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Original article

RT-PCR detection of lentiviruses in milk or mammary secretions of sheep or goats from infected flocks

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Summary — In this study we evaluated a reverse transcriptase polymerase chain reaction (RT-PCR) technique for detecting lentiviral infection in milk or mammary secretions from small ruminants. Initial observations on seven goats infected with cloned caprine arthritis-encephalitis virus (CAEV) showed that RT-PCR on milk cells is as reliable as coculture for detecting viral infection, and is quicker and simpler. With a suitable choice of redundant primers followed by a semi-nested amplification, it proved possible to detect the virus in milk samples from naturally infected French sheep (8/8) or goats (9/9), and viral sub-groups could be identified by hybridization with discriminatory probes. All seropositive animals gave positive amplifications, as did one seronegative goat from a contaminated herd, suggesting greater sensitivity for RT-PCR. None of eight goats from a long-established seronegative herd ever gave a positive RT-PCR amplification. This technique provides a simple means for rapidly identifying potentially infectious animals and for epidemiological investigations, as long as the primers are selected according to the genetic structure of the local viral population.

retrovirus / small ruminants / RT-PCR / coculture / serology

Résumé — Détection de lentivirus dans le lait de chèvre ou de brebis par amplification en chaîne par polymérase après rétrotranscription. Nous avons testé une technique d'ampli-

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fication en chaîne par polymérase après rétrotranscription (RT-PCR), pour rechercher dans le lait ou les sécrétions mammaires la présence des lentivirus des petits ruminants. Une analyse préliminaire effectuée sur sept chèvres infectées par le clone CAEV Cork, a permis de montrer que la recherche de virus sur les cellules de lait par RT-PCR est aussi fiable que la détection par coculture, tout en étant plus simple et plus rapide. Utilisant des couples d'amorces adaptés et deux amplifications successives, cette méthode nous a permis de détecter le virus dans des échantillons de lait de brebis (8/8) et de chèvres (9/9), infectées naturellement. De plus, nous avons identifié différents sous-groupes viraux par hybridation avec des sondes spécifiques. Tous les animaux séropositifs ont donné des amplifications positives, de même qu'une chèvre séronégative issue d'un troupeau contaminé, ce qui suggère une sensibilité plus grande de la méthode de détection par RT-PCR. Aucune des huit chèvres d'un troupeau séronégatif de longue date n'a donné d'amplification positive. Cette technique apporte un moyen simple pour l'identification rapide d'animaux infectés et pour des recherches épidémiologiques, pour autant que les amorces soient sélectionnées en relation avec la structure génétique de la population virale locale.

rétrovirus / petit ruminant / RT-PCR / coculture / sérologie

INTRODUCTION

Lentiviruses are non-oncogenic retroviruses which cause chronic inflammatory and degenerative diseases in several mammalian species. Goats and sheep harbour related small ruminant lentiviruses (SRLV) which typically cause respiratory distress, mammary induration and, rarely, neurological disease in infected sheep, while adult goats generally present arthritis of the carpal, or more rarely the tarsal joints. These infections may have adverse economic consequences (Smith and Cutlip, 1988), and are common in French flocks. The viruses replicate principally in cells of the macrophage / monocyte lineage found in the lungs, bone marrow (Gendelman et al, 1985a), peripheral blood (Gorrell et al, 1992), brain (Georgsson et al, 1989), synovium (Gendelman et al, 1985b) and mammary glands (Lerondelle et al, 1989). Infection occurs mainly from dam to offspring through ingestion of infectious colostrum or milk (Kennedy-Stoskopf et al, 1985; Rowe et al, 1992a, b). Diagnosis of SRLV infection in animals relies on serological testing or viral detection by coculture. Serological testing appears to underestimate the incidence of infection, particularly in young animals which may seroconvert tardily (Johnson et al, 1992).

The virus may be detected by coculturing permissive cells with cell samples, preferably using peripheral blood mononuclear cells, broncho-alveolar lavage cells or bone marrow cells (Brodie et al, 1995) from the possibly infected animals. Coculture is however tedious, time-consuming and costly, and a method for the direct detection of virus in biological samples would be useful for the rapid diagnosis of SRLV infection. We developed a reverse transcriptase-polymerase chain reaction (RT-PCR) technique for the detection of the virus in cells from milk or mammary secretions because these samples are easily obtained by non-invasive procedures, and reflect the major route of natural infection.

