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EVALUATION OF LACTATION MILK YIELD AND POLYMORPHISM OF ALPHA-LACTALBUMIN GENE IN CROSSBRED CATTLE OF KERALA

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Thesis submitted in partial fulfilment of the requirement for the degree of

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2007

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DECLARATION

I hereby declare that this thesis, entitled **"EVALUATION OF LACTATION MILK YIELD AND POLYMORPHISM OF α-LACTALBUMIN GENE IN CROSSBRED CATTLE OF KERALA"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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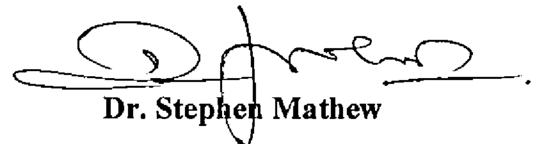

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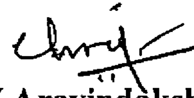

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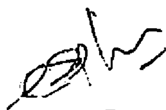

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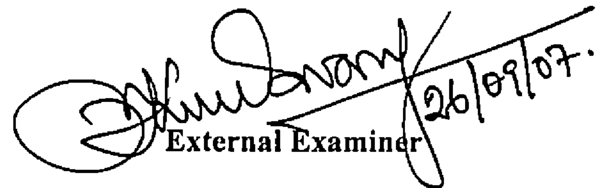

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Reshmi R. Chandran

Dedicated to

My Beloved Parents

and Sister

CONTENTS

Chapter	Title	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	3
3	MATERIALS AND METHODS	15
4	RESULTS	28
5	DISCUSSION	34
6	SUMMARY	40
	REFERENCES	42
	ANNEXURES	
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
2.1	Average 305 day milk yield of crossbred cattle in Kerala	3
2.2	Year wise milk production in Kerala for the period from 1992-1993 to 2004-2005	4
2.3	Gene frequencies of α-lactalbumin gene in various *Bos indicus* breeds	12
3.1	Regression of test day milk yield at fortnightly intervals on 305-day lactation interval and their correlations	17
3.2	Classification of data and no. of observations based on parity	17
3.3	Volume of ingredients for PCR assay	24
3.4	Optimum PCR conditions standardised for α-LA locus in crossbred cattle of Kerala	24
4.1	Average milk yield of crossbred cows from different zones of Kerala	29
4.2	Least squares analysis of variance for zone and parity on 305-day milk yield of crossbred cattle of Kerala.	29
4.3	Zone-wise least squares means of 305-day lactation milk yields of crossbred cattle in Kerala	31
4.4	Parity-wise least squares mean for 305-day milk yield of crossbred cattle in Kerala	31
4.5	Genotype and allele frequencies of α-LA/Bsp12861 polymorphisms in crossbred cattle.	32

LIST OF FIGURES

Figure No.	Title	Between pages
1	The *MnlI* polymorphism of Alpha-lactalbumin gene detected by PCR-RFLP.	30&31
2	The *Bsp* 12861 polymorphism of Alpha-lactalbumin gene detected by PCR-RFLP.	30&31

Introduction

1. INTRODUCTION

In Kerala, even though cattle rearing is considered as a subsidiary occupation, it has demonstrated great potential in sustaining farmers with regular income during climatic situation with adverse effect on agricultural crops as occurred in the recent past. Half of the households in Kerala are engaged in cattle rearing. Thus livestock development plays a vital role in the economic development of Kerala.

Crossbreeding was taken up in 1960's as a tool to increase milk yield of cattle in Kerala. Initially Jersey and Brown Swiss were used for crossbreeding with local cattle. In later part of 1980's, Holstein Friesian breed was introduced. Kerala has got an approved policy for cattle breeding. The continuous crossbreeding programme implemented has resulted in the transformation of more than 80 % of the cattle in Kerala to crossbreds. In the present scenario, cattle in Kerala have a mosaic genetic make up of Jersey, Brown Swiss, Holstein Friesian and local cattle.

Cattle breeding programme is to be evaluated periodically by studying the production performance of the cattle population covering all regions. There have been a number of studies on the production performance of crossbreds in Kerala. (Chacko *et al.*, 1984; Stephen *et al.*, 1985; Iype *et al.*, 1985; Iype *et al.*, 1986; Thomas *et al.*, 1987; Iype *et. al.*, 1993; Iype *et al.*, 1995; Radhika, 1997; Iype and Stephen, 2002; Shaiju *et al.*, 2002; Stephen, 2006). All these studies are mostly limited to certain regions. A comprehensive evaluation covering all regions/zones will be of great value in assessing the progress made and in formulating strategic plans for the future.

To date, most genetic progress for economic traits in livestock has been made by selection on phenotype in conventional breeding programme. During the past decade advances made in molecular genetics has opened new vistas for incorporating information at DNA level in the selection programme. Research

has shown that some genetic variants of specific genes are associated in a positive way with a given trait. It is therefore possible to genotype an animal using a DNA based genotyping test and select individuals carrying the preferred genetic variant. Marker-assisted selection is the process of using the results of DNA testing to assist in the selection of individuals to become parents in the next generation. The genotypic information provided by the DNA testing should help to improve the accuracy of selection and increase the rate of genetic progress by identifying animals carrying desirable genetic variants for a given trait at an earlier age.

Alpha-lactalbumin (α-LA) gene is a promising quantitative trait locus whose polymorphism is greatly associated with milk volume. Its product alpha-lactalbumin is a major whey protein of bovine milk. Within the mammary epithelial cell, α-LA complexes with galactosyl transferases, to form the enzyme lactose synthase. Lactose synthase then synthesizes lactose, the production of which is associated with the movement of large quantities of water into the mammary lumen. Thus α-LA plays a functional role in modulating milk volume. This suggests α-LA as major gene in control of milk volume. The polymorphism can be used as a genetic marker for evaluation of the phenotypic variability. The polymorphism at the α-LA locus in association with the milk volume has been studied in various breeds of cattle.

Realizing the importance of evaluating performance of existing cattle population and discovery of markers linked to economically important traits such as milk production, present study was undertaken with the following objectives.

1. To assess the milk yield of crossbred cattle of Kerala
2. To identify different allelic variants of α-LA gene in crossbred cattle of Kerala.
3. To study the association of different α-LA genotypes with milk production.

Review of Literature

2. REVIEW OF LITERATURE

2.1. MILK YIELD

Milk yield is the most important economic trait and the aim of any dairy cattle improvement programme is to increase the milk production potential of the cows. A number of reports are available on the milk yield of crossbred cattle of Kerala in different periods. The average 305-day milk yield of crossbreds reported from Kerala are summarised in Table 2.1, which ranged from 1140 ± 46 kg (Nair, 1973) to 2502 ± 71.2 kg (Anon, 2000).

Year-wise milk production in Kerala for the period from 1992-1993 to 2004-2005 is given in Table 2.2. (Economic Review, 2005). The milk production of the state was increased from 18.89 to 27.8lakh tonnes in the period from 1992-93 to 2001-02 and thereafter declined to 20.52 in the year 2004-05.

Table 2.1 Average 305 day milk yield of crossbred cattle in Kerala

Breed groups	Average 305 day milk yield (kg)	References
½ Jersey x ½ Non-descript	1140 ± 46	Nair (1973)
½ Jersey x ½ Non-descript	1359 ± 57	Stephen *et al.* (1985)
Jersey x Local	1566.5 ± 101.0	Iype *et al.* (1986)
BrownSwiss crosses	1549 ± 32.8	Chacko *et al.* (1984)
½ BrownSwiss x ½ Non-descript	1482 ± 20	Stephen *et al.* (1985)
BrownSwiss crosses	1445.5 ± 77.9	Iype *et al.* (1985)
BrownSwiss crosses	1513.3 ± 130.2	Thomas *et al.*(1987)
Cows with mosaic inheritance	1479.5 ± 10.3	Iype *et. al.*(1993)
Mosaic inheritance (on the basis of 3633 cows)	1517 ± 12.5	Iype *et al.* (1995)
Crossbred progenies born to test bulls of FPT scheme of KAU	1905.7	Radhika (1997)
Sunandini	2502 ± 71.2	Anon (2000)
Crossbred progenies of test bulls of ICAR-FPT scheme	2069.3	Iype and Stephen (2000)
Crossbred cattle of Kerala	2295.16 ± 6.2	Shyju *et al.* (2002)
Sunandini cows in Thrissur	1965.7 ±12.59	Stephen (2006)

Note : FPT – Field Progeny Testing Scheme

Table 2.2 Year wise milk production in Kerala for the period from 1992-1993 to 2004-2005. (Anon, 2005)

Year	Milk production in Kerala (lakh tones)
1992-93	18.89
1993-94	20.01
1994-95	21.18
1995-96	21.92
1996-97	22.58
1997-98	23.43
1998-99	24.2
1999-2000	25.25
2000-2001	26.05
2001-2002	27.18
2002-2003	24.19
2003-2004	21.11
2004-05	20.52

2.2 NON GENETIC FACTORS AFFECTING MILK YIELD

Non-genetic factors influencing milk yield such as location (zone) and parity were considered for the present study. Important literature related to this aspect are reviewed here.

2.2.1 Location (Zone)

Among the non-genetic factors affecting milk yield of cattle, differences in the location is considered very important. Several investigators reported that different locations influence the lactation milk yield under field conditions of Kerala.

Chacko *et al.* (1984) studied the effects of genetic and environmental factors on first lactation milk records of 1357 animals from 3 units of Progeny testing scheme namely Mavelikkara 1, Mavelikkara 2 in the coastal zone and Kattappana located in the high ranges of Kerala. Of the effects considered, artificial insemination centre, type of dam and sex of calf under the existing management practice contributed maximum to the variation.

