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Simulation of normal, carrier and affected controls for large-scale genotyping of cattle for factor XI deficiency

P.N. Mukhopadhyaya[1], M. Jha[2], P. Muraleedharan[1], R.R. Gupta[1], R.N. Rathod[1], H.H. Mehta[1] and V.K. Khoda[1]

[1]Genomics Laboratory, Productivity Enhancement Group, National Dairy Development Board, Anand, Gujarat, India
[2]Department of Biosciences, Sardar Patel University, VVNagar, Gujarat, India
Corresponding author: P.N. Mukhopadhyaya
E-mail: pnm6619@yahoo.co.in

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ABSTRACT. An insertion mutation within exon 12 of the factor XI gene has been described in Holstein cattle. This has opened the prospect for large-scale screening of cattle using the polymerase chain reaction (PCR) technique for the rapid identification of heterozygous animals. To facilitate such a screening process, the mutant and normal alleles of factor XI gene, represented by 244- and 320-bp PCR amplified fragments, were individually cloned in *Escherichia coli* using a multicopy plasmid cloning vehicle to generate pFXI-N and pFXI-M, respectively. The authenticity of the inserts was confirmed by nucleotide sequencing. A nested PCR method was developed, by which PCR amplicons generated from primers with annealing sites on the recombinant plasmids and by flanking the insert were used as templates for amplification of the diagnostic products using factor XI gene-specific primers. An equimolar mixture of both PCR amplicons, originating from pFXI-N and pFXI-M, constituted the carrier control while the individual amplicons were the affected and normal controls. The controls were used as

references for in-gel comparison to screen a population of 307 cattle and 259 water buffaloes; the frequency of the mutant allele was found to be 0. No DNA size standards were required in this study. The simulated control DNA samples representing normal, carrier and affected cattle have the potential to help in large-scale screening of a cattle population for individuals that are carriers or affected by factor XI deficiency.

Key words: Cattle, Factor XI, Blood coagulation, Carrier DNA sample, Recessive DNA sample

INTRODUCTION

Factor XI (a plasma thromboplastin antecedent) is a serine protease synthesized in the liver that participates in an early phase of blood coagulation (Seligsohn and Griffin, 1995). An inherent deficiency of this enzyme has been observed in Holstein-Friesian cattle. Animals that are deficient in factor XI may be asymptomatic, or they may have several indicators such as prolonged bleeding after injections, production of bloody milk, and anemia (Brush et al., 1987). Furthermore, homozygous and heterozygous deficient animals may have lower calving and calf survival rates, as well as increased susceptibility to infectious diseases (Liptrap et al., 1995). Therefore, this genetic defect can have an economic impact on the dairy industry (Marron et al., 2004). Biochemical tests, such as the activated partial thromboplastin time, are available for determining factor XI activity. Although this test can effectively identify deficient and normal animals, it produces ambiguous results for heterozygous (carrier) individuals.

The molecular basis of factor XI deficiency has recently been discovered. It is a 76-bp insertion of an imperfect poly-adenine tract occurring in exon 12, followed by a repeat segment of 14 bp, which corresponds to the normal coding sequence immediately preceding the insertion (Marron et al., 2004). This finding led to development of a convenient DNA-based test to identify carriers for factor XI deficiency. We cloned, in *Escherichia coli*, normal and mutant alleles, mapping to a region within exon 12 of the factor XI gene in cattle, using a multicopy plasmid DNA as the cloning vehicle. We used these clones, individually and in combination, as reference samples, representing affected, carrier and normal DNA for factor XI deficiency.

