ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 8 (5): 493-499, 2009
ISSN 1682-8356
© Asian Network for Scientific Information, 2009

Nucleocapsid Gene Sequence Analysis and Characterization of an Indian Isolate of Avian Infectious bronchitis virus

Monika Kaul[1], Megha Kadam[1], Yashpal Malik[2], Ashok Kumar Tiwari[3],
Jawaharlal Vegad[4] and Bikash Chandra Sarkhel[1]
[1]Biotechnology Centre, Jawaharlal Nehru Krishi Vishva Vidyalaya, Jabalpur, India
[2]College of Veterinary Science and Animal Husbandry,
Jawaharlal Nehru Krishi Vishva Vidyalaya, Jabalpur, India
[3]Department of Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareily, India
[4]Laboratory of Phoenix Poultry Diagnostics, Jabalpur, India

Abstract: Avian Infectious bronchitis virus belongs to the family *Coronaviridae*. It is an enveloped virus with large positive stranded RNA genome. In the present study RNA was isolated from viral suspension and transcribed into cDNA. Poultry postmortem cases showing lesions of visceral gout were collected and infectious bronchitis virus were isolated. About 1.2 kb Nucleocapsid gene of virus was amplified by RT-PCR from four clinical samples. The amplified product was cloned and the nucleotide sequence of the N gene of an Indian field isolate was determined. The Indian IBV isolate exhibited 95% homology with Korean isolates and Chinese vaccine strains indicated conserved nature of N gene. Haemagglutination assay and chicken embryo inoculation was carried out for antigenic studies of the virus. The virus titre was confirmed using haemagglutination assay and IBVN2 showed the 1:2048+ titre. Propagation of virus was done by chorioallantoic method of inoculation of virus suspension in embryonated eggs. Characteristic curling and dwarfing of embryos was noticed in CAM inoculated embryonated eggs. Inoculated eggs showed teratogenic changes and deposition of urates as indication of naphropathogenic nature of virus.

Key words: Infectious bronchitis virus, chorioallantoic method inoculation, N gene, accession no. EF025537, nucleotide sequence, amino acid, virus isolate

INTRODUCTION

Avian Infectious Bronchitis Virus (IBV) is an acute viral disease of chicken caused by avian infectious bronchitis virus and results in a highly contagious disease of great economic importance to the poultry industry (Calnek, 1997). Initially, IB was recognized as a disease of young chicks. However, it was later observed commonly in semi-mature and laying flocks, hence, all ages are susceptible to this infection. The virus drastically affects poultry industry by causing respiratory disease, reduced performance, reduced hatchability and fertility, retardation in sexual maturity, nephrosis, irreparable damage to reproductive organs, reduced egg quality, increased susceptibility to infections, decreased profit at slaughter and costly vaccination programme. The inclusion of IBV in Office International Des Epizootes (OIE) list B diseases signifies its economic importance. The disease is usually controlled by using live and inactivated vaccines through spray/ocular or intra-muscular injection. The vaccination is further complicated through the occurrence of multiple serotypes of IBV. IBV has always been something of a moving target for many reasons, such as wide variations in the serotypes and virulence of strains that have developed from time to time, a highly contagious nature and the evaluation of specific tissue tropism and recombinants due to simultaneous infection of multiple virus types and use of live vaccines. Vaccination is only partially successful due to continual emergence of antigenic variants and requires the application of multiple vaccines at many sites due to the simultaneous presence of multiple antigenic types (Bayry *et al*., 2005). Avian infectious bronchitis virus is the Prototype of the family *Coronaviridae* (Bouqdaoui *et al*., 2005). The virus has a single stranded positive sense RNA genome of 27.6 Kb in size (Boursnell *et al*., 1987). Being a positive strand virus the viral RNA can serve directly as mRNA. It is an enveloped virus and is thus, more sensitive to treatment with ether and other lipid solvents. Some virus specific proteins have been identified; the Spike glycoprotein (S), the Membrane glycoprotein (M) and the phosphorylated Nucleocapsid protein (N). The N protein varies from 377-455 amino acids in length and is the most abundant virus derived gene produced throughout infection (Hiscox *et al*., 2001). Size of the N gene ORF is 1230 bases in length (Liu *et al*., 2005) which, lies within the typical range for most IBV strains. The N protein plays a role in viral replication, assembly and immunity. It interacts with leader RNA sequences facilitating viral mRNA synthesis and also binds to the viral RNA forming a helical nucleocapsid (Williams *et al*., 1992). The N protein of Coronavirus is overall very basic and contains

