



## A1 milk and its controversy-a review

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Received for publication: October 21, 2015; Accepted: November 12, 2015

**Abstract:** Milk is divided into two groups type A1 and type A2 on the basis of betacasomorphin-7 (BCM-7) production. Type A1 milk on enzymatic digestion produces BCM-7 but A2 does not. This is because of mutation that changed proline to histidine at position number 67 in  $\beta$ -casein of type A1 milk. BCM-7 is a seven amino acid long peptide and speculated to be involved in aggravating non-communicable disorders such as juvenile diabetes, autism and schizophrenia. Gut enzymes such as pepsin, leucine aminopeptidase, elastase, trypsin and pancreatin could produce BCM-7 from  $\beta$ -casein of A1 milk on its digestion, as infant gut system has more intestinal permeability for nutrient; the passage of BCM-7 in serum is possible. The same could also happen in patients having leaky gut syndrome. Although presence of dipeptidyl peptidase (IV) enzymes on the surface of enterocytes and serum degrades BCM-7 to smaller peptides, further less permeability of gut in adults reduces the chance of passage of BCM-7 from gut to serum. Statistical data are correlating BCM-7 for non-communicable disorders, but animal studies and human studies are still not conclusive in this regard. India is largest producer of milk and majority of milk is of A2 type. However, crossbreds having high milk producing capacity have A1 allele in their genome, which requires its careful monitoring. Further, research is needed in knowing A1 milk effects on Indian population.

**Key words:** A1 milk; A2 milk;  $\beta$ -casein; Betacasomorphin-7; Opioid.

### INTRODUCTION

Milk is the base of all forms of dairy products that are consumed by majority of people. Milk contains a significant source of energy, protein, and micronutrients such as calcium, magnesium, phosphorus and vitamin B<sub>12</sub>. In addition to supplying healthy nutrients, inclusion of milk in the diet has been positively associated with a variety of health conditions. However, in past few years' bovine milk comes under scrutiny of its possible role in some non-communicable disorders. Milk is divided into two groups A1 and A2. A1 milk produces BCM-7 on its digestion which is believed to be involved in autism, schizophrenia and other non-communicable disorders. India is number one producer of milk in world and most of the Indian cattle breeds are A2 in nature. It seems that A1 milk is not a major problem for India. Despite of being largest producer of milk in world, milk production is still not able to fulfill the growing demands of milk. To increase milk production government of India has taken several initiatives and one of the areas is cross breeding program in bovines. Crossbreds of bovine has definitely contributed in increasing the overall milk production, but at the same time A1 allele has also entered in their genome, creating concerns of production of BCM-7 from their milk after human consumption. Although it is still not clear whether A1 milk is harmful, more animal and human studies are required to get any conclusion on it. Methods to detect A1 or A2 milk are primarily based on amplification of PCR product from DNA using specific primers. DNA from blood, hair, skin and even milk somatic cell is isolated from bovine. Some of these methods have acquired commercial dimensions and even patented. This review is written to give information on A1 milk and to highlight growing concerns in South-East Asian countries especially in India on A1 milk.

### A1 milk

$\beta$ -casein accounts for 24-28% of total milk protein. Molecular weight of  $\beta$ -casein is 24 kilo Dalton. It comprises of 209 amino acids. At least 12 variants of this protein are known<sup>1</sup>. Each variant differs from other variant in terms of

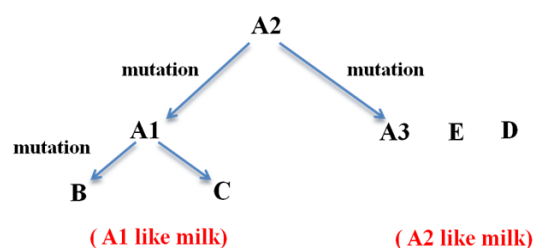
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amino acid substitution at fixed position. For example,  $\beta$ -casein A1 has histidine amino acid at 67<sup>th</sup> position while  $\beta$ -casein A2 has proline at same position.

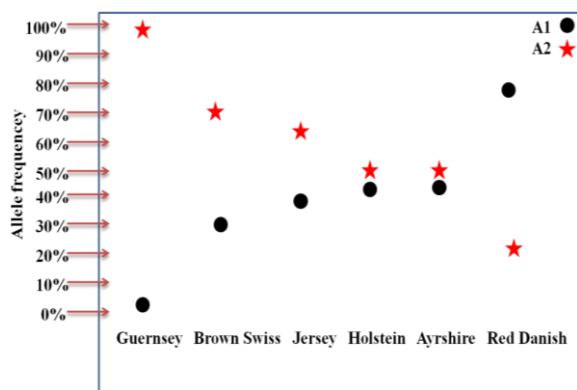
$\beta$ -casein protein is coded by chromosome number 6 of bovine genome. Histidine is coded by CAT (cytosine-adenosine-thymine) bases whereas proline is coded by CCT (cytosine-cytosine-thymine); here due to mutation adenosine is replaced by cytosine. Molecular biology methods can determine this difference of single nucleotide in  $\beta$ -casein gene and can identify individual animal whether they carry gene for  $\beta$ -casein A1 or  $\beta$ -casein A2 or both.  $\beta$ -casein protein is co-dominantly expressed in A1A2 genotype where allele A1 and A2 are equally expressed.  $\beta$ -casein A1 is the result of a genetic mutation in cattle, believed to have occurred about 5000 years ago in Anatolia, Turkey<sup>1</sup>, (Figure 1).



