STUDY POLYMORPHISM MYOSTATIN GENE IN MEHRABAN'S SHEEP USING PCR-RFLP METHOD.

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ABSTRACT:The purpose of this study involves the analysis of mutations in the coding region that was potentially alter myostatin gene expression. Hundred and twenty sheep blood samples were taken and derived DNA used for 337-matereproducer. Shear fragment length polymorphism of PCR products was performed by adding the restriction enzyme Haiti. The frequency of genotypes mm, Mm, respectively, 947 / 0 and 053 / 0 were diagnosed with the MM genotype was observed. The allele frequencies for the alleles M and m , respectively estimated, 026 / 0 and 974 / 0. Comparison of allele M (favorablealleles) was calculated as the gentle sheep breeds with similar studies worldwide , showed that the frequency of this allele in sheep 's kind is not in proper level . It also became clear that the Hardy - Weinberg equilibrium in the study population are connected with this place.

Keywords:Polymorphism , gentle sheep gene, MSTN gene, PCR-RFLP[iiii].

1. INTRODUCTION

Production traits are important economic traits in sheep . Therefore, understanding the genetic aspects and major genes affecting meat production has recently attracted the attention of researchers in genetics and breeding. The detection of major genes (Major gene) and quantitative trait nucleotides controller (QTN[1]) affecting muscle traits and carcass traits can be improved through the use of genetic information. Candidate genes arethe genes that their performance traits has been proven. According to estimates by the use of molecular markers in the selection of beef cattle, genetic improvement can speed up 30 % in 24 months to improve and reduce the generation gap [1].

Molecular studies of candidate genes indicate the polymorphisms of these gene and their effects on most traits. One of the most important candidate genes on production traits of livestock is myostatin gene, which leads to increased muscle phenotype in beef cattle . So are known as candidate genes for meat production

Quantitative trait nucleotides

Traits in different breeds of livestock and poultry. Myostatin (MSTN) which also called as 8 growth and differentiation factor involved in beta conversion growing groups to change factors that play a role in reducing the growth in mammals , as skeletal muscle Inhibitiory.Comparative analysis between existing Myostatinprotein shows a high degree of sequence conservation between species of sheep , a single nucleotide polymorphism (SNP) in the untranslatable region 3 (3UTR) as a cause muscle hypertrophy in myostatin gene to chromosome 2 (OAR2)in European races have been detected , which could be used in breeding programs .

Mehraban's sheep is one kind of Heavy -tail sheep .tail in these animal typically have a slope that starts from the back of the animal and the tail has droop mode.

In 1988 , the World Health Organization and the Department of Energy synchronized their efforts in preparing the Human Genome Project. The project aims to integrate genetic and physical plan of chromosomes with internal nucleotide sequencing of the human genome [2]. Genomic projects have been published in several species of farm animals. Construction of linkage maps with DNA , these projects are able to identify the genetic basis for example the quantity and quality of meat , growing characteristics and susceptibility to disease [3].

Gene planning and construction of genetic planning is an essential part of modern genetics. And The reason is the major part of understanding and manipulating the genetic inheritance of certain traits relationship. If statistical evidence for this relationship is found, it can be concluded that the trait gene may be in the same chromosomal region of marker genes [4]. A genetic linkage map determines the relative position of markers or genes on chromosome . Set them accordingly depending on the frequency of recombination between the loci , multiple alleles that transmitted together [5].

physicalplanning of cloned DNA fragments assigned to a specific location is based on a chromosome. This method of planning complete the planning linkage [6]. Physical planning of genes or markers assigned to chromosomal regions that are identified by the following methods :

1. in situ fluorescent hybridization (FISH) [7],2.With locating the genome sequence [8] .

Comparative mapping results can be used for evolutionary studies [9]. Identify markers and genes affecting quantitative traits in livestock and then help these markers (MAS) or genes (GAS) is a useful strategy for low heritability traits that are important in difficult and impossible measuring [10]. Traits including carcass quality and quantity attributes that identify markers and genes affecting them and applying MAS or GAS can have a significant impact on genetic progress . Thus, if a region of the chromosome containing genes known to be valuable , markers can be used to identify animals with desirable alleles of a gene transfer [11].

