EVALUATION AND COMPARISON OF POLYMORPHISM OF BETA CASEIN GENE IN VECHUR AND CROSSBRED CATTLE OF KERALA

MUHAMMED E. M.

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Department of Animal Breeding, Genetics & Biostatistics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

I hereby declare that this thesis, entitled **"EVALUATION AND COMPARISON OF POLYMORPHISM OF β-CASEIN GENE IN VECHUR AND 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.

Mannuthy,
3-5-2010.

MUHAMMED E.M.
(2008-13-143)

CERTIFICATE

Certified that this thesis, entitled **"EVALUATION AND COMPARISON OF POLYMORPHISM OF β-CASEIN GENE IN VECHUR AND CROSSBRED CATTLE OF KERALA"** is a record of research work done independently by **Dr. Muhammed E. M. (2008-13-143),** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Mannuthy,
3/5/2010

Dr. Stephen Mathew,
(Chairman, Advisory Committee)
Professor,
Department of Animal Breeding,
Genetics and Biostatistics,
College of Veterinary and Animal Sciences,
Mannuthy – 680 651.

CERTIFICATE

We, the undersigned members of the Advisory Committee of Dr. E. M. Muhammed, a candidate for the degree of Master of Veterinary Science in Animal Breeding, Genetics and Biostatistics agree that the thesis entitled **"Evaluation and Comparison of Polymorphism Of β-Casein Gene in Vechur and Crossbred Cattle of Kerala"** may be submitted by Dr. E. M. Muhammed, in partial fulfilment of the requirement for the degree.

Dr. Stephen Mathew,
(Chairman, Advisory Committee)
Professor,
Department of Animal Breeding, Genetics and Biostatistics,
College of Veterinary and Animal Sciences,
Mannuthy- 680 651.

Dr. K.V. Raghunandanan,
Director,
Centre for Advanced Studies in
Animal Genetics and Breeding
College of Veterinary and Animal
Sciences, Mannuthy-680 651
(Member, Advisory Committee).

Dr. T. V. Aravindakshan,
Professor,
Department of Animal Breeding,
Genetics and Biostatistics,
College of Veterinary and Animal
Sciences, Mannuthy-680 651
(Member, Advisory Committee).

K.A. Mercey

Dr. K. A. Mercey,
Professor,
Department of Statistics,
College of Veterinary and Animal
Sciences, Mannuthy-680 651.
(Member, Advisory Committee).

External Examiner
CH. R. JAYASHANKAR
Professor & Head
Dept. of Animal Genetics & Breeding
Veterinary College
KVAFSU, Hebbal
BANGALORE - 560 024

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"We must add the fuel of interest to the fire of genius in the discovery of new and useful things"

Abraham Lincoln

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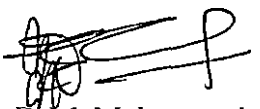

E. M. Muhammed.

Dedicated

to

My Family

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Introduction

1. INTRODUCTION

India has vast resources of livestock. It plays a vital role in the Indian economy and in improving the socio economic conditions of rural masses. Agriculture and livestock sector share 16.74% and 4.36% in the economy of India. As per Livestock census 2003, India has total livestock population of 485 million with 185.2 million cattle and 97.2 million buffalo which are 65% and 34% respectively of total bovine population (283.1million). In Kerala 96% of livestock population is cattle producing 98.50% of total milk (Anon, 2009). More than 82% of the cattle in Kerala are crossbreds except very few indigenous breeds like Vechur. Vechur cattle once thought to be extinct due to extensive crossbreeding are the first native cattle of Kerala to be approved as a distinct breed and the smallest breed in the world. Nowadays, Vechur cattle are maintained by a few farmers and at Kerala Agricultural University.

Milk is a common source of animal protein and associated micro elements for vegetarians. Cow's milk contains two major protein groups: caseins and whey proteins and out of which caseins account for 80% of milk proteins. Casein is a good source of amino acids, phosphates, calcium and biologically active peptides. Bovine milk contains 4 caseins: alpha S1 (CSN1S1 39-46% of total casein), alpha S2 (CSN1 S2 8-11%), beta (CSN2, 25-35%), and kappa (CSN3 8-15%). β Casein (β-CN) is the second most abundant protein in cow's milk that contains 209 amino acids. Bovine β-CN gene belongs to the cluster of 4 casein genes located on chromosome 6. There are 12 genetic variants of β-CN: A1, A2, A3, B, C, D, F, H1, H2, I and G out of which A1 and A2 are the most common (Farrel *et al.*, 2004).

Within β-CN, the A2 variant was the first to be completely sequenced. Using the sequence of β casein A2 as reference, variants A1 and others differ by having a histidine instead of proline at position 67. The natural mutation

that gave rise to this difference is a result of single nucleotide polymorphism (SNP) at codon 67 of β-CN gene: CCT (Proline) ⟶ CAT (Histidine). Milk proteins release bioactive peptides called betacasomorphins (BCMs) upon proteolytic digestion by pepsin, pancreatic elastase and leucine amino peptidase. Beta casomorphine-7 (BCM-7) (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was first isolated as a peptide having morphine like activity. It acts by binding to opioid μ receptors, which are found principally in the central nervous system and the gastro intestinal tract. Presence of histidine at position 67 in A1 β-CN forms cleavage site for pancreatic elastase yielding BCM-7 by the successive gastrointestinal digestion. Human β-CN also contains amino acid sequence (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro) similar to that of bovine β-CN A2 but positions starting from 51-58. Therefore a peptide equivalent to BCM-7 cannot be formed upon digestion of human β-CN (Elliot *et al.,* 1997). No polymorphism of β-CN gene has been observed in human β-CN.

Consumption of β-CN A1 producing BCM-7 is reported as a risk factor of type 1 diabetes mellitus and ischaemic heart disease in humans (Elliot *et al.*, 1999). Relation of BCM-7 to sudden infant death syndrome (SIDS), atherosclerosis, autism and schizophrenia has also been suggested (Sun *et al.*, 2003; Tailford *et al.*, 2003). β-CN A1 is the most frequent variant in Holstein Friesian and Ayrshire whereas high frequency of β-CN A2 is observed in Jersey and Guernsey (Kaminski *et al.*, 2007). The A2 Corporation was set up in the New Zealand in the late 1990s to test cows and market A2 milk at premium price. It is assumed that the β-CN A2 is most probably of *Bos indicus* origin. Most of the Indian cattle and native buffalo breeds are found to posses only β-CN A2 (Mishra *et al.*, 2009).

The hypothesis that high consumption of A1 β-CN increases the risk of several disease syndromes is very intriguing and interesting for basic as well as

application studies and potentially very important for population health. β-CN gene is a promising SNP locus whose polymorphism is greatly associated with milk volume. The use of relatively easy and cost effective molecular technique –AS-PCR (Allele Specific PCR) - enables us to shed light on this (SNP).

Realising the importance of β-CN gene polymorphism in public health and discovery of markers linked to economically important traits such as milk production, present study was undertaken with following objectives:

1. Characterisation of the β-CN gene (A1 and A2) of Vechur and Sunandini crossbred cattle of Kerala by Allele Specific – Polymerase Chain Reaction (AS-PCR)

2. Comparison of allelic frequencies of β-CN gene(A1 and A2) in Vechur and crossbred cattle of Kerala

3. To analyse the relationship between different genotypes with milk yield

Review of Literature

2. REVIEW OF LITERATURE

2.1 MILK PROTEINS

In milk there are two major protein groups: caseins (CNs) and whey proteins. CNs account for 80% of bovine milk protein, where as both major whey proteins constitute about 14%. Bovine milk contains 4 CNs: α S1 (39-46% of total CNs), α S2 (8-11%), β (25-35%) and κ (8-15%) (Martin *et al.*, 2002).

2.1.1 Structure and Function of Milk Protein Genes

More than 95% of the proteins contained in ruminant milk are coded by 6 well characterized structural genes. The 4 CNs- α S1, α S2, β and κ CNs are coded by CSN1S1, CSN1S2, CSN2 and CSN3 genes respectively. The milk protein nomenclature considering only protein polymorphisms includes 8 α S1- CNs, 4 α S2-CNs, 12 β-CNs, and 11 κ-CN variants within the genus *Bos*. CN loci have been assigned to chromosome 6 in cattle. CN genes appear to occur as a single copy per haploid chromosome set because no related sequence or pseudogenes has been reported (Farrel *et al.*, 2004). Mercier and Vilotte (1993) demonstrated that although inter species comparisons of cDNA and mosaic milk protein genes confirmed their high rate of evolution; the overall gene organisation was found to be conserved.

Ceriotti *et al.* (2004) explained milk protein loci polymorphism in taurine (*B. taurus*) and zebu (*B. indicus*) populations bred in hot climate. All loci were polymorphic. The differences in the distribution of alleles and haplotypes observed between zebu and taurine cattle showed that milk protein polymorphism was suited for discriminating *B. taurus* from *B. indicus* efficiently, and also taurine breeds.

2.1.2 Milk Protein Gene Polymorphism

Genetic polymorphism has significance to find association between variants and production traits, reproduction efficiency, adaptation capacity of cattle and also influences on nutritional and technological properties. The best alleles of the major milk protein genes are selected using genetic markers (Farrel *et al.*, 2004).

Sarbour *et al.* (1996) studied the association between milk protein variants and milk yield traits. The results suggested that β-CN loci had no significant effects on breeding value of the bulls for milk, fat and protein.

Rauw *et al.* (1998) described undesirable side effects of selection for high production efficiency in farm animals and found that metabolic, reproduction and health traits are negatively correlated with milk yield.

Ikonen *et al.* (1999) estimated genotypic effects of β–CN, κ – CN, and β – lactoglobulin on milk production. Composite CN genotypes including the A2 allele of β – CN were associated with the highest milk production.

Frajman and Dves (2004) found that frequencies of CN variants are breed specific.

Singh (2006) described that extensive crossbreeding has resulted in less genetic variation in farm animals.

Braunschweig (2008) suggested that the association between CSN2 A1 and A2 alleles and milk protein yields might be a consequence of CSN2 heterogeneity or a closely linked gene.

Cole *et al.* (2009) found that high density scan SNP markers confirmed two major genes on *Bos taurus* autosome 6(BTA6) on which CN gene cluster is located.

Toosi *et al.* (2010) explained genomic selection in admixed and crossbred populations.

2.1.2.1 Indian Breeds

Jairam *et al.* (1983) studied genetic variants of milk proteins in Sahiwal, Red Sindhi, Tharparker and crossbred cows using electrophoretic techniques. Milk proteins from crossbred cows behaved differently from those in zebu milk due to influence of exotic genes.

Jeichitra *et al.* (2003) found that in Kangayam cattle of south India all the four milk protein components studied, αS1–CN, β–CN, β–lactoglobulin and α–lactalbumin exhibited polymorphism. The mean heterozygosity estimated over all the four milk protein loci was 0.242. Genetic equilibrium was observed among the loci studied.

2.1.2.2 Holstein Friesian

Ng-Kwai-Hang *et al.* (1985) demonstrated relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. Higher test-day milk production was associated with, β -CN A1A2.

Ng-kwai-Hang *et al.* (1990) reported that β-CN A2A2 cows produced more milk than β -CN A1A1 cows for the first, second and third lactation.

Bovenhuis *et al.* (1992) studied associations between milk protein polymorphisms and milk production traits in Holstein Friesian cattle and found that effects of β-CN genotypes on milk production were significant.

Ron *et al.* (1994) found that the genotype frequency of β-CN A1A1, A1A2 and A2A2 was 0.29, 0.42, and 0.11 respectively in Israeli Holsteins. They also found a positive correlation between A1A1 genotype and milk production.

Ojala *et al.* (1997) studied the effect of milk protein genotypes on the variation for milk production traits of Holstein. They found that neither the β - CN A2 allele nor the k-CN B allele alone had a positive effect on milk, but the joint effect was strongly positive.

Oner *et al.* (2006) studied milk protein polymorphism in Holstein cattle and found significant deviation from Hardy- Weinberg equilibrium in κ-CN locus while all other CN showed no significance.

Heck *et al.* (2009) showed that in the Dutch Holstein-Friesian population, the allele frequencies changed in the past 16 years.

2.1.2.3 Other Exotic Breeds

Lien *et al.*-(1995) compared milk protein allele frequencies in Nordic cattle breeds. Significant frequency of CSN2(A2) was were found in Icelandic cattle, Swedish Mountain cattle, Northern Fin cattle and western Fjord cattle, which indicated common origin of these populations.

Caroli *et al.* (2004) studied genetic structure of milk protein polymorphisms and effects on milk production traits in local dairy cattle (Reggiana cattle) and compared with Italian Friesian and Italian Brown cattle.

