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MOLECULAR SCREENING OF COMPLEX VERTEBRAL MALFORMATION AND CITRULLINEMIA CARRIERS IN PAKISTANI NILI-RAVI BUFFALO (*BUBALUS BUBALIS*) BREEDING BULLS

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ABSTRACT

Inherited disorders have serious implications for cattle production and breeding programs. Structural or physiological abnormalities and neonate lethality have negative impacts on breeding populations. Complex vertebral malformation (CVM) and bovine citrullinemia (BC) are heritable congenital syndromes having autosomal recessive basis among cattle breeds worldwide. CVM affected malformed foetuses either get aborted or have evident skeletal deformities upon birth and die during early postnatal period. CVM is caused by a missense substitution (G→T) in uridine diphosphate N-acetylglucosamine transporter encoded by *SLC35A3* gene (at position 559). Citrullinemia is a heritable metabolic disorder of urea cycle enzyme argininosuccinate synthetase deficiency which occurs due to a transition (C→T) within exon 5 (codon 86) of *ASS1* gene with neurological complications during first week after birth. Both of these fatal disorders have been reported from all over the world in *Bos taurus* but there is a lack of literature on buffaloes. The present study was carried out to detect CVM and BC carriers among the Pakistani indigenous trans-husbandry water buffalo breed, Nili-Ravi (*Bubalus bubalis*). In this study, the genetic screening for the target point mutations was carried out using healthy elite buffalo bulls (n=152). Genomic DNA was extracted from the blood and *SLC35A3* gene target sequence (281 bp) and *ASS1*gene target sequence (505 bp) were amplified using PCR. Amplified PCR products were visualized by agarose gel electrophoresis and Sanger sequencing was performed. No carriers were detected among the study sample, however, a novel transversion (c.250C>A) was detected in amplified *ASS1* gene fragment. Although, findings of this study confirmed absence of CVM and citrullinemia carriers among the Nili-Ravi buffalo bulls but the presence of carrier animals cannot be ruled out in studies involving larger sample sizes. This genetic screening was carried out for the first time in Pakistani buffaloes which can be used in genetic screening of CVM or BC carrier animals in the future. Further research is recommended in order to enhance the existing data regarding CVM and BC carriers among *Bubalus bubalis*.

Key words: Complex vertebral malformation, citrullinemia, Nili-Ravi buffaloes.
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INTRODUCTION

Livestock bolsters the growth and stability of agrarian economies by contributing substantially to the gross domestic product directly and through value addition. This sector is considered the mainstay of food security and sustainable development due to its essential contribution towards the provision of meat and dairy products. Together with other utilities, livestock products supply around 12.9% of the calories consumed worldwide (FAO, 2009a). With the increase in human population, the demand for food products has increased many times and the development of resilient food processing systems has become inevitable. Livestock resources, as an alternative source of income and food, can act as a buffer against the loss of livelihood and food resources when catastrophic losses of crops occur (FAO, 2009b). It is obvious that an increased investment in the livestock sector in terms of money and technology will lead to better outputs of products, ensuring secure livelihoods and food supply chains (Adil *et al*., 2004; Cruz, 2007; Rehman *et al*., 2017).

Livestock farming in Pakistan generates 56% of the total agricultural revenue. Livelihoods of an estimated 67.5% of the Pakistanis are directly or indirectly connected to livestock (Dhanda, 2005). Being such an important economic activity in the country, the livestock sector of Pakistan holds a diverse collection of

domesticated fauna. With numbers far exceeding those of the dairy cows, buffaloes are regarded as a very important component of this fauna (Raza *et al*., 2000). Buffaloes (*Bubalus bubalis*) are a part of the traditional small mixed farming systems that are integrated with crop production. About 61% of the total milk produced in the country comes from buffaloes (Government of Pakistan, 2017). Of all the buffalo breeds employed locally, the Nili-Ravi buffalo is considered the most important water buffalo breed native to Pakistan and India. Its populations are concentrated in Punjab, Pakistan providing a major portion of the milk produced from buffalo sources (Alexiev, 1998). Nili-Ravi buffalo produces an average of 4-8 kg of milk per day, although its potential can go beyond 30 kg of milk per day. It is evident that the full production potential of the Nili-Ravi buffalo is yet b nto be achieved and exploited. Pakistan, for instance, can emulate the Indian model of "green, white and red revolution" which was implemented for the improved production of animal feed, milk and meat (Pasha and Hayat, 2012).