MATERIAL AND METHODS

DNA from carrier and affected animals for factor XI deficiency was a gift from Dr. J.E. Beevers, University of Illinois, USA. Gene-specific oligonucleotide primers for bovine factor XI gene were synthesized as described by Marron et al. (2004). SP6 and T7 primers were purchased from Promega, USA. Polymerase chain reaction (PCR) was carried out in a 50-µL volume with 100 ng each of cattle genomic DNA/plasmid DNA/PCR amplicon(s), primers (20 ng each), dNTPs (200 µM) and 0.5 units of Taq DNA polymerase in a standard 1X Taq DNA polymerase buffer (Promega, USA). The PCR cycling conditions for amplifying the exon 2

region of factor XI gene were as described by Marron et al. (2004). For amplification of the recombinant plasmid inserts using SP6 and T7 primers, the thermal cycling conditions were as follows: an initial round of denaturation at 94°C for 2 min, then 35 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were resolved in 2% agarose gels, stained with ethidium bromide (0.5 µg/mL in 1X TAE buffer) and visualized under UV light (260 nm).

Molecular cloning of the PCR amplicons was performed in pGEM-H_3 plasmid DNA (Nagee et al., 2004) at the *Not*I site, using *E. coli* Prat 201 as the host strain (Nagee et al., 2001), and recombinants were selected by alpha complementation. Plasmid DNAs (pFXI-N and pFXI-M) were extracted by the alkaline lysis procedure, followed by phenol-chloroform purification (Sambrook et al., 1989). Sequencing reactions were performed according to the protocol of the ABI Prism BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA), modified by one-quarter reaction. The extension products were purified with the GeNei Quick PCR Purification Kit (Bangalore, India) and run on an ABI 3100 DNA sequencer.

ABI DNA Sequence Analysis 3.0 was used for lane tracking. The forward and reverse sequences were assembled, and the assembled files were carefully checked by eye. Fluorescent traces for each variant were rechecked again in all individuals. The sequences were aligned by GeneDoc (Nicholas and Nicholas Jr., 1997).

The kit formulation comprised three different components: A) PCR concentrate, consisting 2 µM each of forward and reverse primers, all four dNTPs at a final concentration of 2 nM each, and Taq DNA polymerase buffer (10 mM Tris, pH 9.0, 50 mM KCl, 0.01% gelatin, and 1.5 mM $MgCl_2$); B) Taq DNA polymerase (3 units/µL), and C) control DNA consisting of SP6-T7 flanked PCR amplicons generated from: a) 50 ng/µL pFXI-N (normal); b) 100 ng/µL pFXI-N and pFXI-M each (carrier), and c) 50 ng/µL pFXI-M (affected). Sterile deionized water was added to the reaction mixtures. For a typical PCR reaction setup with a final volume of 25 µL, 2.5 µLof PCR concentrate (A) was used along with the template DNA, 0.5 µL Taq DNA polymerase and water to make a final volume of 25 µL. To generate a control-amplification profile, template DNA was substituted by 2 µL of control DNA solution in individual PCR reactions.

To demonstrate the usefulness of the kit, assays were conducted on DNA samples from 307 cattle and 259 water buffaloes (*Bubalis bubalis*) from various farms in India (Table 1).

RESULTS AND DISCUSSION

It was previously hypothesized that factor XI deficiency was due to complete absence of the protein (Gentry, 1984). The determination of the molecular basis of the disease (Marron et al., 2004) as an insertion mutation that creates a stop codon suggested that factor XI protein may not be missing, but rather truncated prematurely, resulting in a non-functional protein. The discovery of the mutation made the development of a DNA-based diagnostic test to detect possible carriers. Because of the large size of the insertion sequence, the difference in size between normal and mutant alleles allows unambiguous classification of animals as normal, carrier or affected, using agarose gel electrophoresis after PCR. DNA-based diagnosis of a carrier animal became feasible. Due to the large size of the insertion sequence, the allele difference in a carrier animal is enough for unambiguous identification, using agarose gel electrophoresis after PCR.

Table 1. Breeds of cattle and water buffalo from different farms in India screened for factor XI deficiency.

