SEQUENCE ANALYSIS OF SEGMENT A OF A FIELD VIRUS ISOLATE FROM AN OUTBREAK OF INFECTIOUS BURSAL DISEASE IN INDIA

M.V. BAIS, R.S. KATARIA, A.K. TIWARI*, INDERVESH, V.V S. SURYANARAYANA, S.K. DAS

National Biotechnology Center, Indian Veterinary Research Institute, Izatnagar, 243122 UP, India

Received November 15, 2002; accepted April 3, 2003

Summary. – Sequence analysis of genome segment A of an Indian Infectious bursal disease virus (IBDV) field isolate (KT1/99) revealed total 95 nucleotide substitutions, resulting in 17 amino acid changes. Of these, five amino acid changes, namely F60S, T137I, I374V, V519I and E682D were unique to the KT1/99 isolate. The amino acid change P222A and the proposed hot mutation spot 680Y, reported to be present in very virulent IBDV isolates were also found in KT1/99. This isolate had nucleotide divergence of 1.1% to 4.95% from the other reported serotype 1 IBDV isolates and 19.6% from serotype 2 strain OH in polyprotein gene sequence, while divergence at amino acid level was 0.6% to 2.9% and 11.4%, respectively. Based on both nucleotide and amino acid sequence analysis, KT1/99 was grouped phylogenetically with the reported Bangladesh isolate BD3/99 in one cluster along with other reported very virulent isolates in same lineage.

Key words: Infectious bursal disease virus; nucleotide sequence; amino acid sequence; segment A

Introduction

Infectious bursal disease (IBD), an acute and highly contagious immunosuppressive viral disease of chickens, is responsible for heavy economic losses to poultry industry worldwide. The causative agent of the disease, IBDV destroys B lymphocytes in the bursa of Fabricius, rendering birds more susceptible to secondary microbial infections and with poor response to vaccines (Allan *et al.,* 1972). IBDV (the *Birnaviridae* family, the *Avibirnavirus* genus, the *Infectious bursal disease virus* species*)* is characteristic by non-enveloped icosahedral virions containing a double-stranded, bi-segmented RNA genome (van Regenmortel *et al.,* 2000). Out of the two distinct serotypes of the virus, the serotype 1 is pathogenic to chickens, while the serotype 2 is avirulent (Ismail *et al.,* 1988).

Based on pathogenicity and antigenicity, the serotype 1 strains/isolates are further grouped as classical virulent, antigenic variant, very virulent and cell culture adapted attenuated strains. In late 1980s, emergence of very virulent strains of IBDV, responsible for more than 70% mortality in chickens in Europe and Asia including India (Chettle *et al.,* 1989*;* Nunoya *et al.,* 1992; Sah *et al.,* 1995) has resulted in huge economic losses.

The IBDV genome segment A (3.3 kb) has two overlapping open reading frames (ORFs). The large ORF encodes a 110 K polyprotein, which is auto-processed into mature VP2, VP3 and VP4 proteins (Kibenge and Dhama*,* 1997). Whereas VP2 and VP3 are structural proteins, VP4, a viral protease, is a non-structural protein. The small ORF present at the 5' end of segment A encodes a non-structural VP5 protein, reported to play role in viral pathogenesis (Lombardo *et al.,* 2000). The segment B (2.8 kb) encodes a multifunctional 90 K VP1 protein, a viral RNA polymerase (Azad *et al.,* 1985).

Phylogenetic analysis based on nucleotide and deduced amino acid sequences of various genes has been exploited

*Corresponding author. E mail: aktiwari63@yahoo.com; fax: +91581-2303284.

