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Original scientific article

GENOTYPIC DISTRIBUTION OF *MSTN* GENE POLYMORPHISMS INVOLVED IN RACING PERFORMANCE IN *Camelus dromedarius*

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Camel racing is one of the popular sports around the world and is growing rapidly especially in Gulf countries. Camel has adapted itself to harsh and draught climate of desert. This quality of camel makes it the best choice in racing industry. Pakistani breed

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Dromedarius camels are as good as Omani and Sudanese camel breeds in their racing potential. Myostatin (Growth differentiation factor 8) is a protein coded by *MSTN* gene. Polymorphism in *MSTN* play a significant role in growth of muscle, increasing fast glycolysis Type IIB muscle fiber, forming tubular aggregates in type IIB fiber by increasing the body strength and racing ability. This gene is also responsible for double muscle phenotype in bovines. In present study, Marecha and Brela camel breeds of Pakistan were studied for genomic characterization of *MSTN* gene. Blood samples were collected from the healthy animals between the age group of 2-4 years. Genomic DNA was extracted, amplified by using specific sets of primers, purified and sequenced by Sanger's dideoxy chain termination method. No single nucleotide polymorphisms (SNPs) were found in the exon-3 of *MSTN* of Marecha & Brela, which shows that the gene is highly conserved among species while phylogenetic data of the dromedarius *MSTN* gene showed highest similarity with *Bos taurus* and least similarity with *Gallus gallus*. Genes that are similar to the camel *MSTN* are myostatin of *Homo sapiens, Pan troglodytes, Bos taurus, Canis lupus, Rattus norvegicus*. The significance of this study was to identify the genetic potential of Pakistani camel for racing that will help in the socioeconomic uplift of the local community. It will also help the camel breeders to select the best breed of camel & enhance their genetic potential by using least operating cost. That will in turn provide opportunity to the camel breeders to produce the best breed that will be selected by the trainers for racing that will provide another source of income for the local community and Pakistan's camels will also be used in the racing industry.

Keywords: Camelus Dromedarius, conserved gene, *MSTN*, SNP, muscle mass

INTRODUCTION

Camel racing is a popular sport in Pakistan, Saudi Arabia, Egypt, Bahrain, Jordan, Qatar, United Arab Emirates, Oman, Australia, and Mongolia. Myostatin or growth differentiation factor 8 β family member is a protein secreted by *MSTN* gene that is a negative regulator of muscle mass growth in mammals (THOMAS *et al*., 2000). Mutation that results in reduced myostatin is responsible for muscle hypertrophy (SCHUELKE *et al*., 2004). The gene sequence for *MSTN* has been shown to be highly conserved across vertebrate species (MCPHERRON and LEE, 1997).

Myostatin is expressed in satellite cells and in the adult myoblasts (MCCROSKERY *et al*., 2003). Loss of functional mutations in *MSTN* gene have increased skeletal muscle mass due to an increase in number of muscle fibers (hyperplasia) (LEE, 2007) and thickness of fibers (hypertrophy) (MCPHERRON and LEE, 1997). *MSTN* gene also have effect on other multiple phenotypic characteristics that have been detected in several species, including in child (SCHUELKE *et al*., 2004), mice (SZABO *et al*., 1998), sheep (CLOP *et al*., 2006), dogs (MOSHER *et al*., 2007), double muscled cattle (GROBET *et al*., 1997), chickens (YE *et al*., 2007; MCFARLAND *et al*., 2007; YANG *et al*., 2003) and pigs (STINCKEN *et al*., 2008). Lack of myostatin in mice and double-muscle cattle can specifically increase Type IIB (fast glycolysis) muscle with shortening in relaxation and contraction time in *MSTN*, muscle that is consistent with enhanced racing ability. These fibers also contain tubular aggregates that increase force output of muscle

(AMTHOR *et al*., 2007; DEVEAUX *et al*., 2001; HENNEBRY *et al*., 2009). Several studies have shown the role of myostatin as it inhibits hyperplastic muscle growth in zebra fish by using myostatin prodomain that could inhibit myostatin in skeleton muscles (XU *et al*., 2003).

The gene chosen for the present study is *MSTN*. The position of gene on chromosome is unknown in *Camelus dromedarius*. The single nucleotide polymorphism in exon-3 of *MSTN* gene in the present study may be used to make prognosis about the genetic potential of a camel for racing in two most common Pakistani camel breeds. The present study involves two most common camel breed Marecha and Brela in Pakistan. The genetic diversity of these two breeds for racing is identified by characterizing *MSTN* gene.

