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Genetic Variation of BIV Isolates Characterized by PCR Using Degenerate Primers

Oh-Sik Kwon* and John J. Sninsky[1]

Department of Biology, College of Natural Science, Keimyung University, Daegu 704-701, Korea
[1]Roche Molecular Systems Inc., 1145 Atlantic Ave., Alameda, CA 94501, USA

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The PCR was employed to detect and characterize the bovine immunodeficiency-like virus (BIV), which is a newly recognized member of the *Lentivirinae* of the retroviruses. Degenerate primers representing the conserved regions in the *pol* genes of the *Lentivirinae*, were used to detect proviral DNA obtained from the bovine embryonic spleen cell cultures infected with BIV. The PCR amplified DNA fragment was molecularly cloned and sequenced. The BIV DNA fragment contained a sequence identical to that reported by Garvey *et al.* (Garvey *et al.*, 1990. *Virology*, 175, 391-409). With the degenerate primers, peripheral blood mononuclear cells (PBMCs) of sick cattle and cells cultured with BIV were tested to determine genetic variation of BIV *pol* conserved sequence. We found the sequence heterogeneity within cultures and most variations occurred at the third base of codons that would not lead to amino acid substitutions. Another change was GAG (Glu) to AAG (Lys) within the BIV isolates. Interestingly, the altered sequence is also found in other lentiviruses such as HIV-2, SIVmac, CAEV and EIAV.

Key words: bovine immunodeficiency-like virus (BIV), PCR, degenerate primers, *pol* gene

Bovine immunodeficiency-like virus (BIV) is a newly recognized member of the *Lentivirinae* subfamily of the retroviruses (7), which is originally isolated by Van Der Maaten *et al.* (21) as a bovine visna-maedi virus. The BIV has been identified as a lentivirus with similar molecular characteristics as human immunodeficiency virus (7, 23). The *pol* gene, a segment that encodes the reverse transcriptase of *Retroviridae* family of viruses, has been shown to share clearly defined regions of homology (4). Comparisons of amino acid sequences of various retroviral reverse transcriptases have been utilized to determine their relatedness at the molecular and evolutionary level. Studies revealed that important invariant amino acids are at functional regions of the *pol* encoded polypeptides (3, 4, 13). Upon mutation using standard molecular techniques, one of these regions has been shown to be critical for reverse transcriptase activity (11).

The polymerase chain reaction (PCR) employs a pair of synthetic oligonucleotide primers flanking the region of DNA to be amplified (14, 17). Even if the sequence of the intervening region between the primers is not known, amplification with the appropriate primers and conditions may allow the identification of previously uncharacterized pathogens or genes (12). The appropriate primers derived from amino acid sequences of enzymes are coined as degenerate primers. The term "degenerate primers" means a mixture of oligonucleotides equalled in number of bases but containing various base sequences that differed by at least one residue (9).

In this study, it is demonstrated that degenerate primers can be used to amplify the conserved region of the *pol* gene of other retroviruses. For this reason, BIV was especially selected because 1) BIV shows the same viral morphology of other lentiviruses (2); 2) BIV is a relatively uncharacterized retrovirus whose nucleotide sequence has been published (6); 3) BIV is known to share some biological characteristics with HIV (7); and since the highly conserved nucleotide sequences encode a portion of functionally important protein (e.g. reverse transcriptase) in the retroviral replication system (22), BIV may also contain similar nucleotide sequences in the highly conserved region of the *pol* gene like other retroviruses, thereby allowing genetic insight into structure-function studies.

*To whom correspondence should be addressed.

Materials and Methods

Source of viruses

BIV infected embryonic spleen cell culture was obtained from Dr. M. Van Der Maaten (Ames, Iowa). The virus was originally isolated from a Louisiana cow (R29) with a lymphoma (20) and the virus was usually passaged in bovine embryonic spleen cells (BESC, refer to 10) and inoculated into calves. After 10 passages in BESC, the infected cells, confirmed by indirect immunofluorescent antibody test, were the source of the BIV used in this experiment. The presence of virus particles was confirmed by transmission electron microscopy (10).

Preparation of BIV proviruses

BIV was propagated in bovine first trimester embryonic spleen cells. Supernatant fluid containing BIV was added to a culture flask (75 cm^2) for inoculation. Seven to 10 days later, cells showed syncytia formation when observed with a phase contrast microscopy. Reverse transcriptase activity of the supernatant was measured as high as 70,000 cpm/ml using a technique modified from Gonda *et al.* (7). Genomic DNA was extracted from BIV infected BESC (18) and uninfected BESC served as the control.