Numbers of animals	Breed	Farm/dairy union
Cattle		
15	Holstein Friesian (HF)	Aditya Dairy Farm, Khambhat, Anand, Gujarat, India.
25	HF crossbred	Aditya Dairy Farm, Khambhat, Anand, Gujarat, India.
48	Sahiwal	Sabarmati Ashram Gaushala, Bidaj Farm, Kheda, Gujarat, India.
11	Gir	Sabarmati Ashram Gaushala, Bidaj Farm, Kheda, Gujarat, India.
16	Kankrej	Sabarmati Ashram Gaushala, Bidaj Farm, Kheda, Gujarat, India.
7	Red Sindhi	Sabarmati Ashram Gaushala,Bidaj Farm, Kheda, Gujarat, India.
85	HF crossbred	Sabarmati Ashram Gaushala, Bidaj Farm, Kheda, Gujarat, India.
50	HF crossbred	Baroda Dist. Co. Op. Milk Union, Baroda dairy, Vadodara, Gujarat, India.
50	HF crossbred	Sabar Dairy Milk Union, Himmatnagar, Gujarat, India.
25	HF crossbred	Panchmahal Dairy Milk Union, Godhra, Gujarat, India.
25	HF crossbred	Animal Breeding Centre, Salon, Raibarelly, UP, India.
Buffalo		
50	Murrah	Baroda Dist. Co. Op. Milk Union, Baroda dairy, Vadodara, Gujarat, India.
46	Toda	Nilgiri Dist. Co. Op. Milk Union, Ooty, Banglore, Karnataka, India.
63	Pandharpuri	Kolhapur Milk Union, Kolhapur, Maharastra, India.
50	Mehsani	Doodhsagar Milk Union, Mehsana, Gujarat, India.
50	Surti	Sumul Dairy Milk Union, Surat, Gujarat, India.

For routine screening of cattle, it is desirable to have control DNA samples with profiles identical to that of carrier, affected and normal animals. This allows direct, in-gel comparison of test samples and obviates the use of DNA size standards, thereby making the test more economical and simple. Furthermore, in case an animal is suspected to harbor the mutant allele, the control PCR amplicon should generate a nucleotide sequence identical to that of the mutant allele to allow direct comparison of the test and the control sequences. We previously developed such controls, albeit by different methods, for large-scale screening of cattle populations for the genetic disorder of bovine leukocyte adhesion deficiency syndrome (BLAD) (Mukhopadhyaya et al., 2000).

Molecular cloning of DNA in *E. coli* via a multicopy plasmid-cloning vehicle offers a straightforward option for archiving fragments from its genome of origin. PCR amplification of normal and affected reference, genomic DNA with primers flanking the factor XI exon 12 sequence and containing the mutation site resulted in 244- and 320-bp PCR amplicons. Molecular cloning of these two fragments generated recombinant plasmids pFXI-N and pFXI-M, harboring the normal and mutant alleles, respectively. PCR amplification of recombinant plasmids generated with SP6 and T7 vector primers increased the size of the amplicons by 182 bp, corresponding to the additional nucleotides of the multiple cloning site between the 5'-end of T7 and that of the SP6 primer, respectively (Figure 1). The authenticity of the inserts was confirmed through sequencing. The point of insertion of the 76-bp fragment (Figure 2A) and the imperfect poly A repeat (Figure 2B) in the mutant allele were identified. These PCR amplicons were then used individually, or in combination, as templates for formulating the control DNA solution, using the factor XI-specific primers.

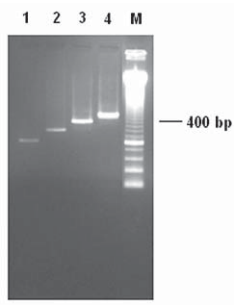
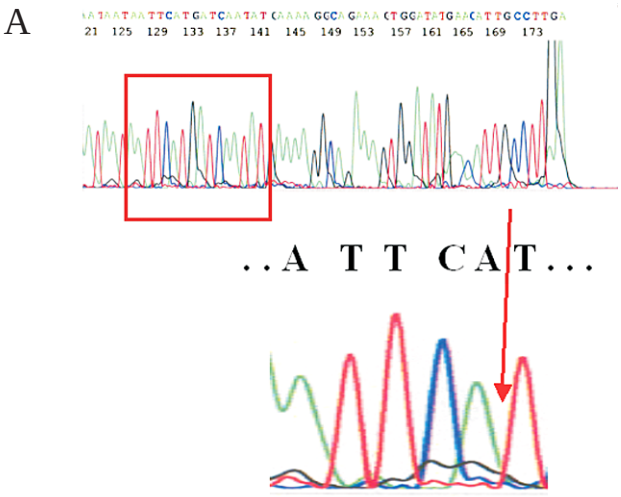