MATERIALS AND METHODS

The present study was conducted to identify the genetic distribution of *MSTN* gene polymorphisms that may play an important role in the racing performance of camels. The research was performed in Postgraduate Molecular Biology and Genomics Laboratory, Institute of Biochemistry and Biotechnology (IBBT), University of Veterinary and Animal Sciences, Lahore.

Animal selection criteria

A total of 150 camels blood samples, 60 from Marecha and 90 from Brela camel breed, were collected from Camel Breeding and Research Station (CBRS) at Rakhmahni Bhakhar.

Sampling strategy

Camel age at the time of selection was 2- 4 years. The selected animals were ensured to have different families or no blood relation.

DNA extraction and quantification

Extraction of genomic DNA from blood cells was performed by using standard organic Phenol-Chloroform-Isoamyl alcohol extraction method (SAMBROOK and RUSSEL, 2001). Genomic DNA concentration was evaluated with the Nanodrop 2000C spectrophotometer (Thermo Scientific). DNA was also run on the gel for their quantification and was stored at -20°C until use.

Primer designing

Sequence of *MSTN* gene was retrieved from NCBI with the accession number [NW_011590997.1]. Gene consist of 6732 bp DNA having 3 exons: exon 1 (379bp), exon 2 (371bp), exon 3 (381bp) and 2 introns: intron1 (363bp), intron 2 (811bp). Primers were designed using primer 3 software from the coding regions of the *MSTN* gene i.e. exon 3 respectively (Table 1). A total three sets of primers were selected on the basis of GC content and melting temperature (Tm).

Table 1. Primer sets used to amplify the exon-3 of the MSTN gene

PRIMERS	SEQUENCES	Annealing temp. (C°)	Amplicon size
STF1	5′- ATG AGT CCC TGA GGT AGG AAA G - 3′	62.1	758bp
STR1	5′ - TCA CCA GAA CAC AAG GAG AAT TG - 3′	61.1	
STF2	5′ - TCG AGC TAG GAG ATC AAA TTC CA - 3′	61.1	799bp
STR2	5′ - TCA TAC ATT ACA TGT TTC TGT GCC T - 3′	60.9	
STF3	5′- AGC TTG CCT TTG CAA CAC TTC - 3′	59.4	791bp
STR3	5′ - AAC CAA ACT TTT GTG CTA AGT TT - 3′	55.7	

Polymerase chain reaction

Amplification of the selected segments of *MSTN* gene from the genomic DNA was performed using PCR. The PCR thermal profile for all the three primers of exon-3 consist of touch down PCR of an initial step at 95°C for 10 min, followed by 10 cycles of 30 s at 95°C, 45s at 65°C, 60 s at 72°C; with the remaining 25 cycles at 55 with the gradual decrease in temperature. The final extension step was carried out at 72°C for 10 min. PCR product was run on 2% Agarose gel to identify the amplified DNA. Amplicons were analyzed with DNA reference ladder for the confirmation of amplicon size.

Amplicon purification and sequencing

Amplicons were precipitated with absolute ethanol and template was washed with 70% ethanol to remove all the contaminants including salts. DNA sequencing of selected PCR amplicons was done by commercial facility. The purified PCR product was sequenced in both directions using Sanger sequencing method (Di-deoxy chain termination method), on ABI Prism 3100 Genetic Analyzer and results were analyzed by doing BLAST of the sequenced product with reference sequence.

Bioinformatics and statistical analysis

Sequenced DNA was compared with the *MSTN* exonic reference sequence by using CHROMAS software. Reference sequence was retrieved from NCBI in FASTA format and BLAST was used to align the reference sequence, every nucleotide position which was not aligned to reference sequence was used to locate the SNP. Population statistics were analyzed by POPGENE32 software and Vesserstat. Protein structural conformation was observed by using Phyre2Seq. Using MEGA software version 7.0, a phylogenetic tree was constructed based on maximum likelihood after aligning *MSTN* gene of different species to study evolutionary genetics. All the required sequences for phylogenetic analysis were retrieved from NCBI in the FASTA format.

