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Genetic Polymorphism of Kappa Casein (κ-CN) in Iraqi Buffalo Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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Abstract: This study was carried out the animal production department, genetic engineering lab, college of agriculture, (UoB), Iraq. The aim of this study was to use the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as a fast, efficient and low cost method to detect the genetic variants of kappa-casein gene (κ-CN) in Iraqi buffalo using three different primers specific for bovine κ-CN to amplify the gene segment, followed by digestion using restriction enzyme (Hind III) for genotyping. DNA from 50 Iraqi buffaloes was extracted by phenol chloroform method. PCR was carried out in a final reaction volume of 25 μL and the reaction mixture was subjected to standard PCR protocol. The results of this work show that among the examined 50 Iraqi Buffalo were homozygous for the κ-CN and genotyped as BB for all three primers but gave different bands. Thus PCR-RFLP using Hind III revealed all the samples to be monomorphic for this locus. The restriction digestion analysis of 397 bp PCR product of κ-CN indicates the presence of two fragments of 154 bp and 225 bp for BB-genotype. A 437 bp fragment of the bovine genomic κ-CN gene was amplified. One Hind III restriction site is found in position 346 of the amplified fragment of allele κ-CN B, yielded 91 bp and 346 bp. Amplified products from Iraqi buffalo (530), after being digested with Hind III, yielded two separate DNA fragments of different sizes i.e., 160 bp and 370 bp. For the first time completed research such specifications in Iraq, for the first time using molecular biology in genetic identification. Our objectives of this study have been to aid in understanding domestication, Buffalo origin and their history and evolution, to identify genetically unique breeds, to provide an objective basis for conservation decisions and to aid the formulation of breeding plans.

Key words: Polymorphism, kappa casein gene, Iraqi buffalo, Hind III, PCR-RFLP.

1. Introduction

The development of DNA markers and molecular biological techniques is fundamental tool for applications in animal breeding. Also has created new possibilities for the genetic markers, genetic improvement and selection of animals. Moreover, the discovery of the PCR had a major impact on the research of animals' genomes and contributed to the development and application of various DNA markers. During the last 15 years, DNA markers gave more benefits of genetic improvement in animals' breeds [1-3]. Buffalo is the first agricultural animal to be domesticated in the Southern part of Iraq because of their high resistance to diseases and high production of milk. Iraqi buffalo characterized long productive age where the females remain up to 15 years. Limited numbers of studies has shown that the production rates of milk varies depending on several factors including the geographical area, feeding systems, health care, reproductive and genetic improvement through using selected pulls. The range of production was between 680 L of milk per season in the marshlands and 1,800 L in the areas near to cities particularly White Gold village, Abu Ghraib region, Baghdad. Production has reached in some populations to 2,500 L in the lactation of 229 days to 245 days [4]. There are few studies reported the superiority of Iraqi

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buffalo to grow compared with the world's breeds [5] and with local calves cow [4], but this ability has not incorporated well because of the absence of identification of genetic background of Iraqi buffalo, therefore must take utmost importance. Recently, a new department of buffalo development has been established by the ministry of agriculture focusing on the genetics and buffalo breeding.

Other studies have focused on the description of Iraqi buffalo through phenotypic markers [6], followed by chromosomal markers [7]. Aljammas [7] studied numbers of chromosomes in the Iraqi buffalo. Another study has focused on the traits of the Iraqi buffalo productivity without focused on genetic material. The first study that began on the genetic aspects was Jaayid et al. [8], as they began study the buffalo through biochemical markers. They have been described that transferrin protein obtained five genotypes, then followed by using molecular markers in the description of Iraqi buffalo using microsatellites technique [9]. Iraqi buffalo divided into two types according to its geographical spread to swamp buffalo and rivers buffalo. Rivers buffalo live near to the banks of rivers and around the big cities. Iraqi buffalo characterized by different properties and there is great diversity in color, which varies from slate black to almost white, white patches on the head, legs and tail are common, horns are generally sickle shaped, the face is slightly dished, the withers are high, and the croup and haunch bones prominent, the body is generally elongated, the udder in good specimens, is large, carried well back and has large well-placed teats.

