Development and comparison of strain specific gag and pol real-time PCR assays for the detection of Visna/maedi virus

Maria Luisa Carrozza a*, Maurizio Mazzei b, Patrizia Bandecchi b, Christophe Fraisier c, Marta Pérez d, Marie Suzan-Monti c, Damian de Andrés e, Beatriz Amorena e, Sergio Rosati f, Valgerdur Andrésdottir g, Lluis Lujan d, Michel Pepin h, Barbara Blacklaws i, Francesco Tolari b, Gordon D. Harkiss j

*Corresponding author contact information, Tel.: +39 050 2216964; fax: +39 050 2216941.

a Scuola Normale Superiore, Piazza dei Cavalieri 7, 56126 Pisa, Italy
b University of Pisa, Dipartimento Di Patologia Animale, Viale delle Piagge 2, 56124 Pisa, Italy
c Unite des Rickettsies, CNRS UMR 6020, IFR 48, Faculte de Medecine, Universite de la Mediterranee, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France
d Universidad de Zaragoza, Departamento de Patologia Animal, Facultad de Veterinaria, Miguel Servet 177, 50013 Zaragoza, Spain
e Instituto de Agrobiotecnologıa CSIC-Universidad Pública de Navarra-Gobierno de Navarra, Car. de Mutilva Baja s/n, 31192 Mutilva Baja, Navarra, Spain
f Universita di Torino, Dipartimento di Produzioni Animali Epidemiologia ed Ecologia, Via L. Da Vinci 44, 10044 Grugliasco, Torino, Italy
g Institute for Experimental Pathology, University of Iceland, Keldur, IS-112 Reykjavík, Iceland
h AFSSA-LERPAZ, 22 Avenue du Général de Gaulle, 94706 Maison Alfort, France
i University of Cambridge, Department of Veterinary Medicine, Madingley Road, Cambridge CB3 0ES, United Kingdom
j University of Edinburgh, Division of Veterinary Pathology, Easter Bush Veterinary Centre, Easter Bush, Midlothian EH25 9RG, United Kingdom

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Abstract

The aim of this study was the development of gag and pol dual labelled probe real-time PCR and RT PCR assays to quantify the proviral load and the transcripts of the British Visna/maedi virus EV1 strain. Primers and probes were chosen based on the consensus sequences of gag and pol clones representative of EV1 genetic variants. Both PCRs had a detection limit of 3 copies of target gene, with a linearity over 6 orders of magnitude. The performances of the two PCRs in vivo were evaluated and compared on a panel of DNAs extracted from blood of sheep infected experimentally with EV1. The pol assay detected in most cases lower numbers of viral molecules than gag assay, yielding some false negative results. The gag real-time RT PCR had a detection limit of 100 RNA molecules with a linearity over 5 orders of magnitude. This did not result in a lower performance of the RT PCR compared to the PCR in cells permissive for virus replication, which contain higher numbers of viral transcripts than proviral genomes. The real-time assays developed in this study, particularly the gag assay, provide a sensitive tool which can be used to quantify the viral load in experimental infections.

Keywords Lentivirus; Visna/maedi virus; EV1; Real-time PCR; Proviral load; Viral transcript

