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1 **Brief Note:**

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3 **Short Title: Beta casein A¹ genotype incidence**

4

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

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24

1 **Abstract**

2

3 Two genetic variants of the bovine *β-casein* gene (A¹ and B) encode a histidine residue at
4 codon 67, resulting in potential liberation of a bioactive peptide, β-casomorphin, upon
5 digestion. An allele-specific PCR (AS-PCR) was evaluated to distinguish between the β-
6 casomorphin-releasing variants (A¹ and B) and the non-releasing variants. AS-PCR
7 successfully distinguished β-casein variants in 41 of 42 animals as confirmed by sequence
8 analysis. Overall, while the incidence of the homozygous A¹ + B animals (homozygous for
9 the histidine residue; 21.4 %) was lower than the homozygous A² animals (30.9 %
10 respectively), 69 % of animals carried at least one A¹ allele.

11

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

1 **Introduction**

2 Milk protein has the potential to yield opioid-like peptides following proteolysis. These
3 peptides have been termed “food hormones” and have been reported to elicit their bioactive
4 effects when released from food constituents (Teschemacher, 2003). The major exogenous
5 milk protein-derived opioid peptides are the β -casomorphins, which are liberated upon
6 digestion of certain variants of β -casein, namely the A¹ and B variants (Svenberg *et al.*, 1985;
7 Meisel, 1986). The *β -casein* gene has 12 known genetic variants in the coding sequence of
8 the gene (Farrell *et al.*, 2004). Genetic variants A¹, A², A³ and B are universally distributed in
9 almost all *Bos taurus* and *Bos indicus* populations. The base changes encoding the amino
10 acid differences between these four variants are located in exon VII that encodes the major
11 part of the mature protein (Bonsing *et al.*, 1988). The β -casein A¹ and B variants differ from
12 the A² variant at position 67 where a histidine replaces a proline. In addition, the B variant
13 differs from the A¹ variant in a substitution of arginine for serine at position 122. (Other
14 variants - C, F & G – also have a histidine residue at position 67). Importantly, it is the
15 change to histidine at position 67 that has the potential to result in cleavage occurring upon
16 digestion and a bioactive peptide, beta-casomorphin potentially being liberated (Lien *et al.*,
17 1992; Stewart *et al.*, 1987; Damiani *et al.*, 1992).

18
19 Consumption of the A¹ and B protein variants of bovine β -casein has been implicated in the
20 development of diabetes and coronary heart disease (CHD). It has also been shown that β -
21 casein protein from animals with the position 67 base change (A¹ or B allele) induced
22 diabetes in NOD mice (Elliott *et al.*, 1988). Elliott *et al.* (1999) compared the incidence of
23 Type 1 diabetes in 0 to 14 year-old children from 10 different countries with the national
24 consumption of protein from cows’ milk. While total protein consumption was not correlated
25 with diabetes incidence ($r = 0.40$), the consumption of the β -casein A¹ variant was associated

1 with the incidence of diabetes ($r = 0.73$). There was an even closer relationship between the
2 combined consumption of A¹ and B variants and diabetes ($r = 0.98$; Elliott *et al.*, 1999).

3

4 It has been hypothesised that consumption of the β -casein A¹ and B protein variants are a
5 specific risk factor for the development of CHD based on a study using data measured for
6 dairy breeds in several countries (McLachlan, 2001). Results for France vs. Northern Ireland
7 were particularly striking where disease mortality in middle-aged men was shown to be
8 between three and four times higher in Belfast than in Toulouse, as was their consumption of
9 the β -casein A¹ and B protein variants. Cardiovascular disease is one of the biggest killers in
10 Ireland: it accounts for 42 % of all deaths in Ireland – the highest of any European country
11 and twice the EU average. Ireland has also been reported to have a high incidence of Type 1
12 diabetes within Europe (Roche *et al.*, 2002). In recent years human consumption of β -casein
13 A¹ and B protein variants has been brought to the public's attention, in particular in New
14 Zealand, where A² milk is marketed by the A² Corporation as being a lower risk factor for
15 disease development.

16

17 This study investigated the cytosine to adenine base change which results in the amino acid
18 change at position 67 and the potential release of a bioactive peptide. The A¹ and B variants
19 (and others with the histidine residue at position 67) were therefore combined and referred to
20 as A¹ throughout, while other variants are referred to as A². Allele-specific primers were
21 evaluated for their use in assessing the presence of the A¹ base change. In addition, a number
22 of genetically unrelated (non-dairy) animals were studied.

