Detection of proviral genomic sequence of bovine immunodeficiency virus in Indian cattle

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Bovine immunodeficiency virus (BIV) infection is an emerging disease of cattle and buffaloes. Though the infection is reported in several countries of the world, the status of this infection in India is not known. In this communication, we report the detection of proviral DNA sequence of BIV in ten blood samples and one milk sample of cattle by Southern hybridization followed by PCR conducted using oligonucleotide primers specific for the p26 genomic region of the virus. The specificity of the amplicons was confirmed by semi-nested PCR, restriction analysis and nucleotide sequencing. Nucleotide sequence homology of 96–97% was observed between three of the p26 amplicons sequenced and the reference (American) strain R-29. The results obtained indicate the necessity of undertaking a detailed study on the prevalence of BIV infection in the country and more particularly, its association with different non-specific (could be immunologic) disease conditions prevalent in dairy animals, including poor response to certain vaccines.

LENTIVIRUSES are a widely disseminated group of exogenous, non-oncogenic retroviruses (family Retroviridae), which include visna-maedi virus of sheep, equine infectious anaemia virus (EIAV), caprine arthritis encephalitis virus (CAEV), bovine immunodeficiency virus (BIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), human immunodeficiency virus (HIV), and Jembrana disease virus (JDV). These viruses are genetically related and share certain biologic and pathologic characteristics. There is also cross-reactivity between antigens of different lentiviruses. A distinguishable feature of lentiviruses is their ability to induce persistent infection in spite of strong host immune response. The two primary sites of virus replication are immune system and brain. BIV causes a persistent viral infection in cattle, and has been reported in the US, Canada, Europe, Pakistan, Korea, Japan, New Zealand, Australia and several other countries[1,2]. The virus is morphologically, antigenetically and genetically related to HIV[3,4].

The original isolation of BIV was made in 1969 from an eight-year-old Holstein cow from Lousiana with lymphocytosis and lymphadenopathy[5]. The virus has been associated with decreased milk yield, clinical immunodeficiency, encephalitis, bovine paraplegic syndrome, skin infection and emaciation[6,7]. Immunocompromised cattle, arising from BIV infection, can develop untreatable secondary disease conditions upon exposure to stress associated with parturition and environmental conditions[8]. Immune dysfunction following BIV infection in calves may result in poor antibody response to viral vaccines[9].

All retroviruses are capable of catalysing the flow of genetic information from RNA to DNA. The mature virions of BIV are bar/cone-shaped and 120–130 mm in diameter. As in other retroviruses, the BIV particle contains two copies of a single-stranded positive-sense RNA genome[10]. Inside the infected cell, the viral RNA genome is converted to double-stranded (ds) DNA by the action of viral reverse transcriptase and ribonuclease[1]. The dsDNA is transported to the cell nucleus and gets incorporated into the host genome. The replication intermediates of lentiviruses can be ds-linear and/or circular DNA molecules[10]. In the form of the linear DNA intermediate, the genome contains the obligatory retrovirus structural genes in the order *gag*, *pol* and *envelope*, flanked on the 5′ and 3′ ends by a *long terminal repeat* (LTR). The core protein of the virus is coded by the *gag* gene, which encodes a 53 kDa precursor protein that is further processed into the matrix (p17), capsid (p26) and nucleocapsid (p15) functional domains in the mature virus[11].

There are difficulties in the isolation of BIV from field cases[12]. Attempts to culture virus from cattle identified as being seropositive or PCR-positive for BIV have been unsuccessful[13]. Therefore, virus isolation remains a limited tool for identifying animals naturally infected with BIV[13]. Serological tests like immunofluorescence and Western blot have been used in detecting BIV infection[6,14–17]. Antibodies to p26 and gp110 are targetted for serological detection of BIV infection[16]. However, there are difficulties in relying solely on serological tests as antibodies to p26, the major gag protein, can be undetectable in persistently infected animals and in animals infected for a long time[12,15]. On the other hand, the peak antibody response to gp110, the surface envelope protein, may take several months post-infection[15]. Therefore, diagnosis by PCR is a more sensitive method than serology in identifying BIV-infected cattle[12,13].

Though BIV infection is an emerging disease of cattle worldwide, and the infection is prevalent in Pakistan our neighbouring country, its status in India is not known. Even though the virus has not yet been linked to any specific disease condition in cattle and buffalo, it certainly can aggravate certain illness in the animals, including impairment of the immune system[6,7,9]. The present investigation was undertaken to look for the possible presence of proviral genomic sequence of the virus (BIV) in randomly collected blood/milk samples of cattle and buffaloes.

