SEQUENCE ANALYSIS OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM INDIA: PHYLOGENETIC RELATIONSHIPS

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Received February 14, 2003; accepted June 30, 2003

Summary. – Prevalence of infectious bursal disease (IBD) among chickens in different parts of Tamil Nadu, India, has been studied by collection of bursal samples from suspected flocks and by performing reverse transcription–polymerase chain reaction (RT-PCR) for amplification of a specific product of 474 bp from the variable region of the VP2 gene. Among 53 bursal samples examined by RT-PCR, 40 showed a positive reaction. The amplified products were subjected to nucleotide sequencing and the obtained sequences were compared with those of IBD virus (IBDV) vaccine strain Georgia, the classical virulent strain 52/70 and the very virulent Japanese OKYM strain. Nucleotide homology data indicated that all the Tamil Nadu isolates showed homology ranging from 91 to 99.6% among themselves. When compared with the very virulent Japanese OKYM strain, four isolates grouped with that strain. Majority of the isolates clustered with the very the virulent OKYM strain as evident from phylogenetic analysis performed using the MEGA program. Comparison of the deduced amino acid sequences of IBDV isolates with those of the vaccine strain Georgia, the classical virulent strain 52/70 and the very virulent strain OKYM also revealed the presence of conserved serine-rich heptapeptide sequence in most of the isolates. Results of this study indicate that majority of the IBDV isolates are very virulent, which is evident from heavy mortality that has been reported in few flocks of poultry in spite of regular vaccination.

Key words: Infectious bursal disease virus; sequence and phylogenetic analysis; Tamil Nadu

Introduction

IBDV affects the bursa of Fabricius of young chickens, resulting in mortality, morbidity and/or immunosuppression. Immunosuppression enhances the susceptibility of chickens to other infections and interferes with vaccination against other diseases. IBDV is a member of the *Birnaviridae* family, which contains two segments, A and B, of double-stranded RNA. Genome segment B is approximately 2800 bp long and encodes VP1, the putative dsRNA polymerase whereas the genome segment A of approximately 3300 bp encodes a precursor poly-protein that is processed into mature VP2, VP3 and VP4. VP2 contains the antigenic regions responsible for neutralizing antibodies and serotype specificity. It is also highly conformation-dependent and is a major host-protective antigen of IBDV. Out of two serotypes of virus reported, only the serotype 1 virus produces a clinical disease in chickens. Very virulent and classical pathotypes as well as antigenic variants of serotype 1 virus are also prevalent world wide (Jackwood and Saif, 1987; Snyder, 1990; Sapats and Ignjatovic, 2000) and in Tamil Nadu (Nachimuthu *et al*., 1993). In India, very virulent pathotypes of IBDV have emerged in late 1992, devastating the poultry industry due to heavy mortality even in vaccinated flocks (Sah *et al*., 1995).

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Abbreviations: IBD = infectious bursal disease; IBDV = IBD virus; RT-PCR = reverse transription–polymerase chain reaction

Materials and Methods

In this study, the VP2 gene of IBDV isolates obtained from different parts of Tamil Nadu, Southern India during the period 1999–2002 has been amplified and sequenced and the nucleotide sequences have been compared with a classical virulent strain, a very virulent Japanese strain OKYM and a vaccine strain (Georgia) to understand the nature of prevalence of IBDV.

Bursal specimens. A total of 53 bursal specimens from outbreaks occurring in different geographic regions of Tamil Nadu over the period of 1999–2002 were used in this study.