RESULT AND DISCUSSION

In the present study we report the nucleotide sequence of the third exon of the *MSTN* locus for the *Camelus dromedarius* species obtained after *in vitro* amplification of genomic DNA using oligonucleotide primers designed from the sequence of *Camelus dromedarius* (Arabian) available at NCBI. After amplification and sequence analysis using CHROMAS, single

nucleotide polymorphisms (SNPs) were identified in the *MSTN* gene by performing the nucleotide BLAST of the product sequence with the reference sequence in NCBI. BLAST result shows that both the sequence have almost 99% homology with primer-1 in Marecha camel breed, the reference also show 99% similarity with the subject sequence for primer-2 in Marecha camel breed, for Primer-3 in Marecha both the sequence on BLAST show 100% homology while in case of Brela camel breed, BLAST analysis shows 99% homology with the reference sequence of Arabian dromedary camels. Similarly, no SNP was found in *MSTN* of Egypt and Tunisia camel breed that shows the highly conserved nature of this gene in different regions of the world except in one in which Blast analysis showed SNP in the *MSTN* genome of Algerian camel breeds (C to G) at 674 position in Brela camel breed and a similar change from (G to C) was identified at 572 position in Marecha camel breed (Fig 1). Protein Analysis shows an amino acid change from E to D at position 176 that result in change from aspartic to glutamic acid in the final protein in Marecha camel breed hence effecting the protein structure and functionality (Fig 2 and 3). Similar change was identified in Brela camel breed as well. No other variation was identified in Blast analysis of Marecha and Brela with each other that show *MSTN* has higher similarity with each other in these two breeds as well.

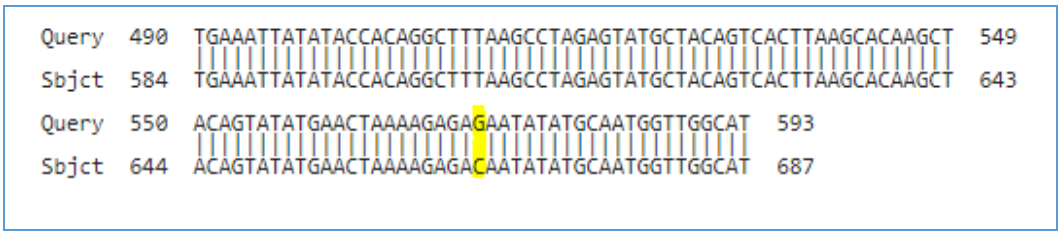

Figure 1. Single Nucleotide Polymorphism was identified on comparison of Marecha MSTN genome with MSTN of Algerian Camel Breed

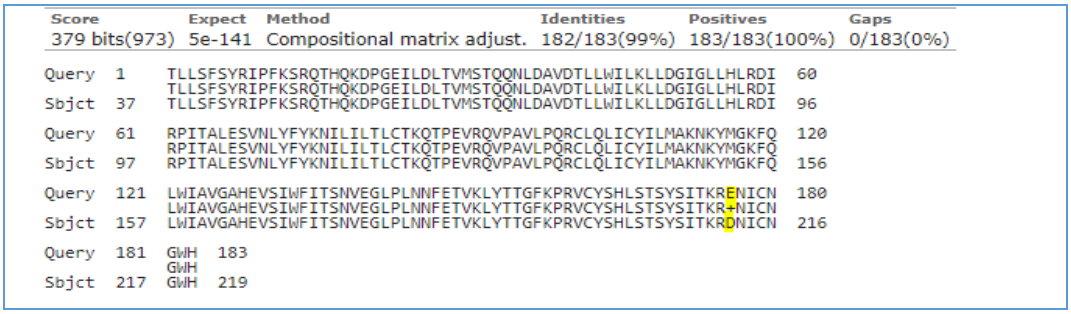

Figure 2. Change in the amino acid due to the SNP at position 176

Figure 3. Protein Structure analysis of wild type allele of MSTN gene (A) of Algerian camel with altered allele (B) in Marecha camel breed.

For this SNP, the maximum likelihood frequency for the Hardy-Weinberg equilibrium is greater than 0.05 (non-significant) showing that the distribution frequency of the single nucleotide polymorphism is similar in each generation (Table 2). Allele and Genotypic frequency analysis shows that genotypic distribution of BB (homozygous mutant) genotype for the polymorphism p. 179E is much higher than AB or AA genotypes in our specie. By performing ANOVA analysis for population, the P-value for the polymorphism (p. D179E) is less than 0.05, the result shows that BB i.e. mutant homozygous genotype is highly significant (Table 3).