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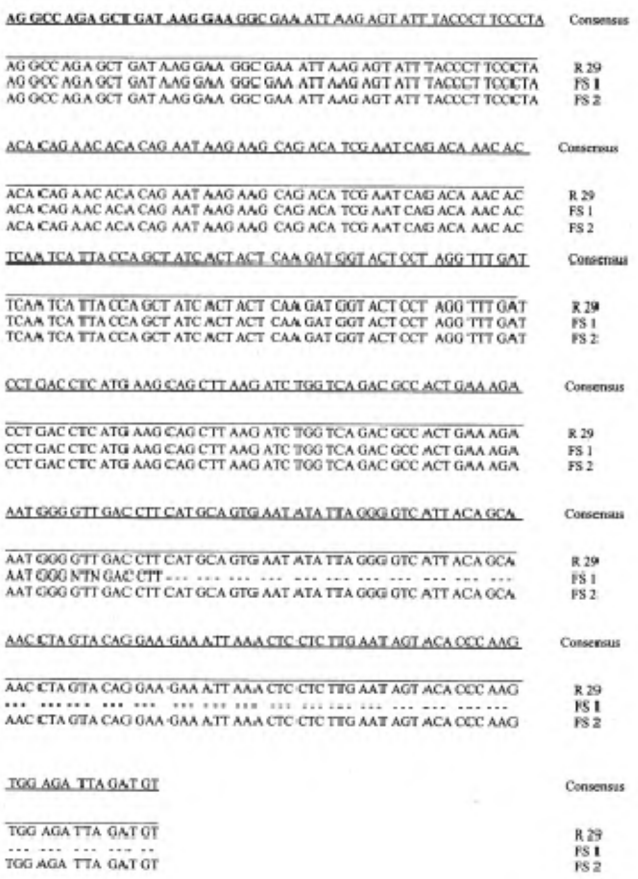
Whole blood (63) and milk (79) were collected from 135 cattle (pure bred, cross-bred, and nondescript) and seven buffaloes (Murrah and crosses; only blood) from Uttar Pradesh, Rajasthan, Gujarat, Maharashtra, Andhra Pradesh (AP), Orissa and Madhya Pradesh (MP). All animals were more than four years of age. Only one sample, either blood or milk, was collected from each animal. Blood of lactating cows was not collected. The milk samples were collected from two dairy farms (one Holstein Friesian and the other Jersey) located in MP. The cattle blood samples originated from 51 bulls and 12 dry (non-lactating) cows. Sterile disposable syringes were used for bleeding the animals. Blood samples in Na-EDTA and freshly-collected whole milk samples were transported on cool-pack (10–15°C) to the laboratory for processing. Total DNA was extracted from the blood/milk leucocytes using QIAamp DNA Mini Kit (Qiagen) and stored at –40°C for further use. All the DNA samples in 10 µg quantity were electrophoresed on 1% agarose gel, denatured with 1 M NaOH, and vacuum transferred onto BiodyneB (Gibco) membrane. The DNA blots were subjected to Southern hybridization[18] using ^{33}P-labelled probe nick translated[18] from a reference BIV gag clone (kindly provided by Charles Wood, University of Nebraska, USA). The DNA samples (total 11) giving clear hybridization signal, in relation to the known positive and negative (DNA) controls (kindly provided by Linda Scobie, Glasgow University, UK), were subjected to PCR for amplification of proviral genomic sequence of BIV. The oligonucleotide primers specific for the p26 region of the virus and reported earlier[19,20] were used. According to the proviral DNA sequence of R29 strain of BIV, clone 127 (GeneBank accession no. M 32690)[21], the forward(+) primer (HSBIV17) corresponded to nucleotides (nt) 653 to 672, whereas the reverse(–) primer (HSBIV18) corresponded to 1393–1374 nt. A 25 µl PCR reaction mix contained 25 pmol of each primer, 200 µM of each dNTP, 1.25 U Taq DNA polymerase (Promega), 2.5 mM $MgCl_2$ (concentration fixed by titration tests), reaction buffer (Promega), 1 µl (50–75 ng) DNA template. The PCR was performed for 36 cycles in two stages as reported earlier[13], with modifications. In the first stage, amplification was done for one cycle comprising denaturation at 94°C for 1 min, annealing at 51°C for 45 s and extension at 72°C for 1 min. This was followed by the second-stage amplification of 35 cycles at 94°C for 45 s, 51°C for 30 s and 72°C for 45 s, with a final extension for 5 min at 72°C. Single amplicon of expected size (approximately 750 bp; actual 740 bp) could be obtained from ten blood samples and one milk sample. All these samples were positive in Southern hybridization, and originated from six pure-bred (Jersey/Holstein Friesian/Gir/Sahiwal), four corss-bred (Holstein Friesian × Sahiwal), and one nondescript adult female cattle. None of the buffalo blood samples was positive. The amplicons were subjected to semi-nested PCR using a forward primer (HSBIV15) internal (700 to 714 nt)[19] to the primer HSBIV17 and the reverse primer HSBIV18. The cycling conditions were same. All the 11 amplicons obtained with the primer set HSBIV71/18, yielded single product of expected size (approximately 700 bp; actual 693 bp) in the semi-nested reaction indicating specificity of the amplification reaction and the amplicons. The identity of the semi-nested 693 bp amplicons was further confirmed by restriction using *Pst*I enzyme, which yielded two fragments of < 300 bp (actual 263 bp) and > 400 bp (actual 430 bp). The p26 region has a single *Pst*I restriction site at the nucleotide position 1130 (^{1126}CTGCAG^{1131}).