a high proportion of Serine residues, which act as sites for phosphorylation. In addition, the N gene is highly conserved among different IBV strains (Williams *et al*., 1992). Several functions have been postulated for the *Coronavirus* N Protein throughout the virus life cycle (Hiscox *et al*., 2001). Primarily, it complexes with the *Coronavirus* genomic RNA to form a ribonucleocapsid structure and it has been observed, together with the M protein, to be a component of the viral core and moreover its association with leader RNA Sequence leads to the conclusion that it has a role in replication of genomic RNA, in transcription of *coronavirus* subgenomic RNAs and in translation from the subgenomic RNAs (Hiscox *et al*., 2001). Seo *et al*. (1997) reported for the first time that specific CTL epitopes to nucleocapsid of IBV can play a central role in protecting chickens from acute viral challenge. So, the N gene is thought to be an appropriate candidate, which should be explored for possibilities to develop diagnostics and vaccine against infectious bronchitis.

In India Verma first reported the prevalence of IBV infection in chicks in 1964. Since then, IB has showed its presence in almost all parts of the country but no significant study has been carried out for N gene. On the basis of S1 genotype study emergence of IBV variant has been reported (Bayry *et al*., 2005) and also an effort for development of DNA vaccine using S1 been (Sylvester, 2005) gene has been reported. But no such studies have been done with N gene from Indian isolates, in spite of the fact that N gene is highly conserved in nature and has immunopotential (Hiscox *et al*., 2001). In India IB usually goes un-noticed as naphropathogenic strain becomes an etiological agent of visceral gout and IB is confused with metabolic visceral gout. Therefore, the present investigation was planed to detect IBV from clinical cases of visceral gout by cloning and sequencing of N gene and characterization of virus by virological methods.

MATERIALS AND METHODS

Isolation of avian infectious bronchitis virus from clinical cases: The present study was carried out for unvaccinated flock in central part of India, which is supposed to be free from IBV disease. Since, IBV has been reported to be one of the etiological agent to cause visceral gout in poultry so the cases of visceral gout were studied and virus was isolated from visceral gout affected kidneys, lungs and trachea. The virus was isolated by the method of Zhou *et al*. (2004), with some modifications. Specimens of kidneys, lungs and trachea were collected from postmortem cases of affected birds. To isolate the virus, tissue sample of kidney, lung and trachea was homogenized (50% w/v) and the suspension was subjected to freeze-thawing for three times. The homogenate was centrifuged at 7500 rpm for 20 min at 4°C. The supernatant was collected and filtered through 0.45 µm syringe filter. The supernatant collected was used for isolation of viral RNA and also stored at -20°C until further use.

Isolation of viral RNA: RNA Isolation. All the glassware used for RNA work was treated with DEPC. RNA isolation was done by using TRIZOL (Sigma, U.S.A) reagent as per manufacturer's instructions with some modifications. All the RNA samples isolated were used for RT-PCR.

Reverse transcriptase PCR (RT-PCR): cDNA synthesis from different IBV isolates. Reverse transcription was carried out following the protocol standardized for vaccine strain (Mass, Venkateshwara hatchery). For cDNA synthesis a 20 µL reaction volume comprising of 7 µL of total RNA, 2 µL each of forward and reverse primers, 3 µL DEPC (Sigma, U.S.A) treated water were added and incubated at 70°C for 5 min and then snap chilled on ice for 2 min. The 1 µL dNTP, 4 µL of 5x RT buffer were added and incubated at 37°C for 5 min. The 1 µL M-MuLv reverse transcriptase (MBI, Fermentas, Germany) was finally added. The reaction mixture was incubated at 42°C for 1 h, followed by incubation at 70°C for 10 min to arrest the further enzyme activity and then stored at -20°C until further use. Specific primer set containing forward primer IBF having the sequence 5'-CCCGGATCCATGGCAAGCGGTAA-3' with restriction site of *Bam*HI and reverse primer IBR having the sequence 5'-CCCGTCGACTCAAAGTTCATTTTCAC-3' with restriction site *Sal*I were designed using Lasergene DNA STAR software with the help of published sequences and little modification was done to obtain a desired restriction sites in the primer sequence. These specific primers were used to amplify the gene for cloning in InsT/A cloning vector (MBI Fermentas, Germany).

