# “PCR based analysis of single nucleotide polymorphsim in beta-casein A1 and A2 gene of bovine in Bangladesh”


A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

Submitted by

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# DECLARATION

This to declare that the research work embodying the results reported in this thesis entitled "PCR based analysis of single nucleotide polymorphism in beta-casein A1 and A2 gene of bovine in Bangladesh" submitted by Olema Taj Rahman, has been carried out under the supervision and able guidance of Dr. M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources has been duly cited and referenced.

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**Dedicated**

# To
# Almighty and
# My parents

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Olema Taj Rahman

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**Abstract**

In the present study "PCR-based analysis of single nucleotide polymorphism in beta-casein A1 and A2 gene of bovine in Bangladesh" was conducted to differentiate between beta-casein-containing types A1 and A2. Casein contributes80% of the bovine milk protein and has four fractions (alpha S1-CN, alpha S2-CN, beta-CN, and k-CN). Beta casein contributes 25-35% of milk protein and many variants reported in various cattle breeds (A1, A2, A3, B, C, D, E, F, G, H1, H2 and I). The beta-casein variants A1 and A2 differ in the position of 67$^{th}$amino acid, the substitution of proline in type A2 with Histidine(in A1) is primarily due to the replacement of nucleotide "C" with nucleotide "A" in the corresponding position of nucleotide. For the detection of polymorphsim of A1, A2 beta-casein gene from genomic DNA, thirty cattle (including both local and cross-bred) were selected. Allele-Specific PCR and Amplification Created Restriction Site PCR amplified the beta-casein gene. AS-PCR, ACRS-PCR and subsequent agarose gel electrophoresis could differentiate between A1, A2 types of beta-casein genes in these animals. The results of the screening showed three animal genotypes in these 30 animals. The number of animals with genotypes A1A1, A2A2 and A1A2 are 5, 13 and 12 respectively. The A2 and A1 allele frequencies are 0.63 and 0.37 respectively.

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# CHAPTER 1:

# INTRODUCTION

# Introduction

## 1.1 Background:

Milk is a complex mixture of proteins, carbohydrates, vitamins, minerals, and other constituents dispersed in water (Harding, 1999).Bovine milk is a resource of lipids, amino acids, vitamins and minerals, immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and alternative bioactive peptides (Haug et al., 2007).The total milk protein component is composed of numerous specific proteins. Caseins and whey proteins (ß-lactoglobulin and alpha-lactalbumin) are the two major milk protein groups. Bovine milk contains four caseins (alpha s1-CN 15-18%, alpha s2-CN8-11%, beta-CN25-35% and k-CN 18-15%, Eigel, et al., 1984; Roginsiki 2003).Bovine beta-casein contains a complete of 209 amino-acid residues.Bovine beta-casein gene is highly polymorphic of which a total of 12 protein variants is known so far. Initially, three variants of bovine beta-casein were discovered and denoted as A, B and C.It were later found that A variants can be more resolved into A1, A2, and A3 by gel electrophoresis.The 12 genetic variants of bovine beta caseins are A1, A2, A3, B, C, D, E, F, H1, H2, I and G (Kaminiski et al., 2007). In dairy cattle, variant forms A1 and A2 are important (Farrell et al., 2004). The variants A1 and A2 are distinguished by the modification of 1 ester in position 67 of the chain (A1 essential amino acid and A2 proline). Initial studies have indicated that the entire bovine population contained only the A2 allele, and A1 allele was created from a mutation (Rangel et al., 2006). The nucleotide sequence change in 67th amino acid position of the beta-casein reading frame, from CCT to CAT, causes substitution of proline (A2) by histidine (A1,B) in the amino acid sequence which might cause a change of secondary conformation in the protein structure and affect the physical properties of casein micelle and vulnerability to enzymatic digestion. During this enzymatic process, beta-casein opioid peptide beta-casomorphin 7 is released exclusively from A1 and B variants (Hartwig, 1997, Jinsmaa and Yoshikawa, 1999, Cieoelinska et al., 2007 and De Noni, 2008) The B-Casomorphin-7 (BCM-7), by the digestion of A1 beta-casein with pepsin, leucineaminopeptidase, and elastase can be released in the small intestine. The term „opioid“ refers to chemical substances that have morphine-like activity in the body.Some of them are noted to play a very important role within the response to worry and pain, and the control of food intake.These agents act by binding to opioid μ-receptors, that are found in the main within the central system and therefore the GI tract (Teschemacher 2003).Such opioid activities are possessed by BCM-7.

## 1.2 Bangladeshi Cattle population:

Cattle any large, even-toed hoofed, ruminant mammal of the genus Bos, family Bovidae. Domestic cattle mostly descended from the wild aurochs (European Ox, now extinct). Domestication of cattle and their propagation at the world level started in the Neolithic ages in about 2000 BC. Livestock population in Bangladesh is currently estimated to comprise 25.7 million cattle ("Livestock", Banglapedia, 2015). In Bangladesh the total cattle population is about 23.4 million of which 11.91 million are males and 11.49 million are females. Included among the cattle population are about 3.53 million milking cows, 2.61 million dry cows (cows without milk), 2.13 million draught cattle, and 4.20 million improved cattle ( "Cattle",Banglapedia, 2014). Since the 1960s, the people of Bangladesh have been rearing three categories of the cattle viz pure breed, crossbreed, and local. The pure breed and the crossbreed cattle havethe high nutritional requirement, less adaptability, and are susceptible to parasitic infestation and diseases compared to the local variety. On the other hand, the local variety is less prone to diseases and is heat tolerant. In Bangladesh the best local cattle are available in some selected areas vizPabna, Sirajganj, Chittagong, and Munshiganj areas. In Pabna and Sirajganj area medium type cattle are seen, known as Pabna cattle. To improve the production potentialities of the local cattle, efforts were made to cross breed with different exotic breeds several times in the past. The introduced breeds are Holstein-Friesian, Jersy, Sahiwal, Harians, Sindhi, Australian, Sahiwal-Friesian, etc. A number of exotic pure breeds, their crossbreeds, and up-graded cattle are found in the government dairy farms, commercial dairy farms, milk pocket area, and in urban and semi-urban areas of Bangladesh.

## 1.3 Milk production in Bangladesh:

The dairy system of Bangladesh is characterized by small-scale operations, integrated with crops and other off-farmactivities. Along with providing a major source of nutrition and income, dairying also offersgood opportunities for both farm families and non-farm rural and urbanemployment. Dairy animals comprise cows, buffaloes, sheep andgoats. Bangladesh has one of the highest cattle densities(Karim, 1997), but with the lowest productivity of milk.In Bangladesh, 10 million dairy cattle, including 4 millioncross-breeds produce 2.82 million tons of milk. This ismuch lower than in Pakistan, where only 5.5 million dairycattle produce 25 million tons of milk (Raja, 2001; DLS,2010; CLDDP, 2008;

Hemme, 2010).The per capita availability of milk in Bangladesh is 52 g/day (Ser-Od et al., 2008), against the requirement of 250 g/day. The informal sector in Bangladesh dominates, with only 5–10% ofmilk being delivered to the processor (Rao and Odermatt,2006; Haque, 2009). However, the number of processorsincreased from two in 1990 to 20 in 2007 (Hussainet al.,2008). Among the larger ones are Milk Vita, BRAC Dairy,Pran Dairy, Ammo Milk, Akij Group, Aftab Milk and MilkProducts Ltd, Tulip Milk Products and RangpurDairy.The sweetmeat 3 processors dominate within the informalsector, with a market share of 50% of the total raw milkproduced in the country (Rao and Odermatt, 2006). About40% of the milk is consumed as liquid raw milk. Withinthe formal sector, over 90% of the milk is processed aspasteurized packaged milk and only 10% is processed intobutter, ghee and milk powder.

## 1.4 Milk protein

The proteins of milk are classified into caseins and wheyproteins, on the premise of their structure and physicochemical behavior (Foxet al., 1975). Casein accounts for 80% of the overall macromolecule within the milk. Thereare four caseins derived directly because of the sequence products; αs1, αs2, β, and κ-caseins (Swaisgood et al., 1975). Each of the four caseins exhibits variabilityreferred to as small non-uniformity owing to variability in the degree ofphosphorylation, glycosylation, disulfide bonding and geneticpolymorphism (Fox et al., 1992). The whey proteins (20% of the overall milkprotein) represent an immense supply of biologically active molecules that able to influence a spread of physiological functions like its role in the uptake oftrace components and vitamins. However, increasing attention is being centeredon physiologically active peptides derived from these proteins. Milk-derivedbioactive peptides are thought of as distinguished candidates for different health-promotingfunctional foods targeted the heart, bone, and digestive system, in addition, to increasing immune defense(Korhonen et al., 1998). Such peptides are inactive among the sequence of the parent macromolecule and may be released by biological process enzymes throughout GI transit or by fermentation or ripening throughout the food process.

**Table 1.1: Protein composition of Ruminant milk**

| Component(g/100g) | Bovine[1] | Ewe[1] | Goat[1] | Buffalo[2] |
|---|---|---|---|---|
| Total Protein | 3.2 | 4.6 | 3.2 | 4.6 |
| Casein | 2.6 | 3.9 | 2.6 | 4.5 |
| Whey Protein | 0.6 | 0.7 | 0.6 | 0.1 |

1.Bovine, ewe, goat (Velisek, 1999)

2.Buffalo milk (Formaggioni, 1999).

## 1.4.1 Casein

Casein is the biggest protein in the milk of cows. Casein is the name assigned to the fraction of phosphoprotein precipitated at $200^0$C at a pH of 4.6 by acidifying milk. The four main types of casein α-s1 Casein, α-s2 Casein, β-Casein, and π-Casein make up about 80 percent of the total bovine milk protein. Caseins in cow's milk are 3-3.5 percent present. The figure in human milk is around 0.3 - 0.6 %. Whey proteins or serum proteins are the remaining 20 percent protein components.