Iype *et al.* (1986) reported that farms, years and farm x season interaction had significant influence on milk yield.

Thomas *et al.* (1987) suggested that 50 per cent and 62.5 per cent Brown Swiss crossbreds under field conditions in Kerala did not differ significantly in lactation milk yield, but centre difference was significant.

Iype *et al.* (1993) reported significant influence of centre (location) on milk yield and observed a higher milk-yield in locations close to townships.

Deb *et al.* (1998) reported mean first lactation milk yield of crossbred cattle under different AI centres ranged between 1200.9 kg (Kalketty) to 2407.4 (Nooranad) and also observed that cows raised in areas attached to AI centres under Mavelikkara and Kottayam progeny testing units showed higher first lactation milk yield than those under Kattapana unit.

Rajeev *et al.* (2002) studied 15012 first lactation records of crossbred cattle spread over nine years reared in places attached to 54 AI centres spread

through out Kerala and found that centres had highly significant effect on milk yield.

In the ICAR filed progeny testing scheme implemented by the Centre for Advanced Studies in Animal Genetics and Breeding, Kerala Agricultural University, also revealed that locations have highly significant effect on milk production (Stephen, 2006).

2.2.2 Parity

Reports on parity-wise milk yield of crossbred cattle of Kerala is scarce. For making comparison of milk yield of dairy cows it is necessary to study the influence of parity on milk yield. The following reports on Indian cattle reveal significant influence of parity on milk yield.

Reddy *et al.* (1991) reported that the maximum milk yield in 305-day lactation was found during the third lactation, in case of Deoni x Holstein-Friesian cows.

Kirankumar *et al.* (2003) in his studies on Ongole cattle observed that the lactation milk yield and service period were significantly influenced by the order of lactation. The lactation milk yield which was lowest (472.44 kg) in first lactation, gradually increased and reached the highest average of 639.70 kg in sixth lactation. The lactation milk yield then declined and reached 577.64 kg in ninth lactation.

Kulkarni *et al.* (2003) reported that parity and genetic groups showed significant effect on total lactation milk yield in crosses between Holstein Friesian, Jersey, Brown Swiss X Gir.

Bhadoria *et al.* (2004) studied the effect of parity on total lactation yield, and lactation period on Gir cows and observed that total lactation yield

increased from first to third parity where as lactation period decreased from first to fourth parity.

The total lactation milk yield, 305-day lactation milk yield, milk yield per day of lactation, milk yield per day of calving interval, service period, dry period and calving interval were significantly affected by parity. The least squares means for total lactation milk yield, 305-day lactation milk yield, milk yield per day of lactation increased with increase in parity from first to sixth lactation (Vinod *et al.*, 2005).

2.3 PREDICTION OF MILK YIELD

Milk yield of cows in the field is usually predicted from the test day milk yields at monthly intervals.

Iype (1991) developed regression equations to predict 305-day milk yield from a single day milk yield at different fortnightly intervals and reported very high correlation (0.89 to 0.92) for the regression equation of any/single day milk yield during 7-13 fortnights after calving.

2.4 PCR – RFLP ANALYSIS

Polymerase chain reaction (PCR) technique was invented by Kary Mullis in 1983. The introduction of polymerase chain reaction permitted the amplification of minute amount of DNA. Several research workers have used PCR – RFLP method to detect genetic polymorphism at various loci (Beckmann *et al.*, 1986; Theilman *et al.*, 1989; Hoj *et al.*, 1993; Aravindakshan *et al.*, 2003; Badola *et al.*, 2004).

The thermostable properties of the DNA polymerase activity from *Thermus aquaticus* have contributed greatly to the yield, specificity, automation and utility of the PCR method for amplifying DNA. For most amplification

reactions 1 to 2.5 units of enzyme per 100 μl reaction mixture was recommended (Lawyer *et al.* 1989).

The final concentration of each dNTP in a standard amplification reaction mixture is 200 μM. The optimal concentration of dNTPs depends on several factors including $MgCl_2$ concentration, reaction stringency, primer concentration, length of amplified product and the number of cycles (Eckert and Kunnel, 1990).

2.5 ALPHA-LACTALBUMIN POLYMORPHISM

Much of the genetic progress for quantitative traits in livestock has been made by selection on phenotype or on estimates of breeding values derived from phenotype, without any knowledge of the number of genes that affect the trait or the effects of each gene. But the genetic architecture of quantitative traits can be treated as a 'black box' and its analysis is considered as a powerful tool for genetic improvement of quantitative traits.

A large number of genes affect the traits of economic interest and are collectively known as Quantitative trait loci (QTL). During the past decades advances in molecular genetics have led to the identification of multiple genes or genetic markers associated with quantitative trait loci (QTL). This has enabled opportunities to enhance genetic improvement programs in livestock by direct selection on genes or genomic regions that affect economic traits through marker-assisted selection (MAS). Alpha lactalbumin is a potential gene among the genes in the quantitative trait loci of dairy cattle.

2.5.1 Alpha Lactalbumin

Alpha lactalbumin is a major whey protein of bovine milk. The primary sequence of α-lactalbumin was determined in bovine (Brew. *et al.,* 1970). It consists of 123 aminoacids with a molecular weight of 14175dalton.

Within the mammary epithelial cell, α-lactalbumin complexes with galactosyl transferase form enzyme lactose synthase. Lactose synthase then synthesizes lactose in the golgi apparatus. Lactose is packaged in secretory vesicles of mammary epithelial cell where it acts as a major osmole of milk by draining water into the vesicles. Both α-lactalbumin and lactose are secreted into the milk (Larson, 1985).

Several works have been done on α-lactalbumin present in both human (Rosen *et al.,* 1999; Lein, 2003; Lonnerdale and Lein, 2003; Booij, 2006) and bovine (Yadav and Brew, 1991) milk.

Fitzgerald *et al.* (1970) reported that the physiological action of α-lactalbumin is to lower the K_m of glucose so that it is used maximally for the synthesis of lactose.

Lactose is synthesized in the golgi complex by lactose synthase, a heterodimeric complex composed of α-lactalbumin and β-1,4 galactosyl transferase (Kuhn *et al.* 1980).

The complete aminoacid sequence of α-lactalbumin was determined from a marsupial by Shewale *et al.* (1984).

Yadav and Brew (1991) investigated on the interaction of α-lactalbumin, the lactose synthase regulatory protein with the regions of bovine galactosyl tansferase and reported that the amino terminal region of the catalytic domain of galactosyl transferase, and possibly the part of proline-rich "stem" region, is affected by the association with α-lactalbumin and is therefore implicated in the binding of acceptor substrates.

α-Lactalbumin is produced only in the mammary gland and has no catalytic function itself. However α-lactalbumin binds to β-1,4 galactosyl transferase and reduces the Km of β-1,4 galactosyl transferase for glucose 1000

fold, allowing the production of lactose under physiological conditions (Brew and Grobler, 1992).

Lactose is a major osmole of bovine milk and its production is associated with movement of large quantities of water into mammary lumen (Bleck and Bremel, 1993b).

Boston and Bleck (2001) demonstrated that increasing α-lactalbumin expression in transgenic mice may increase pup growth and milk production marginally.

α-Lactalbumin is the major protein in breast milk and participates in lactose synthesis, thereby creating an osmotic drag to facilitate milk production and secretion. (Lonnerdale and Lien, 2003). It suggests that α-lactalbumin acts as a major regulator in lactose synthesis, which in turn regulates milk volume.

2.5.2 Alpha Lactalbumin Gene

Sequence of gene encoding whey proteins were first reported for rat α-lactalbumin (Qasba and Safaya, 1984). Structure of α-lactalbumin gene is very simple. It is a 2kb transcriptional unit divided into 4 exons and 3 introns (Mercier and Vilottee, 1993). α-lactalbumin loci are situated on the chromosome No. 5 in bovine (Thread grill and Womack, 1990), chromosome No. 3 in ovine (Imam-Ghali *et al.*, 1991), chromosome No. 5 in caprine (Hayes *et al.*, 1993) and chromosome No. .12&13 (Davis *et al.*, 1987) in human genome.

The complete nucleotide sequence of human α-lactalbumin gene and its immediate flanking sequences were determined and compared with those of rat α-lactalbumin gene by Hall *et al.* (1987). The studies showed that a high degree of homology (67 per cent) exists in the 5′ flanking region, extending as far as 655 nucleotide residues upstream of the transcriptional initiation site.

Hurley and Schuler (1987) identified a cDNA clone for bovine α-lactalbumin and suggested that the clone will be a valuable tool for studying the control of α-lactalbumin expression and milk synthesis in mammary gland of economically important species.

The isolation and characterization of a genomic clone encoding goat α-lactalbumin was described and its sequence was compared with that of its bovine counterpart (Vilotte *et al.*, 1990).

The 5′ flanking region of Holstein α-lactalbumin was cloned and sequenced, which contains 1952 bp of the 5′ flanking region and 66 bp of the protein-coding region (Bleck and Bremel, 1993a).

2.5.3 Genetic Polymorphism in Alpha-Lactalbumin Gene

Genetic variants occur as a consequence of mutation that results in change in nucleotide sequence of particular region leading to substitutions or deletions of amino acid in the concerned milk protein (Ng-Kwai Hang, 1998). Different electrophoretic techniques have been employed to identify the genetic variants for several species at protein as well as nucleotide level. The genetic polymorphism in α-lactalbumin gene was first discovered in South African white Fulani (*Bos indicus*) by paper electrophoresis and two genetic variants namely A and B in order of decreasing electrophoretic mobility were observed (Blumberg and Tomb, 1958). B variant is the only α-lactalbumin, present in all western breeds of cattle (*Bos taurus*). Gene frequencies of α-lactalbumin gene in various *Bos indicus* breeds were shown in the Table 2.3.