$\beta$ -casein variants	Change in amino acid at different positions of $\beta$ -casein						
A2	SerP(18)	SerP(35)	Glu (36)	Glu (37)	His (67)	His (106)	Ser(122)
A3	SerP(18)	SerP(35)	Glu (36)	Glu (37)	His (67)	<b>Gln (106)</b>	Ser(122)
E	SerP(18)	SerP(35)	<b>Lys(36)</b>	Glu (37)	His (67)	His (106)	Ser(122)
D	<b>Lys(18)</b>	SerP(35)	Glu (36)	Glu (37)	His (67)	His (106)	Ser(122)
A1	SerP(18)	SerP(35)	Glu (36)	Glu (37)	<b>Pro (67)</b>	His (106)	Ser(122)
B	SerP(18)	SerP(35)	Glu (36)	Glu(37)	<b>Pro (67)</b>	His (106)	<b>Arg(122)</b>
C	SerP(18)	<b>Ser(35)</b>	Glu (36)	<b>Lys(37)</b>	<b>Pro (67)</b>	His (106)	Ser(122)

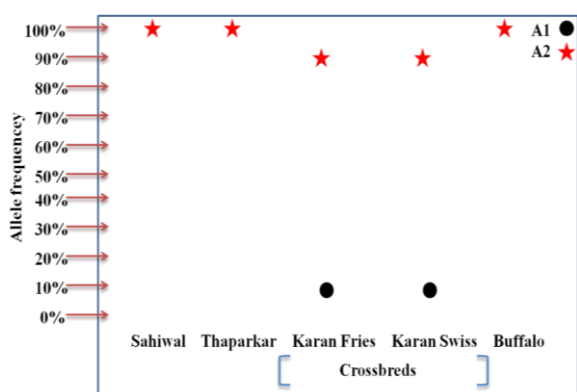
**Figure 1:** Polymorphism in bovine  $\beta$ -casein, amino acid substitution in variants at various positions in  $\beta$ -casein is highlighted in red color.

Milking animals have been screened for  $\beta$ -casein A1 and  $\beta$ -casein A2 in many countries. There is difference in occurrence of  $\beta$ -casein A1 or  $\beta$ -casein A2 amongst species, breeds and geographical locations. The results are denoted in terms of frequency of occurrence. The frequency for A2 allele in Guernsey, Brown Swiss, Jersey, Holstein, Ayrshire and Red Danish bovine breeds is about 96-98%, 66-70%, 50-63%, 44-53%, 40-49% and 23% respectively<sup>2</sup>, (Figure 2A).



**Figure 2A:** Allele A1 and A2 frequency in European bovine breeds.

A limited work has been carried out in India for screening of milking animals for variants of  $\beta$ -casein. In a study conducted by Misra and co-workers, 2009<sup>3</sup>, (Figure 2B) it has been observed that out of 231 buffaloes (8 breeds of river buffalo), all were of A2 type. Also, the allele frequency of A2 in 15 breeds of Zebu cattle (total of 618 animals screened) was 98.7%. In another study, Sahiwal (total of 15 animals screened) and Tharparkar (total of 14 animals screened) breeds of bovine was found to be of A2 type, while in crossbred (Karan Fries and Karan Swiss; total of 38 animals screened), A2 allele frequency was 79-89%<sup>4</sup>, (Figure 2B). A2 Milk Corporation was set up in 2009 in New Zealand with the aim to sell A2 milk only.

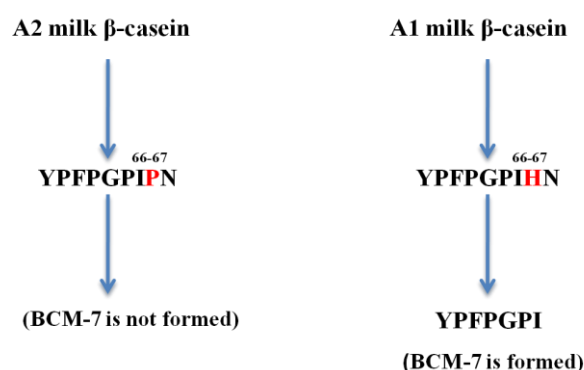


**Figure 2B:** Allele A1 and A2 frequency in Indian cattle breeds and crossbreds.

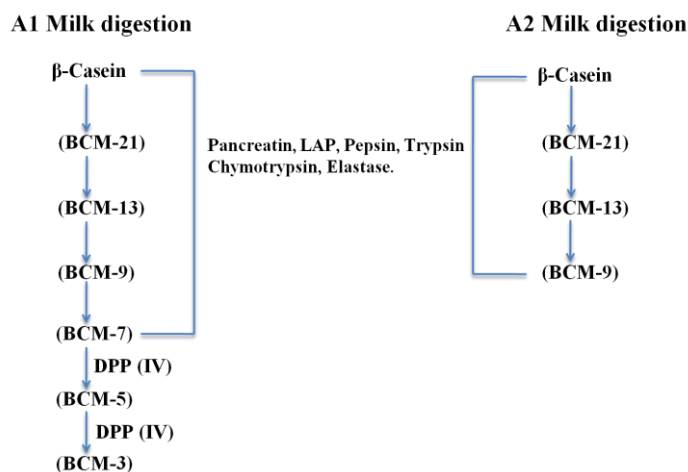
**Betacasmorphin-7 (BCM-7) and its metabolism**

