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Evaluation of lentiviral genetic mutations

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Abstract The small ruminant lentiviruses (SRLV) have the potential to change genetically and switch from an host to another. In this paper, we studied genetic evaluation that accompanied infestation and adaptation of SRLVs in hybrids. Three blood samples, one year apart, were taken from *Hybrid*s living in two sites in French Alps. Antibodies to SRLV were sought in serum using a commercial ELISA to p28 antigen, and ficoll-isolated monocytes were cultured in macrophage differentiation medium to detect active viral infection. DNA extracted from non-cultured monocytes was amplified by PCR and Nested PCR. Amplicons were sequenced and analysed. The Nested PCR technique specifically amplified a 513 nucleotide fragment of the *gag* gene including the 3' portion. The sequences alignments shows two types of genetic mutations: deletion and replacement. The sequences obtained from the samples taken in first year showed a diversity comparable to that observed in the reference virus. After one year of cohabitation, the sequences of the virus showed very important genetic mutations including a deletion of 6 nucleotides. Finally, after 3 years of cohabitation, the virus sequences included more genetic mutations. The percentages of discrepancies between the proviral sequences obtained and that of reference virus were on average from 6.1 to 8.8% in nucleotides. Compared with proviral sequences obtained in first year, the average percentages of divergence was 2.6% in nucleotides in second year and 2.9% in third year. The average of the internal divergence percentages was 0.56 - 2.79%. These differences were also shown by the phylogenetic tree.
Keywords: lentivirus, *gag* gene, SRLV, hybrids, genetic mutations.

تطور الطفرات الوراثية للفيروسات البطيئة (Lentivirus)

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ملخص لفيروسات الـــ SRLV القدرة على التحور وراثيا والانتقال من مضيف إلى آخر، في هذه الورقة قمنا بدراسة التطور الجيني المصاحب لانتقال هذه الفيروسات وتكيفها في بعض العوائل الجديدة (الهجائن). تم أخذ ثلاث عينات دم، بفارق سنة واحدة بينهم، من الهجائن التي تعيش في موقعين مختلفين في جبال الألب الفرنسية. تم البحث بعدها عن الأجسام المضادة لـــ SRLV في المصل باستخدام تقنية الـــ ELISA المعتمدة على المستضد p28، ولعزل الفيروس النشط قمنا بفصل الخلايا الليمفاوية الاحادية على اوساط تمايز مختلفة ومضاعفة الحمض النووي (DNA) للفيروس المعزول من الخلايا الليمفاوية وحيدة النواة الغير مزروعة بواسطة تقنيتي الـــ PCR والـــ NestedPCR وأخيرا قمنا بدراسة وتحليل التركيب الوراثي لسلاسل الحمض النووي المعزول والبالغ حجمها 513 نيوكليوتيد من الجين *gag* بما في ذلك الجزء ذو الاتجاه 3'. اظهر تحليل السلاسل المتحصل عليها نوعين من الطفرات الوراثية: حذف واستبدال نيوكليوتيدات. كما أظهرت السلاسل المتحصل عليها في السنة الاولى تركيبا وراثيا مشابهًا لذلك الموجود في الفيروس المرجعي. بعد عام واحد من الإصابة، أظهرت السلاسل الفيروسية طفرات جينية مهمة للغاية بما في ذلك حذف عدد 6 نيوكليوتيدات. وأخيراً، بعد مرور 3 سنوات من الإصابة، اظهرت السلاسل الفيروسية المزيد من الطفرات الجينية مقارنة بتلك الموجودة في السنة الاولى. كان متوسط النسب المئوية للاختلافات بين السلاسل المعزولة والفيروس المرجعي ما بين 6.1 إلى 8.8٪ بالنسبة للنيوكليوتيدات. بالمقارنة مع السلاسل الوراثية المعزولة في السنة الاولى من الإصابة، كان متوسط النسب المئوية للاختلافات في السنة الثانية 2.6٪ و2.9٪ في السنة الثالثة. وكان متوسط النسب المئوية للاختلافات الداخلية بين السلاسل 0.56 – 2.79٪ كما ظهر ذلك واضحا على شجرة السلالة الوراثية.
الكلمات المفتاحية: الفيروسات البطيئة، جين *gag*، SRLV، الهجائن، الطفرات الوراثية.

