/IBV/South /1 and 2 versus selected avian IBV reference strains

Molecular Detection of 16s rRNA gene of *Mycoplasma gallisepticum*, NP gene of influenza A virus and H9 gene of avian influenza

Infectious bronchitis Virus was isolated from two cases out of total 10 cases (16.39%) which were found positive for IBV by real time RT-PCR amplification of 5'-UTR and RT-PCR amplification of N gene. In remaining 8 cases (From Raigad and Nasik region of MS) wherein isolation was not successful, 4 samples were positive for *Mycoplasma gallisepticum* and 2 were positive for H9 of avian influenza virus by PCR and RT-PCR respectively i.e. samples from 6 farms out of 10 positive cases showed mixed infections of tested pathogens. Gross lesions noted in birds from 4 out of above 6 cases also suggested *E. coli* infection although not confirmed by laboratory methods. As stated by Yashpal *et al.* (2004) avian pneumovirus (APV), avian influenza virus (AIV), infectious bronchitis virus

(IBV), Newcastle disease virus (NDV), and *Mycoplasma gallisepticum* (MG) are important respiratory pathogens. Roussan *et al.* (2008) also regarded same microorganisms as important respiratory pathogens. Some authors have also reported *Ornithobacterium rhinotracheale* and *Bordetella avium* as respiratory pathogens. Detection of other respiratory pathogens was not attempted in this experiment; however possibilities of their involvement in causation of disease are also there. Roussan *et al.* (2008) recorded 13 and 14.8% of these flocks positive for NDV and IBV, respectively, whereas 5.2, 6.0, 9.6, 10.4, 11.3, and 15.7% of these flocks were infected with both NDV and MG; MG and APV; IBV and NDV; IBV and MG; NDV and AIV; and IBV and AIV respectively. Similarly, Hassan *et al.* (2016) reported mixed infection of Infectious bronchitis virus along with avian influenza (H9N1) in 41.7% flocks tested. This clearly indicated that, respiratory form of IB may be complicated with other respiratory pathogens under field conditions. Isolation of the virus from such cases is challenging. Hence, studies involving treatment of inocula with neutralizing antibodies against organisms identified is necessary to get isolation successful. Whilst in case of nephropathogenic form, it is easy to suspect and even get isolation of virus in SPF embryonated eggs.

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