Both  $\beta$ -casein A1 and A2 respond differently towards proteolytic degradation by enzymes of human gut. This is possibly because of one amino acid difference at 67<sup>th</sup> position in  $\beta$ -casein. A1  $\beta$ -casein has histidine at 67<sup>th</sup> position while A2  $\beta$ -casein has proline (Figure 3A). The

difference in amino acid at 67<sup>th</sup> position results in differences in susceptibility of peptide bond between amino acid 66 and 67. The peptide bond between isoleucine and histidine (A1 milk) is cleaved by elastase, while bond between isoleucine and proline (A2 milk) is not hydrolysed. The digested product contained a seven amino acid long peptide having sequence Tyr<sup>60</sup>-Pro<sup>61</sup>-Phe<sup>62</sup>-Pro<sup>63</sup>-Gly<sup>64</sup>-Pro<sup>65</sup>-Ile<sup>66</sup> and is referred as betacasmorphin-7 or popularly called as BCM-7 which is not formed on digestion of  $\beta$ -casein A2 (Figure 3A). In one of the *in vitro* study, A1  $\beta$ -casein was digested with combination of enzymes such as pepsin, elastase, leucine aminopeptidase (LAP) and pancreatin<sup>5</sup>. Along with BCM-7, other degradation products such as BCM-9, BCM-13 and BCM-21 were also produced. BCM-7 is further breakdown to BCM-5 and BCM-3 by dipeptidyl peptidase IV (DPP IV) enzyme present on surface of enterocytes and in blood<sup>6</sup> (Figure 3B).



**Figure 3A:** Formation of BCM-7 from  $\beta$ -casein A1, not from  $\beta$ -casein A2. The difference in  $\beta$ -casein A1 and  $\beta$ -casein A2 is shown at position number 67. Proline at 67<sup>th</sup> position in  $\beta$ -casein A2 is replaced with histidine in  $\beta$ -casein A1.



**Figure 3B:** Breakdown of  $\beta$ -casein by pepsin, elastase, leucine aminopeptidase (LAP), chymotrypsin, trypsin and DPP (IV) enzymes and formation of BCM-21, BCM-13, BCM-9, BCM-7, BCM-5 and BCM-3 peptides.

Even if it survives there due to high permeability of intestine specifically in neonates or improper function of DPP (IV) enzymes (autistic patient), blood brain barrier (BBB) is next stopping point for BCM-7. BBB has also tight

junction like structure which stops the flow of large peptides. Also, presence of peptidases (in BBB) further inhibits its progression to brain<sup>7,8</sup>. Peptides are generally transported across the intestine by two pathways, transcellular or paracellular. As most of opioid peptides are hydrophobic in nature it is difficult for them to transport by diffusion or paracellular pathway. The PEPT1, H<sup>+</sup>-coupled transporters, which are found in intestinal epithelium can only transport di to tri-peptides<sup>9</sup>. In 2003 a new transporter was identified in epithelial cell of retinal pigment (RPE) and was called as ARPE-19 (Na<sup>+</sup>-dependent active transport). This transport system can transport opioid peptides of length of 4 to 13 amino acids<sup>10</sup>. However, its presence in intestine is still unknown. *In vitro* and *in vivo* both studies have demonstrated that BCM-7 can pass through intestinal barrier. Caco-2 cell line is popularly used in *in vitro* study for peptide transportation. This cell line was developed from human colon adenocarcinoma cells<sup>11</sup>. Caco-2 cells differentiate to enterocytes very quickly. These cells are joined by tight junction, mimicking true intestinal system<sup>12</sup>. Iwan *et al.*, 2008<sup>13</sup>, showed BCM-7 can pass through Caco-2 cell line. In *in vivo* studies, dog pups were used as a model and was found that by feeding bovine casein formula, BCM-7 immunoreactive material (BCMIR) could pass through intestinal barrier in new-borns but failed to pass in adults. The length of BCMIR was much higher than BCM-7, consists of 13 amino acids residue<sup>14</sup>. This clearly shows that long peptides can pass through intestine in newborns.