Polymerase chain reaction

PCR was employed to detect the conserved region of BIV *pol* gene. The PCR procedure was described in Mack *et al.* (12). About 0.1~1.0 μg of the BIV infected BESC DNA was added to 50 μl of the PCR-reaction mixture, which contained a 5 μl of 10×*Taq* DNA polymerase buffer, 40 mM dNTPs, primers (100 pmol each) and 1 unit of *Taq* DNA polymerase (Perkin-Elmer). The 10×*Taq* DNA polymerase buffer contained 500 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$ and 0.1% gelatin. Fifty μl of mineral oil was added to every reaction tube before employing an automatic thermocycler (Perkin-Elmer). The PCR thermocycling profile included a 95℃ denaturation step, 37℃ to 55℃ annealing step and 72℃ extension step (30 seconds for each step). After 30 to 35 cycles of amplification, the samples were held for 10 minutes at 72℃ to ensure complete extension.

Southern blotting analysis

Ten μl of the reaction mixture was fractioned on Nu-Sieve composite agarose gel. This gel contained 3% Nu-Sieve agarose and 1% SeaKem agarose (FMC) in Tris-Borate buffer and was stained with an ethidium bromide solution (10 mg/l). The gel was subjected to Southern blot transfer (18) with minor modifications. First, it was submerged in solution containing 1.5 M NaCl and 0.5 M NaOH, with agitation for 15 to 20 minutes. DNA was transferred from the gel to a nylon filter (Plasco) with a 20×SSPE buffer overnight. After 2 hours pre-hybridization, the filter was hybridized with 0.5 pmol/ml [^{32}P]-labelled probe JS32 (Table 1) which was end-labelled with [γ-^{32}P] ATP (6,000 Ci/mmol, DuPont) by using T4 polynucleotide kinase (New England Biolabs). Hybridization was carried out for at least 5 hours in an agitating incubator set at 42℃. The hybridization solution contained 5×SSPE, 5×Denhardt's solution, 10 to 30% formamide (v/v) and 0.5% SDS. The filter was washed at moderate stringency (2×SSPE/0.1% SDS at room temperature) for 30 minutes or at higher stringency (2×SSPE/0.1% SDS at 55℃) for 15 minutes. The autoradiography

Table 1. Sequences of synthetic oligonucleotide primers and probes used in this study.

Oligonucleotides	Sequences	Remarks
Primers		
MD20	5'-gcgggatccTNCCNCARGGNTTYHWNAA-3'	Deg. Onco
MD22	5'-cttggatccTNCCNCARGGNTTGGA-3'	Deg. Lenti
MD35	5'-cttggatccTACCACAAGGTTGGA-3'	CAEV Spec.
MD36	5'-ctcaagcttRTCRTCCATRTA-3'	Deg. YMDD
MD51	5'-ctcctgcagRTCRTCCATRTA-3'	Deg. YMDD
MD72	5'-cttggatccGGNGAYGCNTAYTTY-3'	Deg. GDAYF
MD73	5'-cttggatccTNCCNCARGGGGNTGG-3'	Deg. LPQGW
BLV1	5'-TTTGTGCATGACCTACGAGCTACA-3'	BLV Spec.
BLV2	5'-AAGCGGTCTTCGACTGGAATCT-3'	BLV Spec.
Probes		
JS32	5'-TGGGTNTGYWSNCCNGCNATH-3'	Deg. BIV
HIV1	5'-ATCATCCATGTATTGATAGATAA-3'	HIV Spec.
BLV3	5'-TTTGAGATCTAGGCAAATGATATGTGGAGGGTGCGT-3'	BLV Spec.

The bases of the primer sequences denoted in lowercase letters represent 5' extensions that contain the recognition sequences for specific endonucleases of *Bam*HI (ggatcc), *Hin*dIII (aagctt) and *Pst*I (ctgcag). Signs of mixed bases: R(A+G), Y(C+T), S(C+G), W(A+T), H(A+C+T), N(A+C+G+T).

was performed with an intensifying screen in a −70℃ freezer.

Molecular cloning and DNA sequencing

The PCR-generated DNA fragment using MD72/MD51 (243 bp) was extracted from the agarose gel as described in Sambrook *et al.*(18). The 243 bp DNA was then subjected to reamplification using the same conditions. Ten μl of the reamplified reaction mixture was rerun on the agarose gel as described and the reconfirmed PCR DNA was molecularly cloned (19). The DNA sequences were determined by the dideoxy chain termination procedure and all procedures were followed as described in the supplied sequencing kit (United States Biochemicals).

