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DETECTION OF ENDOGENOUS RETROVIRUS SEQUENCES IN BOVINE MILK

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INTRODUCTION

Retroviral elements and endogenous retroviruses (ERVs) have been found in the genome of all examined vertebrates. These elements are retroviruses that have been integrated into germ line cells during the evolution and they are vertically transmitted as stable inherited Mendelian genes. ERVs have been found in multiple copy numbers in all mammals. It was estimated that approximately 8% of the human genome contains retroviral sequences. This kind of estimation is available also for other species, for example is known that the long terminal repeat (LTR) retrotransposons, which include ERVs, account for 3.20% of the bovine genome.
Most of the known ERVs sequences are incomplete, largely deleted and/or interrupted by stop-codons. However, complete ERVs have been described and some of them are capable of forming virus-like particles.
The biological significance of ERVs has been debated for several decades and were generally thought to be "junk-DNA". However recent studies suggest that ERVs have a variety of beneficial and negatives roles to their host. Many ERVs are often expressed in tumors other brings benefit to the host organism, for example the envelope proteins of endogenous retroviruses drive differentiation to the placental cytotrophoblast into syncytiotrophoblast. One of the more important observation is that a recombination between ERVs and exogenous retroviruses may create new exogenous retroviruses.
Analyses of the ERVs in the genomes of several mammals, including humans, pigs and sheep, have revealed the presence of full-length proviruses which consist of the typical retroviral structure 5'-LTR-*gag-pro-pol-env*-LTR-3'. In mammals, ERVs are classified into the retroviral β (B/D type) and γ (C type) genera. In particular, the bovine ERVs can be classified into at least four families (β3, γ4, γ7, γ9) relative to the *pro/pol* nucleotide sequences of OERV β and γ families (Fig. 1). The aim of this study was to analyze the conserved *pro/pol* region of bovine ERVs by PCR using specific primers on a number of commercial UHT and raw fresh milk samples for human consumption.

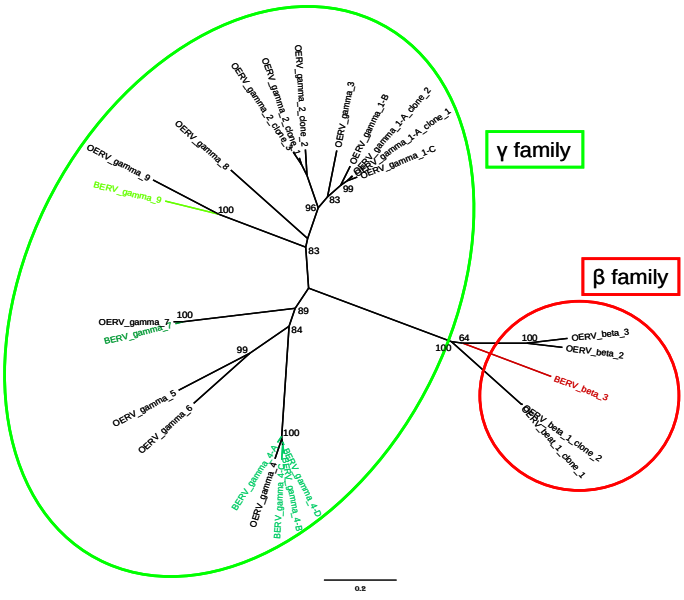

Fig.1. Classification of the bovine endogenous retroviruses (BERV) β3 and γ families relative to the *pro/pol* nucleotide sequence of the ovine endogenous retroviruses (OERV) β and γ families .

MATERIALS AND METHODS

A number of commercial UHT and raw fresh milk samples for human consumption were collected to separate the somatic cells in them. After centrifugation, the lipid phase was sucked, the supernatant was collected for the following analysis of Reverse Transcriptase (RT) activity; the pellet, containing the somatic cells, was used for DNA and RNA purification using respectively QIAamp DNA Mini Kit (QIAGEN) and RNeasy Mini Kit (QIAGEN) according the manufacturer's instructions. Successful amplification of DNA and RNA of an housekeeping gene such glyceraldehyde 3-phosphate dehydrogenase (GAPDH) indicated that the samples were adequate for PCR and RT-PCR analysis and that no PCR inhibitors were present. For amplification of BERV β3, γ4, γ7, γ9 *pol* sequences, specific primers were deduced from bibliography and they are reported in Table 1. PCR consisted of 2 min at 94℃, followed by 40 cycles o f a 94℃ denaturation step for 30 s, annealing at 55℃ for 30 s, extension at 72℃ fo r 1 min, and finally, one step at 72℃ for 1 min. and a *Taq platinum* polymerase (Invitrogen) was used.
The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). To verify the DNase efficiency, for all samples replicate Reverse Transcription was performed: the first one including the enzyme RT (+RT), the second one without it (-RT). Than the amplification was performed as previous described.
The Reverse Transcriptase (RT) activity in the supernatant was tested by the HS-Mn RT Activity Kit (Cavidi).

