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MOLECULAR SCREENING OF *FXI* DEFICIENCY GENE FRAGMENT IN NILI-RAVI BUFFALO BULLS OF PUNJAB, PAKISTAN

K. Zahra[1], M. Y. Zahoor[1], M. Imran[1], K. Ashraf[2], A. Nadeem[1], I. Rashid[2], Habib-ur-Rehman[3], K. Hassan[4], M. Younas[5], M. Akhtar[6] and W. Shehzad[1*]

[1]Institute of Biochemistry and Biotechnology, [2]Department of Parasitology, [3]Department of Physiology, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan; [4]Department of Zoology, University of the Punjab, Lahore 54600. Pakistan; [5]Semen Production Unit, Qadirabad, [6]Buffalo Research Institute, Pattoki, Livestock & Dairy Development, Government of the Punjab.
Corresponding Author's e-mail: wasim.shehzad@uvas.edu.pk

ABSTRACT

Nili-Ravi buffalo breed has an immense potential for productivity and can be a part of effective breeding programs. However, with only a few reports detailing the genetic diseases of the breed, the burden such diseases place on it remains poorly evaluated. Buffalo populations can be afflicted with autosomal recessive disorders that require genetic screening to be detected and prevented. One such disease, Bovine plasma thromboplastin (Factor XI) deficiency, has been well documented in cattle breeds from all over the world. Screening for this disease in native Pakistani buffalo breed was carried out to evaluate its occurrence in the breeding sires. In this regard, blood samples were randomly collected from the breeding buffalo bulls (n=152) reared at Government owned buffalo farms in the Punjab Province. Subsequently, whole genomic DNA extraction followed by PCR amplification of *FXI* gene was carried out. The resulting 244 bp PCR product was sequenced by the Sanger method. No carriers were detected among the studied population. It is proposed that extensive screening of this disorder is required in the country in order to develop a complete picture of its molecular epidemiology. This study holds the merit of first report on Nili-Ravi buffalo *FXI* gene mutational screening from Pakistan.

Keywords: recessive traits, FXI deficiency, Nili-Ravi buffalos.

INTRODUCTION

Livestock capacity building is imperative to meet the future demand of burgeoning populations in terms of food and livelihood in agriculture based countries. The input of livestock in food production delivers essential protein sources. The success of livestock farming is linked to increased production via improving animal health. With proper monitoring of disease dynamics in livestock, the losses of valuable breeding animals can be reduced. Among various health concerns in buffalos, genetic disorders are of apprehension as the diagnosis and treatment is difficult. Genetic disorders adversely affect wild and domesticated animal populations both qualitatively and quantitatively and they can turn out to be notable economic concern (Albarella *et al.,* 2017). Rudimentary data is available on the congenital defects in *Bubalus bubalis*. With the focus on livestock genetics, existing information can be updated through targeted research projects on key species.

Congenital bleeding disorders had been identified in dairy cattle either as functional and structural defects in hemostatic pathways. Although, biochemical assays are present for many disorders but these can overlap positive and negative predictive values of carrier detection. FXI deficiency is one such disorder which has been extensively reported in cattle breeds worldwide. Factor XI is a plasma glycoprotein involved in the intrinsic pathway of hemostasis (Gentry and Black, 1980; Gentry, 1984). It generates additional thrombin by a positive feedback mechanism. First described in humans by Rosenthal (1953), the deficiency was also identified in other mammals. Human *FXI* gene is located on chromosome 4 which was first traced by Asakai *et al.* (1987) and murine *FXI* gene resides on chromosome number 8. In cattle, *FXI* gene is located on chromosome 27 (NCBI RefSeq: NC_007328.3). The total size of the gene is 19150 bp in length (from nucleotide 17607721 to 17626871) and it comprises 15 exons and 14 introns (Kumar *et al.,* 2011). FXI deficiency in Holstein cattle is concomitant with a 76 bp segment insertion [AT$(A)_{28}$TAAAG$(A)_{26}$GGAAATAATAATTCA] within exon 12. This insertion ends up into a stop codon that results in non-functional and inactive protein with a missing functional protease domain encoded by exons 13, 14 and 15. Structurally, FXI is a homodimer; each subunit contains four apple domains (which form a disk structure with binding sites for platelets, HMW kininogen, and factor IX) and a protease domain (Gailani and Smith, 2009). In Japanese black cattle, a mutation in exon 9 was also linked to the disease. Same was reported for Wagyu cattle (www.wagyuinternational.com). The