α-Lactalbumin: ~2%
β-lactoglobulin: ~ 10%
Serum albumin: ~ 1%
Immunoglobulins: ~ 2%
Other Proteins: ~ 2%

Caseins exist in milk as the calcium salt arranged in particles of micellar surrounded by soluble kappa-casein. The casein component of milk is relatively heat-stable, capable at ~62-71 ° C of surviving pasturization. In contrast, at these temperatures the whey protein component is denatured. For casein, the IEP is about 4.6 and acid casein is precipitated at the pH value. The casein micelles have a net negative charge in milk, which has a pH of about 6.6, and are quite stable. In general, the secondary structure of caseins is limited to α-helix and β-sheet. They tend to be rich in content with proline residues and have very few bonds with disulfide. Casein solubility depends on pH and is also affected by composition and ionic strength. Adding sodium

chloride will have a different effect on solubility depending on when added to the solution during pH adjustment (Strange et al., 1994).

## 1.5 Bovine beta casein

Bovine milk contains four main groups of casein, namely alpha s1-, alpha s2-, beta-and kappa-casein, representing 38%, 10%, 36% and 13% of total milk protein, respectively (Rahimi et al., 2015). Beta-casein with 13 variants viz is the most polymorphic milk protein gene. In dairy cattle breeds, A1, A2, A3, B, C, D, E, F, H1, H2, I, G and mostly observed forms of beta-casein are A1 and A2 (Kamiński et al., 2007). Beta-casein has thirteen genetic variants: A1, A2, A3, A4 B, C, D, E, F, H1, H2, I, G. The most common forms are A1 and A2, whereas B is less common, and A3 and C are rare (SharmaV et al., 2017). The Beta-casein composition of milk and milk products has become an important milk animal economic trait(Boro et al., 2016).Variants A1 and A2 are differentiated by changing one nucleotide (A1 histidine and A2 proline) in chain position 67. Initial studies indicated that only the A2 allele was contained in the entire bovine population, and a mutation created A1 allele (Rangel et al. 2016). The nucleotide sequence change in the beta-casein read frame's 67th amino acid position from CCT to CAT causes the amino acid sequence to replace proline (A2) with histidine (A1,B) (Figure 1). This could result in a change in the protein structure's secondary conformation and affect the physical properties of casein micelle and vulnerability to enzymatic digestion.Beta - casein opioid beta - casomorphin-7 (BCM 7) is released during this enzymatic process exclusively from variants A1 and B (Jinsnaa et al., 1999, Jaiswal et al., 2014).

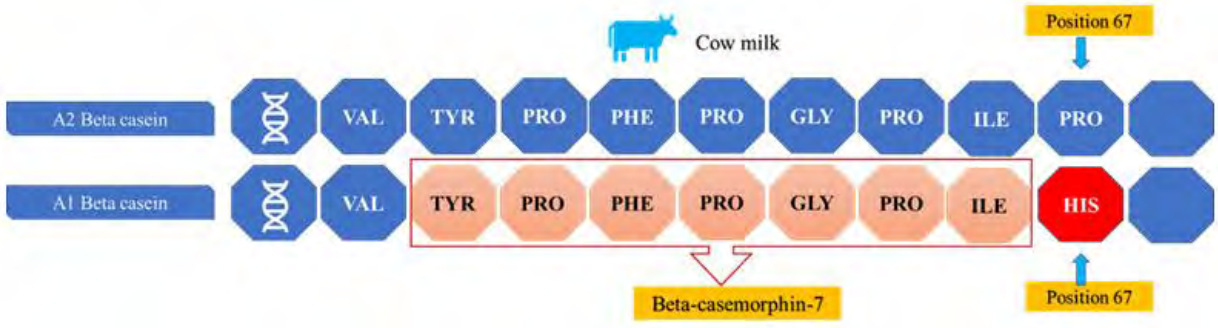



**Figure 1.1: Difference between A1 and A2 variants and formation of beta-casemorphin-7.**

## 1.6 BetaCasomorphin and its metabolism

Both β-casein A1 and A2 react diversely towards proteolytic debasement by enzymes of the human gut. This is conceivably a result of one amino acid distinction at 67th position in β-casein. A1 β-casein has histidine at 67th position while A2 β-casein has proline (Figure 1). The distinction in the amino acid at 67th position results in contrasts in the vulnerability of the peptide bond between amino corrosive 66 and 67. The peptide bond among isoleucine and histidine (A1 milk) is cut by elastase, while bond among isoleucine and proline (A2 milk) is not hydrolyzed. The processed item contained a seven amino acid long peptide having sequence Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66 and is alluded as beta-casomorphin-7 or prominently called BCM-7 which is not created on the absorption of β-casein A2 (Figure 1). In one of the in vitro examination, A1 β-casein was processed with a mix of enzymes, for example, pepsin, elastase, leucineaminopeptidase (LAP) and pancreatin5. Alongside BCM-7, other degradation items, for example, BCM-9, BCM-13, and BCM-21 were likewise formed. BCM-7 is further breakdown to BCM-5 and BCM-3 by dipeptidyl peptidase IV (DPP IV) chemical present on the surface of enterocytes and in the blood (Figure 1.2).

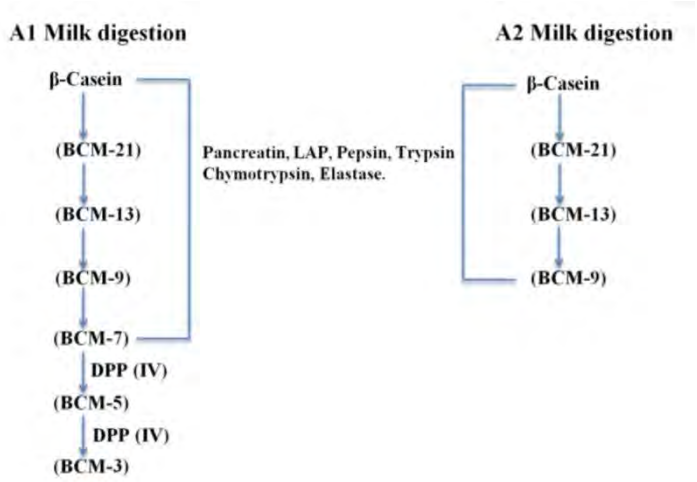



**Figure1.2: Breakdown of β-casein by pepsin, elastase, leucineaminopeptidase (LAP), chymotrypsin, trypsin and DPP (IV) enzymes and formation of BCM-21, BCM-13, BCM-9, BCM-7, BCM-5 and BCM-3 peptides.**

## 1.7 BCM-7 and it's health implications:

Opioid peptides play a role in different biological processes, including breathing, analgesia, constipation, and human behavior(Ng-Kwai-Hang 2003). BCM-7, opioid or narcotic, as well as being an oxidant, is associated with milk intolerance and plays a role in the development of certain human diseases such as cardiac ischemia, diabetes mellitus, atherosclerosis, schizophrenia, autism, coronary heart disease, diabetes mellitus, atherosclerosis, schizophrenia, autism, coronary heart disease, Autistic Spectral Disorder (ASD) and Sudden Infant Death Syndrome (SIDS) (Jaiswal 2014, Sodhi et al., 2012). It may also be involved in a further range of autoimmune conditions. Some people also associate this protein with milk intolerance[Woodford 2008]. Because of its relationships with milk production characteristics, milk composition and milk quality, genetic polymorphism of bovine milk proteins has a great interest in animal breeding (Roginski 2003, Jaiswal 2014).It has been shown that bovine BCM-7 is absorbed into the circulation of formula-fed infants (Kostyra, et al., 2004), where the BCM-7 elimination rate among babies appears to vary. In addition, while one of these infant studies reported a negative correlation between exposure to BCM-7 and child development aspects (Kostyra et al., 2004),The other suggested that higher levels of bovine BCM-7 in some infants could be a risk factor for apnoea and that bovine BCM-7 from milk ingested by lactating mothers could be transferred directly to mother's milk (Kostyra et al., 2004). Human digestion by the enzyme dipeptidyl peptidase-4 can break down Casomorphins into inactive dipeptides (Puschel, 1982).This enzyme is found in some endocrine cells and in the digestive tract. There is also the possibility of casomorphins being released from human milk. Human BCM7 (Tyr-Pro-Phe-Val-Glu-Pro-Ile) differs in two positions of amino acids from the bovine form (Tyr-Pro-Phe-Pro-Gly-Pro-Ile). However, there is still incomplete scientific understanding of casomorphin biochemistry and pharmacology.

## 1.8 Single Nucleotide Polymorphism:

A mutation is defined as any change away from normal in a DNA sequence. This implies that there is a normal allele prevalent in the population and the mutation transforms this into a rare and abnormal variant. A polymorphism, on the other hand, is a variation in the DNA sequence common in the population.No single allele is considered the standard sequence in this case. There are two or more alternatives that are equally acceptable. The arbitrary point of cut-off

between a mutation and a polymorphism is 1 %. That is, the least common allele must have a frequency of 1% or more in the population to be classified as a polymorphism. The allele is considered a mutation if the frequency is lower than this.

Usually, variants of polymorphic sequence do not cause overt debilitating diseases. Many are found outside genes and are in effect totally neutral. Others may be found within genes, but may affect features such as height and hair color rather than medical significance characteristics.However, variation in the polymorphic sequence contributes to the susceptibility of disease and can also influence drug responses (single nucleotide polymorphisms). SNPs occur approximately once in every 1000 base pairs in the genome, representing the majority of the 3 million variations found in the genome.Unlike the other, more rare types of variations, many SNPs occur in genes and genome surroundings that control their expression. The effect of a single SNP on a gene may not be large-perhaps subtly affecting the activity of the encoded protein-but even subtle effects may affect the susceptibility to common diseases such as heart disease or Alzheimer's disease.