Introduction:

The lentiviruses (genus of retroviruses), which cause persistent infections in the human and other mammalian species, include various genotypes that regularly cross the species barrier between hosts and often exhibit high genetic variability. Most hosts infected with lentiviruses do not have clinical disease, but remain persistently infected and can transmit the virus. The first symptoms of the disease are insidious and have a slow progression. The best known lentivirus is the Human Immunodeficiency Virus (HIV), which causes AIDS. The small ruminant lentiviruses (SRLV) infections cause a systemic infection that can affect various target organs such as the lungs, central nervous system, udders and joints. These viruses have the potential to change genetically and switch from an host to another.

One of the main problems of viral pathologies is the genetic evolution of lentiviruses, and the risks of interspecific passage leading to the emergence of new pathogens. SRLVs were chosen as a model for the study of inter-species passage of lentiviruses. Indeed, SRLV are able to infect the cells of several species of wild ruminants (ibex, deer, chamois, mouflons, etc.) *in vitro* [1,2] and *in vivo* [3,4].

The genetic variability of SRLVs during inter-species passage can become a clinical and diagnostic problem. The problem seems to be that a limited number of mutations in regions of the genome may, by their consequences on the structure and function of the corresponding proteins, influence the pathogenesis of viruses. The diagnostic problem can be observed insofar as some lentiviruses are able to very quickly generate new variants that can escape the classical techniques used for their detection [5].

Lentivirus transmission has been observed between different species. The transmission is made exclusively after contacts, which allow exchanges of fluids containing infected cells (blood, milk, nasal fluid, seminal fluid [6]. Studies carried out on the genetic evolution of SRLV after their passage, have shown that these viruses are able in their hosts to select positive mutations to the genome, or to generate recombinant genes [7]. SRLVs infecting domestic ruminants are able to pass and naturally infect several wild ruminant species coexisting on the same geographical site [8].

The lentivirus *gag* gene (for antigen group) encodes a polypeptide precursor where protease cleavage results in CA capsid (p24), MA matrix (p17) and NC nucleoprotein (p7) proteins as well as other proteins (small proteins in the case of the HIV-1 virus like the p6 protein). The MA protein is responsible for the association of the *gag* precursor with the membrane of the cell. CA protein forms the capsid. The NC protein forms the viral component that surrounds the RNA molecule.

The objective of this research was: isolate viruses, study the genetic characteristics of viruses and study the genetic evolution over the years of isolated viruses. Indeed, the genetic variability of SRLVs in hybrids (cross products between the wild ibex and the domestic goat) during *inter-species* passage can become a clinical and diagnostic problem. The problem seems to be that a limited number of genetic mutations in regions of the genome can, through their consequences on the structure and function of the corresponding proteins, influence the pathogenesis of viruses. To better understand the mechanisms involved in the natural transmission and genetic evolution of SRLVs, we studied the characteristics and genetic evolution of the *gag* gene for three years after viral infection.

Materials and methods

Animals and blood samples:

This study was carried out in the research center UMR754/INRA/ENVL/ Claude Bernard-Lyon1 university, Lyon-France. Genetic analyzes of the isolated viruses were carried out in spring 2019. Three blood samples, one year apart, were taken from *Hybrid* living in two sites in French Alps. Serological samples were routinely tested for the presence of circulating antibodies against various potential pathogens such as pestivirus, infectious abortive agents and SRLV. SRLV antibodies were evaluated using a commercial ELISA-test based on detection of specific IgG against capsid and transmembrane SRLV-antigens (Pourquier, Montpellier, France) as previously described by Guiguen [3].

Virological testing:

Ten to fifty milliliters of blood from each animal were collected in EDTA vacutainer. PBMC (peripheral blood mononuclear cells) were isolated from blood by centrifugation through Ficoll gradient [9] and then used for cellular culture and DNA extraction. When viable white blood cells could be obtained, infectious virus was sought by simple culture or co-culture with susceptible cell cultures (GSM) as previously described [10]. Supernatants of cultures were tested for infectivity on GSM and macrophages-derived monocyte (MDM). The cultured were monitored for appearance of of syncitia and the supernantant were archived.

SRLV amplification from genomic DNA:

Genomic DNA was extracted from $5x10^6$ PBMC cells using the DNeasy blood and tissue kit (Quiagen, Courtaboeuf, France) according the manufacturer's instructions. DNA concentration and quality were determined spectrophotometrically and stocks were stored at -70°c until use.

