



Original Article

Isolation and Culture of Fibroblast Cells of Capra Aegagrus Hircus in Central India

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ABSTRACT

Background: Sirohi (Capra hircus) is an important dual-purpose (milk and meat) goat breed, found in the Sirohi, Ajmer, Udaipur, Rajsamand and Bhilwara districts of Rajasthan (India). Genetic profiling is important for the verification and characterization of Sirohi goat breed.

Materials and Methods: This study aimed to extract high-quality genomic DNA from blood samples, demonstrating the importance of a reliable method for successful implementation of genetic resourcing methods.

Observation and Results: Genomic DNA was isolated from fresh blood samples and quality was assessed using agarose gel electrophoresis. The study aimed to identify the housekeeping gene (GAPDH) in the Sirohi breed of goat blood DNA. The isolated gene was confirmed using gel electrophoresis and PCR amplification, resulting in a single clear band of 156bp on a 2% agarose gel.

Conclusion: The current work effectively showed that high-quality genomic DNA could be isolated and characterized from Sirohi breed goat blood samples. Agarose gel electrophoresis verified the isolated DNA's purity and integrity by displaying clear, unbroken bands.

Keywords: Sirohi (Capra hircus), DNA extraction, whole blood, housekeeping gene (GAPDH).

INTRODUCTION

Genetic profiling of Sirohi goat (Capra hircus) is very important for the verification and characterization of goat breed. It's additionally very important for identification of the genetic variability between totally different goats supported their DNA profile for the conservation of its valuable genetic resources. Genetic science incorporates a key role to play in serving to animal farming to meet the world's growing demand for goats and to boost the performance of distributed goats. Goat genetic resources additionally facilitate to determine the productivity of goat populations and their ability to environmental stresses, like temperature change and human development. A necessity step in making a genetic profile and also the foremost step involves in biology is that the ability to isolate a decent quantity of prime quality genomic DNA from goats. Goats genetic profiling needs a reliable supply of biological material.

Molecular genetic characterization-

About 12 alleles were ascertained for the locus OarAE101 all told the eight goat breeds, of that Barbari and Marwari showed ten alleles every. At this locus, the very best genetic variability was ascertained in Barbari, Marwari and Sirohi goats. The locus OarHH56 showed a complete of fourteen alleles all told the analysed samples, during which Jakhrana goats showed 12 alleles and the highest genetic variability was ascertained in Jakhrana, Sirohi and Pashmina goats during this locus. Thus BM4621 marker exhibited the very best gene diversity in Black geographical area, Barbari and Jamunapari goats as compared with alternative 2 markers indicating the simplest appropriate marker for fecundity traits of Indian goat breeds. Beside this, NBAGR, Karnal, Haryana conjointly started molecular characterization work on completely different Indian goat breeds using microsatellite markers (Dixit et al., 2008, 2010, 2011; Kumar et al., 2009; Mishra et al., 2012,

2013). In our study we are using Sirohi goat (*Capra hircus*) blood for Molecular genetic characterization.

DNA isolation from blood sample

Deoxyribonucleic acid (DNA) isolation is an extraction process of DNA from different sources. Strategies utilized to isolate DNA are subordinate on the source, age, and measure of the sample. In spite of the wide assortment of strategies utilized, there are a few likenesses among them. Sources for DNA isolation are exceptionally different. Fundamentally it can be isolated from any living or dead organism. Common sources for DNA isolation is whole blood. Blood is the most common tissue use to isolate the DNA, therefore many methods, available to isolate DNA from different body fluids, and tissues, a new method for isolation to allow for the high amount of quantity, and purity of genomic DNA. This is most significant in livestock genetic research, sufficient amount of DNA was obtained from the whole blood samples and several reports were already made (Chacon-Cortes et al., 2012; Ghaheri et al., 2016). Nonetheless, the extraction of total genomic DNA from these sources is troublesome. Blood contains high concentrations of proteins like heme, electrolytes, enzyme inhibitors which may interfere with the standard of DNA for downstream analysis. Although there are several established protocols which may with success isolate quality DNA like phenol-chloroform methodology and salting-out methodology. On the opposite hand, the toxic nature of phenol and chloroform it remains a most vital concern whereas it is used for DNA extraction caution ought to be taken wearing the gloves and nose mask. The current technique we tend to applied regular Phenol: Chloroform: Isoamyl alcohol and differing kinds of detergents technique utilize. It's frequently used for extraction of genomic DNA from blood samples and additionally used various sorts of biological samples (Goldenberger et al., 1995; Hassani and Khan, 2015; Murray et al., 2016; Qamar et al., 2017). Various ways have been delineated for the isolation of genomic DNA from tissue and peripheral blood. A number of these ways need great amount of samples and aren't appropriate for micro isolation. Further, these ways square measure lit bit overpriced and need specific reagents whose preparation is time intense. In this order to beat these issues, some works are used laundry detergent. To evaluate the probability of DNA integrity, quality of DNA isolated was assessed by agarose gel electrophoresis.