The prevalent haplotypes for CSN1S1-CSN2-CSN3 were CA2B (23%; Reggiana), BA2A (48%; Italian Friesian) and BA2B (51%; Italian Brown).The genotype frequencies observed were in Hardy-Weinberg equilibrium.

Kucerova *et al.* (2006) found significant differences between genotypes of loci CSN1S1, CSN2 and CSN3 and breeding values for milk production in Czech Fleckvieh cattle.

Dinc *et al.* (2007) found the spread of β-CN A1 allele and indicated the frequency of undesirable A1 allele in native Turkish cattle breeds.

Nilson *et al.* (2009) explained that CSN2 A2 was associated with higher protein and milk yield and by selective breeding; there was an increase in population with CSN2 A2 allele among Norwegian red cattle.

2.2 β-CASEIN (β-CN)

The reference protein for this family, β-CN A2-5P is a single-polypeptide chain with no Cys residues containing 209 residues. It consists of Asp4, Asn5, Thr9, Ser11, Ser P5, Glul9, Gln20, Pro35, Gly5, Ala5, Val19, Met6, Ilel0, Leu22, Tyr4, Phe9, Lys11, His5, Trp1 and Arg4 with a calculated molecular weight of 23,983. The most common variant used as reference is variant A2; its ExPASy entry name and file number are CASB_Bovin and P02666, respectively (Farrell *et al.*, 2004).

2.2.1 Structure

β-CN is the most hydrophobic of the CNs. The N terminal sequence codes for charged amino acids as well as a phosphoserine cluster. This molecule presents a high contrast in its sequence, one-tenth of the amino acids at the N-terminus of the protein contain one-third of the total charge, while 75%

of the residues at the C terminal one-tenth consist of hydrophobic amino acids-characteristic of an amphiphile.

Greenberg *et al.* (1984) studied human β-CN amino acid sequence and identified phosphorylation sites. Sequence comparison of human β-CN with the bovine revealed 50% identity. Martin *et al.* (2007) compared CN micelles in raw and reconstituted skim milk. Lencki (2007) suggested evidence for fibril-like structure in bovine CN micelle. The observed fibers closely resembled protofibrils, intermediate structures that is observed during the formation of amyloid fibrils. Qi *et al.* (2007) studied pepsin-induced changes in the size and molecular weight distribution of bovine CN during enzymatic hydrolysis. There are 12 genetic variants of β-CN (Table 2.1) (Kaminski *et al.*, 2007). McMahon *et al.* (2008) described Supramolecular Structure of the CN micelle. Lachkar *et al.* (2008) developed experimental approach for removing CNs from bovine colostrum using anionic polysaccharides.

Table 2.1 Changes in the amino acid sequence of β-CN variants

β-CN	Change in amino acid sequence													
	18	25	35	36	37	67	72	88	93	106	117	122	137	138
A2	Ser	Arg	Ser	Glu	Glu	**Pro**	Glu	Leu	Gln	His	Gln	Ser	Leu	Pro
A1						**His**								
A3										Gln				
B						His						Arg		
C			Ser		Lys	His								
D	Lys													
E				Lys										
F						His								
G						His								Leu
H1		Cys						Ile						
H2							Glu		Leu					
I									Leu					Glu

2.2.2 Properties

Jinsmaa *et al.* (1999) studied enzymatic release of neocasomorphine and BCMs from bovine β-CN. Considine *et al.* (1999) studied the proteolytic specificity of elastase on bovine β-CN. They determined the cleavage specificity of elastase. Gaucheron (2001) studied influence of pH on the heat-induced proteolysis of CN molecules. They characterized eighteen low molar mass peptides, of which nine came from β-CN. Bitri (2004) experimented optimization study for the production of an opioid-like preparation from bovine CN by mild acidic hydrolysis. Yousefi *et al.* (2009) explained micellisation and immunoreactivities of dimeric β-CN.

2.3 β-CN GENE

Bovine β CN gene (CSN2) belongs to the cluster of 4 CN genes: α S1, α S2, β and κ, located on chromosome 6.These CN genes reside in a 250-300 kb region of the same chromosome in the form of a complex arranged in the order α S1-, β- , α S2- and κ- CN.

2.3.1 Structure

Stewart *et al.* (1987) demonstrated complete nucleotide sequences of bovine αs2- and β -CN cDNAs and compared with related sequences in other species. Bonsing *et al.* (1998) performed complete sequencing of bovine β – CN gene.

2.3.2 Characteristics of β –CN Gene

Lien *et al.* (1993) studied Bovine CN haplotypes, their number, frequencies and applicability as genetic markers in Holstein Friesian crossbred cattle. They found that the frequencies of the various CN haplotypes were

mainly influenced by the low number of sires and varying number of sons within each sire. Choi *et al.* (1996) Compared cDNA sequences of β-CN and demonstrated that the N-terminal region is conserved.

Rijnkeles (2002) studied evolution of CN gene family by multispecies comparison of the CN gene loci. They described that, CNs were present in genomic cluster spanning 250-350 kb. Comparative analysis of genomic sequences harbouring the CN gene cluster region of various species (with equal evolutionary distances 79-88Myr) showed that the organisation and orientation of the genes were highly conserved.

2.3.3 β –CN Gene Polymorphism

Malik *et al.* (2000) analysed κ-CN and β-CN alleles in crossbred and Zebu cattle from India using PCR and sequence-specific oligonucleotide probes. They found that in cross bred cattle the frequency of heterozygous A1 A2 genotype was 58%. Kearney *et al.* (2005) explained cumulative discounted expressions of sire genotypes for β-CN loci in commercial dairy herds. Kaminski *et al.* (2007) studied polymorphism of bovine β-CN and its potential effect on human health. Occurrence of β-CN gene variants in various cattle breeds and countries are given in Table 2.2. Mishra *et al.* (2009) studied status of milk protein β-CN variants among Indian milch animals. They reported the frequency of β-CN variants in 15 zebu cattle breeds (Table 2.3).

Table 2.2. Polymorphism of β-CN alleles in cattle breeds of different countries (Kaminski *et al., 2007).*

Breed	Country	A1 allele	A2 allele
Guernsey	USA	0.01	0.98
	USA	0.06	0.97
Jersey	Germany	0.093	0.721
	Denmark	0.070	0.650
	New Zealand	0.123	0.591
	USA	0.22	0.540
Brown Swiss	Germany	0.108	0.71
	USA	0.15	0.72
	USA	0.18	0.72
Simmental	Croatia	0.19	0.63
	Germany	0.34	0.57
HF	USA	0.66	0.30
	USA	0.49	0.50
	Hungary	0.42	0.47
	Germany	0.47	0.47
	Poland	0.4	0.60
	New Zealand	0.47	0.50
	Norway	0.4	0.50
Ayrshire	New Zealand	0.43	0.53
	Finland	0.51	0.49
	U K	0.60	0.40
	USA	0.72	0.28
	Denmark	0.71	0.23

Table 2.3. Allelic and genotypic frequency of β-CN in Indian cattle breeds

Breed	Utility	A1	A2	A1A1	A1A2	A2A2
Kangayam	Draught	0	1	0	0	1
Nimari	Draught	0	1	0	0	1
Red Kandhari	Draught	0	1	0	0	1
Malnad Gidda	Draught	0.096	0.904	0	0.191	0.809
Kherigarh	Draught	0.109	0.891	0	0.2180	0.783
Malvi	Draught	0	1	0	0	1
Amrithmahal	Draught	0	1	0	0	1
Kankrej	Milch	0	1	0	0	1
Gir	Milch	0	1	0	0	1
Sahiwal	Milch	0	1	0	0	1
Hariana	Dual	0	1	0	0	1
Tharparker	Dual	0	1	0	0	1
Rathi	Milch	0	1	0	0	1
Mewati	Dual	0	1	0	0	1
Red Sindhi	Milch	0	1	0	0	1
Mean		0.013	0.987	0	0.026	0.974

2.3.4 β–CN Gene Polymorphism and Milk Yield

Lien *et al.* (1995) found QTL in the region of CN genes for milk yield in Norwegian red cattle.

According to Velmala *et al.* (1995) β-CN A1 was associated with an increase in milk yield in Finnish Ayrshire.

Freyer *et al.* (1999) showed that the main differences between genotypic effects on yield traits were caused by β-CN genotypes in favour of genotype β – CN A2A2 in Holstein Friesian

Ikonen *et al.* (2001) studied the associations between CN haplotypes and first lactation milk production traits in Finnish Ayrshire cows. The β-CN A2A2 was associated with high milk yield, and those that included the β-CN A1 allele were associated with low yield.

Boettcher *et al.* (2004) studied effects of CN haplotypes on milk production traits in Italian Holstein and Brown Swiss cattle. For the Brown Swiss; effects of haplotypes were significant for milk yield.

Comin *et al.* (2008) found that CSN2 locus was associated more with milk and protein yields. The most frequent CSN2 genotypes were A1A2 and A2A2and for milk yield, the best genotype was the most frequent genotype A2A2.

Nilsen *et al.* (2009) found that CSN2 A2 variant was found to be associated with increased milk yield in Norwegian Red cattle.

Miluchova *et al.* (2009) analysed polymorphism of β-CN of Slovak Pinzgau cattle by PCR-RFLP for alleles A1 and A2.

2.3.5 β–CN Geographical Distribution

Pereira *et al.* (2002) described evidence for a geographical cline of CN haplotypes in Portuguese cattle breeds. Their data suggested that high genetic similarity among neighbouring Portuguese breeds was mainly caused by gene flow. The comparison of African *B. taurus* and *B. indicus* breeds allowed the identification *B. indicus* specific haplotype-CSN2A2-that was not found in pure taurine breeds. The occurrence of such haplotype in southern European breeds suggested an introgression of indicine genes into taurine breeds could have contributed to the distribution of the genetic variation observed and this could

be caused by strong selection pressure on milk production traits (Jann *et al.*, 2004).

2.4 RELEASE OF BIOACTIVE PEPTIDES FROM β-CN

Genetic variants A1 and A2 are universally distributed in almost all *B. taurus* and *B. indicus* populations. The β-CN A1s differ from the A2 variant at position 67 where a histidine replaces a proline. Importantly, it is the change to histidine at position 67 that has the potential to result in cleavage occurring upon digestion and a bioactive peptide, β-casomorphine potentially being liberated (Keating *et al.*, 2008).

2.4.1 Mechanism of release

Milk contains a unique component that, along with a low copper status, reduced the dopamine pool size (Miller *et al.*, 1987). Daniel *et al.* (1990) while studying the effect of CN and BCMs on gastrointestinal motility in rats, found that from the CN fraction of bovine milk, different peptides with opioid activity were released during digestion. These bioactive substances are significant to the human neonate also (Weaver *et al.*, 1997).

2.4.2 Bioactive Peptides and Diseases

Scott (1990) described relationship between cow milk and insulin dependent diabetes mellitus. Study revealed a significant positive correlation between consumption of unfermented milk protein and incidence of IDDM in data from various countries.

Cavallo (1996) explained cell-mediated immune response to β-CN in onset of insulin-dependent diabetes and its implications for disease pathogenesis.

Elliott *et al.* (1997) described the role of β-CN variants in the induction of insulin-dependent diabetes in the non-obese diabetic mouse and humans.

Elliot *et al.* (1999) studied type I (Insulin dependent) diabetes mellitus and cow milk CN variant consumption.

Thorsdottir *et al.* (2000) explained different β-CN fractions in Icelandic versus Scandinavian cow's milk influenced diabetogenicity of cow's milk in infancy and explained low incidence of Insulin-Dependent Diabetes Mellitus in Iceland. The lower fraction of A1 β-CNs in Icelandic cow's milk explained why there is a lower incidence of IDDM in Iceland than in Scandinavia.

Beales *et al.* (2002) explained a multi-centre, blinded international trial of the effect of A1 and A2 β-CN variants on diabetes incidence in two rodent models.

Oliveira *et al.* (2003) explained that β-CN-derived peptides, produced by bacteria, stimulated cancer cell invasion and motility.

Tailford *et al.* (2003) explained that a CN variant in cow's milk was atherogenic.

Laugesen *et al.* (2003) explained the relation of ischaemic heart disease, Type 1 diabetes, and cow milk A1 β-CN. They raised the possibility that intensive dairy cattle breeding emphasized a genetic variant in milk with adverse effects in humans.

Trompette *et al.* (2003) explained that β-casomorphins induced mucus release in rat jejunum. Their findings suggested that μ opioid neuropeptides, as well as β -casomorphins after absorption, modulate intestinal mucus discharge.

Monetini *et al.* (2003) described establishment of T cell lines to bovine β-CN and β-CN-derived epitopes in patients with type 1 diabetes. The mechanism by which cow's milk proteins triggered an autoimmune response to β-cells was molecular mimicry.

Swinburn (2004) implicated A1 β-CN as a potential etiological factor in type 1 diabetes mellitus (DM-1), ischaemic heart disease (IHD), schizophrenia, and autism.