animals heterozygous of the trait appear to be normal phenotypically, while homozygotes exhibit hemophilia-like disorder referred to as hemophilia C in humans. The incidental bleeding manifestations can present during horning, elective surgery or chance injury. Occasionally, affected calves may continue to bleed from the umbilical cord, or during dehorning, injections and castration and bleeding may be fatal in some cases (Brush *et al.,* 1987). Symptomatic cows may have hints of blood in colostrum and milk; which also became the basis of disease identification in United Kingdom. The affected animals can become anemic susceptible to pneumonia, mastitis and metritis. The heterozygotes have low calving and survival rates with high morbidity and mortality (Liptrap *et al.,* 1987; Marron *et al.,* 2004). In cattle, FXI deficiency was first reported in US Holsteins (Kociba *et al.,* 1969). Since then, further studies have been reported from United Kingdom (Brush *et al.,* 1975), Canada (Gentry and Ross, 1993), Japan (Kuneida *et al*., 2005) and many other countries of the world.

The accurate genetic analyses provide reliable measure of disease phenotype, even in the prospective progeny. The DNA-based detection tests make comprehension of phenotype-genotype correlates more convenient than the conventional methods and enhance the accuracy of carrier identification. To date, there were few reports available on bovine inherited disorders from Pakistan which targeted *Bos taurus* and *Bubalus bubalis* (Nasreen *et al*., 2009; Mehmood *et al.,* 2011, Imran *et al*., 2012). No work has been done previously on *FXI* gene in Pakistan. In line with this, the current study was planned to identify the FXI deficient sires among Nili-Ravi buffalos of Pakistan through molecular based screening.

MATERIALS AND METHODS

A total of 152 healthy breeding buffalo bulls (Nili-Ravi breed) were randomly selected for this cross-sectional study. Blood samples (10 mL each) collected from the jugular vein were preserved in EDTA containing vacutainers to avoid blood clotting. Phlebotomy was carried out under standard veterinary regimes with prior permission from the ethical review committee for sampling. Whole genomic extraction was carried out by organic method (Sambrook and Russel, 2001) and DNA yield was quantified by 0.8% agarose gel and spectrophotometer (NanoDrop 2000C, Thermo Fisher Scientific, Massachusetts, USA). The genetic screening of *FXI* gene was done by amplifying the gene fragment containing an insertion mutation; the primers sequence was as reported by Marron *et al.* (2009). The PCR reaction was optimized using touchdown PCR strategy (-1 °C decrease for first 10 cycles), followed by gradient PCR. The optimized PCR conditions were followed for further *FXI* gene amplification. The final reaction mixture contained 1x PCR buffer, 1.5 mM $MgCl_2$, 400 µM of each dNTPs, 1 unit of *Taq* DNA polymerase (Thermo Fisher Scientific, Massachusetts, USA), 0.4 µM each of forward (5'-CCCACTGGCTAGGAATCGTT-3') and reverse (5'-CAAGGCAATGTCATATCCAC-3') primers and 50 ng/µL of genomic DNA. The final volume of reaction mixture was adjusted to 25µL. Temperature profile of the thermocycler (Bio-Rad, California, USA) was as follows: initial denaturation at 95 °C for 5 minutes, 35 cycles of 94 °C for 1.5 minutes, annealing of primers at 55 °C for 1 minute and extension at 72 °C for 2 minutes, followed by a final extension at 72 °C for 10 minutes. The PCR amplicons were analyzed on 2% agarose gel. The precipitated PCR products were subjected to sequencing PCR and resolved on Genetic Analyzer 3130 (Applied Biosystems, CA, USA). The DNA sequence chromatogram data were analyzed by FinchTV® and the pair-wise homology analysis was done by NCBI-BLAST (https://www.ncbi.nlm.nih.gov /BLAST/), while, multiple sequence alignment was carried out by Clustal Omega software (Sievers *et al.* 2011).

RESULTS AND DISCUSSION

A total of 152 Nili-Ravi buffalo bulls from Punjab, Pakistan were screened for FXI deficiency. EBV data and pedigrees of all the bulls were recorded at the start of study. Phenotypically, all the bulls were healthy and no bleeding episodes were recorded during blood collection. DNA PCR amplification generated a 244 bp sequence of the *FXI* gene (Exon 12) from Nili-Ravi buffalo bulls (Figure 1). The sequence of the amplicon was aligned using NCBI-BLAST (https://www.ncbi.nlm.nih.gov/BLAST/) which revealed 98% homology with the *FXI* gene of *Bos taurus* followed by *Bubalus bubalis*. The results of this study provided evidence that there were no heterozygous bulls among the studied lot. The sequence of final 244 bp Nili-Ravi *FXI* gene fragment was analyzed for single nucleotide polymorphisms (SNPs) compared with that of *FXI* sequences of *Bos taurus* and *Bubalus bubalis* (Pandharpuri buffalo breed; NCBI_003104605.1). A transversion of adenine into thymine at position 1782 (c.1782A>T) resulted into a silent conversion of glutamine into Asparagine (Figure 3).

