A note on the evaluation of a beta-casein variant in bovine breeds by allele-specific PCR and relevance to beta-casomorphin

Brief Note:

Short Title: Beta casein A^1 genotype incidence

A note on the evaluation of a beta-casein variant in bovine breeds by allele-specific PCR and relevance to β-casomorphin

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Abstract

Two genetic variants of the bovine *β-casein* gene (A^1 and B) encode a histidine residue at codon 67, resulting in potential liberation of a bioactive peptide, β-casomorphin, upon digestion. An allele-specific PCR (AS-PCR) was evaluated to distinguish between the β-casomorphin-releasing variants (A^1 and B) and the non-releasing variants. AS-PCR successfully distinguished β-casein variants in 41 of 42 animals as confirmed by sequence analysis. Overall, while the incidence of the homozygous A^1 + B animals (homozygous for the histidine residue; 21.4 %) was lower than the homozygous A^2 animals (30.9 % respectively), 69 % of animals carried at least one A^1 allele.

Keywords: β-casein; bioactive peptides; allele-specific PCR.

Introduction

Milk protein has the potential to yield opioid-like peptides following proteolysis. These peptides have been termed "food hormones" and have been reported to elicit their bioactive effects when released from food constituents (Teschemacher, 2003). The major exogenous milk protein-derived opioid peptides are the β-casomorphins, which are liberated upon digestion of certain variants of β-casein, namely the A^1 and B variants (Svenberg *et al.,* 1985; Meisel, 1986). The *β-casein* gene has 12 known genetic variants in the coding sequence of the gene (Farrell *et al*., 2004). Genetic variants A^1, A^2, A^3 and B are universally distributed in almost all *Bos taurus* and *Bos indicus* populations. The base changes encoding the amino acid differences between these four variants are located in exon VII that encodes the major part of the mature protein (Bonsing *et al.,* 1988). The β-casein A^1 and B variants differ from the A^2 variant at position 67 where a histidine replaces a proline. In addition, the B variant differs from the A^1 variant in a substitution of arginine for serine at position 122. (Other variants - C, F & G – also have a histidine residue at position 67). Importantly, it is the change to histidine at position 67 that has the potential to result in cleavage occurring upon digestion and a bioactive peptide, beta-casomorphin potentially being liberated (Lien *et al.,* 1992; Stewart *et al.,* 1987; Damiani *et al.,* 1992).

Consumption of the A^1 and B protein variants of bovine β-casein has been implicated in the development of diabetes and coronary heart disease (CHD). It has also been shown that β-casein protein from animals with the position 67 base change (A^1 or B allele) induced diabetes in NOD mice (Elliott *et al.,* 1988). Elliott *et al*. (1999) compared the incidence of Type 1 diabetes in 0 to 14 year-old children from 10 different countries with the national consumption of protein from cows' milk. While total protein consumption was not correlated with diabetes incidence ($r = 0.40$), the consumption of the β-casein A^1 variant was associated

with the incidence of diabetes (r = 0.73). There was an even closer relationship between the combined consumption of A^1 and B variants and diabetes (r = 0.98; Elliott *et al.*, 1999).

It has been hypothesised that consumption of the β-casein A^1 and B protein variants are a specific risk factor for the development of CHD based on a study using data measured for dairy breeds in several countries (McLachlan, 2001). Results for France vs. Northern Ireland were particularly striking where disease mortality in middle-aged men was shown to be between three and four times higher in Belfast than in Toulouse, as was their consumption of the β-casein A^1 and B protein variants. Cardiovascular disease is one of the biggest killers in Ireland: it accounts for 42 % of all deaths in Ireland – the highest of any European country and twice the EU average. Ireland has also been reported to have a high incidence of Type 1 diabetes within Europe (Roche *et al.*, 2002). In recent years human consumption of β-casein A^1 and B protein variants has been brought to the public's attention, in particular in New Zealand, where A^2 milk is marketed by the A^2 Corporation as being a lower risk factor for disease development.

This study investigated the cytosine to adenine base change which results in the amino acid change at position 67 and the potential release of a bioactive peptide. The A^1 and B variants (and others with the histidine residue at position 67) were therefore combined and referred to as A^1 throughout, while other variants are referred to as A^2. Allele-specific primers were evaluated for their use in assessing the presence of the A^1 base change. In addition, a number of genetically unrelated (non-dairy) animals were studied.

Materials and Methods

Blood sampling and DNA extraction

Approximately 15 ml of blood was collected into heparinised tubes from the coccygeal vein of 42 cattle representing ten breeds. Breeds involved were high genetic merit Holstein-Friesian, low genetic merit Holstein-Friesian, Irish-Friesian, Dutch-Friesian, Limousin, Montbeliarde, Charolais, Normande, Norwegian Red and Kerry. With the exception of the Limousin, Charolais, and Kerry animals, these subjects were part of the herd at Teagasc, Moorepark, Fermoy, Co. Cork. Limousin and Charolais samples were from Teagasc, Grange, Co. Meath, while Kerry samples were obtained from the herd at Muckross House, Killarney, Co. Kerry. DNA extraction was carried out using the Gentra Capture Column™ (Gentra, Minneapolis, MN55441, USA) system for approximately 200 μl of whole blood per animal. Blood was stored at –80 ^{o}C and DNA was stored at –20 ^{o}C until further use.