Ferranti *et al.* (2004) described CN proteolysis in human milk and traced the pattern of CN breakdown and the formation of potential bioactive peptides.

Sofia *et al.* (2005) explained CNs as source of bioactive peptides and described that biologically active peptides are of particular interest in nutrition.

Bell *et al.* (2006) described the various health implications of milk containing β-CN with the A2 genetic variant.

Ledesma *et al.* (2007) performed identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin.

Haug *et al.* (2007) studied Bovine milk in human nutrition and suggested the possibility of adjusting feeding regimes develop milk with increased content of healthy components.

Woodford (2007) described A2 milk, farmer decisions, and risk management. Sito *et al.* (2008) studied synergistic effect between different milk-derived peptides and proteins.

Freyer *et al.* (2008) reported increased incidence of calf mortality in Holstein Friesian (8.87%) than Jersey (2.97%).

Korhonen (2009) described milk-derived bioactive peptides; its science and applications.

Phelan *et al.* (2009) described potential bioactive properties of CN hydrolysates.

2.5 MOLECULAR TECHNIQUES FOR SNP IDENTIFICATION

Mutation mechanisms result either in transitions: purine-purine (A to G) or pyrimidine–pyrimidine (C to T) exchanges, or tranversions ; Purine-pyrimidine or pyrimidine–purine (A to C, A to T, G to C, G to C, G to T) exchanges. With twice as many possible tranversions than transitions, the transitions over tranversions ratio should be 0.5. However, observed data indicate a clear bias towards transitions. The SNP corresponding to β-CN A1/A2 polymorphism is C to A transversion. Among other marker types SNPs share the advantages of being 1) widespread in genome 2) So close to or with in coding regions 3) codominant, hence heterozygotes can be identified 4) amenable to the use of PCR and hence for automation (Flint and Woolliams, 2008).

2.5.1 Molecular Studies on β-CN Gene

Lien *et al.* (1992) detected multiple β-CN (CASB) alleles by amplification created restriction sites (ACRS).

Velmala *et al.* (1994) genotyped bovine β-CN C allele by amplification created restriction site.

Lindersson *et al.* (1995) genotyped bovine milk proteins using allele discrimination by primer length and automated DNA sizing technology.

Barroso *et al.* (1999) demonstrated a multiplex PCR-SSCP test to genotype bovine β-CN alleles A1, A2, A3, B and C.

Einspanier *et al.* (2001) characterised β-CN variants using real-time PCR approach.

Rincon *et al.* (2003) studied single nucleotide polymorphism genotyping of bovine milk protein genes using the tetra primer ARMS-PCR.

Chessa *et al.* (2007) developed SNP genotyping microarray platform for the identification of bovine milk protein genetic polymorphism.

Keating *et al.* (2008) evaluated bovine β-CN variants by AS-PCR.

Miluchova *et al.* (2009) analysed polymorphism of β CN of Slovak Pinzgau cattle by PCR-RFLP for alleles A1 and A2.

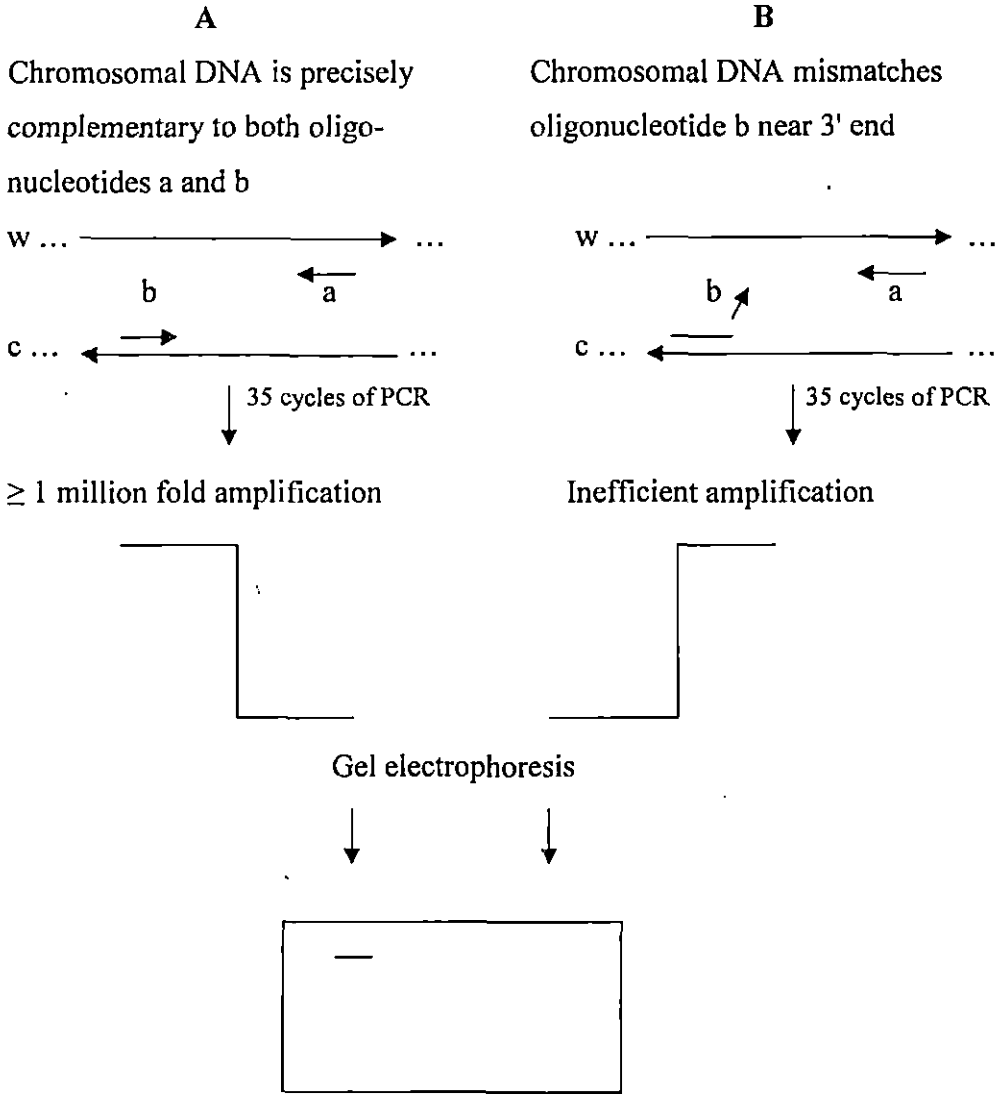
Mishra *et al.* (2009) studied status of milk protein β-CN A1 and A2 among Indian milch animals by PCR-RFLP.

PCR-RFLP (Polymerase Chain Reaction –Restriction Fragment Length Polymorphism) offers a straight solution for SNP identification, when the restriction pattern of the region is affected by mutation under examination. However the two step approach has the inconvenience of being labour intensive and inapplicable to mutations that do not modify the restriction pattern or that affect it with scarcely detectable changes (Wangkumhang *et al.*, 2007).

2.5.1.1 AS-PCR Analysis

AS-PCR is modification of PCR where the DNA polymerase is used to amplify the target DNA only if the PCR primers are perfectly complementary to the target DNA sequence (Fig.2.1).

Fig.2.1 Allele Specific PCR (AS-PCR) reaction (Bottema *et al.*, 1993)



AS-PCR is modification of PCR where the DNA polymerase is used to amplify the target DNA only if the PCR primers are perfectly complementary to the target DNA sequence (Fig.2.1). It relies on the use of an oligonucleotide primer for PCR amplification that precisely matches one of the alleles but not the other (Sarkar *et al.*, 1990). AS-PCR makes use of the fact that product formation in PCR is sensitive to mismatching of the 3' residue in one of the two primers (forward or reverse) used (Bottema *et al.*, 1993). When a mismatch occurs at or near the 3' end of the PCR primer, amplification is inefficient. AS-PCR requires the use of a polymerase that lacks a 3'-5' exonuclease activity because such activity would remove the 3' terminal mismatch. Generally, T*aq* polymerase has been used. The AS-PCR approach has been modified in different ways in order to increase sensitivity. Unfortunately, each of these modifications has been given a different designation resulting in a bewildering array of acronyms *viz.*, Amplification Refractory Mutation System (ARMS) (Newton *et al.*, 1989), PCR Amplification of Specific Alleles (PASA) (Bottema *et al.*, 1993) or Tetra primer ARMS PCR (Rincon and Medrano, 2003). AS-PCR has the advantage of combining amplification and detection without requirement of additional probes or enzymes (Wangkumhang *et al.*, 2007).

2.5.1.2 Principle

All the above methods use the same principle which is based on DNA amplification by using an allele specific primer at the 3' position, which binds to the SNP allele. PCR product containing the polymorphic site serves as template, and the 3' end of the primer consists of the allelic base (Kwok *et al.*, 1990). However; it shows some restricted specificity that may hamper the accuracy of the results. This limitation derives from the low capacity of Taq polymerase to discriminate single 3' mismatches, when one allele specific primer anneals the wrong target (Bottema *et al.*, 1993). One relies on the DNA polymerase to extend a primer only when its 3' end is perfectly complementary

to the template. When this condition is met, a PCR product is formed. Since Taq DNA polymerase lacks 3'-5' exonuclease activity, a primer with a mismatch at the 3' terminal with regard to the template will be amplified with reduced efficiency, allowing discrimination between matched and mismatched templates (Pettersson *et al.*, 2003). By determining whether a product is formed or not, one can infer the allele found on the target DNA.

2.5.1.3 The Design of Primers

AS–PCR involves designing a PCR oligonucleotide primer that amplifies one allele but not the other. Primers as short as 13 nucleotides are effective (Sarkar *et al.*, 1990). In the process of primer designing single mismatch 3 residues from the 3' terminal base of primer efficiently extended without modification of amplification reaction condition (Kwok *et al.*, 1990). The design of allele specific primer may encounter difficulties because only a few mismatches (*i.e.*, A/G, G/A, C/C, and A/A at the 3' primer extreme reduce the yield of PCR amplification. The effects of mismatches on PCR were symmetrical (*i.e.*, both A: G and G: A mismatches were equally detrimental to PCR under these conditions (Kwok *et al.* 1990). It has been reported that Taq DNA polymerase extends mismatched allele specific DNA primers, thereby generating false positive results (Lien *et al.*, 1992). Once a mismatched product has been originated it undergoes perfectly efficient amplification because its sequence anneals to a primer present in the amplification mix.

Another complication is that reliable discrimination between alleles may be difficult to achieve in single reaction (Ayyadevara *et al.*, 2000). The interpretation is purine –purine mismatches do not extend efficiently while pyrimidine–pyrimidine and purine-pyrimidine mismatches did extend efficiently. Size of the primer also affects the primer duplex stability (Waterfall *et al.*, 2002). Several automated bioinformatics tools are available for

specifically designing AS primer, such as, Tetra-primer, Primo SNP, commercial software Visual-OMP and WASP (Wangkumhang *et al.*, 2007). With mismatched primers β-CN variants in bovine breeds were evaluated by AS-PCR (Keating *et al.*, 2008).

The term mispriming is used when PCR products are generated through primer annealing to partially complementary sequences. Priming probabilities of a mismatched primer are good and universal features to assess the performance because they can be measured with any primer pair, on any given target, with any master mix and in any PCR conditions (Boyle *et al.*, 2009). Priming probabilities are essential to describe analytical specificity as required in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin *et al.*, 2009). The strength of destabilization for all combinations of nucleotide pairing is given below (Bui *et al.*, 2009).

Base pairing	Destabilising strength
GA,CT,TT	Maximum
CC	Strong
AA,GG	Medium
CA,GT	Weak
AT,GC	None

Mismatch combination in β-CN A1/A2 AS-PCR in present study

Alleles	A2	A1
Template base	C **C** T	C ***A*** T
Mismatched 3' primer	**G**	***T***
Mismatch combination	***T C***	**G *A***
Destabilising strength	Maximum	Maximum

2.5.1.4 Variation in Reaction Components and Annealing Temperature

The absence of detectable product reflects small differences in amplification efficiency rather than complete inhibition of extension (Kwok *et al.*, 1990). Minor differences in template and primer concentrations did not account for the dramatic effects on PCR (Ayyadevara *et al.*, 2000). Mismatches occur preferentially in the first cycles of amplification, when the DNA template is subjected to an excess of PCR components; hence the specificity of priming during the initial rounds of PCR is crucial for the success of the method. Michelis- Menten kinetics has been used to determine the variation of the rates of many enzyme catalyzed reactions as the substrate or effector concentration is varied (Waterfall *et al.*, 2002). It is necessary to optimize the PCR conditions such as cycle number, annealing temperature and Mg2+ concentration for each primer sequence to avoid mismatched amplification (Petterson *et al.*, 2003).