Polymerase chain reaction (PCR) & GAPDH gene

Molecular methods are wide wont to study the genetic variance among livestock. Polymerase chain reaction (PCR) technique is widely wont to study the specific genetic material (DNA) in an exceedingly controlled and exponent fashion. The polymerase chain reaction (PCR) may be a sensitive technique by that one DNA molecule will serve as a templet for amplification (Azevedo et al. 2003). The polymerase chain reaction (PCR) is a technique that enables the amplification of specific sequences of nucleic acids. Although invented by Kary Mullis¹ and described originally by Saiki et al, ² the principle had been described over a decade earlier by Khorana and colleagues, ³⁴ and has received widespread application in many diverse fields such as forensic science, histopathology, and prenatal diagnostics. A variant of this technique is real-time quantitative PCR (qRT-PCR) that permits quantification of a selected region of DNA. Quantitative real-time PCR (qPCR) technique is taken into account to be the foremost correct and reliable methodology for gene expression analysis. It's the benefits of sensitivity, real time detection of reaction progress, speed of research and precise quantification of the fabric within the sample (Gachon et al. 2004). The qPCR may be a time period process and also the accuracy of obtained results depends on many factors together with the standard, stability and input of RNA, the potency of reverse transcription, primer performance, reference genes, PCR steps and methodology chosen for knowledge analysis (Bustin, 2002; Bustin and Nolan, 2004; Pfaffl, 2001; Skern et al. 2005; Fleige and Pfaffl, 2006; Derveaux et al. 2010). Among them, the selection of appropriate reference genes to normalize knowledge may be a nice importance to get correct results. An appropriate reference gene ought to be expressed at a continuing level among samples, and its expression is assumed to be unaffected by the experimental conditions (Bustin, 2002). The employment of unsuitable reference genes might result in errors in quantification and, then, the expression knowledge might result in misunderstanding. Reference gene validation was disbursed in several organs of dairy farm and beef cattle, like animal tissue (Saremi et al. 2012), liver, kidney, pituitary and thyroid (Lisowski et al. 2008), milk corporeal cells (Varshney et al. 2012; Verbeke et al. 2015), mammary gland (Bionaz and Looor, 2007; Bougarn et al. 2011), gametocyte (Macabelli et al. 2014; Mahdipour et al. 2015) and blood samples of goat. Reverse transcription quantitative real-time PCR (RT-qPCR) may be a speedy and powerful technique for the examination of the expression levels of transcripts. Relative quantification by RT-qPCR can confirm if the transcript level changes in given samples relative to manage samples (Chen et al. 2015). The accuracy of RT-qPCR mostly depends on the soundness of the reference gene for normalization, that permits for the elimination of potential variants in RNA quality (Huggett et al. 2005), transcription activity (Vandesompele et al. 2002), PCR potency (Rekawiecki et al. 2012), and run-to-run variation throughout time period experimental processes (Zeng et al. 2016). The properties of the PCR technique are usually employed in the quantitative analysis of the transcript level. However, the extent of target gene expression in dynamic conditions shows genetic instability and forces the choice of the simplest reference gene during a specific experimental model to validate the experiment by mistreatment connected expression of target gene (Reboucas et al., 2013). Candidate reference genes ought to be elite on the premise of the various biological functions they play, biological pathways they're concerned in and expression abundance levels they show. The expression of the Housekeeping genes (HKGs) looks to be the best management. The most frequently used reference genes embrace glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In our work we also amplified GAPDH gene with the help of PCR amplification. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is one amongst the vital glycolytic catalyst that has tremendous role not solely in supermolecule metabolism but conjointly in transcriptional activation, cell death initiation, ER to Golgi sac shuttling, and axoplasmic transport (Tarze et al., 2007). Due to its glycolytic and anti apoptotic result, this catalyst is over expressed and completely