We could use correlate QTL markers in selection and this information increase the standard of accuracy selection and pursuit that increase the response to the selection [12].

Assisted selection, genetic markers genetic polymorphism data with a combination of MAS and phenotypic information is desired, it can increase the accuracy of selection and genetic progress is slow in the selection strategy of specific polymorphisms or specific chromosomal

segments used . When the desired trait is controlled by different alleles , allele information can be used in animal genetic selection .

Efficiency of genetic markers depends on whether structural changes or genetic polymorphisms , and direct action are effective gene or QTL affecting the trait under study is continuous . However, since these genetic markers can be located anywhere in the genome , finding useful markers is like finding a needle in a haystack [13].

Identification of genomic regions or genes of major effect for the different characteristics of the three methods of scanning the genome , candidate genes and integration can be done in two ways [14].

Generally, markers with high PIC 7.0 are considered as markers very efficiently [15] . Access to a large number of DNA markers, genetic mapping has provided full development of all regions in the genomes of various organisms in the identity [16]. Single nucleotide polymorphism (SNP) markers that are standard for a wide range of biological studies such as genome scans are used [17].

Major advantage of candidate gene is the application in any population that has phenotype recapitulates record especially in species those have long generation distance and have fewer breeds, discovering various limited enzymeBy smith and Vilkooks (1970) , as well as the invention of the polymerase chain reaction (PCR) by Carrie Mollies (1987) provided a unique opportunity to study diversity of organisms at the DNA level is possible with the use of enzyme reaction (PCR) is a method in which DNA polymerase in vitro at the level of a specific piece of DNA replication is significant . In This system only part of the DNA amplification and the other does not exist in living systems . Such an approach facilitates variation in DNA sequence data, and thus has revolutionized the methods of genetic analysis . These DNA markers can be used without using the polymerase chain reaction .

In this system only effective parts in reproducing DNA segment exist and other parts in live systems don't exist. This method simplify the diversity of DNA repeat and as a result evolutes genetic analysis methods. This set of DNA indicators applied without the use of polymerase chain reaction. Significant group of this set of indicators RFLP[2]or diversity in cut segments and indicator VNTR[3] or different diversityof repetitive case that is comprised based on the difference of normal repeated row in being DNA genome.

The technical indicators and use of radioactive materials is based on the use of synthetic probes [10, 16 , 14].

Williams (1991) convert RFLP markers from the ends of rows issue of DNA genomic parallelism oligonucleotide primers designed based on the results row issue, the Oscar-pointers made and then the primers used for amplification of genomic DNA , and also Mitchell Paran Moore (1993) were also convert RAPD markers to scar markers [11] .

In the second RELF method the segment with Polymorphic place ,reproduced with polymerase chain reaction and use of two special primers designed for this purpose and electrophoresis after enzyme digestive, skeletonmuscle as dominant muscle organ is the zebra muscle connected to skeleton system and their works are holding body weight, also moving and do something by contraction [18].

Considering Mehraban's sheep in meat production and also no molecular researches especially on Myostatin gene and it's rule in meat production, make the aim of this research finding Myostatin gene genotype and allele frequencies in Mehraban's sheep and appoint the polymorphism balance inmehraban'ssheep's.

In present paper we study the connection of outcome genotypes polymorphism Myostatin gene ,phenotype data and body weight on Mehraban's sheep.

2. EXPERIMENTAL SECTION

In this kind of study, such as having a pedigree sheep flock reliable , convenient and recorded a wide distribution range of this race has been surveyed . Cattle population sampled is shown in Table 1 .the sampling Herds studied, was a total of 800 sheep. Random samples taken from 120 sheep and 95 samples of DNA samples extracted and analyzed .During the sampling , sample recording animals including sex, age and live weight of cattle were also performed . Restriction Fragment Length Polymorphism -[2,3] -Variable number of tandem repeats

All blood samples from the jugular vein in the neck and 10 ml using shallowtubes (Venoojekt) 5 ml containing EDTA anticoagulant was prepared immediately after sampling until extracted DNA, the temperature was 4 degrees Celsius .

To perform PCR on DNA, extracted blood samples is required . DNA was extracted from whole blood samples was performed using salt extraction [19].

