MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM NEPAL BASED ON HYPERVARIABLE REGION OF VP2 GENE

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Summary. – Two Infectious bursal disease virus (IBDV) isolates, NP1SSH and NP2K were obtained from a severe infectious bursal disease (IBD) outbreak in Nepal in 2002. The hypervariable (HV) region of VP2 gene (1326 bp) of the isolates was generated by RT-PCR and sequenced. The obtained nucleotide sequences were compared with those of twenty other IBDV isolates/strains. Phylogenetic analysis based on this comparison revealed that NP1SSH and NP2K clustered with very virulent (vv) IBDV strains of serotype 1. In contrast, classical, Australian classical and attenuated strains of serotype 1 and avirulent IBDV strains of serotype 2 formed a different cluster. The deduced amino acid sequences of the two isolates showed a 98.3% identity with each other and 97.1% and 98.3% identities, respectively with very virulent IBDV (vvIBDV) isolates/strains. Three amino acids substitutions at positions 300 (E→A), 308 (I→F) and 334 (A→P) within the HV region were common for both the isolates. The amino acids substitutions at positions 27 (S→T), 28 (I→T), 31 (D→A), 36 (H→Y), 135 (E→G), 223 (G→S), 225 (V→I), 351 (L→I), 352 (V→E) and 399 (I→S) for NP1SSH and at position 438 (I→S) for NP2K were unique and differed from other IBDV isolates/strains. NP1SSH and NP2K showed highest similarity (97.8%) with the BD399 strain from Bangladesh as compared with other vvIBDV isolates/strains. We conclude that the NP1SSH and NP2K isolates of IBDV from Nepal represent vvIBDV of serotype 1.

Key words: Infectious bursal disease virus; VP2 gene; nucleotides sequence; deduced amino acids sequence; phylogenetic analysis; Nepal

Introduction

IBD is an acute highly contagious and immunosuppressive disease in young chickens caused by IBDV. The target cell of IBDV is a developing B-lymphocyte located within the bursa of Fabricius (Lukert and Saif, 1997). IBD causes significant economic losses to the poultry industries due to high mortality and immunosuppression (van den Berg, 2000). IBDV belongs to the *Infectious bursal disease virus* species, the *Avibirnavirus* genus, the *Birnaviridae* family and occurs in two serotypes (van Regenmortel *et al*., 2000). Whereas the serotype 1 is pathogenic to chicken and varies in virulence, the serotype 2 is apathogenic to chicken and turkey (McFerran *et al*., 1980). Because of the virulence variability, the serotype 1 includes classical, very virulent and attenuated strains (Lukert and Saif, 1997).

IBDV genome consists of two segments of double-stranded RNA, of approximately 3.3 kb (segment A) and 2.7 kb (segment B) (Dobos *et al* 1979). Segment A encodes two structural proteins (VP2 and VP3) and two non-structural proteins (VP4 and VP5). Segment B encodes non-structural protein VP1, the viral RNA polymerase (Azad *et al*., 1985). Substitution of single amino acid at the HV region (nt 206–

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Abbreviations: HV = hypervariable; IBD = infectious bursal disease; IBDV = IBD virus; vvIBDV = very virulent IBDV

350) of VP2 gene is considered a marker for differentiation of virulence and antigenic variation of IBDV strains (Eterradossi *et al*., 1999). Any change of amino acid in the HV region, namely in hydrophilic region at peak A (nt 214–222) or B (nt 314–324) is responsible for antigenic variation of IBDV strains (Azad *et al*., 1987; Heine *et al*., 1991).

Outbreaks of IBD caused by vvIBDV have been reported from Europe in late 1980's (Chettle *et al.,* 1989) and in early 1990's in Asia (Hair-Bajo, 1992; Nunoya *et al.,* 1992). vvIBDV is antigenically very similar to classical IBDV but causes high mortality even in the presence of maternal immunity (Brown *et al*., 1994; van den Berg *et al*., 1996; Yamaguchi *et al*., 1996; Eterradossi *et al*., 1998). The first outbreak of IBD in Nepal has been reported in 1991 (Dhakal, 1992) and the disease has been found to continue both in vaccinated and non-vaccinated chickens. Molecular characteristics of the IBDV strains involved are mostly unknown.

Therefore we attempted to characterize at molecular level the NP1SSH and NP2K isolates of IBDV obtained from broiler chicken during the IBD outbreak in Nepal in 2002.