The phylogenetic analysis of *MSTN* in different species based on Maximum Likelihood, analysis shows that *Camelus dromedarius* show maximum homology on evolutionary scale with the *Camelus bactrianus* and *Camelus ferus.* Organism *MSTN* gene show 100% homology with *Bos taurus*, *Bos indicus* in a tree with the branch distance of 0.00 next is with Gorilla and *Homo sapiens* (0.01), *Ovis aries* (0.04), *Mus musculus* (0.09) and *Equus caballus* (0.10) with the least similarity shown with *Gallus gallus.*

Table 2. Hardy Weinberg Equilibrium value for MSTN

Polymorphism	Allele Frequency		Genotypic Frequency			P- Value (<0.05)	Hardy-Weinberg Equilibrium
	Wild Allele (C)	Mutant Allele (G)	AA	AB	BB		
p. D176E	0.0500	0.9500	-	0.200	0.800	0.86941	NS

Table 3. Association analysis of polymorphic site

Polymorphism	AA	AB	BB	P- Value (<0.05)
p. D176E	--	0.396 ± 0.19474	0.743804 ± 0.099245	0.008817

Phylogenetic Analysis of *MSTN* in 17 different species was performed based on neighbor joining and maximum parsimony, the tree shows that Marecha and Brela camel breeds

show maximum homology on evolutionary scale with the *Camelus dromedarius, Camelus bactrianus* and *Camelus ferus* of Arabian origin (Fig. 4).

In Pakistan, the potential of camel has not been utilized properly because of this no proper scientific research has been done to evaluate its importance by the scientists. Whereas in Pakistan it is highly recommended for the identification of actual camel breed to develop advance techniques for their farming. By using these advance farming techniques, the life of Pakistani pilgrims can be improved because their main source of income depends on raising the camel (YAQOOB and NAWAZ, 2007). It was reported that the adult female dromedary camel during exercise even on the poor diet can consume less food and produce more energy than at the rest state so have the higher potential for racing (NAGPAL *et al*., 2000).

Camel because of its multiple working abilities can help in improving the living conditions of nomads that are involved in raising the camels, by providing the necessary supplements, improve their conventional management system with latest farming techniques and the health of the camel (KHAN *et al*., 2003).

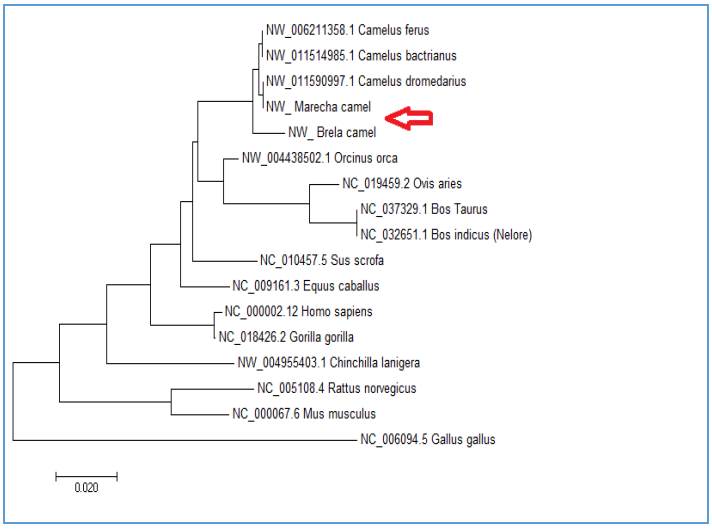

Figure 4. Phylogenetic Analysis of MSTN in species by Neighbor Joining Tree

For the purpose of racing, dromedary camels are largely bred in countries like Arabian Peninsula because they are economically more important in countries with the developing racing industry especially Africa and Arabia. Currently UAE have 200,000 racing camels (YAM and KHOMEIRI, 2015).

Camel racing and adventurous journeys for hunting and other purposes including the camel fairs are the pathways to increase camel production because they are the main reason to attract many tourists in different countries around the globe. *MSTN* gene as a part of sport ability in human that can negatively regulate the muscle mass, the mutation in gene is responsible for increasing muscle mass and provide strength during racing and has already been reported not

only in human but in dog, rabbit, mice and cattle as well (MARCHITELLI *et al*., 2003; MATA *et al*., 2012; MOSHER *et al*., 2007).

Previously no study was reported that highlights the role for racing, except one that only report sequence assembly and SNP of *MSTN* in camel just showing that the gene is conserved among species. The mutation in *MSTN* is responsible for increasing muscle mass, strength and fast glycolysis by Type-IIB muscle fibers as reported by others (AMTHOR *et al*., 2007; MCPHERRON *et al*., 1997).

In Camel, previously a 256 bp region in the first exon of the *Camelus dromedarius MSTN* gene had been published in six different Pakistani breeds without observing any sequence polymorphism (SHAH *et al*., 2006). Another study sequenced more than 3.6 kb of nucleotide sequence in a total of 22 animals from three different geographic regions (Algeria, Tunisia and Egypt), including the three exons, part of intron 1 and intron 2 and part of the 3' and 5' ends of the *Camelus dromedarius* myostatin gene and reported the 3 SNP's two transitions (798_G/A and 799_C/T) and one transversion (486_G/C) only found in intron 1 of the gene. In the present study, it is confirmed that Egypt and Tunisia *Camelus dromedarius* have not shown any genomic variation in coding region (exon-3) of *MSTN* gene in comparison with Marecha & Brela camel breeds of Pakistan. A single nucleotide polymorphism was identified in exon 3 of *MSTN* gene in comparison with Algerian camel breed that would result in increasing muscle mass in camels and as a result our breeds may have the potential for better racing.

