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EVOLUTIONARY RELATEDNESS OF MAMMARY DERIVED KAPPA CASEIN GENE OF RIVERINE BUFFALO (BUBALUS BUBALIS) WITH HUMAN AND RAT FIBRINOGEN Γ CHAIN

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Milk proteins of all mammals can be divided into two classes: the caseins and the whey proteins. The caseins (alpha-S1-casein, alpha-S2-casein, beta-casein, and kappa-casein) comprise the major protein component of ruminant milk and are secreted in the form of stable calcium phosphate micelles. The three calcium-sensitive genes, alpha-S1-casein, alpha-S2-casein, beta-casein, have evolved from a common ancestral gene by events such as gene duplication and exon shuffling whereas the k-casein gene appears to have evolved along a different pathway, since it does not share any common pattern with other casein genes. In the present study the relatedness of kappa- casein gene which is involved in milk clotting with fibrinogen gamma-chain , leucine rich repeat interacting protein 1 gene and chymosin gene involved in blood clotting, was being studied. The mammary derived kappa casein cDNA of Buffalo (Bubalus bubalis) was compared to the other three caseins along with fibrinogen γ chain , chymosin and leucine rich repeat interacting protein 1(LRRIP1) gene. kappa casein and chymosin are involved in milk clotting while fibrinogen γ chain and leucine rich repeat interacting protein 1 gene are involved in blood clotting. Phylogenetic analysis showed co-evolution of kappa casein gene with chymosin, fibrinogen γ chain and leucine rich repeat interacting protein 1 gene as their gene sequences culminated on one node while the other three caseins formed a different composite group.

Key words: Phylogenetics, exon shuffling, gene duplication, Bubalus bubalis, Kappa casein.

Kappa-Caseins, involved in the milk clotting process, and human fibrinogen gamma-chain, involved in the blood clotting process, show structural similarities. Several long kappa-casein sections, together corresponding to 80% of the whole protein molecule, have their counterparts in the gamma-chain of fibrinogen, in that 31-42% of the amino acid residues occupy identical positions. The section of kappa-casein which contains the chymosin-sensitive bond has a counterpart not only in the gamma but also in the beta-chain of fibrinogen. When milk clotting process (interaction kappa-casein/chymosin) is compared to the blood clotting process (interaction fibrinogen/thrombin): a large number of similarities could be noted between both clotting phenomena. There is some evidence indicating that the k- casein gene is evolutionarily related to the fibrinogen γ -chain gene family whose cleavage by thrombin results in blood clotting [3]. This hypothesis is sustained by the structural and functional similarities between the proteins, and by the nucleotide sequence similarities between kappa casein and γ fibrinogen cDNAs [2].
In this paper the four casein genes including kappa casein along with chymosin , Fibrinogen γ chain and leucine rich repeat interacting protein 1 gene sequences, were utilized for phylogenetic analysis so that the evolutionary status of kappa casein gene could be studied in the light of literature available in this context.

MATERIALS AND METHODS

For the present study the coding sequence of kappa casein gene and alpha S2 gene of Buffalo were amplified and sequenced in the laboratory.

Tissue Collection

25-50mg of tissue from mammary gland, of buffalo was collected from slaughter house which was immediately flash freezed and immersed in tubes containing RNA later. Tissue was transported to the laboratory and stored at -80oC for subsequent use.

RNA Isolation

Isolation of total RNA was carried out using Roche high pure RNA tissue kit. 5g of the total RNA thus obtained was immediately processed for cDNA first strand synthesis and rest was stored at -80oC.

First strand cDNA synthesis

First strand cDNA synthesis was carried out by oligo (dT) method from invitrogen kit. The cDNA obtained was stored at -20oC for further used.

Primer Designing

Utilising cDNA sequence of Bos Taurus available at NCBI and ensemble, two pairs of primers were designed to amplify cDNA of the selected candidate genes in buffalo. Primer 3 software [4] was used for primer designing .Degenerate primers were designed where necessary.

Polymerase chain Reaction

PCR amplification was carried out with 20l reaction, 200M dNTP, 5pmol primers, 1 unit Taq DNA polymerase and 1X reaction buffer containing 1.5mM MgCl2 . Amplification was carried out in eppendorf thermocycler with initial denaturation of 95oC for 5 min, followed by 35 cycles of 94oC for 45 s, annealing temperature (55 to 60oC) for 45 s and 72oC for 45 s. The final cycle was followed by an extension step at 72oC for 10 min and hold at 4oC. The PCR products were visualized on 2% agarose gel (EtBr stained) using 1X TAE buffer.PCR product was then purified for sequencing.

Sequencing

Sequencing was carried out on ABI-3100 AVANT automated DNA sequencer. Sequences with chromatogram were collected by ABI PRISM® DNA sequencing Analysis Software. The consensus coding sequence was then obtained for both the genes. Sequences of alpha S1 casein, Beta casein Fibrinogen γ chain , chymosin, and leucine rich repeat interacting protein 1 were retrieved from NCBI.