Nested PCR was used to detect the 513 nt fragments including the region encoding the *gag* gene. All the primers are numbered according to the sequence of the reference strain CAEV-Co (Genbank accession number M33677) [11]. The primers GEX5 (5′-GAAGTGTTGCTGCGAGAGGTCTTG-3′: positions 393-416) and GEX3 (5′-TGCCTGATCCATGTTAGCT TGTGC-3′: positions 1291-1268) were used for the first amplification (PCR). Primers GIN5 (5′-GATAGAGACA TGGCGAGGCAAGT-3′: positions 524-546) and GIN3 (5′-GAGGCCATGCTGCATTGCTACTGT-3′: positions 1036- 1013) were used for the second amplification (Nested PCR). Reaction conditions

for both rounds of PCR were as described previously by Chebloune [12] using the *Pfu* polymerase (Promega France).

The PCR mix in a final volume of 50 µl consisted 1×Pfu DNA polymerase buffer with $MgSO_4$ (200mM Tris-HCL, 100mM KCL, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1 mg/ml nuclease-free BSA), 200µM dNTP : 1.25u/ of *Pfu* DNA polymerase (Promega, France), 0.1-1.0µM of each primer and 1 µg of DNA. For each amplification, a positive control (cells infected by CAEV-Co) and negative control (ultrapure water) were run in parallel. After amplification, PCR products from each sample were controlled by electrophoresis on 1% agarose gel containing 1 µg/ml ethidium bromide in 1× TAE buffer.

Cloning and sequencing:

Amplicons from independent cloning reactions realized from the independent NestedPCRs were sequenced to assess the genetic characteristics of the *gag* gene. Amplicons were purified (Montage PCR kit-UF7PCR50-Millipore). In order to be efficiently cloned into the pGEM-T® Easy Vector System 1 (A1360-Promega), the blunt-ended *gag* amplicons were modified using a standard A-tailing procedure by a 10 minutes incubation at 72°C in the presence of Taq polymerase and dATP The ligation products were used to transform MAX Efficiency DH5α™ Chemically Competent Cells (Invitrogen, France) The plasmidic DNA of the resulting clones were extracted using the Plasmid mini kit (Qiagen) and controlled by EcoRI digest to identify inserts with the expected size. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer on the "Sequencing technical platform" of IFR128, Lyon-France using GIN5′ and GIN3′ primers in the *gag* gene.

Sequence alignment and phylogenetic analysis:

The different sequences obtained were aligned using ClustalW [13] with reference sequence with manual adjustment where necessary. Trees were constructed by the Neighbor-Joining (NJ) method [14], with bootstraps values determined for 1000 iterations [15]. The trees were modified using the Njplot program [16].

Results:

The Nested PCR technique specifically amplified a 513 nucleotide fragment of the *gag* gene including the 3' portion encoding the template protein (MA) and the first 42 nucleotides encoding the capsid protein (CA).

The sequences alignments shows two types of genetic mutations: the first is a deletion of six nucleotides CAAGAG at position 165-170. The second type of genetic mutations is a simple replacement of one nucleotide by another (Table 1).

The sequences of the *gag* gene obtained from the samples taken on the hybrids in first year showed a diversity comparable to that observed in the reference virus. After one year of cohabitation, the sequences of the virus infecting the hybrids showed very important genetic mutations including a deletion of 6 nucleotides. Finally, after 3 years of cohabitation, the virus sequences included more genetic mutations (Figure 1).

Most of the substitutions were observed in two variable regions, the first located from position 133 to 150 and the second from position 164 to 195. The latter contains the most numerous substitutions, especially for sequences which have the characteristic deletion of 6 nt compared to other sequences that do not have it. In the third year, the proviral sequences have larger substitutions than those observed in the first and second year.

These nucleotide changes can lead to amino acid changes. The amino acid (aa) alignment shows the two types of alterations: amino acid substitution and deletion of two amino acids (Q&E) resulting from the deletion of six nucleotides in the virus sequences over the three years (data not shown).

The percentages of discrepancies between the proviral sequences of the *gag* gene of the three hybrids and that of CAEV-Co were on average from 6.1 to 8.8% in nucleotides (4.2 to 7.6% in aa). Compared with proviral sequences obtained in first year, the average percentages of divergence was 2.6% in nucleotides (1.4 in aa) in second year and 2.9% (2.0% in aa) in third year. The average of the internal divergence percentages was 0.56 - 2.79% (± 0.21 - ± 1.12) (Table 2).

