ASSOCIATION BETWEEN POLYMORPHISMS OF CYP19 GENES AND REPRODUCTIVE TRAITS IN SAHIWAL CATTLE*

Amitosh Kumar[1], G.C. Gahlot[2], Urmila Pannu[3] and Sita Ram Gupta[4]

Molecular Genetics Laboratory, Department of Animal Genetics and Breeding, College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences, Bikaner-334 001, Rajasthan, India

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ABSTRACT

The most common cause of infertility in cattle is anestrum. During late maturity the ovaries are in a state of true anestrum. One of the predominant causes of true anestrum is a low level of ovarian estrogens. The key enzyme in estrogen biosynthesis is cytochrome P450 aromatase, encoded by CYP19 gene. Therefore, the present study was conducted in sahiwal cows to investigate the genetic variation in CYP19 gene through RFLP marker and to observe their association with reproductive trait parameters. Genomic DNA was extracted whole blood of 60 unrelated Sahiwal cows through Spine Column method. The 406 bp fragment of CYP19 was amplified and digested overnight with PVU II restriction Enzyme to explore the genetic Variability in CYP 19 gene. The reproductive traits of selected sahiwal cow were tested for significance of association with genetic marker. All the analyzed samples showed polymorphic patterns. The study concluded that CYP19 gene was polymorphic in Sahiwal Cattle and no association could be established in Sahiwal cows between CYP 19 gene and reproductive trait.

Key words: Sahiwal, CYP 19 Gene, Aromatase cytochrome P450, polymorphism, PCR-RFLP, reproductive gene

Introduction

Genetic improvement of the reproductive traits in dairy cattle is crucial not for ensuring quantitative aspect of production but mainly for the efficient course of lactogenesis and lactopoesis. It is extremely difficult process, regulated at many stage in a multifactorial manner, Moreover, coefficients characterizing reproductive traits are of low heritability and there is a small number of described genetic markers associated with reproduction that could be used as potential factors supporting the classical selection methods. Among indigenous breed, Sahiwal is one of the best milch breed of India (Nivsarkar *et al*., 2000) producing high milk on average of 2585 kg per lactation (Tomar *et al*.,1996). It is comparatively a heavy breed with symmetrical body and loose skin. It is considered as one of the best Zebu milch breed in the tropics with higher disease resistance and heat tolerance properties (Ahmad *et al*., 2000). Among the indigenous cattle Sahiwal, play important role in milk production/economics of Rajasthan.

A good reproductive performance positively affects cattle herd active life and plays an important role in dairy herd economics. Reproduction is influenced by many environmental and genetic components, as are most complex traits. In contrast, long generation interval and the low heritability of reproduction trait can cause limited success in the selection for reproductive traits, such as fertility.

The estrus behavior and productivity in farm animals, are essential regulated by status of ovarian hormones, the important being estrogen. The hormone is essential for the development of dominant follicles in the ovary. A low estrogen level is one of the predominant cause for true anestrum (Hafez and Hafez, 2000). Most notably high estrogens level is found to be a marker in selection of dominant follicle in bovine (Evans *et al*., 2004) buffalo (Onteru *et al*., 2009). Aromatase cytochrome P450, product of the CYP19 gene, is the enzyme responsible for estrogen biosynthesis from androgen precursors. The regulation of aromatase expression and maintenance of 17 β-estrodiol (E_2) levels is one of the prerequisite for follicular maturation. These characteristics make CYP19 a strong candidate gene for reproductive traits. The bovine CYP19 is mapped on chromosomes 10 (Furbass *et al*., 1997). The polymorphic status of CYP 19 gene and their association with AFC, gestation period, service period and calving interval has amply reported in exotic cattle, however, there have been scant studies in Indian cattle.

The identification of specific pattern of allele and genotype frequencies in indigenous cattle breeds and association with reproductive performance may result in detection of causal factors responsible for variation in performance for reproductive traits. Restriction fragment length polymorphisms (RFLP's) is one of the most abundant type ideal polymorphic genetic marker for detection of genetic variation in polygenetic traits (Jalving *et al*., 2004)

Therefore, the present investigation was undertaken to with an objective to estimate the gene and genotypic frequency of CYP 19 gene in Sahiwal cattle and their association with reproductive traits.