Abbreviations: IBDV = Infectious bursal disease virus; IBD = infectious bursal disease; RT-PCR = reverse transcription–polymerase chain reaction; CEF = chicken embryo fibroblasts; ORF(s) = open reading frame(s)

Table 1. Primers used for amplification and sequencing

Primer pair No.	Primer	Sequence	Strand	Nucleotide position
1	MB3	5'-CCGGTACCCCACGCGTCGATCGCAGCGATGACAAACC-3'	+	120–141
	Mash 10	5'-AATAGCATGCGAATTCTGTAGTTCATGGCTCCTGGG-3'	–	1323–1304
2	MB9	5'-CCGGTACCCCACGCGTTATGTGAGGCTTGGTGACCC-3'	+	660–679
	MB8	5'-GGGATACTCCATAGACTCTGTGTCCGG-3'	–	1942–1961
3	MB7	5'-CCGGTACCCCACGCGTCGTGCGAGAAGACCTCCAAC-3'	+	1886–1905
	MB4	5'-GGGATATCAGACTCCCAGGAGCCTCACTCAAGG-3'	–	3161–3185
4	VL/NBC/67	5'-TAGCTAGCAACTCCTCCTTCTACAACGCTATC-3'	+	35–58
	VL/NBC/68	5'-GGATCCCGACGGTGACCCCTTCCCCTACTA-3'	–	594–572

to study evolutionary relationships among different viruses. Earlier studies, based on sequence analysis of the VP2 gene hyper-variable region amplified by reverse transcription–polymerase chain reaction (RT-PCR) have revealed close genetic resemblance of the Indian field isolates with very virulent isolates from Europe, Japan, China and Israel (Kataria *et al.,* 2001). Partial and complete nucleotide sequence of both segments have been used to characterize very virulent isolates originating from different countries (Brown and Skinner, 1994; Yamaguchi *et al.,* 1997).

In the present study, the complete genome segment A of the Indian IBDV field isolate KT1/99 was sequenced and compared with known sequences of other IBDV isolates in order to assess their relationships.

Materials and Methods

RNA extraction and RT-PCR. A field isolate of IBDV, KT1/99, collected from a southern province of India, was obtained as infected bursal tissue. Total RNA from 200 µl of 20%(w/v) infected bursal tissue homogenate in PBS was extracted using the TRIZOL reagent (Life Technologies) as described by the manufacturer, and resuspended in 20 µl of nuclease-free water. Approximately 5 µg of total RNA was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Promega) and four pairs of specific primers. Details of these primers are given in Table 1.

Table 2. Details of IBDV strains/isolates used in the study

No.	Designation	Type of strain/isolate	Acc. No.	Geographic origin
1	KT1/99	Field isolate	AJ427340	India
2	BD3/99	Very virulent strain	AF362776	Bangladesh
3	HK46	Very virulent strain	AF092943	China
4	Harbin1	Very virulent strain	AF454945	China
5	UPM 97/61	Very virulent strain	AF247006	Malaysia
6	OKYM	Very virulent strain	D49706	Japan
7	UK661	Very virulent strain	X92760	UK
8	D6948	Very virulent strain	AF240686	Netherlands
9	52/70	Classical virulent strain	D00869	UK
10	Cu1-wt	Classical virulent strain	AF362747	Germany
11	STC	Classical virulent strain	D00499	USA
12	Cu1	Attenuated classical strain	D00867	Germany
13	CEF94	Attenuated strain	AF194428	Netherlands
14	GLS	Antigenic variant	M97346	USA
15	OH	Non-pathogenic serotype 2 strain	M66722	USA

Nucleotide sequence analysis. The PCR products generated were purified by the QIAquick Gel Extraction Kit (Qiagen) and sequencing of both strands was carried out by the *f*mol DNA Sequencing System (Promega) according to the manufacturer's instructions. After completion of sequencing, samples were electrophoresed in 6% polyacrylamide gel under denaturing conditions. The sequences were read manually and entered in the EditSeq Programme of the Lasergene Software (DNASTAR, USA). The sequence submitted to EMBL database is available under Acc. No. AJ 427340. Nucleotide and deduced amino acid sequences were aligned by the Clustal Method using the MegAlign Programme. After aligning clusters pairwise, all the sequences were then aligned in groups. The sequence of KT1/99 was subjected to phylogenetic analysis after comparison with known sequences of different IBDV strains or isolates (Table 2).