related in progression of assorted tumours in human likewise as in animals (Ramos et al., 2015). Furthermore GAPDH enzyme is employed as a novel therapeutic target to regulate the tumoregenesis by exploiting the protecting power towards the telomere shortening (Phadke et al., 2011). It's been shown that several neurodegenerative diseases in animals are connected to the interaction of GAPDH with alternative proteins (Allen et al., 2012). Moreover, this enzyme has conjointly vital role in inducing mitochondrial elimination underneath aerophilic stress (Qvitet al., 2016), DNA repair (Kosova et al., 2017) and also the development and progression of diabetic retinopathy in human likewise as animals (Kanwar and Kowluru, 2009). Furthermore, thanks to its constant expression within all mammalian cells, GAPDH sequence is taken into account as housekeeping sequence, used as internal relevancy normalize the target genes in numerous molecular techniques like immunoblotting, assay and real time PCR (Greer et al., 2009). Due to on top of importance of GAPDH enzyme in animals, this enzyme must be characterised with respect to its structural and physiochemical properties at molecular level for higher understanding throughout drug development. Roger et al. (1996) showed a proof by phylogenetic analysis of genes encoding GAPDH for the Heterolobosea. So GAPDH will be used as a candidate gene for the establishment of organic process relationship at intervals mammalian species. With this in mind, within the gift study, the GAPDH genes of various class species with additional emphasis on the domestic species (cattle, water buffalo, sheep, goat, pig, and dog) as compared to GAPDH gene of human were analysed with relevancy identity, similarity, physiochemical properties, secondary and tertiary structure through in silico platform. What is more, phylogenetic analysis was done among eighteen species to expand the information regarding evolutionary relationships of the gene.

Hence, this study aimed to isolate the genomic DNA from blood samples of the Sirohi goat (*Capra hircus*), with the help of standard DNA isolation protocols. Here we also discussed about GAPDH gene of Sirohi goat (*Capra hircus*) which is amplified by PCR amplification.

AIM & OBJECTIVE

DNA isolation and gel electrophoresis from Sirohi Goat (*Capra hircus*) blood.

PCR amplification of GAPDH gene of Sirohi Goat (*Capra hircus*).

REVIEW OF LITERATURES

Genotyping Studies on Goats

In the genome research of goats, Vaiman et al. (1996) reported a first genetic linkage map of the goat genome, which was established by using the cattle and sheep microsatellites. This map consisted of 219 markers, of which 204 markers were incorporated into linkage groups and 15 were mapped by the fluorescence in situ hybridization (FISH). The study showed that there are 55 polymorphic markers can be used and defined a panel of 223 microsatellites which is suitable for goat. Besides that, a linkage map of goat genome was also generated. Pariset et al. (2009) showed the recent evolutionary history of domestic goats by using 26 single nucleotide polymorphism (SNP) for a total of 12896 genotyped assayed, which revealed that the breeds were not similar in terms of genetic variability. Besides that, the Mantel test also showed that there were correlation between genetic diversity and geographic distance.

Luikart et al., (1999) carried out a study about the parentage testing in goats by using power of 22 micro satellite markers in fluorescent mUltiplexes. In this case, the multiplexes provide a very high power of individual identification as the probability of finding 1.10¹⁵ in two identical genotypes for the 22 loci is smaller than 1 in each breeds (Mongolian Native Cashmere, Turkish Angora, Swiss Saanen and Spanish Murciana Grenadina). This research is very useful for the study of population structure, history and diversity in goats. The variability in genetic makeup of sirohi goats still because the completely different management leads to variation in milk yield. In step with Kumar et al., (2010), genotyping studies on sirohi goats has been dispensed to evaluate the genetic variability in exon-I of the ex lactalbumin milk gene exploitation the non-radioactive PCR Single-strand Conformation Polymorphism (PCR-SSCP) technique on molecular marker-assisted choice for improvement in milk yield and quality in Indian goat genetic resources.