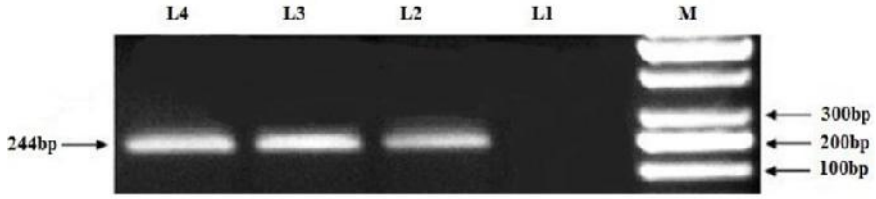

Figure 1. Agarose gel showing 244 bp PCR amplicons of *FXI* gene (lanes 2, 3 and 4) against 100 bp DNA size marker (Lane M).

CTTACTATGCAATTCTGCTTCGCCGTGGATGTATCGCCTTCTCCTTGGAATTCTCTTTTCCTCTCTTG
CTTACTCCTTAGGGTCGAGTCACCTAAAGTGTTGCGTGTCTATAGCAGCATTTTGAATCAATCAGAA
ATAAAAGAGGATACATCTTTCTTTGGGGTTCAAGAAATAATAATTCATGATCAATATGAAAAGGCAG
AAAGTGGATATGACATTGCCTTGA

Figure 2. The sequence (5'-3') of the *FXI* gene fragment from exon 12 in *Bubalus bubalis* (Nili-Ravi breed).

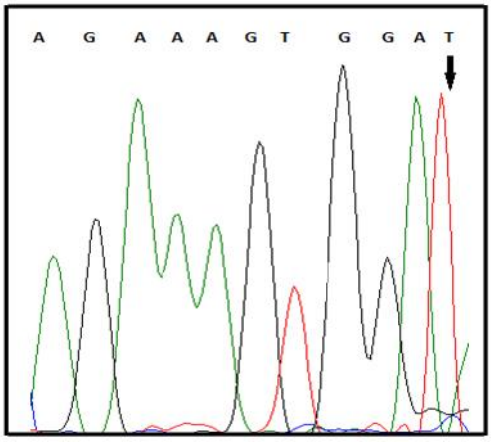

Figure 3. A transversion (A>T) at position 1782 in *FXI* gene (exon 12) in *Bubalus bubalis.*

FXI deficiency was initially studied by Kociba *et al.* (1969) in American cattle with complete absence of coagulation protein. Later on, Marron *et al.* (2004) investigated and confirmed that a 76 bp insertion mutation in the *FXI* gene had truncated some essential functional part of the protein in Holstein cattle leading to hereditary *FXI* deficiency. They investigated a total of 419 animals and detected five carriers with 1.2% frequency of mutant alleles. Since then, various studies on incidence of FXI deficiency in dairy cattle breeds such as Holstein, Friesian and their cross-breds have been reported from different countries. Kociba *et al*. (1969), Robinson *et al*. (1997) and Marron *et al.* (2004) described this disorder in Holsteins of USA, Gentry *et al.* (1975) from that of Canada, Brush *et al.* (1975) in those of England with high incidence. Citek *et al*. (2008) studied FXI deficiency in Czech Republic but the incidence was low; a single carrier out of 309 animals was found. From Poland, Gurgul *et al.* (2009) reported the carriers of this disease in 3 out of 128 studied cows. They also reported that this disorder was associated with udder health in cattle. Studies from Turkey also reported the occurrence of thisdisorder. Meydan *et al.* (2009) found 4 carriers among 225 Holsteins in Turkey with 0.9% mutant allele frequency. In another report by Meydan *et al*. (2010), the mutant allele frequency was 0.006%, with 4 being carrier out of 350 cattle studied. Similarly, Oner *et al*. (2010) studied the incidence of FXI carriers in Turkey. Among 170 studied animals, 2 carriers were detected with 0.06% mutant allele frequency. Karsli *et al.* (2011) studied 504 cattle and they detected 0.4% FXI carriers. Akyuz *et al.* (2012) estimated 0.85% carrier frequency in 161 Holstein bulls from Anatolia, Turkey. Agaoglu *et al.* (2015) reported 1.8% carrier frequency of Factor XI deficiency in Holsteins in Burdur, Turkey. Avanus and Altinel (2016) studied the FXI deficiency in Thrace region of Turkey. No carriers were found among 287 Holsteins studied by them. Studies from Asian countries also identified the occurrence of this hereditary disease in their livestock. Kuneida *et al*. (2005) studied the complete coding region of the *FXI* gene in Japanese black cattle. Ghanem *et al*. (2005) found 5 carriers out of 500 studied cows in Japan (0.5% mutant allele frequency).Watanabe *et al.* (2006) reported high incidence of FXI deficiency in stunted Japanese black cattle. The incidence of heterozygosity was up to 50%