BCM-7 production is pH dependent and pH 4.0 is optimum for its production from bovine A1 milk. B allele (A1 like milk, very low frequency of this allele is found in animals) produced maximum amount of BCM-7 at pH 2. However, at pH 4, BCM-7 amount in B type (A1 like milk) was still higher as compare to A1 milk when milk was digested in *in vitro* condition<sup>15</sup>, (Figure 4A). In neonates for few months of their life stomach pH is found in the range of 2 to 5 as compared to 2 in adults<sup>16,17</sup>. Further milk stays longer period of time in neonates, creating more favorable condition for BCM-7 production. The processing of milk does not affect *in vitro* production of BCM-7, but the amount of BCM-7 was found high in case of cheese (Gouda, Cheddar), 15 to 21 mg/kg as compared to UHT or pasteurized treated milk, infant formulas, unprocessed milk and fermented milk which was in the range of 0.04 to 1.16 mg/l during *in vitro* digestion study<sup>15</sup>, (Figure 4B).

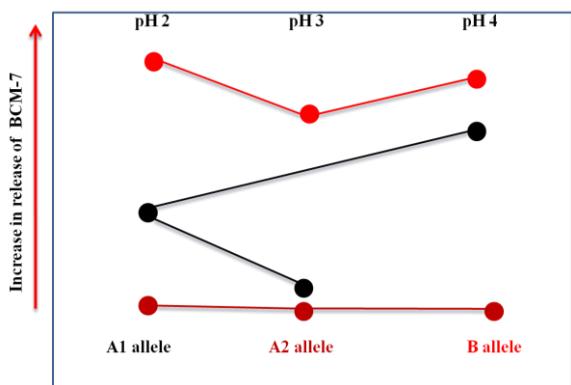


Figure 4A: Release of BCM-7 during *in vitro* digestion of milk (type A1, A2 & B) at different pH.

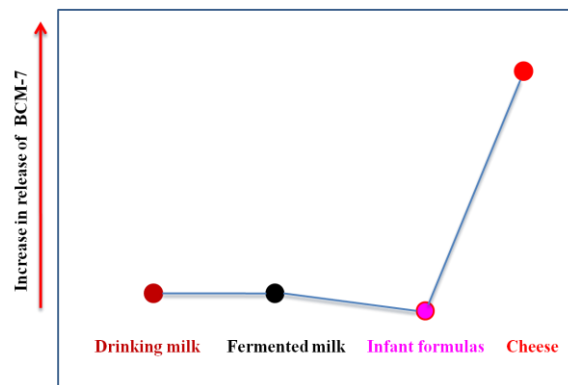


Figure 4B: Release of BCM-7 during *in vitro* digestion of drinking, fermented milk, infant formulas and cheese.

Overall it seems that BCM-7 can pass through intestinal barrier in neonates as they have increased permeability for improved nutrient absorption. However intestinal permeability reduces as the age progresses. Also in adults with compromised digestive health or conditions such as celiac disease, stomach ulcers or autism have increased intestinal permeability which means that BCM-7 can enter the bloodstream more easily in adult's also<sup>6</sup>.

**Biological effects of BCM-7**

**Effect of A1 milk consumption on human health (Ecological or population studies):** Ecological studies conducted in nineteen countries (U.K, Finland, Ireland, Sweden, Denmark, France, Germany, Iceland, Norway, Austria, Switzerland, USA, Japan, Israel, Australia, New Zealand, Hungary, Venezuela and Canada) revealed that there was strong relation between consumption of  $\beta$ -casein A1 and incidence of type 1 diabetes mellitus (DM1). The correlation coefficient ( $r$ ) value was 0.92, 0.68 and 0.47 for A1 milk, total milk protein and A2 milk respectively<sup>18</sup>. Similar results were also found in earlier studies conducted at smaller scale<sup>19,20,21</sup>. Ecological analysis conducted in 22 countries has also shown a close relationship between cardiovascular disease and consumption of A1 milk. The correlation coefficient value of 0.79-0.81 was found in ischaemic or coronary heart disease (IHD) mortality and consumption of A1 milk<sup>18</sup>.

Epidemiological studies presented in one of the patent applications reveals that 'statistically relevant' correlation exists between  $\beta$ -casein A1 consumption and aggravation of neurological disorders such as autism and schizophrenia<sup>6</sup>.

**Role in neurological disorders**

Opioid receptors are basically found in brain and classified in three groups,  $\mu$  (MOP),  $\kappa$  (KOP) and  $\delta$  (DOP)<sup>22</sup>. MOP or  $\mu$  receptor is found in brain, immune cells, endocrine glands and to some extent in intestine. From guinea pig ileum assay (GPI) it was known that bovine milk has opioid effect<sup>23,24,25</sup> and it acts most probably through MOP receptor<sup>26</sup>. There are several animal experiments carried out in this regard that support the role of BCM-7 in affecting behavior of an animal. BCM-7 was administered in rat and mouse model by intra-cerebro-ventricular or intra-peritoneal where peptide has shown analgesic activity, change in sleep cycle, amnesia which was

reversed by addition of naloxone drug, acts especially on MOP receptor<sup>25,27,28</sup>. Bovine BCM-7 has much higher affinity for MOP receptors as compared to human milk BCM-7 (YPFVEPI)<sup>29</sup>. Also BCM-7 level in human milk reduces drastically after 2 to 4 months of delivery of new born<sup>30</sup>, reducing overall effect of opioid peptides such as human BCM-7. This kind of reduction in BCM-7 level occurs in bovine milk is still unknown. According to Meisel and FitzGerald, 2000, bovine milk proteins also produces opioid antagonist like casoxin A, B and C during its digestion. But overall maximum theoretical yield (mg/g protein) of opioid agonist can be higher as compared to opioid antagonist<sup>31</sup>.