Total RNA was extracted after homogenizing bursal tissues in TNE buffer (100 mmol/l Tris.HCl pH 8.0; 150 mmol/l NaCl, 10 mmol/l EDTA) as suggested by Lin *et al*. (1993) using the RNA isolation reagent (ABgene, UK). Briefly, 300 µl of bursal extract was mixed well with 900 µl of the RNA isolation reagent and left to stand on ice for 10 mins. Chloroform (200 µl) was added, mixed and kept on ice for 10 more mins. Then the mixture was centrifuged at 10,000 rpm for 15 mins at 4°C. The supernatant (about 750 µl) was transferred to another 1.5 ml eppendorf tube and an equal volume of cold isopropanol was added, mixed well and kept on ice for 10 mins. Then it was centrifuged at 10,000 rpm for 20 mins at 4°C. The RNA pellet was washed once with 75% ethanol, air-dried and resuspended in 20 µl of diethylpyrocarbonate (DEPC)-treated water. The tube was kept at 37°C for 30 mins for complete dissolution of RNA.

RT-PCR. RNA samples were subjected to RT-PCR using the Robust RT-PCR Kit (Finzymes, Finland) with a pair of primers specific for VP2 gene as suggested by Lin *et al*. (1993): forward primer 5'-CCA GAG TCT ACA CCA TA-3' and reverse primer 5'-TCC TGT TAC CAC TCT TTC-3'. The RT-PCR mixture contained 10 µl of denatured RNA sample, 5 µl of 10X reaction buffer, 1.5 µl of 50 mmol/l $MgCl_2$, 10 pmoles of each primer in 1 µl, 0.5 µl of an RNase inhibitor, 1 µl of 10 mmol/l dNTPs, 1 µl (5 U) of AMV reverse transcriptase, 2 µl of Dynazyme EXT DNA polymerase containing 1U/µl and 27 µl of DEPC-treated water to give a final volume of 50 µl. cDNA was synthesized at 42°C for 60 mins and was denatured at 94°C for 2 mins. PCR amplification was carried out in 25 cycles, each cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and strand extension at 72°C for 2 mins. The final extension step was carried out at 72°C for 10 mins. The amplified products were analyzed on a 1.5% agarose gel. The presence of 474 bp product was taken as positive.

Fifteen amplified products were sequenced in an automated sequencer. The sequences were compared by computer program-Clustal X for analysis and multiple alignments. Nucleotide sequences of isolates obtained from different parts of Tamil Nadu were compared with an IBD vaccine strain (Georgia strain), a classical virulent strain (52/70) and a very virulent Japanese OKYM strain.

Results

Bursal tissues from infected flocks were collected from different areas of Tamil Nadu. RNAs extracted from 53 bursal tissues were subjected to RT-PCR using primers specific for variable region in the VP2 gene of IBDV. Forty RNA samples were amplified with a specific product of 474 bp (Fig. 1).

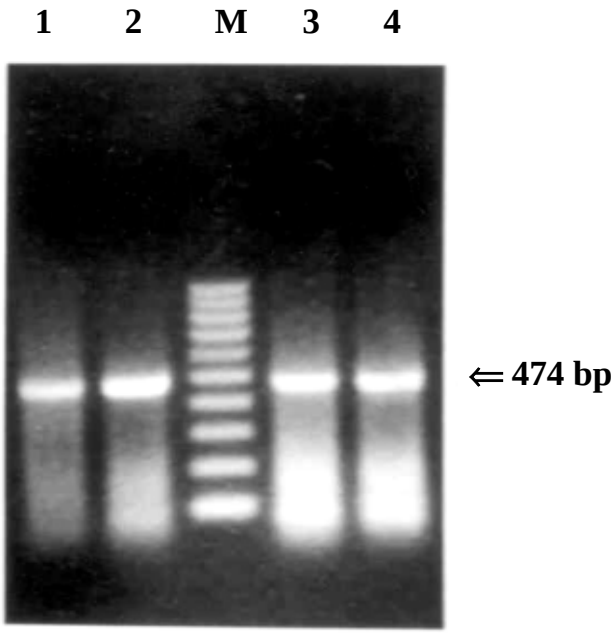

Fig. 1

Agarose gel electrophoresis of RT-PCR products of IBDV isolates

IBDV isolates (lanes 1–4). 100 bp DNA ladder (lane M).