## 1.9 SNP detection techniques:

### 1.9.1 Allele Specific PCR (AS-PCR):

All-specific polymerase chain reaction (AS-PCR), also known as refractory mutation amplification system (ARMS) or specific allele amplification PCR (PASA) is a PCR-based method that can be used to detect known SNPs. In this approach, the specific primers are designed to allow DNA polymerase amplification only if the nucleotide at the 3'-end of the primers complements the base in the variant or wild-type sequences perfectly. The patterns of specific PCR products allow the differentiation of the SNPsafter the PCR and electrophoresis.The concept of allele specific PCR (AS-PCR) has been described by Keating et al., 2008. This concept-based method for animal typing uses bovine blood DNA and finds single nucleotide polymorphisms and mutations. Selective amplification is accomplished by designing a primer to match or mismatch its 3' end with an allele base.Primer strand will only extend if its 3 ' end is a perfect complement to the DNA allele. Thus, if a single base polymorphism occurs, it is possible to observe the genotyping results simply by observing the presence and absence of PCR products on the gel.

A common forward primer (Bwtp3;5'GCCCAGATGAGAGAGG GAAGTGAGG3') and reverse primers with either T or G at the 3 ' end (5'GATGTTTGG GAGGCTGTTAT / G-3')are used to amplify an 854 base pair fragment.In Figure 8A, the DNA fragment of 854 base pairs in L1 well was amplified by DNA forward priming and reverse priming specific to A1 allele. However, A2 allele - specific reverse priming did not amplify the same DNA well in L2. L1&L2 wells represent that animal's A1A1 genotype.In L3&L4, the DNA fragment of 854 base pairs was amplified respectively by A1 allele reverse primer and A2 allele reverse primer, representing that animal's A1A2 genotype. In L5 well, A1 allele-specific priming does not amplify the 854 base pair DNA fragment while in L6, A2 allele-specific priming amplified the desire product, representing the animal's A2A2 genotype in L5&L6 wells.The method can detect whether milk type A1A1, A2A2 or A1A2 will be produced by the bovine.

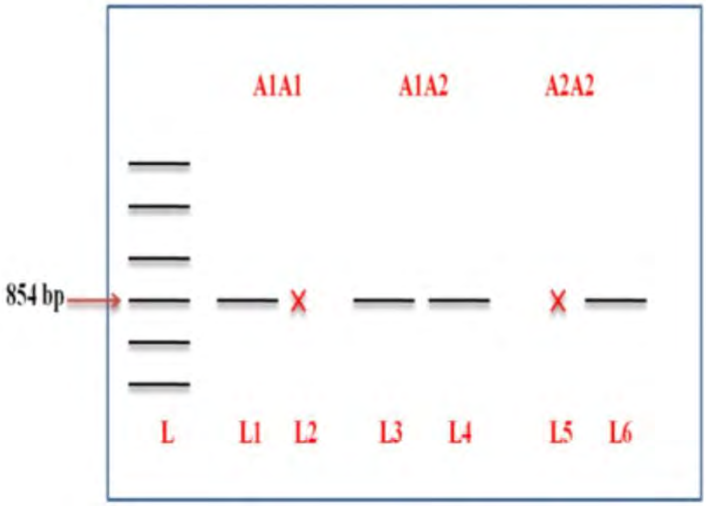



**Figure 1.3: Amplification by AS - PCR method of A1, A2 allele. L refers to DNA ladder, L1&L2 refers to animal genotype A1A1, L3&L4 refers to animal genotype A1A2, and L5&L6 refers to animal genotype A2A2 (X refers to absence of band).**

## 1.9.2 Amplification created restriction site PCR (ACRS-PCR):

Raies et al., 2009 described the method used by the concept of amplification created restriction site (ACRS-PCR) to type animals using bovine blood DNA. It is very similar to PCR allele. DNA length of 121 base pairs is amplified.The forward primer sequence is made of the same sequence, but reverse primers are made so that one base penultimate to the 3' end cannot form a hydrogen bond with base present at the complementary amplifying DNA strand out of 18

bases.Due to a base difference between A1 and A2 beta-casein, a restriction site is created in A2, but not in A1 gene that is recognized by an endonuclease enzyme which cuts the A2A2 genotype DNA into two parts that appear as two bands on the gel, but appears as one band in the A1A1 genotype. In the genotype of A1A2, three bands are observed.The method Amplification created restriction site PCR is performed using two types of primers,

Casein4 (Forward):5'CCTTCTTCAGGATGAACTCAGG-3' and

Casein Dde2 (Reverse):5'GAGTAAGGAGGGATGTTTGGAGGCTCT-3' which amplifies 121 bp DNA product, the penultimate nucleotide mismatch (cytosine underlined) at the 3 ' end of Casein Dde2 primary (Reverse) creates a DdeI enzyme restriction site.Due to the presence of CCT (proline) codon in the A2 allele of β-casein, the specific digestion resulted in 86 base pairs and 35 base pair fragments (Figure 1.4).

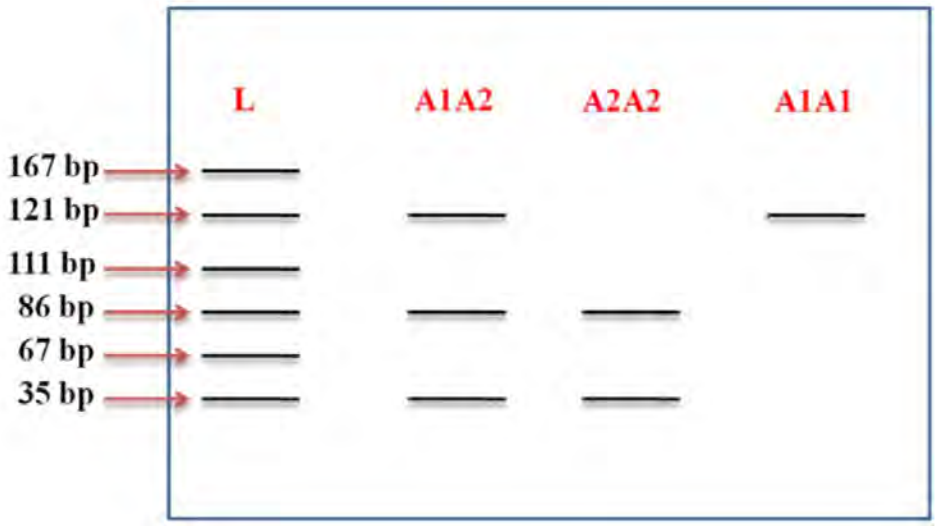



**Figure 1.4: ACRS-PCR method for animal typing, L indicates DNA ladder.**

## 1.10 Rationale of the study:

Among many dairy cattle breeds, the beta-casein locus with various allelic forms of A1 and A2 was reported. These allelic forms produce variants of A1 and A2 in beta-casein. Beta casomorphin-7 a bioactive peptide is produced from beta casein A1 variant by proteolysis. Beta casomorphin-7 has morphine like activity and results in some diseases including type 1 diabetes mellitus, cardiovascular disease, sudden death and madness. The significance of this BCM-7 occurrence has created an urge to determine the frequency ofbeta-casein variants in native cattle.

## 1.11 Objectives of the study:

The objective of the study is to analyze the single nucleotide polymorphism in beta casein A1 and A2 of bovine in Bangladesh.

1. To establish a convenient method of manual genomic DNA extraction from blood.
2. To perform a PCR based method to distinguish A1 and A2 type of beta casein.
3. To genotype cattles for A1 and A2 beta casein type.

# CHAPTER 2:

# MATERIALS AND METHOD

# Materials and Methods

## 2.1 Study place:

The laboratory works of this research was done in the laboratory at Advanced Seed Research and Biotech Centre, ACI.

## 2.2 Study Period:

This research work was carried out from December 2018 to March 2019.

## 2.3 Materials:

### 2.3.1 Chemicals and Equipment:

Chemicals used in the investigation are included in the annexed text.The annex also lists the composition of the various solution and reagent used in the procedure.

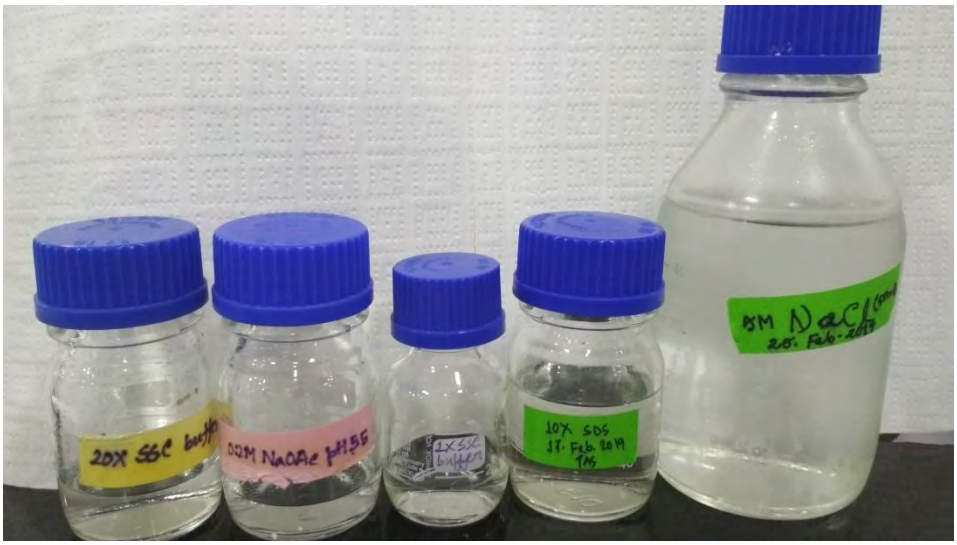
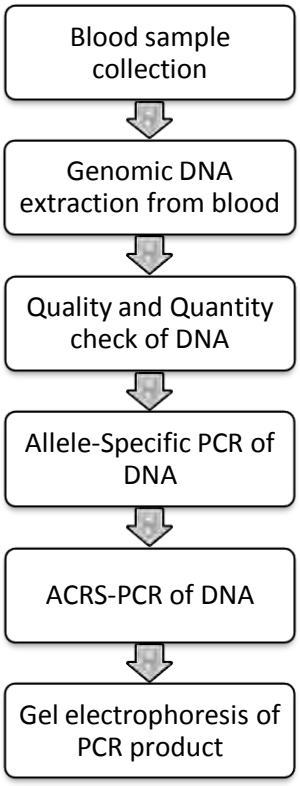



**Figure 2.1:Prepared Chemicals**

## 2.4 Flowchart of the study design:




## 2.5 Methods:

### 2.5.1 Sample Collection:

Thirty cows (18 local and 12 crossbred) from two local dairy farms in Dhaka were included in the study. The blood samples (5ml each) were collected from the jugular vein in 10ml EDTA tubes under aseptic conditions and transported to the laboratory in a box containing ice. The blood samples were then stored at -80$^0$C until further use.