Buffalo bred in Iraq for thousands of years and spread in most provinces except Dohuk, where there are 60% of the Iraqi buffalo in the Southern region. Despite the unique traits of Iraqi buffalo such as production and along term of their productivity, however, they did not receive considerable research interest and all Iraqi buffalo wherever it reared have the strength, harshness and brutality that are not available in the world buffalo breeds [10]. The genetic diversity and evaluation are mutually connected. In farm animals, many breeds well adapted to different conditions. On the other hand, man has decreased the genetic diversity. Lately different methods are used to evaluate genetic diversity: different in body structure or the coefficients of inbreeding are studies, polymorphism of blood groups, milk and blood proteins and molecular markers, especially microsatellites are evaluated. Recent molecular techniques are needed to characterize buffalo breeds or strains genetically and to estimate the genetic variability between them in order to enhance selection and breeding programs. Modern molecular techniques will help in conservation of our buffalo resources by depositing new sequence data as bio-information in gene bank.

The purpose of this proposal project is to give an overview of our current knowledge on the polymorphisms occurring in genes coding for milk proteins in Buffalo, responsible for quantitative variability in their gene expression and to analyze the genetic polymorphism of the kappa casein gene (κ-CN), which plays a key role in micelle stabilization and milk coagulation in buffalo in the Southern part of Iraq. We will detect the polymorphism at DNA level. Our objectives will be made the first stage of animal genetic resources conservation in Iraq.

2. Material and Methods

2.1 Animals

Data was collected from 50 Iraqi Buffaloes from three regions of Iraqi southern: Maisan (130 KM) and Thy Qar (200 KM) from Basrah in additional to Basrah city.

2.2 DNA Extraction

Genomic DNA was extracted from the whole blood by phenol-chloroform method described by John et al. [11] with minor modifications. Method described as follows: Portion of each blood sample, collected on EDTA, was immediately stored at -5 °C. Then after thawing, to an aliquot of 50 μL blood, 700 μL of lysis

buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% SDS) and 60 μL of proteinase K (20 mg/mL) were added. The mixture was vortexed and incubated at 37 °C overnight. DNA was extracted with equal volumes of phenol:chloroform (1:1) and chloroform:isoamylalcohol (24:1). DNA was precipitated by adding 0.1 (by volume) of 3 M sodium acetate and 2 volumes of chilled ethanol. The pellet was washed with 70% ethanol, air-dried and subsequently dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA). The concentration of DNA and its relative purity were determined using a spectrophotometer based on absorbance at 260 nm and 280 nm, respectively. The integrity of extracted genomic DNA was checked by electrophoresis in 0.2% agarose gels using DNA as a molecular weight marker. The quality and concentration of extracted DNA was assessed by NanoDrop from Fisher Scientific Company (USA).

2.3 PCR Reaction and DNA Amplification

Selection of markers and primers design: the primers used in this study are basically of cattle origin but because of very high degree of nucleotide sequence conservation between cattle and river buffalo, these primers are likely to give amplification in buffalo too [12-14]. This particular primers have been used successfully for PCR amplification from buffalo DNA and these primers have been used in this work to detect polymorphism at the κ-CN locus in Iraqi buffaloes (Table 1). A PCR cocktail consists of 1.0 μM forward, reverse primers, 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100 and 1.25 units of Taq polymerase. The cocktail was aliquot into tubes with 100 ng buffalo DNA. The reaction mixture was overlaid with sterile mineral oil and is run in a Applied Biosystems Veriti^{TM} thermal cycler. The 100 bp ladder marker was used as molecular size marker. The amplified product was visualized as single band under UV light and photographed by gel documentation system (S 140, Votorenix, USA).