Results

Application of HIV probe for BIV detection

A strategy employing degenerate primers to detect previously uncharacterized DNA was proposed by one of us (13). Since BIV is a member of the retroviral subfamily *Lentivirinae*, degenerate primers were designed for lentivirus (MD22/MD36, Table 1) and synthesized. With various samples, the PCR using degenerate primers successfully generated a 131 bp DNA band when BIV provirus was present (Fig. 1A; lanes, 2, 4, 5 and 7). Since multiple bands were generated, a Southern blot was used to verify the BIV DNA by applying a radioactive probe. Initially, an HIV-1 probe (refer to Table 1) was used in an attempt to detect the amplified DNA because this animal virus was shown to be closely related to HIV-1 using immunological cross reaction studies (7).

The autoradiogram in Fig. 1B showed specific bands at 131 bp, confirming that the degenerate primers (MD 22/MD36) were capable of detecting BIV provirus. Amplification of DNA from samples of the BIV infected BESC with 60 thermocycles of PCR (lanes 4 and 5) revealed much stronger radioactive signals at the 131 bp region than the DNA from samples of that with 30 thermocycles of PCR (lane 2). This presented a firm evidence that the HIV-1 probe could detect amplified BIV DNA, and confirmed that relatedness of the *pol* conserved sequences between HIV-1 and BIV.

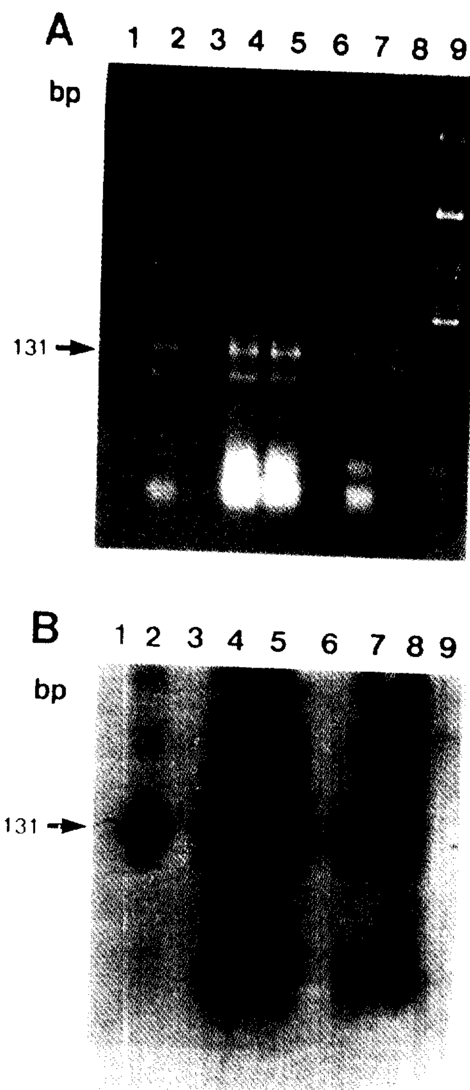

Fig. 1. (A) Results of gel electrophoresis of PCR using various degenerate primers. Lane 1, marker ϕX174 RF DNA *Hae*III cut; lanes 2,7,8 and 9, high copy BIV provirus used; lanes 3 and 6, blank; lanes 4 and 5, reamplification of low copy BIV provirus (60 cycles). Used primers are MD22/MD36 (lanes 2, 4, 5 and 7), MD20/MD36 (lane 8) and MD35/MD36 (lane 9). (B) Application of HIV specific probe (HIV1) to detect BIV DNA. Lane descriptions are same as above. The expected DNA band size is 131 bp.

Design of BIV probe and effect of primer annealing temperature

The stringency of hybridization indicated incomplete homology of the HIV-1 probe with the amplified BIV DNA. An oligonucleotide probe (JS32) with a sequence more likely to be a more full complement to the conserved region of the BIV *pol* gene was designed. We attempted to design degenerate probes by nucleic acid sequences that encoded the short stretch of the amino acid sequences of the BIV reverse transcriptase reported by Gonda *et al.* (7).

The only available region that could be used was the sequence N′-WVCSPAI-C′ (Table 2). The short stretch of amino acids shared significant homology with the *pol* gene of other lentiviruses. Particularly, the carboxyl terminal portion (SPAI) was identical to HIV sequences that were previously published (22).