1. Introduction

The ovine LentivirusVisna/maedi virus (VMV) is the causative agent of a chronic inflammatory disease of sheep affecting primarily lungs, central nervous system, mammary glands and joints. VMV was the first lentivirus to be isolated (Gudnadottir and Palsson, 1967 and Sigurdsson, 1954). Since then, several other members of this genus of the family Retroviridae have been identified, including caprine arthritis encephalitis virus (CAEV), equine infectious anemia virus (EIAV) and human, simian, feline and bovine immunodeficiency viruses (HIV, SIV, FIV and BIV respectively). VMV has long been considered the prototype lentivirus (Carey and Dalziel, 1993 and Narayan and Clements, 1989) and has been widely employed to study many aspects of lentiviral infections, such as the molecular mechanisms underlying viral tropism (Agnarsdóttir et al., 2000, Barros et al., 2005, Chebloune et al., 1996 and Óskarsson et al., 2007), persistence and pathogenesis (Torsteinsdottir et al., 2007), routes of viral transmission (Blacklaws et al., 2004) and strategies to eradicate or prevent the infection (de Andres et al., 2005 and Reina et al., 2009). The British VMV strain EV1 was isolated from a sheep displaying symptoms of arthritis and pneumonia and its complete genome was cloned and sequenced (Sargan et al., 1991). This strain has been and is currently used for in vivo and in vitro studies focusing on diverse topics such as the identification of viral promoter elements, or cytokines that control the expression and replication of the virus (Sargan et al., 1995, Sutton et al., 1997 and Zhang et al., 2002), the dissection of the initial steps of the infection (Bird et al., 1993, Blacklaws et al., 1995 and Ryan et al., 2000), the characterization of the immune response (Bird et al., 1993 and Singh et al., 2006), the routes of viral entry and dissemination (Blacklaws et al., 1995, McNeilly et al., 2007 and Niesalla et al., 2008) and the development of immunization strategies (Gonzalez et al., 2005, Niesalla et al., 2009 and Reina et al., 2008). In some of these studies the presence of the EV1 genome has been investigated by conventional PCR (Bird et al., 1993, Niesalla et al., 2008 and Ryan et al., 2000) or by quantitative competitive PCR (QC PCR) (Zhang et al., 2000). The aim of this work was the development of a highly sensitive dual labelled probe real-time PCR for the absolute quantitation of EV1 viral load in vivo and in vitro. Two viral genes, gag and pol, were chosen as targets for amplification and DNA and RNA based real-time PCR assays were developed and evaluated.

2. Materials and methods

2.1. Viral strain

A low passage viral stock of VMV strain EV1 (5th passage, 3.6 × 106 TCID50/ml) was grown and titred as described previously (Bird et al., 1993 and Sargan et al., 1991).

2.2. Cloning and sequencing of EV1 gag and pol gene fragments

Primary lung fibroblasts were infected with EV1 at a m.o.i. of 0.4 TCID50/cell. Two days later genomic DNA was extracted (DNeasy Tissue Kit, Qiagen, Hilden, Germany) and the presence of EV1 provirus was confirmed by nested pol PCR (Zhang et al., 2000).

A 567 bp gag and a 492 bp pol fragment were amplified with proof reading DNA polymerase (Proofstart DNA Polymerase, Qiagen, Hilden, Germany). The primers (Table 1) were designed on the published EV1 sequence (Sargan et al., 1991) using Primer 3 (Rozen and Skaletsky, 2000). Amplicons were A tailed (A Addition Kit, Qiagen, Hilden, Germany) and cloned in pDRIVE cloning vector (PCR Cloning Kit, Qiagen, Hilden, Germany). Eleven gag and eleven pol plasmid clones were sequenced on both strands (Thermo

Sequenase Cy5 Dye Terminator Cycle Sequencing kit, Amersham Biosciences, Little Chalfont, UK). Consensus sequences (Fig. 1A and B) were generated with Bioedit Sequence Alignment Editor (Hall, 1999).

Table 1.

EV1 *gag* and pol specific primers and probes.

Primer/probe	Sequence (5′ → 3′)	Nucleotide position	Amplicon lenght bp
EV1 gag F	GGAGAAGTTGGAAGGCGGTAG	963–983	567
EV1 gag R	TTCCCAAGACTCTGTCCATTTGC	1529–1507	
EV1 pol F	AGGAGATAGAGCGTTACAAAG	3002–3022	492
EV1 pol R	CCTAAACTCCCTATTCCATTC	3493–3473	
GAG real-time F	TCAACAGGCATCACAGGCTAATA	1249–1271	106
GAG real-time R	GTTACCTGGCCTATGCGACAT	1354–1334	
GAG real-time R probe	*6-FAM*-ACCGCTCTCAAGGCTGTTATGACCCA-*BHQ1a*	1326–1301	
POL LUX F	*GACCAC*-CGATGGAAGAGAAGAAAT-*GTGG5C*	3403–3420	77
POL LUX R	CCATTCTTCTTGCCACCATCAGTA	3479–3456	
POL real-time F	AGATTGGGGAAATAAAGCAATAGAAT	3134–3159	
POL real-time R	TTATTACCTCTTGTGTAAGCTTTTGT	3279–3254	146
POL real-time R probe	*6-FAM*-CGCTTTAATGATCTGCTGTGCTTGAC-*BHQ1a*	3251–3226	