Figure 1. PCR product generated using pFXI-N and pFXI-M plasmid DNA as templates, with different PCR primers. *Lanes 1* and *2*: 244 and 320 bp, using factor XI gene-specific primers; *lanes 3* and *4*: 426 and 502 bp, using SP6 and T7 primers.



B

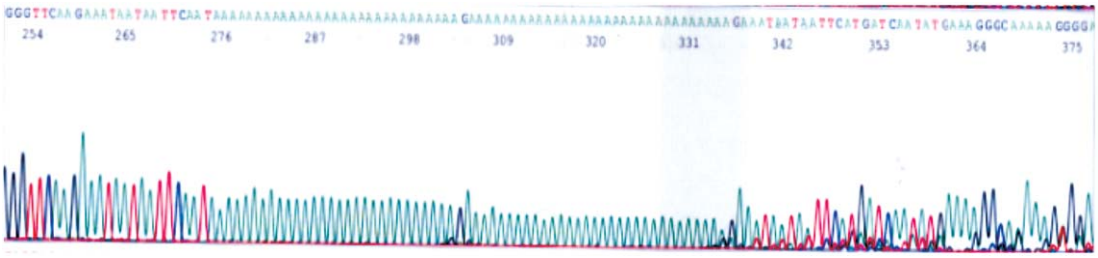


Figure 2. Nucleotide sequence of the normal and mutant alleles cloned as 244- and 320-bp inserts in pGEM-H_3 to generate recombinant plasmids pFXI-N and pFXI-M, respectively. The points of insertion of the 76-bp repeat in the normal allele (A) and the poly A repeat sequence in the mutant allele (B) are shown.

SP6-T7 flanked PCR amplicons generated from pFXI-N and pFXI-M served as templates for the amplification of the 244- and 320-bp fragments. Regions of non-specific priming were reduced in these templates when factor XI gene-specific primers were used for PCR. The PCR products thus generated were devoid of amplification artifacts and could be amplified over a wide range of annealing temperatures.

Normal and affected control DNA of the kit yielded 244- and 320-bp PCR amplicons, respectively. The carrier control produced both fragments in a single-PCR reaction. The PCR amplicon profiles were identical to those obtained with cattle genomic DNA samples from normal, affected or carrier individuals (Figure 3). The advantage of having cloned control DNAs is that these reference samples can be generated in the laboratory in large amounts for use as in-gel size standards for determining size, for sequence comparisons, and as probes generated by PCR.

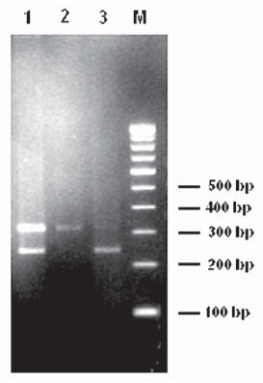


Figure 3. Recombinant control DNA for genetic testing for factor XI deficiency, using gene-specific primers. The template DNAs for PCR were amplicons generated using SP6-T7 primers and the following as template DNA: *lane 1*, combination of pFXI-N and pFXI-M; *lane 2*, pFXI-M; *lane 3*, pFXI-N; M, 100-bp DNA size standard.

The carrier, affected and normal control DNA, along with other components of the kit, were effectively used for screening 307 cattle and 259 water buffalo samples (Table 1). The mutant allele frequency was found to be 0. Molecular size standards were replaced by the newly developed recombinant DNA controls in the screening process.

