

The Complete Nucleotide Sequence of Segment L2 of Ibaraki Virus Encoding for the Antigen Recognized by Neutralizing Antibodies

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ABSTRACT. The complete nucleotide sequence of cDNA clones representing the L2 dsRNA from Japan isolate of epizootic hemorrhagic disease serotype 2 (EHDV-2JPN) was determined. The EHDV-2JPN L2 gene is 3002 base pairs long with a single open reading frame of 2949 bp which predicts a polypeptide of 982 amino acid residues. Comparison of VP2 sequence between Japan and North American Isolates of EHDV-2 showed a 72% homology in spite of the same serotype, although those among the North American isolates showed a high genetic identity (>97%).—**KEY WORDS:** cloning, Ibaraki virus, VP2 RNA gene.

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Ibaraki virus is grouped into epizootic hemorrhagic disease virus Japan serotype-2 (EHDV-2 JPN), which is classified as Orbiviruses in the family *Reoviridae*. The Orbiviruses have ten segments of dsRNA genome as bluetongue virus (BTV) [11] and contain a double-layered capsid which comprises an outer coat of two viral proteins (VP2 and VP5), two inner core proteins (VP3 and VP7) and three minor proteins (VP1, VP4 and VP6) within the core particles [13]. The most outer capsid protein, VP2, is encoded by genome RNA segment L2 and is the main determinant of the neutralization-specific and serotype-specific response in Orbiviruses [5, 7, 9]. Several other properties such as hemagglutination and cell adsorption have also been ascribed to VP2 [2]. Being located on the outer most surface of the virion, VP2 is subject to constant immunological pressures from the host and alterations within its genome are vital if these Orbiviruses are to remain viable within their host system [14]. Therefore, the sequence from the L2 has extensive variability among the serotypes of BTV. This variability has also been demonstrated among field isolates of BTV-17 [3] and between Australian and South African isolates of BTV-1 [14]. In EHDV-2, the sequence of the genome segment coding for VP2 of EHDV-2 field isolates in North America has been reported and showed a high degree of genetic identity (>97%) [1]. However, the L2 sequence of EHDV-2 JPN has not been determined and its genetic relationship with North American isolates is not known. To clarify genetic relationship between the EHDV-2 isolates, we determined the complete nucleotide sequence of segment L2 of EHDV-2 JPN, Ibaraki virus, which encodes the VP2 antigen recognized by neutralizing antibodies.

The viral dsRNA was purified from HmLu cells infected with Ibaraki virus No. 2 strain, which was kindly supplied

from National Institute of Animal Health, Japan. The segment L2 RNA of Ibaraki virus was separated in agarose gel and recovered as described elsewhere [8]. The L2 segment was transcribed into cDNA following the Marathon™ cDNA Amplification Kit protocol (CLONTECH, U.S.A.). Adaptor-ligated L2 cDNA library was amplified by polymerase chain reaction (PCR) using adaptor primer and cloned into pCR 2.1 vector by original TA Cloning Kit protocol (Invitrogen, U.S.A.). The clones were randomly selected and screened by *Hinf* I restriction pattern and sequence analyses of both termini of the insert by using Thermo Sequenase dye terminator sequencing kit protocols (version 2.0, Amersham Life Science, Inc., U.K.). Some clones which could not be obtained by the above method were generated by PCR using specific primers. The sequences of both strands were determined by the cycle sequencing protocols as described above using appropriate DNA fragments or deletion derivatives subcloned into pCR2.1. Deletion clones were generated following the ExoIII/Mung Bean Nuclease Deletion Kit protocol (Stratagene, U.S.A.). To determine both terminal sequences of L2 segment, 5' Full RACE Core Set (Takara, Japan) was used for cDNA cloning of each terminus. To confirm sequence data, at least two overlapped or PCR-derived clones were analysed.