Amplification of N gene by PCR standardization: Specific primer set containing forward primer IBF and reverse primer IBR were used to amplify the gene for cloning in InsT/A cloning vector. PCR was performed using 25 µL reaction volume, containing 1 µL of cDNA, 2.5 µL of Taq DNA polymerase buffer, 1 µL of dNTP mixture, 1 µL of forward and 1.5 µL of reverse primer, 0.5 µL of $MgCl_2$ and 1 µL of Taq DNA polymerase. The final volume was made with sterile milli-Q water. The thermo cycling steps were carried out in Px2 thermal cycler (Thermo, India) consisting of initial denaturation at 95°C for 5 min followed by 30 cycles, each cycle consisting of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 20 min. The PCR products generated were confirmed for their size in 1% agarose gel in 0.5x TBE buffer as per the method of Sambrook and Russel (2001).

Cloning of N gene in InsT/A cloning vector: Puration of PCR production. After amplification of N gene by specific primers, the amplified product was subjected to purification using Gen Elute™ Gel extraction kit (Sigma, USA). Protocol was followed as per the manufacturer's instruction with some modifications.

Ligation: The PCR amplified 1.2 Kb gene encoding nucleocapsid protein was ligated in InsT/A cloning vector in a ratio of 3:1. About 10 µL of the purified PCR product and 1 µL of InsT/A cloning vector (MBI Fermentas) were ligated in 25 µL reaction with 2.5 µL ligase buffer (MBI Fermentas), 1 µL of T4 ligase enzyme and 2.5 µL PEG. The final volume was made with nuclease free water. The ligation reaction was carried out in a thermomixer (Eppendorf, Germany) at 16°C for 18 h and the ligated product was subsequently used for transformation.

Transformation: For transformation 10 µL ligation mixture, was diluted to, 200 µL TCM (1x). To this, 200 µL of the competent cells were added and mixed gently. The mixture was kept on ice for 1 h. Heat shock was given for 45 sec at 42°C and rapidly chilled on ice for 10 min. The 1 mL SOB, 20 µL glucose and 10 µL $MgCl_2$ was added and incubated at 37°C for 2 h with shaking. The transformed cells were spread on LB agar plate containing ampicillin (75 µg/mL), X-gal and IPTG under sterile conditions. Appropriate positive and negative controls were processed simultaneously. On transformation in *E. coli* DH5α transformed cells characteristically produced white and blue colonies on agar containing X-gal and IPTG and ampicillin.

Screening of recombinant InsT/N clones: Screening of the colonies was done by picking ten white colonies in LB broth containing ampicillin (75 µg/mL). The plasmid DNA was extracted by miniprep plasmid isolation method (Sambrook and Russel, 2001).

Agarose gel electrophoresis: Plasmid was confirmed on 0.8% Agarose in 0.5x TBE buffer as per the method of Sambrook and Russel (2001) using horizontal submarine electrophoresis and staining with Ethidium Bromide. The gel was examined on UV-transilluminator and photographed by gel documentation system.

Restriction analysis of recombinant clones: The plasmid DNA isolated from positive clones was subjected to restriction digestion. *Bam*HI and *Sal*I restriction enzymes were used for digestion as the primers designed for amplification was having site for these two restriction enzymes. After digestion 1.2 Kb insert was released from 2.8 Kb vector.

Colony PCR: Clone was also confirmed by colony PCR using the specific primers. Colony PCR was done according to the PCR standardized for amplification of N gene. Single colony was picked as template DNA for amplification and PCR was performed.