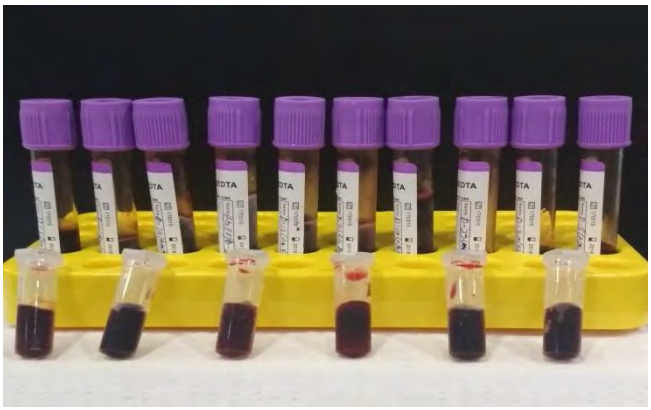


**Figure 2.2: Collected samples in EDTA tube and divided into eppendorf tube.**

### 2.5.2 Isolation of Genomic DNA from blood by Phenol:Chloroform:Isoamylalcohol (PCI) method:

About 1ml of each blood sample was divided into eppendorf tubes and stored at -80$^0$C. After thawing the tubes 1ml of 1X SSC buffer was added and mixed well by proper vortexing. The samples were then centrifuged at 12000 rpm for 3 min and after that, the supernatant was discarded. This was repeated 2-3 times and all the supernatant was discarded. Then 375µl of 0.2M Sodium Acetate was added to each pellet and mixed briefly. 25µl of 10% SDS and 5µl of proteinase K was also added, mixed well and incubated for 1 hour at 55$^0$C. After the protein digestion 120µl of Phenol:Chloroform: Isoamyl alcoholwas added and mixed well while inverting the tube until a uniform suspension was obtained. The tubes were then centrifuged at 12000 rpm for 20 min at room temperature. The aqueous phase was collected without disturbing the interphase and equal volume of Phenol: Chloroform: Isoamyl alcohol was mixed gently for 5-10 min. and centrifuged at 12000 rpm for 10 min at room temperature. The aqueous phase was again collected in a fresh micro centrifuge tube and equal volume. of Chloroform: Isoamyl alcohol was added and gently mixed for 5 min. Centrifuging at 12000rpm for 10 min at room temperature, the aqueous phase was then transferred to a fresh micro centrifuge tube and 1ml of chilled 100% ethanol was added and incubated for 20 min at -20$^0$C. The tubes were then centrifuged at 12000 rpm for 10 min and the supernatant was discarded. 180µl of 1X TE buffer was added to the pellet, vortexed and incubated at 55$^0$C for 10 min. After incubation 20µl of 5M NaCl and 500µl of chilled 100% Ethanol was added and centrifuged at 12000 rpm for 10 min. The supernatant was discarded and the pellet was rinsed with 1ml 70% Ethanol and centrifuged at 12000 rpm 2 min. The pellet was air dried after discarding the supernatant. The pellet was re-suspended in 100µl 1X TE buffer and stored at -20$^0$C.

**Figure 2.3: DNA pellet after extraction**

### 2.5.3 Quality check and Quantification of genomic DNA:

### 2.5.3.1 Spectrophotometric quantification of DNA:

Using (nano-drop) spectrophotometer, spectrophotometric quantification of isolated DNA was done. The diluted DNA sample's optical density was taken as blank at 260/280 nm using DNA storage solution (Mili-Q water). The DNA conversion factor per OD 260/280 units was taken as µg/µl (Sambrook et al., 2001). OD ratio was 1.8-1.7 at 260 nm and 280 nm which indicated that the DNA was pure.


**Figure 2.4:Nano-drop spectrophotometer**

### 2.5.3.2 Quality checked by Agarose gel electrophoresis of DNA

By running the DNA in 0.5% agarose gel, the integrity of the isolated DNA was examined electrophoretically. The 0.5 % agarose gel of high-quality agarose grade molecular biology was prepared by dissolving the agarose in 1X TAE buffer (pH 8.0) followed by microwave heating. Before casting the gel, a solution of ethidium bromide stock was added directly to the molten aga rose solution at a rate of 5 µl /100 ml of gel volume. Before pouring the gel, the surface of the ge l casting tray and comb was lifted. The comb was carefully removed after complete gel setting and the gel casting tray was placed in the 1X TAE (pH 8.0) buffer electrophoresis tanks. Then 2 µl of the DNA sample was mixed with 2 µl of 6X loading buffer and then slowly loaded with a micropipette into the submarine gel wells. At 100V, electrophoresis was performed for 30 min.

The gel was examined under the UV transilluminator / Gel documentation system after electrophoresis had been completed. (Sambrook et al., 2001).

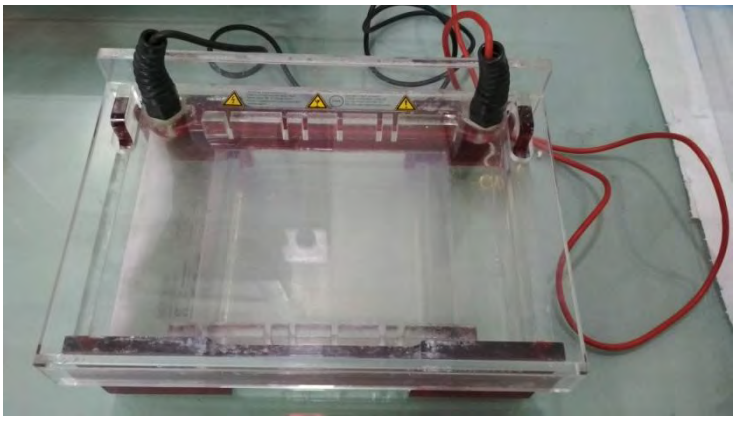


**Figure 2.5:GelElectrophpresis**

## 2.5.4 Polymerase chain reaction:

### 2.5.4.1 Primer:

Primers used in this study are presented in table along with expected PCR product size and reference. The primers are supplied by Invent Technologies Ltd. Bangladesh.


**Figure 2.6: PCR machine**

**Table 2.1: Selected Primers for PCR**

| PCR | Primer name | Sequence 5‟-3‟ | Reference |
|---|---|---|---|
| AS-PCR | Bwtp3 | 5'GCCCAGATGAGAGAGG GAAGTGAGG3' | Rahimi (2015) |
| | Reverse for A1 | 5′- GAT GTT TTGTGG GAG GCT GTT AT -3′ | |
| | Reverse for A2 | 5‟-GAT GTT TTG TGG GAG GCT GTT AG -3′ | |
| ACRS-PCR | Casein4 | 5'CCTTCTTCAGGATGAACTCAGG-3' | McLachlan (2006) |
| | Casein Dde2 | 5'GAGTAAGGAGGGATGTTTGGAGGCTCT-3' | |

## 2.5.4.2 AS-PCR:

A fragment with 854 base pairs was amplified by allele specific-polymerase chain reaction (AS-PCR). The PCR was conducted by Master Cycler Gradient- Eppendorf (Germany). The PCR mastermix was prepared in a 1.5ml tube by mixing nuclease free water,10XTaq buffer, dNTPs, primers and Taq DNA Polymerase. Sufficient master mix for the number of reactions plus one extra for a no-template control was prepared. The master mix was aliquoted into individual 0.2ml PCR tubes. Then 2µl of genomic DNA was added to each aliquot of mastermix using a clean micropipette. The PCR reagents were mixed gently and spun down. The tubes were then placed onto the PCR machine. AS-PCR program wasrun after which it was stored at -4$^{o}$C.The PCRwas conducted using the forward primer Bwtp3 and the reverse primer 1 for A1 and the reverse primer 2 for A2 allele (Keating et al. 2008). The obtained PCR product with 854-bp was electrophoresed on 3% agarose gel.