We also changed degenerate primers to MD72/MD51 for PCR, which was deduced from amino acid sequences

Table 2. Conserved amino acid sequence alignment of lentivirus pol gene with BIV.

Viruses	5′ Upstream AA Group specific AA Generic AA
HIV-1	GDAYFSVPLDEDFRKYTAFTIPSINNETPGIRYQYMVLPQGWKGSPAIFQSSMTKILEPFKKQNPDIVIYQYMDD
HIV-2	. . . . . . .I. . H . . . .P. . . . .L. . V. . AE . . K . . Y . K . . . . .HT. RQV. . . .R. A. K. VY . I . . . . .
SIVmac	. . . . . . .I. . . . E . .Q. . . . .L. . V. . AE . . K . . I . K. . . . . .YT. RHV. . . .R. A. . . VT L V. . . . .
SIVagm	. . . . Y .I. . . P N . .K. . . . . . .TV. . QG . . . . . . F . C . . . . T . . .NTAAS. . .EIRRNL.ALT . V . . . . .
BIV	K . . . . TI. . H . . . .PF. . .SVVPV.R.G . I E . F . W. . . . . . . V C . . . . . .TTTQK.I.NI. .SH. .VM L . . . . . .
CAEV	. . . . . TI. . YKPY.E. .C. .LL.P. .LG.CK. . YWK. . . . . . . L. . .SVY.FT.QE. .GEW.QEH.E.QFRI. . . .
VISNA	. . . . . TI. . Y . PY.Q. .C. .ML.P. .LG.CV. . YWK. . . . . . . L. . . VY.FT.Q. . .RGWIEEH.M.QFGI. . . .
EIAV	. . . . . TI. . . PG. .P. . . . . .HQE.DK. . VW.G. . . .FVL. . Y.Y.KTLQE. .Q. .IERY.EVQ L . . . . . .
FIV	. . . . . TI. . . P . Y AP. . . . .L.RK. . AG. . R. FVWCS. . . . .IL. . L. Y. .TLDN.IQ. .RR. . .QLD I . . . . . .

[1] Aligned sequences of the viruses are all published.
[2] The dots indicate the same sequences in HIV-1.

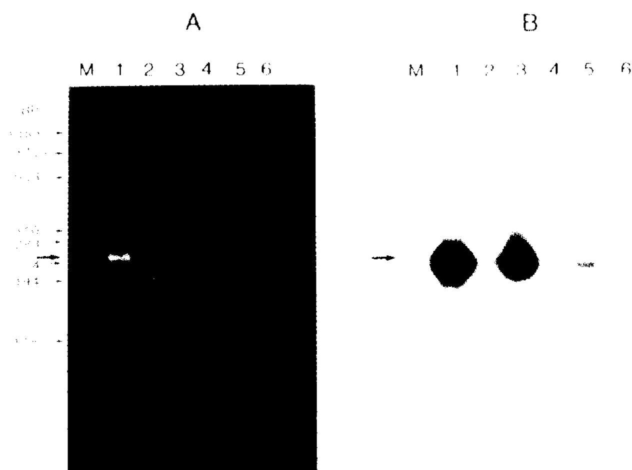

Fig. 2. (A) Effects of primer annealing temperature on PCR for detecting BIV. M, marker ϕX174 RF DNA *Hae*III cut; lanes 1 and 2, 37℃; lanes 3 and 4, 2 cycles at 37℃, then 28 cycles at 55℃; lanes 5 and 6, 55℃. Odd number lanes are BIV positive controls and even number lanes are BIV negative controls. (B) Application of degenerate BIV probe JS32. Its description is same as above. The expected DNA band size is 243 bp.

of the lentivirus reverse transcriptase conserved region (N′-GDAYF-C′). The primer set produced 243 bp DNA comparing to 131 bp DNA of MD22/MD36 set. A significant result was obtained when we tried to change the primer annealing temperature from 37℃ (Fig. 2A; lanes 1 and 2) to 55℃ (Fig. 2A; lanes 5 and 6). We also shifted the primer annealing temperature from 37℃ for the first 2 cycles to 55℃ for the remaining cycles of thermocycling. As shown in Fig. 2A (ethidium bromide stained gel), the treatment produced several PCR DNA bands from the BIV positive samples (lanes 1, 3 and 5) and negative samples (lanes 2, 4 and 6). However, differences from the previous PCR result were twofold, i.e., less non-specific DNA band was appeared and the target DNA band (243 bp) was visually distinguishable from the BIV negative samples. Since the PCR results showed non-specific DNA bands, the gel was subjected to Southern blot analysis with the radioactive probe JS32. After overnight exposure (14 hours) of the radioactive filter, an autoradiogram was obtained as shown in Fig. 2B. Surprisingly, only the lanes from BIV positive samples (lanes 1, 3 and 5) were vividly marked with strong radioactive signals at the predicted band size. It was noticed that the low primer annealing temperature (lane 1) produced more target DNA than the higher primer annealing temperature applied (lane 5). It may be an important evidence on PCR mechanisms using degenerate primers. That is, under the low primer annealing condition (37℃), deprivation of a single kind primer is not significant because similar degenerate primer species could help to overcome deprivation by allowing random annealing.