Table 1 - The demographic composition of cattle sampled

livestock	number of samples take	total number of Mehraban's sheep
ewe	100	**400**
rams	20	**50**
Male and female lambs	-	**350**
Total	120	**800**

Because of the purity of the genomic DNA is studied, so check the quality and quantity of extracted DNA is done because in some studies, genomic DNA of high purity is required . The amount of DNA replication is also very important. To determine the amount of DNA on agars gel electrophoresis and spectrophotometer methods commonly used either of these two methods, shows information about the quality of the extracted DNA [20] .

DNA samples with DNA of phage λ DNA as determined by standard concentration 1 mg / ul was digested with Hind III enzyme on agars gel 0.8 is the percentage of electrophoresis .When samples make distance of 4-5 CM electrophoresis suspend and after coloration gel with ethidium bromide the gel under ultraviolet surveyed and the severity of connected bands to samples must compared to connected band λ .

The quality of the DNA bands was found with SEE .

DNA samples were stored at - 20 ° C was used for PCR . Materials needed for the PCR reaction are shown in Table 2

. Based on the number of samples needed materials were provided . For your convenience, the original mix was prepared containing DNTP, ddH2O, Buffer, MgCl2 and the enzyme DNA polymerase . Materials were mixed in a laminar hood under sterile micro tubes .

The scale of master mix[4] for one reaction was 35 micro liter that was divided in each special micro tube then DNA samples and primers were adding to it. For controlling the activity accuracy one control sample have regarded that in this sample in place of DNA,5 micro liter distilled water were used.

Master mix

Table 2 - Material Requirements for polymerase chain reaction (PCR)

Densityrequired	Response parts
1 X	**PCR buffer**
mM4	**$MgCl_2$**
uM0/25	**Primer F**
uM0/25	**Primer R**
uM200	**dNTPs**
Unit/reac0/5	**Taq**
Ng/reac150	**DNA**
-	**Distilled water**

Primers used in this study was synthesized by ferments in Germany . Primer sequences used in this study were as follows :

Forward: 5'-CCGGAGAGACTTTGG GCTTGA -3 '

Reverse: 5'-TCATGAGCACCCACAGCGGTC -3 '

In this study, for all positions of a standard PCR was used to heat the program is shown in Table 3.

Table 3 - Thermal Cycle PCR reactions

row	PCR processes	Temperature**(C°)**	**time**
1	**competence**	**95**	**4´**
2	**competence**	**95**	**30´**
3	Connecting primer	**-**	**30´**
4	Expansion	**72**	**45´**
5	Final extension	**72**	**5´**
6	Maintenance	**4**	Pause

Steps 2 to 4 were repeated 35 times for all positions - the fusing temperature of any place is different .

The PCR products from 8% polyacrylamide gels for electrophoresis of PCR products competence was used for each gel and prepared from a solution of 40 cc.

Loading and electrophoresis of PCR products

After installing electrophoresis gel buffer tank, top and bottom of the device (X 5/0 TBE) is filled . Then, using a syringe containing buffer tank washing wells so as to be prepared for the PCR products for loading . After PCR tube containing the PCR products using the ratio of loading buffer 2.5 to 1. (7 micro liters to 15 micro liters of loading buffer and the mixture is added to the PCR product) was obtained , for samples for 2.5 minutes heated in temperature of 95 ° C is inside thermo cycler to be competence. Then the sample is loaded into each well .

Loading Buffer

At the end connecting wire between electrical resource and electrophoresis is connected and allow the samples to move on gel after adjusting the voltage.

In this research, Voltage 100 for 19 hour were used for electrophoresis ofproducts . After electrophoresis , the gel is prepared for staining and staining done by using ethidium bromide.staining withethidium bromide caution is one gram of powder in 100 ml of distilled water and sealed in a container and hold in special place in darkness . The 10 ml of this solution (with a concentration mg/ml10) can be diluted in 1 liter of distilled water in a closed basin , away from the light can be used for DNA staining .

The polymerase chain reaction products using enzyme Hae III for 12 h at 37 ° C. digestion was used . Shear enzymes derived from bacteria are able to identify specific regions of the DNA and cutting it in different ways .