**Table 2.2: Composition of AS-PCR master mix**

| Component | Stock Concentration | Working Concentration |
|---|---|---|
| Nuclease-free water | | |
| Buffer | 10X | 1X |
| dNTPs | 10mM in each 1ml | 0.2 mM |
| Primer | 100 µM | 10 µM |
| Template | 100 ng- 320 ng | 150 ng/ 10 µl |

**Table 2.3: AS-PCR thermal cycle parameters**

| Step | Temperature, $^0$C | Time | |
|---|---|---|---|
| Initial Denaturation | $95^0$ C | 2 min | X 1 cycle |
| Denaturation | $95^0$C | 1 min | X 35 cycle |
| Annealing | $58^0$C | 1 min | |
| Extension | $72^0$C | 1 min | |
| Final extension | $72^0$C | 5 min | |

## 2.5.4.3 ACRS-PCR:

A fragment with 121 base pairs was amplified by ACRS-PCR.The PCR mastermix was prepared in a 1.5ml tube by mixing nuclease free water, 10X Taq buffer, dNTPs, primers and Taq DNA Polymerase. Sufficient master mix for the number of reactions plus one extra for a no-template control was prepared. The master mix was aliquoted into individual 0.2ml PCR tubes. Then 2µl of genomic DNA was added to each aliquot of mastermix using a clean micropipette. The PCR reagents were mixed gently and spun down. The tubes were then placed onto the PCR machine. ACRS-PCR program wasrun after which it was digested using DdeI enzyme.The PCRwas conducted using the forward primer Casein4 and the reverse primer Casein Dde2. The obtained PCR product with 121-bp and the digested product was electrophoresed on 3% agarose gel.

**Table 2.4: Composition of ACRS-PCR master mix**

| Component | Stock concentration | Working concentration |
|---|---|---|
| Nuclease free water | | |
| Buffer | 10X | 1X |
| dNTPs | 10mM in each 1ml | 0.2 mM |
| Primer | 100µM | 10µM |
| Template | 100 ng- 320 ng | 150 ng/ 10 µl |
| Enzyme | 10U/ µl | 5U/ µl |

**Table 2.5: ACRS-PCR thermal cycle parameters**

| Step | Temperature, $^0$C | Time | |
|---|---|---|---|
| Initial Denaturation | 95$^0$C | 5 min | X 1 cycle |
| Denaturation | 95$^0$C | 40 sec | X 30 cycle |
| Annealing | 58$^0$C | 1 min | |
| Extension | 72$^0$C | 90 sec | |
| Final extension | 72$^0$C | 10 min | |
| Enzyme digestion | 37$^0$C | 1-4 hours | |

### 2.5.5 Agarose Gel Electrophoresis of PCR product:

The 3% agarose gel of high quality molecular biology grade agarose(Sigma Chem. Co., USA) was prepared by dissolving the agarose in 1XTAE buffer (pH 8.0) followed by heating in a microwave oven. Ethidiumbromide stock solution was added directly to molten agarose solution at therate of 0.5 µg/ml of gel volume before casting the gel. The surface of the gelcasting tray was leveled before pouring the gel. After complete setting of thegel, the comb was removed carefully and the gel casting tray was placed inthe electrophoresis tanks containing 1X TAE (pH 8.0) buffer. The amplifiedDNA samples were mixed with 5 µl of tracking dye and then loaded slowlyinto the wellsof the submarine gel using micropipette. Electrophoresis was carried out at 100 V for half an hour. After completion of electrophoresis,the gel was examined under UV transilluminator /Gel documentation system.

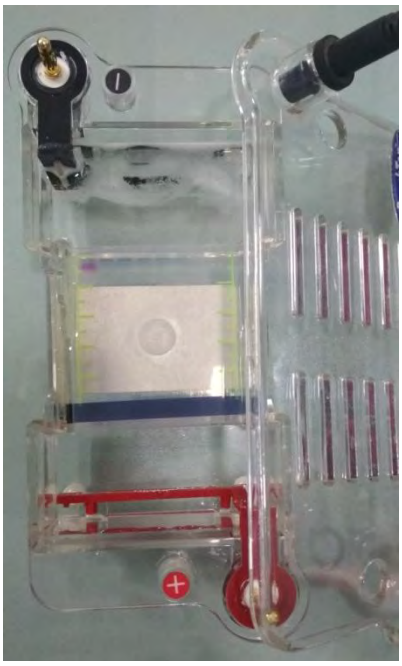


**Figure 2.7: Gel Electrophoresis of PCR product.**

# Chapter 3:

# Result

# Results

### 3.1 DNA Isolation:

**G**enomic DNA was isolated from peripheral blood leukocyte of cattle by standard Phenol chloroform methodology as mentioned in Material & Method. A total of 30 DNA samples were included in the present study from cattle as given in the Table 3.1. The concentration of the DNA isolated is provided in Table 3.1.

### 3.2 Quality and quantity of genomic DNA

Quality and quantity of isolated genomic DNA were evaluated by horizontal Agarose gel electrophoresis (0.5 % of agarose gel) and measuring absorbance at 260 and 280 nm in nanodrop spectrophotometer. Isolated DNA showed a distinct band of genomic DNA under UV trans-illuminator indicating good quality of isolated DNA Fig-3. The ratio of optical density at two wavelengths (OD260/OD 280) indicate the quality of isolated DNA. OD ratio between 1.7 to 1.9 indicates that the purity of DNA was good.OD260/OD280 ratio if less than 1.7 indicate protein contamination and more than 2.0 indicate RNA contamination. In this study OD 260/280 ratio of most of DNA samples ranged from 1.7 to 1.9. The concentration DNA varied from 414 to 90.6 ng/µl. The working solution contained approximately 150 ng of DNA.These diluted DNA samples were used subsequently as template DNA for PCR amplification.

**Table 3.1: Quantity and Quality of isolated DNA using Nanodrop Spectrophotometer**

| Animal no. | 260/280 ratio | DNA concentration(ng/µl) | Animal no. | 260/280 ratio | DNA concentration(ng/µl) |
|---|---|---|---|---|---|
| CS-1 | 1.87 | 119.7 | CS-17 | 1.88 | 121.1 |
| CS-2 | 1.87 | 126.1 | CS-18 | 1.88 | 140.7 |
| CS-3 | 1.9 | 329 | CS-19 | 1.89 | 414.4 |
| CS-4 | 1.86 | 283.7 | CS-20 | 1.93 | 90.6 |
| CS-5 | 1.91 | 100.4 | D-1 | 1.83 | 124.3 |
| CS-6 | 1.89 | 98.2 | D-3 | 1.79 | 105.7 |
| CS-7 | 1.88 | 126.4 | D-4 | 1.85 | 148.5 |
| CS-8 | 1.87 | 330.7 | D-5 | 1.78 | 320.8 |
| CS-9 | 1.86 | 390.3 | D-7 | 1.75 | 295.5 |
| CS-10 | 1.89 | 170.7 | D-9 | 1.87 | 256.6 |
| CS-11 | 1.87 | 199.4 | D-10 | 1.79 | 209.5 |
| CS-12 | 1.92 | 151.6 | D-24 | 1.82 | 183.2 |
| CS-13 | 1.96 | 185.3 | D-25 | 1.85 | 227.7 |
| CS-15 | 1.9 | 403.3 | D-26 | 1.89 | 210.2 |
| CS-16 | 1.89 | 186.1 | D-28 | 1.79 | 228.5 |

# Agarose Gel Electrophoresis

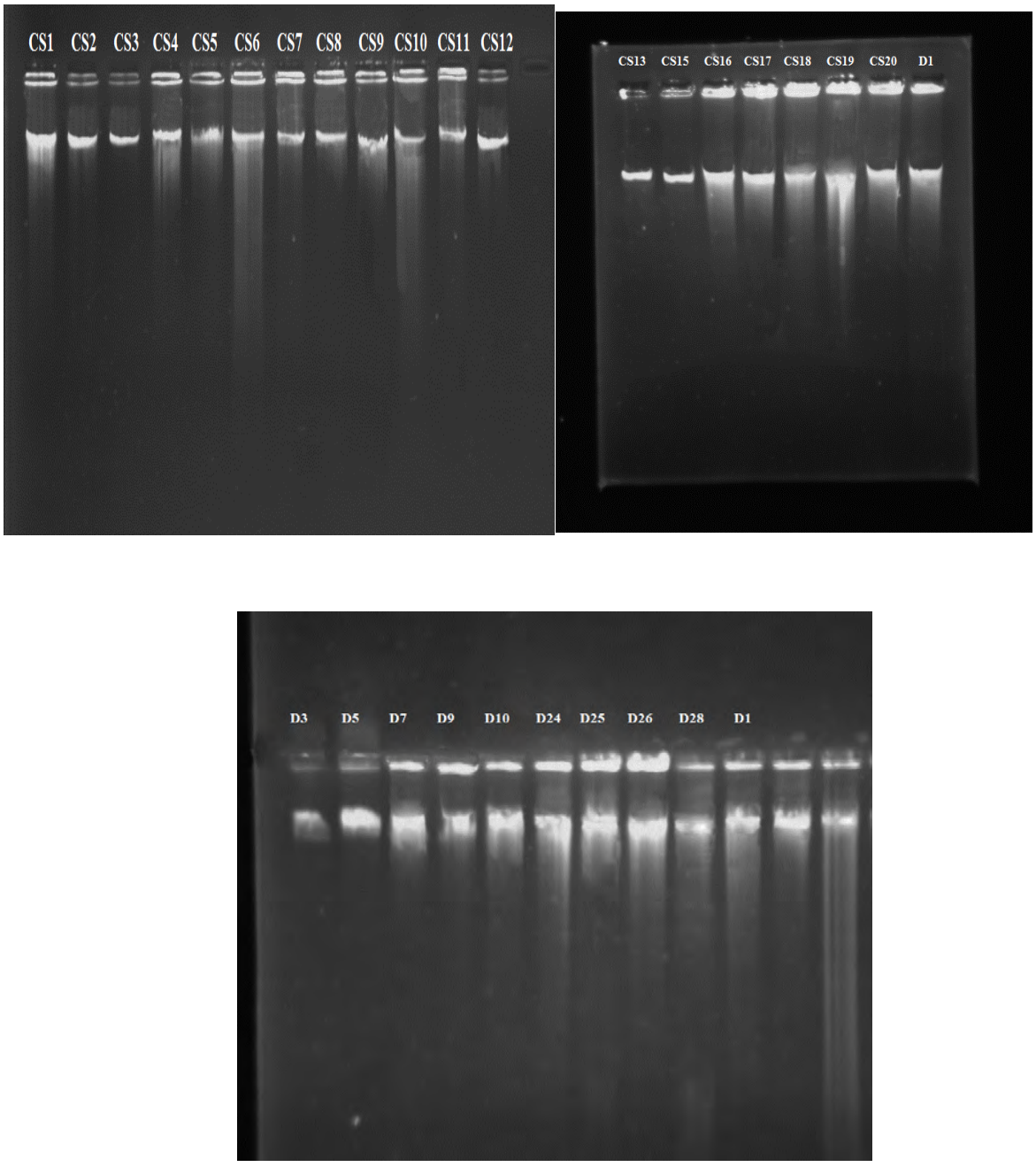



**Figure 3.1: Agarose gel electrophoresis in (0.5%) of isolated genomic DNA in 1X TE buffer.**

## 3.3 PCR amplification of beta casein variants

After analyzing the mutation point in the bovine beta casein gene, primers were designed to amplify bovine beta casein variants. The optimum primer concentration and cycling parameter results in an efficient amplification of the desired beta casein variants fragment size by PCR. For both A1 allele ("A" allele) and A2 allele ("C" allele), the amplified AS-PCR product was 854 bp. In case of the ACRS-PCR the amplified primer product was 121 bp and after restriction enzyme digestion for A1 allele was 121 bp and A2 allele 86 bp and 35 bp.