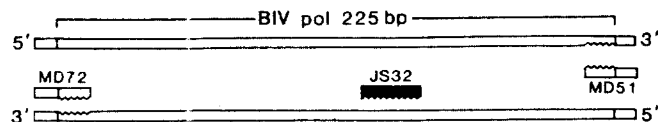

Fig. 3. Diagram of the region of the BIV reverse transcriptase gene for cloning and DNA sequencing. The primer MD72 (left) and MD51 (right) contain each 9 nucleotide linker sequences as described in Table 1. The probe (JS32) binding site is located in the middle of the PCR amplified DNA.

Thus, use of the degenerate primers and probe made it possible to reveal presence of BIV successfully. The specific detection by the JS32 probe confirms that there are invariant sequences in the BIV *pol* gene as shown for other lentiviruses.

Molecular cloning and DNA sequencing of the conserved region of BIV *pol* gene

Since the primers used for PCR were degenerate primers, it was necessary to demonstrate the real sequences amplified by PCR, which was designated as BIV *pol* conserved region. It would verify that the PCR using the degenerate primers (MD72/MD51) was correct and specific enough in detecting BIV because the PCR using

```
          Gly*Asp Ala Tyr Phe Thr Ile Pro
5'-cttggatcc GGG GAT GCC TAT TTT ACT ATA CCT

     Leu His Glu Asp Phe Arg Pro Phe Thr Ala
     TTA CAT GAG GAC TTT AGA CCC TTT ACA GCC

     Phe Ser Val Val Pro Val Asn Arg Glu Gly
     TTT TCT GTA GTC CCT GTA AAT CGA GAA GGA

     Pro Ile Glu Arg Phe Gln Trp Asn Val Leu
     CCT ATA GAG AGG TTC CAG TGG AAT GTT CTA

     Pro Gln Gly Trp Val Cys Ser Pro Ala Ile
     CCA CAA GGA TGG GTA TGT AGC CCT GCC ATT

     Tyr Gln Thr Thr Thr Gln Lys Ile Ile Glu
     TAT CAG ACT ACC ACC CAG AAG ATT ATA GAA

     Asn Ile Lys Lys Ser His Pro Asp Val Met
     AAC ATT AAA AAG AGT CAC CCA GAT GTC ATC

     Leu Tyr Gln Tyr Met Asp Asp
     TTG TAT CAA TAC ATG GAT GAC ctgcaggag-3'
```

Fig. 4. Nucleotide sequences of the conserved region in the BIV reverse transcriptase gene. The first five amino acid sequences are in the region of the primer MD72 and the last four amino acid sequences are in the region of the MD51. According to Gonda *et al.* (7), the first amino acid (*) would be lysine. The lowercase letters are 5' extensions of the primers.

