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Phylogenetic relationships of bovine immunodeficiency virus in cattle and buffaloes based on surface envelope gene sequences

Brief Report

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Summary. Isolates of bovine immunodeficiency virus (BIV) exhibit a striking genomic diversity, most of which are located in the viral envelope gene. Since this property of the BIV group of viruses may play an important role in the pathobiology of the virus, the surface envelope gene, particularly the conserved (C) 2, hypervariable (V)1, V2 and C3 regions, of eleven different isolates from different environments with different bovine breeds naturally infected with BIV, including dairy cows in Japan, buffaloes in Pakistan and draught animals in Cambodia, were sequenced. When compared to the nucleotide sequence of American BIV isolates, all Asian BIV field isolates seem to be smaller, several base substitutions were observed in the V1 region, and deletions were also found in the V2 region of *env* gene in these samples. However, deduced amino acid sequences were not so different among isolates from different bovine breeds, suggesting that bovine susceptibility to BIV infection may not depend upon bovine breed or buffaloes. Moreover, phylogenetic analysis revealed that genotypes were distinct between Asian and American BIV isolates and these results also provide an information on the molecular epidemiology of naturally occurring BIV infection in cattle and buffaloes

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The *Lentivirinae* subfamily of retroviruses is a group of exogenous, nononcogenic viruses which cause chronic, multi-system disease in susceptible host [16]. The bovine immunodeficiency virus (BIV) was originally isolated from an 8-year-old dairy cow in Louisiana which was suspected to have lymphosarcoma with persistent lymphocytosis, lymphadenopathy, central nervous system lesions, progressive weakness and emaciation [23]. BIV is known to be genetically, antigenically and structurally similar to human immunodeficiency virus type 1

(HIV-1) and simian immunodeficicency virus (SIV) [5, 6, 8]. Although several pathological changes have been reported in BIV-infected cattle, including, dysfunction of monocyte [17], encephalitis, lymphadenopathy, and immunodeficiency [8], the pathogenesis of BIV still remains unknown in infected cattle and buffaloes.

Besides the three large open reading frames (ORFs) encoding for the *gag*, *pol*, and *env* proteins, which are common to all replication-competent retroviruses, five additional small ORFs were found in the BIV genome. Numerous point mutations and deletions were found mostly in the *env* region of the viral genome isolates from different organs within one host. These data suggest that, within a single virus isolate, BIV displays extensive genomic variation [5]. The nucleotide sequences of the 298-bp DNA fragment corresponding to a part of the *pol* gene were compared, and it was found that the Japanese BIV isolates are very similar to strain R29 [12], but the *pol* sequences are highly conserved among lentiviruses. The sequence analysis of the surface envelope (SU) gene of nine different isolates from cattle in the USA suggested that strain R29 may not be representative of BIV currently present in cattle populations in the USA [20, 21]. Hypervariable (V1–V6) and conserved (C1–C6) regions of the SU gene have been identified in BIV as well as other lentiviruses including HIV-1 and feline immunodeficiency virus (FIV), and the BIV SU gene starts from C1 followed by C2, V1, V2, C3, C4, V3, C5, V4, V5, V6 and C6 regions [20]. In comparing different BIV isolates, differences in the length of the SU genes were commonly observed among different isolates, and these differences are mainly due to the size variations in the three hypervariable regions of the *env* gene, especially in the second hypervariable region (V2) among American (Florida and Oklahoma) BIV isolates in experimentally and naturally infected cattle [20, 21]. Moreover, a present report has suggested that the V2 domain may play an important role in the BIV replication in vivo and in vitro [3]. However, there has been no demonstration that genetic diversity around the C2, V1, V2 and C3 regions of the BIV *env* protein in naturally infected cattle and buffaloes in Asian countries. Therefore, the current study was designed to determine the sequences of the C2, V1, V2 and C3 regions of the *env* gene obtained from BIV-infected dairy cattle in Japan, draught animals in Cambodia, and buffaloes from Pakistan. These sequences were compared to those of American BIV isolates, and their phylogenetic relationships were determined.

Total cellular DNA samples were extracted from peripheral blood mononuclear cells of dairy cattle in Japan [12], buffalo in Pakistan [13], and draught animals in Cambodia [14] by the methods reported by Meas et al. (1998). These samples are listed in Table 1.