So it is concluded from our results that *MSTN* is highly conserved gene in our breeds of Marecha and Brela as our results on BLAST and sequence analysis show only one SNP identified in comparison with Algerian camel breed in the third exon of *MSTN* gene. As Marecha are the camels used for racing so Brela having similar genome similarity can be used for racing as well but the potential and capabilities for racing cannot be reported on *MSTN* basis. Future study on other genes is required to identify racing ability of these two popular camel breeds in Pakistan.

CONCLUSIONS

This work represented the characterization of *MSTN* gene by identifying the single nucleotide polymorphism of the myostatin gene in the two most widely present dromedary camel breeds Marecha and Brela of Pakistan. Interestingly, a very low diversity was observed at the *MSTN* locus in our population sample, which may reflect the distinctive evolutionary history of this species. The results show that exon 3 of *MSTN* gene is highly conserved among species. Further ongoing studies from recent available whole genome sequences on the three *Camelus* species (*C. bactrianus*, *C. ferus* and *C. dromedarius*) will help to better clarify the *MSTN* patterns of evolution.

It is highly recommended from the study that for improving the racing trait this gene need more to be explored in future in Pakistani camel breeds with the other genes like *PDK4*, *COX4i2*, *DMRT3* and *PPARCG* gene that are involved in and responsible for racing as already reported in thoroughbred and human athletic performances. Our work will be a guideline for future genetic analysis in camel that would be better promoted for racing, increase their export to other countries like UAE, increasing tourist attraction in order to increase the income and living of nomads of desert and Northern area of Pakistan. As a result, it will help in uplifting Pakistan's economy in the current state.

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GENOTIPSKA DISTRIBUCIJA POLIMORFIZMA *MSTN* GENA UKLJUČENOG U TRKAČKE PERFORMANSE *Camelus dromedarius*

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Izvod

Trke kamila su jedan od popularnih sportova u svetu koji se brzo razvija, posebno u zemljama Zaliva. Kamila se prilagodila oštroj pustinjskoj klimi i nedostatku vode, tako da ovaj kvalitet kamile čini najboljim izborom u trkačkoj industriji. Kamile pakistanske rase *Dromedarius* su dobre kao omanske i sudanske rase kamila u svom trkačkom potencijalu. Miostatin (faktor diferencijacije rasta 8) je protein koji je pod kontrolom *MSTN* gena. Polimorfizam u *MSTN* ima značajnu ulogu u rastu mišića, povećavajući brzu glikolizu mišićnih vlakana tipa IIB, formirajući tubularne agregate u vlaknima tipa IIB povećanjem snage tela i trkačke sposobnosti. Ovaj gen je takođe odgovoran za fenotip dvostrukog mišića kod goveda. U ovoj studiji, pasmine kamila Marecha i Brela iz Pakistana su proučavane za genomsku karakterizaciju *MSTN* gena. Uzorci krvi su uzeti od zdravih životinja u starosnoj grupi od 2-4 godine. Genomska DNK je ekstrahovana, amplifikovana korišćenjem specifičnih setova prajmera, prečišćena i sekvencionirana Sangerovom metodom. Nijedan SNP nije pronađen u egzonu-3 *MSTN* Marecha i Brela, što pokazuje da je gen visoko konzerviran među vrstama, dok su filogenetski podaci *Dromedarius MSTN* gena pokazali najveću sličnost sa *Bos taurus*-om i najmanju sličnost sa *Gallus gallus*-om. Geni koji su slični *MSTN* kamile su miostatin *H. sapiens, P. troglodites, B.taurus, C. lupus, R. norvegicus*. Značaj ove studije je bio da se identifikuje genetski potencijal pakistanske kamile za trke koji će pomoći u socioekonomskom podizanju lokalne zajednice. Takođe će pomoći uzgajivačima kamila da odaberu najbolju rasu kamila i poboljšaju njihov genetski potencijal korišćenjem najmanjih operativnih troškova. To će zauzvrat pružiti priliku uzgajivačima kamila da proizvedu najbolju rasu koju će treneri odabrati za trke, što će obezbediti još jedan izvor prihoda za lokalnu zajednicu, a pakistanske kamile će se takođe koristiti u trkačkoj industriji.

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