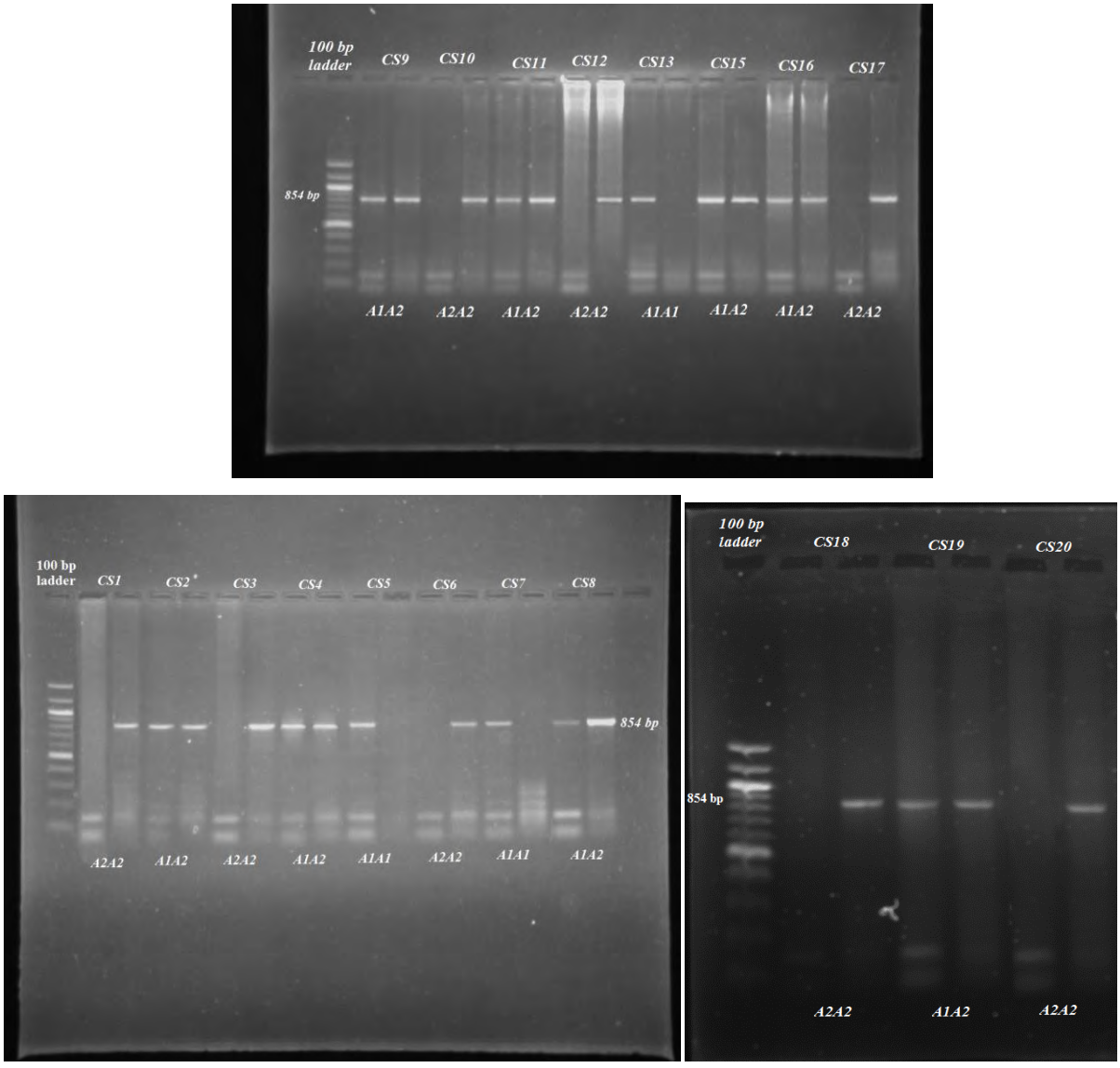
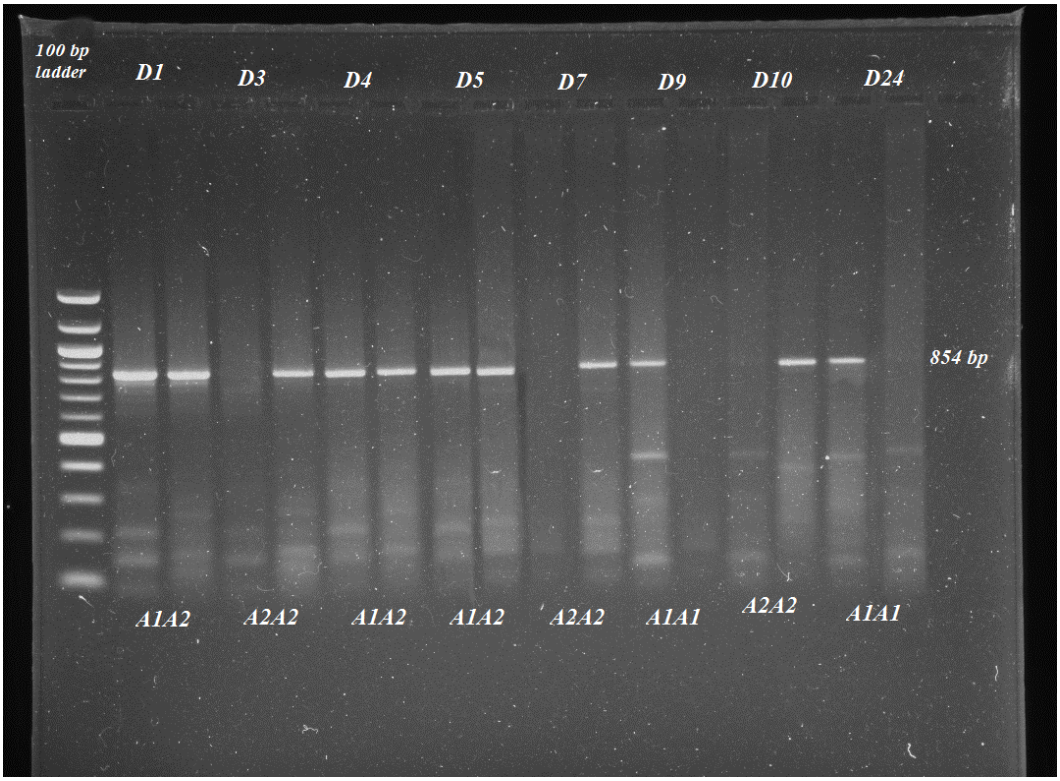