Table 2.3 Gene frequencies of α-lactalbumin gene in various *Bos indicus* breeds.

Breeds	Gene frequency		References
	Variant A	Variant B	
Hariana	0.22	0.78	Bhattacharya *et al.*, 1963
Sahiwal	0.37	0.63	Bhattacharya *et al.*, 1963
Gir	0.38	0.62	Jeichitra *et al.*, 2003

2.5.4 PCR-RFLP Analysis in Alpha-Lactalbumin Gene

Three single base pair (bp) polymorphisms were identified in Holstein-Friesian within the 5′ flanking region at positions +15, +21 and +54 relatively to the mRNA transcription start point. The +15 and +21 variations were in the zone encoding the 5' untranslated region of the mRNA sequence, while the +54 polymorphism was a silent mutation in the coding region of the gene. The α-lactalbumin (+15) A allele and α-lactalbumin (+15) B allele are characterized respectively by an adenine and a guanine in position +15 (Bleck and Bremel, 1993a).

Mao (1994) reported that an *Mnl*1 Polymerase chain reaction-Restriction fragment length polymorphism exists in the bovine α-lactalbumin gene and detected 3 different patterns after *Mnl*1 digestion. He detected codominant inheritance of the *Mnl*1 digested fragments in four paternal half sib families, including 12 cows and 12 calves.

Voelker *et al.* (1997) found a single base pair difference, located at position –1689 from the transcription start point in Holstein and Brown Swiss breeds. This polymorphism is reported to be a variation of adenine and guanine. An adenine at position -1689 was designated as the A allele, and a guanine at

this position was designated as B allele. The allele frequency for A allele was 63.6 per cent for Holstein and 21.4 per cent for Brown Swiss.

Kazmer *et al.* (2001) found a single base pair (bp) difference at position -1691 relative to mRNA transcription start point in the bovine α-lactalbumin gene with restriction enzyme *Bcg*I and detected the presence of either guanine at position -1691 which agrees with previously published sequence for bovine α-lactalbumin gene or cytosine which represents novel polymorphism.

Bojarojc-Nosowicz *et al.* (2005) detected the polymorphism of α-lactalbumin at position –1689 from the starting point of transcription by PCR-RFLP method using restriction enzyme *Sdu*I in European dairy cattle breed. Adenine at position -1689 was designated as A allele and guanine at the position as B allele.

Kaczmarczyk *et al.* (2005) studied on the polymorphism of α-lactalbumin gene in the –1689 bp point by PCR-RFLP method using enzyme *Sdu*I and its relation with the population and subpopulation of peripheral blood lymphocytes in young heifer naturally infected with Bovine Leukaemia virus.

2.5.5 Polymorphism of Alpha-Lactalbumin Gene Related to Milk Volume

As a component of lactose synthase, an increase in α-lactalbumin expression could lead to an increased formation of the lactose synthase complex and an increase in lactose production (Kuhn *et al.*, 1980).

α-lactalbumin is the most lactation specific of all bovine milk protein and its expression has been used as a lactation specific marker in both mammary explant and cell culture systems (Akers *et al.*, 1988; Vega *et al.*, 1988).

Bleck and Bremel (1993b) reported that animals having the α-lactalbumin (+15) AA genotypes had higher milk production and lower fat and

protein percentages, and the α-LA(+15) **BB** animals have lower milk production and higher fat and protein percentage.

The expression of α-lactalbumin mRNA and protein was shown to increase dramatically at parturition, remain elevated throughout lactation, then increase within two days of the start of involution. (Goodman and Schanbacher 1991; Mao *et al.*, 1991)

The levels of α-lactalbumin expression could be used as an indicator for selection of animals to produce high volume of milk (Bleck and Bremel, 1993b).

Stacey *et al.* (1995) detected that mice carrying the human α-lactalbumin gene in place of the murine α-lactalbumin gene produces more α-lactalbumin and there is evidence of increase in milk volume.

Materials and Methods

3. MATERIALS AND METHODS

3.1. MILK YIELD

3.1.1 Collection of Data

In order to study 305-day lactation milk yield, one-day milk yield of crossbred cattle at a lactation stage of 7-13 fortnights after calving were recorded from four different zones of Kerala. The recorded data were used for the prediction of 305-day milk yield of the animal using regression equation (Iype, 1991). Regression of test day milk yield at fortnightly intervals on 305-day lactation interval and their correlations are given in Table 3.1.

Different zones in this study were southern zone (Thiruvananthapuram, Kollam, Alappuzha, Pathanamthitta), central (Kottayam, Ernakulam, Thrissur, Palakkad), northern (Malappuram, Kozhikode, Kannur, Kasargode) and highlands (Idukki, Wayanad). Five hundred animals selected at random from different centres of each zone were studied. Centre in this study refers to a region coming under one milk co-operative society.

In the southern zone, data were collected from 96 animals reared in the area coming under six different centres in Thiruvananthapuram, 237 animals from eleven centres in Kollam, 118 from five centres in Alappuzha and 49 animals from one centre in Pattananthitta. In the central zone, data were collected from 38 animals from two different centres in Kottayam, 49 from two centres in Ernakulam, 342 from fifteen centres in Thrissur and 72 animals from two centres in Palakkad. Data from northern zone were collected by selecting 40 animals of a single centre in Malappuram, 186 animals of six centres in Kozhikode, 227 animals of four centres in Kannur and 47 animals of a single centre of Kasargode. Data of high-lands constitute 328 animals from seven centres of Idukki and 172 animals from 3 centres in Wayanad. Exact places/centres from where the milk yield of cows where collected are given in Appendix 1.

3.1.2 Analysis of Data

3.1.2.1 Classification of Data

Zone and parity were considered as non-genetic factors on 305-day milk yield and data were classified based on zone and parity.

3.1.2.1.1 Location (Zone)

The animals were grouped into four groups based on location (zone).

3.1.2.1.2 Parity

The animals were grouped into five groups based on their parity. Classification of data based on parity is presented in Table 3.2

Table 3.1 Regression of test day milk yield at fortnightly intervals on 305-day lactation yield and their correlations.

Fortnights	Regression formula	Correlations
7	$254.22+229.87X_7$	0.915
8	$272.41+227.12\ X_8$	0.911
9	$326.94+225.77\ X_9$	0.892
10	$321.13+233.29\ X_{10}$	0.899
11	$327.41+242.60\ X_{11}$	0.914
12	$376.65+241.10\ X_{12}$	0.905
13	$343.71+255.63\ X_{13}$	0.904

Table 3.2. Classification of data and number of observations based on parity

Sl. No.	Parity	No. of observations
1	1	534
2	2	729
3	3	548
4	4	156
5	5 and above	33
Total number of observations		2000

3.1.3. Analytical Methods

Least squares analysis of variance using the following model was employed to detect the effect of zone (location) and parity on lactation milk yield (Harvey, 1986).

$$Y_{ijk} = \mu + Z_i + P_j + E_{ijk}$$

Y_{ijk} = The observation of the kth animal belonging to z^{th} zone, and of pth parity.

μ = over all mean

Z_i = effect of zth zone (z =1to 4)

P_j = effect of pth parity (p = 1 to 5)

E_{ijk} = Random error associated with Y_{ijk}. Random error was assumed to be independently and normally distributed with mean zero and variance σ^2_e.

Duncans multiple range test (DMRT) (Kramer, 1957) was used for comparison of means, in case of significant non-genetic effects.

R_p = Table value (Standardised range test value) X $[0.5 (1/n_1 + 1/n_2) MSE]^{1/2}$

3.1.3.1 Adjustments of Records

For efficient genetic analysis adjustment of data for non-genetic effects was needed. The data was adjusted for significant non-genetic factors.

3.2. DETECTION OF ALPHA-LACTALBUMIN ALLELES

3.2.1 Collection of Blood

Five ml of blood sample was collected from the jugular vein aseptically using EDTA (1mg/ml of blood) as anticoagulant in 15 ml sterile disposable centrifuge tubes, from 100 animals (25 animals selected at random from each zone). The samples were brought in ice to the laboratory and stored at 4°C until processing.

3.2.2 Extraction of Genomic DNA

DNA was extracted from whole blood using the standard phenol chloroform extraction procedure with modifications.

3.2.2.1 Phenol:chloroform extraction procedure

1. To 5 ml blood, double the volume of ice-cold RBC lysis buffer (150 m*M* NH_4Cl, 10 m*M* KCl, 0.1 m*M* EDTA) was added, mixed well and kept on ice with occasional mixing for 10 minutes for complete lysis of red blood cells.

2. The leucocytes were pelleted by centrifuging at 3500 rpm for 15 min. and the supernatant containing lysed RBC's was discarded.

3. The pellets were resuspended in ice-cold RBC lysis buffer and the above two steps were repeated till a clear pellet without any unlysed erythrocytes were obtained.

4. The pellet was then washed twice with 10 ml of Tris buffered saline (TBS-140 m*M* $NaCl_2$, 0.5 m*M* KCl, 0.25 m*M* Tris) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes.

. Resuspended the washed white cell pellet in 5 ml saline EDTA buffer (SE-75 m*M* NaCl, 35 m*M* EDTA) completely by vortexing so that no cell clumps remain. To this mixture 0.25 ml of 20 per cent SDS and 25 μl of Proteinse-K (20 mg/ml) were added, mixed well and incubated at 50°C in water bath with occasional mixing for a minimum of three hours.

i. Cooled the digested samples to room temperature, 300 μl of 5 M NaCl was added and mixed by vortexing. An equal volume of phenol (pH 7.8) saturated with Tris-HCl, was added, mixed thoroughly by inversion of the tubes for 10 minutes and centrifuged at 3500 rpm for 15 minutes.

The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added. The contents were mixed thoroughly by inversion for 10 minutes and centrifuged at 3500 rpm for 15 minutes.