1 **Materials and Methods**

2 *Blood sampling and DNA extraction*

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

13

14 *Primer design and Polymerase chain reaction (PCR)*

15 Allele-specific PCR was carried out using a common forward primer (Bwtp3; 5'-
16 GCCCAGATGAGAGAAGTGAGG-3'), and reverse primers with either T or G at the 3' end (5'-
17 GATGTTTTGTGGGAGGCTGTTATG-3') to amplify an 854 bp fragment (Figure 1A). The
18 polymorphisms represented by the A3 variant and the second polymorphism (codon 122)
19 associated with the B variant were not present in this amplicon and therefore could not
20 interfere with the interpretation of the results. Additional primers were used to amplify a 730
21 bp fragment encompassing the polymorphism for sequence validation (5'-
22 GGCCATTGTTAAGGAACTCC-3';5'-AAGGTGCAGATTTTCAACAT-3'). Primers were synthesised
23 by MWG Biotech (90 Long Acre, Covent Garden, London, WC2E 9RZ). PCR was carried
24 out from a starting template of approximately 200 ng of genomic DNA in a final volume of
25 50 µl containing 1X *Taq* DNA polymerase buffer (Invitrogen Ltd, 3 Fountain Drive,

1 Inchinnan Business Park, Paisley, UK, PA49RF), 1.5 mM MgCl₂, 200 μM dNTPs (Promega),
2 0.3 μM each primer and 1U *Taq* polymerase (Invitrogen). After an initial incubation at 95 °C
3 for 2 min, samples were amplified for 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C
4 for 1 min. PCR product sequencing was carried out by MWG Biotech. The resulting
5 sequences were analysed using the Vector NTI® Suite of software (Invitrogen).

6

1 **Results**

2
3 An examination of animals predicted their genotype through the use of AS-PCR (Figure 1),
4 and these results are listed in Table 1. These PCR products were sequenced and their
5 genotype determined. Table 1 shows the genotypes predicted by AS-PCR and the sequencing
6 results for each of 42 animals. With the exception of one animal as indicated, the results
7 matched perfectly. Use of the AS-PCR method distinguished the A¹ from A² base change
8 with a success rate of 97 %. Results of the investigation (combining AS-PCR and sequencing
9 data) were as follows: A¹A¹ – 21.4 %, A²A² – 30.9 %, A¹A² – 47.6 %. Overall the incidence
10 of A¹ allele carriers (either one or two copies of A¹ allele) was 69 %.

11

12 **Discussion**

13 The incidence of the A¹ variant of β -casein has been the focus of much interest in the last
14 decade due to the possibility that a bioactive peptide is liberated from these variants upon
15 enzymatic digestion. While evidence for a clear link between this bioactive peptide and a
16 disease state has not been demonstrated, it is desirable to have some indication of the
17 incidence of this genetic variant in the Irish herd. Neither the base change encoding these
18 variants nor the “wild-type” leads to any recognition site for a restriction enzyme, ruling out
19 the use of restriction fragment length polymorphisms (RFLP) to distinguish between the
20 variants. Therefore, the use of allele-specific primers, with a single base change (T/G) at the
21 3' end complementary to the base change of the specific β -casein variant (A¹, B primer - T, A²
22 primer - G) was evaluated. Allele-specific PCR uses two allelic-specific primer sets differing
23 from each other in their 3' terminal nucleotide and was first described by Wu *et al.* (1989). A
24 single band of 854 bp should only result for one or other of the allele-specific (AS) reactions
25 if the template is homozygous or a band should appear with both allele-specific reactions in
26 the case of a heterozygous template. Genotypes were predicted for each animal using the AS-

