A PCR-RFLP assay to detect genetic variants of kappa-casein gene in cattle and buffalo

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ABSTRACT

Caseins are milk proteins existing in several molecular forms alpha S1, alpha S2, beta and kappa with variant alleles of each. Out of five known kappa-casein genetic variants, the A and B are the most common in the majority of cattle breeds. Kappa-casein variant B is reported to be favorable for milk quality and is considered to be included in breeding strategies of dairy animals. The aim of this study was to use the PCR-RFLP as a fast, efficient and low cost method to detect the genetic variants of kappa-casein gene in cattle and buffalo using K1 and K2 primers to amplify the gene segment followed by digestion by two restriction enzymes (Hind III & Hinf I) for genotyping. The results of this work showed that among the examined Holstein cattle (20 cows), 17 were found to be of the genotype AA and 3 were of the AB genotype. The BB genotype could not be detected among the studied animals. Higher protein percentage in milk is associated with kappa-casein genotype AB compared to AA, but there was no difference in the fat percentage. All buffalo samples (20 animals) were homozygous for the kappa-casein and genotyped as BB. On the basis of these results it is concluded that kappa-casein variant B may be used as a DNA marker in bovine selection programs for production of milk with improved properties.

***Key words:** PCR-RFLP, genetic variants, kappa-casein gene, cattle, buffalo.*

INTRODUCTION

Studies on milk protein are in progress for more than 100 years. Milk proteins are usually divided into two fractions. The soluble fraction, named "whey protein", constitutes the α-lacto albumen and β-lacto globulin. The insoluble fraction, named "whole casein", constitutes 4 different caseins (alpha S1, alpha S2, beta and kappa caseins). The casein fraction of milk proteins significantly influences the composition and physico-chemical properties of the milk (Grosclaude, 1988). The genetic variants of these proteins are resulting as a consequence of substitution or deletion of amino acids within the polypeptide chain (Eigel, 1984). These genetic variants are of economic importance as they can be used as a DNA marker for milk yield, fat and protein percentages (NG-Kwai-Hang, 1990). The DNA markers offer two possible future applications in animal selection; the combination of best alleles of two or more breeds, or the selection of best alleles within a breed (Haley, 1995). Moreover, DNA markers can be used to decrease generation interval by allowing selection at earlier stage in life (Kinghorn *et al.*, 1991). Also, markers can be used for selection of young dairy bulls prior to

progeny testing as a selection criterion for dairy sires (Soller and Beckmann, 1983). The genetic variants of kappa-casein gene are the most important milk markers for protein percentage. This genetic marker assisted selection (MAS) acts as an initial step to increase the frequency of favorable alleles and decrease the frequency of unfavorable alleles within a population (Brascamp *et al.*, 1993 and FitzGerland, 1997). Classical selection methods used in bovine for milk quantitative traits improvement depend on the milk as a substrate and so restricted only to mature lactating females. On the other hand, with the advent of molecular techniques, through polymerase chain reaction, greater genetic gain can be obtained by determining the potentials of an animal even before being expressed phenotypically by determining the genetic variants of any gene at the DNA level, independent of age and sex (Otaviano *et al.*, *2005*).

The aim of this study was to use the PCR-RFLP technique as a fast efficient and low cost method, independent of age and sex, to identify the genetic variants of kappa-casein in both cattle and buffaloes. At the same time, fat and protein percentages of milk from the same animals were also evaluated.

MATERIALS AND METHODS

Animals

The study was performed on a total of 40 animals from different herds. It included 20 Holstein cows and 20 Egyptian buffaloes, from three different farms at Al-Ismailia and Giza governorates.

Sampling

Both milk and blood samples were taken from all animals. Blood samples were taken on EDTA and stored at - 20° C until time of DNA extraction. Milk samples were collected from individual animals for estimation of somatic cell count (SCC) using Soma count 150 from Bentley to exclude any sub clinical mastitic cases from the studied samples. Fat and protein percentages were estimated using Infra red milk analyzer. This analysis was performed in the Mastitis and Neonates Diseases Research Department, ARRI.