The nucleotide sequences of the variable region in the VP2 gene of the IBDV isolates were obtained and compared with those of the vaccine strain Georgia. The homology ranged from 87.2% to 98.3% and only one isolate (TNK 1997) was found closer to the vaccine strain (98.3%). The nucleotide sequences comparison with the classical virulent IBDV strain 52/70 resulted in homology ranging from 89.3% to 95.7%. The isolates TNK 2002/1 (95.7%), TNK 2001/3 (95.3%), TCB 2001/2 (95.3%), and TTN2001 (95.3%) showed high homology with the strain 52/70 (Table 1).

When nucleotide sequences of the isolates were compared with the very virulent Japanese strain OKYM, the homology ranged from 91.5% to 98.7%. The isolates TCB 2001/2 (98.7%), TSL 2001 (98.7%), TNK 2001/3 (97.9%), TCN 2001 (97.9%), TNK 1999/1 (97.5%), TNK 2002/2 (97.5%) and TNK 2002/4 (97.5%) showed a higher homology with the very virulent strain OKYM (Table 1). The phylogenetic tree constructed on the basis of the nucleotide sequence alignment of the VP2 gene variable region also showed relatedness of these isolates with the vaccine strain Georgia, the classical virulent strain 52/70 and the very virulent Japanese strain OKYM. However, TNK 2002/3 showed the least homology with the strains OKYM (91.5%), 52/70 (89.3%) and vaccine strain (87.2%) and clustered out from these strains (Fig. 2).

Table 1. Homology of IBDV isolates from Tamil Nadu (1999–2002) with the classical virulent strain and the highly virulent OKYM strain

Isolates	Nucleotide homology (%) with the strains			Amino acid homology (%) with the strains		
	Georgia	52/70	OKYM	Georgia	52/70	OKYM
TNK 2000/1	89.4	91.9	96.6	92.3	96.2	98.7
TTN 2000	90.2	92.3	97.0	92.3	96.2	98.7
TNK 2001/3	92.8	95.3	97.9	93.6	97.4	100
TCB 2001/2	93.6	95.3	98.7	93.6	97.4	100
TRP 1999/2	94.0	94.0	97.0	92.3	96.2	98.7
TNK 1999/1	94.5	94.4	97.5	93.6	97.4	100
TTN 2001	94.9	95.3	97.9	93.6	97.4	100
TNK 1997	98.3	94.9	96.2	97.4	96.2	93.6
TSL 2001	92.8	94.4	98.7	93.6	97.4	100
TCN 2001	93.2	94.4	97.9	92.3	96.2	98.7
TCB 2001	89.4	93.2	97.0	92.3	96.2	98.7
TNK 2002/1	94.9	95.7	96.6	92.3	96.2	96.2
TNK 2002/2	91.1	93.6	97.5	92.3	96.2	98.7
TNK 2002/4	91.1	93.2	97.5	92.3	96.2	98.7
TNK 2002/3	87.2	89.3	91.5	83.3	85.9	87.2
OKYM	94.0	95.3	100	93.6	97.4	100
52/70	95.3	100	96.6	96.2	100	97.4
IBD Georgia	100	95.3	95.7	100	96.2	93.6