Sequencing: Plasmid InsT/N1 containing the insert was selected for nucleotide sequencing and sequencing reaction was done using IBF and IBR forward and reverse primers, respectively and a complete sequence of 1230 bp was sequenced. Sequencing was done on 310 Genetic Analyzer (ABI). Purified Plasmid designated as IBVN1 was used for sequencing. Following sequencing reaction was used for Extension

Reaction

Sequence terminator ready reaction mix	4 µL
2.5x sequencing buffer	4 µL
DNA template	100 ng
Primer	3.2 pm
Deionized water	to make final volume 20

Two reactions were performed using IBF and IBR primers, respectively. Following Protocol was used for sequence extension in thermal cycler.

95ºC for 5min	
95ºC for 30 sec	
Rapid thermal ramp to 50ºC	Repeated 30 cycles
50ºC for 10 sec	
Rapid thermal ramp to 60ºC	
60°C for 4 min	
Rapid thermal ramp to 4°C	

Purification: After completion of reaction, reaction mixture was precipitated and extracted with 75% isopropanol to remove dDNTPs and salts.

Setting of sequencing reaction: The pellet was dissolved in 20 µL of Hi di Formamide, heated at 95°C for 5 min and immediately chilled on ice. The Sequencing mix was then put for Sequencing in 60 mm capillary containing POP6 polymer (ABI). The Sequence was analyzed using Genetic analyzer. The sequence reported here has been submitted to the Genbank nucleotide database and has the Accession no. EF025537. A nucleotide sequence portion of 1230 bp containing the complete coding region of N gene of the isolate IBVN1 was aligned with other isolates in the Megalign programme of Lasergene software (DNA STAR, USA). Sequence similarity with foreign isolates was also confirmed by Clustal V method. The amino acid sequence was deduced from 1230 bp nucleotide sequence and a complete coding sequence was generated. Amino acid sequence was aligned with other sequences and sequence similarity report was generated.

Antigenic studies of virus

Haemagglutination assay: Total 50 µL of normal saline was added in all the wells in row of 96 well microtitre plate. The 50 µL of IB virus (Antigen) was added to the first well and a two fold dilution was carried out. The 50 µL of 1% chicken RBC was added to all the wells in a row. The plate was kept at RT for 40 min and observed for haemagglutination reaction.

Virus propagation in embryonated eggs using Chorio-Allantoic Membrane (CAM) route: Characterization of virus was done by injecting 120 µL of virus suspension through CAM inoculation in nine days old embryonated eggs. On the 4th-5th day of the post-inoculation eggs were chilled at 4°C for 1 h and embryos were collected taking all sterile precautions avoiding egg yolk. Allantoic fluid was also collected for further viral passage.

RESULTS AND DISCUSSION

Out of 43 clinical samples, which were processed for viral RNA isolation and RT-PCR four samples showed positive amplification of 1.2 kb N gene Fig. 1a. On transformation in *E. coli* DH5α, transformed cells characteristically produced white colonies on agar containing X-gal and IPTG. Plasmid from white colonies were isolated and confirmed by agarose gel electrophoresis. On digestion of InsT/N with *Bam*HI and *Sal*I Restriction enzymes 1.2 Kb insert was released Fig. 1b. Clone was also confirmed by colony PCR using specific primers. Plasmid InsT/N1 containing the insert was selected for nucleotide sequencing and sequencing reaction was done using IBF and IBR forward and reverse primers, respectively and a complete sequence of 1230 bp was sequenced. The plasmid containing the insert was selected for sequencing and the sequence reported was submitted to the Genbank nucleotide database and has the Accession no. EF025537. Sequencing was done with both forward and reverse primers and both

Table 1: Description of the IBV strains with the Gene bank accession numbers used in this study for comparison

Isolate/strain	Origin	Accession no.
Jilin	China	AY839145
JP8147	Japan and Taiwan	AY363966
K43401	Korea	AY790346.1
K507-01	Korea	AY790355
K203-02	Korea	AY790355
IBN	China	AY856349
HB-CH	China	DQ473615
h52	China	AF352310
Ckch031	China	AY839137
Ckch411	China	DQ352156
Ckch991	China	AY842864
J	China	DQ084440
JAAS	China	AY839138