**Figure 3.2: Agarose gel electrophoresis of AS-PCR product (854 bp, CS1-CS20).**



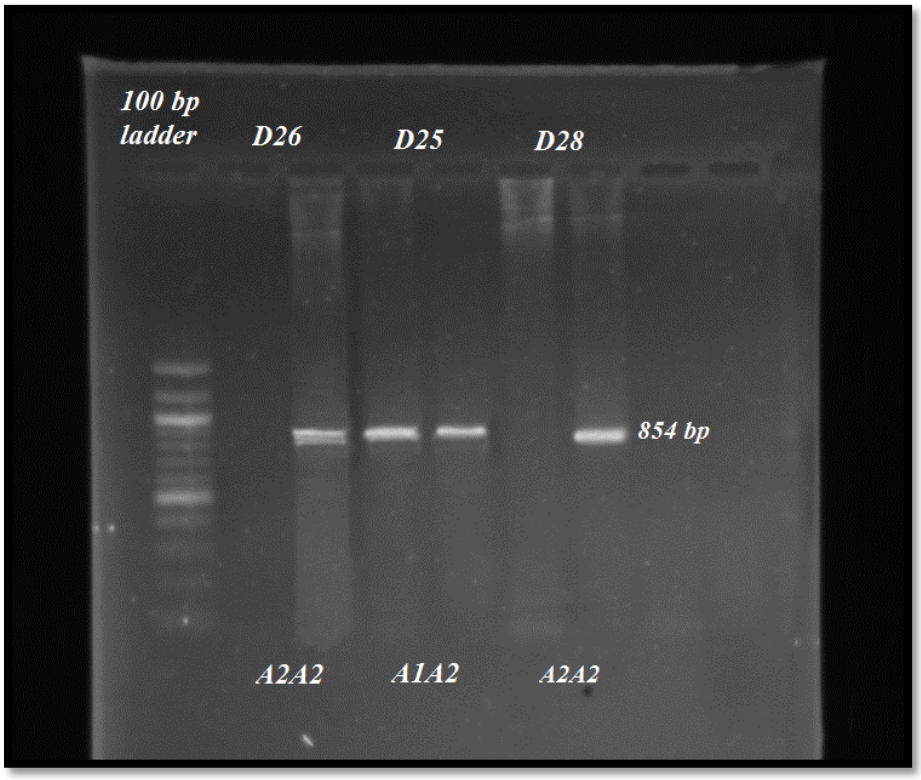




**Figure 3.3:Agarose gel electrophoresis of AS-PCR product (854 bp, D1-D28)**

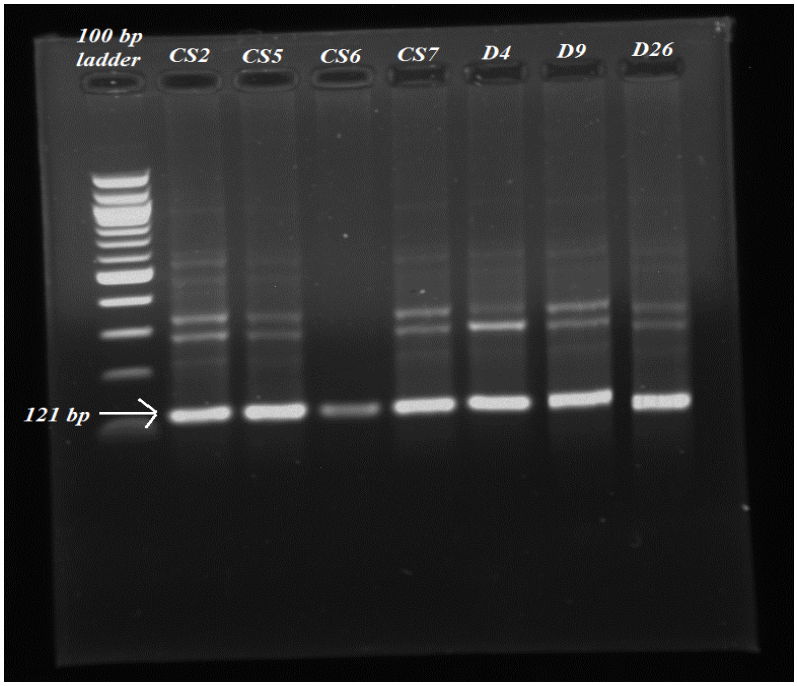



**Figure 3.4:Amplified PCR product (121 bp) of ACRS-PCR**

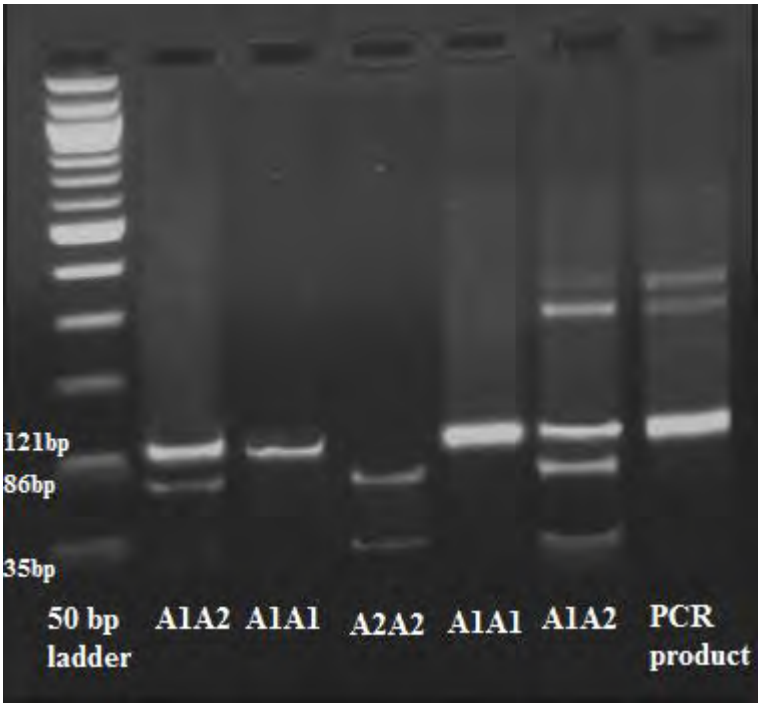



**Figure 3.5:Agarose gel electrophoresis of restriction enzyme digested PCR products (121 bp, 86 bp, 35 bp)**

## 3.4 Gene and Genotypic frequency:

In the experimented bovine beta-casein gene, three genotypes were revealed namely, CC, AC, and AA (Fig- 3.2, Fig-3.3, Fig-3.4). At this particular SNP location, two nucleotide present that is A (A1) and C (A2). The genotype and allele frequency for both these alleles were calculated and presented in Table-3.4. The homogenous genotype CC was found to be the predominant genotype in local cattle breed. Overall frequencies of CC,AC,AA genotypes were 0.43,0.4, and 0.17 respectively. In the experimented cattle the wild type allele i.e. "C" nucleotide is prevalent. Out of 30 animal tested, 13 animals (43.3%) showed A2A2 genotype containing "C" nucleotide (wild type allele; A2 allele). Twelve animals were having A1A2 genotype containing "C" and "A" nucleotide (Both A1 and A2 allele respectively) in the same animals. While five animals (16.7%) showed "A" nucleotide. Table -3.4 shows the inheritance pattern of the variant allele (A1 allele containing "A" nucleotide) was present in both heterozygous and homozygous conditions. Only five animals were found homozygous for A1A1genotype containing only "A" allele (A1 allele).

**Table 3.2: Genotype of the tested animals**

| Animal no. | Genotype | Animal no. | Gwnotype |
|---|---|---|---|
| CS-1 | A2A2 | CS-17 | A2A2 |
| CS-2 | A1A2 | CS-18 | A2A2 |
| CS-3 | A2A2 | CS-19 | A1A2 |
| CS-4 | A1A2 | CS-20 | A2A2 |
| CS-5 | A1A1 | D-1 | A1A2 |
| CS-6 | A2A2 | D-3 | A2A2 |
| CS-7 | A1A1 | D-4 | A1A2 |
| CS-8 | A1A2 | D-5 | A1A2 |
| CS-9 | A1A2 | D-7 | A2A2 |
| CS-10 | A2A2 | D-9 | A1A1 |
| CS-11 | A1A2 | D-10 | A1A2 |
| CS-12 | A2A2 | D-24 | A1A1 |
| CS-13 | A1A1 | D-25 | A1A2 |
| CS-15 | A1A2 | D-36 | A2A2 |
| CS-16 | A1A2 | D-28 | A2A2 |

**Table 3.3: A1, A2 genotypes and their frequencies in local and cross breed cattle**

| Breed | Number of samples | Genotype frequency | | | Allele frequency | |
|---|---|---|---|---|---|---|
| | | A1A1 | A1A2 | A2A2 | A1 | A2 |
| **Local** | 18 | 0 | 0.33 (n=6) | 0.67 (n=12) | 0.17 | 0.83 |
| **Cross** | 12 | 0.42 (n=5) | 0.33 (n=4) | 0.25 (n=3) | 0.58 | 0.42 |

Among the 18 local samples, AA genotype was not found, the genotype frequency of AC and CC were 0.33 and 0.67 respectively. However, five cross breed sample out of twelve were reported to have AA genotype. Consecutive (AA, AC, CC) genotype frequency in crossbreed cattle were 0.42, 0.33, 0.25. From the above table, it can be seen that for local breed samples A2 (wild type) allele was predominant. On the other hand, for the crossbreed cattle, the A1 allele was predominant.

**Table 3.4: Overall A1, A2 genotypes and their frequencies in thirty cattle sample**

| A1, A2 Genotype | Number of variants | Genotype Frequency | Allele Frequency | |
|---|---|---|---|---|
| **A1A1** | 5 | 0.17 | **A2** | 0.63 |
| **A1A2** | 12 | 0.4 | | |
| | | | **A1** | 0.37 |
| **A2A2** | 13 | 0.43 | | |

From the above result it can be seen that frequency of "A" nucleotide is 0.37 and "C" nucleotide is 0.63. Indicating higher frequency of A2 allele.

# Chapter 4:

# Discussion

## Discussion

Bovine milk and dairy products are the main nutritional and physiological ingredients of the human diet (Keating et al., 2008). Various major biological activities are carried out by casein and major milk proteins. These proteins provide critical nutrients and immunological protection as positive regulators of human health (Clare &Swaisgood, 2000). Nowadays, the focus is on biologically active peptides (Beta Casomorphin-7) derived from milk proteins. These peptides are inactive in the parent protein molecule (β casein) sequence and may be released through gastrointestinal milk digestion, milk fermentation with proteolytic starter cultures, or proteolytic enzyme hydrolysis (Korhonen&Pihlanto, 2006). Beta - casein variants A1 and B differ from the variant A2 at position 67, resulting in the peptide bond cleavage following digestion and release of the β-casomorphin-7 bioactive peptide (Kaminski et al., 2007).