Despite having adequate resources for the support of livestock and holding a herd of 31.7 million buffaloes alone, Pakistan is yet a non-exporter of dairy products. The obstacles to successful exploitation include inadequate finances, fluctuating climatic conditions, defective farming facilities and importantly, potential threat of infectious, metabolic or hereditary diseases inflicting the animals. A number of inherited diseases afflicting buffaloes have been reported including: transverse hemimelia, arthrogryposis, dystocia, perosomus elumbus, umbilical hernia, schistosoma reflexus, hydrocephalus, polycephaly, etc. (Nasreen *et al*., 2009; Mehmood *et al*., 2014; Albarella *et al*., 2017). Despite the availability of these reports, comprehensive information on genetic disorders of buffalo breeds still needs to be made available. The situation in Pakistan is no different with only a few reports available on bovine inherited malformations.

Complex vertebral malformation (CVM) is a noteworthy problem of the cattle breeds worldwide (Duncan *et al.,* 2001). CVM is caused by a missense mutation in a uridine diphosphate N-acetylglucosamine transporter coding *SLC35A3* gene. A single base substitution G→T has been located in the defective allele (position 559) in the *SLC35A3* gene (Thomsen *et al*., 2006). CVM can affect multiple organ systems resulting in anomalies of the skeletal system in calves, which in most cases are aborted. The neonates appear to have craniofacial dysmorphism, axial skeletal deformities, short forelimbs and necks because of contracted tendons. Contractions can be observed in fetlocks and carpal joints as well, along with rotation of distal limbs, ankylosis of cervico-thoracic vertebrae, elongation of tarsus and severe deformation of the spinal region hemivertebrae, scoliosis, symmetric arthrogryposis of the lower limb joints and cardiac anomalies (Nielsen *et al*., 2003; Agerholm *et al.,* 2004; Rusc and Kaminiski, 2007). In *Bos taurus*, the gene responsible for the ailment is located on chromosome 3, while the chromosome location in buffaloes is still to be detected (Konersmann, 2003; Agerholm *et al*., 2004). Previous reports confirmed that the use of the proband called Penstate Ivanhoe Star (US1441440) and his son Carlin-M Ivanhoe Bell (US1667366) were the cause of dissemination of defective alleles, when both were extensively used worldwide for selective breeding programs (Healy, 1996; Revell, 2001; Agerholm, 2007; Windsor and Agerholm, 2009). Studies for the identification of the mutant allele carriers were conducted in Denmark (Agerholm, 2007), Australia (Healy, 1996; Windsor and Agerholm, 2009), United Kingdom (Revell, 2001), United States (Robinson *et al*., 1993b; Duncan *et al.,* 2001), Sweden (Berglund *et al.,* 2004), Czech Republic (Citek *et al.,* 2006), Japan (Ghanem *et al*., 2008) and are still being carried out by various scientists globally.

Bovine citrullinemia is a congenital metabolic disorder of cattle which is inherited in an autosomal recessive manner. Its lethality for the affected animal is certain during the early postnatal period (Robinson *et al.,* 1993a; Windsor and Agerholm, 2009). Citrullinemia is caused by a C→T transition in exon 5 of argininosuccinate gene, leading to a CGA/arginine into TGA/stop codon transition. This mutation has been traced to codon 86 of the argininosuccinate synthase coding gene leading to a truncated peptide that gives rise to a defective Argininosuccinate synthetase enzyme. Argininosuccinate synthetase (ASS, EC 6.3.4.5) is a urea cycle enzyme which is expressed at high levels in the liver. The enzyme catalyzes the conversion of citrulline, aspartate, and ATP to yield argininosuccinate, the immediate precursor of arginine (Husson *et al.,* 2003). The affected animals experience a defective urea cycle and can survive up to the first 7 days of their life, showing symptoms of distress along with feeding inabilities. The citrullinemia gene is located on chromosome 11 of *Bos taurus* (Grupe *et al.,* 1996; Patel *et al*., 2006; Sun *et al.,* 2011). Earlier reports of the condition in breeds of cattle include those from the United States (Robinson *et al*., 1993a), Germany (Grupe *et al*., 1996), India (Padeeri *et al.,* 1999), Hungary (Fesus *et al*., 1999), Taiwan (Lin *et al*., 2001), Czech Republic (Citek *et al*., 2006) and China (Wang *et al.,* 2009). Furthermore, in the present decade, a number of studies have been published by scientists, based on molecular screening of citrullinemia in the cattle populations of their native regions, from all over the world. But reports were lacking from Pakistan.