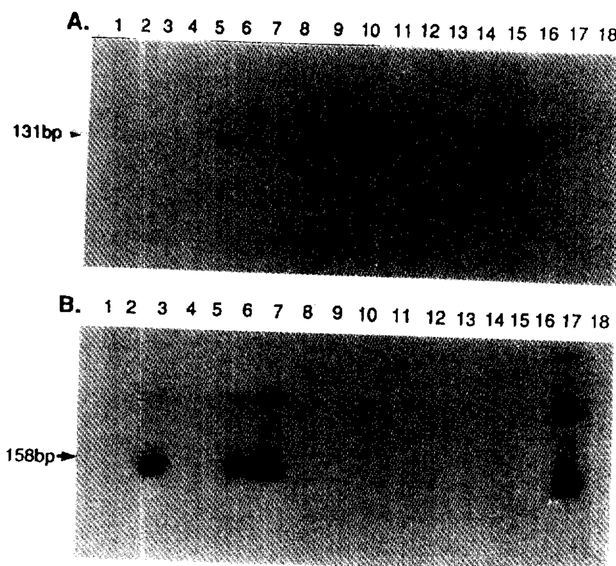

Fig. 5. PCR detection for naturally occuring bovine retroviruses. (A) Hybridization results with probe JS32 detecting BIV. Used primers were MD73 and MD51. The expected DNA band size is 131 bp. Sample DNA is from: lane 1, marker ϕX174 RF DNA *Hae*III cut; lane 2, normal BESC as BIV negative control; lanes 3, 4, 5, PBMC from experimental cattle (PBL7079, PBL7112 and PBL7116 respectively); lanes 6, 7, 8, PBMC from other cattle as BLV positive by agar gel immunodiffusion assays (PBL Aunt, PBL 49, PBL1152 respectively); lane 9, BIV positive BESC from R29 strain; lanes 10, 11, BESC cocultured with PBL Bull, PBL625; lanes 12, 13, PBMC from BIV infected male calf JB (4 month P.I.) and female calf JY (ditto); lane 14, PBMC from sick cow (PBL Turlock); lane 15, BIV positive BESC; lane 16, normal BESC; lane 17, BLV infected bat lung cells (BLV positive control); lane 18, normal bat lung cells (BLV negative control). (B) Hybridization results with probe BLV3 detecting BLV. Used primers were BLV1 and BLV2. The expected DNA band size is 158 bp. Lane descriptions are same as above.

MD72/MD51 amplified a portion of the *pol* gene of the BIV provirus. This was assumed to be the conserved region of the reverse transcriptase known in other lentiviruses. Its nucleotides consisted of 225 bp in the *pol* gene of BIV (Fig. 3). The PCR amplified DNA of MD72 /MD51 generate a 243 bp DNA with 9 additional base pairs to provide convenient cloning sites were present at both termini.

The purified 243 bp PCR DNA was directly cloned and ligated into the vector ϕM13mp18 RFI through *Hin*cII restriction site which provided the blunt end at both ends. One of these recombinant vectors (WP2-7) revealed the conserved region of the BIV pol gene as shown in Fig. 4. The WP2-7 clone includes the sequences of primers MD72/MD51 and the sequence of probe JS32. When the nucleotide sequences are translated into amino acid sequences, the sequences at the amino terminal portion of the conserved region of reverse transcriptase are exactly the same as the sequences reported by Gonda *et al.* (7). The preserved text sequence at the region of the primer MD72 is 5'GGTGATGCCTATTTT3', which can be found in one sequence of the MD72 (5'GGNGAYGCNTAYTTY3', refer to Table 1). Also the true sequence at the carboxyl terminal end (region of primer MD 51), as it turns out, is 5'TACATGGATGAC 3', which is complementary to the degenerate MD51 primer 5'RTCRTCCATRTA3'. These sequences allow us to have predictable and effective PCR (for lentivirus) with the degenerate primers and the power of degenerate probe JS32.

More interesting finding is the sequence of the JS32 probe which was found in the middle of the sequence. The detection of the sequence 5'TGGGTATGTAGCCCTGCCATT3' was one of the 6,144 kinds of oligonucleotides possible with the JS32 probe. That is, the other 6,133 kinds of the JS32 must differ from the real sequence by at least one to eight nucleotides. The successful detection might be due to the non-stringent condition

```
BIV  9A GGC GAT GCC TAC TTC ACT ATC CCT TTA CAT GAG GAC TTT AGA CCC TTT ACA GCC TTT TCT
     9B --G --- --T --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9C --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9D -AT --- --- --T --- --C --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BIV 10A --- --- --G --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    10B --A --- --T --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    10C --T --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    10D --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BIV GAR AAA --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --C ---

BIV  9A GTA GTC CCT GTG AAT CGA GAA GGA CCT ATA AAG AGG TTC CAG TGG AAT GTT CTA CCA CAA
     9B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BIV 10A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G ---
    10B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G ---
    10C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G ---
    10D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G ---
BIV GAR --- --- --- --A --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- ---

BIV  9A GGA TGG GTA TGT AGC CCT GCC ATT TAT CAG ACT ACC ACC CAG AAG ATT ATA GAA AAC ATT
     9B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     9D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BIV 10A --- --- --- --- --- --- --- --- --- --+ --- --- --- --- --- --- --- --- --- ---
    10B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    10C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    10D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BIV GAR --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

BIV  9A AAA AAG AGT CAC CCA GAT GTC ATG TTG TAT CAA TAC ATG GAC GAT
     9B --- --- --- --- --- --- --- --- --- --- --- --T --- --- ---
     9C --- --- --- --- --- --- --- --- --- --- --- --- --- --T --C
     9D --- --- --- --- --- --- --- --- --- --- --- --T --G --- --C
BIV 10A --- --- --- --- --- --- --- --- --- --- --- --T --- --- ---
    10B --- --- --- --- --- --- --- --- --- --- --- --T --- --- ---
    10C --- -G- --- --- --- --- --- --- --- --- --- --T --- --- ---
    10D --- --- --- --- --- --- --- --- --- --- --- --T --- --- ---
BIV GAR --- --- --- --- --- --- --- --- --- --- --- --T --- --T ---
```