Enzyme used for restriction enzyme Hae III for this position is able to identify and cut at the (AG / CT) position. Polymorphic position , size and details bands digested by restriction enzyme Alu1 are presented in Table 3-2 . There is a special buffer for restriction enzyme . For restriction enzyme Hae III, R is the buffer containing (BSA b mg / ml 0.1, 10 Mm Tris-HCIPH = 8.5, 10 Mm Mgcl_2, 100 Mm KCl). Most enzymatic digestion were obtained at 37 ° C for 12 - 16 h. enzyme digest fragments by PCR amplification of the following substances in micro tubes 0/2 ml were collected . quantities and concentrations used for enzymatic digestion (Table 3-2) are presented.

Table 3-4 - polymorphic position , size and details bands digested by restriction enzyme Hae III

polymorphic place	polymorphic position	Band size(BP)	Polymorphism standing position as polymorphic bands (BP) of digested fragments
Myostatin	Exon3	337	MM: 337 Mm: 337, 131, 123, 83 mm: 131, 123, 83

Restrition Enzyme

Table 3-5 - Appropriate amounts of material to digest theMyostatin position

PCR reaction product	8 micro liters
10X buffer supplied by the enzyme	2 micro liters
Distilled water	9/4 micro liters
Enzyme Hae III	0/6 micro liters
total	20 micro liters

To test the reproducibility of this indicator , 20 random DNA samples were used to genotype were genotyped again . All samples tested showed the same genotype previously . Chi-square test for Hardy - Weinberg equilibrium in the study population were performed using Pop Genesoftware . Fixed effects influencing variables and the method of least squares analysis using the GLM procedure of the software (9.1) SAS was performed .

RESULTS AND DISCUSSION

Figure 1 shows bands on agars gel electrophoresis of extracted 5/2 % DNA.Extracted DNA were not the same on quantity and quality level that after loading samples in agars gel in some samples band sharpen and in other samples were band were observed. Samples showed weak bands extracted again, perhaps the cause of DNA extracted difference are the pipes containing EDTA and the kind of syringe for taking blood samples and maybe because of the of EDTA used in depriving clod were not enough.

It can also be caused by lack of protein and RNA samples .sharp bands are due to high concentrations DNA extraction . Purity of DNA extracted spectrophotometer was confirmed by the results of the DNA by agars gel . OD280/OD260 than extracted DNA samples ranging from 1.8 to 2.0 were too high , resulting in pure DNA and RNA or proteins lacking .

Figure 2 show PCR products on agars gelelectrophoresis 2/5 %. Fragment of 337 BP is seen in this image . Molecular weight standards used alongside the PCR products confirmed the authenticity of the amplified fragment

In order to detect the digestion pattern of MSTN gene fragment of 337 BPit was digested with HaeIIIenzyme . A point mutation in Exxon 3 occurred due to restriction enzymes HaeIII cut and not detecting the enzymatic digestion of different genotypes provided. Enzymatic digestion of the volume of 20 ml of buffer and temperature conditions was performed using the restriction enzyme HaeIII . When enzymatic digestion products of PCR, the heterozygous nature (Mm) Parts 337 , 131 , 123 and 83 BP is obtained and the wild homozygous (mm) of parts 131 , 123 and 83 BP is obtained , while the homozygous mutant (MM) remains intact fragment of 337 BP in Figure 3.

Genotype frequencies are given in Table 4 . Genotypes mm, Mm , and MM , respectively, with a frequency of 0.947 , 0.053 and 0.000 were observed inMehraban's sheep . As with most genotype frequencies,mm genotype were observed to have more frequencies . The results ofDooraket.al (2002) on theKaroolyas cattle breed correspond but conflicts with the results of Weller et al (2001) in Pydmantscattle breed . it is Due to the high incidence of double muscle phenotype in cattle Pydmants. The results of this study with results Belzhyn Blue cattle breed , the double muscle phenotype that is observed frequently contradicts [21]. For alleles M and m , respectively, 0/026 and 0/974 in the Mehraban's sheep was calculated (Table 4).

Table 4 : Prevalence and genotypes of MSTN gene inMehraban's sheep

genotype	observed frequencies	Allele frequencies	
		m	M
mm	0/947	0/974	0/026
Mm	0/053		
MM	0/0		

Mm is the most frequent genotype heterozygous for the allele M can only reach this allele frequencies to 0/ 026 (Fig. 4).