Materials and Methods

Virus. The IBDV isolates NP1SSH and NP2K were obtained from the bursa of Fabricius of broiler chickens during recent outbreak of IBD in Nepal in 2002. Clinical signs of the affected chickens were anorexia, diarrhea, depression, ruffled feather, drowsiness and reluctance to move. Post mortem findings showed hemorrhages in the bursa of Fabricius, thigh and pectoral muscles and the mucosa region at the junction of proventriculus and gizzard. The bursa of Fabricius was homogenized with PBS pH 7.4 (1:3 w/v). The homogenate was clarified first at 3000 rpm for 15 mins and then at 10,000 rpm for 20 mins, each time at 4°C. The supernatant was filtered through 0.45 µm filters and supplemented with antimycotic.

Viral RNA was extracted from chicken bursal homogenates originating from field outbreaks using Trizol Reagent (Gibco BRL, Life Technologies) by a modified method of Cao *et al*. (1998).

RT-PCR for detection of the HV region of VP2 gene of IBDV (1326 bp) was performed by using the Gibco BRL Superscript Preamlification System. The primers described by Cao *et al*. (1998) were employed. In the reaction, the Platinium *Taq* polymerase from Life technologies (USA) was used. The PCR products were subjected to agarose gel electrophoresis and purified using the Geneclean II^{R} Kit (Bio 101, USA). The products were sequenced using an ABI PRISM^{TM} 377 automated DNA sequencer and Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkins Elmer).

Sequence analysis. The deduced amino acid sequences for NP2K and NP1SSH and other IBDV isolates/strains, accessible at the EMBL database, were compared using the Bio-edit package (version 3.57c) of Clustal W multiple alignment program (Thomson *et al*., 1994).

Phylogenetic tree inclusive of the percentage of homology for the isolates NP2K and NP1SSH and other IBDV isolates/strains was constructed on the basis of the 1326 bp sequence of the HV region of VP2 gene using DNA Star MegAlign software version 5.10 (http://www.dnastar.com).

Results

Sequence analysis

The 1326 nt long sequences of the HV region of VP2 of the IBDV isolates NP1SSH and NP2K were determined and

Table 1. Characteristics of IBDV isolates/strains subjected to sequence analysis

Isolate/strain	Serotype	Pathotype	Country	Reference	Acc. No.
NP1SSH	1	Very virulent	Nepal	This paper	AY605264
NP2K	1	Very virulent	Nepal	This paper	AY367560
BD3/99	1	Very virulent	Bangladesh	Islam *et al.* (2001)	AF362776
UPM97/61	1	Very virulent	Malaysia	Chong *et al*. (2001)	AF 247006
UPM94/273	1	Very virulent	Malaysia	Kong *et al.* (2004)	AF527039
Tasik 94	1	Very virulent	Indonesia	Rudd *et al.* (2002)	AF322444
HK46	1	Very virulent	China	Cao *et al.* (1998)	AF051838
KK1	1	Very virulent	South Korea	Kwon *et al.* (2000)	AF165150
KSH	1	Very virulent	South Korea	Kwon *et al.* (2000)	AF164151
OKYM	1	Very virulent	Japan	Yamaguchi *et al*. (1996)	D49706
D6948	1	Very virulent	Netherlands	Boot *et al.* (2000)	AF240686
UK661	1	Very virulent	UK	Brown *et al.* (1994)	X 92760
TO9	1	Very virulent	Nigeria	Unpublished	AY099456
STC	1	Classical	USA	Kibenge *et al.* (1990)	D00499
52/70	1	Classical	UK	Bayliss *et al.* (1990)	D00869
002-73	1	Classical	Australia	Hudson *et al.* (1986)	X03993
Variant E	1	Variant	USA	Rosenberger and Cloud (1986)	AF133904
P2	1	Attenuated	Germany	Nick *et al.* (1976)	X84034
CT	1	Attenuated	France	Lejal *et al.* (2000)	AF1924429
Cu-1M	1	Attenuated	Germany	Spice *et al.* (1989)	X16107
23/82	2	Avirulent	UK	Cursiefen *et al.* (1979)	AF362773
OH	2	Avirulent	USA	Jackwood *et al.* (1982)	U30818