We are confident that service-providing laboratories that are engaged in routine testing of farm animals will be encouraged by our work and initiate blind screening of large numbers of cattle (particularly the Holstein Friesian/Jersey crossbreds) and eventually come out with more refined data on the incidence of this genetic disorder within our cattle population in the dairy sector of India.

The water buffalo is an important source of milk in India. Although various nutritional studies have been made to improve milk production traits (Paul et al., 2002), genetic studies are still in their infancy. Therefore, we considered screening for factor XI deficiency in water buffalos from various farms in India to get an overall picture of the disease profile, as we have done for other genetic disorders, such as BLAD, citrullinemia and DUMPS (Mukhopadhyaya and Mehta, 2005). No sequence information for the factor XI gene in water buffalo is available in the Genebank data library. Therefore, we sequenced the 244-bp amplicons from exon 12 of factor XI gene in 10 individuals each of Toda (Genebank accession No. DQ233653) and Pandharpuri (Genebank accession No. DQ232885) breeds of water buffalo and compared it with the published cattle sequence (Genebank accession No. AY570504). Analysis revealed single-base substitutions at positions 47 and 66 in all the 20 water buffalo individuals sequenced of the two breeds (Figure 4). This corresponded to positions 1243 and 1262 of the cattle reference sequence. The base substitutions resulted in two non-synonymous mutations within the

factor XI protein of water buffalo at amino acid positions 393 (Asparagine → Lysine) and 400 (Glycine → Serine). These substitutions were not present in any of the 10 Holstein Friesian x Sahiwal crossbred cattle analyzed concomitantly in this study (Figure 5A and B). It therefore appears that these changes are specific to the water buffalo, although this needs to be further confirmed by analyzing a larger number of animals.