The complete nucleotide and amino acid sequence data (DDBJ/EMBL/GenBank Accession No. AB030735) of the cDNA of L2 segment in its coding sense are shown in Fig. 1. The L2 segment is 3,002 base pairs in length and its coding strand has a calculated base composition of 35.01% A, 26.38% T, 23.72% G and 14.89% C, comparable to those of EHDV-1 [6] and EHDV-2 Alberta isolate (strain sv 124 [1], EHDV-2 CAN). An open reading frame of 2949 nucleotides was flanked by 17 base pairs with a presumed initiation codon (ATG) at position 18–20, and a 3' noncoding region of 36 bases excluding stop codon. The 5' and 3' termini of the clone were found to contain characteristic consensus motifs of Orbivirus RNA segments, namely 5' GTTAAA... and ...ACTTAC 3', whereas EHDV-2 CAN has

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1  gttaaattgttccagaaatggaggagatttttataagcgaattgatagcagtcagcgcgtaccgaaacagttgtacaagattatccag
   M E E I F I S V I D S S Q R V P K Q L Y K D Y P
91  taatttatagatgtcggacaacgggaaggtgaaatagattaccagtggaacgattggaagccaaacaccatcgaactgattcaggcgag
   V I I D V G Q R K G E N R L P V E R L E G K N T I E L I Q A
181 aagcgagagattttatttcaatatgactcaaaagatgattatgagataatttttccagatgcgttatcaataggccatcgcgtccatgatgact
   E A R D L F Q Y D S K D D Y E I I F P D A L S I G I R P Y D
271 ggagacataaagagttctcagggaatcaaaatcacggcgatggtcgcggattgatttcatcagatgatgcgtttgaagagtttaagcggtt
   W R H K E F S R N Q N T T G D G R G L I S S D D A F E E L M R
361 gctcaaatgcaaacgtcaggttaaaaacgggttttaaatgaggagcatattcatcacgaattgtcatattgtgacgtttatgtaaatgcga
   C S N A N V S L K T D F N E E H I H H E L S Y C D V Y V N A
451 ccatagcggaaactatagaanttagcgcctcataatagtgaagaaggattgttttcacggtgaagagacagcggttatataatcatatgc
   I I A E T I E I S A H N S E K K D C F H G E E T A V Y N H M
541 taactgaggcgctatgcacgcgtcagggaacatggtatgatttgaagaacatgtccagctcacaacgatcggggaggtaggcttacgtc
   L T E A L C I G S G T C Y D L E E E H V Q L K T I G E V G L R
631 caecgcacccattgttgcgtttcagggaagacatcccaaggaggagaagatgataacaaggagattcggaggtggcgagattaaagcgc
   P R D H V D V S G R T H P K G E K M I T R R F G G G E I K T
721 tgcccaacagttataaaccagatcaatttgacttaaaaggagagatttttaattgaggagatcgctatcacggttagaaaaacgcgactaa
   L T T S I N P D Q F D L K E E I F N E E I A I T V E K R D L
811 ttaaatatgatgatgaaataatgcagcttgatgaaatagctgtgaaatggattcgggaatcagaacgcgcatgacctggaaaaaatcatat
   I K Y D D E I M Q L D E I A V K W I R N Q N A D D L E K I I
901 tattgtcggagcaatagagaaaaagataaacgtgtgcgaacccgctaatcagaagatatccgcaataaattcaaaaggaattacaag
   L L E L E K D K R V E P A N T E D I R N K F K R L Q
991 ttaattctccagaaacgggatggtgaaatcagaacataaggaattatcatcagcatggtgaacccgaacgggtggcgcaatattaatat
   V N L Q K T D G E I R N I R N Y H Q H G E P K R L A A I L I
1081 taacaaatgtcggagtaactgaacccgtgcctatggggagaaataggttttaactagtttagaggagtgtaacaaatagggaatatagaa
   L T M C D V M N R A I W G D N R F K L V R G V Y N Y G K Y R
1171 tggggctggttttatcacgcaatgcgaacagatgatgatgtgcgaactcgcgatcaagttatctagatcacgtgtcctctgtattttgtatagaa
   M G S V Y H A M R T D M M H W Q L R S S Y L D T C P C I C D R
1261 gaaaatatttaaatgtaccgctataaatttttcttttaggcgggagacgggagatttttatataaatgggacatcaggactatgcgag
   R K Y L M Y R Y N Y F S L G R E T G D F I V K W D I R T H R