Results and Discussion

Comparison of nucleotide sequence of KT1/99 with those of other IBDV strains/isolates, demonstrated 95 nucleotide substitutions resulting in total 17 amino acid changes from the consensus (majority). At nucleotide level, KT1/99 diverged in polyprotein gene by 1.1–4.9% from other serotype 1 isolates and by 19.6% from serotype 2 isolate, while at amino acid level it diverged by 0.6–2.9% and 11.4% from other serotype 1 isolates and the serotype 2 strain OH, respectively. In the polyprotein, five amino acid changes, namely F60S, T137I, I374V, V519I and E682D were found to be unique to KT1/99. The five unique amino acid changes in KT1/99 point towards continuous mutations going on in IBDV in the nature. Important amino acid changes in different IBDV isolates are shown in Table 3.

Similarly to the reported very virulent isolates, the amino acid change P222A in the hydrophilic region 1 was present in the Indian field isolate KT1/99. The hydrophilic regions 1 (aa 212–224) and 2 (aa 315–324) in VP2 are reported to play an important role in formation of virus neutralizing

conformational epitopes (Brown and Skinner, 1994; Yamaguchi *et al.,* 1997).

Besides the specific very virulent amino acid changes also V213I and L294I were present in KT1/99. Kataria *et al.* (2001) have reported the same amino acid changes in other Indian field isolates of IBDV.

In this study, amino acids Q253, D279 and A284, which have been reported to be involved in virulence and cell tropism (van Loon *et al.,* 2002), indicated that KT1/99 is of virulent phenotype. Earlier, we have also demonstrated importance of amino acids 279 and 284 in cell culture adaptation (Toroghi *et al.,* 2001).

The heptapeptide sequence 326-SWSASGS-332, conserved in virulent strains or isolates of IBDV was also found in the isolate KT1/99. The amino acid position 680 in the VP4 region of polyprotein in very virulent strains or isolates, suggested to be a "hot mutation spot" (Pitcoviski *et al.,* 1998), was also present in the isolate KT1/99.

The polyprotein cleavage sites 511-LAA-513 and 754-MAA-756, essential for processing of VPX-VP4 and VP4-VP3 precursors, respectively, were conserved among all IBDV strains or isolates including KT1/99 (Sanchez and Rodriguez, 1999). The changes of amino acids L451, S715 and D751 in flanking regions of cleavage sites, found in very virulent strains or isolates inclusive of KT1/99, have been suspected to increase the processing efficiency of the polyprotein (Yamaguchi *et al.,* 1997). However, the exact role of these amino acid changes needs to be assessed by site-directed mutagenesis.

In the phylogenetic tree, based on nucleotide sequence of the polyprotein, the isolate KT1/99 grouped with very virulent strains or isolates originating from other countries in one cluster, while variant, attenuated and classical strains or isolates formed another cluster in the same lineage (Fig. 1). This indicates that all very virulent, variant and attenuated strains or isolates of IBDV originated from a common ancestor of classical serotype 1. Based on complete nucleotide and amino acid sequences of the polyprotein,