There is a substantial interest within the application of molecular genetic technologies within the form of specific DNA markers that are related to various QTL to promote more efficient and comparatively easy selection and breeding of domestic animals with a bonus for the inheritable traits of rate of growth, weight, carcass merit, feed intake furthermore as milk yield and composition (Spelman and Bovenhuis, 1998; Othman et al., 2011).

Sirohi is a large size breed and is best known for meat and milk production, and it is resistant to a number of diseases. a-LA (LAL) locus exhibited a monomorphic pattern in all the Indian goat breeds except Sirohi and Jakhrana. CSN2 O (null allele) was observed in Barbari, Sirohi, and Local up goats; the frequency was highest in Sirohi. AAA haplotype had the highest frequency in all the analyzed genetic groups followed by AOA, AAB, and FAA. AOA haplotype was observed in Barbari, Jamunapari, and Sirohi goats and the frequency ranged from 0.174 to 0.257. (P. K. Rout, A. Kumar, A. Mandal, D. Laloe, S. K. Singh & R. Roy 2010).

Parameters Influencing DNA Isolation

Lysis Buffer

Cell disruption is the preliminary step in DNA isolation protocol. Inaccessibility of cells to lysis forces will result in reduced

yield and reproducibility. (Ginny et al. , 1999) The cells are disrupted by adding lysis buffer which will destabilizes the cell membrane and causes the breakdown of cellular structure so that the DNA will be released from the nucleus. Lysis buffer consists of Tris-HCl pH 8.5, ethylenediaminetetraacetic acid (EDT A) and Sodium Dodecyl Sulfate (SDS) detergent. (Hogan et al. ,1994)

Proteinase K

Proteinase K is a type of endolytic enzyme which is isolated from the saprophytic fungus *Tritirachium album*. It has high stability in wide range of pH and temperature but denatures above 65°C. Proteinase K has high activity in the presence of SDS and EDT A whereby SDS can enhance the activity of Proteinase K by unfolding the proteins substrates causing them become more easily to be degraded. The incubation temperature of 55°C is to increase the activity of the enzyme. (Kieleczawa, 2006)

Incubation Time and Temperature

The incubation of samples with lysis buffer and Proteinase K is a phase in which the cell lysis take place. The incubation time is highly depends on the cell type of the specimens used. The presence of cell wall or peptidoglycan require more time for lysis reaction. The optimization of this parameter is essential because if the incubation time is too long, it will lead to partial degradation of DNA. (Khosravinia et al. , 2007) On the other hand, short incubation time will causes incomplete lysis of the cell membrane and restrain the release of DNA from the nucleus resulting in significantly low yield of DNA obtained. (Khosravinia et al., 1997) The optimal incubation temperature for Proteinase K lies within the range of 55°C to 65°C. Temperature above 65°C will causes denaturation and inactivation of Proteinase K. Hence, incubation temperature is an important factor in affecting DNA isolation.

Mammalian Blood Components

Blood could be a specialised humor that delivers essential substances like nutrients and oxygen to the body's cells and transports waste merchandise aloof from the cells. Mammalian blood contains inorganic parts as well as the most important electrolytes metallic element, chloride, and potassium ions. Plasma contains soluble proteins, the foremost exuberant of that is serum albumin. different plasma proteins embody immunoglobulins, fibrinogen, and natural action factors. The blood cells present in blood are in the main red blood cells that conjointly known as erythrocytes. There is only tiny concentration of nucleate white blood cells, as well as leukocytes and platelets present in mammalian blood. (Taylor et al., 1997)