Screening of livestock disorders and their underlying causes is vitally important for the welfare of the stake holders and procurement of benefits from livestock agriculture. Existing approaches used to detect

the mutations or genetic polymorphisms include phenotypic, cytological, biochemical and PCR-based techniques. Among the various strategies utilized, PCR-based methods are far better, as careful design of gene-specific primers allow direct relative quantification. These advancements can be adopted for significant progression in livestock welfare (Henriques *et al.,* 2012). The present study was aimed at the detection of two such disorders; complex vertebral malformation and citrullinemia in Nili-Ravi buffalo bulls. This is the first report on inherited disorders in Pakistani elite breeding buffalo bulls and will contribute to the ongoing research in this area.

MATERIALS AND METHODS

Nili-Ravi bulls (n=152) reared at Semen Production Unit (Qadirabad, Punjab, Pakistan) and Buffalo Research Institute (Pattoki, Punjab, Pakistan) were randomly sampled for the present study. The sampling was carried out during January, 2016 to April, 2017. Blood samples were obtained from the jugular vein of the bulls, transported to the laboratory and stored at -20 °C. DNA was extracted using phenol-chloroform (organic method) and was stored at 4 °C. PCR amplification was carried out using newly designed primers for *SLC35A3* gene (codon 559) and *ASS1* gene (Exon5; codon 86). The primer sequences and PCR temperature profiles are given in table. The amplicons were visualized on 1.2% Ethidium Bromide stained agarose gel. The PCR products were precipitated and the CVM and Citrullinemia genotypes were identified by Sanger sequencing using ABI 3100 Avant Automated DNA sequencer (Applied Biosystems, CA, USA). The sequences were read using FinchTV®. DNA Sequencing was facilitated by Center of Applied Molecular Biology, University of the Punjab, Lahore, Pakistan.

Table 1. Primer sequences and temperature profile for PCR amplification of *SLC35A3* and *ASS1* genes.

Genes	Primer Sequence (5'→3')	Temperature cycling profile	PCR Product (bp)	Reference
SLC35A3	Forward: CAGATTCTCAAGAGCTTAATTCTA Reverse: TATTTGCAACAACAAGCAGTT	95 ºC = 05 minutes 94 ºC = 45 seconds 54 ºC = 45 seconds 72 ºC = 60 seconds 72 ºC = 10 minutes	281	Meydan *et al.* (2010)
***ASS1* (Newly Designed)**	Forward: AGGCCATCGGAGACTCTGT Reverse: AGTCAGGGACAGCTCCACTC	95 ºC = 05 minutes 94 ºC = 30 seconds 55 ºC = 45 seconds 72 ºC = 45 seconds 72 ºC = 10 minutes	505	This study

RESULTS

The primers amplified the partial sequences of *SLC35A3* gene (281 bp; Figure 1) and *ASS1* gene (505 bp; Figure 2) for CVM and citrullinemia, respectively. Possible point mutations of both disorders were screened in all the samples by sequencing analyses. The sequences from electropherogram were aligned using NCBI-BLAST (https://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was done by Clustal Omega. 98% homology was detected with *Bos taurus*. Single nucleotide polymorphisms (SNPs) were not detected in *SLC35A3* gene fragment; however, a novel SNP was detected as a transversion in *ASS1* gene fragment (c.250C>A).

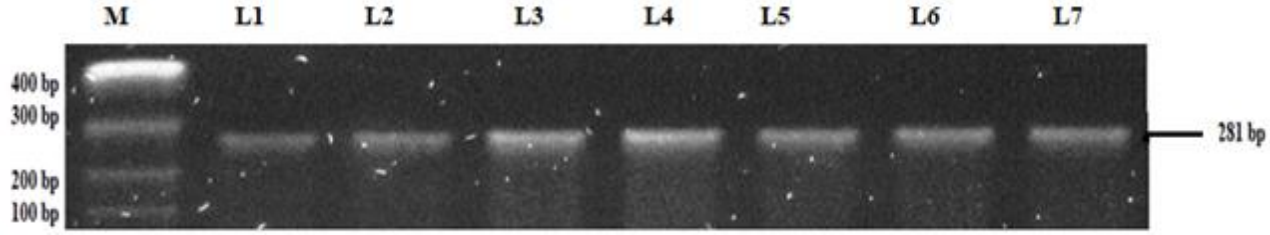

Figure 1. 2% agarose gel showing PCR products of *SLC35A3* gene target sequence from Nili-Ravi buffalo bulls. Lane 1, 2, 3, 4, 5, 6, 7: *SLC35A3* (281 bp) gene fragment. M: 1 KB DNA size marker.