2.4 RFLP Technique and Agarose Gel Electrophoresis

After amplification gene fragment (10 μL of PCR product), they were subjected to digestion with 5 units of the restriction enzyme (Hind III) in a final reaction volume of 25 μL. The reaction mixture was incubated at 37 °C in water bath for at least 3 h. After restriction digestion, the restricted fragments were analyzed by electrophoresis on 2.5% agarose gel/1X TBE gel stained with ethidium bromide. The 100 bp ladder was used as molecular size marker. The bands were visualized under UV light and photographed with yellow filter on black and white film. The band size were judged by comparing with molecular size markers and recorded.

3. Results

3.1 Analysis of PCR Amplified κ-CN Gene Fragment

PCR amplification of the all fragments of the κ-CN gene produced a clear and distinct band on the gel, which is major importance in the digestion step since the presence of PCR artifacts could compromise the scoring of the fragments (Fig. 1).

Table 1 The DNA sequences and information of the primers used for κ-CN.

Primers for κ-CN	Primer sequence 5'---------- 3'	PCR conditions	Reference
Primer 1	CAC GTC ACC CAC ACC CAC ATT TAT C TAA TTA GCC CAT TTC GCC TTC TCT GT	1 min, 94 °C 2 min, 56 °C 2 min, 72 °C	[12]
Primer 2	TGA GCA GGT ATC CTA GTT AT TTT GAT GTC TCC TTA GAG T	1 min, 94 °C 2 min, 45 °C 2 min, 72 °C	[13]
Primer 3	ATA GCC AAA TAT ATC CCA ATT CAG T TTT ATT AAT AAG TCC ATG AAT CTT G	1 min, 94 °C 2 min, 57 °C 2 min, 72 °C	[14]

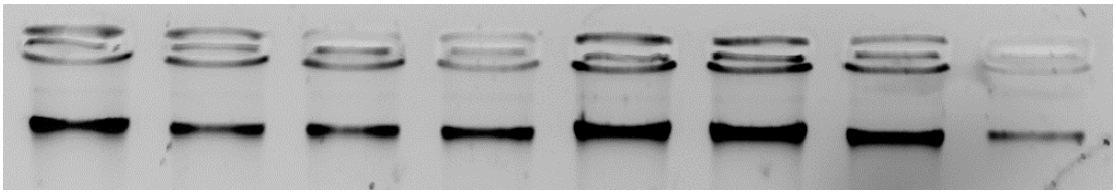

Fig. 1 The DNA profile for Iraqi buffalo.

Buffalo genotyping using the first primer (379 pb): the first primer is amplified with DNA fragment which is used as template for PCR reaction. The PCR amplification was confirmed by running 5 μL of PCR product along with 100 bp DNA ladder in 1% agarose gel. PCR amplification using primer yielded a 379 bp DNA fragment of κ-CN gene. Presence of a single band visible under UV light with ladder (Fig. 2).

3.2 RFLP Analysis of Amplified DNA Fragment of κ-CN Gene

Amplified products from Iraqi buffalo, after being digested with Hind III, generated two separated DNA fragments of different sizes. Cumulative analysis of the results depicted the existence of only B allele (Fig. 3). When length of each fragment compared with the marker lane and fragment size was estimated. All the samples revealed two fragments of 154 bp and 225 bp = 379. Only B allele with BB genotype was found in Iraqi buffalo.

Buffalo genotyping using the second primer (437 pb): A 437-bp fragment of the bovine genomic κ-CN gene was amplified. One Hind III restriction site is found in position 346 of the amplified fragment of κ-CN allele B (Fig. 4).

Buffalo genotyping using the third primer (530 pb): PCR amplification of the κ-CN gene in Iraqi buffalo flocks yielded fragment of 530 bp. Amplified products from Iraqi buffalo, after being digested with Hind III, generated two separate DNA fragments of different sizes i.e., 160 bps and 370 bps. Cumulative analysis of the results depicted the existence of only B allele (Fig. 5).