DNA extraction

DNA was extracted by phenol-chloroform method described by Sambrook *et al.*, (1989) with minor modifications. It was based on removal of erythrocytes by successive washing in lysis buffer and subsequent lysis of the leucocytes using SDS (sodium duodecyle sulphate) and proteinase K followed by extraction with phenol chloroform. Briefly, 800 μl of the buffy coat were transferred to 1.5 ml microcentrifuge tube; after centrifugation of the blood samples, the volume was completed to 1.5 ml with erythrocyte lysis buffer. The solution was mixed by vortexing and then centrifuged for 15 min at 14000 rpm. The supernatant was discarded and the pellet was resuspended in 800 μl of lysis buffer. The lysis step was repeated till the pellet lost all reddish coloration. The resultant pellet was then resuspended in 400 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 1 % SDS and 0.3 mg / ml proteinase K and incubated at 37° C overnight. DNA was extracted once with phenol followed by another extraction with chloroform – isoamyl alcohol (24:1). To each extraction, equal volume of the solvent was added, followed by gentle mixing and centrifugation for 10 min at 14000 rpm. The top layer was carefully transferred to another clean tube for the second extraction. To the final aqueous phase, 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol were added and the solution was stored at- 20° C overnight. DNA was precipitated by centrifugation at 4°

C for 30 min at 14000 rpm. DNA pellet was washed by 70% ethanol, dried and then dissolved in TE buffer. DNA concentration was adjusted by measuring the OD at 260 nm.

Polymerase chain reaction (PCR)

The primers used in this study are basically of cattle origin, but because of the very high degree of nucleotide sequence conservation between cattle and river buffalo; these primers are likely to give amplification in buffalo too. These primers were those described by Mitra *et al.* (1998) and re-evaluated by Pipalia *et al.* (2001) and Othman (2005) with the following sequence K1 (5'-CAC GTC ACC CAC ACC CAC ATT TAT C-3') and K2 (5'-TAA TTA GCC CAT TTC GCC TTC TCT GT-3'). Besides the 5 ng of template DNA, the final 50 μl volume of PCR amplification cocktail contained 30 pmol of each primer and 1 X PCR master mix (DYNAzyme). The amplification cycles were carried out in a PTC-100 Thermocycler (MJ Research USA). Reaction conditions were 95°C for 5 min as initial denaturation followed, by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min was followed.

RFLP and electrophoresis

Fifteen microliters of each PCR product were digested separately with 7.5 units of each restriction enzyme (*Hind III* and *Hinf I*) in a final reaction volume of 20 μl containing 1X of the enzyme buffer. The digestion mixtures were incubated at 37°C for 3 hr in the thermocycler. After digestion, the resulting fragments were analyzed by electrophoresis on 2 % agarose gel using 1X TBE buffer containing 0.5 μg / ml ethidium bromide at 50 volts until complete separation of the bands. A 100 bp ladder DNA marker was used as a molecular size marker. The bands were visualized under UV-light to observe the polymorphic locus by the size change of DNA fragments.

Statistical analysis

The effect of kappa-casein variants on milk fat and protein percentages were analyzed by the T- test according to Petrie and Watson (1999).

RESULTS AND DISCUSSION

Caseins are milk proteins existing in several molecular forms, *alpha S1*, *alpha S2*, *beta* and *kappa* with variant alleles of each. Genetic variants of all caseins have been previously reported by Eigel *et al.* (1984). Out of 5 known kappa-casein genetic variants, the A and B alleles are the most common in the majority of cattle breeds (Erhardt, 1989). Kappa casein variant B is reported to be favorable for milk quality and is considered to be included in breeding strategies of dairy animals. The PCR-RFLP technique has been used to study the frequency of kappa-casein gene in bovine (Damiani *et al.*, 2000 and Soria *et al.*, 2003) and buffalo (Mitra *et al.*, 1998 and Othman, 2005).

In order to detect the polymorphism of kappa-casein gene, we used PCR for gene amplification, and RFLP analysis for allele type identification. A 379 bp fragment of Kappacasein gene was successfully amplified from the DNA extracted from each sample using both K1 and K2 primer pair (Fig.1). Following amplification, genotyping was performed by the restriction digestion of the amplified products using both *Hind III* and *Hinf I*, each in a separate digestion reaction, followed by agarose gel electrophoresis for analysis of the digestion pattern.