MATERIALS AND METHODS

Animals

Eight multiparous 4 to 5-year-old Saanen goats obtained from a consistently seronegative herd with no clinical history of arthritis were used as negative controls. Seven seronegative multiparous 4 to 5-year-old Saanen goats from a separate herd were experimentally infected with the Cork strain caprine arthritis-encephalitis virus (CAEVCo) by instilling of in vitro infected

allogeneic monocytes into the milk canal 3 times at 3–5 day intervals (Lerondelle et al, 1995). None of these animals developed clinical symptoms during the experimental period. Milk or mammary secretions were also obtained from nine naturally infected primiparous Alpine breed goats (2 years old) with no clinical symptoms, and from eight Lacaune breed milking sheep (2 to 7-year-old), among which four showed signs of arthritis. These two groups of animals originated from seropositive commercial herds. The mammary secretions were obtained from three Alpine goats after the period of lactation.

Serology

Serum antibodies to SRLV were evaluated for each animal by the agar gel immunodiffusion test (AGID) (using the kit commercialized by Institut Pourquier, Montpellier, France), and by ELISA (Chekit CAEV/MVV, Behring, France).

Detection of the infectious virus

Milk cells from the experimentally infected animals were checked for the presence of infectious virus by coculture at weekly intervals as described previously (Lerondelle et al., 1995). Briefly, cells from de-fatted milk were cultured with caprine fibroblasts and observed for the appearance of syncytia over 10 days. Supernatants from syncytium-positive cultures were tested for retroviral reverse transcriptase activity (Lyon and Huppert, 1983).

RT-PCR detection of viral sequences

Cells were prepared from 30 mL milk samples taken 1–2 weeks after parturition, or from 10 mL of mammary secretions in the case of the three non-lactating goats. The experimentally infected goats were sampled at 9, 10 and 11 weeks post infection; one animal was further

sampled until 21st week post-infection (at weeks 12 to 17, 19 and 21). Total RNA was recovered from the cells as described in Leroux et al (1995a), dissolved in 50 μ L diethyl pyrocarbonate-treated water, and 3 μ L were reverse transcribed with 0.1 μ g random hexamers, 20 units of RNase inhibitor (Rnasin inhibitor, Promega, France) and 50 units of Moloney virus reverse transcriptase in a final volume of 20 μ L containing 5 mM $MgCl_2$, 0.5 mM each deoxynucleotide, 10 mM Tris HCl pH 8.8, 50 mM KCl and 0.1% triton X100. The mixture was incubated for 10 min at 20 °C, 15 min at 42 °C, then denatured for 5 min at 99 °C, followed by 5 min at 4 °C. PCR reactions used 5 μ L of this cDNA, 20 pmol of each primer (see below), and 1.5 units Taq polymerase (Eurobio, France) in 50 μ L (1.5 mM $MgCl_2$, 40 μ M each deoxynucleotide triphosphate, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8.8 and 0.1% Tween 20). The preservation and retrotranscription of cellular mRNA was controlled by amplifying a 332 bp fragment of a housekeeping gene, ovine glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Leroux unpublished data, Genbank accession number U39091). Both the ovine and caprine samples produced clear signals after 35 rounds of amplification (30 s each at 95 °C, 55 °C and 72 °C successively) using the primers OG1 (sense), 5'-CCAGAA-CATCATCCCTGC-3', and OG2 (antisense), 5'-CCAGGAAATGAGCTTGAC-3'. CAEVC_o was detected in the experimentally-infected animals using primers CPS519 (sense), 5'-ACA-GAAGAGAAATTTAAAGG-3', and CPAS994 (antisense), 5'-CATC-CATATATATGCCAAATTG-3', corresponding to nts 2236–2256 and 2710–2688 in the *pol* gene of the CAEVC_o genomic sequence (Saltarelli et al, 1990). A 475 bp fragment was amplified after 35 cycles of 1 min each at 95 °C, 50 °C and 72 °C successively. To allow for genetic variation in the viruses taken from the naturally infected animals we used the corresponding degenerate *pol* gene primers P1 (5'-DSAAGARAAATTARARGG-3') and P2 (5'-ATCATCCATRTATATBCCAAATTG-3'), where B = C, G or T, D = A, G or T, R = A or

G, and S = C or G, previously found to amplify many natural samples (Leroux et al, 1995b), for 35 cycles of 1 min each at 95 °C, 45 °C and 72 °C successively. This was followed by semi-nested amplification replacing the sense (P1) primer with P3 (5'-GATTTAACAGAG-GCACA-3'), selected by comparing several natural French isolates. After a further 35 cycles for 30 s each at 95 °C, 50 °C and 72 °C, both the ovine and caprine samples generated the expected 303 bp fragment.