4. Discussion

Owing to the importance of κ-CN, the technological properties of milk, the polymorphism in κ-CN gene have been extensively studied in the ruminant and use of DNA polymorphic markers allows the determination of individual genotypes at many loci and provides information on population parameters such as allele frequencies as well as improving selection by marker assisted selection. In this study, PCR-RFLP technique was used to identify the κ-CN genotype in Iraqi buffalo using set of primers and one restriction enzyme. Our results clearly indicate uniform and homozygous population of Iraqi buffalos for κ-CN B allele. Restriction analysis of κ-CN amplified fragment with Hind III generated different DNA length fragments specific for BB genotype. The present findings was in similar to the finding of Mitra et al. [15], who reported monomorphism for κ-CN gene showing similar bands pattern of 225 bp, 154 bp and 288 bp, 91 bp by using restriction endonuclease Hind III and Hinf I, respectively in Murrah and Nili-Ravi buffalo breeds. Some authors [12, 16-18] also found monomorphism (BB) for this gene in buffalos. These results further confirm that *B. indicus* cattle are predominantly of κ-CN B type as compared to *B. taurus* cattle.

These results differ from other studies, which have reported A and B alleles for κ-CN, with B having the highest frequency for the Murrah and Bhadawari breeds but they have reported monomorphism in Surti and Mehsana breeds of buffalo [19]. On the other hand, Allmere et al. [20], Oner and Elmaci [21] reported the presence of A and B alleles for K-Ca, with highest frequency B allele in Holstein breed. Cows with AB and BB genotypes showed significant higher milk proteins and fat content when compared with that of AA genotype [22]. The absence of other alleles than B allele in our study may be attributed to the lower number of studied animals comparing with

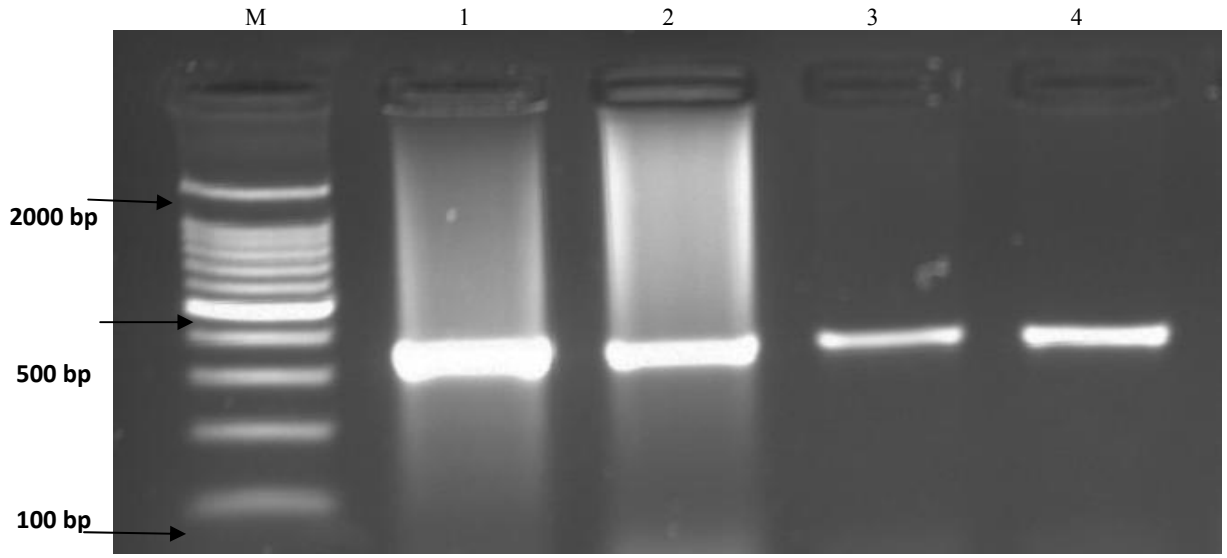

Fig. 2 Analysis of amplified product of κ-CN gene after PCR amplified with the first primer for buffalo κ-CN gene on 1% agarose gel (lanes 1-4), M: 100 bp marker.