Table 3. Important amino acid changes in different IBDV isolates

IBDV strains/ isolates	Amino acid change at position													
	60	137	222	294	299	374	519	680	682	715	751	865	990	1005
Consensus (majority)	F	T	A	I	S	I	V	Y	E	S	Y	T	A	A
KT1/99	S	I	A	I	S	V	I	Y	D	S	Y	A	V	A
BD3/99	F	T	A	I	S	I	V	Y	E	S	Y	T	V	A
HK46	F	T	A	I	S	I	V	Y	E	S	Y	T	V	A
Harbin1	F	T	A	I	S	I	V	Y	E	S	Y	T	V	A
UPM3/97	F	T	A	I	S	I	V	Y	E	S	Y	T	V	A
OKYM	F	T	A	I	S	I	V	Y	E	S	Y	A	V	A
UK661	F	T	A	I	S	I	V	Y	E	S	Y	T	V	A
D6948	F	T	A	I	S	I	V	Y	E	S	Y	T	A	A
52/70	F	T	P	L	N	I	V	C	E	P	H	T	A	T
Cu1-wt	F	T	P	L	N	I	V	C	E	P	H	T	A	T
STC	F	T	P	L	N	I	V	F	E	P	H	T	A	T
Cu1	F	T	P	L	N	I	V	C	E	P	H	T	A	T
CEF-94	F	T	P	L	N	I	V	C	E	P	H	T	A	T
GLS	F	T	P	L	N	I	V	C	E	P	H	T	A	T
OH	F	T	P	L	S	I	V	–	E	P	H	T	A	T

Yamaguchi *et al.* (1997) have also reported similar type of grouping of very virulent strains or isolates.

In the phylogenetic tree, prepared on the basis of nucleotide sequence, two major lineages were found, in which the isolate KT1/99 grouped with the Bangladesh isolate BD3/99 in the same lineage. Similarly, in the cladogram based of nucleotide sequences of individual VP2, VP3 and VP4 proteins also two major genetic lineages were found (data not shown). In the first lineage, all very virulent

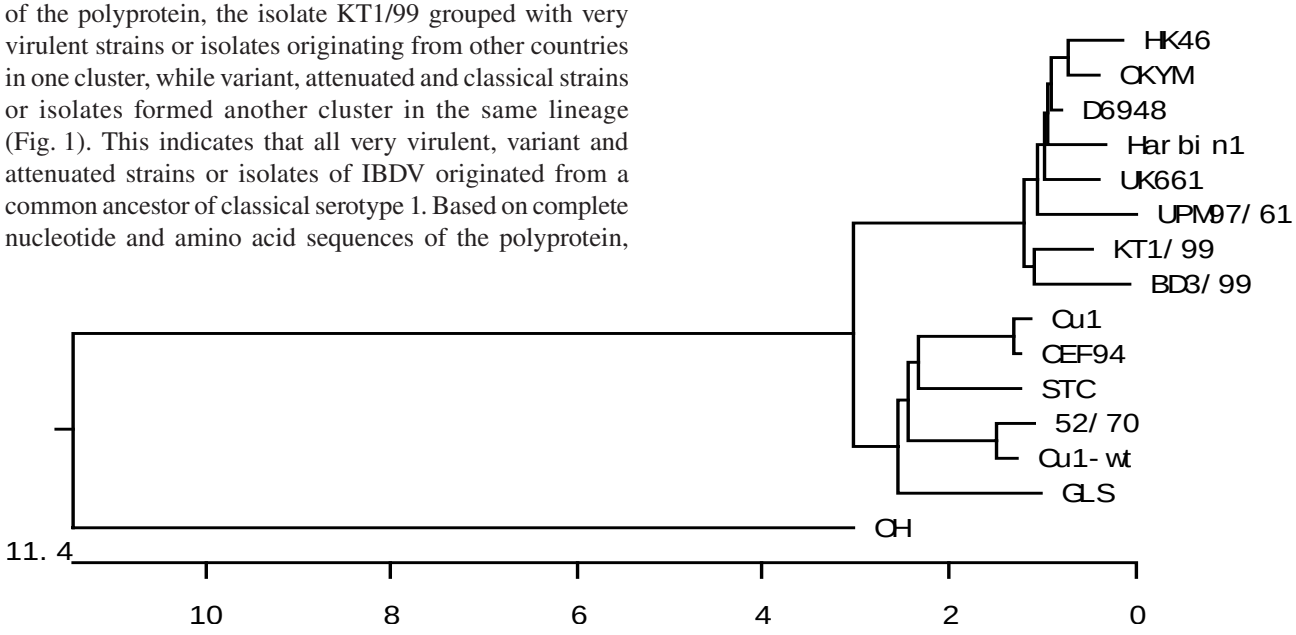

Fig. 1

Phylogenetic tree based on nucleotide sequence alignment of the polyprotein gene of different IBDV strains/isolates

Branched distances correspond to sequence divergence. Scale: distances between sequences, units indicating number of substitutions.