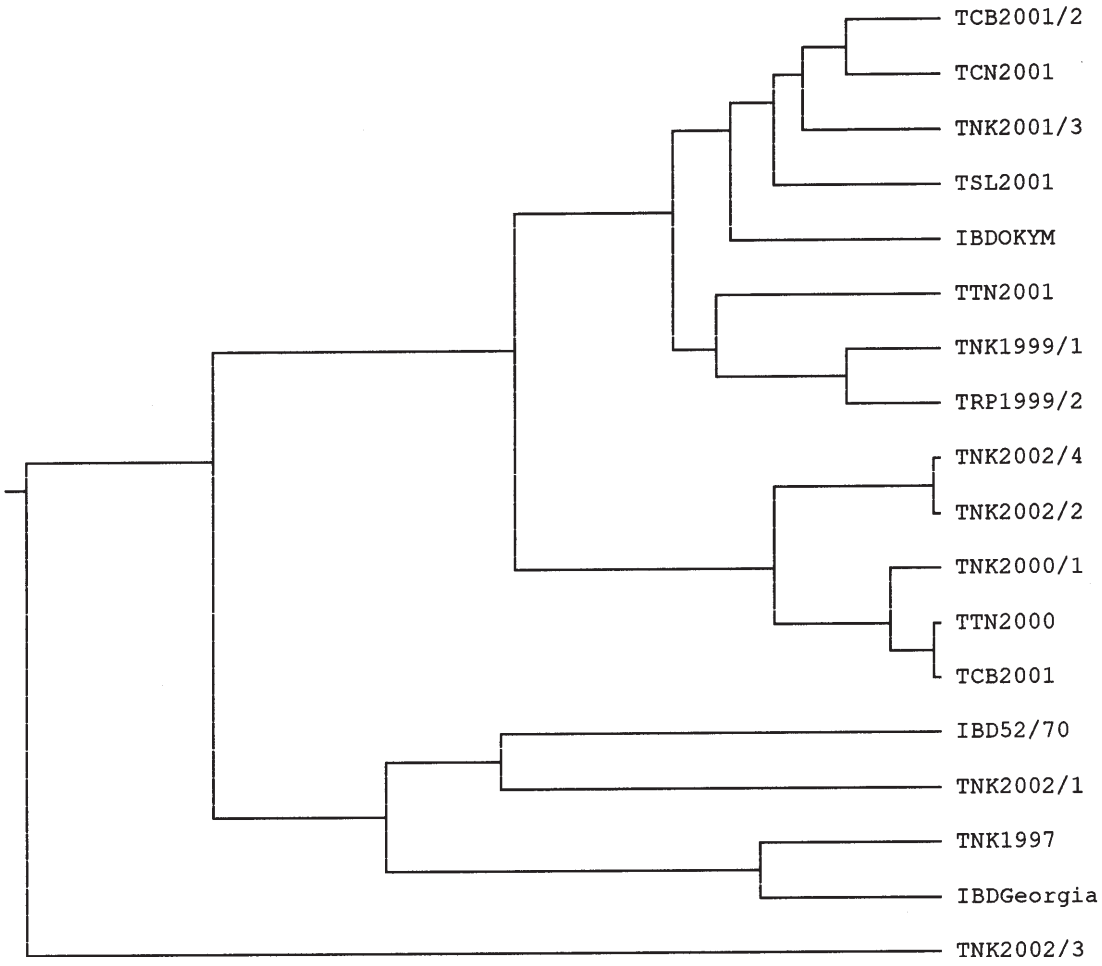

Fig. 2

Phylogenetic tree of IBDV isolates based on VP2 gene homology with the vaccine strain Georgia, the classical strain 52/70 and the very virulent strain OKYM

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IBD52/70   LIGFDGTAVI TRAVAADNGL TAGTDNLMPF NLVIPTNEIT QPITSIKLEI VTSKSGGQAG   DQMSWSASGS LAVTIHGG
TNK1997    .......... ......N... .T........ .......... .......... ..........   .......R.. ........
TTN2001    .......... .......... .......... .I....S... .......... ..........   .......... ........
TNK1999/1  .......... .......... .......... .I....S... .......... ..........   .......... ........
TCB2001/2  .......... .......... .......... .I....S... .......... ..........   .......... ........
TCN2001    .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TNK2001/3  .......... .......... .......... .I....S... .......... ..........   .......... ........
TSL2001    .......... .......... .......... .I....S... .......... ..........   .......... ........
TNK2000/1  .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TTN2000    .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TCB2001    .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TRP1999/2  .....R.... .......... .......... .I....S... .......... ..........   .......... ........
IBDOKYM    .......... .......... .......... .I....S... .......... ..........   .......... ........
TNK2002/3  .......... .I....Y... ...P...... .I....RA.. .....F..D. ..........   .......C.. .S....C.
TNK2002/4  .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TNK2002/2  .......... .......... .......... .I....SA.. .......... ..........   .......... ........
TNK2002/1  .......T.. .......... .......... .IA....... .......... ..........   .......... ........
Georgia    .......... ......N... .T........ .......... .......... ..........   ......V... ........
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Fig. 3