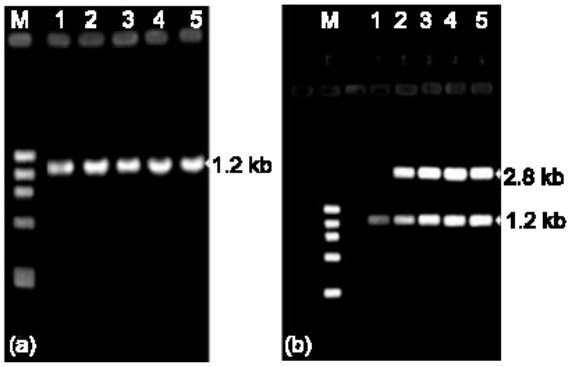

Fig. 1: a): About 1.2 kb PCR amplified N gene from Indian isolate of IBV. Lane M-DNA ladder Phi X174 DNA/HaeIII Digest (Bangalore Genei), Lane1-4: 1.2 Kb N gene from IBVN isolates, Lane5- Positive control; PCR amplified fragment from Mass strain, b): Restriction digestion analysis of InsT/N clones containing 1.2 Kb N gene insert digested by *Bam*HI and *Sal*I enzymes, Lane M-DNA ladder Phi X174 DNA/HaeIII Digest (Bangalore Genei), Lane1-PCR amplified N-gene fragment, Lane2-5 N-gene fragment released from InsT/N1 plasmid

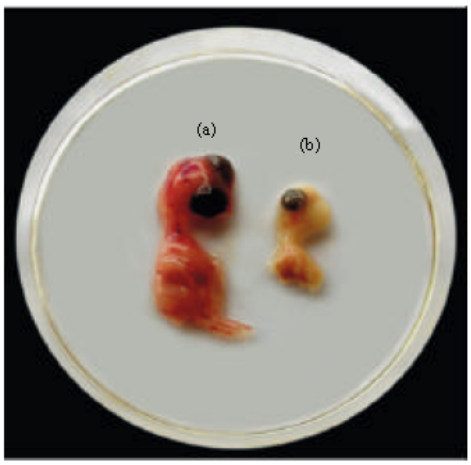

Fig. 2: Showing 16 days old embryonated eggs infected with IB virus, a): Uninfected embryo, b): Infected embryo showing dwarfing and curling

primers amplified around 800 bp sequence from 5 and 3, respectively with high degree of confidence, the sequences was then joined using Seascape software ABI. Sequence was aligned with other published sequences Table 1 and pairwise sequence distance and phylogenetic study was done for both nucleotide Table 2a and b and aminoacid sequences Table 3a and b (Fig 1a and b, Table 2a and b, Table 3a and b).

(a) Percent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		86.9	87.6	88.4	93.5	93.0	93.3	87.3	89.9	95.6	95.5	92.8	92.8	94.1	1	IBVN1
2	13.6		91.2	88.2	86.3	86.7	84.7	89.4	86.9	86.8	86.7	86.0	87.3	83.2	2	chck0031
3	12.8	8.6		91.6	87.3	87.6	86.4	86.6	88.4	87.9	87.7	87.3	87.6	88.2	3	chck411
4	11.5	11.4	8.4		86.7	86.7	87.3	85.0	88.0	88.0	87.8	87.3	87.3	88.0	4	chck911
5	6.6	14.1	13.0	13.1		97.7	89.9	87.1	89.7	93.4	83.4	90.7	91.1	91.6	5	h52
6	7.1	13.7	12.7	13.1	2.2		90.2	87.3	89.0	93.0	93.0	90.1	90.9	91.3	6	HB-CH
7	6.4	15.6	13.8	12.6	9.9	9.6		85.7	88.0	92.8	92.7	90.1	90.5	91.1	7	Ibn
8	13.4	10.3	13.3	14.8	13.4	13.0	14.6		86.8	87.4	87.6	86.8	87.2	87.9	8	J
9	10.4	13.3	11.8	12.3	10.1	10.9	12.1	13.5		89.6	89.4	87.2	88.0	88.3	9	JAAS
10	4.6	13.6	12.4	12.0	6.7	7.1	6.7	13.1	10.7		99.4	93.0	94.2	94.4	10	Jilin
11	4.6	13.7	12.6	12.1	6.6	7.0	6.9	13.0	10.9	0.6		93.1	94.1	94.5	11	JP8147
12	7.7	14.5	13.3	12.9	9.6	10.1	9.8	13.8	13.1	7.0	7.0		95.5	96.4	12	K203-02
13	6.9	13.6	12.3	12.3	8.7	9.0	8.8	12.8	11.8	5.3	5.3	4.1		96.7	13	K507-01
14	6.3	13.2	12.1	12.1	8.7	8.8	8.4	12.6	11.8	5.5	5.5	3.6	2.7		14	K43401
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Divergence