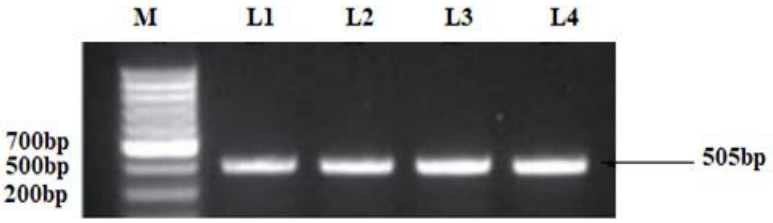

Figure 2. 1.5% agarose gel showing PCR products of *ASS1* gene target sequence from Nili-Ravi buffalo bulls. Lane 1, 2, 3, 4: *ASS1* (505 bp) gene fragment. M: 1 KB DNA size marker.

DISCUSSION

The occurrence of genetic disorders causes direct economic losses in breeding cattle populations in terms of animal mortality, reduced reproductive ability and milk production (Khan *et al.,* 2007). Despite the fact that the dairy animals have been well exploited for their economic traits, information available on the congenital defects of buffaloes is not ample enough as compared to that on cattle breeds such as *Bos taurus*. As artificial insemination is widely used in cattle breeding, it is recommended to screen prospective sires to minimize horizontal dissemination of defective alleles. Bulls represent half of the herd and a single insemination can affect hundreds of off-springs, thus employment of genetic testing approach can subsequently be helpful in screening of carrier bulls. With the advent of molecular-based technologies, evaluation of the status of carriers among breeding animal populations has been made easy. Although, biochemical tests may be available for few disorders but DNA based testing can replace conventional methods that gave unreliable or overlapping results. DNA testing for genetic disorders in animals has made a significant contribution to the control and eradication of some devastating conditions among dairy and beef cattle (Al-Haggar, 2013).

More than fifty hereditary disorders have been well documented in *Bos taurus* breeds worldwide but little information regarding hereditary disorders in *Bubalus bubalis* is available especially from Pakistan. The actual number of clinical cases of CVM and citrullinemia are unreported in the Pakistani buffalo breeds. Thus, the current study was designed in an attempt to detect the prevalence of CVM and citrullinemia carriers among Pakistani Nili-Ravi buffaloes through molecular screening.

A total of 152 healthy elite bulls were randomly selected from Buffalo Research Institute, Punjab, Pakistan and Semen Production Unit, Qadriabad, Pakistan. Blood samples were collected from the jugular vein and preserved in EDTA containing vacutainers. Extraction of blood was carried out under standard veterinary regimes. Whole genomic extraction was carried out by organic method and DNA yield was quantified by 0.8% agarose gel and spectrophotometer (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA). The genetic screening of CVM and BC carriers was done by amplification of *SLC35A3* gene partial sequence (281 bp) using already reported primers by Meydan *et al.,* 2010) and *ASS1* gene partial sequence (505 bp) by primers newly designed for this study. The findings of the current study reported that carriers of CVM and citrullinemia were not detected among buffalo breeding sires at the government managed breeding institutes in the Punjab province.

Previously, a number of studies have been carried out for the detection of CVM carriers, with the earliest being that on Danish Holsteins (Agerholm *et al.,* 1993). Later studies in other countries include those from the United States (Duncan *et al.,* 2001), Australia (Healy, 1996), United Kingdom (Revell, 2001), Czech Republic (Citek *et al.,* 2006) and Japan (Nagahata *et al.,* 2002; Ghanem *et al.,* 2008).The fact that no carriers of CVM were found was in accordance with a number of studies reporting either no carriers or a low frequency of carriers in cattle. Meydan *et al.* (2010) reported 12 CVM carriers among 350 animals (at a low frequency; 0.017%) in Turkish Holstein population, while, Avanus and Altinel (2017) detected 3.2% carriers in Turkey. With a screening technique of high resolution melting analysis (HRMA) used for SNP detection in Slovak cattle by (Gabor *et al.,* 2012), the genotyping of mutant allele "T" was carried out with 47 samples and out of those tested animals, 4 were heterozygous. Rezaee *et al.* (2009) screened 144 Iranian Holsteins but found no carriers among the lot while, Alaie *et al.* (2012) screened 100 Holsteins and 100 Guilan cows by PCR-SSCP method but found no carriers among Iranian cattle. Wang *et al.* (2011) detected 10 CVM carriers out of 342 studied animals (2.9% carrier frequency). In another study, Zhang *et al.* (2012) used real-time PCR assay and detected 56 out of 587 animals studied in Chinese cattle, with 1.36% heterozygous carrier frequency. Paiva *et al.* (2013) studied Brazilian Girolando cattle (783 animals) and found the carrier frequency as 1.53%. Similarly, Adamov *et al.* (2014) identified one CVM carrier out of ninety Macedonian Holstein-Friesian cattle. It is notable that the results of the present study coincide with the findings of two neighbouring countries, Iran and China. Some other studies also reported a high prevalence of the condition. Agerholm (2007), pointed out that the disorder was of high prevalence worldwide and was a result of