A phylogenetic tree (*Neighbor-Joining*) has been constructed to determine genetic variation over time. The phylogenetic tree has shown four groups, the first includes the virus sequences in the first year, the second includes the viral sequences in the second, the third group includes the sequences in the third year and the fourth group presents the sequence of the reference virus (CAEV- Co) (Figure 2).

Table 1: Replacement of nucleotides: Po.=Position, Nt= Nucleotide, Rep. by= Replacement by.

Mutations of 1st year			
Po.	**Nt**	**Rep. by:**	**Sequences**
121	A	G	All sequences
128	A	C	UHB1.2, UHB1.3
134	T	A	All sequences
142	T	C	All sequences
151	A	G	UHA1.1, UHA1.4
161	G	A	All sequences
179	G	A	All sequences
185	C	G	All sequences
190	G	A	All sequences
Mutations of 2nd year			
Po.	**Nt**	**Rep. by:**	**Sequences**
158	A	G	UHB2.3, UHB2.5
173	A	G	UHB2.3, UHB2.4. UHB2.5, UHB2.6
175	A	G	UHB2.3, UHB2.4. UHB2.5, UHB2.6

178	A	G	All sequences
195	A	C	All sequences
		Mutations of 3rd year	
Po.	**Nt**	**Rep. by:**	**Sequences**
120	A	G	All sequences
134	T	G	All sequences
137	A	G	All sequences
148	A	T	All sequences
154	G	C	All sequences
157	A	G	All sequences
175	**A**	G	All sequences
178	**A**	G	All sequences
182	**A**	C	All sequences
188	**A**	C	All sequences
194	**A**	G	All sequences

Table 2: Gag sequence diversity of isolated viruses from hybrids. Percentage divergence values were obtained by comparisons of nucleotide (plain numbers- upper right part) and deduced amino acid (numbers in bold- lower left part).