'. Collected the aqueous phase in fresh tubes, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed for 10 minutes and centrifuged at 3500 rpm for 15 minutes.

}. The supernatant was transferred to a sterile 50 ml beaker and one-tenth volume of 3 M Sodium acetate (pH 5.5) was added and mixed.

). To this mixture, equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out using a fresh micropipette tip, washed in 70 per cent ethanol and air-dried.

:0. Dried DNA was resuspended in 0.5 ml of Tris buffer (TE-10 m*M* Tris base, 0.1 m*M* EDTA) and stored at –20°C.

3.2.2.2 Agarose Gel Electrophoresis for Checking Quality of Genomic DNA

To determine the quality and molecular weight of DNA samples, approximately 0.5 to 1 µg DNA were checked electrophoretically using 0.7 per cent agarose in 1 X TAE buffer in a horizontal submarine gel electrophoresis unit. The agarose gel electrophoresis was carried out as follows.

1. Washed the appropriate sized gel tray and comb, stick the cello tape on both sides of the tray, place the comb at a level of one cm from the end of the tray and leave it on an even surface.

2. Weighed 0.7 g of agarose and transfered into 250 ml conical flask.

3. Added 100 ml of 1X TAE (prepared by diluting 10 ml of 10 X TAE buffer to100 ml with distilled water), mixed gently and boiled in a microwave oven.

4. Cooled down the agarose to 50°C, added 5µl of ethidium bromide (10mg/ml) and poured carefully on the gel tray avoiding air bubbles and allowed to set the gel.

5. After setting the gel, removed the cello tape from the gel tray and placed it in electrophoresis tank, so that the wells are near the negatively charged electrode.

6. Poured 1 X TAE buffer into the tank till the gel surface is covered with the buffer.

7. Removed the comb carefully from the gel.

8. From DNA stock solution 0.5 to 1µg DNA was mixed with one sixth volume of 6X gel loading buffer and the samples were loaded into the wells carefully.

9. Connected the electrodes. Electrophoresis was carried out at 2V/cm at room temperature until the bromophenol dye migrated more than two third of the length of the gel.

10. After disconnecting the electrodes, removed the tray along with the gel from the electrophoresis tank.

11. The gel was visualized under UV transilluminator.

3.2.3 PCR – RFLP Typing

PCR was done at the α-lactalbumin locus. The PCR amplified product was digested with restriction enzyme to analyse the polymorphic patterns.

3.2.3.1 Template DNA preparation for PCR

Working solutions of DNA samples were prepared from the DNA stock solution by diluting with sterile triple distilled water to get a final concentration of 50 ng/μl and was stored at -20°C. One μl of this working solution was used in every 10 μl PCR reaction.

3.2.3.2 Selection, reconstitution and dilution of primers

The primers for the present study were selected from published reports and were custom synthesized. The sequences of the forward and reverse primers for each locus were as follows.

Locus	Primer sequence (5′-3′)
Ala 1	F 5′-CTCTTCCTGGATGTAAGGCTT-3′
Ala 2	R 5′-AGCCTGGGTGGCATGGAATA- 3′

The primer Ala I (Acession number: X06366) was used to amplify a 429 base pair fragment corresponding to -1951 to -1523 from the α-LA transcription start site.

Locus	Primer sequence (5′-3′)
Ala 3	F 5′- GATCAGTCCTGGGTGGTCATT-3′
Ala 4	R 5′-CAGTGGGTACCCATCCTAAGT-3′

The primer Ala II (Voelker et al., 1997) was used to amplify a 166 base pair fragment corresponding to 657 to 822 of the bovine α-lactalbumin gene.

The primers obtained in lyophilized form were reconstituted in sterile ultra pure distilled water to make a stock solution of 100 pM/μl concentration. The solutions were incubated at room temperature for one hour and then stored at -20°C. Working solutions of the primers were prepared by diluting the stock solution.

3.2.3.3 Setting up of PCR

A master mix was prepared just before setting up the PCR assay combining 10X PCR buffer, 10 m*M* $MgCl_2$, dNTP mix, primer pairs, *Taq* DNA polymerase and sterile water. Volume of ingredients for PCR assay is given in Table 3.3

To each reaction tube 9 μl of master mix and 1 μl of template DNA were added. The tubes were spun briefly and placed in the thermal cycler. One negative control without template DNA was also included to monitor contamination, if any. Optimum PCR conditions standardized for α-LA locus is given in Table 3.4

Table 3.3 Volume of ingredients for PCR assay

Ingredients	Volume	
	Ala I (μl)	Ala II (μl)
10 X PCR buffer	1.0	1.0
dNTP (2000μM/μl)	1.0	1.0
$MgCl_2$ (10 m*M* /μl)	1.5	1.0
Primer Forward (10p*M*/μl)	0.5	0.5
Primer Reverse (10p*M*/μl)	0.5	0.5
Taq DNA polymerase (5IU/μl)	0.1	0.1
Sterile water	4.4	4.9
Template DNA (50 ng/μl)	1	1
Total	10	10

Table 3.4 Optimum PCR conditions standardized for α-LA locus in crossbred cattle of Kerala

Primer Sequences	Initial denaturation	Denatu-ration	Primer annealing	Primer Extension	Number of Cycles	Final extension
Ala I	94°C for 3 min	94°C for 1 min	63.5°C for 1 min	72°C for 1 min	35	7 min at 72°C
Ala II	94°C for 3 min	94°C for 1 min	61.7°C for 1 min	72°C for 1 min	35	7 min at 72°C

3.2.3.4 Checking of Target DNA Amplification

The PCR products were checked by agarose gel electrophoresis to confirm the amplification before analyzing for polymorphism.

Two microlitre aliquots of the PCR products were checked electrophoretically using 1 per cent agarose gel in 1 X TAE buffer using pBR322 DNA/*Hae* III digest as DNA size marker.

3.2.3.5 Analysis of PCR Amplified Products

For restriction digestion, the amplified products were digested in a total volume of 25 µl as follows.

Locus Ala I:

Amplified product (Amplified with Ala I)	10µl
10 X reaction buffer	2.5µl
Enzyme *Mnl*I	0.4µl (2 units)
Distilled water	12.1µl
Total	25µl

The reaction mix was incubated at 37°C for 4 hours.

Locus Ala II:

Amplified product (Amplified with Ala II)	15µl
10 X reaction buffer	2.5µl
Enzyme *Bsp*12861	0.2µl (1unit)
Distilled water	7.3µl
Total	25µl

The reaction mix was incubated at 37°C overnight.

Following the digestion the enzyme was inactivated by incubating the tubes at 65°C for 30 min and the digested products were stored at 4°C till analysed.

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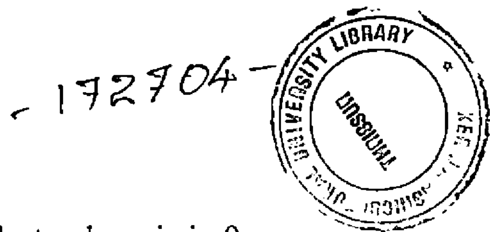


3.2.3.6 Separation of Restriction Fragments

The restricted PCR products were seperated by electrophoresis in 9 per cent and 6 per cent polyacrylamide gels (PAGE) in 1 X TBE buffer for Ala I and Ala II respectively. As DNA size markers pBR 322 DNA/*Hae* III digest and pBR 322 DNA/*Msp*I digest were used.

3.2.3.6.1 Non Denaturing Polyacrylamide Gel Electrophoresis (PAGE) for DNA Samples

Polyacrylamide gel electrophoresis was done following the standard procedure.

1. Cleaned the glass plates of size 18 x 16 cm of the vertical electrophoresis system of suitable dimension with detergent solution and water. Dried it using a clean bloating paper and wiped the inside surfaces with chloroform to remove grease or oils.

2. Assembled the glass plates with Teflon spacers (1.5 mm) in between using cello tape and mounted it over the gel casting assembly.

3. Mixed the components of appropriate per cent non-denaturing polyacrylamide gel. Polymerisation will begin as soon as the TEMED has been added. Without delay swirled the mixture rapidly and poured into a gap between the glass plates. Immediately inserted a clean Teflon comb (1.5 mm thickness) into the gel solution between the glass plates. Placed the gel assembly in a vertical position at room temperature and allowed the gel to polymerise for about 30-45 minutes.

4. After polymerisation is completed (30 min), removed the Teflon comb carefully. Washed the wells immediately with deionised water to remove any unpolymerised acrylamide.

5. Prepared the samples by mixing about 5µl of the digested product with 1µl of the 6X gel loading dye

6. Loaded the samples into the bottom of the wells. Filled the remaining portion of the wells with 1X TBE buffer (prepared by diluting 10 ml of 10 X TBE to 100 ml).

7. Mounted the gel sandwich in the electrophoresis apparatus.

8. Added 1 X TBE buffer to the top and bottom reservoirs

9. Attached the electrophoresis apparatus to the power pack and adjusted the voltage to 100. Electrophoresis was carried until the bromophenol blue reaches the bottom of the gel. Then turned of the power supply.

10. Removed the glass plates from the electrophoresis apparatus and separated the gel from the glass plates.

11. Stained the gel by immersing in 1X TBE buffer containing ethidium bromide (0.5 µg/ml) and allowed staining for 45 minutes.

12. Examined the gel under UV light in a UV transilluminator and analysed the restriction patterns. Documented the gel in a gel document system.

3.2.4 Alpha-lactalbumin Polymorphisms and Milk Yield

The effect of different genotypes of alpha-lactalbumin on 305-day milk yield was analysed using one-way analysis of variance (Snedecor and Cochran, 1967).