Primer design and Polymerase chain reaction (PCR)

Allele-specific PCR was carried out using a common forward primer (Bwtp3; 5′-GCCCAGATGAGAGAAGTGAGG-3′), and reverse primers with either T or G at the 3’ end (5′-GATGTTTTGTGGGAGGCTGTTAT/G-3′) to amplify an 854 bp fragment (Figure 1A). The polymorphisms represented by the A3 variant and the second polymorphism (codon 122) associated with the B variant were not present in this amplicon and therefore could not interfere with the interpretation of the results. Additional primers were used to amplify a 730 bp fragment encompassing the polymorphism for sequence validation (5′-GGCCATTGTTAAGGAACTCC-3′;5′-AAGGTGCAGATTTTCAACAT-3′). Primers were synthesised by MWG Biotech (90 Long Acre, Covent Garden, London, WC2E 9RZ). PCR was carried out from a starting template of approximately 200 ng of genomic DNA in a final volume of 50 μl containing 1X *Taq* DNA polymerase buffer (Invitrogen Ltd, 3 Fountain Drive,

Inchinnan Business Park, Paisley, UK, PA49RF), 1.5 mM $MgCl_2$, 200 μM dNTPs (Promega), 0.3 μM each primer and 1U *Taq* polymerase (Invitrogen). After an initial incubation at 95 ^{o}C for 2 min, samples were amplified for 30 cycles at 95 ^{o}C for 1 min, 58 ^{o}C for 1 min and 72 ^{o}C for 1 min. PCR product sequencing was carried out by MWG Biotech. The resulting sequences were analysed using the Vector NTI® Suite of software (Invitrogen).

Results

An examination of animals predicted their genotype through the use of AS-PCR (Figure 1), and these results are listed in Table 1. These PCR products were sequenced and their genotype determined. Table 1 shows the genotypes predicted by AS-PCR and the sequencing results for each of 42 animals. With the exception of one animal as indicated, the results matched perfectly. Use of the AS-PCR method distinguished the A^1 from A^2 base change with a success rate of 97 %. Results of the investigation (combining AS-PCR and sequencing data) were as follows: A^1A^1 – 21.4 %, A^2A^2 – 30.9 %, A^1A^2 – 47.6 %. Overall the incidence of A^1 allele carriers (either one or two copies of A^1 allele) was 69 %.

Discussion

The incidence of the A^1 variant of β-casein has been the focus of much interest in the last decade due to the possibility that a bioactive peptide is liberated from these variants upon enzymatic digestion. While evidence for a clear link between this bioactive peptide and a disease state has not been demonstrated, it is desirable to have some indication of the incidence of this genetic variant in the Irish herd. Neither the base change encoding these variants nor the "wild-type" leads to any recognition site for a restriction enzyme, ruling out the use of restriction fragment length polymorphisms (RFLP) to distinguish between the variants. Therefore, the use of allele-specific primers, with a single base change (T/G) at the 3' end complementary to the base change of the specific β-casein variant (A^1, B primer - T, A^2 primer - G) was evaluated. Allele-specific PCR uses two allelic-specific primer sets differing from each other in their 3' terminal nucleotide and was first described by Wu *et al.* (1989). A single band of 854 bp should only result for one or other of the allele-specific (AS) reactions if the template is homozygous or a band should appear with both allele-specific reactions in the case of a heterozygous template. Genotypes were predicted for each animal using the AS-

PCR method. A further PCR was used to amplify a 730 bp fragment spanning the polymorphic site. These samples were purified and sequenced. Predicted genotypes were compared with sequenced products (n = 42; Table 1). The predicted genotypes from AS-PCR were correct for 97 % of the samples - one animal was predicted incorrectly. Some further optimisation of the amplification conditions may be appropriate to ensure a 100 % success rate. Regardless, we feel that the use of these allele-specific primers successfully distinguished between the genetic variants.

The genotypes of the group of animals studied were A^1A^1 – 21.4 %, A^2A^2 – 30.9 %, A^1A^2 – 47.6 %. Overall incidence of A^1 allele carriers was 69 %. When the different bovine breeds were examined and divided into dairy (Holstein Friesian (high or low genetic merit), Irish Friesian, Norwegian Red, Dutch Friesian), dual-purpose (Normande, Montbeliarde, Kerry) and beef (Charolais, Limousin) animals, it was observed that the highest incidence of the β-casein A^1 (or B) allele occurred in the dairy breeds at 77 %, compared with the dual-purpose at 69 % and beef breeds at a lower incidence of 50 %. This study, however, involved a small number of animals and thus may not be indicative of the overall incidence in the Irish cow herd. A large examination of the Irish national dairy herd may therefore be desirable to determine the exact extent of the β-casein A^1 and B variant incidence, for example in either milk or semen samples. Removal of this genotype from the national herd might prove beneficial for both the health and dairy industries; however the consequences of removal of this milk protein variant should first be carefully considered.