Comparison of deduced amino acid sequences of VP2 genes of IBDV isolates with those of the vaccine strain Georgia, the classical strain 52/70 and the very virulent strain OKYM

Deduced amino acid sequences showed 100% identity in 11 out of 15 isolates compared (Fig. 3). All the 11 isolates shared amino acid sequence homology with the very virulent strain OKYM. The serine-rich heptapeptide sequence SWSASGS was also conserved among the isolates compared. The isolate TNK 2002/3 showed substitutions at 8 amino acid positions (including one in the heptapeptide sequence) that were not observed in other isolates.

Discussion

In the control of the disease it is important to ascertain antigenic variation and virulence of IBDV field isolates. To study this problem, bursal tissues collected from different parts of Tamil Nadu were subjected to RT-PCR and the variable region of VP2 gene was amplified and analyzed. The amplicons 474 bp in length from different isolates were sequenced and the nucleotide and amino acid homologies compared with the vaccine strain Georgia, classical virulent strain 52/70 and the very virulent Japanese strain OKYM.

Although these isolates originated from outbreaks in different parts of Tamil Nadu like Namakkal, Coimbatore and Thirunelveli, the nucleotide homology studies of the VP2 variable region revealed that they were very closely related, indicating the possibility of their common origin.

High homology of a few Indian isolates (TCB 2001/2, TSL 2001, TNK 2001/3, TCN 2001, TTN 2001, TRP 1999/2, and TNK 1999/1) with the very virulent Japanese strain OKYM indicated that in Tamil Nadu also very virulent IBDV strains circulate. The conserved serine-rich heptapeptide sequence SWSASGS adjacent to the second hydrophilic region was found in most of the isolates. Conservation of this sequence has been earlier reported as an indication of existence of a pathogenic IBDV in nature (Vakharia *et al*., 1994). The conserved serine-rich heptapeptide sequence was not present in the isolate TNK 1997 which clustered with the vaccine strain Georgia. However, the isolate TNK 2002/3 that exhibited a highest number of mutations when given to IBDV-seronegative chicks, mortality was not observed upto 10 days post infection. On histopathological examination of the bursa, lymphoid depletion in the follicles resembling the classical virulent IBDV strain was noticed as already reported (Lukert and Saif, 1997). However, this isolate did not produce mortality rates of the classical virulent strain.

Previous studies have indicated that few of Indian IBDV field isolates are similar to a very virulent IBDV from European and Asian countries (Kataria *et al*., 1999). It has been postulated that very virulent IBDV strains would have entered India from northern and eastern parts, probably from China and South East Asia (Kataria *et al*., 2001).

Phylogenetic studies of the Tamil Nadu isolates showed that most of the local isolates are more closely related to the very virulent Japanese OKYM strain than to the classical virulent or vaccine strains. Among the isolates, TNK 2002/1 alone clustered with the strain 52/70 and TNK 1997 with the vaccine strain indicating the occurrence of classical virulent and vaccine strains. However, TNK 2002/3, which clustered out from these standard strains, produced bursal lesions resembling the classical virulent IBDV strain in the pathotyping studies. The sequence analysis indicated the presence of a mixed type of IBDV infection in Tamil Nadu posing problems in the institution of vaccination regimen in the control of IBD.

Acknowledgement. The authors acknowledge the National Agricultural Technology Project, ICAR, Government of India, New Delhi, India for funding this study and Tamil Nadu Veterinary and Animal Sciences University, Chennai, India for providing all the facilities.

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