and that of homozygosity was 5.2%. Ohba *et al*. (2008) surveyed the Japanese cattle and out of 123 animals (both dams and sires), 7 were detected as homozygous for the mutant allele. The frequency of mutants was higher up to 26.4%. In contrast, Eydivandi *et al.,* (2011) investigated the *FXI* gene frequency in Iranian cattle and out of 330 animals none were found to be carriers. Also, Bagheri *et al.* (2012) reported no carriers in Khuzestan buffalo breed of Iran out of 300 studied buffalos. Similarly, in a study by Siswanti *et al.* (2014), the incidence of FXI deficiency was studied in Indonesian Bali cattle. Among 325 samples, no carriers were detected. India harbors large populations of *Bos taurus* and *Bubalus bubalis.* FXI deficiency was reported from India in *Bos taurus* but the incidence was low. Mukhopadhaya *et al.* (2006) studied 307 cows and 259 water buffaloes and found no carrier. In another study, Patel *et al.* (2007) carried out a thorough investigation on 1001 Indian cows and buffaloes of various breeds; the FXI deficient cattle were detected with 0.6% mutant frequency. Patel and Patel (2014) reviewed that the FXI deficiency gene had been extensively studied in Indian Holstein cattle. In a recent study by Mondal *et al.* (2016), Indian Sahiwal bulls were tested for FXI deficiency. They screened a total of 120 bulls with 0.025% mutant allele frequency. The results of these studies from Asian countries were in accordance with the findings of current study, as no carriers were detected in native Pakistani buffalo breed. It is noteworthy that no carriers of FXI deficiency were detected in *Bubalus bubalis* to date. The results of our study are concordant with the findings of Patel *et al*. (2007). They reported no carriers from India in *Bubalus bubalis* (Surti, Jaffarabadi and Murrah buffalo breeds). In the current study, a silent nucleotide change was observed in the *FXI gene* of Nili-Ravi buffalo. There was a transversion of adenine into thymine (c.1782A>T) which resulted into an amino acid change of glutamine into Asparagine (Figure 3). The Similarly, Azad *et al.* (2010) screened 200 crossbred Karan Fries in Haryana, India and found no carriers. However, a non-synonymous nucleotide change (G→A) was observed at position 105. Phenotypically, no bleeding episodes were reported in the study animals. Factor XI deficiency disorder is a rare hematological disorder inherited in an autosomal recessive manner. Such disorders may express phenotypically in homozygous form, thus, in most of the cases carrier animals are difficult to identify unless the disease appears itself in affected homozygous animals. As discussed by Ramesha *et al*. (2017), there was very low incidence of FXI deficiency in Poland, Turkey, Iran, and India (0.006%-0.6%) which is in agreement with our findings. It is also recommended that the screening for carrier identification for the recessive disorders should be extensive with increased sample size. Congenital genetic disorders significantly limit the success of selective breeding programs as the phenotypic identification of carrier animals is not possible until appearance of affected animals among the subsequent populations (Al-Haggar, 2013). It becomes difficult to distinguish between the FXI deficient normal and carrier bulls phenotypically, but molecular screening is convenient as normal and carrier bulls can be identified by the position of amplicons on agarose gel; as the amplicon of normal animal is of 244 bp and that of carrier animal is 322 bp in size because of an insertion of 76 bp fragment in mutated animals. Thus, this test can be commercialized for the farmers at large. In order to avoid the dissemination of deleterious alleles from prospective sires or dams, it becomes inevitable to reduce the disease burden by genetic screening of breeding animals.

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