BCM-7 has also shown increase in the expression of genes responsible for producing inflammatory enzymes such as myeloperoxidase<sup>32</sup> that could further increase the expression of MOP receptors in intestine<sup>33</sup>. The overall process further enhances the action of this peptide. However information regarding similar type of action is not available for brain.

Both autism and schizophrenia are mental disorders and it is believed that the symptoms of these disorders can be reversed by changing in food habits. In mental disorder children generally show behavioral problems such as social isolation, mutism, slow learning skills, difficult to speech, hyper-activity, self-mutilation, panic attacks and no eye to eye contact. Patients suffering from autism and schizophrenia (leaky gut syndrome) have shown large amount of BCM-7 in their urine<sup>6</sup>. Such patients have shown dramatic improvements in their symptoms following gluten free and casein free diet (GFCF)<sup>6,34</sup>. Professor Cade of University of Florida, who has contributed to a number of studies on autism, has provided preliminary results from a behavioral study of 70 patients with childhood autism who were put on a gluten free, casein free diet and he concluded that GFCF diet had helped in reducing autism disorder symptoms. However, the diet failed to fully cure these disorders.

It is believed that mercury toxicity inactivates cysteine amino acid present in active site of DPP (IV) enzyme which is required during digestion of casein<sup>6,35,36</sup>. In autistic and schizophrenic patient it has been seen that they not only have high level of casomorphin in their blood samples but also have high level of gliadorphin peptide (gliadorphin comes from gluten digestion) in their serum<sup>6,37</sup>. This proves that DPP (IV) normal activity is very important for protein digestion (milk protein or wheat protein), (Figure 5A & 5B) and its impaired function could be involved in mental disorders. In neonates high level of mercury toxicity could arise through vaccination, mother's dental amalgams or other environmental factors<sup>6,38</sup>. Further, high intestinal permeability; lack of breast feeding & immature BBB (blood brain barrier) enhances the movement of BCM-7 from gut to brain which it shows its effect. Gluten and casein both are very similar peptide and their digestion process is also similar. By making change in food habits, reduction in pollution can eliminate these disorders.

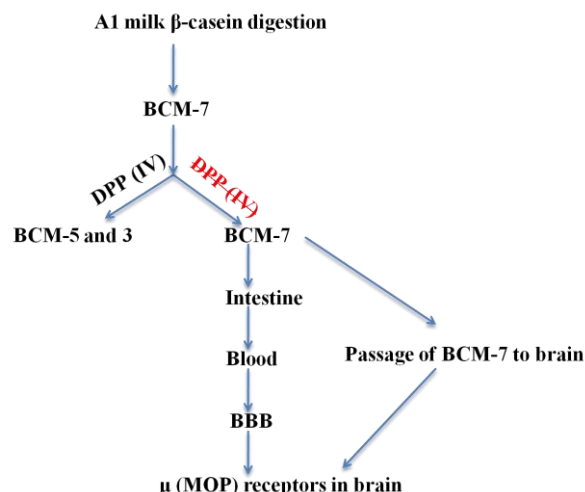


Figure 5A. Passage of BCM-7 from intestine to brain.

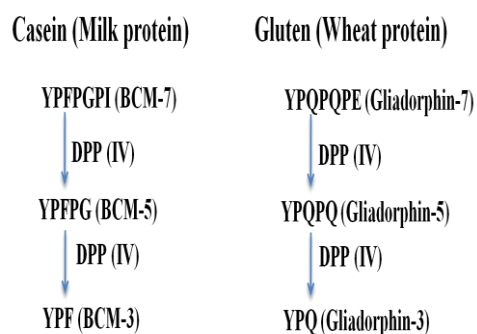


Figure 5B. Action of DPP (IV) on milk protein (Casein) and wheat protein (Gluten).

High amount of BCM-7 has also been found in sudden infant death syndrome (SIDS) patient's sera and such individual had low activity of DPP (IV) enzyme<sup>39</sup>.

**Role in type 1 diabetes**

Type 1 diabetes is an autoimmune disorder in which beta cells of pancreas is destroyed by immune system of host. This disorder mostly effects children and requires daily insulin injection to control glucose level. The potential role of cows' milk in diabetes is still debated and there is no consensus on the diabetogenicity of individual milk proteins. It is believed that BCM-7 suppresses body's immune system that may enhance the survival of pathogens such as enteroviruses<sup>21,40</sup> or bacteria (*Mycobacterium avium*)<sup>41</sup>. These pathogens are ultimately involved in trigger of type1 diabetes like symptoms. Second theory says autoantibody generation against beta cells. According to this theory some of the peptide sequences of β-casein which are produced during digestion, mimic the sequence of GLUT-2 transporter<sup>42,43,44</sup>(involved in glucose transportation in cell). In this response T cells recognize them as an antigen and activate B cells for production of antibodies. Antibodies not only targets the digested segment of β-casein product but also to insulin producing beta cells, causing type 1 diabetes.