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                190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Tasik94   GDPIPAIGLD PKMVATCDSS DRPRVYTITA ADDYQFSSQY QSGGVTITLF SANIDAITSL
NP1H      .......... .......... .......... .......... .AS.I..... ........
NP2K      .......... .......... .......... .......... .A........ ..........
BD399     .......... .......... .......... .......... .A........ ..........
UPM94273  .......... .......... .......... .......... .A........ ..........
UPM9761   .......... .......... .......... .......... .A........ ..........
HK46      .......... .......... .......... .......... .A........ ..........
KK1       .......... .......... .......... .......... .A........ ..........
KSH       .......... .......... .......... .......... .A........ ..........
OKMY      .......... .......... .......... .......... .A........ ..........
D6948     .......... .......... .......... .......... .A........ ..........
UK661     .......... .......... .......... .......... .A........ ..........
T09       .......... .......... .......... .......... .A........ ..........
STC       .......... .......... .......... .......... .P........ ..........
5270      .......... .......... .......... .......... .P........ ..........
00273     .......... .......... .......... .......... .P........ ........N.
Variant E .......... .......... .......... ..N....... .T........ ..........
P2        .......... .......... .......... .......... .P........ ..........
CT        .......... .......... .......... .......... .P........ ..........
CU1M      .......... .......... .......... .......... .P........ ..........
2382      ......T... ..LI...... .......... ..E......L IPS..KT... ......L..F
OH        ......T... ..LI...... .......... ..E......L IPS..KT... T.....L...


                310        320        330        340        350        360
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Tasik94   SIGGELVFQT SVQGLILGAT IYLIGFDGTA VITRAVAADN GLTAGTDNLM PFNIVIPISE
NP1H      .......... .......... .......... .......... .......... .......T.A
NP2K      .......... .......... .......... .......... .......... .......T.A
BD399     .......... .......... .......... .......... .......... .......T.A
UPM94273  .......... ...S...... .........E .......... .......... .......T..
UPM9761   .......... .......... .......... .......... .......... .......T..
HK46      .......... .......... .......... .......... .......... .......T..
KK1       .......... .......... .....L.... .......... .......... .......T..
KSH       .......... .......... .......... ........N. .......... .......T..
OKMY      .......... .......... .......... .......... .......... .......T..
D6948     .......... .......... .......... .......... .......... .......T..
UK661     .......... .......... .......... .......... .......... .......T..
T09       .......... .......... .......... .......... .......... .......T..
STC       .......... .....V.... ..F......T .......... .......... ...L...TN.
5270      .......... .....V.... .......... .......... .......... ...L...TN.
00273     .......... .......N.. ...V.....T .T........ .......... ...L...T..
VariantE  ........K. ...S...... .......... ........N. .....I.... ...L...TN.
P2        .......... ..H.I..... .......... ........N. ...T...... ...L...TN.
CT        .......... ..H....... .........T ........N. ...T...... ...L...TN.
CU1M      .......... ..H....... .........T ........N. ...T...... ...L..STN.
2382      ........NQ ITIQN.EVDI TIHFIKFDKT D..IKAV.T. FKLTTKT.NL IPFNL.IPTN
OH        ......I.NQ ITIHN.EVDI TIYFIKFDKT E..IKAV.T. FKLTTKT.NL IPFNLNNPTN
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Fig. 1

Comparison of amino acid sequences (aa 180–360) of HV region of VP2 gene of NP2K, NP1SSH and other isolates/strains of IBDV

Dots indicate identity. Amino acids are numbered according to Bayliss *et al*. (1990).

submitted to the EMBL database. They were assigned Acc. Nos. AY605264 and AY367560, respectively. The deduced amino acid sequences at positions 1–446 in the HV region of VP2 of the isolates NP2K and NP1SSH and other isolates/strains of IBDV were aligned and compared (Table 1, Fig. 1). The two Nepalese isolates showed a 98.3% identity. No insertions or deletions both at nucleotide and amino acid level were observed.