Amplification of the C2, V1, V2 and C3 region of the *env* gene from different Cambodian BIV uncultured field isolates and Japanese field isolates, and from buffaloes in Pakistan (Table 1) were carried out by nested polymerase chain reaction (PCR) system using primers 04 (nt 5413: 5′-CTATGGATCAGGACCTAGAC-3′) and 14 (nt 7192: 5′-CTCTCCACAACCTTAGCCGT-3′) in the first step of the reaction and primers 04 and 92 (nt 6305: 5′-ATCATTCACTATGCGGTACC-3′) in the second step reaction. To amplify the C2, V1, V2 and C3 regions of the BIV

Table 1. BIV isolates are used in this study

Isolate/strain	Animals	Database no.[b]
JapanPL	Dairy cow	AB040419
Japan212	Dairy cow	AB040420
Japan234	Dairy cow	AB040421
Pakistan4	Water buffalo	AB040422
Pakistan7	Water buffalo	AB040428
Pakistan10	Water buffalo	AB040423
Cambodia2	Draught cattle[a]	AB040424
Cambodia15	Draught cattle	AB040425
Cambodia48	Draught cattle	AB040426
Cambodia759	Draught cattle	AB040427
Cambodia21	Draught cattle	AB040429
R29	Dairy cow	M32690[c]
FL112	Dairy cow	L43126[d]
OK40	Dairy cow	L43129[e]

[a]Draught cattle were mix-breed with American Brahman, Haryana and local breed

[b]The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with a accession number in this column

[c]Garvey et al. [22]

[d] and [e]Suarez and Whetstone [20]

proviral DNA, 2 μl of 100 fold dilutions of the second step reaction was PCR-amplified by using primers 45 (nt 5533: 5′-ATCAATAACGGTGAGATCCA-3′) and 01 (nt 5965: 5′-TCTATGGTATCTCTGGCTGC-3′) [5, 20]. All PCR was done in a buffer containing 22.5 mM Tris-HCl (pH 8.8), 5.5 mM ammonium sulfate, 2.25 mM $MgCl_2$, 3.35 mM 2-mercatoethanol, 2.2 mM EDTA (pH 8.0), 56.5 mg/ml BSA, 0.5 mM dNTPs, 20 pM of each primer and 1.25 U of *Taq* polymerase (Phamacia, Sweden). Cycling conditions for the first step as well as the second and third step PCR were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 57 °C for 45 sec and 72 °C for 30 sec, with a final extension step at 72 °C for 7 min.

The amplified fragments were separated on an agarose gel (1.5%) and visualized by the staining with ethidium bromide. The PCR products were excised from the gel, purified by using the Geneclean II kit (Bio101, La Jolla, USA), and cloned into the pGEM-T vector (Promega, Madison, USA). The pGEM-T plasmids containing the inserts corresponding to the C2, V1, V2 and C3 regions from different BIV isolates were purified by a standard mini-prep method, and sequenced by using the BigDye terminator cycle sequencing kit (Perkin Elmer-Applied Biosystems, Foster City, USA) and Model 310 genetic analyzer (Perkin Elmer-Applied Biosystems, Foster City, USA). At least 2 individual plasmid clones per each BIV isolate were used for sequencing. DNA sequence analysis was done using

the GENETYXMAC 10.1.2 package (Software Development Co., Ltd, Tokyo, Japan) in combination with the BLAST program at the GenBank for homology search with known BIV gene sequences.

Nucleotide sequences of the *env* genes of the BIV isolates were translated into the amino acid sequences, and initial multiple sequence alignments were performed with the Clustal W [22]. The phylogenetic tree was constructed by Neighbor joining (NJ) [18] with the Kimura's two parameters method [11] with floating scale based on amino acid sequences corresponding to the C2, V1, V2 and C3 regions with those of American BIV isolates (Table 1). The bootstrap values are replicated 1,000 times.