(b)

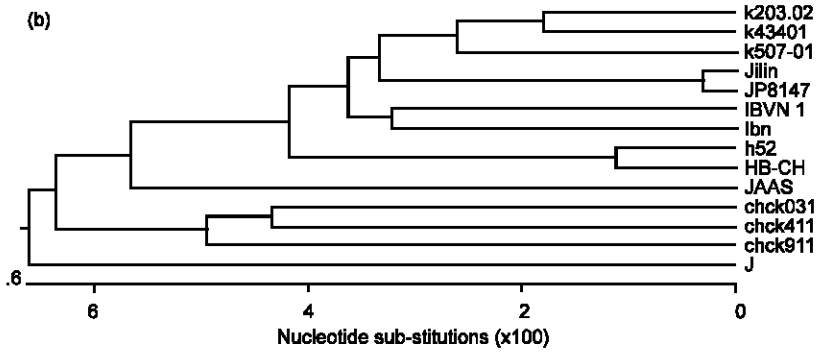

Table 2: a): Percentage nucleotide similarity and divergence based on the nucleotide sequences of 1230 bp coding region of N gene from different Infectious bronchitis viruses, b): Phylogenetic tree (Cladogram) based on the nucleotide similarity of 1230 bp coding region of N gene from different Infectious bronchitis viruses

Antigenic studies of the virus were also carried out byvirus propagation in embryonated eggs. All the IBV field isolates and the vaccine strain were adopted in Embryonated Chicken Eggs (ECE). Majority of the isolates showed teratogenic changes like curling and dwarfing of embryo five days post inoculation. Thinning of CAM and deposition of urates were also noticed in all the four positive samples. Haemagglutination properties of the virus were studied using haemagglutination assay of all four positive samples, which resulted a highest titre of 1:2048+ in IBVN2 sample (Fig. 2).

The present study was carried out with the object of developing a more rapid and confirmatory diagnosis for the detection of IBV. More than 50% of the isolates showed the signs of infectious nephritis rather than respiratory lesions. This clearly indicates the shifting of pathogencity and tissue tropism from the predominantly respiratory to nephropathogenic. The investigation also revealed that the amplified N gene is highly conserved in nature. Specific primers were used for the amplification of N gene. The selection of appropriate primers for use in the RT-PCR is essential for its specificity (Zwaagastra *et al*., 1992). Sequencing of amplified and cloned N gene revealed conserved nature of N gene when compared with different N gene sequences of various foreign strains. The sequence showed around 95% similarity with many Asian isolate sequences especially with Jilin, which are vaccine strain of China and with Korean strains. Phylogenetic analysis of nucleotide show close relation ship of IBN a Chinese isolate. Size of the coding sequence found in present study was similar to finding of (Zhang *et al*., 2005; Liu *et al*., 2005). Deduced amino acid showed variations of around 17 amino acids, which may be involved in changing the nature of N gene. Amino acid analysis again revealed high percentage of sequence similarity among Asian strains. Results of phylogenetic analysis of aminoacid sequences differed little from that of nucleotide. This indicated that variation in amino acid sequence by 17 amino acids may result in alteration in pathogenicity of virus. Different strains of IBV isolated