Results

4. RESULTS

4.1 MILK-YIELD

The arithmetic mean of 305-day milk yield of crossbred cattle from different zones of Kerala are given in Table 4.1. The over all arithmetic mean of 305-day milk yield of crossbred cows in Kerala was found to be 2406 ± 16.6 kg.

The least squares mean of 305-day milk yield of cattle according to location was 2351 ± 28.9 kg. The highest average 305-day milk yield (2638 ± 40.1 kg) was in southern zone, followed by highlands (2454 ± 38.9) and then by northern zone (2232 ± 39.6 kg) and the least average was observed in central zone (2080 ± 40.6 kg).

The least squares means of 305-day milk yield according to parity was highest in second parity (2525 ± 26.2 kg) and second highest in third (2406 ± 30.2 kg) parity. The lowest least squares mean of 305-day milk yield was for fifth parity (2260 ± 123.0 kg).

4.1.1 Effect of Location (Zone) and Parity on Milk Yield

The least squares analysis of variance for the effect of zone and parity on 305-day milk yield of crossbred cattle of Kerala is presented in Table 4.2. The analysis revealed that different zones and parity exerted significant effect on 305-day milk yield.

The least squares mean of 305-day milk yield of cattle at different locations (Zone) along with Duncan's multiple range tests are given in Table 4.3. Duncan's multiple range test (DMRT) has revealed significant difference between zones.

Table 4.1 Average milk yield of crossbred cows from different zones of Kerala

Zone	No. of observations	305-day milk yield (kg)
South	500	2697 ± 25.8
Central	500	2140 ± 35.2
North	500	2289 ± 28.0
Highlands	500	2499 ± 27.5
Total	2000	2406 ± 16.6

Table 4.2 Least squares analysis of variance for zone and parity on 305-day milk yield of crossbred cattle of Kerala.

Source	D.F.	F	P
Centre	3	60.328	0.00
Parity	4	10.448	0.00
Remainder	1992	-	-
Total	1999	-	-

$P \leq 0.01$

Mean = 2406.12

Error standard deviation = 706.335

Coefficient of variation = 29.36

R squared = 0.264

The least squares mean of 305-day milk yield of cows at different parity along with Duncan's multiple range tests are presented in Table 4.4. The effects of parity were highly significant. Duncan's multiple range test (DMRT) has revealed that second parity is significantly different from all others.

4.2 PCR-RFLP ANALYSIS

4.2.1 Amplification of Alpha-Lactalbumin Gene by PCR

The DNA samples isolated from blood samples of cattle were amplified by PCR at the alpha-lactalbumin locus without any major non specific amplified products. The size of amplified product was 166 bp for Ala I and 429 bp for Ala II.

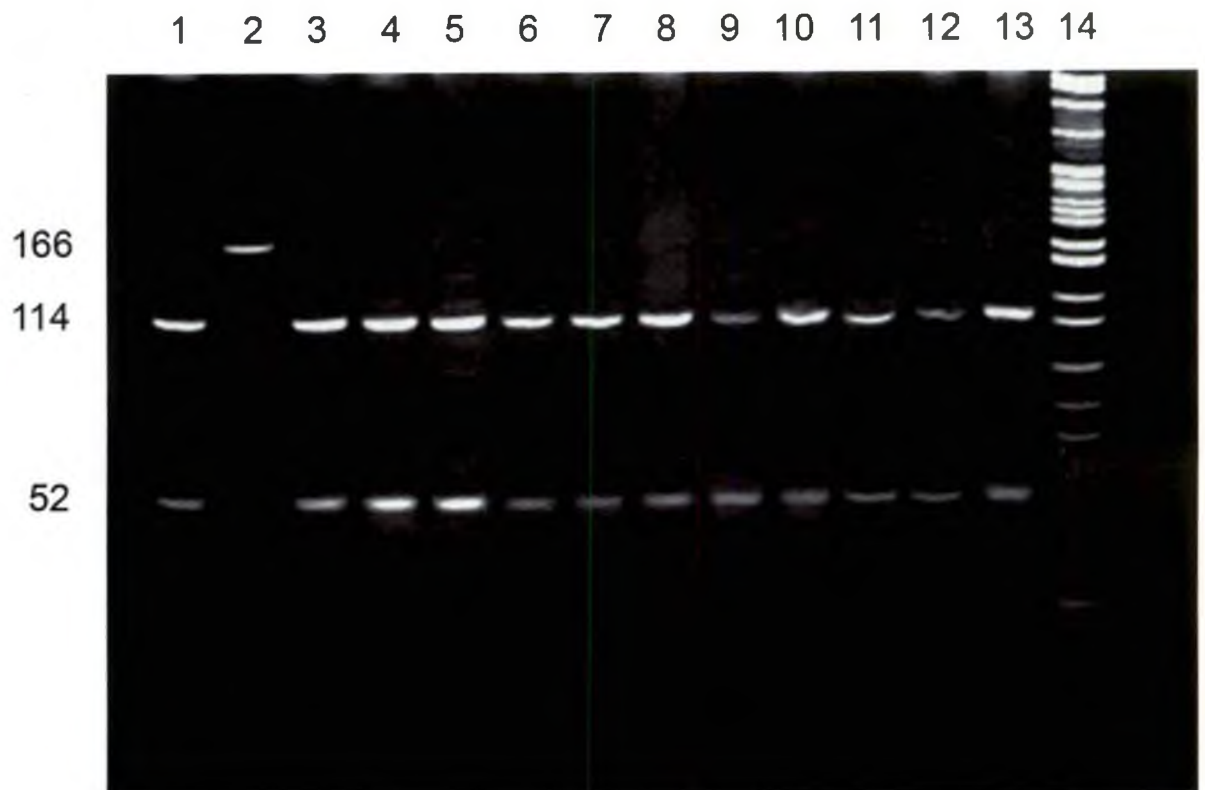
4.2.2 RFLP Analysis

*4.2.2.1 Analysis Using Mnl*1 *Enzyme*

The 166 base pair amplified DNA was digested with restriction enzyme *Mnl*1 and the restriction fragments were resolved by 9% acrylamide gel electrophoresis. All the 95 samples analysed showed same pattern of digestion (114 and 52 bp fragments), indicating absence of any α-LA/*Mnl*1 polymorphism in the population studied (Fig 4.1). The genotype obtained was BB type [α-LA/*Mnl*1 (-/-)].

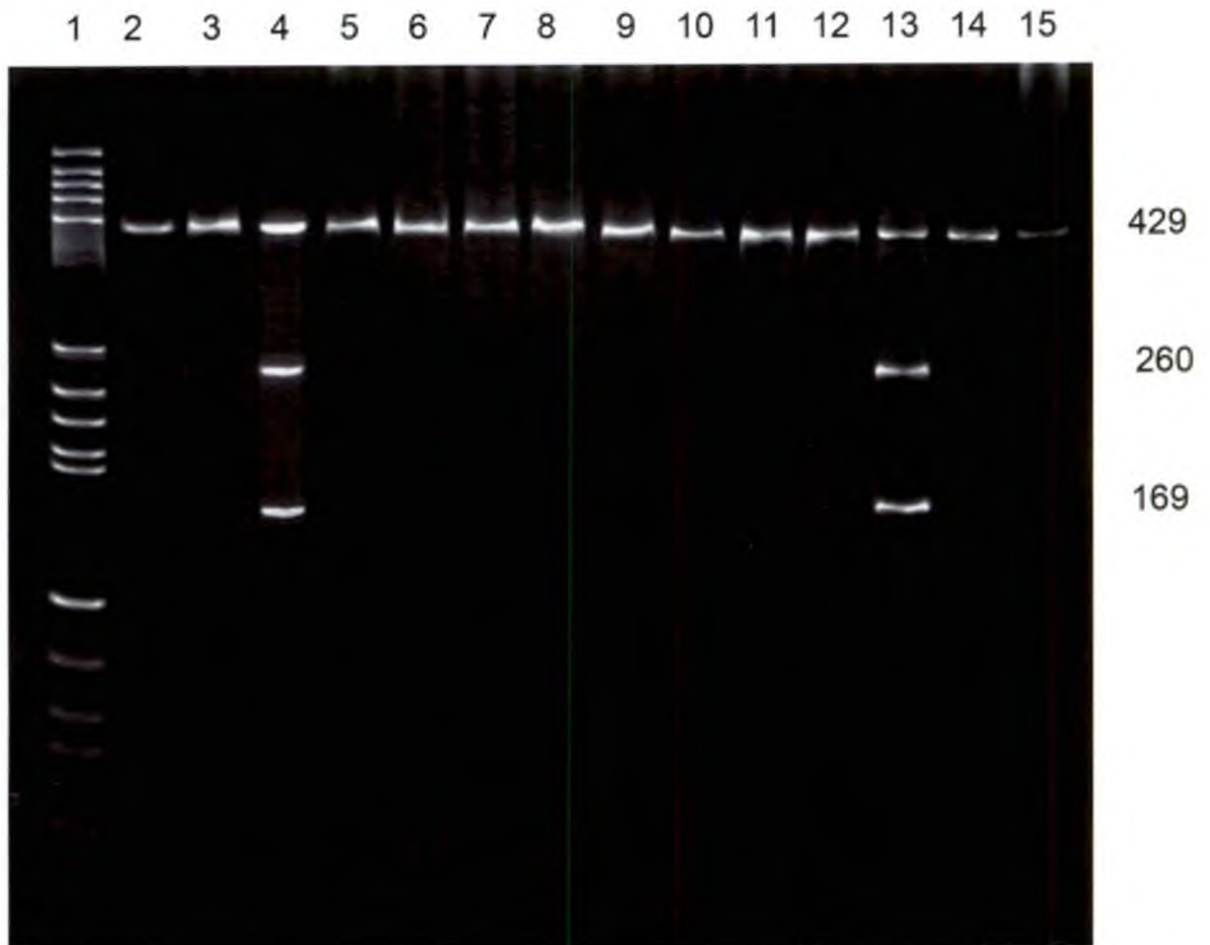
*4.2.2.3 Analysis Using Bsp*12861 *Enzyme*

The 429 base pair amplified DNA was digested with restriction enzyme *Bsp*12861 and the restriction fragments were resolved by 6% acrylamide gel electrophoresis. Upon electrophoresis two restriction digestion patterns could be observed. (Fig 4.2) This allowed the identification of two alleles viz., A allele [α-LA/*Bsp*12861 (-)] (429bp fragment) and B allele [α-LA/*Bsp*12861 (+)] (260 &169 bp fragments). The gene and genotype frequencies of α-LA/*Bsp*12861 polymorphism in the four population groups as well as in the pooled population are presented in Table 4.5.