Materials and Methods

Milking Sahiwal cows (N=60) were selected randomly from Livestock Research Station, Kodamdesar, RAJUVAS, Bikaner Rajasthan. The biological material such as blood for analysis was collected from the same animals included in the study in accordance with the standard ethical procedures.The reproductive parameters such as age at first calving, gestation period, service period and calving interval were collected from farm records and were standardized to reduce systematic

*[1]Part of Ph.D Thesis submitted to RAJUVAS, Bikaner by first author and corrosponding author present address: Associate Professor, Department of Animal Genetics and Breeding, Faculty of Veterinary and Animal sciences, RGSC, Barkachha, BHU, Mirzapur (U.P) -231001; email-amitosh@bhu.ac.in; [2]Rtd. Professor RAJUVAS Bikaner; [3]Professor & Head AGB Dept RAJUVAS, Bikaner; [4]Assistant professor Department of Clinical Veterinary Medicine, E & J, CVAS, RAJUVAS, Bikaner.

errors and to make the data set uniform.

About 2 ml of blood was taken from jugular vein in a sterile vacutainer tube containing Ethyene diamine tetraacetic acid (EDTA) as an anticoagulant under strict aseptic conditions from all the selected animals of sahiwal cattle. Genomic DNA from the whole blood sample was extracted through spine column method as per standard method (Sambrook and Russell, 2001) under manufacturer's protocols with slight modification. The quality and quantity of extracted genomic DNA was assessed through Nano-drop spectrophotometer and 0.8% agarose electrophoresis. The well-established 5'-flanking region of CYP19 gene was selected as genetic marker for Sahiwal population study. Species specific primers were used to amplify the selected genomic regions 5'-flanking region of CYP19 gene. The sequences of primers and expected fragment length of different selected region are represented in Table 1.

The selected 405 bp region of the cattle CYP 19 gene was amplified by PCR in a reaction mixture containing 5X PCR buffer (5 µl), 3 µl $MgCl_2$ (25 mM), 1µl of dNTPs (10 mM), 0.5 µl of each forward (10 pmoles/µl), reverse primer (10 pmoles/µl) and 0.25 µl of Taq DNA Polymerase and target DNA (2 µl) was separately added in tube. The PCR programme used for the amplification of CYP19 gene is presented in Table 2.

The quality and size of the PCR amplicons for studied locus was on 1.5 % agrose gel containing ethidium bromide (1% solution) (Fig. 1). The 405 bp amplicons for the respective CYP19 gene were digested with 5 U alu (1 µl) restriction enzyme in a 40 µl reaction mixture containing 10X buffer(5µl) and nuclease free water (35 µl). The reaction mixture was mixed properly through spinning and kept under water bath at 37^{0}C for overnight digestion. The different CYP19 genotype were analyzed as per method of Winter *et al*. (2002) on 8 % polyacrylamide gel. The results of electrophoretic separation were visualized and documented through GelDOC Unit after staining with ethidium bromide dye. The reproductive trait records of selected Sahiwal cows were tested for significance of association with genetic marker information using least square method of SPSS *ver*. 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

Statistical analysis was carried out under single gene model to estimate the effect of genotype of the traits. The following univerate analysis of variance was carried out through general linear modal (GLM) procedure to analyse the differences among genotypes:

$Y_{ij} = \mu + A_i + e_{ij}$

Where,

Y_{ij}= Performance record of the j^{th} progeny of i^{th} genotype

μ = Overall mean

A_i= effect of i^{th} genotype

e_{ij}= Random error NID (0,σ^2).

Results and Discussion

The genetic variation in 5-flank region of CYP19 gene in sahiwal cattle (*Bos indicus*) was reported in the present study. The 405 bp amplified products of CYP 19 gene were digested with *Pvu*II RE enzyme as per the protocol. The *Pvu*II/PCR-RFLP assay revealed three types of banding pattern,one of them was of 405 bp (AA genotype);second of 327 and 78 bp (BB genotype) and heterozygous pattern had 405, 327 and 78 bp bands(AB genotype)in all the 60 animals studied (Fig. 1). This revealed that the Sahiwal Breed of cattle used in the present study were polymorphic in nature with two types of alleles A and B. The AA genotype was the most frequent (48.33%) in all the screened samples, followed by the heterozygote AB (41.66%), whereas the BB genotype was the least frequent (10.0%). The frequency of CYP19/*Pvu*II A and B alleles was 0.69 and 0.31, respectively. The selected population of Sahiwal cattle was found in Hard-Weinberg law Equilibrium.