inbreeding as a proband (Penstate Ivanhoe Star; US 1441440) and his son (Carlin-M Ivanhoe Bell; US 1667366) were the founders carrying defective alleles. Berglund *et al.* (2004) identified high carrier frequency of CVM (23%) in Swedish cattle. Rusc and Kaminiski (2007) determined the status of CVM carriers in a total of 605 Polish Holstein-Friesians. A high incidence was detected among them with 150 heterozygotes, by using polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP). Similarly, 10% carrier frequency was detected by Betka *et al.* (2008) in Slovenia. Ghanem *et al.* (2008) identified 26 carrier cows out of 200 in Japan (10% carrier frequency). Mahdipour *et al.* (2010) studied Indian Karan Fries and out of 52 animals, 12 were carriers of the CVM allele. According to the findings of our study, no carriers of either CVM or citrullinemia were detected in buffalo bulls. As it is noticeable that the previous studies indicated a high prevalence of CVM concern with *Bos taurus* rather than with buffaloes (Meydan *et al.,* 2010).

Nili-Ravi buffaloes were also screened for citrullinemia using newly designed primers. A 505 bp *ASS1* gene fragment was amplified for the first time in Pakistani buffaloes. Sanger sequencing revealed a novel transversion in bubaline *ASS1* gene (c.250C>A) but this SNP had no phenotypic effects when it was compared with Ensembl genone browser. The results of our study suggest that citrullinemia occurs with perhaps a very low incidence in buffaloes. Our study found no carriers but a further study with a much larger sample size might be able to report a few, if at all, carriers. A number of previous studies support this supposition. Initially, Robinson *et al.* (1993a) detected citrullinemia for the first time in US bulls, followed by Grupe *et al.* (1996) in Germany. The incidence of carriers was low in both the countries. It was disseminated throughout the Australian Holstein population following importation of semen from the US sire Linmarck Kriss King (Healy *et al.,* 1991). Padeeri *et al.* (1999) studied the incidence of citrullinemia in Indian cows and buffaloes. According to their findings, one Holstein bull was detected as a carrier with 0.67% frequency and no carriers were detected among the studied buffalo breeds. Fesus *et al.* (1999) reported a few carriers in Hungarian Holstein populations. Lin *et al.* (2001) and Wang *et al.* (2009) studied the citrullinemia carrier in Taiwan and China, respectively. From the Czech Republic, Citek *et al.* (2006) found no incidence of citrullinemia. Similarly, Meydan *et al.* (2010), Oner *et al.* (2010) and Eydivandi *et al.* (2012), screened the cattle populations in Turkey and Iran respectively, but no carriers were detected in these regions. JianBin *et al.* (2011), identified citrullinemia carriers in Chinese Holstein by PCR-RFLP analysis. Among the 615 animals, one citrullinemia carrier was identified with a 0.16% carrier frequency. Gaur *et al.* (2012), found a few carriers in Indian Holstein population. Incidence of citrullinemia was altogether absent in Turkish Holsteins as reported by Agaoglu *et al.* (2015) and Avanus and Altinel (2017). Branda *et al*. (2016), carried out PCR-RFLP for detection of citrullinemia in Uruguay where no carrier was detected among the study population (n=190). Similarly, Ramesha *et al.* (2017) screened Indian native cattle and buffalo populations and reported no citrullinemia carriers among the studied animals. In a study carried out in India, Kotikalapudi *et al.* (2014) identified a carrier bull among the Indian Holstein population. They also reported a novel polymorphism within exon 3 of *ASS1* gene. It was however, a silent mutation at codon 80 with no change in amino acid (Serine AGC→AGT) at 240 bp position (NCBI accession No. KF933365). The findings of the present study are supported by these previous studies that citrullinemia is more prevalent in *Bos taurus* breeds and the incidence is low in *Bubalus bubalis*.

In conclusion, it is emphasised that the DNA-based detection analyses provide reliable detection of carriers even in the prospective populations. These methods are rapid and more convenient than the conventional methods with enhanced accuracy of carrier identification. As the existing data on carriers of hereditary disorders among dairy animals in Pakistan needs to be upgraded this study can be helpful in PCR-based genetic screening of CVM and BC carriers with extended sample sizes. This study was an effort to generate first hand data on hereditary disorders among the Nili-Ravi buffalo breed through molecular screening.

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