By using the BLAST program, all of the eleven nucleotide sequences shown to be highly homologous to the respective sequences of a molecular clone, BIV R29, and other known BIV sequences in the database. The nucleotide sequences from difference BIV isolates were adjusted their lengths according to the location of primers around the C2, V1, V2 and C3 regions [5, 20], translated into amino acid sequences, and were compared each other (Fig. 1). Multiple alignments indicated that the sequences of Asian BIV isolates (Japanese, Pakistan and Cambodian BIV isolates) seem to be smaller than those of American BIV isolates. Nucleotide substitutions which alter amino acid sequences identified in field isolates were mainly observed between the V1 and V2 regions, and deletions were also found in the V2 region. These findings are in agreement with a recent study [3].

Based on the neighbor joining method, a phylogenetic tree was constructed as shown in Fig. 2. There are two main branches, one leading to the American BIV isolates group (a Florida genotype FL112, Oklahoma40), another large branch is Asian BIV isolates. BIV sequences of Japanese, Pakistan and Cambodian BIV isolates were clustered together with different BIV strains, a Florida genotype FL112, Oklahoma40 and Louisiana genotype R29. BIV R29 shown to be located at the different branch from genotypes FL112 and OK40, in agreement with a previous report [20]. Amino acid sequences of BIV isolates from Japanese dairy cattle were clustered to the isolates from Pakistan water buffaloes, and were more closely related to those from Cambodian draught animals. Two Cambodian BIV isolates, Cam2 and Cam48, were diverged from other Cambodian BIV isolates. Taken together, this tree topology of BIV field isolates indicated that genotypes were distinct between Asian and American BIV isolates.

It has been shown that several distinct strains of BIV may exist worldwide [9, 10]. In this study, BIV isolates were obtained from different bovine breeds with different environmental co-factors in Asian countries. However, deduced amino acid sequences from all of these BIV field isolates from clinically healthy animals

Fig. 1. Comparison of the amino acid sequences of representative clones of the Japanese (Jpn), Pakistan (Pak, B-buffalo) and Cambodian (Cam) isolates of BIV with American BIV isolates (R29, FL112, OK40). Dots indicate identity to R29 isolate, and dashes indicate amino acid deletions

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                          C2                    V1
                    ______________________  .............

R29              1 INNGEIHPWVLAGMLSMGVGMLLGVYCQLPDTLIWILMFQLCLYWGLGETSRELDKDSWQ   60
FL112            1 S.........T..M.I........M.NI.GPVT.L.I....I.LAF.....R..VN..N   60
OK40             1 ...............I.........TP.SE....L.IS...I.........K..RE..H   60
Jpn212           1 ............................N...........I........K......H   60
Jpn234           1 ........................................I........K.YFGELA   60
JpnPL            1 .........M...................HFGYCFNYA.FGV.VKHLENTRIVGSGSE   60
Pak.B4           1 ..............FH.SRNATRSILSVYQ.H..........I..S......T.FFDQL   60
Pak.B7           1 ........................LSVTRHTDLDTNVSIMP.L.F.RNIEKIRQGELA   60
Pak.B10          1 ........................................I..F.RNIEKIRHGESG   60
Cam2             1 ........................H................I........K.......   60
Cam15            1 ..........T...............SVTRHTDLDTNVSIMP.L.F.RNIEKIRQGLVR   60
Cam21            1 .........P.RNAIH.SRNATRSILSVTRHTGFGY.......I........K..MGEL   60
Cam48            1 ........................................I........K.Y.....   60
Cam759           1 ...............V...............DLDTNVSIMP.L.F.RNIEKIRQGQLA   60
                   .*********.................... .. .  ...  .. .     .