Fig. 6. Nucleotide sequences of the partial pol gene of different BIV isolates and clones. The first 15 nucleotides are parts of the primer MD72 and the last 12 nucleotides are from complementary sequences of the primer MD51. Sequences of BIV 9A, B, C, D are derived from PCR DNA of BESC co-cultured with BIV obtained from Dr. Van Der Maaten (R29) and BIV 10A, B, C, D are from the PCR DNA of BESC co-cultured with PBL Bull (refer to Fig. 5A, lane 10). "A, B, C, D" imply different clones. Sequence of BIV GAR is from the published BIV nucleotide sequences (BIV R 29-127, see reference 6).

of the probe during hybridization, which allowed for more hybridization between the probe (degenerate oligonucleotides) and the BIV DNA. The variable sequence of molecular clones in the primer regions are informative. The data suggest that multiple primers initiate amplification.

Genetic variation among BIV isolates

After successfully detecting BIV in infected BESC by PCR and revealing the partial BIV nucleotide sequence, we tested some peripheral blood mononuclear cells (PBMCs) DNA of sick cattle and co-cultured BESC DNA for BIV surveillance. From the results of the PCR using degenerate primers, MD73 and MD51 (Fig. 5A), BIV proviral sequences were present as shown in lanes 9 and 15 (as BIV positive controls) by their strong radioactive signals. Also, PCR DNA obtained from the PBMCs of a 4 year old male Holstein-Friesian bovine co-culturing with BESC (lane 10) showed a very weak signal under stringent washing conditions (2× SSPE, 0.1% SDS in 55℃), while all others failed to show radioactive signals after hybridizing with probe JS32. It is noteworthy that experimentally inoculated animals JB (lane 12) and JY (lane 13), were negative for BIV. It was not demonstrated until 5 months later using PCR probe that JB was BIV positive, while JY was positive at 4 months using OK01 and OK02 primers (specific primers) with 4 μg of PBMC DNA (10).

The samples were also screened for bovine leukemia virus (BLV) infection using BLV specific primers and probe (refer to Table 1). Some blood samples collected from sick cattle revealed presence of BLV (Fig. 5B), which is frequently found among 50% of the dairy cattle herds in the United States (1). Thus, PCR testing with this BLV primer set also discriminated between BLV infection when BLV negative control samples were used. As shown in Figure 5B, three samples (lanes 3,6 and 7) showed radioactive signals at the expected PCR DNA band (158 bp), which indicated natural BLV infection.

PCR DNA (MD72/MD51) was subjected to cloning and sequencing in order to characterize genetic variances among different BIV isolates to examine, if indeed dissimilar sequences existed. Fig. 6 gives a summary on DNA sequencing of BIV9 (from BIV-R29) and BIV10 (from Fig. 5A, lane 10). There are many nucleotides changes in primer regions due to the use of degenerate primers at both end of the *pol* gene region. Regions of nucleotides compared between the two primers were relatively unchanged, although there were some base substitutions, such as G→A, A→G and C→T.

However, significant sequence heterogeneity within BIV isolates was also noticed between region sequences such as TT<u>C</u> (Phe)→TT<u>T</u> (Phe), GT<u>A</u> (Val)→GT<u>G</u> (Val), CC<u>A</u> (Pro)→CC<u>G</u> (Pro) and <u>G</u>AG (Glu)→<u>A</u>AG (Lys). Two possible reasons for these observations are that (1) it happened in different clones, or (2) the change from <u>G</u>AG (Glu) to <u>A</u>AG (Lys) gave a different amino acid sequence. Interestingly, the changed sequence can also be found in the conserved region of *pol* gene of different lentiviruses such as HIV-2, SIVmac, CAEV and EIAV (Table 2). Some other nucleotide changes could have been caused by *Taq* DNA polymerase mistakes during PCR such as A<u>A</u>G→A<u>G</u>G.