One of the animal experiments carried out by Beales group in 2002<sup>45</sup> gave information that consumption of A1 milk is not diabetogenic in nature as it was previously predicted. In the experiment carried out on NOD/Ba (non-

obese diabetic mouse, New Zealand) and BB (bio breeding rat, Canada) animal model, wheat containing diet NTP 200 (control) was highly diabetogenic. While pregestimil (PG) diet containing casein hydrolase as a major ingredient had lowered diabetes incidence in NOD/Ba and BB animals as compared to control ones. When PG diet was mixed with 10%  $\beta$ -casein A1, diabetes incidence was same in NOD/Ba mice but less in BB rats, compared to PG diet given alone to these animals. On the other hand, PG with 10%  $\beta$ -casein A2 diet had similar effect as of PG diet on animals. ProSobee (PS) diet was also tested which had soya protein as a major component; it caused very low incidence of diabetes in NOD/Ba mice but had similar effect (PG diet alone) on BB rats. PS+10%  $\beta$ -casein A1 and A2 both had increased the diabetes incidence in NOD/Ba mice but PS+A1 had little effect on (BB) rats for diabetes, while PS+A2 had reduced diabetes incidence in BB rats when compared to PS diet used alone (Figure 6). The above data is not conclusive in blaming A1 milk for diabetes. Also, it was clear from above experiment that genetic aspect of diabetes making some of the animals prone to diabetes than others at the same diet (PS dietary effect on BB and NOD/Ba), wheat containing diet is more diabetogenic as compared to milk protein (casein). Further soya protein can be good for health.

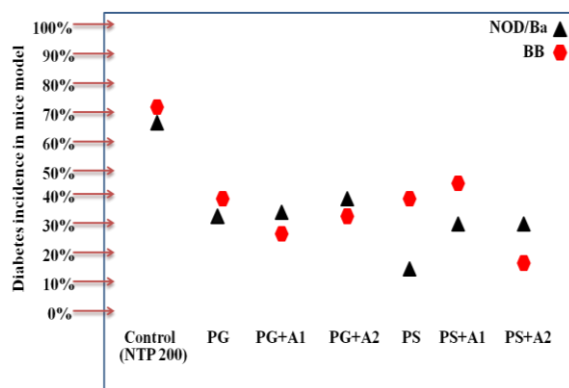


Figure 6: Effect of different diets on diabetes incidence in mice model.

**Role in cardiovascular disease (CVD)**

It is believed that BCM-7 could be involved in oxidation process of low-density lipoprotein (LDL)<sup>46</sup>. Oxidized form of LDL is engulfed by macrophages by specific receptors present on their surface. These macrophages ultimately converted into foam cells and start the process of atherosclerosis in heart (Figure 7). It is long before known that high fatty diet can increase the oxidation process in LDL. However, food containing fish (rich source of omega-3 fatty acids) or bovine whey protein is believed to be inhibitors of oxidation of LDL. Lactoferrin of bovine whey protein prevents the accumulation of cholesterol in macrophages by oxidized LDL<sup>47</sup>. Also, omega-3 fatty acid with supplement of vitamin E or C is very helpful in inhibiting oxidation of LDL<sup>48</sup>. Tailford et al., 2003<sup>49</sup> published a paper where it was shown that A1 milk is more responsible in causing atherosclerosis as compared to A2 milk. In rabbit model, an artificial injury was made in carotid artery of animal and was fed on A1 and A2 milk diet respectively. After 6 weeks of diet it was found that rabbits

fed on A1 milk diet had thicker fatty streak on injured area as compared to animals fed on A2 milk. However, data is not very conclusive as formation of foam cells in fatty streak during tissue repair and in atherosclerosis process are two different events.

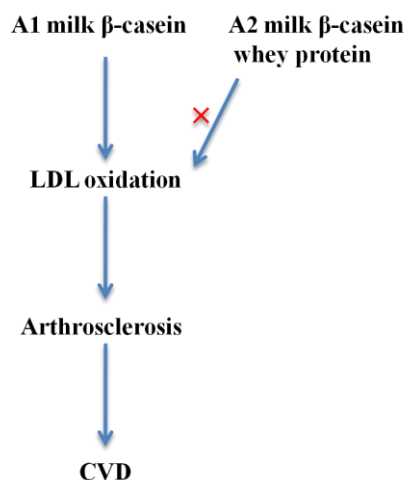


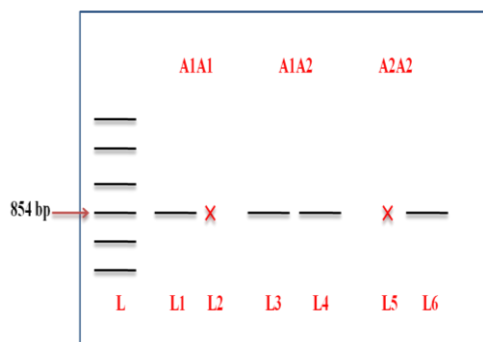
Figure 7: A1 milk and its predicted role in cardiovascular disease.