	CAEV-Co	1st year	2nd year	3rd year
CAEV-Co	-	6.1	8.2	8.8
1st year	4.2	-	2.6	2.9
2nd year	4.8	1.4	-	2.8
3rd year	7.6	2.0	1.9	-
Internal diversity		0.56 ±0.21	1.84 ±0.16	2.79 ±1.12

```
                130       140       150       160       170       180       190
CAEV-Co AAGATGCAATGGCTACAGTAATCATGAAAGATGGGTTACTGGAACAAGAGGAAAAGAAGGAAGACAAAAGAGAAA 195
UHA1.1  .G...........A.......C.........G.........A..................A.....G....A..... 195
UHA1.2  .G...........A.......C.....................A..................A.....G....A..... 195
UHA1.3  .G...........A.......C............A......A..................A.....G....A..... 195
UHA1.4  .G...........A.......C.........G.........A..................A.....G....A..... 195
UHA2.1  .G...........A.......C.....................A...------.......GA.....G....A....C 189
UHA2.2  .G...........A.......C.....................A...------.......GA.....G....A....C 189
UHA2.3  .G...........A.......C................G..A...------..G.G..GA.....G....A....C 189
UHA2.4  .G...........A.......C.....................A...------..G.G..GA.....G....A....C 189
UHA2.5  .G...........A.......C................G..A...------..G.G..GA.....G....A....C 189
UHA2.6  .G...........A.......C.....................A...------..G.G..GA.....G....A....C 189
UHB1.1  .G...........A.......C.....................A..................A.....G....A..... 195
UHB1.2  .G.....C.....A.....T.C.....................A..................A.....G....A..... 195
UHB1.3  .G.....C.....A.....T.C.....................A..................A.....G..C.A..... 195
UHB1.4  .G...........A.......C.....................A..................AA....G....A..... 195
UHB1.5  .G...........A.......C.....................A..................A.....G....A..... 195
UHB2.1  .G...........A.......C.....................A...------.......GA.....G....A....C 189
UHB2.2  .G...........A.......C.....................A...------.......GA.....G....A....C 189
UHB3.1  GG...........A..G..T.C............G...G..A...------....G..GA..C..G..C.A...GC 189
UHB3.2  GG...........G..G..T.C............G...G..A...------....G..GA..C..G..C.A...GC 189
UHB3.3  GG...........G..G..T.C............G...G..A...------....G..GA..C..G..C.A...GC 189
UHB3.4  GG...........G..G..T.C............G...G..A...------....G..GA..C..G..C.A...GC 189
Consen  **GATGC*ATGGC**C+GT*A*CATGAAAG*TG*GTT*CT*GAA......GA*A:GA+**A*GA*AA*A*AGA*+
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Figure 1: Alignment of nucleotide sequences (fragment) with the sequence of prototype CAEV-Co (Genbank accession number M33677 [11]: (·) homology, (−) deletion, Consen= Consensus.

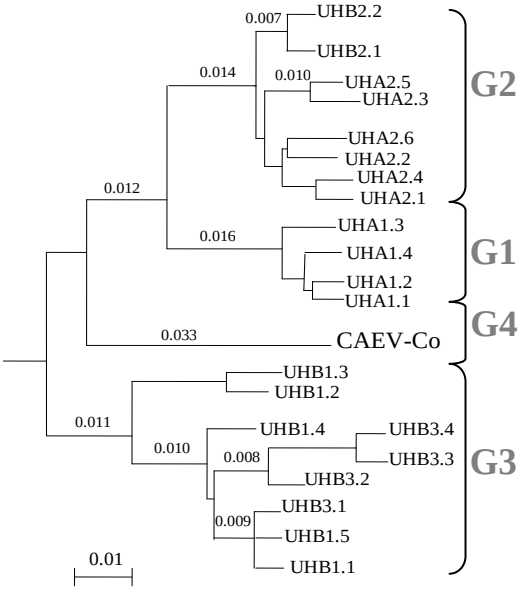

Figure 2: *Neighbor-joining* tree of an approximatively 0.5 kb fragment of the *gag* gene of the isolated proviruses. Horizontal lengths are proportional to the estimated genetic distance between the sequences; the bar marker represents 1% divergence. Bootstrap values derived from 1000 bootstrap replicates are shown on the phylogenetic tree. The isolated viruses form 4 groups: G1=group1, G2=group2 G3=group3 and G4=group4.

Discussion:

Infection and the passage of SRLV are widespread in most countries of the world. The economic consequences are important and their infections deserve consideration in terms of veterinary and human health. We have studied the genetic properties that accompany SRLV adaptation in hybrids. The biological and genetic properties will make it possible to evaluate the correlation between the bio-pathological and genetic properties of the virus following its adaptation in new species [6].

The analyzes carried out consisted in comparing the different sequences obtained (*gag* gene) over time using the CAEV-Co as a reference. In a second step, we compared the viral sequences obtained during the three years. The alignment performed shows a number of characteristic nucleotide substitutions including the deletion of six nucleotides from a year of cohabitation.

The first isolated proviral sequences (first year) are genetically heterogeneous as the reference virus. This heterogeneity may reflect that of the viruses that infected their mothers, a consequence of the classical transmission of SRLVs by milk and colostrums [17]. Recent data support the possibility of transmission of SRLV through genital tract and secretion [18] including the presence of viruses in sperm and genitals [19], and also the transmission of viruses through respiratory pathways and secretions [20].

At the end of one year of cohabitation (second year), the isolated proviral sequences showed strong similarities. The very great homology between the proviral sequences of the isolated *gag* gene suggests that SRLV has been passed, this homology between the isolated sequences supposes in this case that only viruses with these common characteristics would be able to infect the hybrids effectively [21]. The sequences of the *gag* gene obtained from the samples taken in the second year showed quite a large diversity comparable to that observed in the first year. Indeed, these genetic modifications in the viral genome are essential to be able to adapt in these new species. Finally, after 3 years of cohabitation, the virus sequences had very high genetic mutations. these modifications would correspond to a selection of genotypes that have a selective advantage of replication in the cells of these new species [22]. Indeed, a deletion of six nucleotides has also been observed in MVV [23]. Recently, SRLVs were classified, according to their viral sequences, into four main groups from A to D which differ from 25 to 37% for the *gag* and *pol* genes. Groups A and B are further divided into different subtypes which are 15-27% different from each other. Group A includes seven subtypes (A1 to A7) and group B includes two subtypes (B1 and B2) [24].

Conclusion:

Genetic analyzes of the gag gene in hybrids have shown an increase in the variability of the sequences over time, notably with the appearance of a deletion of six nucleotides after one year of cohabitation. The first proviral sequences of the hybrids are heterogeneous. After one year of cohabitation, the proviral sequences of the hybrids showed strong similarities with those of the hybrid in the first year, suggesting possible transmission of the virus. This possibility is consistent with the heterogeneity of the viral sequences after 2 years of cohabitation. The very high homology between the proviral sequences of the gag gene suggests that there has been passage of SRLVs accompanied by genetic evolution for the virus to adapt to the new host.

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