Discussion

In the present study, we determined the conserved sequence of BIV *pol* gene by using PCR. The gene of reverse transcriptases of lentiviruses shared short but clearly homologous region known as highly conserved sequences (4, 13). The sequence is very critical for the reverse transcriptase function and a mutation in the region usually decrease or nullify the enzymatic activity (11). Thus, it is assumed that BIV could share the same

homologous region in pol gene as in other lentiviruses because BIV is a new member of *Lentivirinae* (7).

The PCR technique was used to detect the BIV DNA from the BIV provirus. Since nucleotide sequences of BIV was not available yet, degenerate primers were tested for PCR amplification. Although the used degenerate primers contained 128 different sequence isomers, it was expected that there were sufficient single kind oligonucleotides for PCR amplification. Previous use of degenerate primers for detecting Hepadnaviruses gave sufficient amplification (13). Although each primer contains 9 nucleotides at 5′ end for the purpose of linker, the final result did not effect the efficiency of PCR amplification. The linker sequences attached at the 5′ region of primers did not result in the different stability during PCR (13, 19). In addition, we applied a degenerate probe which revealed presence of the BIV pol gene from the PCR using degenerate primers.

The nucleotide sequence of the conserved region of BIV *pol* and resulting amino acid sequence, are compared to other known conserved regions of the reverse transcriptases of lentiviruses (Table 2). To state briefly, the amino acid sequences of all the lentiviruses reveal very short stretches of the conserved amino acid sequences of reverse transcriptases in different areas, such as GDAYF (Gly-Asp-Ala-Tyr-Phe) in the upstream of amino terminal end, LPQGW (Leu-Pro-Gln-Gly-Trp) in the central portion, and YMDD (Tyr-Met-Asp-Asp) at the carboxyl terminal end. These sequences allow us to have a predictable and effective PCR (for lentiviruses) with the degenerate primers. These regions are regarded as core sequences in reverse transcriptases (22). That is, if those regions are mutated, the reverse transcriptase activity would be greatly reduced (3). From *in vitro* mutagenesis studies with the reverse transcriptase (11), changing the sequences of some nucleotides in YMDD nullified its reverse transcriptase activity.

Therefore, the results successfully support the idea that PCR can be used to detect BIV in the following ways: 1) There are conserved amino acid sequences in the BIV reverse transcriptase similar to other known lentiviruses. Some amino acid sequences are strictly preserved as predicted, such as LPQGW (Leu-Pro-Gln-Gly-Trp) and YMDD (Tyr-Met-Asp-Asp). 2) The PCR amplification proved to be very specific, even when degenerate primers were used and with alteration of some PCR conditions. The successful detection and the gene amplification of BIV relied on the modification of PCR conditions.

The same methods and logic were applied to characterize BIV infectivity among sick cattle discussed previously. PCR was used as a primary method to screen BIV infection among those samples collected from sick cattle. A BLV screening test was also attempted on those samples by PCR using BLV specific primers, BLV1 and BLV2 (Fig. 5B). From such PCR screening of cattle with natural exposure, there were no doubly infected BLV-BIV cattle found, however, an experimentally BIV infected calf (JB, lane 12) was not detected to be infected with BIV until 9 month post infection (10). Thus, our study provides a corroborating evidence on the possibility of double infection (BLV and BIV) in dairy cattle as reported by Amborski *et al.* (1) and Cockerell *et al.* (5).

Further investigation was directed toward a sample which showed a vague BIV signal from Southern blot (Fig. 5A). It may be that this animal had an infection with a new strain of BIV. One can make an assumption that every different isolate or strain of newly discovered lentiviruses gives typical nucleotide variances (8). However, we found that the BIV conserved pol gene sequence was very close to the sequence of BIV (R29) published by Garvey *et al.* (6). The possible explanation is that conserved domain of the reverse transcriptase of many lentiviruses may be preserved for their viral replication and infectivity by means of reverse transcription. There is also a report that the same nucleotide variation occurred in other lentiviruses such as HIV-1 and HIV-2 (8).

In fact, the in between sequences are strongly conserved among different clones (e.g. clone BIV10A, B, C and D) and even in different isolates such as BIV9 and BIV 10. This finding leads us to a conclusion that some nucleotide sequence changes may come from genetic mutation by BIV reverse transcriptase infidelity identical to HIV (15, 16).

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