The present study is in agreement with the findings of Jedrzrjezak *et al*. (2011) in Black and White cattle's, Szatkowska *et al*. (2011) in Polish-Holistein-Friesian, Keskin *et al*. (2015) in Holistein-Friesian. Jadrzejczak *et al*. (2006), reported the no

Table 1: Primer sequences and expected fragment size of PCR products of CYP 19 gene

Selected region	Primer Sequences	Expected Fragment Length	References
CYP 19 region	Forward	405 bp	Jedrzwjczak *et al*. (2011)
	5'- CTCTCGATGAGACAGGCTCC-3		
	Reverse		
	5'- ACAATGCTGGGTTCTGGACT-3'		

Table 2: PCR programming for amplification of CYP19 gene

Steps	Temperature	Time	No of cycle
I. Initial Denaturation	94^{0}c	5 min	1 cycle
II. Cycle			
(i) Denaturation	94^0 c	1 min	
(ii) Annealing	61^0 c	40 sec	35 cycles
(iii) Synthesis	72^{0}c	30 sec	
(III) Final extension	72^{0}c	10 min	
IV. Hold	4^{0}c	5 min	1 cycle

Table 3: Reproductive performance (LSM ± SE) of studied Sahiwal animals

Age at first calving	Gestation period	Service period	Calving interval
1583.49± 62.07	279.9±0.72	287.47±62.73	560.73±64.36

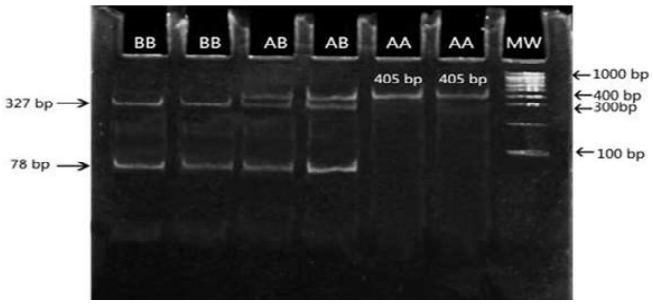

Fig. 1: PCR-RFLP pattern obtained after digestion of amplified CYP 19 gene With *Pvu* II in indigenous Sahiwal cattle. Lane 1-6 (genotypic pattern of Sahiwal cattle), Lane 7 MV (Molecular Weight Marker 100 bp ladder)

polymorphism in the CYP19 gene in Jersey cattle in contrast to present findings where polymorphism was reported in the CYP 19 gene in Sahiwal cattle.

The mean reproductive performance of selected milking animals for reproductive traits was analyzed results are displayed in Table 3. The average age of first calf, gestation period, service period and calving interval were observed to be 1583.49 days, 279.9 days, 287.47 days and 560.73 days, respectively.

Associations between CYP19 variants and reproductive traits have been reported by various authors (Szatkowska *et al*., 2011 and Lobo *et al*., 2009). Szatkowska *et al*. (2011) analyzed the significant association of calving to conception intervals and the average calving intervals in Polish Holstein-Friesian cows. Kowalewska-Luczak (2010) detected polymorphisms in CYP19 gene using PCR-RFLP in Black and White cattle and observed association between CYP19 variants and milk production traits

Variation in CYP19 is reported to be non significantly associated with milk production trait in Jersey (Kowalewska-Luczak *et al*., 2013). Jedrzejczak *et al*. (2011) detected the relationships between the SNPs of CYP19 gene and milk production traits of Black and white cattle. The present study however did not find the association between CYP19 variants with Reproductive traits in Sahiwal cattle.

Further investigations should be conducted with large groups and more phenotypic data to exhibit more realistic results regarding possible associations.

Conclusion

The genomic DNA extracted from 60 healthy Sahiwal cattle and was amplified using primer that showed the polymorphism in CYP19 gene using PCR-RFLP method and PvuII restriction enzyme. It was detected from the blood sample that the A allele was more frequently present than the allele B.

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