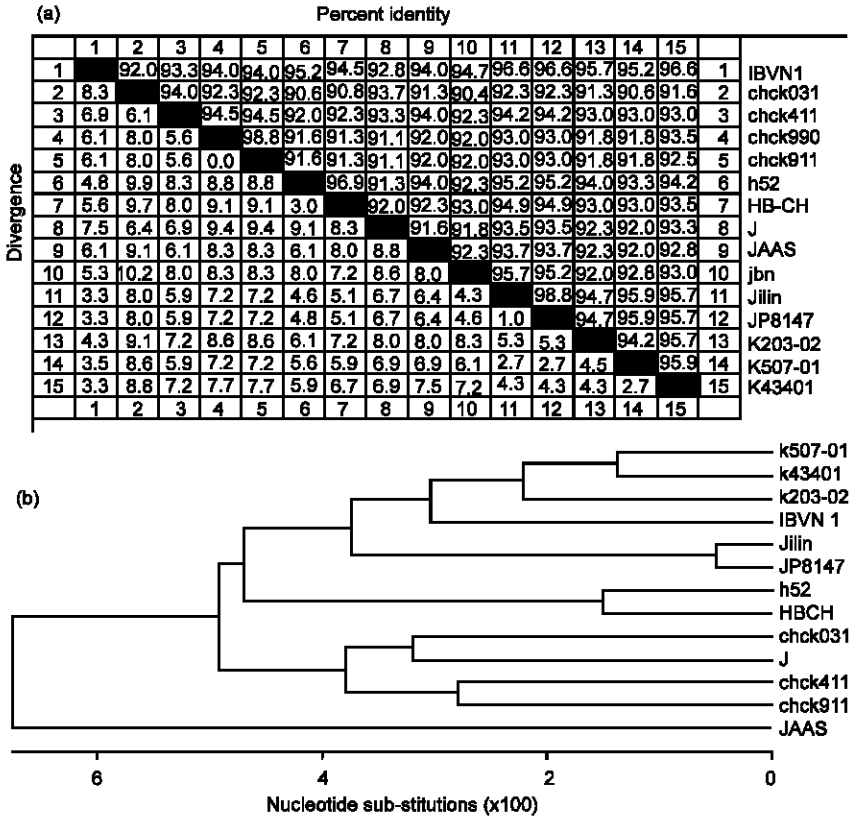

Table 3: a): Percentage amino acid similarity and divergence based on the deduced amino acid sequences of nucleocapsid protein from different Infectious bronchitis viruses, b): Phylogenetic tree (Cladogram) based on the deduced amino acid sequence of nucleocapsid protein from different Infectious bronchitis viruses

from diverse location for N protein has shown to be highly conserved (Sapats *et al*., 1996). Pairwise distance analysis of nucleotide sequence showed higher similarity with Jilin strain and Korean strains. While, showed more divergence with Tiwan, Japanese and other Chinese isolates. Phylogenetic analysis indicated close relationship with Korean isolates and Jilin. In central India vaccination against IBV is not being practiced so, it is assumed that the virus isolated in present study is an emergent of IBV, which is causative agent of acute nephritis and visceral gout. Sequence homology of N gene with Chinese vaccine strain indicates that the N gene of Indian isolate can be exploited as a vaccine agent against IBV in India. Antigenic studies of the virus were also indicated nephropathogenic nature of virus and lesions found in IBV were also reported by Fabricant (1949) as pathognomonic lesion of IB. The clinical manifestations, gross lesions of the infected Chicken embryo were the same as those infected with nephropathogenic IBVs (Calnek, 1997; Zhou *et al*., 1998). HA is described to be specific, very sensitive, accurate, highly reproducible, simple and economical test. The test was found to have a good correlation with RT-PCR (Ruano *et al*., 2000). So, the virus was subjected to haemagglutination assay and the highest titre of 1:2048+ was found in IBVN2 sample. For knowing the effect of sequence variation on protein expression, characterization of protein is required. The present investigation documents several outbreaks of emerging IBV virus infection in this region of India. High genetic drift and shift in the IBV genome may be the cause of multiple genotypes of IBV. The study can be concluded by saying that N gene shows potential to be used as subunit vaccine but further characterization of N protein is essentially recommended.

ACKNOWLEDGEMENTS

We sincerely thank Dr. B.C. Sarkhel (Professor-Incharge BTC, JNKVV, Jabalpur) for providing necessary facilities to carry out this study. We are also thankful to Phoenix Diagnostic Laboratory (Jabalpur) for supplying IBV field samples used in this study.