Nucleotide positions refer to the VMV EV1 sequence published by Sargan et al. (1991) (GenBank accession no. S51392).

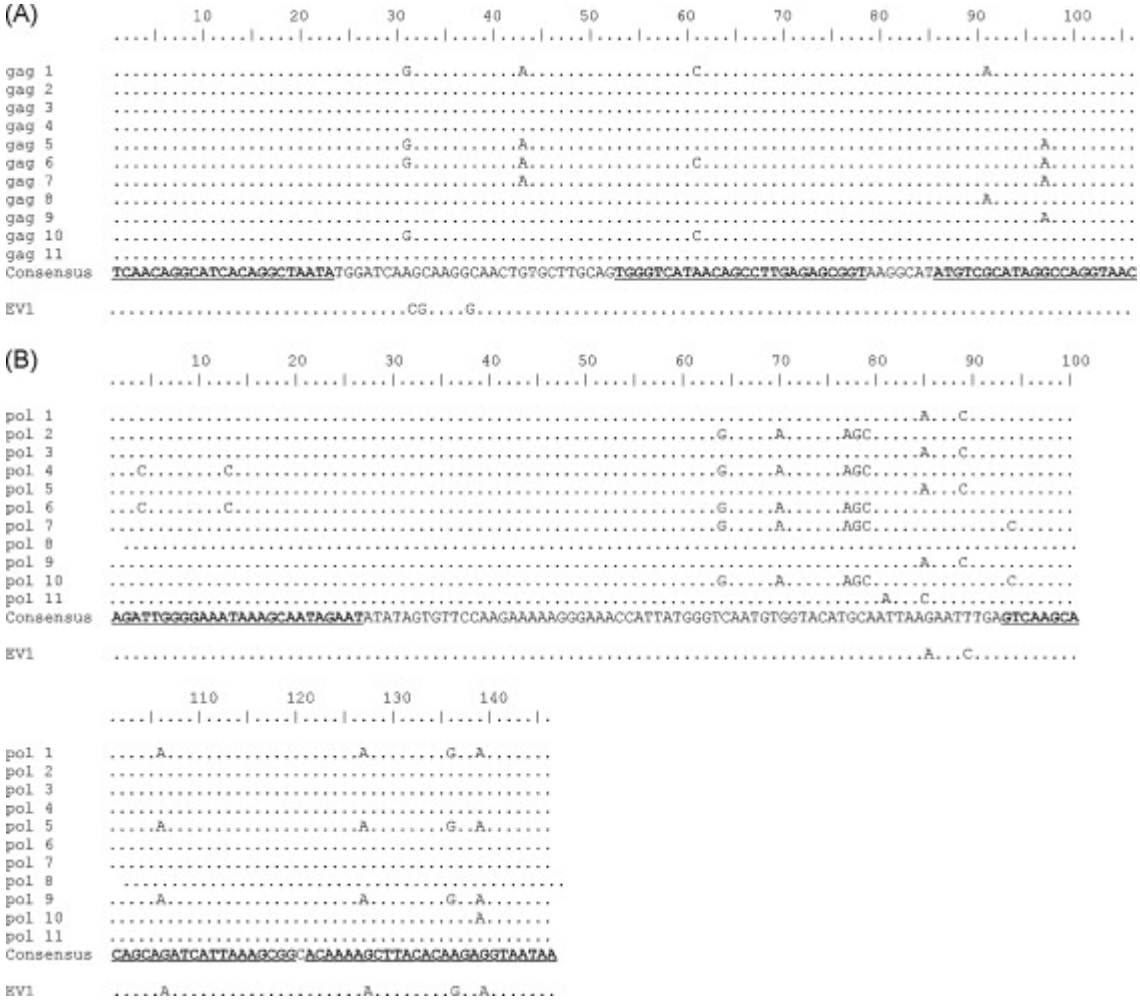

Fig. 1.