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

8
9 The genotypes of the group of animals studied were A^1A^1 – 21.4 %, A^2A^2 – 30.9 %, A^1A^2 –
10 47.6 %. Overall incidence of A^1 allele carriers was 69 %. When the different bovine breeds
11 were examined and divided into dairy (Holstein Friesian (high or low genetic merit), Irish
12 Friesian, Norwegian Red, Dutch Friesian), dual-purpose (Normande, Montbeliarde, Kerry)
13 and beef (Charolais, Limousin) animals, it was observed that the highest incidence of the β -
14 casein A^1 (or B) allele occurred in the dairy breeds at 77 %, compared with the dual-purpose
15 at 69 % and beef breeds at a lower incidence of 50 %. This study, however, involved a small
16 number of animals and thus may not be indicative of the overall incidence in the Irish cow
17 herd. A large examination of the Irish national dairy herd may therefore be desirable to
18 determine the exact extent of the β -casein A^1 and B variant incidence, for example in either
19 milk or semen samples. Removal of this genotype from the national herd might prove
20 beneficial for both the health and dairy industries; however the consequences of removal of
21 this milk protein variant should first be carefully considered.

22
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24

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

2

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				

3 * Sample that was incorrectly identified.

4

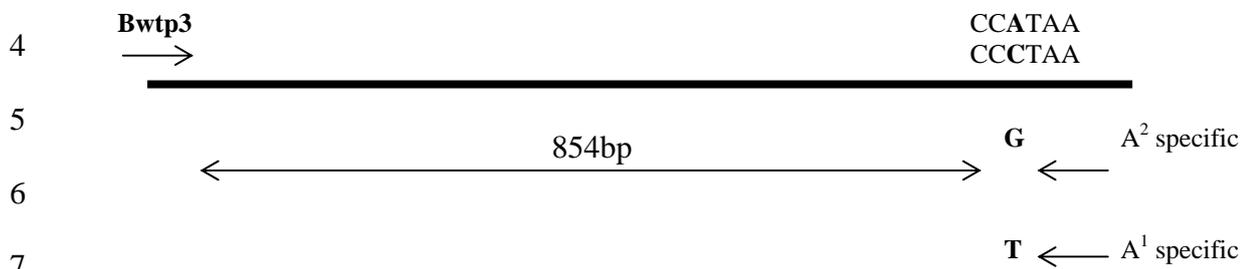
5

6

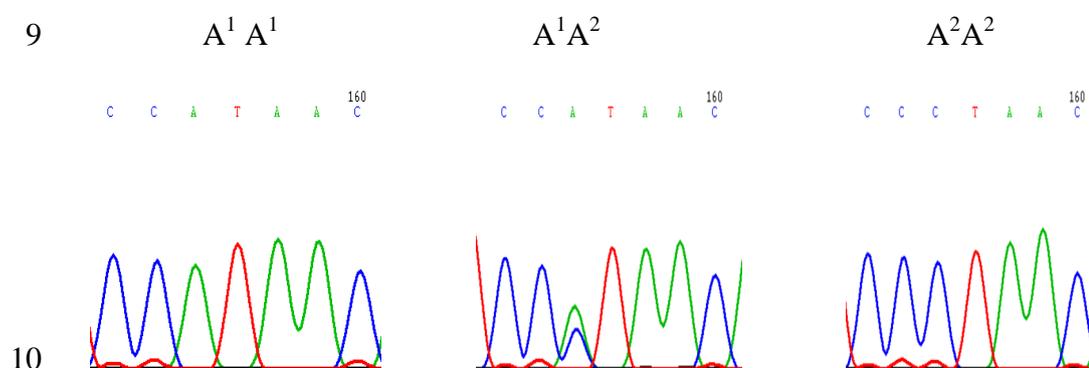
7

1 **Figures**2 **Figure 1.**

3 (A)



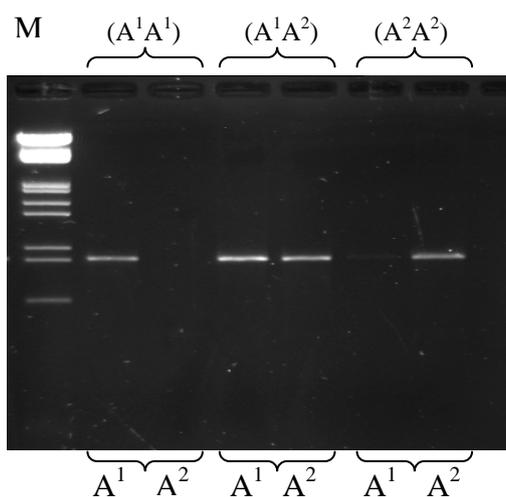
8 (B)



11

12 (C)

13



1 **Figure Legends.**

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

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

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