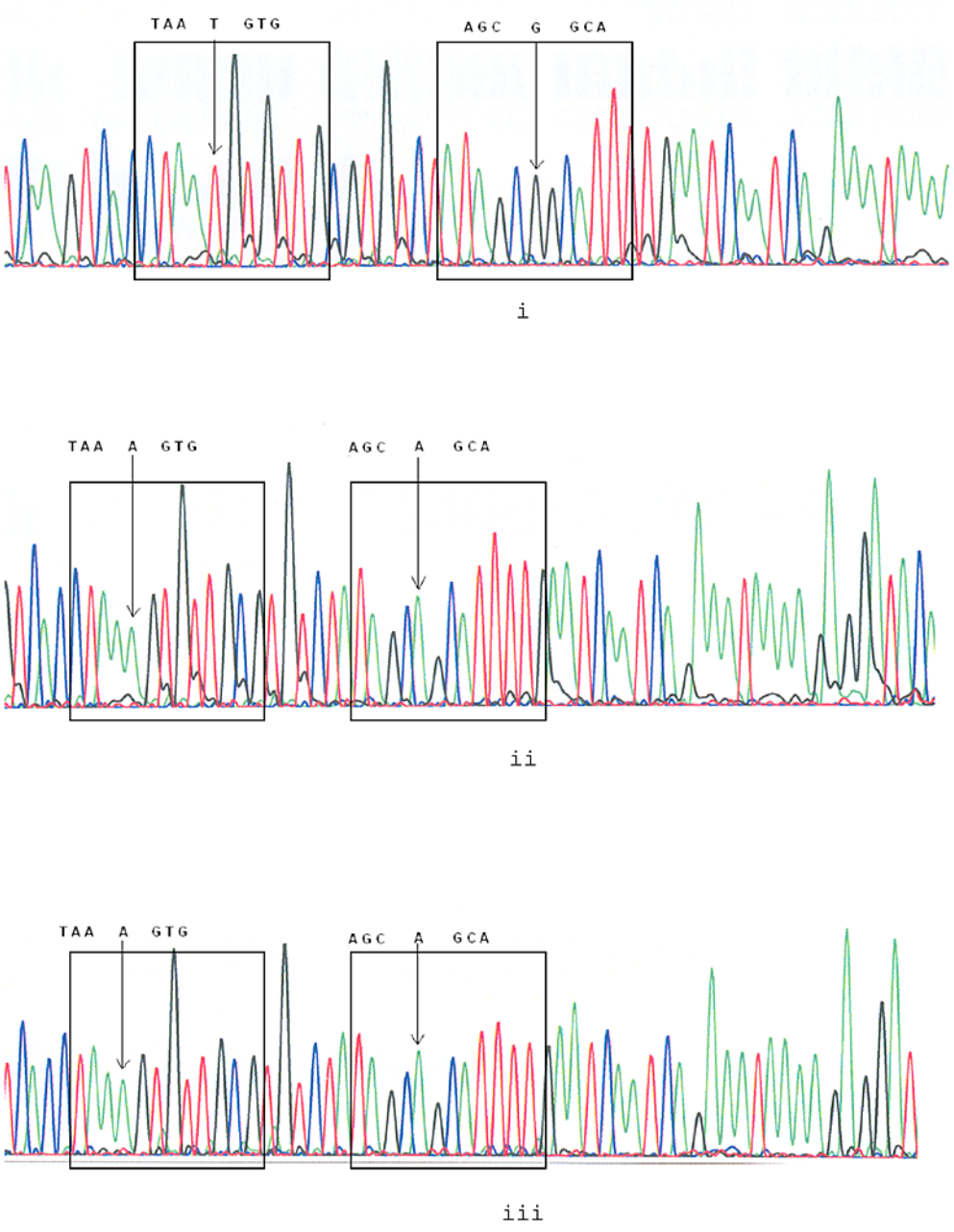
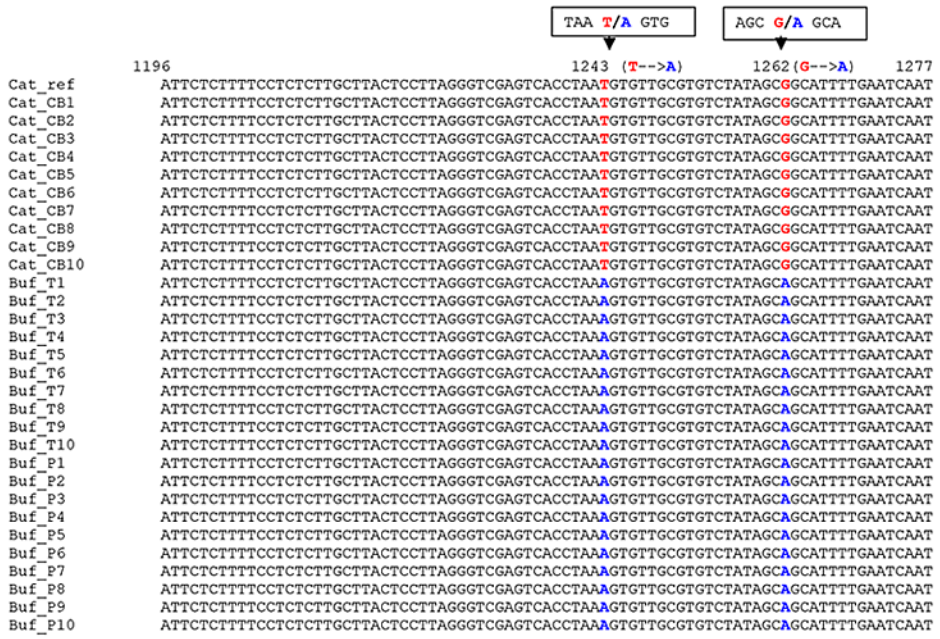


Figure 4. Electropherogram showing base substitutions in water buffalo within exon 12 of factor XI gene. i) Holstein Friesian x Sahiwal crossbred cattle, ii) Toda breed of water buffalo, iii) Pandharpuri breed of water buffalo.