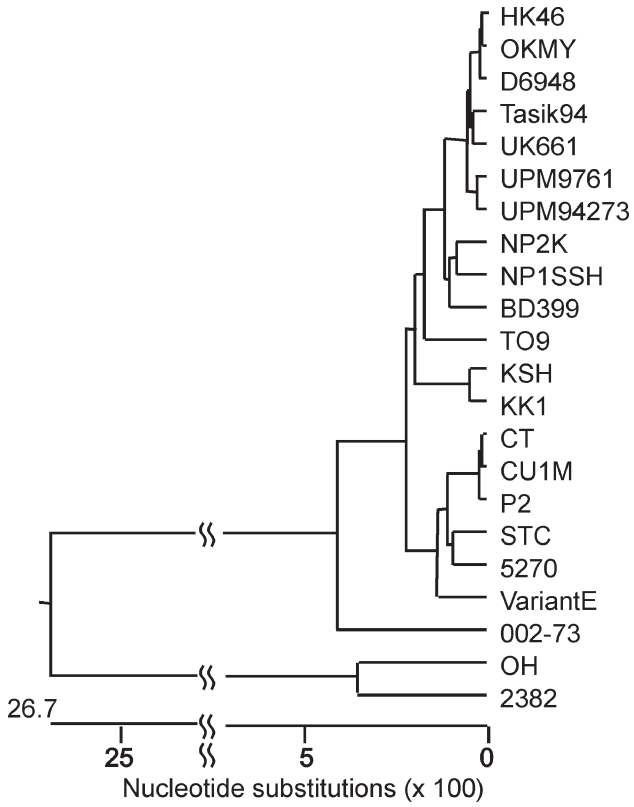

Fig. 2

Phylogenetic relationships based on 1326 nt sequence of HV region of VP2 gene of the NP2K, NP1SSH and other isolates/strains of IBDV

Amino acid substitutions were observed at 222A, 256I, 294I, and 299S in all isolates/strains analyzed. No amino acid substitutions were observed in both the hydrophilic peak A (aa 214–222) and B (aa 314–324) in the isolates NP2K and NP1SSH. The amino acid substitutions at the HV region at positions 300 (E→A), 308 (I→F) and 334 (A→P) were common for both the isolates. Amino acid substitutions outside the HV region were observed at positions 27 (S→T), 28 (I→T), 31 (D→A), 36 (H→Y), 135 (E→G), 223 (G→S), 225 (V→I), 351 (L→I), 352, (V→E) and 399 (I→S) in NP1SSH and at position 438 (I→S) in NP2K. The serine-rich heptapeptide SWSASGAS at the positions 326–332 was also conserved in both the isolates.

Phylogenetic analysis

Phylogenetic analysis of NP2K and NP1SSH and other isolates/strains of IBDV was based on the 1326 nt sequence of the HV region of VP2 gene (Fig. 2). NP2K and NP1SSH were related most closely to the vvIBDV strain BD399 from Bangladesh, thereby forming a small cluster within a large cluster of vvIBDV isolates/strains. On the other hand, classical, Australian variant classical, attenuated and avirulent strains formed a separate cluster.

Discussion

The study has demonstrated that both the IBDV isolates obtained from broiler chickens during the IBD outbreak in Nepal in 2002, NP2K and NP1SSH have a molecular characteristics of vvIBDV strains of serotype 1. The amino acid substitutions in the HV region of VP2 at 222A, 256I, 294I and 299S in both NP1SSH and NP2K were conserved similarly to other vvIBDV isolates/strains (Cao *et al*., 1998; Pitcovski *et al*., 1998 Eterradossi *et al*., 1999). Also the SWSASGS heptapeptide (aa 326–332), a marker of pathogenicity (Vakharia *et al*., 1994; Kataria *et al*., 2001) was conserved in both of the isolates. Mutations in this region are common in less virulent or attenuated strains (Chen *et al*., 1998; Heine *et al*., 1991).

Mutations in the HV region of VP2 have impact on the characteristics of IBDV strains (Bayliss *et al*., 1990). Amino acid substitutions in this region may increase the virulence of the virus (Eterradossi *et al*., 1998) In the NP2K isolate, they were found at three positions, namely 300 (E→A), 308 (I→F) and 334 (A→P). In the NP1SSH isolate, they were observed at five positions, namely 223 (G→S), 225 (V→I), 300 (E→A), 308 (I→F), and 334 (A→P). The substitution in the first hydrophilic region at the position 233 (G→S) in the NP1SSH isolate corresponds to the increased virulence of the virus (Heine *et al*., 1991). Also a substitution at 253Q is considered to play a critical role in the increased virulence of the virus (Yamaguchi *et al*., 2000).

The deduced amino acids sequences at positions 1–446 in the HV region of VP2 of the isolates NP2K and NP1SSH showed a 98.3% identity, while their comparison with other vvIBDV isolates/strains revealed identities of 97.1% and 98.3%, respectively. In the phylogenetic tree, the NP1SSH and NP2K isolates clustered together with vvIBDV isolates/strains, especially with the BD399 strain from Bangladesh. The classical, Australian variant classical, attenuated and avirulent strains formed a separate group. Thus, vvIBDV isolated in Nepal could originate from Bangladesh, China, South East Asia, Japan or Europe. We consider NP1SSH and NP2K IBDV the first isolates from Nepal characterized at molecular level.

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