Lane 2	: 166 base pair (bp) amplified product.
Lane 1, 3-13	: BB [-/- Alpha LA/*Mnl*1] genotype (114 bp and 52 bp fragments)
Lane 14	: Marker - PBR 322DNA/*Msp*1 digest.

Figure 1 : The *Mnl*1 polymorphism of Alpha-Lactalbumin gene detected by PCR-RFLP in 9% non-denatured PAGE.



Lane 1	: Marker-PBR 322 DNA/*Hae*III Digest
Lane 2, 3	: 429 base pair (bp) amplified product.
Lane 4, 13	: AB [+/- Alpha LA/*Bsp* 12861] genotype (429, 260, 169 bp fragments)
Lane 5-12, 14, 15	: AA [-/- Alpha LA /*Bsp* 12861] genotype (429 bp fragment)

FIgure 2 : The *Bsp* 12861 polymorphism of Alpha-Lactalbumin gene detected by PCR-RFLP in 6% non-denatured PAGE.

Table 4.3 Zone-wise least squares means of 305-day lactation milk yields of crossbred cattle in Kerala

Sl. No.	Zone	No. of observations	Mean ± SE kg
1	Southern	500	2638 ± 40.1[a]
2	Central	500	2080 ± 40.6[b]
3	Northern	500	2232 ± 39.6[c]
4	Highlands	500	2454 ± 38.9[d]
5	Total	2000	2351 ± 28.9

Note: Zone-wise mean were highly significant ($P \leq 0.01$)

Means with different superscript are significantly different.

Table 4.4 Parity-wise least squares mean for 305-day milk yield of crossbre cattle in Kerala

Sl No.	Parity	No. of observations	Mean ± SE, kg
1	1	534	2290 ± 30.6[b]
2	2	729	2525 ± 26.2[a]
3	3	548	2406 ± 30.2[b]
4	4	156	2275 ± 56.7[b]
5	5	33	2260 ± 123.0[b]

Note: Parity-wise mean were significant. ($P \leq 0.01$)

Means with different superscripts are significantly different.

Table 4.5 Genotype and allele frequencies of α-LA/Bsp12861 polymorphisms in crossbred cattle

Population	Genotype frequency			Allele frequency	
	(+/+) BB	(-/+) AB	(-/-) AA	(+) B	(-) A
Population 1 (n=25)4	0	0.16 (4)	0.84 (21)	0.08	0.92
Population 2 (n=20)	0	0.15 (3)	0.85 (17)	0.075	0.925
Population 3 (n=25)	0	0.2 (5)	0.8 (20)	0.1	0.9
Population 4 (n=25)	0	0.16 (4)	0.84 (21)	0.08	0.92
Pooled Population (n=95)	0	0.17 (16)	0.83 (79)	0.08	0.92

The frequencies for AA genotype [α-LA/*Bsp*12861(-/-)] and AB genotypes [α-LA/*Bsp*12861(+/-)] in the pooled population were 0.83 & 0.17 respectively. Of the 95 animals typed, none of the animals were of BB genotype. The gene frequencies for A and B alleles in the pooled population were 0.92 & 0.08 respectively.

4.3 POLYMORPHISM OF ALPHA-LACTALBUMIN AND MILK VOLUME

4.3.1 Adjustments of Records

The predicted 305-day milk yield was adjusted for the effect of non-genetic factors. Since zone and parity was found to be significant, the predicted milk yield was adjusted to eliminate the effect of both the factors.

4.3.2 α-Lactalbumin/Mnl1 Polymorphism and Milk Yield

Since only one allele was obtained in this study comparison could not be made to detect the association of α-LA/*Mnl*1 alleles with milk yield.

4.3.3 α-Lactalbumin/Bsp12861 Polymorphism and Milk Yield

The average adjusted milk yield (305-day) detected in this study for the two genetic groups are given below.

Genotype	Milk yield
AB	2363 ± 215.9 kg
AA	2779 ± 298.1kg

Statistical analysis (One Way Analysis of Variance) revealed difference between milk yield of two genetic groups non significant.

Discussion

5. DISCUSSION

5.1 MILK YIELD

The overall arithmetic mean of 305-day milk yield of the crossbred cattle observed in the present study was 2406 ± 16.6 kg. This estimate is in close proximity with the average first lactation 305-day milk yield reported by Sajeevkumar (2005) and the average milk yield, 2502 ± 712 kg, in the annual reports of KLD Board (Anon 2000). Shaiju *et al.* (2002) also had reported the average first lactation milk yield of crossbred cattle of Kerala as 2295± 6.2kg. The present estimate is higher than the reports of Chacko *et al.* (1984) who conducted studies in Mavelikkara (Alappuzha) and Kattappana (Idukki), Stephen *et al.* (1985) who conducted studies in Kattappana (Idukki), Kanjirappally (Kottayam), Chalakkudy (Thrissur) and Mavelikkara (Alappuzha), Iype *et al.* (1985) who conducted studies in Mannuthy, Thiruvazhankunnu (Pallakkad) and Vellayani (Thiruvananthapuram) farms, Radhika (1997) who conducted studies in Mannuthy, Thumburmuzhi (Thrissur) and Thiruvazhamkunnu (Palakkad) farms. As per the annual progress report of ICAR-FPT scheme for the year 2006, the overall average 305-day milk yield of Sunandini cows in Thrissur district was 1965.7 kg (Stephen, 2006).

The least squares mean of 305-day milk yield of cattle at different zones are given in the Table 4.3. Out of the total four zones studied, the highest 305-day milk yield, 2638 ± 40.1 kg, was in southern zone followed by high lands, 2454 ± 38.9 kg. The least average, 2080 ± 40.6 kg, was observed in central zone. The least squares mean of 305-day milk yield of cattle in central zone, 2080 ± 40.6 kg, is in close proximity with the 305-day milk yield, 1965.7 kg, of Sunandini cows in Thrissur district (central zone) reported by Stephen (2006). Since no studies have been conducted earlier taking different zones through out Kerala at a time no literature was available to substantiate the result.

Since no breed variation exists between cattle in different zones, significant variation between zones obtained in the present study might be due to differences in management practices between the zones. Higher milk yield in the southern zone might be due to higher nutritional status of the animals as a result of more concentrate feeding practiced in this area compared to other zones. Second highest milk yield in high-lands might be due to the more amount of green fodder available in Idukki and Wayanad areas.

The aim of cross breeding policy introduced in Kerala was to achieve 305-day milk of 2500 kg/animal by the year 2000. The present study has revealed that only south zone has covered the target milk yield where as high lands and north zone is nearing the target. Central zone is far behind the expected milk yield.

The least squares means for 305-day milk yield of cows at different parity are presented in the Table 4.4. The least squares means for 305-day milk yield according to parity was highest in second parity as 2525 ± 26.2 kg and second highest in third parity as 2406 ± 30.2 kg. The lowest least squares means for 305-day milk yield was for fifth parity, 2260 ± 123.0 kg. In the present study, the least squares mean for 305-day milk yield according to parity increased from first to second parity and then declined to fifth parity. The result obtained is contradictory to the early reports of Kirankumar *et al.* (2003) who reported in Ongole cattle that the lactation milk yield was lowest in first lactation, gradually reached the highest average in sixth lactation and Vinod *et al.* (2005), who reported that 305-day lactation milk yield increased with increase in parity from first to sixth lactation and Reddy *et al.* (1991) reported a maximum milk yield in 305-day lactation during the third lactation in Deoni X Holstein-Friesian cows.

5.1.1 Effect of Location (Zone) and Parity on Milk Yield

The least squares analysis of variance for the effect of zones and parity on 305-day milk yield of crossbred cattle of Kerala is presented in Table 4.2.

The analysis revealed that different zones and parity exerted significant effect on 305-day milk yield.

The result showed that different zones exerted significant effect on 305-day milk yield. Duncan's multiple range test (DMRT) has revealed that each zone is significantly different from each other. This is in agreement with the findings of earlier workers, Deriaz (1981), Chacko *et al.* (1984), Thomas *et al.* (1987) and Deb *et al.* (1998) who conducted studies in crossbred cattle of Kerala, Iype and Stephen (2002) who conducted studies in crossbred cattle of Thrissur area and Rajeev *et al.* (2002) who conducted studies on different progeny testing units of KLD Board.

The effect of parity was highly significant. This is in close agreement with the reports of Rahumathulla (1992), Sreemannarayana *et al.* (1996), Kirankumar *et al.* (2003) and Kulkarni *et al.* (2003), Bhadoria *et al.* (2004) and Vinod *et al.* (2005). Duncan's multiple range test (DMRT) has shown that second parity is significantly different from all others.

5.2 PCR-RFLP TYPING

In this study, PCR-RFLP typing of α-lactalbumin gene was done using two sets of bovine specific primers. The primer sequence of Ala I and Ala II used were obtained from earlier reports of Medrano (2005) and Voelker (1997) respectively.