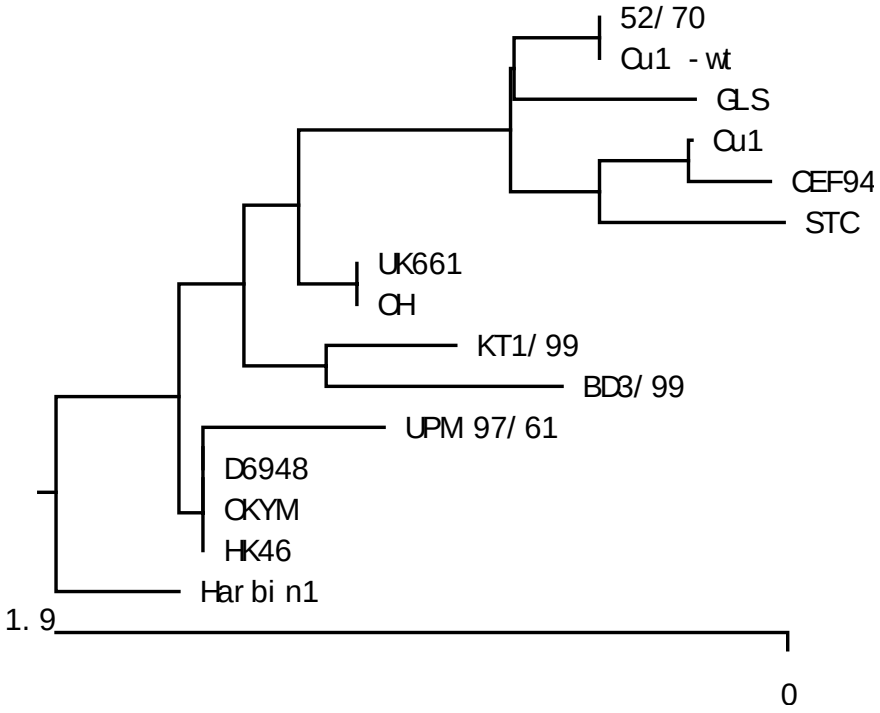

Fig. 2
Phylogenetic tree based on nucleotide sequence alignment of VP5 gene of different IBDV strains/isolates
Branched distances correspond to sequence divergence. Scale: distances between sequences, units indicating number of substitutions.

isolates including KT1/99 clustered together, while classical and attenuated vaccine strains grouped in the second cluster of the same lineage. The serotype 2 strain OH was placed in another lineage away from all the serotype 1 strains or isolates. Brown and Skinner (1996) and Yamaguchi *et al.* (1997) have reported a similar phylogenetic grouping of different IBDV strains or isolates.

The non-structural protein VP5, encoded by small overlapping ORF of the genome segment A, reported to play a role in viral pathogenesis and apoptosis (Lombardo *et al.,* 2000; Yao and Vakharia, 2001) had a unique amino acid change of H134N at the C-terminal region of the KT1/99 isolate and the BD3/99 isolate from Bangladesh. This amino acid change may be a unique feature of field isolates prevalent in the Indian subcontinent. This needs to be further confirmed by sequence analysis of a higher number of field isolates. In the cladogram, an unique feature of the VP5 gene phylogeny was that the isolate KT1/99 along with all very virulent strains or isolates clustered together with the serotype 2 OH strain (Fig. 2). The significance of unique amino acid changes, present in the polyprotein of very virulent strains or isolates, needs to be assessed by site-directed mutagenesis combined with reverse genetics. Phylogenetic analysis of the isolate KT1/99 confirms the common origin of very virulent IBDV strains or isolates, which have probably spread from Europe to other parts of the world.