Sequence Analysis

The sequences were edited in Chromas [7] and aligned by CLUSTAL W [6].

Traditional Phylogenetic analysis

Phylogenetic analysis was carried out using MEGA software [5]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Poisson correction model. Initial trees for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used otherwise BIONJ method with MCL distance matrix was used.

Bayesian Phylogenetics

The MPD (Maximum Posterior Decoding) alignment was estimated using Phylogeny café. The phylogenetic tree was reconstructed using the bayesian inference method implemented in the MrBayes program v3.1.2 [1]. The number of substitution types was fixed to 6 (GTR). The Poisson model was used for amino acid substitution, while rates variation across sites was fixed to "invariable+gamma". Four Markov Chain Monte Carlo (MCMC) chains

were run for 10000 generations, sampling every 100 generations, with the first 100 sampled trees discarded as burn-in. Finally, a 50% majority rule consensus tree was constructed.

RESULTS AND DISCUSSION

Kappa casein and Alpha S2 casein sequences were submitted to Genbank and accession number HQ677596 and HQ840513 respectively were obtained.

Phylogenetic Analysis

The Phylogenetic tree derived from the amino acid sequence data (Figure1) forms two major clades. The tree with the highest log likelihood -3778.1724 is depicted (Figure1). One clade comprised of caseins excluding kappa casein which formed a group with genes involved in milk and blood clotting. Before the addition of chymosin and Leucine rich repeat interacting protein1 in the dataset , kappa casein culminated on the same node with fibrinogen (Figure2). After the addition of chymosin and Leucine rich repeat interacting protein1 in the dataset kappa casein clustered with them along with fibrinogen, As the Figure1 shows the genes involved in clotting process culminated on one node.

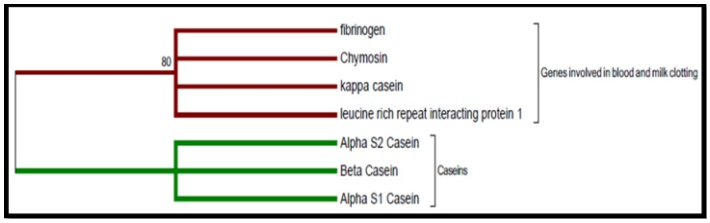

Figure1. Molecular Phylogenetic analysis by Maximum Likelihood method

Figure2: Molecular Phylogenetic analysis by Maximum Likelihood method (Nodes showing ancestral states)

Bayesian Phylogenetics

Figure3 shows the phylogeny of the genes (amino acid sequence) as derived from Bayesian analysis of the gene sequences. The MPD (Maximum Posterior Decoding) alignment was estimated using only alignments from the after-burn-in period. kappa casein gene formed a sister group with LRRIP1 with a clade credibility value of 50% which clustered together with chymosin and fibrinogen with a clade credibility value of 50%.The caseins(alpha-S1-casein, alpha-S2-casein and beta-casein) formed a monophyletic group with a clade credibility of 50%.

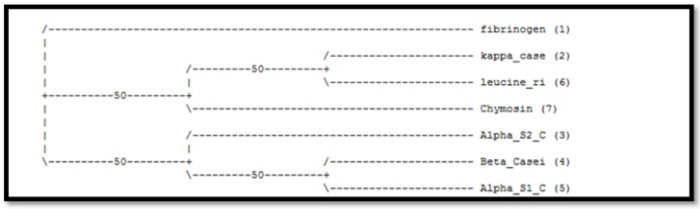

Figure3. Tree topology based on Bayesian analysis

SUMMARY

The hypothesis of kappa casein evolving along with fibrinogen-γ chain was brought about by Jollès et.al (1978) after the comparative analysis of the gene sequences in goat. Our effort was to reconstruct the phylogeny in buffalo so as to study the evolutionary relatedness of kappa casein gene with genes involved in milk and blood clotting including fibrinogen γ chain. The results summarize that kappa casein may have co evolved with genes involved in milk and blood clotting rather than with the casein cluster. Homology has been observed at the cDNA and AA level between κ-casein and different parts of human and rat γ -fibrinogen . γ –Fibrinogen (GFF) is involved in the blood-clotting process, and the gene encoding it is part of the Fibrinogen gene family also located on HSA4 (4q31; casein gene cluster, 4q13). The structure of the genes in the fibrinogen gene family is poorly conserved . The regions of homology between CSN3 and GFF border at least one splice junction in GFF . A peptide encompassing the first 11 AA of the κ-casein GMP (AA 126–137 in bovine) exhibits similar behavior as a structurally related γ -fibrinogen peptide by inhibiting platelet aggregation and fibrinogen binding . Taken together, these data support the notion that the CSN3 and GFF are evolutionarily related and have evolved via exon shuffling and intron deletions as previously suggested

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