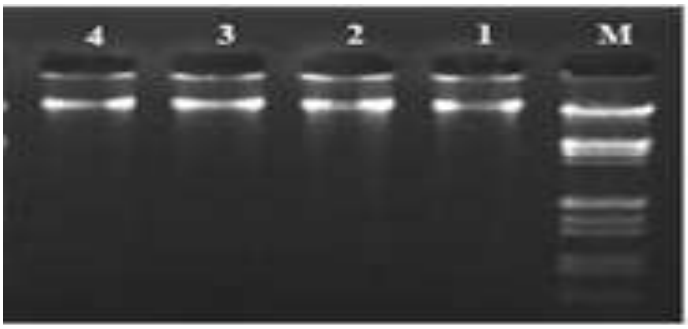

Figure 1 - bands on agars gel electrophoresis of DNA extracted 2/5 %..

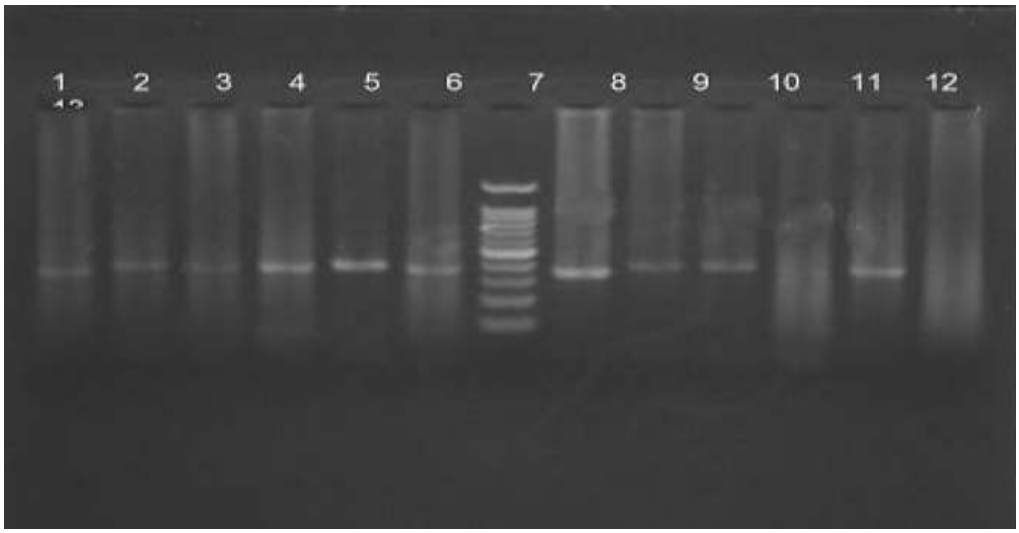

Figure 2 - MSTN gene amplification BP 337 in Mehraban's sheep

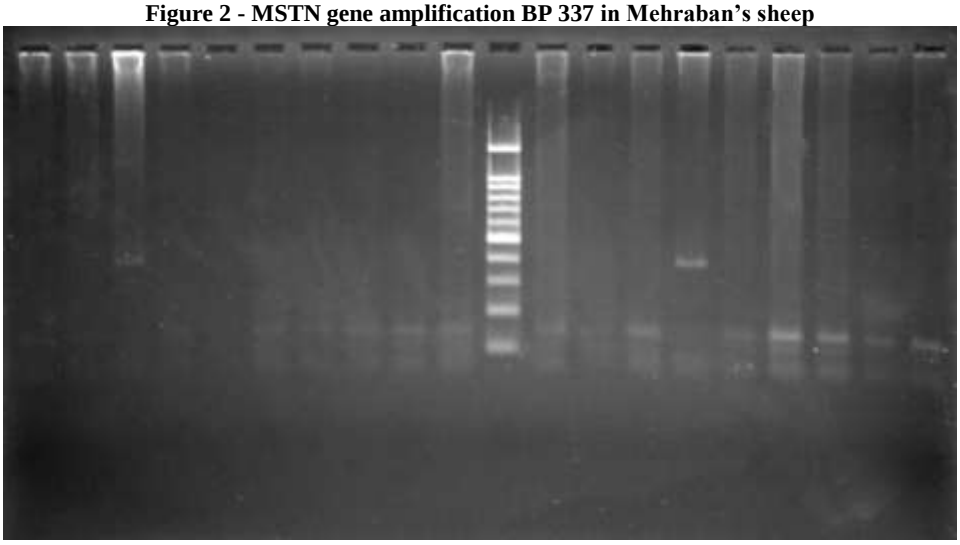

Figure 3 - Results of digestion with restriction enzyme HaeIII digestion products by electrophoresis on agars gel (2/5 %).