                                        ....................................
R29             61 WVRSVFIIAILGTLTMAGTALADDDQSTLIPNIT---------------KIPTKDTEPGC  120
FL112           61 ...G.L..G.............A.P.DTHSDPENITYKVDVPP---------------  120
OK40            61 .........LL.......I.F...EVY.AS.ATAPLDFNETVHPVSSTL.T..N....PV  120
Jpn212          61 VGQKCIYNSNI.-NSHYGRNCFGRRRSKYFNPQYHKNNT----------------------  120
Jpn234          61 VGQKCIYNSNI.-NSHYGRNCFGRQRSKYFNPQYHTNNT----------------------  120
JpnPL           61 MYLQYWEFSLWQELLW-----.............---------------KI---------  119
Pak.B4          61 ASG.DNV.VSQQYW.IL........-----------------------------------  120
Pak.B7          61 LGSEMYLEQHYWRILTMVGTCFWQTTFKVPLIPHYHPLFYLCPET--------------  120
Pak.B10         61 SGSEMYLEQQYWEF-SLWQEL.WQTTIKVLESPISQNEYLSRDTATS------E.LLQAI  120
Cam2            61 ...N..........................--ISKKEYLSRDTAT-------------  120
Cam15           61 L.G..MHLEEHYWELSLWQE.......R.....--------------------------  120
Cam21           61 AAGQKCIYNSNIGNSHY.RNCFGRTTIKVLESPISQKEYSSRDAATP-------------  120
Cam48           61 ...N...TA........................-------------------------  120
Cam759          61 VGQKCICNSNI.-NSHY.RNL.WPATIKVLESPISQKEYLSL-------------------  120
                              .
                             V2
                   ......................................................___
R29            121 TYPWI--------------------------------LILLILAFILGILGIILVLRRSN  144
FL112          121 ------SVPENITREVINGSVDTDPCTGPCHTNMQDT..F.G.VC...F..V.IAM....  169
OK40           121 -FST.KTLTNDNEPPVSSTIKTPTNGTESCHNYIQGT..G.ICVST.V...V..AM....  180
Jpn212         121 ----------------------------------------------------LSRDTATS  155
Jpn234         121 ----------------------------------------C.ETQQLRRI.FGSQ.SIE  168
JpnPL          121 ----------------------------------------------------........  119
Pak.B4         121 ----------------------------------PVLSSPAFTTQQT------------  180
Pak.B7         121 --------------------------------------------------------....  159
Pak.B10        121 ------------------------------------------------------------  168
Cam2           121 -------------------------------------------------------RR--  156
Cam15          121 -------------------------------------------HYLPNESLSRVH.H  163
Cam21          121 ------------------------------------------------------------  149
Cam48          121 ----------------------------------------------------........  154
Cam759         116 -------------------------------------------------------..H..  116
                        C3
                    __________
R29            144  SEDILAARDTI
FL112          169  PGG..S...AV                                                   169
OK40           181  S.G........                                                   199
Jpn212         155  .Q..FGTP...                                                   155
Jpn234         168  ITSAA.C.S..                                                   168
JpnPL          119  ..N....K..N                                                   119
Pak.B4         181  W...P.....                                                    182
Pak.B7         159  ----F...LL                                                    159
Pak.B10        168  -------...                                                    168
Cam2           156  --.IGSQRS.                                                    156
Cam15          163  E..FG....L                                                    163
Cam21          149  P.........                                                    149
Cam48          154  ..........                                                    154
Cam759         116  ..........                                                    116
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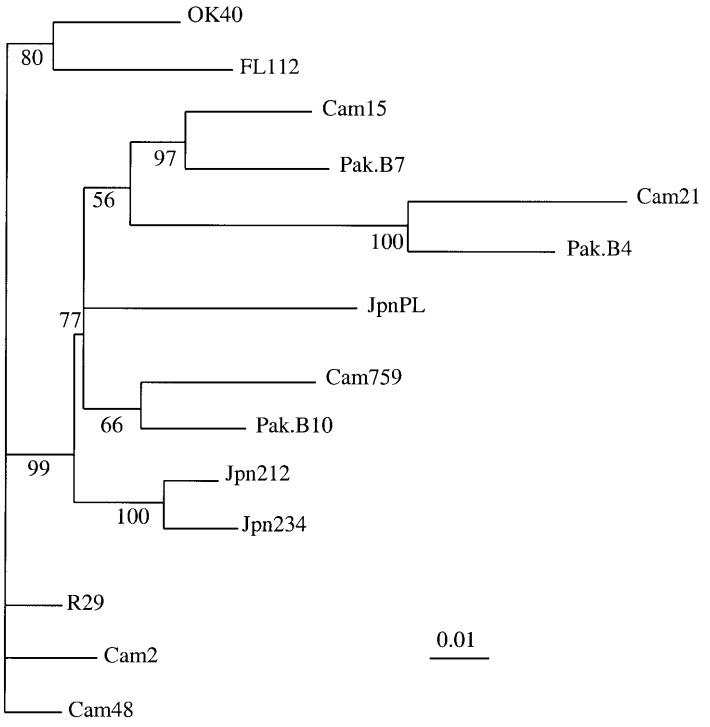