2.5.1.5 Heterozygous samples

In case of heterozygous samples, A1 and A2 reactions should give amplification. Heterozygous sample may give differential amplification due to sequence dependent differences in mispairing discrimination (Ayyadevara *et al.*, 2000). The ability of a primer to prime on a DNA template is governed by two kinetic variables: the rate at which annealed primer dissociates from the template before initiating polymerization (r_{off}) and the rate at which the DNA polymerase extends the primer (r_{pol}). Efficient priming in PCR occurs whenever $r_{pol} > r_{off}$. Addition of first few nucleotides to the primer then greatly stabilizes the primer –template complex and allows continued extension of the primer. From a given primer r_{pol} is an intrinsic property of the polymerase. It allows one to take advantage of the difference between r_{pol} of the perfectly matched and mismatched primers as well as to optimise variation in reaction

concentration and annealing temperature which will affect the difference in the r_{off} of the two allele specific primers. Set of conditions should exist such that $r_{pol} > r_{off}$ for the perfectly matched primer, while $r_{pol} < r_{off}$ for the mismatched primer (Wu *et al.*, 1989).

2.5.2 Modifications of AS-PCR

Ehlen (1989) Detected Ras point mutation by Polymerase Chain Reaction using mutation specific inosine containing oligonucleotide primers. Newton *et al.* (1989) described analysis of any point mutation in DNA by amplification refractory mutation system (ARMS). Pastinen *et al.* (1997) established minisequencing principle applied to oligonucleotide array format. Tyagi *et al.* (1998) developed hybridization technique with allele specific probes with multicolour molecular beacons. Hirschhorn *et al.* (2000) described SBE-TAGS: An array –based method for efficient single nucleotide polymorphism genotyping.

Ye *et al.* (2001) developed tetra primer ARMS –PCR combined with microplate array. Akada *et al.* (2001) described detection of a point mutation in fas2 gene by allele-specific PCR amplification. Myakishev *et al.* (2001) demonstrated high-throughput SNP genotyping by Allele-Specific PCR with universal energy-transfer-labeled primers. Petterson *et al.* (2003) evaluated AS-PCR and pyro sequencing. Wittwer *et al.* (2003) developed amplicon melting analysis using LC green. Singh *et al.* (2004) differentiated members of the *Anopheles fluviatilis* and *Anopheles culicifacies* species complex by AS-PCR. Zhou *et al.* (2004) described closed tube genotyping with unlabelled oligonucleotide probes and a saturating DNA dye.

Nasis *et al.* (2004) described that improvement in sensitivity of allele-specific PCR facilitates reliable noninvasive prenatal detection of cystic

fibrosis. Liew *et al.* (2004) described high resolution melting of small amplicons. Shah *et al.* (2005) described AS-PCR method based for distinguishing *Salmonella gallinarum* from *Salmonella pullorum*. Koizumi *et al.* (2005) investigated the specificity and sensitivity of PCR with primers containing the ENA residue at the 3' end.

Sztankoova *et al.* (2006) developed a new allele specific polymerase chain reaction method (AS-PCR) for detection of the goat CSN1S1 allele. Hermann *et al.* (2006) explained amplicon DNA melting analysis. Corless *et al.* (2006) described Allele AS-PCR for the imatinib-resistant mutations in mastocytosis and acute myelogenous leukemia. Canadas *et al.* (2007) described molecular haplotyping of tandem single nucleotide polymorphisms by allele-specific PCR. Diaza *et al.* (2007) described individual single tube genotyping and DNA pooling by allele-specific PCR.

Hayashi *et al.* (2008) described genotyping by allele-specific L-DNA-tagged PCR. Kaminski *et al.* (2008) developed MilkProtChip - oligonucleotide micro array allowing bovine genotyping based on SNPs in genes influencing milk protein biosynthesis. Dettori *et al.* (2009) developed a reliable AS-PCR method for the reliable characterisation of the goat CSN1S1 allele. Hoti *et al.* (2009) described an allele specific PCR assay for screening for drug resistance among *Wuchereria bancrofti* populations. Bui *et al.* (2009) explained simple allele discriminating PCR (SAP).

2.6 VECHUR CATTLE

Girija (1994) reported the diploid chromosome number in Vechur cattle as 60. The acrocentric nature of Y chromosome establishes that the Vechur cattle belong to the species of the zebu cattle (*Bos indicus*) as different from European cattle (*Bos taurus*), which has the metacentric chromosome. The

percentage of fat and total solids in the milk of Vechur cows was higher compared to crossbred cow (Venketachelapathy, 1996). Vechur cows are considered the pride of Kerala. These animals derived their name from the village of origin, Vechur, a small place by the side of Vembanattu lake near Vaikom in Kottayam district of central Kerala. The FAO has listed this breed in their Domestic Animal diversity Information system (Iype and Venketachalapathy, 1997).

Venketachelapathy and Iype (1997) reported that Vechur cattle has unique characteristics of its own and have separate identity from other breeds of cattle not only by its small size but also due to its milk protein variants, composition of milk, size of fat globules and level of saturated fatty acids. Vechur cattle of Kerala are small sized with an average weight of about 130 kg for cows and 170 kg for bulls. These animals need less feed as compared to crossbreds with an yield of 2-3 kg milk per day and they are adapted to hot and humid local environment (Iype and Venketachalapathy, 2001).

2.6.1 Molecular Studies on Vechur Cattle

Aravindakshan *et al.* (2003) analysed the genetic polymorphism at the third exon of bovine prolactin (PRL) gene in Vechur cattle by PCR based RFLP. Aravindakshan *et al.* (2004) carried out investigations on the genetic polymorphism at the β lactoglobulin locus in vechur cattle using PCR-RFLP. Suprabha (2005) used the band sharing (BS) values in RAPD-PCR analysis of dwarf cattle of Kerala of each group and between groups as an analytical tool to find out genetic variation among animals. The Vechur, Vatakara and Vechur-Vatakara combinations were found to be more uniform with high BS value. The Vechur cattle were screened for the presence of the bovine leukocyte adhesion deficiency (BLAD) genetic defect using the PCR-RFLP and DNA sequencing methods and found that none carry the BLAD allele

(Aravindakshan *et al.*, 2006). Aravindakshan *et al.* (2007) reported the gene frequencies of GH/Msp I polymorphism in Vechur cattle. Aravindakshan and James (2008) studied κ- CN gene polymorphism in Vechur and Kasargode cattle. Anand (2009) studied leptin gene polymorphism in Vechur and crossbred cattle of Kerala.

2.7 MILK PRODUCTION

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 with a given trait. It is therefore possible to genotype an animal using a DNA based genotyping test and selects individuals carrying the preferred genetic variant. β-CN gene is a promising quantitative trait locus whose polymorphism is greatly associated with milk volume.

2.7.1 Vechur cattle

Girija (1994) reported the mean total lactation yield of 471 kg in Vechur cattle while the average daily milk yield reported was 2.17 kg. According to Venketachelapathy and Iype (1997), the fat and total solids percentages of Vechur milk showed increasing trend as the lactation advanced. Iype and Venketachelapathy (2001) recorded average daily milk yield (2.2 kg) and yield (3.6 kg) in Vechur cattle.

2.7.2. Crossbred cattle

To improve milk production, local cattle of Kerala were crossed with exotic breeds Jersey, Brown Swiss and Holstein Friesian. Now Kerala has a mosaic population of exotic and local cattle called Sunandini cattle. Chacko and Jose (1988) reported the average first lactation milk yield 2500 kg.

Radhika (1997) evaluated the production performance of crossbred cattle population maintained in Kerala Agricultural University farms and reported the average 305 day milk yield as 1829.68 kg. The average daily milk yield of pooled population consisting of purebreds and crosses of Sahiwal, Jersey and Friesian breeds was reported as 8.64 kg by Kant and Prasad (1997).

Kannan *et al.* (2000) studied the production performance of cross bred dairy cattle and reported a mean 305 day lactation yield of 2113 kg. Thomas (2005) associated microsatellite markers with milk production traits in crossbred dairy cattle and reported that 305 day milk yield of crossbreds of Kerala was 2070.5 kg.

According to Thirumurugan and Saseendran (2006), the average daily milk yields of crossbred cattle maintained in various housing systems in Kerala Agricultural University livestock farms ranged from 7.78 to 8.86 kg. Reshmi and Stephen (2007) reported the mean 305 day milk yield of cattle from different zones of Kerala as 2406 kg. Lali (2009) reported the average milk yield standardized to 305 days as 348.01Kg in Vechur and 2106 kg in crossbred cattle.

Materials and Methods

3. MATERIALS AND METHODS

Blood samples collected from 72 Vechur cattle, 100 crossbred (Sunandini) cows and 14 Kasargode Dwarf cattle formed the materials for the present study on β-CN gene polymorphism. Of the total 72 Vechur cattle, 62 animals were from KAU farm and the remaining 10 numbers were obtained from animals maintained by Vechur Conservation Trust, Vechur.

Vechur is a small breed of cattle which were very popular in Vechur area of Kottayam district, Ernakulam and Alappuzha. Though they were very small of about 150 kg adult body weight they had the capacity to produce 2-3 Kg milk daily with fat content above 5%. They were thought to be extinct by the aggressive crossbreeding policies followed in Kerala by using exotic germplasm on local female cattle. In the year 1989 a search for the existence of legendary Vechur cattle was launched by the students of Veterinary College under the leadership of Anil Zachariah and Prof. Sosamma Iype. Their search became successful and they could identify and locate a handful of Vechur and Vechur like small animals, thanks to a few poor illiterate farmers who conserved this germplasm resisting all actions exterminating the local breeds. That was the beginning of Vechur Conservation programme at Kerala Agricultural University. The present population of Vechur animals will be around 200 including 78 animals in the KAU Vechur farm. The crossbreds used in the study were from different parts of Kerala *viz*, Kozhikode (22), Kannur (15), Malappuram (14), Wayanad (15), Thrissur (24) and Kottayam (10). Most of the crossbred cattle in Kerala are having exotic inheritance ranging between 50-62.5% of Jersey/ Brown Swiss/Holstein Friesian or a combination of these breeds. The 14 Kasargode Dwarf cattle were from the animals maintained at KAU farm unit.

3.1 COLLECTION OF SAMPLES

From each animal, 5 ml of blood was collected from the jugular vein into sterile 15 ml polypropylene centrifuge tube (vacutainer) containing Ethylene Diamine Tetra Acetic acid (EDTA) as anticoagulant (1 mg/ml of blood). The samples were brought to the laboratory at 4°C, temperature being maintained with the aid of ice packs and stored at -20°C until needed for DNA extraction.

3.2 ISOLATION OF DNA FROM WHOLE BLOOD

DNA was extracted from whole blood using the standard phenol chloroform extraction procedure (Sambrook and Russell, 2001) with modifications. Though the minimum incubation period recommended for proteinase K/sodium dodecyl sulphate digestion was three hours, in this study the samples were kept overnight. The procedure followed was

1. To 5 ml blood, double the volume of ice cold RBC lysis buffer (150 m*M* ammonium chloride, 10 m*M* potassium chloride and 0.1 m*M* EDTA) was added and kept in ice cold condition with occasional mixing for 10 min for complete lysis of red blood cells.
2. The leukocytes were pelleted by centrifuging at 4000 rpm for 10 min, and the supernatant containing lysed RBCs was discarded.
3. The pellet was resuspended in ice-cold RBC lysis buffer and the above two steps were repeated till a clear pellet was obtained.
4. The pellet was then washed twice with Tris buffered saline (TBS-140 m*M* sodium chloride, 0.5 m*M* potassium chloride and 0.25 m*M* Tris base) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes.

5. The white blood cell pellet was resuspended in 3 ml saline EDTA buffer (SE-75 m*M* sodium chloride and 35 *mM* EDTA). The cell suspension was incubated at 50°C in water bath with 0.25 ml of 20 % sodium dodecyl sulphate (SDS) and 25 µl of proteinase-K (20 mg/ml) for overnight. Swirled the viscous solution occasionally.
6. The digested samples were cooled to room temperature, 300 µl of 5 *M* sodium chloride was added and mixed. An equal volume of phenol (pH 7.8) saturated with Tris-hydrochloride was added, mixed by gentle inversion for 10 min and centrifuged at 4500 rpm for 10 minutes.
7. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol: chloroform: isoamyl alchohol (25:24:1) was added. The contents were mixed and centrifuged at 4500 rpm for 10 minutes.
8. The aqueous phase was transferred in fresh tubes, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 4500 rpm for 10 minutes.
9. 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.
10. 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% ethanol and air-dried.
11. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE-10 m*M* Tris base, 0.1 *mM* EDTA) and stored at -20°C

3.3 ASSESSMENT OF DNA SAMPLES

3.3.1 Assessment of Yield and Purity

From the DNA stock solution, 20 µl was diluted with 1980 µl of sterile triple distilled water. Optical densities (OD) were measured at 260 nm and 280

nm wavelengths using disposable cuvettes in an UV spectrophotometer (Jenway, UK) using sterile triple distilled water as blank. The yield and purity of DNA sample were estimated as follows.