1351 acgacaagatgaccacaagatcaaaaggatggcagttatggagcaatagaggtgagagaggaaagtgtgaagtgttaatacatgatgtttg
   D D K M T T R S K G W Q Y G A I E D E E E S D E V L I D F
1441 atgaaggttaaatatgcagaatatatgcagcggataattcagggaacatggattgagaagatggaattgggattctaatgaaagagcaag
   D E G K Y A E Y M Q R I I Q G F W I E K D G I G I L M K E Q
1531 cagcgattgagctgtttgatatttacggggatgcttatgtagatgaagcgggttttctcagggtgcgccatattacaataagctaat
   A A I E L F D F T R D A Y V D E A G F L R L P A N K L I
1621 aatcaacttttatatgaattcgttttaaggtaagacgggttagagattaccacaaggttaagagaccagatccttggacccaacaaacggagg
   K S T L Y E S A F K V R R V E I T Q G K R P D P W T Q K T S
1711 atgaactgaaggaaggaacagagatgtggcttttaactgtgtattcaagttgttggaacggagcgttctgtatgactggcaatatattaaaga
   D E L K K K E N E M W L L P V Y S V V D R A F C M T G N I L S
1801 ctgcaaaacagggaacaaagtgcaacgttttacagccataattgaggttttaagaaagagagagagaagtagagaaagatattcacgta
   T A K Q E Q S A R P T A I I E A L K K E K R E V R S R
1891 atagatttacactgtcccaaatgttaaacgtatttaattatcacaggatataggcagcgaaggttcgtttttcaattataaaastcaoc
   N D S Y T C P M L N V F N Y T G Y R Q R R F V F S I L K N H
1981 tgcaaaaaaatctactcatagacatgtacccagatgaggcctatagaatacagatcctcgcgattatacagactgtatgggaaagaagaga
   L P K L L I D M Y P D E I E Y D P R D Y T D C M G K E
2071 ttttgatgaagatgaaatcgatatttgaggttaatactttatctaattcasataggtctcgaacaaagggttctgtgtctgagcgaagagg
   I L M K M K S I F E V I L Y L I Q I G F E K E V V V L S E E
2161 agatcccggtgtgaaacatagaatgataaagagagagacaccgaacacgacatcatgagtaacgttttgcgggaattttgcggattataa
   E I P G V K H R M I K R E H R N D I M S T L L P E F S R I I
2251 ggagagggcagagaasatgcagaggtggagagaagatgaggacattaccaatgtatttctatcaatcactcattttatcaacgaattga
   R R G E K M Q E V E K N E D L L P M Y F Y Q S L I L S N E L
2341 tatatgaaaacgcgaataaatcgcacacagttctttatgtttttgtgaaaaaagagtaaggtatcgtccctattcagacgaacggtttggcgaa
   I Y E N A N K S H P V L M F C E K R V R I V P I Q T N V M R
2431 aagatgtgcctctgtctttctttttttgttttgaatatcatgcatggatggcggaagcaggtgaaactattgaaagagcgttttcgaa
   K D V P L L S F L P V L K Y H A G W R R R G E T I E D V R
2521 cagtatggcccttttaacgaagtatttggttaaatattgaattccacgcaggggaatccacgatttaacggttgatgagaatgcacat
   T V W P L L T K Y W L N I E F P R R E I T D L T L M R N H P
2611 tgaatacgcattttagcacatattgcttgctatgttttgaggtatatagttttcggttacctatagatcattccatcgaaggttatgtag
   L N T H F S T Y C L R M F E V Y S F A L P I V H P S K G I V
2701 ttataggtatcattcccgatactatctcgaatgcocaaaggtttttcggttatataacaaagattttcattctgttgaaacgtatgtacatg
   V I G I I P D T I S N A Q G F S V I K Q R F N S V E Q Y V H
2791 cgcgaataatttaagagtggttggaagtggtcagtgagtggtttacgacgatggcgacgtcaagtgtaatttattagagagattttgtt
   A R I I L R V L E N G H V S V Y D D G D V K C N L L E K F C
2881 ggggaaggaggtcgaagattgtaagagtttaactaaatggaaaagtatacgcggatccagaataatctcacaatttaattgaattaaacoc
   C G R K S K I V R V K L N G K V Y A D P E I I S K L M N *
2971 ctgacccgggtttactgggaacaaacattac 3002

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Fig. 1. Complete nucleotide sequence of Ibaraki virus L2 gene shown as the cDNA of the message-sense RNA. Amino acids are shown below their respective DNA codons. Possible N-glycosylation sites are underlined and an asterisk indicates the termination.