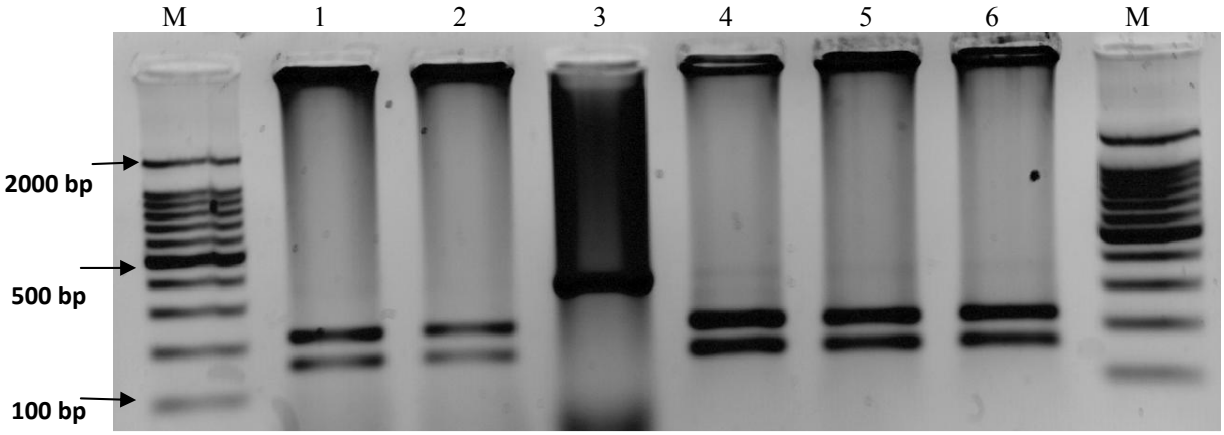

Fig. 3 Separation of Hind III digested PCR products on 2.5% agarose gel. M: 100 bp marker; Lanes 1-2, 4-6: restriction digested PCR products showed two fragments of 154 bp and 225 bp (BB genotype); Lane 3: 530 bp undigested PCR product.

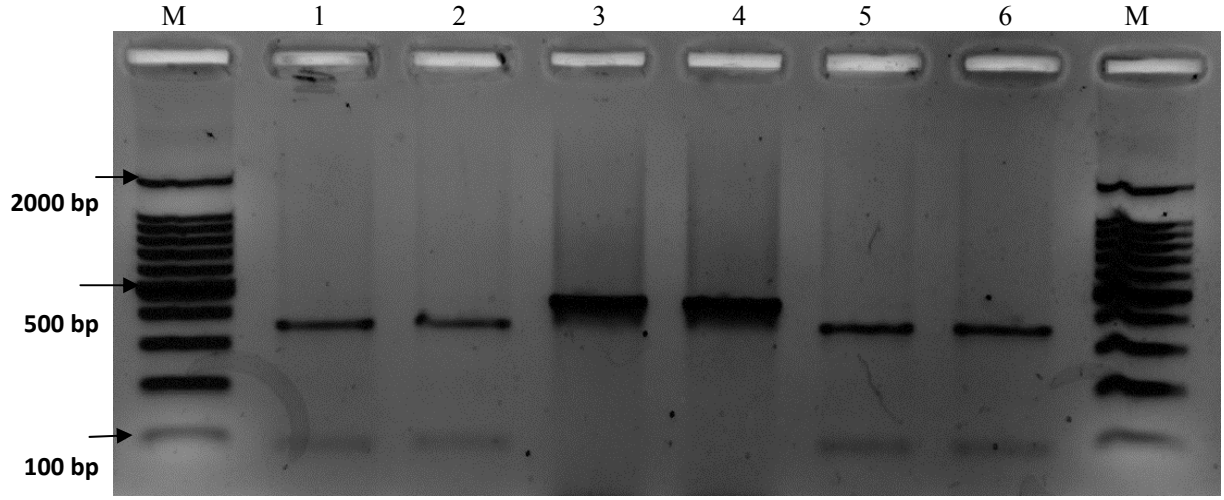

Fig. 4 Separation of Hind III digested PCR products. M: 100 bp marker; Lanes 1-2, 5-6: restriction digested PCR products showed two fragments of 91 bp and 346 bp (BB genotype); Lane 3: 437 bp undigested PCR product.