3.3.1.1. Yield

An OD of 1 at 260 nm wavelength is equivalent to 50 µl/ml of double stranded DNA. Concentration of DNA stock solution per ml was found out by multiplying OD 260 with a factor of 5000. The total yield of DNA was calculated by multiplying the concentration and the volume of DNA stock solution. Concentration of DNA stock solution (µl/ml) = OD_{260}* Dilution factor*50.

3.3.1.2 Purity

Purity of DNA stock solution was estimated by finding the ratio between the optical density readings at 260 nm and 280 nm wavelengths (OD_{260} / OD_{280}).

3.3.2 Quantification and quality check of DNA by agarose gel electrophoresis

The quality and molecular weight of DNA were measured electrophoretically using 0.8% agarose. An appropriate sized clean gel tray was selected and set with suitable combs. The agarose gel was prepared in 1X Tris EDTA buffer (TBE- 0.045*M* Tris borate and 0.001 *M* EDTA), boiled and allowed to cool to 50°C. Five microliter of ethidium bromide (10 mg/ml) was added into the molten agarose and then poured into the tray to polymerize. After solidification, the sealing and comb were removed gently and the gel with tray was immersed in the 1X TBE buffer in the electrophoresis tank with the wells towards the negatively charged electrode. One microliter of each DNA

sample was mixed with one-sixth the volume of 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose) and loaded into the wells. Horizontal electrophoresis unit was set at 80 V for 2 hours and the gel was visualized after the electrophoresis under UV Transilluminator (Hoefer MacroVue^{TM}).

3.3.3 Template DNA

Template DNA for PCR was prepared by diluting the DNA stock solution with sterile triple distilled water to a final concentration of 50 ng/µl.

3.4 AS-PCR ANALYSIS

AS-PCR is a modification of PCR in which the DNA polymerase is used to amplify the target DNA only if the PCR primers are perfectly complementary to the target DNA sequence. It relies on the use of an oligonucleotide primer for PCR amplification that precisely matches one of the alleles but not the other. AS-PCR makes use of the fact that product formation in PCR is sensitive to mismatching of the 3' residue in one of the two primers (forward or reverse) used. When a mismatch occurs at or near the 3'end of the PCR primer, amplification is inefficient.

3.4.1 Classification of Alleles and Primers Used

As already reviewed there are 12 alleles (A1, A2, A3, B, C, D, F, G, H1, H2, I and G) of β-CN in bovines. Of all the 12 β-CN variants all except A2 produce BCM-7 upon enzymatic digestion in the gut. Among BCM-7 yielding variants A1 is the predominant type and other alleles are very rare. Therefore in this study alleles are classified as A1 and A2 only. Based on this, two allele specific reverse primers each one matched to one of two alleles at 3' end (G for

β-CN A2 and T for β -CN A1) along with a common forward primer were used (Keating *et al.*, 2008). The sequences of primers are given in Table 3.1.

The specificity of the primers was checked by NCBI blast programme (http://www.ncbi.nlm.nih.gov/BLAST/). Primers were checked for possible hairpin, self dimer and cross dimer using software PRIMER EXPRESS.

Oligos supplied in freeze –dried powder form were reconstituted in 0.3X TE buffer to make primer concentration 100 pmol/µl and further diluted in MiliQ water to give a final concentration of 10 pmol/µl.

3.4.2 Setting up of PCR

For setting up the PCR a master mix was prepared (Sambrook and Russell, 2001). Components of master mix along with their final concentration in PCR, which gave the desired amplification, are given in Table 3.2 below. Difference in the reverse primer used in the final reaction volume of 10 µl in each PCR tube decided the product formation (either A1 or A2) in PCR. All reactions were carried out in 200 µl thin walled PCR tubes. Master Mix was prepared for one additional sample to cover pipetting error. DNA was diluted to 10 to 150 times according to the quantity of DNA in the sample and was used as template for AS- PCR reaction. Components along with final concentration in PCR are given in Table 3.2. PCR tubes containing mixture were tapped gently and quickly spinned @10,000 rpm for few seconds. The tubes were placed in thermal cycler and subjected to PCR. PCR was standardised for discrimination of two alleles (Table 3.3).

Each sample was amplified with A1 and A2 reverse primer in separate tubes for detecting A1 and A2 alleles. Amplification in these two tubes containing either A1 or A2 primer was checked by running 5 µl of AS–PCR

product from each tube along with 100 bp molecular weight marker (Gene Ruler, MBI Fermentas) on 2% agarose gel.

3.5 AGAROSE GEL ELECTROPHORESIS

The AS–PCR products were checked electrophoretically using 2% agarose as described in chapter 3.3.

3.6 ALLELE AND GENOTYPE FREQUENCIES

The allelic and genotype frequencies at A1/A2 locus were calculated by direct counting method for Vechur, Kasargode and crossbred cattle separately. The variations of the allelic frequencies among the three groups were analyzed by the *Chi*-square test of significance as described by Snedecor and Cochran (1994) considering the allelic frequencies in a 2×2 table using the formula,

$$\chi^2 = \frac{(ad-bc)^2 N}{(a+c)(b+d)(a+b)(c+d)}$$

where a, b, c and d are allelic frequencies in different groups and N is the total number of alleles observed.

The distribution of A1/A2 genotypes in Vechur and crossbred cattle population was checked for Hardy-Weinberg equilibrium by *Chi*-square test comparing the observed and expected frequencies.

3.7 MILK PRODUCTION TRAITS

The information regarding the milk yield for Vechur cattle was collected from the records maintained in the farm. Since the lactation length of Vechur cows was less than the standard lactation length, average daily milk

yield was calculated for studying the influence of β-CN gene polymorphism on milk yield. For crossbred animals the peak yield was obtained from the farmers as milk recording was not done by them. *In Kasargode Dwarf cattle milk yield* is very less and therefore the information from them was not considered.

3.7.1 Influence of β-CN Gene Polymorphisms on Milk Production Traits

There were only two genotypes (A1A2 and A2A2) in Vechur cattle and the difference in milk yield of the two genotypes was compared by T-test. In crossbred cattle there were three genotypes- A1A1, A1A2 and A2A2 and the effect of genotype on milk yield was determined by analysis of variance (ANOVA).

Table 3.1 Primer sequence for Allele Specific PCR

Primer Type	Primer sequence	Size of the product
FORWARD FOR A1 AND A2 ALLELES	5' GCCCAGATGAGAGAAGTGAGG-3'	854bp
A2 ALLELE REVERSE	5' GATGTTTTGTGGGAGGCTGTTAG-3'	
A1 ALLELE REVERSE	5' GATGTTTTGTGGGAGGCTGTTAT-3'	

Table 3.2 Components used for AS- PCR

PCR components	Concentration	Volume (µl)		Final Conc. in 10 µl
		A1	A2	
Deionised water		5	5	
10X buffer	20m*M* Tris-HCl & 500m*M* KCl	1	1	1X
$Mgcl_2$	15m*M*	1	1	1.5mM
dNTPs	2.5 m*M*	0.8	0.8	200 µ*M*
Template	50 ng/µl	1	1	
Forward primer	10 pmol/µl	0.5	0.5	5pm
A1 Reverse primer	10 pmol/µl	0.5		5pm
A2 Reverse primer	10 pmol/µl		0.5	5pm
Taq polymerase	5 U/µl	0.2		1U
Total		10	10	

Table 3.3 PCR protocol for AS amplification of β-CN A1 and A2 alleles.

Initial Denaturation	Denaturation[1]	Annealing[2]	Extension[3]
95^0 C for 2 min	95^0 C for 1 min	58^0 C for 1 min	72^0 C for 1 min
Repeat 1,2 and 3 for 30 times			
Final extension at 72^0C for 10 min			

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

Good quality DNA samples were obtained from the blood samples of Vechur, crossbreds of Kerala and Kasargode Dwarf cattle using phenol chloroform extraction procedure. The ratio of optical density at two wavelengths (OD 260/ OD 280) was in between 1.7-1:9. Single, clear band without shearing was observed on agarose gel upon electrophoresis (Fig. 4.1).

4.2 AS-PCR ANALYSIS

Using genomic DNA as template, and under optimised reaction protocol (Table 3.3) desired band of 854 bp was amplified with no nonspecific bands. Thus genomic DNA was found to be suitable template for this AS-PCR reaction.

4.2.1 Heterozygous Samples

The samples with equal band intensity were considered heterozygous and if not the homozygous (with false amplification in A1 or A2). Fig. 4.2 shows sample 2 as heterozygous (equal amplification in A1 and A2) and sample 1and 3 as A1 homozygous (weak amplification in A2) based on band intensities compared to that of heterozygous sample (equal intensity bands in both A1 and A2).

4.3. AGAR GEL ELECTROPHORESIS

PCR products were checked in 2% agar gel along with 100 bp ladder. Clear bands with approximate size of 854 bp were visible (Fig. 4.3).

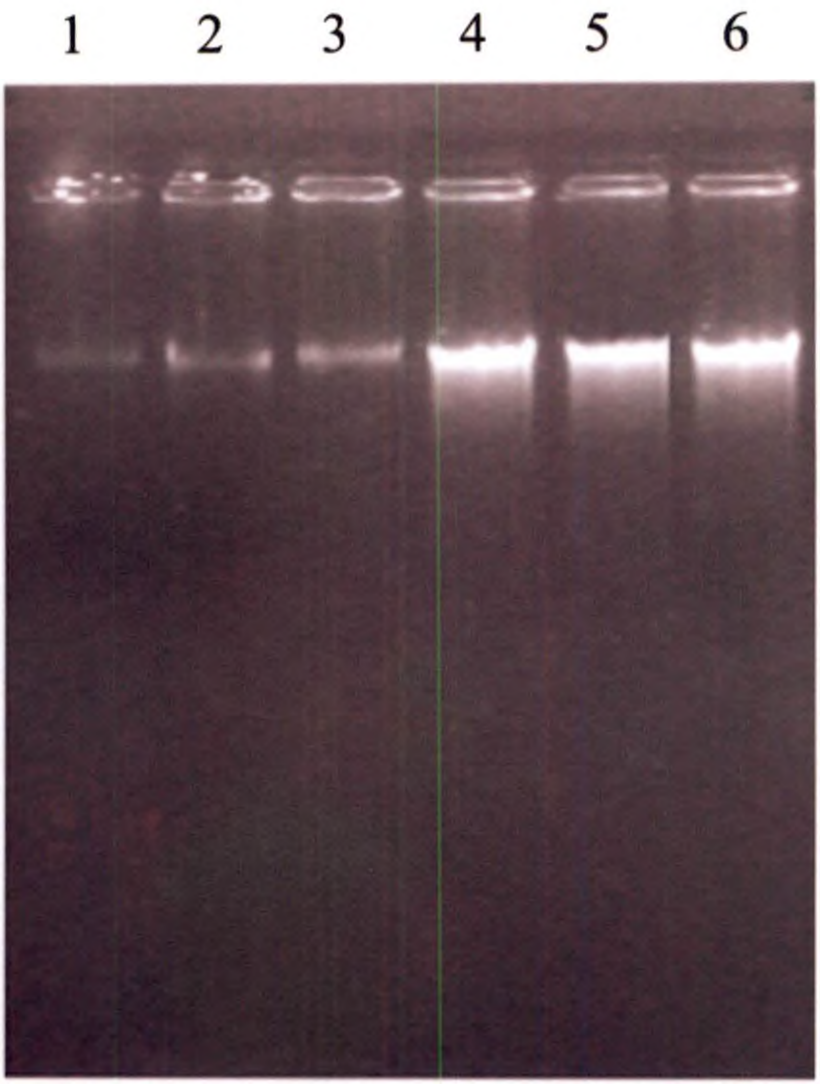


Fig 4.1 DNA isolated by phenol chloroform extraction procedure on 0.8% agarose gel