Genotypes were identified according to Rottmann and Schlee (1992) and Mitra *et al.* (1998) who reported that bovine AA genotype lacks the restriction site for *Hind III* in the 379 bp segment; hence it remains undigested and

yields only one fragment of 379 bp. The BB genotype has one restriction site for the same enzyme and so yielding two fragments of 225 and 154 bp. The AB genotype identified by yielding three fragments of 379, 225 and 154 bp. By using the *Hinf I*, the same authors identified the AA genotype by yielding three fragments of 156, 132 and 91 bp as it has two restriction sites. On the other hand, BB genotype has only one restriction site and generates two fragments of the size 288 and 91 bp. The AB genotype was identified by yielding four fragments of 288, 156, 132 and 91bp.

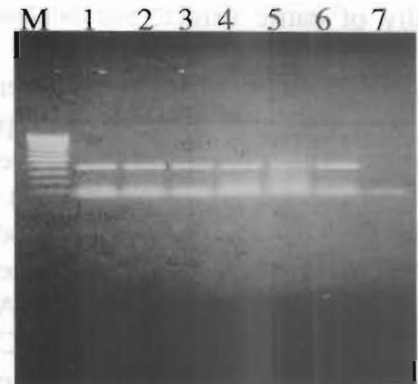


Fig. (1): 379 bp PCR products of kappa-casein gene.
M: 100 bp ladder marker
Lanes 1-3: 379bp PCR products of buffalo kappa casein.
Lanes 4-6: 379bp PCR products of cow kappa casein.
Lane 7: non-template negative control.

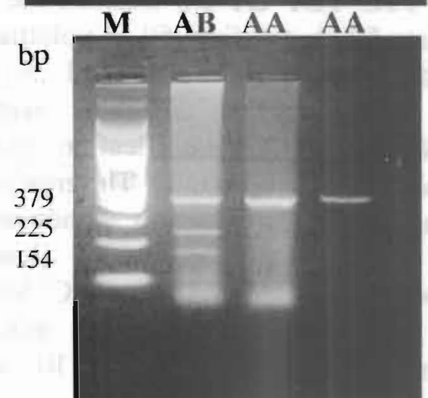


Fig. (2): Separation of Hind III digested 379 bp PCR products of representative samples of cattle kappa-casein with genotypes indicated above (M: 100bp ladder marker).

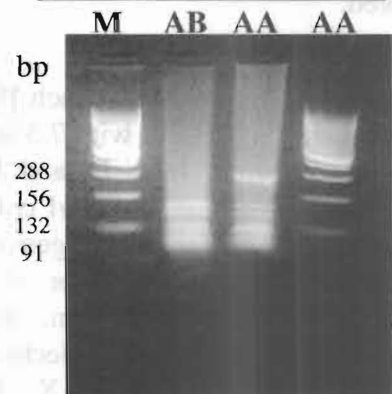


Fig. (3): Separation of Hinf I digested 379 bp PCR products of representative samples of cattle kappa-casein with genotypes indicated above (M: 100bp matker).

Cattle genotyping

In this study, twenty Holstein cows were genotyped by PCR-RFLP. Seventeen cows were found to be AA genotype, where they generate only one band of 379 bp after digestion with *Hind III* and three fragments of 156, 132 and 91bp when digested with *Hinf I*. The other three cows were identified as AB genotype, where they generate three fragments of 379, 225 and 154bp after digestion with

Hind III and four fragments of 288, 156, 132 and 91bp after digestion with *Hinf I* enzyme. (Fig. 2 and 3). Among the examined cows, the BB genotype could not be detected.

The frequency distribution of kappa-casein genotypes shows rare occurrence of homoz-ygous B in Holsteins (NG-Kwai-Hang, 1990) and this is in agreement with the previous studies for the same breed (McLean *et al.*, 1984; NG-Kwai-Hang *et al.*, 1984; 1986). Similar results have been observed by Mitra *et al.*, (1998) among Indian dairy cattle (namely Sahiwal) with low frequency of B allele and no homozygous BB animal. In the Europe breed, the situation is different; Pinder *et al.*, (1991) and FitzGerald (1997) observed low frequency of BB genotype in Northern cattle opposed to that found in Western cattle. The authors mentioned that it may be related to fitness for survival in this breed.