**Methods for typing animals (A1/A2) and detection of BCM-7**

**Typing of animals on the basis of their genetic materials:** It is difficult to ascertain presence of  $\beta$ -casein A1 or  $\beta$ -casein A2 variant in milk. Thus, efforts have been made to identify alleles in  $\beta$ -casein from DNA isolated from animals.

**Allele specific PCR (AS-PCR):** Keating et al., 2008<sup>50</sup> has described concept of allele specific PCR (AS-PCR). Method based on this concept for typing of animals uses DNA from bovine blood and finds single nucleotide polymorphisms (SNPs) and mutations. Selective amplification is achieved by designing a primer such that its 3' end will match or mismatch with base of allele. Primer strand will extend only when its 3' end is a perfect complement to the allele present in DNA. Thus, if a single base polymorphism occurs, the genotyping results can be observed by simply observing presence and absence of PCR products on the gel. Allele specific PCR is carried out by using a common forward primer (Bwtp3; 5'GCCCAGATGAGA GAAGTGAGG-3') and reverse primers with either T or G at the 3' end (5'-GATGTTTTGTGG GAGGCTGTTAT/G-3') to amplify an 854 base pair fragment. In Figure 8A, 854 base pair DNA fragment was amplified in L1 well by DNA forward primer and A1 allele specific reverse primer. However, the same DNA was not amplified in L2 well by A2 allele specific reverse primer. L1&L2 wells represent A1A1 genotype of that animal. In L3&L4, 854 base pair DNA fragment was amplified by A1 allele reverse primer and A2 allele reverse primer respectively, representing A1A2 genotype of that animal. In L5 well, A1 allele specific primer fails to amplify 854 base pair DNA fragment, while in L6, A2 allele specific primer amplified the desire product, representing A2A2 genotype of the animal in L5&L6 wells.

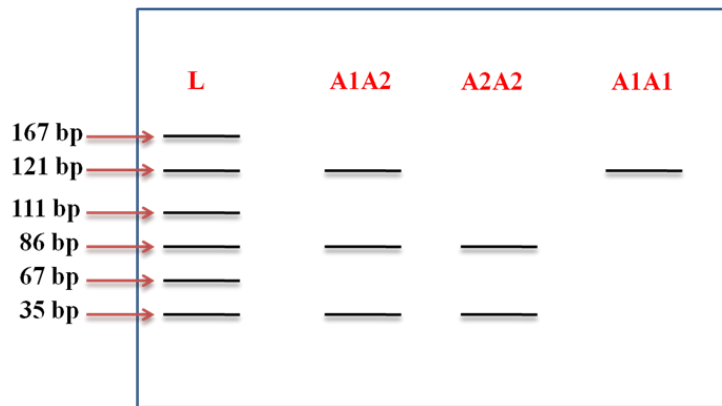
The method can detect whether the bovine will produce A1A1, A2A2 or A1A2 type of milk.



**Figure 8A.** Amplification of A1, A2 allele by AS-PCR method. L indicates DNA ladder, L1&L2 indicates A1A1 genotype of animal, L3&L4 indicates A1A2 genotype of animal and L5&L6 indicates A2A2 genotype of animal (X denotes absence of band).

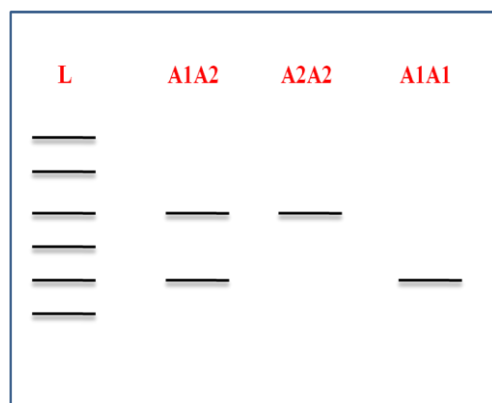
**Amplification created restriction site PCR (ACRS-PCR):** Raies *et al.*, 2009<sup>4</sup> has described the method for typing of animals using DNA from bovine blood using concept of amplification created restriction site (ACRS-PCR). It is quite similar to allele PCR. 121 base pair length of DNA is amplified. Forward primer sequence is made of identical sequence, but reverse primers are made such that out of 18 bases, one base penultimate to the 3' end is not able to form hydrogen bond with base present at complementary strand of amplifying DNA. Because of one base difference between A1 and A2 beta-casein, a restriction site is created in A2 but not in A1 gene which is recognized by an endonuclease enzyme; it cuts the DNA of A2A2 genotype into two parts, which appears as two bands on the gel, but appears as one band in case of A1A1 genotype. Three bands are observed in A1A2 genotype. The ACRS-PCR method is carried out by using two kinds of primers, Casein4

(Forward):5'CCTTCTTTCCAGGATGAACTCCAGG-3'and Casein Dde2  
(Reverse):5'GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT-3'which amplifies 121 bp DNA product, the mismatch at the penultimate nucleotide (cytosine underlined) at 3' end of Casein Dde2 primer (Reverse) creates a restriction site for DdeI enzyme. The specific digestion resulted in the occurrence of 86 base pair and 35 base pair fragments, due to the presence of the CCT (proline) codon in the A2 allele of β-casein (Figure 8B).