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Table 1. Genotypes of animals determined by AS-PCR and sequence analysis.

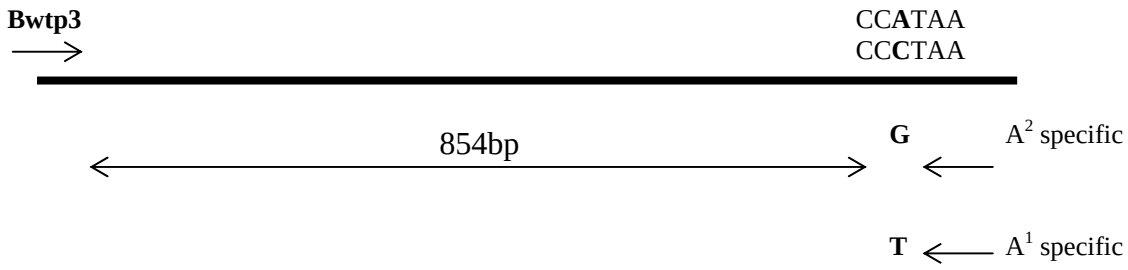
Dairy Breeds	Genotype	Dual Purpose	Genotype	Beef	Genotype
0011	A^1A^2	**0163**	A^1A^1	**0292**	A^2A^2
0026	A^1A^2	**0166**	A^1A^1	**191C**	A^1A^2
3048	A^1A^2	**1226**	A^1A^1	**0183**	A^2A^2
9615	A^2A^2	**1267**	A^1A^2	**0094**	A^2A^2
0050	A^1A^2	**1212**	A^1A^1	**42L**	A^1A^2
0059	A^1A^2	**0130***	A^1A^2	**215W**	A^1A^2
0081	A^2A^2	**1023**	A^1A^2	**0069**	A^2A^2
0876	A^1A^1	**1545**	A^1A^1	**0086**	A^1A^2
0599	A^1A^2	**39**	A^2A^2		
1668	A^1A^2	**40**	A^2A^2		
1270	A^2A^2	**41**	A^1A^2		
1257	A^1A^2	**42**	A^1A^2		
0407	A^1A^2	**43**	A^2A^2		
0287	A^1A^1	**44**	A^2A^2		
0188	A^1A^1	**45**	A^1A^2		
0508	A^1A^2	**46**	A^2A^2		
1535	A^1A^1				
0191	A^2A^2				

* Sample that was incorrectly identified.

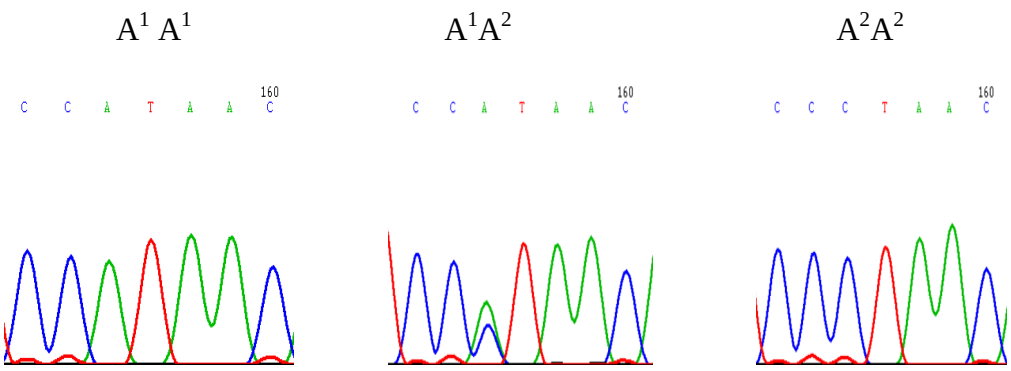
Figures

Figure 1.

(A)



(B)



(C)

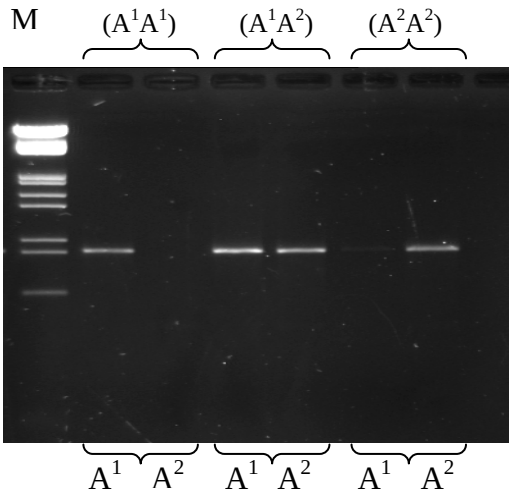


Figure Legends.

Figure 1. Comparison of allele-specific PCR results and sequencing validation.

(A) Primer design and layout. (B) Sequencing chromatograms representing each of the three genotypes (A^1A^1, A^1A^2, A^2A^2). (C) Three animals (genotype in parenthesis) are shown as examples of typical results. Lane 1: Marker (M). Lane 2, 4, 6: A^1-specific primer PCR. Lane 3, 5, 7: A^2-specific primer PCR.