EV1 real-time amplicon sequences: A: gag amplicon; B: pol amplicon. Alignment of 11 clones with consensus and with EV1 sequence (GenBank accession no. S51392). Underlined bold indicates real-time primers and probe positions. Conserved nucleotides are depicted as dots, nucleotides that differ from the consensus are shown.

2.3. Primers and dual labelled probes

Beacon Designer software v2.0 (Premier Biosoft International, Palo Alto, CA) was used to design several primer and probe sets within the consensus sequence of gag and pol clones. Primer and probe combinations to be used in real-time assays were further selected based on the homology with variant sequences (Table 1, Fig. 1A and B). The 5' and 3' end of the probes (Operon, Ebersberg, Germany) were labeled with reporter 6 Carboxyfluorescein (6 FAM) and quencher Black Hole Quencher 1a (BHQ 1a) respectively. The ability of the selected primers to amplify viral DNA was evaluated by conventional PCR on plasmid clones representative of consensus and variant viral sequences.

2.4. DNA standards

Serial dilutions of gag and pol consensus clones (clone 3 for both genes, Fig. 1A and B), corresponding to 1 × 106, 1 × 104, 1 × 103, 1 × 102, 60, 30, 10, 6, and 3 molecules per reaction were used to generate standard curves. To determine whether the presence of background nucleic acid would affect assay performance, two series of reactions were repeatedly carried out in parallel: in one, pure plasmid DNA dilutions were used as template, in the other, plasmid DNA was spiked with 500 ng of VMV free ovine genomic DNA to mimic the biological samples.

2.5. Gag real-time PCR assay

The assay was set up using dilutions of consensus plasmid clone 3 (Fig. 1A) as template. Plasmid was linearized, the DNA concentration was measured with PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA), the number of molecules per microliter was calculated and the dilution series (2 × 105 to 1 molecule/µl) prepared accordingly. The real-time reactions were carried out with the Quantitect Probe PCR Kit (Qiagen, Hilden, Germany), using an iCycler Real-Time PCR Detection System (Biorad, Hercules, CA). All reactions were carried out in triplicate, in a volume of 25 µl, with 5 µl of template. Primers and probe were used at a concentration of 1 µM and 0.2 µM respectively. The amplification programme was: 15 min at 95 °C, followed by 50 cycles of 15 s at 94 °C and 1 min at 58 °C. No template reactions and DNA from VMV free ovine lung fibroblast cell culture were used as negative controls.

2.5.1. Gag assay performance on viral variants

The assay was performed using as template a dilution series of plasmids carrying either consensus (clones 3 and 11) or variant (clones 1 and 6) primer and probe sequences (Fig. 1A). Dilutions and reactions were carried out as described above.

2.5.2. Gag real-time RT PCR assay

A one step assay was developed for the absolute quantitation of viral RNA, with the same gag primers and probe used for the DNA based PCR. The assay was performed in triplicate with the Quantitect Probe RT PCR Kit (Qiagen, Hilden, Germany), with 1 µl of template in a volume of 20 µl. Primers and probe were used at a concentration of 1.0 µM and 0.2 µM respectively. The amplification programme was: 30 min at 50 °C for reverse transcription, followed by 15 min at 95 °C then 50 cycles of 15 s at 94 °C and 1 min at 58 °C.