5.2.1 Amplification of Alpha-Lactalbumin Gene by PCR

When primer set Ala I was used for polymerase chain reaction, an amplified product of size 166 bp was obtained. This is in accordance with the earlier reports of Bleck and Bremel (1993a) and Mao (1994). The amplified product obtained in the case of second set of primers (Ala II), was of size 429 bp as reported by Voelker *et al.*(1997).

5.2.2 RFLP Analysis

5.2.2.1 Analysis Using Mnl1 Enzyme

The amplified DNA was digested with restriction enzyme *Mnl*1 which produced two fragments of size 114 and 52 basepair. All the samples analysed showed same pattern of digestion indicating absence of any α-LA/*Mnl*1 polymorphism in the population studied (Fig 4.1). Bleck and Bremel (1993a) had reported a polymorphic α-LA/*Mnl*1 locus at position +15 relative to mRNA transcription start point in Holstein-Friesian. The polymorphism is considered to be a variation of adenine (+ allele) and guanine (-) allele. However their study could not observe any α-LA/*Mnl*1 polymorphism in Brown Swiss and Jersey breeds. The present result supports this observation.

Present study indicated the absence of A allele [α-LA/*Mnl*1(+)] in the crossbred cattle of Kerala. According to the reports of Iype *et al.* (1993), Jersey, Brownswiss and Holstein bulls were used for crossbreeding local cattle in Kerala. The absence of A allele may be due to allele fixation that had taken place as a result of intense selection at sire level.

5.2.2.2 Analysis Using Bsp12861 Enzyme

The amplified DNA of 429 bp was digested with restriction enzyme *Bsp*12861. Upon electrophoresis two restriction digestion patterns could be observed. (Fig 4.2) These allowed the identification of two alleles viz., A allele [*Bsp*12861(-)] (429bp fragment) and B allele [*Bsp*12861(+)] (260 &169 bp fragments).Voelker *et al.*(1997) reported a polymorphic α-LA/*Bsp*12861 locus at position –1689 relative to mRNA transcription start point that give rise to three patterns of digestion such as 429 bp fragment (AA genotype), 429, 260 and 169 bp fragments (AB genotype) and 260 and 169 bp fragments (BB genotype).

The result obtained showed the existence of A and B alleles. This is in agreement with the findings made by Voelker (1997), but the result indicated the absence of BB genotype in the population studied.

Among the ninety five animals studied, 79 AA and 16 AB genotypes were found giving rise to genotype frequencies of 0.83 and 0.17 respectively. Out of the three possible genotypes AA, AB, and BB, the BB genotype was not observed in the present study. The allele frequencies for A allele and B allele were 0.92 and 0.08. The allele frequency obtained for A allele was higher than the reports of Voelker *et al.* (1997) in Holstein-Friesian and Brown-Swiss and of Bojarojc-Nosowicz *et al.* (2005) in black and white breed cattle.

5.3 POLYMORPHISM OF α-LACTALBUMIN GENE AND MILK YIELD

5.3.1. α-Lactalbumin/*Mnl*1 Polymorphism and Milk Yield

Bleck and Bremel (1993b) reported that A allele is associated with higher milk production and lower fat and protein percentages and B allele is associated with lower milk production and higher fat and protein percentages. The present study indicated an absence of α-LA/*Mnl*1 polymorphism in the crossbred cattle of Kerala. All the samples analysed showed BB genotype. Since only B allele was obtained in this study comparison could not be made to detect the association of α-LA/*Mnl*1 polymorphism with milk yield. Further studies with large population size are needed to make a conclusion about the absence of α-LA/*Mnl*1 polymorphism in the cattle population of Kerala.

5.3.2. α-Lactalbumin/*Bsp*12861 Polymorphism and Milk Yield

The average milk yield obtained in the case of AA genotype (2779 ± 298.1kg) was higher than that of AB genotype (2364.32 ± 215.9 kg). However the statistical analysis revealed non-significant differences between the milk yield of the two genetic groups obtained. Non-significant effect of genotype on milk yield in the present analysis might be due to the small number of

population studied and also due to low number of AB genotypes obtained in this study. Out of the total 95 animals, 79 were AA genotypes and only 16 were of AB genotypes. A study with more number of animals and with comparable number of genotypes is needed to make a conclusion on this aspect. Studies in larger population may also reveal the presence of BB genotype which was not obtained in the present study. No earlier reports could be traced out on the association of α-LA/*Bsp*12861 polymorphism and milk yield.

Summary

6. SUMMARY

The present study was undertaken with the objective of assessing the milk yield of crossbred cattle in Kerala by collecting data from different zones of Kerala and to detect the different α-lactalbumin allelic variants and its association with milk yield in crossbred cattle of Kerala.

The data were collected from four different zones of Kerala, selecting five hundred crossbred cows at random from each zone. One-day milk yield of crossbred animal at a lactation stage of 7-13 fortnights after calving was recorded. The data recorded was used for the prediction of 305-day milk yield of crossbred cattle using the formulae given by Iype (1991).

The predicted lactation milk yield was used for estimating average 305-day milk yield of crossbred cattle in Kerala. The non-genetic factors affecting milk yield considered in this study were zone and parity. The 305-day milk yield obtained was adjusted to remove the effect of significant non-genetic factors. The adjusted data on 305-day milk yield were used to study the association of milk yield with polymorphism of α-lactalbumin gene in crossbred cattle of Kerala.

The overall least squares mean of 305-day milk yield of crossbreds in Kerala was found to be 2351 ± 28.9 kg. Least squares analysis of variance has shown that different zones and parity exerted significant effect on 305-day milk yield.

Highest least squares means for 305-day milk yield, 2638 ± 40.1 kg, was in southern zone, followed by highlands, 2454 ± 38.9 kg, and the lowest was in central zone, 2080 ± 40.6 kg. Duncan's multiple range test (DMRT) has revealed that each zone is significantly different from each other.

The least squares mean for 305-day milk yield according to parity was highest, 2525 ± 26.2 kg, in second parity, followed by third parity,

2406 ± 30.2 kg, then by first parity and the lowest was in fifth parity, 2260 ± 123.0 kg. Duncan's multiple range test (DMRT) has revealed that second parity is significantly different from all others.

Genomic DNA was isolated from crossbred cattle, 25 animals selected at random from each zone. The mean yields of DNA from five ml of whole blood extracted by phenol-chloroform method was 217 ± 13.2 μg.

Two sets of Bovine specific oligonucleotide primers were used for amplification of α-lactalbumin gene at two loci in cattle. Successful amplification of the expected 166 bp and 429 bp fragments were obtained.

Digestion of 166 bp amplified product with *Mnl*1 enzyme revealed same pattern of digestion for all animals studied (114 and 52 bp fragments). This indicated the absence of α-LA/*Mnl*1 polymorphism in the crossbred cattle of Kerala. Since only one allele was obtained in this study, comparison could not be made to detect the association of α-LA/*Mnl*1 alleles with milk yield. The observed α-LA/*Mnl*1 (-) allele is reported to be associated with lower milk yield. Further studies are recommended in large population for confirmation.

Restriction of 429 bp amplified product with *Bsp*12861 enzyme revealed two digestion patterns indicating the presence of two α-LA/*Bsp*12861 alleles. The α-LA/*Bsp*12861 (+) and (-) alleles were obtained with gene frequencies of 0.08 and 0.92 respectively. The frequency of LA/*Bsp*12861 (-/-) and LA/*Bsp*12861 (+/-) genotypes in the pooled population were 0.84 and 0.16 respectively. Out of the 95 animals typed none showed LA/*Bsp*12861 (+/+) genotype. Study on the association of LA/*Bsp*12861 alleles with milk yield in crossbred cattle revealed that the LA/*Bsp*12861 genotypes did not exhibit significant effect on the milk yield of animal. It is suggested to conduct further research in large sample to confirm the findings and to identify other polymorphic loci associated with milk yield.

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Annexures

ANNEXURE 1

LIST OF CENTRES SELECTED FOR THE STUDY

Zone: South

Thiruvananthapuram

Sl.No.	Name of centre	No. of samples
1	Chembakamangalam	24
2	Chittatumukku	10
3	Poojappura	9
4	Peroorkada	20
5	Sreekaryam	9
6	Ulloor	24

Alappuzha

Sl.No.	Name of centre	No. of samples
7	Karuvatta	24
8	Harippad	23
9	Thottapally	24
10	Nangyarkulangara	23
11	Thuravoor	24

Pathanamthitta

Sl.No.	Name of centre	No. of samples
12	Thiruvalla	49

Kollam

Sl.No.	Name of centre	No. of samples
13	Ayoor	24
14	Kanjavelly	26
15	Anchalumoodu	24
16	Eravipuram	24
17	Mangad	4
18	Neendakara	22
19	Kunnicode	24
20	Mulavana	22
21	Paravoor	11
22	Karunagapally	21
23	Thrikkovilvattom	35

Zone: Central

Thrissur

Sl.No.	Name of centre	No. of samples
1	Marottichal	9
2	Pattikad	14
3	Choondal	23
4	Arthat	24
5	Kunnamkulam Municipality & Chowvannur Panchayat	24
6	Porkulam	49
7	Kallampara	49
8	Karikad	6
9	Parakkad	24
10	Eruvathody	22
11	Pallipu	24
12	Panjal	16
13	Thichur	20
14	Thayoor	17
15	Kalappara	20

Palakkad

Sl.No.	Name of centre	No. of samples
16	Vadavannur	48
17	Kottopadam	24

Kottayam

Sl.No.	Name of centre	No. of samples
18	Erattupetta	6
19	Ettumannoor	32

Ernakulam

Sl.No.	Name of centre	No. of samples
20	Edakkatuvayal	23
21	Peppathi	26

Zone: North

Kozhikode

Sl.No.	Name of centre	No. of samples
1	Thottumukkam	43
2	Vellikulangara	24
3	Mayannoor	51
4	Anakamboil	24
5	Cheruvannoor	24
6	Palachuvadu	20

Kasargode

Sl.No.	Name of centre	No. of samples
7	Thalangara	47

Malappuram

Sl.No.	Name of centre	No. of samples
8	Manjeri	40

Kannur

Sl.No.	Name of centre	No. of samples
9	Kannadiparambu	38
10	Palleri	52
11	Kalliaserri	77
12	Chirakkal	60

Zone: High range

Idukki

Sl.No.	Name of centre	No. of samples
1	Purappuzha	23
2	Thankamani	66
3	Pandippara	57
4	Rajakkadu	48
5	Kamakshi	85
6	Thodupuzha	25
7	Neelivayal	24

Wayanad

Sl.No.	Name of centre	No. of samples
8	Mananthavady	79
9	Poothadi	48
10	Cheeral	45

ANNEXURE – 2

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

30% Acrylamide

Acrylamide	29g
N,N – Methylene bisacrylamide	1 g
Water to	100 ml

10% Ammonium persulphate

APS	100mg
Water to	1 ml

EDTA (0.5M, pH 8.0)

Dissolved 18.61g of EDTA (disodium, dihydrate) in 80ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10mg/ml)

100mg ethidium bromide dissolved in10ml distilled water. Solution stored at 4°C in dark coloured bottle.