Southern blot hybridization

PCR products were separated by electrophoresis through 1.5% agarose gels containing ethidium bromide and vacuum transferred to nylon membranes (Hybond N, Amersham, France). Probes were prepared from two wild type French SRLV isolates (Leroux et al, 1995b: 685, 253 bp long, and 676, 160 bp long) and from CAEVCo (221 bp long), which were chosen to represent three major genetic subtypes of SRLV present in France. After labelling with α P³² CTP using a DNA labelling kit (Multiprime, Amersham), the probes were separately hybridized to the transferred fragments as previously described (Mornex et al, 1986).

Sequencing

Several of the amplified products were cloned into a TA-vector (InVitrogen, Abingdon, UK) and sequenced using the Sequenase version 2.0 DNA sequencing kit (US Biochemical, Amersham).

RESULTS

Serology

All 15 Saanen goats initially tested were antibody negative by both AGID and ELISA. The seven animals that were experimentally infected with CAEVCo all seroconverted between 2 and 4 months post-infection by AGID, and were confirmed positive by ELISA. All eight naturally infected Lacaune sheep and eight out of nine Alpine goats from an infected herd had positive AGID tests (table 1). One goat remained negative by AGID and ELISA.

Detection of infectious virus in coculture

All the goats experimentally infected with the cloned viral strain CAEV Co were negative,

Table 1. Lentivirus detection in infected small ruminants.

Animals (No)	Clinical signs ^a	Lentiviral infection		
		Serology ^{b, c}	Coculture ^d	RT-PCR ^d
Uninfected goats (8)	0/8	0/8	nd	0/8
Experimentally infected goats (7) ^e				
Before inoculation	0/7	0/7	0/7	0/7
After inoculation	0/7	7/7	27/28 ^f	28/28 ^f
Natural infection				
Goats (9)	0/9	8/9	nd	9/9
Ewes (8)	4/8	8/8	nd	8/8

^a Arthritis ; ^b AGID and ELISA tests; ^c number of positive animals/number of tested animals; ^d number of positive samples/number of tested samples; ^e with the CAEVCo strain; ^f seven animals were sampled at 9, 10 and 11 weeks post-infection and one animal also at weeks 12, 13, 14, 15, 16, 17, 19 and 21. nd: not done.

by coculture of cells from defatted milk with caprine fibroblasts, before infection (table I). The seven animals showed the presence of virus in all three weekly samples from the 9th to the 11th week after infection. In the goat sampled until 21st week post-infection, infectious virus particles were detected in nine of the ten cocultures. All the cocultures presenting cytopathic effect were positive for reverse transcriptase activity in the supernatant.

Detection of viral genome by RT-PCR

All the samples obtained from 9th to 21st week were positive by RT-PCR (table I). We then attempted detection of viral transcripts by RT-PCR of RNA from milk cells of goats and sheep from flocks with a high incidence of natural infection. Using a semi-nested amplification technique with degenerate primers in the *pol* region for the first amplification, and a consensus upstream primer based on French SRLV sequences for the second, none of the eight negative control goats produced a positive signal despite the presence of copious and well-preserved cellular mRNA, as attested by strong amplifi-

cation of the G3PDH housekeeping sequence. All of the nine goats and eight sheep from naturally infected flocks gave distinct positive signals (fig 1). The specificity of the amplified sequences was confirmed by their hybridization with at least one of the three subgroup probes (fig 2), and in several cases by nucleotide sequencing. The sequences obtained from both sheep and goats were consistent with known SRLV sequences. Pairwise comparisons indicated a mean homology of 87.9% (78.9–98.7%) in nucleotide and 93.8% (83.1–100%) in amino acid between the French isolates.