UV Spectrophotometer Assay

UV-visible spectrophotometry is an analytical technique widely used in the quality control of drugs, being present in official monographs, for identification and quantitation. According to William et al., (2007), DNA absorbs lightweight maximally at 260nm. This feature is utilized to estimate the number of extracted DNA by measuring a range of wavelength from 220nm to 300nm. It may be accustomed assess the number of carbohydrates that has maximum absorbance at 230nm and protein at 280nm that will have co-extracted with the sample.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) refers to an in vitro enzymatic DNA amplification strategy of producing millions of copies of DNA within a short period of time. A PCR technique was developed by Kary Mullis in 1985. Since then, PCR was applied extensively within the field of research project and bioscience. The appliance of PCR include the generation of hybridization probes for southern or northern blotting and DNA cloning which needs great amount of DNA. Since PCR allows the generation of large amount of DNA samples, it's been extensively changed to perform a large array of genetic manipulations, diagnostic tests, and for several alternative uses. (Dennis et al., 2006). It involves a repetitive cycles of template denaturation, primer annealing and the extension of the annealed primer. The denaturation of desoxyribonucleic acid samples into ssDNA was done in high temperature of about 94°C. Next, the hybridization of the oligonucleotides to the targeted desoxyribonucleic acid template was aided by lowering the reaction temperature to about 54°C. The last step that is the extension happens once the reaction temperature was enlarged to 72°C thereby speed up the method of synthesizing cDNA strand of targeted DNA sequence with the addition of dNTPs catalysed by Taq polymerase in the 5'-3' direction forming double stranded copies of desoxyribonucleic acid. In the next cycle, newly synthesised DNA molecules can become the guide desoxyribonucleic acid for subsequent reaction. (Dennis et al., 2006)

METHODOLOGY AND MATERIALS

Location of work

The research work was carried out at the Animal Biotechnology Centre, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh.

Genetic stock

The study was conducted on Sirohi breeds of goat. Blood samples were obtained from Animal Biotechnology center NDVSU, Jabalpur (MP).

Molecular biology chemicals

The chemicals used for present study were purchased mostly from Sigma Aldrich, USA; Hi Media Ltd, India; Merck, USA; MBI Fermentas, Germany; Genei, Bangalore, India and Origene Technologies, USA etc. Detailed list of chemicals along with resource of purchase used in the present study are listed in the table 01.

Table 01: List of chemicals and source of manufacturer

Chemicals/ Kits	Manufacturer
100bp DNA molecular marker, 1kb DNA molecular weight marker, dNTP mixture	MBI Fermentas, GERMANY
Agarose, EDTA, Glucose, Sodium bicarbonate, Tris base, Taq DNA polymerase, Sodium chloride, Sodium bisphosphate, Calcium chloride, Magnesium sulphate	Sigma Chemicals, USA
Chloroform, Ethanol, Hydrochloric acid, Isopropanol, Isoamyl alcohol, Sodium carbonate, Sodium hydroxide, Sodium chloride, Boric acid	Merck Mumbai, INDIA
Bromophenol blue, Ethidium bromide, Magnesium chloride, Potassium chloride, Tris HCL, Nonidet- P40	Hi Media, INDIA
2X PCR master mix.	ABI, USA

● Glassware's and plastic wares

The glass-wares used during the study were procured from Borosil, India. These were thoroughly washed and sterilized as per standard procedure. All the plastic wares used in this study including centrifuge tubes, eppendorf tubes, micropipette tips, PCR tubes and filters, were procured from different reputed firms viz. Corning (USA), Axygen (USA), Tarson (India), Thermo (India), Nalgene and Nichipet (Japan).

● Buffers and reagents

The details of buffers and reagents used in this study are given in the Appendix.

Table 02: List of main instruments used during the research programme

Name of the instruments	Source
PCR machine (Thermal cycler)	ABI, USA
Gel documentation system	Vilber Lourmat, Germany
Electrophoresis station	Bio-Rad Laboratories, USA
Laminar flow cabinet	Streamline Lab, Singapore
Nanodrop ND-2000 spectrophotometer V3.5	Thermo Scientifics, USA
Thermo-mixer	Eppendorf, USA

METHODOLOGY

● Collection of blood samples

Total five ml of goat venous blood kept in EDTA coated vacutainer tube having anticoagulant were collected from Animal Biotechnology Center.

● DNA extraction from blood samples

DNA was extracted from blood samples and was carried out by John et al. (1991) method with minor modifications.

● Procedure of DNA extraction

All the DNA samples were extracted by the method of John et al. (1991) with some modification.