Table 1. Amino acid and nucleotide comparisons amongst VP2 of Orbiviruses

	EHDV-2 JPN	EHDV-2 CAN	EHDV-1	BTV-10	AHSV-4
EHDV-2 JPN	–	72	31	19	10
EHDV-2 CAN	70	–	33	22	11
EHDV-1	51	50	–	18	12
BTV-10	36	37	38	–	13
AHSV-4	42	42	45	38	–

Upper section: percentages of amino acid sequence homology.

Lower section: percentages of nucleotide sequence homology.

a different 3' terminal sequence of ...CCTTAC 3', i.e. it possessed a C residue instead of the usual A residue at sixth position [1]. The 3' noncoding region was somewhat variable between two EHDV-2 isolates. While 16 of 17 nucleotides were conserved in the 5' non-coding region, 27 of 36 nucleotides were conserved in the 3' noncoding region. The deduced amino acid sequence have 982 residues in length with an estimated size of 115,597 Da and an isoelectric point of 5.99. VP2 protein had a hydrophobic residue of 43.44%, neutral residue of 18.51% and hydrophilic residue of 37.95%. These data were also comparable to the other Orbivirus VP2 proteins. In addition, 4 possible N-glycosylation sites were observed at 143–145, 625–627, 637–639 and 780–782 amino acids. Though another outer capsid protein of VP5 was reported to be glycosylated [15], it's not known whether VP2 protein is glycosylated or not.

Comparison of the VP2 sequences of EHDV, BTV and African horse sickness virus (AHSV) serogroups indicated that it was the most variable protein [14]. Sequence alignments were performed by Malign program (DNA Data Bank of Japan, National Institute of Genetics, Japan) [4]. Figure 2 shows the comparison of the VP2 amino acid sequence of EHDV-2 JPN with those of EHDV-2 CAN and EHDV-1. The percentages of homologies between EHDV-2 JPN and other Orbiviruses are also shown in Table 1. The identity of L2 nucleotide sequence between EHDV-2 JPN and EHDV-2 CAN was 70%, but the percentages of identity decreased when comparing EHDV-1, BTV-10 and AHSV-4 L2. Predicted amino acid homologies also showed similar results (Table 1) and the homology between EHDV-2 JPN and EHDV-2 CAN was 72%. While six North American isolates showed a high degree of comparative genetic identity (>97%) in nucleotide and protein levels, the comparison between EHDV-2 Japan isolate and North American isolate showed a 70% nucleotide identity in spite of the same serotype. Similarly, BTV-1 Australian and South African isolates showed the 74% identity of L2 sequence. The phylogenetic analysis of the L2 genes using a CLUSTALW program [12] also suggested that the both EHDV-2 isolates were closely related to each other and showed similar genetic distance from EHDV-1, but the genetic distance between EHDV-2 JPN and North American

isolates was similar to those between Australian and South African isolates of BTV-1 and between BTV-11 and 17. Genetic distinction among EHDV-2 field isolates in North America was reported to be due to random genetic mutation within virus populations or influence of region-specific environmental factors [1]. However, these reasons might not completely explain the lower identity of L2 between the EHDV-2 JPN and North American isolates, which might be evolved independently in the different host animals and/or area as different lineages [14]. In addition, the homology and genetic distance between the EHDV-2 isolates were similar to that between different BTV serotypes as seen between BTV-10 and 11 or between BTV-11 and 17. These data suggested that the classification of viruses by seroreactivity might not be completely corresponding to genetic relationship of EHDV. Further studies on genetic analyses of other segments and EHDV serotypes are needed to clarify these points.