Primers	Sequence	Product lenght
GAPDH For DNA	5'- ATG GTA GGA GTG GTG GGA AAC T -3'	483 bp
GADPH Rev DNA	5'- CAG GTC AGA TCC ACA ACA GAC A -3'	
GAPDH For cDNA	5'- GAA GCT CGT CAT CAA TGG AAA G -3'	338 bp
GAPDH Rev cDNA	5'- CAG TGG TCA TAA GTC CCT CCA C -3'	
β3 For	5'- ACT GAA GAA TGG CCC CTT G -3'	221 bp
β3 Rev	5'- CTG TGG CTT TCG TTT GTT CA -3'	
γ4 For	5'- CTC CTC CCA AAC CTG TAC CA -3'	759 bp
γ 4 Rev	5'- AAT ACT GTC CAA GTC ATC TG -3'	
γ7 For	5'- TGA CTT CTC TGT TCT TCC TT -3'	762 bp
γ7 Rev	5'-TGT TCC CAG GTC CCA CCA CT-3'	
γ9 For	5'- GGT GGG ACA ACA ACC TAC T -3'	700 bp
γ9 Rev	5'- CAG GAG CCA ACA TCC ATA CC -3'	

Table 1: Oligonucleotides and length of amplification products used in the described PCR protocols

RESULTS AND DISCUSSION

All the BERV sequences were detected in the genomic DNA extracted from somatic cells of all the milk samples, as expected.
Concerning the expression, no one heat-treated milk samples revealed amplification products or RT activity. These results could be explained with the RNA and enzyme thermolability. In raw fresh milk samples, on contrary, RT activity was observed and apart from γ9 e β3 families which were identified by PCR analyses, other BERV sequences were not identified (Fig. 2). This shows that some ERVs could be capable to form and release virus-like particles.
These results provide evidence that milk and milk derivatives have the potential to carry the virus and, even if many endogenous retrovirus do not readily re-infect their own host cells, we can not exclude the risks deriving from the exposure of other hosts (including humans) to these retroviruses.
These reasons and findings encourage further examinations in different directions. First of all, to deepen the knowledge regarding the risk for public health consequent milk consumption. Further more, to investigate about presence of other BERV families in bovine genome combining bioinformatics tools and molecular techniques. In this sense we already working on BERV β1 family obtainig, as preliminary results, encouraging data from PCR (using genomic DNA as template) and from the first sequence analysis.

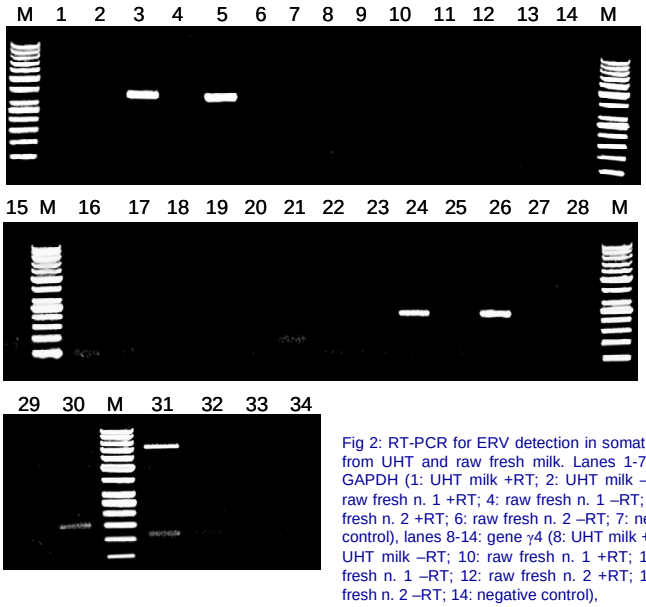

Fig 2: RT-PCR for ERV detection in somatic cells from UHT and raw fresh milk. Lanes 1-7: gene GAPDH (1: UHT milk +RT; 2: UHT milk –RT; 3: raw fresh n. 1 +RT; 4: raw fresh n. 1 –RT; 5: raw fresh n. 2 +RT; 6: raw fresh n. 2 –RT; 7: negative control), lanes 8-14: gene γ4 (8: UHT milk +RT; 9: UHT milk –RT; 10: raw fresh n. 1 +RT; 11: raw fresh n. 1 –RT; 12: raw fresh n. 2 +RT; 13: raw fresh n. 2 –RT; 14: negative control), lanes 15-21: gene γ7 (15: UHT milk +RT; 16: UHT milk –RT; 17: raw fresh n. 1 +RT; 18: raw fresh n. 1 –RT; 19: raw fresh n. 2 +RT; 20: raw fresh n. 2 –RT; 21: negative control), lanes 22-28: gene β3 (22: UHT milk +RT; 23: UHT milk –RT; 24: raw fresh n. 1 +RT; 25: raw fresh n. 1 –RT; 26: raw fresh n. 2 +RT; 27: raw fresh n. 2 –RT; 28: negative control), lanes 29-34: gene γ9 (29: UHT milk +RT; 30: UHT milk –RT; 31: raw fresh n. 1 +RT; 32: raw fresh n. 1 –RT; 33: raw fresh n. 2 +RT; 34: raw fresh n. 2 –RT). M: molecular weight marker 50 bp Generuler.