- 1) For DNA isolation 5 ml of blood was taken along with 5 ml solution-1 and 120 µl Nonidet -P40 in a 15 ml tube to lyse the cells. Tube was inverted several times (15 min.) for proper mixing and centrifuged at 1500 g for 10 min. Details of reagents for DNA extraction are given in Tables 03 and 04.
- 2) The supernatant was discarded and the pellet was resuspended gently in 400 µl of solution-2 for lysis of nuclei. After mixing properly, equal volume of saturated phenol (400 µl) was added in suspension and centrifuged at 11,000 g for 1 min at 24°C.
- 3) The upper phase was transferred to a clean micro centrifuge tube and equal volume (400 µl) of saturated phenol: chlorophorm: isoamyl alcohol (25:24:1) was added. The tube was centrifuged at 11,000 g for 1 min. at 24°C, after which upper phase was transferred to another micro centrifuge tube.
- 4) Equal volume (400 µl) of chlorophorm: isoamyl alcohol (24:1) was added. The tube was centrifuged at 11,000 g for 1 min. at 24°C and then upper phase was transferred to another micro centrifuge tube.
- 5) After that contents of the tube were mixed gently and centrifuged at 11,000 g for 10-15 min at 4°C to 8°C to form DNA pellet.
- 6) The isopropanol was discarded carefully and the pellet was washed with 70 per cent ethanol for 3 times and the pellet

was air-dried for 1-1.5 hours.

- 7) After drying completely, the pellet was resuspended carefully in 50 µl of nuclease free water (Sigma) and incubated at 56°C for 1 hour in water bath.
- 8) DNA samples were then stored at -20°C for further use.

Table 03: Composition of solution 1

S.No.	Composition of solution 1		
1.	Tris	0.303 gm	(10 mM)
2.	Potassium chloride (KCl)	0.186 gm	(10 mM)
3.	Magnesium chloride (MgCl ₂)	0.230 gm	(10 mM)
Volume made to 250 ml with distilled water. Autoclaved and stored at room temperature. pH - 7.6			

Table 04: Composition of solution 2

S.No.	Composition of solution 2		
1.	Tris	0.061 gm	(10 mM)
2.	Potassium chloride (KCl)	0.031 gm	(10 mM)
3.	Magnesium chloride (MgCl ₂)	0.048 gm	(10 mM)
4.	Sodium chloride (NaCl)	1.461 gm	(0.5 M)
5.	Ethylene Diamine Tetra acetic acid (EDTA)	0.037 gm	(2 mM)
Volume made to 50 ml with distilled water. Autoclaved and 0.250 gm SDS was added. pH - 7.6			

Concentration, purity and quality check of genomic DNA

The concentration, purity and integrity of genomic DNA were checked by UV spectrophotometer and agarose gel electrophoresis.

Spectrophotometry

The concentration and purity of all the DNA samples were checked by taking optical densities (OD) at 260 nm and 280 nm by using UV-spectrophotometer (Nanodrop 2000, Thermo Scientific, USA). DNA samples with an OD ratio of 260/280 equal to 1.7 to 1.9 were considered good. They were subjected to agarose gel electrophoresis for integrity check.

Agarose gel electrophoresis

Agarose was melted in 0.5X TBE (pH 8.0) buffer to make 0.8 percent solution. After cooling upto 60°C Ethidium bromide (1per cent) was added @ of 5 µl /100 ml of gel solution. The gel was poured into caster assembly after placing comb properly to formed wells. After setting of gel the wells were carefully charged with 2.0 µl DNA mixed with 1.0 µl 6X gel loading dye. Electrophoresis was carried out at 80V for about 60 min. On completion of electrophoresis, the gel was visualized under UV transilluminator (Gel documentation system) for judging the quality & purity of the genomic DNA. DNA was appeared as single compact bright fluorescent band free from degradation (whole lane fluorescent) / RNA contamination (fluorescent spot migrated away from well).

Table 05: Chemicals used for gel electrophoresis

(A) 10X TBE, pH 8.0	0.9M Tris HCl 0.9M Boric acid 20 mM EDTA
(B) Gel loading dye (6X)	0.25 per cent bromophenol blue 0.25 per cent Xylene cyanol-FF 15 per cent Ficoll Stored at room temperature
(C) Ethidium bromide (1%)	10 mg ethidium bromide, 1.0 ml distilled water

Amplification of GAPDH gene

● Primer dilution

The published primer was synthesized from Integrated DNA technology. Primers were dissolved in nuclease free sigma water and were further reconstituted in nuclease free sigma water to give a final concentration of 10 pmol/µl. PCR condition for amplification was optimized and standardized.