As mentioned above, the complete nucleotide sequence of L2 gene of Ibaraki virus encoding for the antigen recognized by neutralizing antibody was determined and its close relationship with that of Canada isolate of EHDV-2 was shown. However, the homology was only 72% in VP2 sequence between these isolates, suggesting that the structure of neutralizing epitopes of EHDV-2 JPN might be not completely the same as that of North American isolates. Roy *et al.* [10] have developed a more efficient and long-lived protection against the BTV using virus-like particles (VLPs). The data in this report might contribute to studies on genotype classification and recombinant vaccine development.

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EHDV-2	JPN	MEEIFISVIDSSQRPVKQLYKDYPIIDVQQRKGENRLPVERLEGKNTIELIQAEARDLF	60
EHDV-2	CAN	M****F*****LI*****EN***V***H***N*G*****D*H*****N**	60
EHDV-1		M*D*NLTIFSDDALPHAVVD**VIA*K--RNPNGTF*EPHQMYDRY*Q*FL*GK**DVL	58
EHDV-2	JPN	QYDSKDDYEIIFPDALSIGIRPYDWRHKEFSRNQNTGDGRGLISSDDAFEELMRCSNANV	120
EHDV-2	CAN	S**E**E**GV*****R*****RA*Y*K**SVERSN*I**E*****I*S*S**	120
EHDV-1		RQNGR*G*KL*I*E***T**IR*ENKT*--GAQSEVELENQ*R*-----IRHQR*	107
EHDV-2	JPN	ELKTDNFNEEHIHHELSYCDVYVNATIAETIEISAHNSEKKDCFHGEETAVYNHMLTEALC	180
EHDV-2	CAN	K*****SGGR*****IHID*****D***N*EEG*****N*V*****SNY	180
EHDV-1		KPRM*EAHRKLI*RGQQILLHPR***S**F*IISK*NAT*S*TPVNCA*EVL*SGGIN	167
EHDV-2	JPN	IGSGTCYDLEEHVQLKTIGEVLGRPRDHVDVSGRT-HPKGEKMITRRFGGGEIKTLTTSI	239
EHDV-2	CAN	**S*****GD*I**R***D**P*****L***-**R***H*I**Y**D*****M	239
EHDV-1		V*T*****SSRLK*RV**D*DRHR*SMQN*L*VI*TGDP*I*N*VNQI*SQQFIDRA*	227
EHDV-2	JPN	NPDQFDLKEEIFNEEIAITVEKRDLIKYDDEIMQLDEIAVKWIRNQNADDLEKIILLEA	299
EHDV-2	CAN	S**E**E**KK*L*GDV**G***N****SNE*L***D**AS***S***N*****VA**R	299
EHDV-1		G*K*E**R***DRLK*LD*DV*KV*REEEASAE***MGRR*M*D**VNIVND**QS*VK	287
EHDV-2	JPN	IGEKKRVEPANTEDIRNKFKRLQVNL--QKTDGEIRNIRNYHQHGEPKRLAAILITM	357
EHDV-2	CAN	L*****QK***H*SN***ER*R***LQ***--*****QDAT**F**V**V**	357
EHDV-1		K*SRSEKLAHR*EQGMQAR*R*TIAT**RD*RQ GK*VL***GTRGQP*E*KF**V*LM*G	347
EHDV-2	JPN	CDVMNRAIWGDNRFKLVGVYNYGKYRMGSVYHAMRTDMMWQLRSSYLDTCPCICDRKY	417
EHDV-2	CAN	**T*****E*****E*A*****I*YS***VT***T***V*****R*****R*	417
EHDV-1		**IVE***SNEETA*L*L*A*AHDKL*C*R**KK*FV*SI*PT*T*R*AGV**KRT	407