Lane 1-3: PCR Template

Lane 4-6: Stock DNA

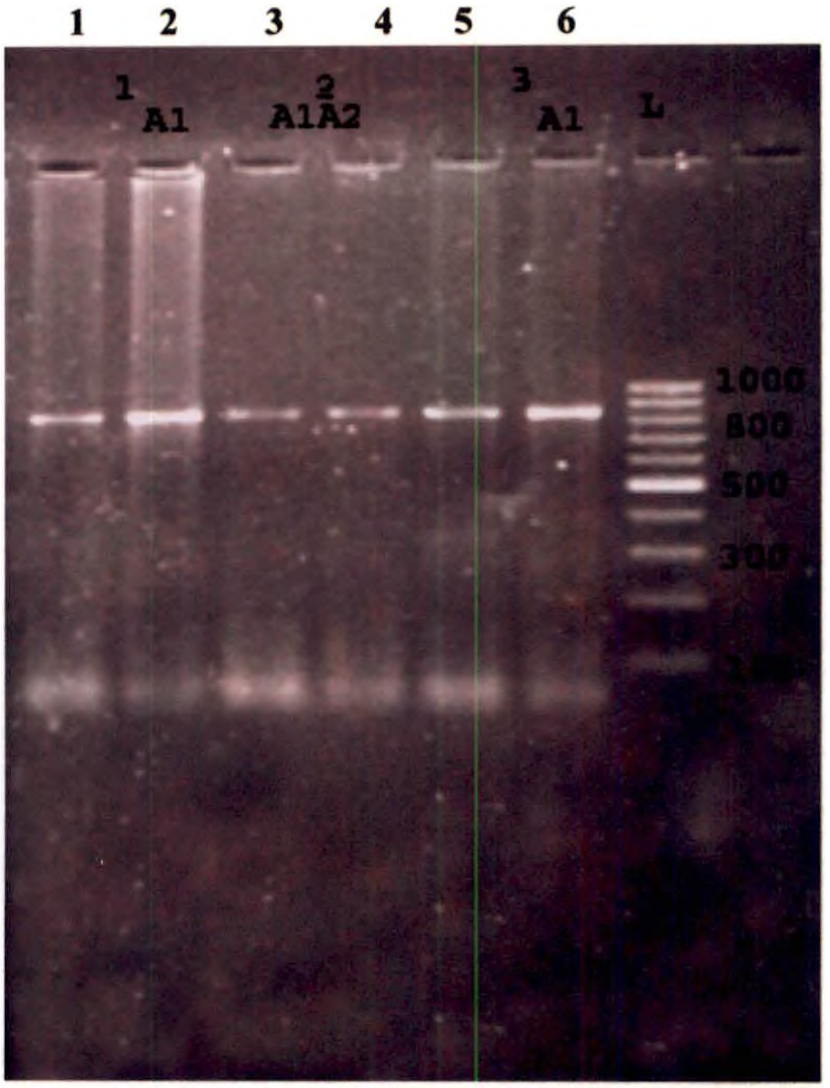


Fig 4.2 AS-PCR amplification of 854 bp fragment rendering heterozygous and homozygous discrimination

Lane 1: Sample 1 &A2 primer; Lane 2: Sample 1 &A1 primer. Interpretation: Lane 1(A2) shows weak amplification, Hence sample 1 is A1 homozygous (A1A1).

Lane 3: Sample 2 &A2 primer; Lane 4: Sample 3&A1 primer. Interpretation: Equal amplification in both Lane 3(A2) &Lane 4(A1).Hence sample 2 is heterozygous (A1A2)

Lane 4: Sample 3&A1 primer; Lane 4: Sample 3&A2 primer. Interpretation: Similar to sample 1, Lane 4(A2) shows weak amplification, Hence sample 3 is A1 homozygous(A1A1).

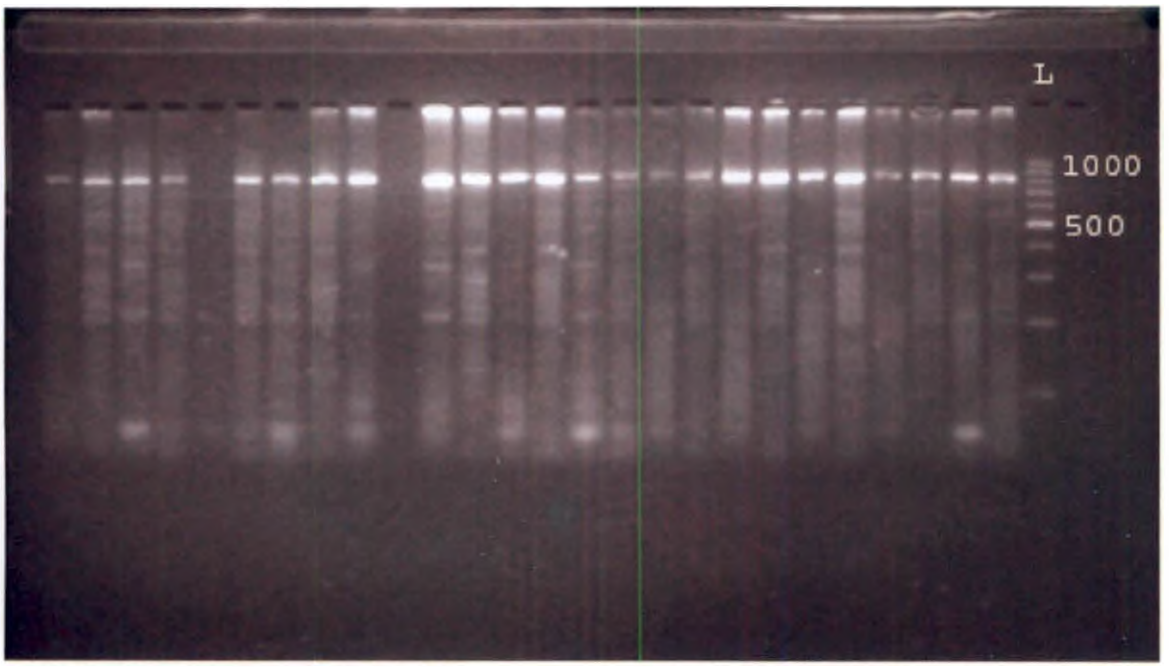


Fig.4.3 Optimisation of AS-PCR amplification of 854 bp fragment, rendering allele discrimination possible showing no extra non specific amplification.

AS-PCR amplification of 13 samples showing allele discrimination with clear bands.

L-100 bp ladder

4.4 GENOTYPE AND ALLELE FREQUENCIES OF β-CN

The allele and genotype frequencies of β-CN A1A2 in Vechur, crossbred cattle and Kasargode Dwarf cattle of Kerala studied are presented in Table 4.1.

4.4.1 Genotype Frequency

In Vechur cattle the genotype frequencies of A1A1, A1A2 and A2A2 were observed as 0, 0.34 and 0.66 whereas in crossbred cattle the corresponding frequencies were 0.32, 0.28 and 0.40 respectively (Table 4.1). Of the 72 vechur cattle typed, none of the animals were of A1A1 genotype. No A1A1 genotypes were found in Kasargode Dwarf animals also and the corresponding frequencies in Kasargode Dwarf cattle was recorded as 0.79 (A1A2) and 0.21 (A2A2).

4.4.2. Allele Frequency

The frequencies for A1 and A2 alleles in Vechur were noted as 0.2 and 0.80 respectively and for crossbreds the frequencies were recorded as 0.46 and 0.54, respectively. In the Kasargode Dwarf cattle A1 and A2 allele frequencies were obtained as 0.39 and 0.61 respectively.

4.4.3. Allele Frequencies at β-CN Locus and Test for Hardy-Weinberg Equilibrium

The *Chi*-square test showed that A1 and A2 alleles were not homogenously distributed in Vechur, crossbred and Kasargode Dwarf cattle (Table 4.2). By comparing the observed and expected frequencies using *Chi*-square test (Table 4.3), crossbreds were not found to be in Hardy-Weinberg equilibrium while Vechur and Kasargode Dwarf cattle were found to be in equilibrium.

4.5. MILK PRODUCTION TRAITS

4.5.1. Average Daily Milk Yield and Peak Yield

The average daily milk yield of 50 vechur cows were found to be 1.27±0.07 kg and the peak yield of 100 crossbred cattle under this study were found to be 10.71±1.046 kg. The milk yield of Kasargode Dwarf cattle were not recorded as the number of observations was very less.

4.5.2. β- CN Polymorphism and Milk Yield

β-CN A1/A2 polymorphism had significant effect on average daily milk yield in the Vechur cattle (Table 4.4) and peak yield in crossbred cattle groups studied (Table 4.5). In t test, Vechur cattle carrying A1A2 genotype showed a significantly higher average daily milk yield (1.52±0.08 kg) compared to A2A2 genotype (1.14±0.04 kg). In ANOVA, crossbred cattle carrying A1A1 genotype showed significantly higher average for peak yield (14.64±3.181 kg) whereas A1A2 and A2A2 genotypes had lower average peak yield (8.54±0.019 kg and 9.09±0.125 kg respectively). In DMRT, the peak yields of A1A2 and A2A2 were not significantly different while the peak yield of A1A1 genotype was significantly higher than the other two genotypes.

Table 4.1 Genotype and allele frequencies of β-CN A1/A2 polymorphism in Vechur, crossbred and Kasargode Dwarf cattle of Kerala

Population	Genotype frequency			Allele frequency	
	A1A1	A1A2	A2A2	A1	A2
Vechur cattle (72)	0	0.34 (29)	0.66 (43)	0.20 (29)	0.80 (115)
Crossbred cattle (100)	0.32 (32)	0.28 (28)	0.40 (40)	0.46 (92)	0.54 (108)
Kasargode cattle (14)	0	0.79 (11)	0.21 (3)	0.39 (11)	0.61 (17)

Figures in parenthesis are actual numbers

Table 4.2 Comparison of frequencies of β-CN A1A2 alleles in Vechur, crossbred and Kasargode Dwarf cattle of Kerala

Population	A1 allele	A2 allele	χ^2 value (df=2)
Vechur	0.20 (29)	0.80 (115)	24.65**
Crossbred cattle	0.46 (92)	0.54 108)	
Kasargode Dwarf	0.39 (11)	0.61 (17)	

** – Significant at 1% level

Figures in parenthesis are number of observations

Table 4.3 Testing of genotypes of β-CN A1/A2 polymorphism for Hardy–Weinberg equilibrium in Vechur, crossbred and Kasargode Dwarf cattle

Population	Source	Genotypes			χ^2 value (df=1)
		A1A1	A1A2	A2A2	
Vechur	Number Observed	0	0.40 (29)	0.60 (43)	4.58
	Number Expected	2.92	23.16	45.92	
Crossbred cattle	Number Observed	0.32 (32)	0.28 (28)	0.40 (40)	19.04**
	Number Expected	21.16	49.68	29.16	
Kasargode Dwarf	Number Observed	0	0.79 (11)	0.21 (3)	5.86
	Number Expected	2.16	6.68	5.16	

** – Significant at 1% level
df – degrees of freedom
Figures in parenthesis are actual numbers

Table 4.4 β-CN A1/A2 polymorphism and average daily yield in Vechur cattle

Sl. No.	Milk production traits	Genotype of Vechur cattle (Mean± SE)	
		A1A2	A2A2
1	Average daily yield (kg)	1.52±0.08[a] (29)	1.14±0.04[b] (43)

Superscript with different alphabet differ significantly at 5% level
Number of observations in parenthesis

Table 4.5 β-CN A1/A2 polymorphism and average daily yield in crossbred cattle

Sl. No.	Milk production traits	Genotype of crossbred cattle (Mean± SE)		
		A1A1	A1A2	A2A2
1	Peak yield (kg)	14.64±3.181[a] (32)	8.54±0.194[b] (28)	9.09±0.125[b] (40)

Superscript with different alphabet differ significantly at 5% level
Number of observations in parenthesis

Discussion

5. DISCUSSION

5.1. ISOLATION OF GENOMIC DNA

DNA isolation from whole blood was carried out using the standard phenol chloroform extraction procedure (Sambrook and Russell, 2001), with the modification of overnight incubation of the WBC suspension with SDS and proteinase K. The ratio of optical density at two wavelengths (OD 260/ OD 280) was in between 1.7-1.9, indicative of the good quality of genomic DNA without contamination of protein. Single, clear band without shearing on agarose gel upon electrophoresis indicated the presence of good quality high molecular weight DNA (Fig. 4.1).