The data of milk analysis (Table1) sugge-sted a higher protein percentage in the milk of AB genotype when compared with that of AA genotype. On the other hand, there was no clear difference in the fat percentage. NG-Kwai-Hang *et al.* (1990) mentioned that kappa-casein phenotypes influence milk concentration of protein and fat but not the milk yield. In a previous study, NG-Kwai-Hang *et al.*, (1986), based on test day data, reported that kappa-casein BB milk was associated with higher fat content than AA variants. While the study of NG-Kwai-Hang *et al.*, (1990) based on lactation averages showed that there was no difference in the fat content for kappa-casein of genotypes AA and BB but AB genotype had less fat. In contrast, Bovenhuis (1993) reported a decreased fat yield for milk containing k-casein BB. In most cases, the results obtained between various studies are conflicting and not comparable due to differences in population size, breed of cows, frequency of occurrence of specific genetic variants under consideration, methods of expressing traits (whether test day or lactation averages) and the effect of other genetic variants.

There is a general consensus that kappa-casein B is associated with higher protein in milk (Ng-Kwai- Hang *et al.*, 1990; Vanden Berg *et al.*, 1992; Bovenhuis *et al.*, 1992). This increase in the protein content is due to the increase in casein concentration in addition to increase in the casein number (FitzGerland, 1997), where as the whey protein fraction is not altered. This may be of economic importance to cheese manufacture, where casein is precipitated in the cheese curd and so, cheese yield is positively related to the casein content of milk. Ng-Kwai-Hang *et al.* (1990) mentioned that the shift from A to B gene for kappa-casein would result in an increase of protein percentage during three lactation periods.

Table (1): Fat and protein % of milk collected from Holstein cows with different genotypes.

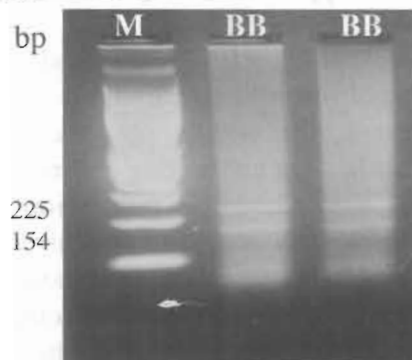
Genetic variant	Fat %	Protein %
AB	3.208 ± 0.070	*3.072 ± 0.318
AA	3.010 ± 0.124	*2.358 ± 0.042

* $P < 0.05$, Values represent mean ± SE

Buffalo genotyping

In this study, twenty Egyptian unreal-ted water buffaloes from different dairy herds were genotyped using the PCR-RFLP tech-nique. All the examined animals were found to be of the BB genotype where they generated two fragments of 225 and 154 bp, when digested with *Hind III* and two fragm-ents of 288 and 91 bp after digestion with *Hinf I* (Fig 4: right and left respectively). These results are similar to those found by Mitra *et al.* (1998); Otaviano *et al.*, (2005) and Othman (2005).

The same genotyping pattern was also reported in all Indian buffalo breeds (Mitra *et al*., 1998 and Pipalia *et al*., 2001). This homology between Egyptian and Indian buffaloes is expected where both belong to the same species (*Bubalua bubalis L.*) and the Indian buffalo is considered to be the source of the Egyptian buffalo (Othman 2005).



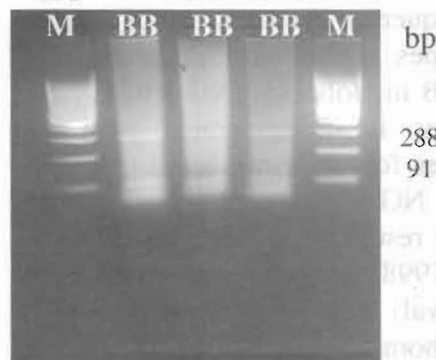


Fig. (4): Separation of Hind III (left) and Hinf I (right) digested 379 bp PCR products of buffalo kappa-casein gene (M: 100 bp ladder marker).