EHDV-2	JPN	LMYRYNYFSLGRETGDFIYKWDIRTMRDDKMTTRSKGWQYGAIEDEEESDEVLIHDFDEG	477
EHDV-2	CAN	I*Q**D***N*****S*****VGDI*GG*K*SRWE**P*KS*****DE*****D	477
EHDV-1		I*V*ED**D*Q**EN*SV***I*TEWDKNDVLIISA*NGYLYSKYSG*DE*DI*V*EI*DR	467
EHDV-2	JPN	KYAEYMQRIIQGPWIEKDGIGILMKEQAAIELDFDTRDAYVDEAGFLRLPAYYNKLIKST	537
EHDV-2	CAN	**TQ****V*****V*****V*****E*****T***S	537
EHDV-1		L*TAMID*LING***E*LSQII**EVRL*S***K*****E**DRV*A*N	527
EHDV-2	JPN	LYESAFKVRVVEITQGRPDPTQKTEDELKKENEMWLLPVYSVVDRAFCMTGNILSTAK	597
EHDV-2	CAN	****S**I*****MH**KA***K**N*****LPT***KTL*L*****I*	597
EHDV-1		I*DCK**IS**S***SSND**DK**A*SIID*QCL*KI*LPNII*VRP*FR*DL*TSNS	587
EHDV-2	JPN	QEQSARFTAIEALKKEKREVRERYSRNDSY-TCPMLNVFNHYGYRQRRFVSILKNHLP	656
EHDV-2	CAN	*****EI*KRK*T*D*T*--*****L*****I*****	656
EHDV-1		**Y*K**SG**DE***D*EIYDDFIPVQGVPR*VQGH*CR*AF***KLTI*T***RY**	647
EHDV-2	JPN	KNLLIDMYPDEDIEYDPRDYDTCMGKEEILMKMSIFEVILYLIQIGFEKEVVVLSEEEI	716
EHDV-2	CAN	*D**MEV*Q*P*E***H*****V*VG*R*****HL***NQITTY*****	716
EHDV-1		IERILELTDE**Y*NLYLDKE*YK**SLILNLR**SL*CF**DF*Y*GREITRGED*Y	707
EHDV-2	JPN	PGVKHRMIKREHRNDIMSTLLPEFSRIIRGEKMQVEKNEDLLPMFYQSLILSNELIY	776
EHDV-2	CAN	RVI*****K***DG*VDI*A*N*****EN**IKI**Y*****A*V*****M**	776
EHDV-1		LKIFNEINYGG*--ARKEAINKY*PQFYQ*LMRVRTS*N*****LA***A*L*DPCTD	765
EHDV-2	JPN	ENANKSHPVLMFCEKRVRIPIQTNVWRKDVPLLSFLFVLKYHAGWRRRGETIEEDVRTV	836
EHDV-2	CAN	**M*****M*****DN*****V***S***S*****T**I***Y***K*E****D*I***	836
EHDV-1		NSEKS**LIL**QDK**V***R*ATQERGL***CCIHIF*F*P*LQM*KKEL*D*IKKT	825
EHDV-2	JPN	WPLLTKYWLNIEFPREITDLTLRMHPLNTHFSTYCLRMFEVYSFALPIVHPSKGIVVI	896
EHDV-2	CAN	**H**R***DV**Q****A*G*VI**Q**K**L***SY*S*****T***I*AV	896
EHDV-1		L*AIFD**IEL*MK*LDTG*RLRT*AQMVELY**NCGGSY*TLNFVF*****N*FIAC	885
EHDV-2	JPN	GIIPDTISNAQGSVVIKQRFHSVEQYVHARIILRVLENGHVSVDGDKCNLLEKFCG	956
EHDV-2	CAN	*V***V*****I*****Y*IDR*****V***IQKD*S*N**GE**I***V*D*****	956
EHDV-1		V*SSKGGMG*LNEDDVRR**RRIQSSIQGISISID*EMEIQLHHS*NIQARI***VFFE	845
EHDV-2	JPN	RKSKIVRVKLNGKVYADPEIISKLMN	982
EHDV-2	CAN	K*T**I**R*****N*****	982
EHDV-1		H*WH**Q*****IFENH*L*T****	971

Fig. 2. Comparison of the Ibaraki virus VP2 (EHDV-2 JPN) amino acid sequence with those of EHDV-2 Alberta isolate (strain sv 124, EHDV-2 CAN) and EHDV-1. Residues identical to those of Ibaraki virus are indicated by asterisks.

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