Table 06: Primer detail used for PCR

Primers	Sequences (5'-3')	Primer size	Amplification size	Reference
GAPDH (F)	AGATGGTGAAGGTCGGA GTG	20	156	XM_088626
GAPDH (R)	CGTGGGTGGAATCATACT GG	20		

● PCR reaction mixture

PCR amplification of GAPDH gene was carried out in a final reaction volume of 25 µl. A master mix for desired number of samples was prepared and aliquoted 24 µl in each PCR tube. 1 µl genomic DNA (50 ng/µl) was added in each tube to make the final volume of 25 µl. (Details of PCR reaction components are given in table 7).

A negative control containing all the reaction components except the template DNA was also made to check any contamination of the foreign DNA in the reaction components.

Table 07: PCR Components used for each reaction volume

S.No.	PCR component	Volume	Final concentration
1.	2X PCR master mix	12.5 µl	1X
2.	Deionised water	7.5 µl	
3.	Forward primer (10 pmole/µl)	2.0 µl	0.4 pmole
4.	Reverse primer(10 pmole/µl)	2.0 µl	0.4 pmole
5.	Genomic DNA (50 ng/µl)	1.0 µl	2 ng/ µl
	Total	25.0 µl	

● Setting of PCR reaction

The PCR tubes were kept in a pre-programmed thermo cycler (Veriti 96 well thermal cycler, ABI, USA) and set the standardized reaction programmes given in table 08.

Table 08: PCR programme for GAPDH gene

S.No.	Steps	Temperature	Time
1.	Initial denaturation	94°C	5 min
2.	Denaturation	94°C	30 secs
3.	Annealing	58°C	30 secs
4.	Extension	72°C	30 secs
Repeat steps 2 to 4 for 35 times			
5.	Final Extension	72°C	10 min

Checking of the amplified product by agarose gel electrophoresis

For confirmation of PCR amplification, agarose gel electrophoresis was done by running 5 µl of PCR product mixed with 1 µl of 6X gel loading dye from each tube along with 100 bp DNA ladder (Gene Ruler Fermentas) on 1.8 per cent agarose gel at a constant voltage of 80-90 V for 60 min in 0.5X TBE buffer. Ethidium bromide was incorporated in 100 ml of agarose gel @ 5 µl of 1 per cent solution. The amplified product was visualized as a single compact fluorescent band of expected size under UV light and documented by gel documentation system.

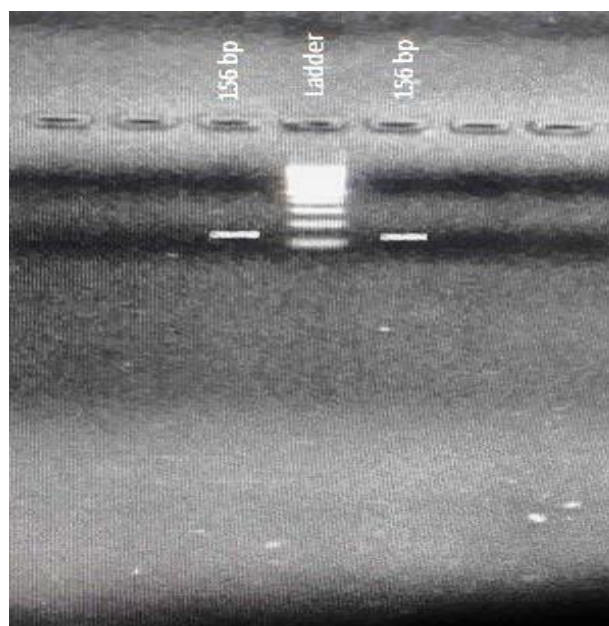
RESULTS

In the present study an attempt was made to identify the housekeeping gene (GAPDH) present in the blood DNA sample of Sirohi breed of goat. Isolated gene was further confirmed by gel electrophoresis, compared with 100bp ladder simultaneously run with the targeted gene.

DNA extraction, Quality and Quantity Analysis

Genomic DNA isolated from fresh blood samples. All the samples yielded appreciable amount of DNA. Quality of isolated genomic DNA samples was assessed by agarose gel electrophoresis (0.8%). All the samples isolated showed clear and distinct bands of genomic DNA under UV transilluminator indicating its good quality (Plate 01).