Gel loading buffer

Bromophenol	0.25%	50mg
Xylene cyanol	0.25%	50mg
Sucrose	40%	8g

Components stirred well in 20ml distilled water and stored at 4°C.

ANNEXURE – 2

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

30% Acrylamide

Acrylamide	29g
N,N – Methylene bisacrylamide	1 g
Water to	100 ml

10% Ammonium persulphate

APS	100mg
Water to	1 ml

EDTA (0.5M, pH 8.0)

Dissolved 18.61g of EDTA (disodium, dihydrate) in 80ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10mg/ml)

100mg ethidium bromide dissolved in10ml distilled water. Solution stored at 4°C in dark coloured bottle.

Gel loading buffer

Bromophenol	0.25%	50mg
Xylene cyanol	0.25%	50mg
Sucrose	40%	8g

Components stirred well in 20ml distilled water and stored at 4°C .

Phenol (Saturated, pH 7.8)

Commerically available crystalline phenol melted at 65°C in a waterbath. Hydroxyquinolone added to a final concentration of 0.1 percent. 0.5M Tris HCl (pH 8.0) added to molten phenol in equal volume. Mixture stirred for 30 min. on a magnetic stirrer and contents transferred into a separating funnel. Lower phenolic phase colleced, mixed with equal volume of 0.1M Tris HCl (pH 8.0) and stirred again for 30 min. The phenolic phase was collected and extraction repeated with 0.1M Tris HCl (pH 8.0) until the pH of phenolic phase was more than 7.8. Finally 0.1 volume of 0.01M Tris HCl (pH 8.0) added and stored in dark bottle at 4°C.

9% Polyacrylamide gel

30% Acrylamide	30 ml
Triple distilled water	49.5 ml
5X TBE	20.0 ml
10 % APS	600µl
TEMED	35 µl
Total	100.0 ml

6 % polyacrylamide gel

30% Acrylamide	14 ml
Triple distilled water	42 ml
5X TBE	14 ml
10 % APS	490µl
TEMED	30µl
Total	100.0 ml

RBC lysis buffer

Ammonium chloride 150mM

8.0235g

Potassium chloride 10mM

0.7455g

EDTA 0.1mM

0.0372g

Dissolved the contents in distilled water and volume made upto 1000ml. Stored at 4°C after filtration and autoclaving.

Sodium acetate (3 M, pH 5.5)

Dissolved 40.824g of sodium acetate in 70ml distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume made utpo 100ml, autoclaved and stored at room temperature.

Sodium chloride (5M)

Dissolved 29.22g of sodium chloride in 80ml distilled water and volume made upto 100ml. Solution filtered and stored at room temperature.

Sodium chloride – EDTA (SE) buffer (pH 8.0)

Sodium chloride	75mM	4.383g
EDTA	35mM	9.306g

Dissolved in 900ml distilled water and pH adjusted to 8.0. Made up the volume to 1000ml, filtered, autoclaved and stored at 4°C.

Sodium dodecyl sulphate (SDS) 20%

SDS 20g

Sodium dodecyl sulphate (SDS) 20%

SDS	20g
Distilled water to make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris-Acetate EDTA (TAE) buffer (50X)

Tris base	48.4g
Glacial acetic acid	11.42ml
0.5M EDTA (pH 8.0)	20ml

Distilled water upto 1000ml

Autoclaved and stored at room temperature.

Tris-Borate EDTA (TBE) buffer(pH 8.3) 10X

Tris base	108.0g
Boric acid	55.0g
EDTA	9.3g

Dissolved in 700ml of distilled water and pH was adjusted to 8.3. Volume made up to 1000ml, autoclaved and stored at room temperature.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140mM	8.18g
Potassium chloride	0.5mM	
0.0373g		
Tris base	0.25mM	
0.0303g		

Dissolved in 900ml distilled water and pH adjusted to 7.4. Made up the volume to 1000ml, filtered, autoclaved and stored at 4°C.

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10mM
1.2114g	
EDTA	0.1mM
0.3722g	

Dissolved in 900ml distilled water and adjusted the pH to 8.0. Made up the volume to 1000ml, filtered, autoclaved and stored at 4°C.

ANNEXURE – 3

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICAL

Acrylamide (Molecular Biology grade)	-	SRL, Bombay
Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Ammonium per sulphate	-	SRL, Bombay
N-N-Methylene Bis acrylamide	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
dNTPs.	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6 X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	BDH-E,Merck (India) Ltd
Hydroxy quinolone	-	Qualigens Chemicals, Mumbai
Isoamyl alcohol	-	Merck
Methanol	-	SRL, Bombay
Potassium chloride	-	SRL, Bombay
Sodium acetate	-	SRL, Bombay
Sodium chloride	-	SRL, Bombay

Sodium dodecyl sulphate (SDS)	-	SRL, Bombay
TEMED	-	SRL, Bombay
Tris base	-	SRL, Bombay

(B) PRIMERS

Imperial bio medics, Chandigarh

(C) MOLECULAR MARKERS

pBR322 DNA /*Hae* III digest	-	Bangalore Genei Pvt. Ltd.

(D) ENZYMES

Taq DNA polymerase	-	Bangalore Genei. Pvt. Ltd
Proteinase-K	-	Bangalore Genei. Pvt. Ltd

Restriction endonuclease

Mnl 1	-	Labmate (Asia) Pvt. Ltd.
Bsp 12861	-	Labmate (Asia) Pvt. Ltd.

ANNEXURE – 4

ABBREVIATIONS

α-LA	Alpha lactalbumin
APS	Ammonium Persulphate
DNA	Deoxy Ribo Nucleic Acid
DMRT	Duncan's multiple range test
EDTA	Ethylene Diamine Tetra acetic Acid
MAS	Marker Assisted Selection
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
QTL	Quantitative Trait Loci
RFLP	Restriction Fragment Length Polymorphism
TEMED	N, N, N′, N′-Tetramethylethylenediamine
cM.	Centimorgan
µl	microlitres
µg	microgram
mg	milligram
m*M*.	millimolar
cm	centimeter
nm	nanometer
bp	basepair
SDS	Sodium Dodecyl Sulphate
dNTP.	Deoxy Nucleotide Triphosphate

EVALUATION OF LACTATION MILK YIELD AND POLYMORPHISM OF ALPHA-LACTALBUMIN GENE IN CROSSBRED CATTLE OF KERALA

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Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

The present study was carried out to assess the milk yield of crossbred cattle of Kerala by studying four different zones of the state viz. northern, southern, central and highlands and to analyse the association of α-lactalbumin gene polymorphism with milk production of crossbred cattle in Kerala.

One day milk yield of 500 animals from each zone, at a lactation stage of 7-13 fortnights after calving was recorded. From this one-day milk yield 305-day milk yield of crossbred cattle was predicted using the formulae given by Iype (1991). The predicted lactation milk yield was used for estimating average milk yield of crossbred cattle in Kerala.

The overall least squares mean of 305-day milk yield was 2351 ± 28.9 kg. Least squares analysis of variance has shown that different zones and parity exerted significant effect on 305-day milk yield ($P \leq 0.05$). The highest least squares means for 305-day milk yield was in southern zone and the lowest was in central zone. The least squares mean for 305-day milk yield according to parity was highest in second parity, followed by third parity and the lowest was in fifth parity.

Genomic DNA samples isolated from 25 crossbred cows selected at random from each zone was subjected for PCR-RFLP of α-lactalbumin gene at two different specific loci. On successful amplification the expected 166 bp and 429 bp fragments were obtained.

Digestion of 166 bp amplified product with *Mnl*1 enzyme revealed similar pattern of digestion for all animals studied, indicating the absence of α-LA/*Mnl*1 (+) in the population.

Restriction of 429 bp amplified product with *Bsp*12861 enzyme revealed two digestion patterns (indicating the presence of two α-LA/*Bsp*12861 alleles). The gene frequencies of α-LA/*Bsp*12861 (+) and (-) alleles were 0.08 and 0.92 respectively. In the crossbred population studied, none of the animal showed α-LA/*Bsp*12861 (+/+) genotype. The average milk yield of 79 α-LA/*Bsp*12861 (+/+) genotypes was 2779 kg and that of 16 α-LA/*Bsp*12861 (+/+) genotypes was only 2364 kg. However, the difference was statistically non-significant. It is suggested to conduct further research in large samples to confirm the findings and to identify other polymorphic loci associated with milk yield.

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