DISCUSSION

RT-PCR detection of SRLV infections in the field is both quicker and more convenient than the classical procedure of coculture. Under experimental conditions, we demonstrated that there is an excellent correspondence between the results from the two techniques, and that RT-PCR is no less sensitive than coculture. However, there may be concern that natural variations in the lentiviral sequences, which also occurs in SRLVs (Leroux et al, 1995b), could limit the applicability

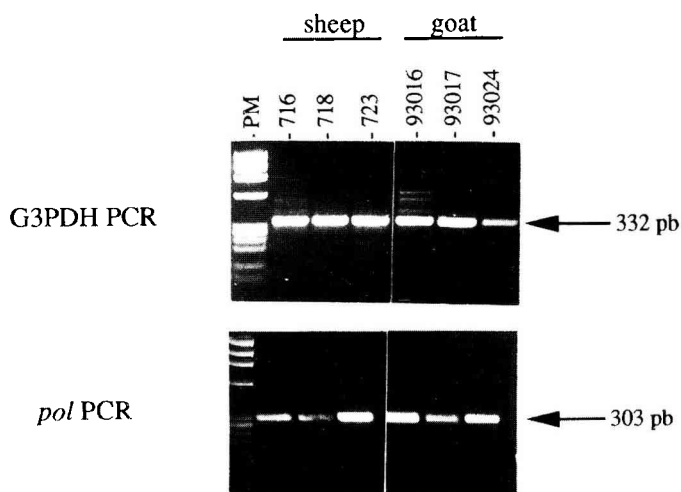


Fig 1. RT-PCR detection of SRLV- and G3PDH RNAs in milk cells. Results typical of the positive reactions are shown for three ewes (716, 718, 723) and three goats (93016, 93017, 93024). Molecular weight marker: FX174 HaeIII digest.

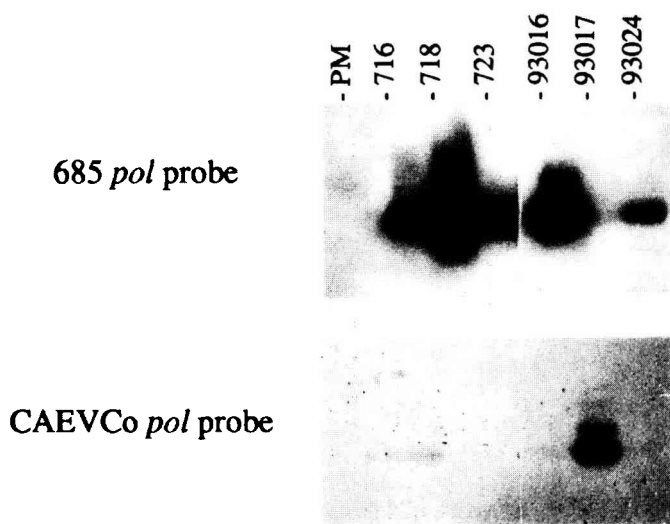


Fig 2. Autoradiograph of Southern blot hybridization of pol PCR products obtained in figure 1 with a 685 probe (upper panel) or a CAEVCo probe (lower panel).

of the technique under field conditions. We show here that the use of a pair of redundant primers in the *pol* region, followed by semi-nested amplification with a primer chosen by consensus among local sequences, can provide consistent positive results from unselected naturally infected sheep and goats. These primers allowed a higher efficiency of amplification from milk cells or mammary secretion cells than that obtained in a previous study (Barlough et al, 1994). Under these conditions we obtained positive signals from all 17 naturally infected and seven experimentally infected animals. In addition, one seronegative goat from a contaminated herd gave a positive signal, suggesting that viral expression in milk cells may precede seroconversion as claimed elsewhere (Lerondelle et al, 1995). The nucleotide sequence of the fragment amplified from this animal is typical of homologous SRLV sequences (not shown). Although this study is preliminary and concerns a small number of animals, RT-PCR from milk samples provides a rapid and efficient method for the diagnosis of lentivirus in the context of both experimental and natural infection of sheep and goats.

Using the RT-PCR approach to detect natural SRLV infections may prove useful in situations where rapid identification of infected animals is desirable. In addition, Brodie et al (1995) showed a correlation between expression of viral proteins and genomic RNA in macrophages in target tissues, so that the presence of RNA transcripts detected by RT-PCR may be taken as a strong indication of the presence of infectious virus. Milk is a major natural transmission vehicle for SRLVs, and like mammary secretions from non-lactating animals, provides a suitable source of infected cells. We demonstrated here that with an adequate choice of primers, RT-PCR can give simple and reliable detection of lentiviral contamination in an animal despite the variability of SRLV genomes. The amplified fragments may be discriminated by Southern hybridization with specific sub-group probes, providing a means for the epidemiological tracing of viral spread in both sheep and goats. In view of the geographical differences in SRLV genomic sequences, we suggest that primers should be selected with reference to local viral populations, and that negative tests in animals of foreign origin be treated with caution.

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