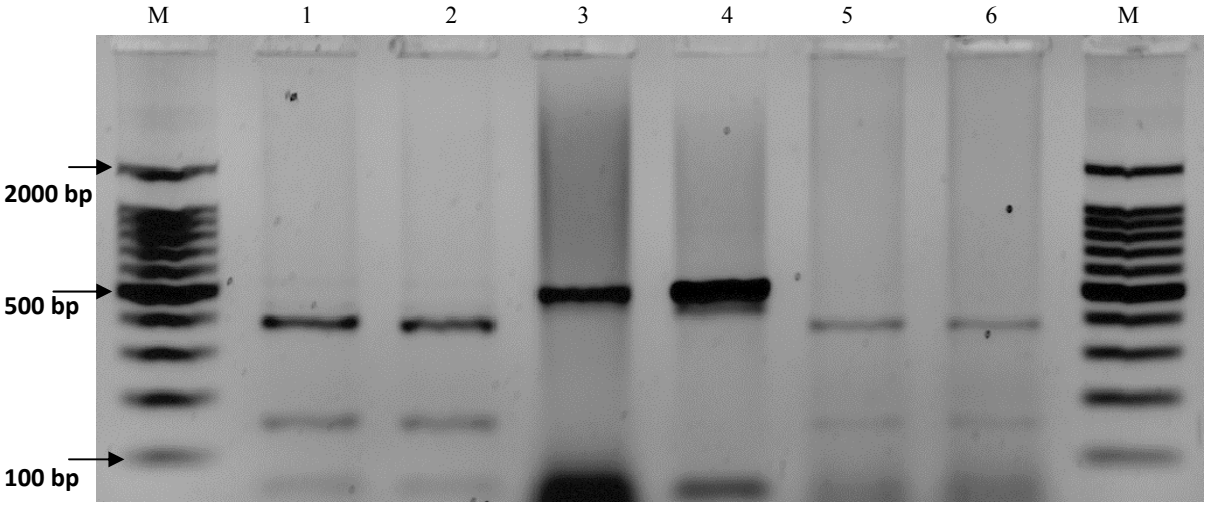

Fig. 5 Analysis of amplified product of κ-CN gene (Exon IV). M: 100 bp marker; Lanes 1-2, 5-6: restriction digested PCR products showed two fragments of 160 bp and 370 bp (BB genotype); Lane 3-4: 530 bp undigested PCR product.

other studies. Moreover, the frequency of B alleles in buffalo is very much higher**.** Similarly, two types of alleles were found in Murrah, Surti and Pandharpuri breeds of buffalo by Patil et al. [23]. Monomorphic form BB of κ-CN is responsible for higher yield in cheese making as well as milk and milk protein yield [24]. The cheese production can be increased by 10% if milk from cows of genotype BB of κ-CN is used [25]. Many researchers reported that κ-CN genotypes especially BB one was related to the quality of milk and cheese making [23, 26]. Therefore, the BB genotype in Holstein cattle seems better suited for improvement of the quality of milk and cheese making in Iraq. Hence, the observed frequency distribution of κ-CN B allele in Iraqi buffaloes confirmed the monomorphism [27].

5. Conclusions

This study shows that the PCR-RFLP test is quite easy and the rapid method of genotyping bovine milk protein loci and the results of this study can also be helpful to study the κ-CN gene polymorphism in other bovine breeds in Iraq. So, the application of molecular marker in conjunction with conventional animal breeding practices can be useful for improvement in animal selection and identification of best alleles to utilize in breed selection. The present study is the first report on κ-CN genotyping of local buffalo in Iraq.

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