REFERENCES

Bayry, J., M. Goudar, P.K. Nighot, S.G. Kshirsagar, B.S.J.G. Ladman Jr. G.R. Ghalsasi and G.N. Kolte, 2005. Emergence of a nephropathogenic avian infectious bronchitis virus with a novel Genotype in Ind. J. Clin. Microbiol., 43: 916-918.

Bouqdaoui, M.E., R.A. Mhand, H. Bouayoune and M.M. Ennaji, 2005. Genetic grouping of Nephro-pathogenic avian infectious bronchitis virus isolated in Morocco. J. Poult. Sci., 4: 721-727.

Boursnell, M.E.G., T.D.K. Brown and I.J. Foulds, 1987. Completion of the Sequence of the genome of the Coronavirus Avian Infectious bronchitis virus .J. Gen. Virol., 68: 55-77.

Calnek, B.W., 1997. Diseases of Poultry, 10th Edn. Iowa State university press, Ames, IO.

Fabricant, J., 1949. Studies on the diagnosis of New-Castle disease and Infectious bronchitis of Fowl II. The diagnosis of Infectious bronchitis by virus isolation in chick embryos. Cornell Vet., 39: 414-431.

Hiscox, J.A., T. Wurm, L. Wilson, P. Britton, D. Cavanagh and G. Brooks, 2001. The Coronavirus infectious bronchitis virus Nucleoprotein localizes to the nucleolus. J. Virol., 75: 506-512.

Liu, S., J. Chen, X. Kong, Y. Shao, Z. Han, L. Feng, X. Cai, S. Gu and M. Liu, 2005. Isolation of avian infectious bronchitis Coronavirus from domestic peafowl (*Pavocristatus*) and teal (*Anas*). J. Gen. Virol., 86: 719-725.

Ruano, M., J. El-Attrache and P. Villegas, 2000. A rapid plate haemagglutination assay for the detection of Infectious bronchitis virus. Avian Dis., 44: 99-104.

Sambrook, J. and D.W. Russel, 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, A8.40-A8.54.

Sapats, S.I., F. Ashton, P.J. Wright and J. Ignjatovic, 1996. Novel variation in the N protein of Avian Infectious bronchitis virus. Virology, 226: 412-417.

Seo, S.H., L. Wang, R. Smith and E.W. Collisson, 1997. The carboxyl-Terminal 120 residue polypeptide of infectious bronchitis virus Nucleocapsid induces cytotoxic T lymphocytes and protects chicken from Acute infection. J. Virol., 71: 7889-7894.

Sylvester, A., 2005. Molecular characterization and development of a DNA vaccine against Avian Infectious Bronchitis Virus (dissertation), pp: 25. Available from: Deemed University Indian Veterinary Research Institute, India.

Verma, K.C., 1964. Studies on Infectious bronchitis virus. (dissertation). Available from Agra University, pp: 3.

Williams, A.K., L. Wang, L.W. Sneed and E.W. Collisson, 1992. Comparative analyses of the Nucleocapsid genes of several strains of infectious bronchitis virus and other Coronaviruses. Virus Res., 25: 213-222.

Zhang, D.Y., J.Y. Zhou, J. Fang, J.Q. Hu, J.X. Wu and A.X. Mu, 2005. An ELISA for antibodies to Infectious bronchitis virus based on nucleocapsid Protein produced in *Escherichia coli*. Vet. Med-Czech, 50: 336-344.

Zhou, J.Y., L. Yu and J. Frong, 1998. Isolation and identification of Chicken's Infectious bronchitis nephritis Virus. J. Zhejiang Agric. Uni., 24: 303-307.

Zhou, J.Y., D.Y. Zhang, J.X. Ye and L.Q. Cheng, 2004. Characterization of an Avian Infectious bronchitis Virus isolated in China from Chicken with nephritis. J. Vet. Med. B,inf. Dis. Vet. Pub. Health, 51: 147-152.

Zwaagastra, K.A., B.A.M. Van der Zeijst and J.G. Kusters, 1992. Rapid detection and identification of avian Infectious bronchitis virus. J. Clin. Microbiol., 30: 79-84.