Purity and concentration of genomic DNA was determined by ND2000 spectrophotometer (Nanodrop InC. USA). DNA samples with O.D.260/O.D.280 ratio ranging between 1.7 to 1.9 indicating high purity and absence of contamination of proteins and other impurities. The genomic DNA concentration ranged from 250-900 ng/µl and diluted upto 50 ng/µl with nuclease free.



PCR optimization

PCR of genomic DNA requires optimization of several parameters. The crucial chemical variables (template DNA, primers, thermostable DNA polymerase, MgCl₂ and de-oxynucleotides) and annealing temperature decide the net synthesis of product during thermal cycling. Hence, efforts to get the possible amplification, optimization of reaction conditions were done by using different combinations of reaction mixture and different annealing temperatures. The reaction combination giving the best result was chosen in the present study (Table 07). Most important step in optimization of PCR programme was the optimization of annealing temperature because it depends on the length and GC content of the primers. Annealing temperature was tested from 58° C for GAPDH gene but consistent results were obtained at

PCR amplification for GAPDH gene

GAPDH gene consists of a total size of 156 bp was the target sequence obtained from the sample. The PCR amplification was carried out by using the published primer sequence as reported by Zhang et al. (2011). PCR amplified products of GAPDH gene were run on 2% agarose gel and found a single clear band of 156bp. (plate 1)

DISCUSSION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a widely used as a housekeeping gene is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme that catalyzes the sixth step of glycolysis, a process in which glucose is converted into pyruvate. Its role in glycolysis is not its only function, since it still participates in DNA repair (Meyer-Siegler et al. 1991) and transcriptional regulation (Sirover 2005). In this study isolated and amplified GAPDH gene from goat blood samples was found similar in all samples having non identifiable difference between them. In gene identification sequence several studies has incorporated with different types of tissue samples used for isolation and amplification of GAPDH gene. These were used as a biomarker for observation of several changes involved in a variety metabolic regulator and has been shown of cellular processes like DNA repair, membrane fusion, and cell death (Winzi et. al. 2018). GAPDH used as one of the most common reference genes, often used to normalize the gene expression data, being used as an endogenous control in the quantitative analysis of RT-PCR, since in some experimental systems, its expression is very constant (Edwards and Denhardt 1985; Winer et al. 1999). This study was carried out with GAPDH gene however some other housekeeping genes like β -ACTIN, β -TUBULIN, UBIQUITINA (UBQ), RNA RIBOSOMAL 18 S - RNAr 18 S, PHOSPHOGLYCERATE KINASE-PGK, RIBOSOMAL PROTEIN-RPL etc. are also the most widely used genes for normalization in the experiments of gene expression (Emanuela et al 2013). The concentrations of GAPDH may vary between different individuals (Bustin and Mckay 1999), for example, during the pregnancy (Cale et al. 1997), with the development stage (Puissant et al. 1994; Calvo et al. 1997) and during the cell cycle (Mansur et al. 1993). According to Robert et al. (2005), studies with qRT-PCR determined the expression of 1,718 genes using GAPDH as reference gene in 72 kinds of normal human tissues. The use of GAPDH in the studies with clinical samples of cervical human tissues HPV-positive appeared as a reliable strategy for the normalization in qRT-PCR using the program Norm Finder (Shen et al. 2010). On the other hand, GAPDH was not suitable as an internal control in rabbit tissues (Deindl et al. 2002) and in nucleated human blood cells (Bustin et al. 1999).

In bovine oocytes, GAPDH was selected as reference gene because its expression showed stability (Van Tol et al. 2007); in zebrafish embryos, the GAPDH gene was considered less stable Lin et al. 2009). In fibroblast cell lineages of mice under different treatment conditions with antidepressants, the levels of GAPDH were found unstable (Sugden et al. 2010).

CONCLUSION

Our study was concluded as non-significant differences found within Sirohi goat breed and no such differences were observed within group. To increase the growth and survival of *Capra aegagrus hircus* fibroblasts, more study is required to optimize the culture conditions and media.

Examining the patterns of gene expression in cultivated fibroblasts can reveal information about their biology and its uses. All things considered, our findings show the media-dependent variation in Fibroblast in vitro dynamics and culture properties, which could be helpful in formulating plans to effectively cultivate these cells for study and subsequent uses.

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