5.2. AS-PCR ANALYSIS

A triumvirate of methods, cloning, DNA sequencing and PCR underlies almost all modern molecular biology. Of these PCR is the oldest in theory and most versatile in practice. The method was first proposed in the early 1970's by H.G. Khorana, and the technique was independently conceived in 15 years later by Kary Mullis. Single nucleotide polymorphisms (SNPs) are the most common source of genetic variation. There are potential uses of SNPs in genetic mapping, pharmacogenetics, medical diagnostics and animal breeding. Such applications require reliable and economical methods for high throughput SNP genotyping (Myakishev *et al.*, 2001). In addition to its simplicity PCR is robust, speedy and most of all, flexible. An enormous number of variations on the method have been described including PCR using mutation specific inosine containing oligonucleotide primers (Ehlen and Dubeau, 1989), primer guided nucleotide or dye-terminator incorporation (Pastinen *et al.*, 1996), hybridization with allele specific probes (Tyagi *et al.*, 1998), Tetra primer ARMS-PCR combined with microplate array diagonal electrophoresis (Ye *et*

al., 2001), high throughput SNP genotyping by AS-PCR with universal energy transfer labeled primers (Myakishev *et al.*, 2001), haplotype determination using AS-PCR and pyro sequencing (Petterson *et al.*, 2003), amplicon melting analysis using LC green (Wittwer *et al.*, 2003), closed tube genotyping with unlabelled oligonucleotide probes and a saturating DNA dye (Zhou *et al.*, 2004), high resolution melting of small amplicons (Liew *et al.*, 2004), AS-PCR using primers modified with an ENA residue (Koizumi *et al.*, 2005), amplicon DNA melting analysis (Hermann *et al* ., 2006), haplotyping of tandem SNPs by AS-PCR (Canadas *et al.*, 2007), WASP: Web-based Allele Specific PCR (Wangkumhang *et al.*, 2007) and simple allele discriminating PCR (SAP) (Bui *et al.*, 2009). Although these methods improve SNP genotyping, they involve multistep sample processing, which is a disadvantage in high throughput tasks. The drawbacks of these methods are that the reactions must be monitored in real time, which requires expensive instrumentation, and/or it is necessary to design and size costly probe for each SNP.

Comparison of the reported cDNA sequences of β-CN in different species demonstrates that the N-terminal region is conserved (Choi *et al.*, 1996). Moreover no pseudogenes are reported, thus chance of non specific amplification is less. AS-PCR provides a rapid method for detecting SNPs. AS- PCR works allele specifically and discriminate wild type or mutant allele at an optimised annealing temperature (Boyle *et al.*, 2009). The simplicity, accuracy and reproducibility of AS-PCR make it highly promising method of population screening. Multiple individuals can be screened simultaneously and once the DNA is on hand the cost of supply and labour is very less per AS-PCR reaction. Partial automation already exists and complete automation which is feasible by combining AS-PCR with robotic and currently available nucleic acid detection technology will favour high throughput.

AS-PCR involves designing a PCR oligonucleotide primer that amplifies one allele but not the other. When the 3' base of the oligonucleotide primer mismatched an allele, no amplification product could be detected. Primers as short as 13 nucleotides are effective. (Sarkar *et al.*, 1990). Designing of AS-PCR primers are based on effects of primer template mismatches on the PCR (Chapter 2.5.1.3). Several automated bioinformatics tools are available for specifically designing AS primer, such as, Tetra-primer, Primo SNP, commercial software Visual-OMP and WASP (Wangkumhang *et al.*, 2007). Keating *et al.*, (2008) evaluated β-CN variants in bovine breeds by AS-PCR with mismatched primers. Priming probabilities of a mismatched primer are good and universal features to assess the performance because they can be measured with any primer pair, on any given target, with any master mix and in any PCR conditions (Boyle *et al.*, 2009). Priming probabilities are essential to describe analytical specificity as required in the MIQE guidelines (Bustin *et al.*, 2009). The strength of destabilization for all combinations of nucleotide pairing was explained by Bui *et al.* (2009). Use of internal mismatches is also successfully employed in goats (Sztankoova *et al.*, 2006; Dettori *et al.*, 2009). Amplification obtained in the present reaction strictly followed these observations.

Several theoretical descriptions of PCR have been put forward that apply different mathematical approaches to simulate various parameters of the system (Kwok *et al.*, 1990). These include statistical estimations of amplification rate, probability of DNA replication, probability of DNA binding rates and derivation of expression of amplification efficiency. Michelis-Menten kinetics has been used to determine the variation of the rates of many enzyme catalyzed reactions as the substrate or effector concentration is varied (Waterfall *et al.*, 2002). Factors that have been attributed to attenuation of PCR include depletion of substrate (dNTPs or primers), thermal inactivation or

limiting concentration of DNA polymerase, inhibition of enzyme activity by increasing pyrophosphate production, reannealing of amplicon at concentrations above 10^{-8} M, reduction in the denaturation efficiency per cycle, destruction of product due to enzyme 5'-3' exonuclease activity, product–to–product reannealing or the chelation of critical metal ions by the substrate depriving the enzyme of a cofactor. Substrate accumulation was more accurate for a match- than mismatch primed PCR (Ayyadevara *et al.*, 2000). Thus it is necessary to optimize the PCR conditions such as cycle number, annealing temperature and Mg^{2+} concentration for each primer sequence to avoid mismatched amplification (Petterson *et al.*, 2003) and the desired amplification could be obtained.

5.2.1 Heterozygous Samples

In case of heterozygous samples, A1 and A2 reactions should give amplification. While typing some of the samples, amplification was observed in both reactions but with unequal intensities. This led to confusion that the sample was really heterozygous one or there was a false positive feeble amplification in one of the reactions. Thus the samples with equal band intensity were considered heterozygous. Even heterozygous sample may give differential amplification due to sequence dependent differences in mispairing discrimination (Ayyadevara *et al.*, 2000).

Wu *et al.* (1989) reasoned that a set of conditions should exist such that $r_{pol} > r_{off}$ for the perfectly matched primer, while $r_{pol} < r_{off}$ for the mismatched primer. The results shown here clearly demonstrate this to be true.

5.3 AGAR GEL ELECTROPHORESIS

Agar gel electrophoresis of the PCR products using 2% agar yielded detectable bands. The same sized PCR product (854bp) was reported earlier by Keating *et al.* (2008) in various purebred cattle using the same primers.

The polymorphism of β-CN gene was investigated in Vechur, crossbred and Kasargode cattle of Kerala by AS-PCR. In all the animals tested, the size of the amplified product was between 800 and 900 bp indicating a product size of 854 bp as expected (Fig.4.3). This suggested the conservation of DNA sequences at the β-CN A1/A2 locus in *B. indicus* and their crossbreds with *B. taurus*. In bovines, rats and humans no nucleotide substitutions, insertions, deletions or other rearrangements were evident in β-CN gene (Stewart *et al.*, 1987). The absence of major sequence rearrangements in the β-CN gene and the maintenance of hydrophilic N-terminal and hydrophobic C terminal domains despite many amino acid replacements, points to the operation of functional constraints acting to conserve the overall architecture of the molecule.

5.4. GENOTYPE AND ALLELE FREQUENCIES OF β-CN

Genotype and allele frequencies are given in Table 4.1. The chi-square test showed that there were significant differences in allele frequency between three groups studied (Table 4.2). Test for Hardy-Weinberg equilibrium revealed that the allele frequency of Vechur and Kasargode Dwarf cattle studied are in equilibrium while, in crossbreds not in equilibrium (Table 4.3).

5.4.1 Vechur Cattle

The genotype frequencies of β-CN A1/A2 polymorphism in Vechur cattle were observed as 0 for A1A1, 0.34 A1A2and 0.66 for A2A2. The

frequencies of A1 and A2 alleles were observed as 0.20 and 0.80 respectively, indicating the predominance of A2 allele in the population. Similar results were reported by Mishra *et al.* (2009) as 0.191, 0.218 for A1A2 and 0.809, 0.783 for A2A2 in Malnad Gidda and Kherigarh cattle respectively. Lower frequency of A1A2 and absence of A1A1 were reported earlier (Table 2.2). Malik *et al.* (2000) earlier reported A1A2 genotype frequency in Sahiwal cattle as 0.458 for A2A2 and 0.50 for A1A2.

In contradiction to the present findings in Vechur cattle with regard to the genotype frequencies of β-CN A1/A2 polymorphism the absence of A1A2 genotype was reported in most of the *B.indicus* cattle except Malnad Gidda and Kherigarh (Mishra *et al.*, 2009).

Mishra *et al.* (2009) made similar findings in Malnad Gidda and Kherigarh cattle and reported A1 and A2 allele frequencies as 0.096, 0.109 and 0.904, 0.891 respectively. Malik *et al.* (2000) reported A1 frequency as 0.25 and A2 frequency as 0.71 in Sahiwal cattle. Perusal of literature revealed that the allele frequencies of β-CN A1/A2 polymorphism were different in various breeds of *B.taurus, B. indicus* and their crossbreds (Table 2.3).

The presence of A1 allele of β-CN was also reported in *B. indicus* breeds outside India. The frequencies of A1 and A2 alleles were 0.18 and 0.80 respectively in Brahman cattle (Jann *et al.*, 2004). Ceriotti *et al.* (2004) reported the allele frequencies in African *B. indicus* breeds Sudanese Zebu Pearl, Azaouak and Adamawa. The A1 allele frequencies in these breeds were 0.12, 0.12 and 0.26 respectively. The A2 allele frequencies were 0.74, 0.8, and 0.69 respectively. Mishra *et al.* (2009) studied β-CN variants of 17 Zebu breeds and 8 buffalo breeds out of which 15 breeds of zebu cattle and all the buffalo breeds were completely A2A2 genotype (Table 2.2). Their results pointed

towards the origin of A2 variants in *Bos indicus* cattle. A1 allele is common in taurine breeds.

The presence of A1 allele in Vechur cattle suggests that the foundation stock (described in Chapter 2.1) of the present Vechur herd had the introgression of A1 alleles of taurine breeds. The very low frequency of A1 allele in the present study indicate that knowingly or unknowingly breeding bulls used could have been predominantly of A2A2 genotype. Another possibility for the low frequency could be sampling fluctuation of A1/A2 alleles that usually happens in small populations.

Present study has covered A1/A2 polymorphism of almost all adult Vechur cattle in Kerala Agricultural University farm. So the knowledge of genotype obtained in the study can be applied judiciously to develop a herd/breed of Vechur cattle with A2A2 genotype only.

5.4.2 Crossbred Cattle

In crossbred cattle of Kerala, the genotype frequencies of β-CN A1/A2 polymorphism were found to be 0.32 for A1A1, 0.28 for A1A2 and 0.40 for A2A2. The A1 and A2 allele frequencies of crossbred cattle of Kerala were obtained as 0.46 and 0.54. Similar results were recorded by Malik *et al.* (2000) in crossbreds of *B. indicus* and *B. taurus* with genotype frequencies of 0.73 for A1A2 and 0.20 for A2A2. The A1/A2 allele frequencies in *B. taurus* purebreds are recorded (Table 2.2) which shows that these breeds have higher frequency of A1 alleles. Ceriotti *et al.* (2004) reported the allele frequencies in *B.taurus* and *B. indicus* crossbred cattle (Borgou cattle) as 0.24 for A1 allele and 0.74 for A2 allele. Malik *et al.* (2000) reported the A1 (0.37) and A2 allele (0.57) frequencies in crossbred cattle in India. They detected an increasing trend in the A1 allele frequencies with the increase in levels of *B.taurus* blood and opined that the A1 allele was contributed by *B taurus* cattle.

As observed in the study, it is quite natural to have A1 alleles in crossbred cattle population as the exotic breeds used (Jersey, HF, and BS) have A1 alleles in their population. Increased selection pressure is the contributing factor for increased A1 allele propagation among crossbreds (Rauw *et al.*, 1998).

5.4.3 Kasargode Dwarf Cattle

The frequency of A1A2 and A2A2 genotypes in Kasargode Dwarf cattle were 0.79 and 0.21 respectively and there was no A1A1 genotype in the Kasargode dwarf cattle studied as in the case of Vechur cattle. The A1 and A2 allele frequency in Kasargode cattle was obtained as 0.39 and 0.61 respectively. But the study was conducted only in 14 animals available in the University Farm. Though the frequency of A1 allele was higher in proportion (0.39), the absence of A1A1 genotype could be due to the use of A2A2 bulls for breeding. All the bulls in current use were found to be of A2A2 genotype.

5.5 MILK PRODUCTION

5.5.1 Milk yield in Vechur and Crossbred Cattle

The recorded average daily milk yield of Vechur cattle was 1.27±0.07 kg in the present study against 2.17 kg reported by Girija (1994) and 2.2 kg reported by Iype and Venketachelapathy (2001).

In crossbred cattle of Kerala the peak yield was recorded as 10.71±1.046 kg. The 305 day milk yield of crossbred cattle of Kerala is reported as 2406 kg by Reshmi and Stephen (2007) and 2106 kg by Lali (2009).

5.5.2 β-CN Polymorphism and Milk Yield

β-CN A1A2 genotypes in Vechur and crossbred cattle of Kerala were found to be associated with milk yield. Vechur animals of A1A2 genotype showed significantly higher average daily milk yield (1.52±0.08 kg) than those with A2A2 genotype (1.14±0.04 kg). In the crossbred cattle studied the average peak yield (kg) recorded for A1A1 genotypes was significantly higher than the other two genotypes, *viz* A1A2 and A2A2 (Table 4.5).