Fig. 2. Phylogenetic relatonships among BIV isolates based on surface envelope gene of amino acid sequences. Phylogenetic tree of BIV was constructed by NJ method based on C2, V1, V2 and C3 regions of amino acid sequences with Kimura's two parameter distance with floating scale and the bootstrap values are replicated 1,000 times. Japanese, Pakistan and Cambodian BIV isolates were clustered together and these genotypes were distinct from Asian and American (OK40, FL112 and R29) BIV isolates

or animals with some clinical symptoms (JapanPL) were found not so different from one another among these bovine breeds and buffaloes. Our observation that Japanese BIV isolates are different from American BIV isolate R29 partly at *env* gene regions contrast with the previous described at *pol* gene size [12]. As well as other lentiviruses such as HIV isolates [1], the hypervariable regions around the C2, V1, V2 and C3 of the *env* gene are not only polymorphic in sequence but also differ in length due to nucleotide insertions and deletions. The hypervariable regions of Asian BIV isolates shown to be shorter than those of American BIV isolates, strain R29, FL112 or OK40. It was not clear that two Cambodian BIV isolates, Cam2 and Cam48, which were diverged from another Cambodian BIV isolates (Table 1, Fig. 2) and the host animals were originally imported and subsequently bred with local breed, another BIV field isolates clustered together. In the cases of two clones BIV R29-127 and R29-106, all of the changes were due to substitutions and deletions, and no obvious duplications were observed. The largest number of amino acid substitutions occurred in *env* region, though biological significance of this observation remains unknown. However, there may be a selective advantage for the *env* region of lentiviruses to be hypervariable [2, 7], and all of the differences between clones 127 and 106 occurred in vivo

prior to the isolation of the parental stock of BIV [5]. Furthermore, R29-derived isolates appeared to be nonpathogenic, not even causing the early increase in mononuclear cell number that was observed in earlier inoculation [4]. Additionally, new Florida BIV isolates (FL112) [19] indicated that, unlike classical immunodeficiency virus such as HIV and FIV, there was no remarkable depletion of $CD4^+$ cells although B-cell proliferation was observed in the calves inoculated with BIV FL112 in short-term studies [24]. Sequences of the V2 regions of these American BIV isolates seem to be larger than those of strain R29 (Fig. 1) [20] and all Asian BIV isolates reported here which is in agreement with a recent study [3]. However, other studies have demonstrated selection of larger genotypes during in vitro replication of isolates containing mixed populations of larger and smaller genotypes [21]. Point mutations may be a common to many retroviruses [15] or BIV [21], and deletion in the V2 regions reported here is in agreement with a previous report [3]. To detect this original or dominant gene type, direct sequences should be done in this samples to reconfirm by using specific primers above, but not modified primers as reported [20, 21]. Further studies are needed to clarify the role of polymorphism in the V2 region in BIV replication in vivo as well as in vitro.

Genetic mutations could play an important role in the development of quasispecies which is a mixture on multiple distinct but related viral genotypes within one host. Since the presence of the quasispecies has been demonstrated frequently in other lentiviruses such as HIV [25], analysis of the relationship of BIV quasispecies in various tissues from JapanPL-infected cow are under investigation. The ability of the BIV genome to rapidly mutate may contribute to the persistence of the virus and its pathogenesis. BIV appears to show a high mutation rate similar to those of other lentiviruses.

Taken together, bovine susceptibility to BIV infection may not depend upon bovine breed or buffaloes, it seems that genotypes of BIV distinct, from USA isolates are distributed in Asian countries and these results provide an information on the molecular epidemiology of naturally occurring BIV infection in cattle and buffaloes. However, their association with other diseases affected or stresses are still not clear. Additional studies on the effects of Asian BIV isolates on the immune system of infected animals are necessary to determine whether BIV has immunosuppressive potential, and the role of cofactors in disease development induction should be studied.

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