The detection of A1 and A2 beta casein variant was done by performing allele-specific PCR. The presence of A1 or A2 gene was confirmed when the samples showed a band at 854 bp with A1 or A2 allele-specific primers. In the case of amplification created restriction site PCR samples with A1 variant showed a band at 121 bp and A2 variant showed two bands at 86 bp and 35 bp. The result of the present study is similar to other studies conducted by Rahimi et al.,(2015) where AS-PCR was used to determine the A1 and A2 variants of beta casein. The objective of this study was to determine the frequency of the beta-casein variant in native and cross cattle of Bangladesh. Blood samples of thirty cattle (eighteen local and twelve crossbred) were taken from two cattle farms in Dhaka. As the DNA was extracted without a DNA extraction kit, it was difficult to establish a manual and convenient DNA extraction method. However, the whole genomic DNA was extracted from blood using a phenol-chloroform method. After the successful extraction of DNA, the quality and quantity of extracted DNA were determined through measurement of OD (A260/A280) and agarose gel electrophoresis. The quality and quantity of DNA were within the desired range. Subsequent to the quality check polymerase chain reaction was done, with different primers specific to A1 and A2 allele, to amplify the DNA. Depending on the amplification result the genotype of the animal was determined. In case of allele-specific PCR if the animal show band with A1 specific primer it indicates A1A1 genotype, similarly with A2 specific primer it specifies A2A2 genotype. If an animal shows band with both the primers it has A1A2 genotype. In the amplification created restriction site PCR after getting the 121bp

amplified PCR product restriction digestion was performed with DdeI enzyme. Following the digestion animal giving two bands (86 bp and 35 bp) indicates A2A2 genotype and on the other hand, one band of 121 bp shows A1A1 genotype. Among the thirty animal studied the genotype frequency of A1A1, A1A2, and A2A2 was 0.17, 0.4 and 0.43 respectively.

In the population included in the study moderately significant differences in frequencies of genotypes were found. When considered gene frequency, A2 was found dominant in local (0.83) and A1 in crossbred (0.58) animals. It is reported that the frequency of A1 allele varies generally among different exotic breeds between 0.01-0.06 (Guernsey), 0.09-0.22 (Jersey), 0.31-0.66 (Holstein), 0.43-0.72 (Ayrshire) and 0.71 (Danish Red) (Kaminski et al., 2007). In the present study, A1 allele frequency was found very diverse in local (0.17) and crossbred (0.58) animals. The origin of crossbred animals could not be identified during sample collection, therefore it was not possible to compare specifically according to breed. However, the finding of A1 allele frequency is within the range of A1 allele frequency in Ayrshire, Black, and White, Red, and White (Germany) breed as reported earlier (Kaminski et al., 2007). AS-PCR and ACRS-PCR were found incredibly useful in distinguishing A2 and A1 allele. Recently, it was reported that Polish Holstein Friesian has comparable A1 (0.35) and A2 (0.65) allele frequencies (Olenski et al., 2010). The predominance of CSN2 A2 allele (0.764) detected by Caroli et al., (2008) in a population of Carora cattle. Manga et al., (2006) presented a lower frequency of allele A1 in a population of Czech Spotted and Czech Holstein breed. The higher frequency of allele A1 was reported by Bech et al., (1990) for Black-and-White breed and Ehrmann et al., (1997) for Red-and-White breed. Ikonen et al., (1997) reported slight superiority of allele A1 for Ayrshire breed and Hanusová et al., (2010) for Holstein bulls. In cross-breed cattle (Karan Fries) of India, a lower frequency of A1 allele of beta-casein (17.5%) was detected (Jaiswal et al., 2014). The higher frequency of the allele A2 in a population of Pinzgau cattle was reported by Beja-Pereira et al., (2003). The finding regarding the higher frequency of A2 allele than A1 allele is complying with the findings reported mentioned above. A moderately high frequency (37%) was found in the present study for beta - casein A1 allele, which indicates that the experimented cattle milk may contain a relatively high percentage of deleterious beta - casein A1 variant and β-casomorphin-7.

Milk has a variety of nutrient composition for people (Islam et al., 2014). Because of the association with a range of illnesses through releasing BMC-7 upon digestion, the detection of genetic polymorphism of milk protein beta-casein in dairy cattle population is really important. BCM-7 can play a functional role in human disease development such as diabetes mellitus and heart disease. New Zealand epidemiological reports show that consumption of A1 beta-casein variant is associated with higher national heart disease mortality rates. However, the benefit of milk containing the variant A2 beta casein for human health has been suggested as compared to milk with the variant A1 beta-casein (McLachlan, 2001). Populations consuming milk with high levels of beta-casein A2 variant appear to have a lower incidence of cardiovascular disease and type 1 diabetes mellitus (Laugesen& Elliott, 2003). However, as a major cause of sudden infant death syndrome and neurological disorders such as autism and schizophrenia, consumption of milk with a high level of BCM-7 has been suggested (Ganguly et al., 2013, Kaminski et al., 2007). Sampled native and crossbreed cattle exhibit a superiority of A2 allele that does not produce BCM-7 and is, therefore, safe for human consumption. In the sampled population, however, there is also the existence of A1 allele that can lead to the genotype of A1A1 if bred with another A1 allele. Therefore, care should be taken to prevent A1 allele breeding.

# Conclusion

## Conclusion

There are 209 amino acids in bovine beta-casein. At position 67, which is histidine in A1 or proline in A2 milk, the variants of beta-casein A1 and A2 differ only. A bioactive seven-amino acid peptide, b-casomorphin-7(BCM-7) may be released from the digestion of milk-containing A1 beta-casein. Thirty cattle of local and cross breed from two cattle farms in Dhaka genotyped for the beta-casein gene type A1 and A2. Allele-specific PCR and Amplification created restriction site PCR was used to detect the A1 and A2 beta casein type. Out of thirty animals tested, 13 animals (43.3%) showed A2A2 genotype containing "C" nucleotide (wild type allele; A2 allele). Twelve animals had A1A2 genotype containing "C" and "A" nucleotide (both A1 and A2 allele respectively) in the same animals. Five animals (16.7%) showed "A" nucleotide (variant allele, A1 allele). The allelic frequencies for "A" and "C" nucleotide containing alleles were 0.37 and 0.63 respectively. In local breed cattle the wild type allele i.e., "C" nucleotide is prevalent. While on the other side variant allele i.e., "A" is pervasive in crossbreed animal. The data presented in the study concludes that the frequency of undesirable "A" nucleotide is 0.37 which is supposed to be responsible for the production of A1 milk.This frequency of undesirable "A" nucleotide allele may be attributed to the fact that crossbreed animal is evolved from a breed with higher A1A1 genotype.Thus, it can be said that Bangladeshi cattle breed are still on the safer side that allele responsible for A2 milk production is comparatively high of "C" nucleotide containing wild type allele.

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# Appendices

## Appendix 1

# Composition of different buffers & reagents used in DNA isolation from blood and gene cloning

### 1. 20X SSC Buffer (500ml)

| Reagent | Amount |
|---|---|
| Sodium chloride | 87.65 g |
| Trisodium citrate | 44.1 g |
| Autoclaved distilled water | Upto 500 ml |

### 2. Proteinase k (20mg/ml) (200µl)

| Reagent | Amount |
|---|---|
| Proteinase k | 4mg |
| TE buffer | 200 µl |

Store at -20$^0$C

### 3. 10% SDS

| Reagent | Amount |
|---|---|
| SDS | 10mg |
| Autoclaved distilled water | 100ml |

### 4. 50X Tris Acetate EDTA(TAE) (500 ml)

| Reagent | Amount |
|---|---|
| 2M Tris base | 121 g |
| Acetic acid | 28.55 ml |
| 0.05 M EDTA | 50 ml/ 18.6 g |
| Autoclaved water | Upto 500 ml |

### 5. Phenol/ Chloroform/ Isoamyl alcohol (25:24:1) (50 ml)

| Reagent | Amount |
|---|---|
| Saturated Phenol (pH-8.0) | 25 ml |
| Chloroform | 24 ml |
| Isoamyl alcohol | 1 ml |

**6. Chloroform/ Isoamyl alcohol (24:1) (25 ml)**

| Reagent | Amount |
|---|---|
| Chloroform | 24 ml |
| Isoamyl alcohol | 1 ml |

**7. 70% Ethanol (100 ml)**

| Reagent | Amount |
|---|---|
| 100% Ethanol | 70 ml |
| Autoclaved distilled water | 30 ml |

**8. 5 M Sodium Chloride (500 ml)**

| Reagent | Amount |
|---|---|
| Sodium Chloride | 146 g |
| Autoclaved distilled water | 500 ml |

**9. TE (10:1) Buffer (pH-8.0)**

| Reagent | Amount |
|---|---|
| Tris-HCL | 10 mM |
| $Na_2EDTA$ | 1 mM |

**10. Ethidium bromide**

| Reagent | Amount |
|---|---|
| Ethidium bromide | 10 mg |
| Autoclaved distilled water | 1 ml |

## Appendix 2

## Instruments

| Instrument | Manufacturer |
|---|---|
| Weighing machine | RADWAG, Model-AS 220, R2 |
| Incubator | BenchTop lab system |
| Laminar flow hood | Biobase Model- BBS-SDC |
| Fume hood | Biobase Model- FH 1000 |
| Nanodrop 2000 spectrophotometer | Thermo scientific, USA |
| UV transilluminator | UV solo TS, Biometra |
| $-20^0$C Freezer | Siemens |
| $-80^0$C Freezer | Thermo Scientific |
| Vortex machine | VWR International |
| Centrifuge machine | Labogene |
| Micropipette | Eppendorf , Germany |
| Disposable micropipette tips | Eppendorf, Germany |

## Appendix 3

## List of abbreviations

| | |
|---|---|
| % | Percentage |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| AS | Allele specific |
| ACRS | Amplification created restriction site |
| BCM-7 | Beta Casomorphin 7 |
| CN | Casein |
| cm | Centimeter |
| SDS | Sodium-dodesyle-sulfate |
| PCI | Phenol:Chlorofrom:Isoamlyalchohol |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTPs | Deoxynucleoside triphosphate |
| OD | Optical density |
| EDTA | Ethylenediaminetetraacetate |
| CSN | Casein |
| kb | Kilo base pair |
| bp | Base pair |
| mg | Milligram |
| ml | Milliliter |
| mM | Millimolar |
| Etbr | Ethidiumbromide |
| PCR | Polymerase chain reaction |
| U | Enzyme unit |
| SNP | Single nucleotide polymorphism |