**Figure 8B.** ACRS-PCR method for animal typing, L indicates DNA ladder.

**Single strand conformation polymorphism PCR (SSCP-PCR):** Barroso *et al.*, 1999<sup>51</sup> has described the method for typing of animals using DNA from bovine blood. The method gives information whether the bovine will produce A1 or A2 milk. SSCP is the electrophoretic separation of single-strand nucleic acid based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through a gel. It detects whether the bovine will produce A1A1, A2A2 or A1A2 type of milk (Figure 8C). SSCP screening only tells that a mutation exists. However DNA sequencing has to be performed to know the nature of the mutation that caused an electrophoretic mobility shift in a given sample. Also, highly standardized electrophoretic conditions are required to obtain reproducible results.



**Figure 8C.** SSCP-PCR method for animal typing, L indicates DNA ladder.

**Taq Man method:** In this method dsDNA is isolated from somatic cells of bovine milk; ssDNA probe (18 bases long) is made specific for A1 and A2 beta-casein gene that contains at 5' end fluorescent agent and at 3' end a quencher. Quencher inhibits the activation of fluorescent agent in native form of probe<sup>52</sup>. During PCR, Taq Man probe (ssDNA labeled with fluorescent and quenching agent) binds to A1 or A2 beta-casein gene at their sense strand along with forward primer and reverse primer. DNA polymerase breaks the probe during amplification process, activating fluorescent agent present on it at specific

wavelength. This method is highly sensitive and could detect whether lactating animal is A1 or A2 in nature.

#### Typing of animals on the basis of presence or absence of BCM-7:

The method requires isolation of  $\beta$ -casein from milk, *in vitro* digestion by gastrointestinal enzymes and separation of generated peptides by HPLC-MS method. The sequence and mass of peptide helps in identification of BCM-7<sup>15</sup> as well as typing of milk.

#### Detection method of BCM-7 in urine / blood:

RIA/ELISA methods are described for assaying BCM-7 in urine or plasma<sup>53,54</sup>. Sokolov group with the help of ELISA method has detected BCM-7 in pg. level in urine of normal as well autistic children. However, the concentration of BCM-7 was much higher in urine of autistic children as compared to normal ones. This clearly suggests that a seven amino acid peptide (BCM-7) can pass through intestinal barrier and survive in blood followed by its excretion into urine. Commercially available kit such as S-1334 (ELISA based kit from Bachem company) can detect BCM-7 in beta- casein digest at ng. level. Aptamer (single strand DNA) has also been selected against BCM-7 that recognized it at IC<sub>50</sub> value of 25 nanogram<sup>55</sup> but aptamer has yet to detect BCM-7 in urine or blood samples, this is due to a very low level of peptide in samples or could require further investigation.

#### CONCLUSION

In last 20 to 30 years several studies were carried on A1 milk and its effect on human health. Statistical data correlates its role in non-communicable disorders (diabetes, autism). At the same time animal studies and even human studies does not give substantial evidence of A1 milk for its role in these disorders. That is why we are still consuming A1 milk and it is not banned. It seems that early consumption could have indirect role in these disorder along with other environmental factors and food habits. South East Asian countries have A2 herd of cattle and their milk is in general of type A2 in nature and it seems that A1 milk is not an issue for them. But as milk demand has surged with increase in population Dr. Verghese Kurien started white revolution in India in 1970. It has three objectives- i) increase milk production, ii) increase in rural income, iii) fair price of milk for consumers. Operation flood or milk revolution has made India self-dependent in milk and its product and at the same time economic condition of farmers has also changed. Currently India is the largest producer of milk in world. Increase in milk production has achieved by taking several measures such as selection of high yielding breed, improve in quality of fodder, crossbreeding program in cattle etc. In crossbreeding program Indian bovine was crossed with exotic European breed. Crossbreeding program has created several high yields producing variety but at the time they are producing type A1 milk which is a matter of concern. In India still majority of milk comes from buffalo which is A2 in nature. In view of this ICAR (Indian Council of Agriculture Research) has taken initiatives to do more research on A1 milk and putting fund to monitor A1 allele

frequency in crossbreds. It is also taken in mind that we also need statistical data and human studies on A1 milk to see whether it has any role in aggravating non communicable disorders in Indian population. All in all, controversy on A1 milk is an issue which needs to be addressed but its harmful effect has yet to be proved.

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**CITE THIS ARTICLE AS:**

Abhishek Parashar and Ram Krishan Saini. A1 milk and its controversy-a review. *International Journal of Bioassays* 4.12 (2015): 4611-4619.

**Source of support:** Nil

**Conflict of interest:** Nil