The results of milk analysis showed that all milk samples characterized by high and good quality of milk (high protein and fat %) (Table 2) where all were homozygous for the kappa-casein B. No analysis for the results was possible where the buffalo k-casein gene was found to be monomorphic. This homozygous did not allow making any inference about the influence of each allele on milk protein and fat percentages.

Table (2): Fat and protein % of milk from individual Egyptian buffaloes.

Genetic variant	Fat %	Protein %
BB	6.947 ± 0.43	3.094 ± 0.038

In any future breeding-selection programme for particular genetic variants, account should be taken, not only on the effect of a particular selection on the quality of the resulting milk, but also on any potential effects on animal health and longevity. For example, countries such as Austria, Switzerland and Italy breed for kappa-casein B specifically to give better quality milk for cheese making. In Italy, the breeding index for dairy sires incorporates a positive weighting for the AB (+2.5%) and BB genotypes (5%). The potential therefore exists to breed for "designer milk", i.e. with specific compositional and functional properties. The reasons for increa-sing the frequency of the B allele of kappa-casein summarized in: increased cheese yield, shorter rennet coagulation times, faster rates of curd formation and higher casein content in milk. Selection for a particular casein variant as raw material is a safe, additive free, and developping higher quality food ingredients /products (FitzGerland, 1997).

In conclusion, kappa-casein gene can be used as a marker gene for milk production in cattle because of its positive effect on milk protein percentage and so, milk manufacturing properties. PCR-RFLP technique can be used as a fast, accurate, low cost method independent of age and sex for genotyping of kappa-casein gene, hence, allowing the selection of animals with the favorable genotypes (BB and AB genotypes) to increase the protein percenttage in milk. On the contrary, buffaloes were found to be homozygous for the kappa-casein

gene where they possess only the allele B, and hence confirming previous literatures data.

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الملخص العربي

استخدام اختبار PCR – RFLP للكشف عن التراكيب الوراثيه المختلفه لجين الكاباكازين في الابقار والجاموس

جليله عبد الباقى الرافعى و سماح فكرى درويش
معهد بحوث التناسليات بالهرم – الجيزة – مصر

الكازين من بروتينات الألبان التى توجد على أشكال جزيئيه متعدده (alpha s 1, alpha s2, beta and kappa) وكل منها مجموعه من الأليلات المختلفة. يوجد خمسة أنواع من الكاباكازين ينتشر منها اثنان فى الابقار على صورة A,B. كاباكازين B من البروتينات المفضلة ولها اهمية خاصة وكبيرة فى نوعية الألبان واستراتيجية أو(نظام) التربية للحيوانات الحلابة, وقد تم فى هذة الدراسة إستخدام اختبار PCR – RFLP للتمييز بين الأليلات المختلفة للكاباكازين. ومن مميزات هذه الطريقة أنها سريعة وتعطى نتيجة مرضية بأقل تكلفة. وقد تم إستخدام البادئات (K1 & K2) للابقار لاكثار هذا الجين , كما تم إستخدام إثنين من انزيمات القطع (*Hind* III & *Hinf* I) للتعرف على التراكيب الوراثية للكاباكازين . أظهرت النتائج أن عينات الأبقار الهولستاين (٢٠حيواناً) ١٧ منهم ذوات تركيب وراثى متشابة AA و٣ ذوات تركيب وراثى هجين AB ولكن لم يظهر التركيب الوراثى BB فى هذة العينات . كما أظهرت نتائج عينات اللبن للأبقار عدم وجود علاقة بين نسبة الدهون فى الألبان واختلاف التركيب الوراثى فى حين وجدت علاقة بين ارتفاع نسبة البروتين فى ألبان الأبقار ذوات التركيب الوراثى AB بالمقارنة بنسبة البروتين فى ألبان الابقار ذوات التركيب الوراثى AA . اما عينات الجاموس (٢٠حيواناً) كلها ذوات تركيب وراثى متشابه BB وذوات إنتاج مميزمن الألبان ولم يتم تحليل النتائج لوجود أليل واحد فى الجاموس بحالة اصيلة. على أساس هذه النتائج يمكن أن نقول أن جين الكاباكازين له تأثير فعال على نسبة البروتين فى الألبان وأنه يمكن زيادة هذه النسبة بإختيار كاباكازين B.