Three of the eleven HSBIV17/18 PCR products (673–1393 nt) obtained from peripheral blood leucocytes (PBLs) of cattle, one each from MP, Gujarat and AP, were cloned (TA cloning) in pTZ57R/T vector (MBI Fermentas) following standard procedure. Partial nucleotide sequence of the insert in recombinant plasmids was determined using fmol cycle sequencing kit (Promega) along with ^{33}P-labelled M13 forward primer, and aligned with the reference sequence of BIV[21] using CLUSTAL method available in the DNAstar software package. Alignment report of the two field strain sequences (FS1 and FS2) with the 5′ end of the reference p26 sequence is shown in Figure 1, and that of the third field strain (FS3) with the 3′ end of the reference p26 sequence is shown in Figure 2. In both the regions (ends), there was 96–97% homology between the field strain and reference sequences, indicating fool-proof identity of the amplicons obtained from PBLs of cattle using BIV p26-specific primers. Other amplicons, including the one from milk leukocytes are under sequence determination and analysis.

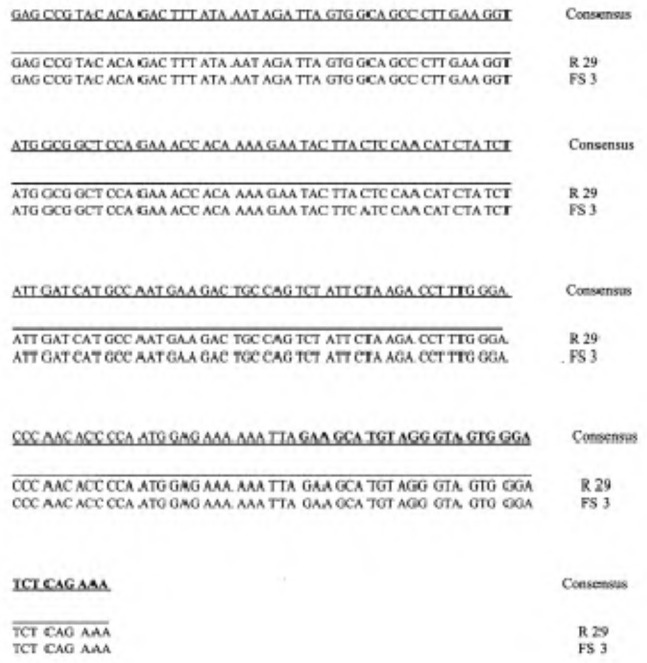
In earlier studies, PCR amplification of env and/or pol region of the virus has been used for detection of BIV infection in cattle[13,22–25]. Total DNA extracted from blood/milk leucotyes and semen has been used for the purpose[22,23]. In the present study, the p26 region of gag has been targetted in PCR as this region is highly immunogenic and conserved among the lentiviruses[19,20]. It has been observed that one stretch of ten amino acids within the p26 of BIV (residues 293–302) has high degree of similarity to HIV-1, HIV-2, EIAV and SIV, and lesser degree of similarity to visna virus[21]. Accordingly, detection of antibodies to p26 antigen, which is the capsid protein of the virus, has been the indicator of BIV infection in cattle, buffaloes and experimentally infected animals[2,20,24,26,27].