A



B

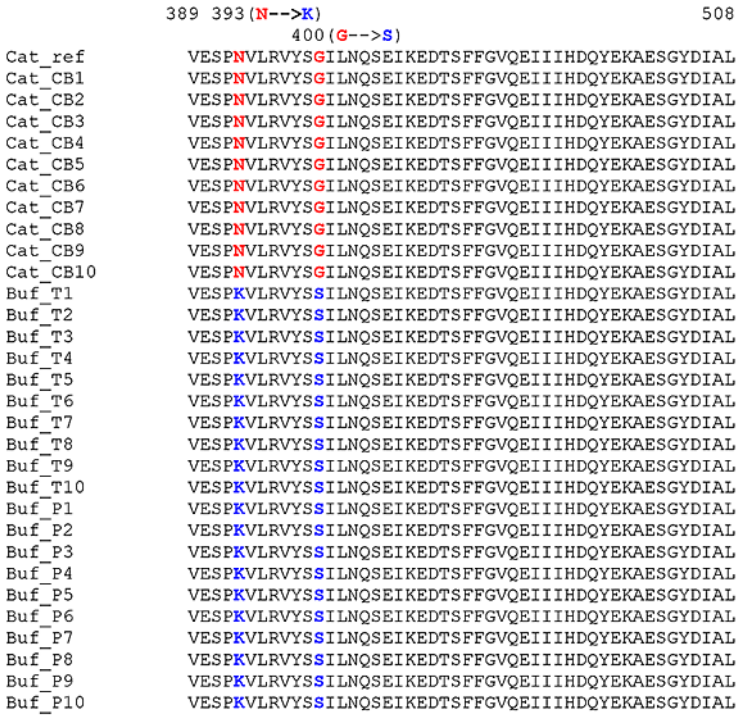


Figure 5. Clustal W (1.60) alignment of nucleotide (A) and amino acid (B) sequences from exon 12 of factor XI gene from 10 animals each of Holstein Friesian (HF) x Sahiwal (S) cattle, Toda and Pandharpuri breeds of buffalo. Cat_ref, published reference cattle sequence (AY570504); Cat_CB1-10, HF x S crossbred cattle; Buf_T1-10 and Buf_P1-10, Toda and Pandharpuri breeds of buffaloes, respectively.

Artificial insemination is an effective method for the rapid, horizontal dissemination of genes into the population. While the propagation of genes linked to positive economic traits, such as milk yield and disease resistance is desirable, the simultaneous propagation of genetic defects needs to be avoided. Since most of these genetic diseases are autosomal recessive, the phenotype of heterozygous (carrier) animals is normal. If such animals are not removed from the breeding stock, 50% of their progeny are expected to inherit the defective allele. A classic example is the spread of the genetic disorder citrullinemia in Australia. This disease is caused by a 'C' to 'T' transition at codon 86 within exon 5 in the gene coding for argininosuccinate synthetase, leading to an impaired urea cycle (Dennis et al., 1989). Healy et al. (1991) reported that 50% of the Australian national Friesian herds and 30% of bulls in artificial insemination centers are descendents of Linmack Kriss King, which was a carrier for citrullinemia. We reported similar cases of artificial insemination bull carriers of citrullinemia and BLAD in the artificial insemination bull farms of India (Muraleedharan et al., 1999). Removal of such heterozygous carrier bulls from the breeding program effectively prevents the propagation of defective genes into our future livestock generation. However, we are currently using these diagnostic tests in the management of breeding programs and selection of breeding stocks, since the removal of carriers eliminates "good" genes as well, and is thus not a very desirable approach.

In our study, the simulation of controls for rapid detection of cattle carrier for the genetic disorder caused by a mutation in the factor XI gene was intended to assist in the development of a disease-free cattle population. The kit formulation, along with the simulated control DNA samples, has the potential to simplify large-scale screening of the cattle and the water buffalo population for carrier and affected animals with factor XI deficiency. It would be helpful in screening livestock and for monitoring for the factor XI defective allele to avoid its entrance into our dairy population in the future.

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