A higher milk yield was reported for heterozygous A1A2 animals by Ng-Kwai-Hang *et al.* (1985) and Ojala *et al.* (1997) in Holstein Friesian cattle. Significantly increased level of milk yield obtained in A1A1 crossbred cattle is in line with findings of Ron *et al.* (1994) in Israeli Holstein cows and Velmala *et al.* (1995) in Finnish Ayrshire cattle. Higher milk production from A2 allele is reported by Ng-Kwai-Hang *et al.*(1990) in Holstein Friesian, Ikonen *et al.* (1999) in Finnish Ayrshire, Freyer *et al.* (1999) in Holstein Friesian, Comin *et al.* (2008) in Italian Holstein Friesian and Nilsen *et al.* (2009) in Norwegian Red cattle. Milk yield was not associated with β-CN A1/A2 polymorphism as reported by Sarbour *et al.* (1996) in Canadian Holstein and Boettcher *et al.* (2004) in Holstein Friesian cattle.

In public health point of view, A2A2 milk is beneficial as intake of A1 milk yielding BCM – 7 is reported to be one of the etiological agent for various diseases (Reviewed in Chapter 2.4). Globally, there is a trend for production of milk containing only β-CN A2 by means of selective breeding. A2 Corporation was set up in New Zealand for production and marketing of A2 milk at a premium price.

As observed in the present study, selection for increasing milk yield may contribute for the higher proportion of undesirable A1 alleles in the

population. Considering the public health implication, adequate weightage should be given to select bulls with A2A2 genotype while making selection for increasing milk yield of crossbreds. The proportion of A2 allele is high in Vechur cattle and almost entire available adult Vechur cattle in the KAU farm were genotyped in the present study. Based on the present results, careful breeding strategies can be adopted to develop a Vechur herd/breed with A2A2 genotype using the information generated and its milk can be marketed at a premium price. Similarly, effort should be made to develop Kasaragode Dwarf cattle with A2A2 genotype.

Summary

6. SUMMARY

Milk is the important source of animal protein. In India, although major share of milk (57%) come from buffaloes, it is cow milk that is widely used in Kerala (98% of total milk). Milk protein polymorphism is widely used as markers for selection. The recent finding that some of the milk proteins yield biologically active peptides upon gastrointestinal digestion has attracted the attention of both public health authorities and animal breeders.

Beta casein (β-CN) is the second most abundant milk protein in bovine milk. There are 12 genetic variants for β-CN identified in bovine milk. Of the 12 variants A1 and A2 are the common types and the rest of the variants are very rare. A2 possess a proline at position 67 and all other 11 variants are having histidine at the corresponding position. The difference in amino acid at position 67 is due to a single nucleotide polymorphism of β-CN gene at codon 67 . The sequence CCT codes proline while CAT codes for histidine. Upon proteolytic digestion variant A1 and other histidine possessing variants at the 67^{th} position of amino acid sequence releases betacasomorphine-7 (BCM-7). BCM-7 is a potent opioid agonist reported to be one of the etiological agent in the development of various diseases in humans such as diabetes mellitus, cardiac diseases, atherosclerosis, schizophrenia, neurological disorders and sudden infant death syndrome (SIDS). Beta casein A1 type is most prevalent in taurine breeds and Holstein Friesian and Ayrshire the major milk producers in the world possess higher frequency of this allele.

Beta casein A2 is predominant in all Zebu populations investigated so far and considered as the original type of genus *Bos*. The proportion of investigated populations is higher for taurines than Zebu. Recently it is reported that β-CN A1 was not prevalent in major Indian breeds of cattle and buffaloes. In view of this the present study was conducted to ascertain the

prevalence of β-CN A1A2 polymorphism in Vechur, Crossbred and Kasargode Dwarf cattle of Kerala. Vechur and Kasargode Dwarf animals included in the study were mainly from the stock maintained at Kerala Agricultural University. Crossbred cattle which constitute major bovine population in Kerala (more than 82% of total population) are having mosaic genetic character of Holstein Friesian, Jersey, Brown Swiss and local cattle. The crossbred cattle under this study were from districts of Kerala

Allele Specific PCR (AS-PCR) was carried out in 182 animals (100 crossbred, 72 Vechur and 14 Kasargode cattle) for screening β-CN A1/A2 polymorphism. The relationship of this SNP with milk yield was also investigated. Blood samples were collected from all these animals and DNA was extracted by standard phenol chloroform method. Two allele specific reverse primers were used with 3' mismatch complementary to A1 and A2 allele. The PCR reaction and protocol was standardised to amplify 854 bp DNA fragment from genomic DNA.

The genotypic frequencies of A1A1, A1A2 and A2A2 were, respectively, 0, 0.40 and 0.60 in Vechur cattle, 0.32, 0.28 and 0.40 in crossbred cattle and 0, 0.79 and 0.21 in Kasargode cattle. Genotypic frequencies were not found to be in Hardy Weinberg equilibrium in crossbred cattle while in Vechur and Kasargode cattle frequencies were found to be in equilibrium.

The A1 and A2 allele frequency was 0.20 and 0.80 in Vechur cattle, 0.46 and 0.54 in crossbred cattle and 0.39 and 0.61 in Kasargode cattle. Comparison of allele frequencies revealed that there is significant variation in allele frequencies among these three groups of animals.

The relationship between β-CN A1/A2 polymorphism with milk yield was also assessed. The average daily milk yield was 1.27±0.05 kg in Vechur

cattle. The peak yield of crossbred cattle was 10.71±1.046 kg. The average daily milk yield for A1A2 genotype in Vechur cattle was 1.52±0.08 kg and 1.14±0.04 kg for A1A2 genotype. The t-test showed the difference in the milk yield of the two genotypes was significantly different at 5% level of significance. In crossbred cattle the peak yield (kg) of A1A1 genotype was 14.64±3.181, 8.54±0.194 for A1A2 genotype and 9.09±0.125 for A2A2 genotype. The A1A1 genotype in crossbred cattle showed significantly higher average peak yield compared to other genotypes and difference between A1A2 and A2A2 genotypes were not significantly different.

A1 allele was found to associate with higher milk production in both Vechur and Crossbred cattle. Hence selection of animals based on milk yield only, has the disadvantage of chance of increasing the A1 allele frequency in our cattle population. Globally, there is a trend for production of milk containing only β-CN A2 by means of selective breeding. Hence immediate efforts are needed to utilize this useful SNP as marker in selection and reduce the frequency of A1 allele from our bovine population. The information generated in this study on the genotypes of Vechur and Kasargode dwarf cattle can be very effectively used for developing a herd/breed of cattle with A2A2 β-CN genotype.

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ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Agarose (0.8%)

Weighed 0.8 g of agarose powder and mixed with 100 ml of 1X TBE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Agarose (2%)

Weighed 2 g of agarose powder and mixed with 100 ml of 1X TAE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

EDTA (0.5*M*, pH 8.3)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 80 ml 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 (10 mg/ml)

Dissolved 100 mg ethidium bromide in 10 ml of distilled water. Solution stored at 4°C in a dark coloured bottle.

Gel loading buffer

Bromophenol blue	0.25 %	50 mg
Xylene cyanol	0.25 %	50 mg

Sucrose	40 %	8 g

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

Phenol (Saturated, pH 7.8)

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

RBC lysis buffer

Ammonium chloride	150 m*M*	8.0235 g
Potassium chloride	10 m*M*	0.7455 g
EDTA	0.1 m*M*	0.0372 g

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

Sodium acetate

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

Sodium chloride (5 *M*)

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

Sodium chloride- EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 m*M*	4.383 g
EDTA	35 m*M*	9.306 g

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

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20 ml
Distilled water up to	1000 ml

Autoclaved and stored at room temperature.

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

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

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

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 m*M*	8.18 g
Potassium chloride	0.5 m*M*	0.0373 g
Tris base	0.25 m*M*	0.0303 g

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

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 m*M*	1.2114 g
EDTA	0.1 m*M*	0.3722 g

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

Tris 1M (pH 8.0)

Tris base	121.14 g

Added distilled water up to 1000 ml, pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Ammonium per sulphate	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
Di-sodium hydrogen orthophosphate	-	SRL, Bombay
dNTPs	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6X 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
Tris base	-	SRL, Bombay

(B) PRIMERS Integrated DNA technologies

(C) MOLECULAR MARKERS

100 bp DNA ladder Bangalore Genei Pvt. Ltd.

(D) ENZYMES

Taq DNA polymerase - Bangalore Genei Pvt. Ltd.

Proteinase-K - Bangalore Genei Pvt. Ltd.

ANNEXURE – III

ABBREVIATIONS

AS	Allele Specific
β-CN	Beta casein
SNP	Single Nucleotide Polymorphism
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single Strand Conformation Polymorphism
PCR	Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
MAS	Marker Assisted Selection
QTL	Quantitative Trait Loci
PIC	Polymorphic Information Content
EDTA	Ethylene Diamine Tetra acetic Acid
APS	Ammonium Persulphate
PNK	Polynucleotide Kinase
µl	microlitres
µg	microgram
mg	milligram
m*M*	millimolar
cm	centimeter
nm	nanometer

pmol	picomols
kb	Kilo basepair
bp	base pair
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
dNTP	Deoxy Nucleotide Triphosphate
ddATP	Dideoxy Adenosine Triphosphate
ddCTP	Dideoxy Cytosine Triphosphate.
ddGTP	Dideoxy Guanosine Triphosphate
ddTTP	Dideoxy Thymidine Triphosphate

EVALUATION AND COMPARISON OF POLYMORPHISM OF BETA CASEIN GENE IN VECHUR AND CROSSBRED CATTLE OF KERALA

MUHAMMED E. M.

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Kerala Agricultural University, Thrissur

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Department of Animal Breeding, Genetics & Biostatistics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

Beta casein (β-CN) is the major milk protein which imparts biological, technological and physical properties to the milk. Recently, the polymorphism of the gene at codon 67 has attracted much public health attention. A single nucleotide polymorphism (SNP) from CCT to CAT leads to an amino acid change in the mature protein from proline to histidine. Among the twelve β-CN variants identified A1 and A2 are the common types and others are very rare. A1 variant has histidine at position 67 of the amino acid sequence while A2 possess proline at this position. This single amino acid change causes the release of bioactive peptides upon gastro intestinal digestion. Morphine like opioid beta casomorphine-7 (BCM-7) thus released from A1 milk is reported to cause various illness like diabetes mellitus, heart diseases, atherosclerosis, schizophrenia and sudden infant death syndrome (SIDS). The original variant A2 does not produce BCM-7 and thus is safe for human consumption. The major taurine milch breeds such as Holstein Friesian and Ayrshire have a high frequency of A1 allele where as Channel Island breeds such as Guernsey and Jersey have more of A2 allele. It is also interesting to note that most of the Indian breeds of animals have only β-CN A2 allele.

The present study was undertaken to characterise the β-CN gene polymorphism in Vechur and crossbred cattle of Kerala considering its public health importance. Blood samples were collected from 72 Vechur cattle, and 14 Kasargode Dwarf cattle available in the KAU farm and 100 crossbred cattle randomly selected from different parts of Kerala. Genomic DNA was isolated by standard phenol chloroform procedure. Beta casein A1 A2 polymorphism was analysed by Allele Specific Polymerase Chain Reaction (AS-PCR).

The genotypic frequencies of A1A1, A1A2 and A2A2 were, respectively, 0, 0.40 and 0.60 in Vechur cattle, 0.32, 0.28 and 0.40 in crossbred cattle and 0, 0.79 and 0.21 in Kasargode cattle. Genotypic frequencies were not found to be in Hardy-Weinberg equilibrium in crossbred cattle while in Vechur and Kasargode cattle frequencies were found to be in equilibrium.

The A1 and A2 allele frequency was 0.20 and 0.80 in Vechur cattle, 0.46 and 0.54 in crossbred cattle and 0.39 and 0.61 in Kasargode cattle. Comparison of allele frequencies revealed that there is significant variation in allele frequencies among these three groups of animals.

The relationship between β-CN A1/A2 polymorphism with milk yield was also assessed. The average daily milk yield was 1.27±0.05 kg in Vechur cattle. The peak yield of crossbred cattle was 10.71±1.046 kg. The average daily milk yield for A1A2 genotype in Vechur cattle was 1.52±0.08 kg and 1.14±0.04 kg for A1A2 genotype. The t-test showed the difference in the milk yield of the two genotypes was significantly different at 5% level of significance. In crossbred cattle the peak yield (kg) of A1A1 genotype was 14.64±3.181, 8.54±0.194 for A1A2 genotype and 9.09±0.125 for A2A2 genotype. The A1A1 genotype in crossbred cattle showed significantly higher average peak yield compared to other genotypes and difference between A1A2 and A2A2 genotypes were not significantly different.

From the present study it can be concluded that selection for enhancing milk production may increase the frequency of harmful A1 allele in our bovine population. So efforts should be taken to enhance the A2 allele with a view of transforming our cattle population capable of producing A2 variant of β-CN which has a global demand. The information generated in this study on the genotypes of Vechur and Kasargode dwarf cattle can be very effectively used for developing a herd/breed of cattle with A2A2 β-CN genotype.