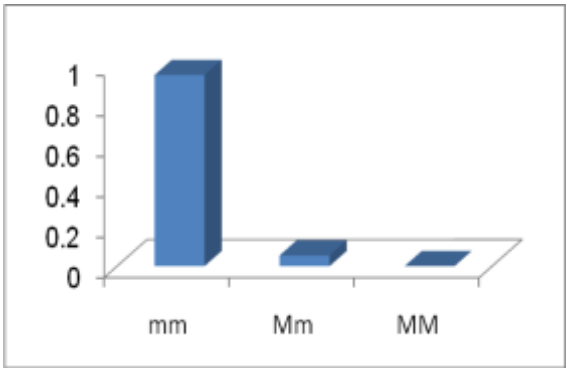

Figure 4 - MSTN gene genotype frequencies observed in the study population

Table 5 shows Chi test in the population studied . Chi-square value is calculated as the square of the table (P= 0.05)which is smaller, so the Hardy-Weinberg equilibrium in the study population is not established . Imbalance in the equilibrium position , possibly indicating the presence of some disruptive factors , including migration and selection. However, the sample size can also be effective .

It should be mentioned that the Hardy-Weinberg equilibrium for MSTN gene in this population was at odds with the results reported by other researchers .Farhadiyan et al 2011, in a study on Makui'ssheep in intron 1 were performed on 417 pieces ,deviation from Hardy - Weinberg equilibrium in this population were reported .

In a study on 337 slices of Exxon 3 was performed in sheep Dalagh All samples were mono- morph (Azari et al 2012). In other research on the breed Zell took by Dehnaviet al polymorphisms in intron 2 and Exxon 3 polymorphism in intron 1 was reported mono absence of Hardy-Weinberg equilibrium and was observed in this population .

Sophie et al also study on chipmunk sheep shows allele m have the most frequency 0.97 and allele m have the low frequency and chi-square showed that Hardy-Weinberg balance in this population for Myostatin gene place is not established.

To investigate the diversity within the population or MSTN gene diversity in the study population , estimating the

observed heterozygosis (H_0) and expected heterozygosis (H_e) found that the results are presented in Table 6 . It is one of the important indicators of the level of heterozygosis and genetic diversity in the population

As can be seen , the observed heterozygosis in 0.052 of the lower amount rather than the amount of observed homozygosis was concluded that MSTN gene variation in the population is low.

The estimated number of alleles and effective alleles are presented in Table 7 . Results showed that the number of effective alleles is less than the number of alleles .number of alleles becomes effective only when all alleles have the same frequency but in most cases less effective allele number of alleles observed.

Also according to the value obtained for the population studied Fis(-0.027) , the population of inbreeding is not alarming

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Table 5 -chi test in the study population

genotype	The observed frequency	The expected frequency	chi-square test
MM	0	0.053	
Mm	5	4.894	P< 0.8142
mm	90	90.053	

Table 6 - Estimates of heterozygosis and homozygosis in the population studied

gene place	**samples**	observed heterozygosis	observed heterozygosis	expected heterozygosis	expected heterozygosis
Myostatin	95	0/9474	0/052	0/9485	0/051

Table 7 - Number of effective allele and Shannon index

place gene	**number of samples**	number of observed alleles	effective number of alleles	Shannon index
Myostatin	95	20000	1.054	0.1217

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4. CONCLUSIONS

Larger populations are needed to be applied to the same study results with more confidence in the selection of animals used .Closer scrutiny of the SNP polymorphism to be used. It should be considered that we only study one mutation that cause double muscels, thus for assuring the conclusion all the mutations must studied. Regarding the quantity characteristics by estimating many genes with low effects and also opposite impression controlled inadequate desirable allele frequencies at one locos is not due to unfavorable work of one breed other characteristics.So check the status of other gene place is required.

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