Out of the blood samples of 12 cows tested in Southern hybridization and PCR, ten were positive for proviral DNA sequence of BIV, whereas none of the male cattle (bull) was positive. This could be a significant observation, but more number of male and female cattle need to be sampled and tested before arriving at any conclusion. Seven of these cows (Holstein Friesian cross/Gir/



Note: FS 1, Field strain 12/2000 from MP
FS 2, Field strain 940/2001 from Gujarat
R 29, Reference sequence (GenBank Accession No. M 32690)
---, Not read
N, Ambiguity/not clear

Figure 1. Alignment of partial nucleotide sequence of 740 bp p26 amplicons obtained from two cattle with reported sequence of BIV strain R 29 (nucleotide position 653–958 is shown). Primer position is shown in bold letters.



Note: FS 3, Field strain 395/01 from Hyderabad, AP
R 29, Reference sequence (GenBank Accession No. M 32690)

Figure 2. Alignment of partial nucleotide sequence of one 740 bp p26 amplicons obtained from cattle with reported sequence of BIV strain R 29 (nucleotide position 1400–1200 is shown). Primer position is shown in bold letters.

Sahiwal) originated from one farm in Gujarat, two cows belonged to a farm in MP, and the remaining one was a nondescript animal from AP. None of the cattle (bull) samples originated from the two farms in MP and Gujarat. Therefore, it is possible that the farms from which the bull blood samples were collected are free from BIV infection. However, attempts are being made to test the bulls from farms in which BIV genomic sequence could be detected in cows. Similarly, more buffaloes need to be sampled and tested for comparison with the BIV infection status in cattle. Out of the 79 cow milk samples tested, only one (a Jersey cow) was positive for BIV (proviral DNA) in both the tests. This animal was from the farm in MP in which BIV sequence was detected in PBLs of two cows tested at random. None of the milk samples collected from 34 Holstein Friesian cows was positive. Detection of pol sequence of the virus in milk leucocytes of BIV-seropositive cows has been reported, and possibility of lactogenic transmission of the virus within a herd underlined[22], as established in case of other lentivirus infections like visna[28], CAE[29] and HIV. In the present sampling condition, blood samples were not collected from the lactating (milk-sampled) cows; only dry cows were blood-sampled. Therefore, presence of the BIV sequence in blood could not be correlated with the dissemination of the virus (viral sequence) into the mammary secretions.

The present investigation reveals that the PCR amplicons obtained from the cattle originate from the p26 region of the BIV proviral DNA. Attempts are being made to isolate BIV from PCR-positive cattle. Further studies are under way to correlate the presence of BIV genomic sequence in the blood with dissemination of the virus into the milk, and different clinical/physiological manifestations like decreased milk yield, repeat breeding, poor response to vaccines, dermatitis and non-responsiveness to treatments, chronic debility and emaciation, etc. in the animals.

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A survey of haplotype frequencies and linkage disequilibrium at the DRD2 locus in the Nilgiri hill tribes, South India

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DNA analysis has made it easier to study haplotypes, arrays of alleles at closely linked loci along the chromosome. These regions are short enough to show little or no recombination, and behave as blocks that might have ancient origins. Scoring these markers as haplotypes, allows analysis both in terms of haplotype frequencies and identity in terms of linkage disequilibrium. The human dopaminergic system is an important focus of study in the fields of neuropsychiatry and pharmacology; it is also a promising nuclear DNA marker in studies of human genome diversity. Haplotype frequencies and linkage disequilibrium for the dopamine D2 receptor gene (*DRD2*) was determined in 250 unrelated individuals from five tribal populations. The three marker systems in this study are highly polymorphic in all the five tribal populations and the haplotype system showed high level of heterozygosities. Out of the possible eight haplotypes, four are commonly shared by all the populations. The ancestral allele B2D2A1 accounts for 0.021 to 0.080, which was present in all the groups consistently. The linkage disequilibrium was statistically significant in all the populations. Data obtained in this study on *DRD2* represent one of the small, but growing number of data sets examining disequilibrium and haplotype frequencies in human populations.

THE human dopaminergic system is an important focus of study in the fields of neuropsychiatry and pharmacology, and therefore genes involved in dopaminergic transmission and metabolism have been extensively studied to identify genotype–phenotype relationships in neuropsychiatric disorders[1–3]. Among the five known human dopamine receptors, the *DRD2* receptor gene has been studied most extensively because it is the site of action of neuroleptic drugs and it is also believed to be involved in the pathophysiology of various neuropsychiatric diseases. Several polymorphisms in the *DRD2* gene have been identified in the DNA encompassing the coding sequences; most are in the introns or downstream-flanking DNA[4–7], but some are in coding regions[8,9] and in the promoter region upstream of exon 1 (ref. 10). Many of

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