Acknowledgement. The authors thank the Director of the Indian Veterinary Research Institute, Izatnagar, India, for providing necessary facilities to carry out the work. Technical assistance rendered by Mr. N. Prasad and B. Pal and financial assistance of NATP is also gratefully acknowledged. The authors are also grateful to Dr. M.A. Skinner, IAH, Compton, UK for providing primers used in this study.

References

Allan WH, Faragher ST, Cullen GA (1972): Immunosupression by infectious bursal agent in chickens immunized against Newcastle disease. *Vet. Rec.* **90**, 511–512.

Azad AA, Barrett SA, Fahey KJ (1985): Characterization and molecular cloning of the double stranded genome of an Australian strain of infectious bursal disease. *Virology* **143**, 35–44.

Brown MD, Green P, Skinner MA (1994): VP2 sequences of recent European "very virulent" isolates of infectious bursal disease virus are closely related to each other but are distinct from those of "classical" strains. *J. Gen. Virol.* **75**, 675–680.

Brown MD, Skinner MA (1996): Coding sequences of both genome segments of European very virulent infectious bursal disease virus. *Virus Res.* **40**1–15.

Chettle N, Stuart JC, Wyeth, PJ (1989): Outbreak of virulent infectious bursal disease in East Anglia.*Vet. Rec*. **125**, 271–272.

Ismail NM, Saif YM, Moorhead PD (1988): Lack of pathogenicity of five serotype-2 infectious bursal disease viruses in chickens. *Avian Dis*. **32**, 757–759.

Kataria RS, Tiwari AK, Butchaiah G, Kataria JM, Skinner MA (2001): Sequence analysis of the VP2 gene hyper-variable region of infectious bursal disease viruses from India. *Avian Pathol.* **30**, 501–507.

Kibenge FSB, Dharma V (1997): Evidence that virion associated VP1 of *Avibirnaviruses* contain viral RNA sequences. *Arch. Virol.* **142**, 1227–1236.

Lombardo E, Maravet A, Espinosa I, Fernandez-Arias A, Rodriguez JF (2000): VP5, the non-structural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. *Virology* **277**, 345–357.

Nunoya T, Otaki Y, Tajima M, Hiraga M, Saito T (1992): Occurrence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in specific-pathogen-free chickens. *Avian Dis*. **36**, 597–609.

Pitcoviski J, Goldberg D, Levi BZ, Castro D, Azriel A, Krispel S, Maray T, Shaaltiel Y (1998): Coding region of segment A sequence of a very virulent isolate of IBDV-comparison with isolates from different countries and virulence. *Avian Dis.* **42**, 497–506.

Sah RL, Kataria JM, Arya SC, Verma KC (1995): Outbreak of acute infectious bursal disease causing high mortality in chicken. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **16**, 7–13.

Sanchez AB, Rodriguez JF (1999): Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology* **262**, 190–199.

Toroghi R, Kataria JM, Verma KC, Kataria, RS, Tiwari, AK (2001): Amino acid changes in the variable region of VP2 in three infectious bursal disease viruses with different virulence, originating from a common ancestor. *Avian Pathol.* **30**, 667–673.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego-San Francisco-New York-Boston-London-Sydney-Tokyo.

van Loon AA, de Hass N, Zeyda I, Mundt E (2002): Alteration of amino acids in VP2 of very virulent IBDV results in tissue culture adaptation and attenuation in chickens. *J. Gen. Virol.* **83**, 121–129.

Yamaguchi T, Ogawa M, Miyoshi M, Inoshima Y, Fukishi H, Hirai K (1997): Sequence and phylogenetic analysis of highly virulent infectious bursal disease virus. *Arch. Virol.* **142**, 1441–1458.

Yao K, Vakharia VN (2001): Induction of Apoptosis *in vitro* by the 17-Kda nonstructural protein of Infectious bursal disease virus; Possible role in viral pathogenesis. *Virology* **285**, 50–58.