2.5.3. Gag RNA standard

Gag plasmid clone 3 was linearized downstream of the insert with BamHI and used as template in a transcription reaction driven by the SP6 promoter of pDRIVE (MAXIscript SP6/T7 In Vitro Transcription Kit, Ambion, Austin, TX, USA). Following plasmid DNA removal by RNase free DNase treatment (RNase Free DNase Set, Qiagen, Hilden, Germany), the transcript was purified and concentrated with the RNeasy Minelute Cleanup Kit (Qiagen, Hilden, Germany) and quantified with the RiboGreen RNA Quantitation Reagent and Kit (Invitrogen, Carlsbad, CA). The number of RNA molecules/µl was calculated and a dilution series was prepared (1 × 106 to 10 copies/µl).

2.5.4. Gag real-time vs. GAG real-time RT PCR assay performance

DNA and RNA were extracted (Dneasy Tissue Kit and RNeasy Mini Kit, Qiagen, Hilden, Germany) from aliquots of the same EV1 infected ovine fibroblast cell culture 2 days post-infection (p.i.) (m.o.i. of 0.4 TCID50/cell). Following fluorometric quantitation, serially diluted DNA (140 ng/l to 0.14 pg/l) and RNA (100 ng/l to 0.1 pg/l) were amplified by real-time PCR and real-time RT PCR respectively. Each sample was tested in triplicate. The results were expressed as viral copy numbers per microgram of DNA and RNA respectively.

2.6. Pol LUX PCR assay

An assay was developed with fluorogenic LUX primers (Invitrogen, Carlsbad, CA), selected with LUX Designer software (Invitrogen, Carlsbad, CA) within the consensus sequence of pol clones. Primer sequences are shown in Table 1. Reactions were carried out in triplicate on serial dilutions (2 × 105 to 1 molecule/µl) of pol consensus clone 3, with 5 µl of template in 25 µl reaction volume (Platinum

Quantitative PCR SuperMix UDG, Invitrogen, Carlsbad, CA). The amplification programme was: 2 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C.

2.7. Pol dual labelled probe real-time PCR assay

The dual labelled probe real-time assay was set up as described for gag, except that the annealing temperature was 56 °C.

2.8. Evaluation of specificity and sensitivity of gag and pol dual labelled probe PCR assays: analysis of blood samples

Both dual labelled probe real-time PCR assays were used to analyze a panel of 204 DNAs extracted from peripheral blood mononuclear cells (PBMCs) of 68 sheep infected experimentally with the EV1 viral stock described above, sampled at increasing time intervals until 3 months p.i. Previous identification of sheep infected successfully was based on the measurement of anti-VMV antibodies in serum, T cell proliferation and anti-VMV T cell reactivity and determination of histopathological changes in lungs and mediastinal lymph nodes. A total of 222 DNAs from PBMCs of 74 uninfected sheep were used as negative controls to determine the specificity of the real-time assays (Niesalla et al., 2009).

3. Results

3.1. Gag real-time primers and probe

The consensus sequence of 11 gag clones amplified from fibroblasts infected with EV1 viral stock shared 94% identity with the published EV1 sequence. Real-time primers and probe (Table 1) were designed in regions that displayed limited interclonal sequence heterogeneity (Fig. 1A): two clones carried a G to A transition at ntd 1339 and four at ntd 1345, in both cases within the reverse primer. Three clones carried an A to C transversion at ntd 1309, within the probe sequence. Amplicon length was 106 bp. The selected primers were able to amplify by conventional PCR plasmid clones carrying the variant viral sequences identified within the EV1 stock (data not shown).

3.1.1. Linear range of amplification of gag real-time PCR assay

The linear range of amplification was determined over 1 × 106 to 3 copies per reaction using consensus plasmid clone 3 dilutions. The amplification of the standard dilutions showed linearity over six orders of magnitude (Fig. 2A). The assay was able to amplify at least one replica sample of one of the low copy standards (6 and 3 copies per reaction). Assay performance was not modified by spiking plasmid dilutions with ovine DNA (data not shown).

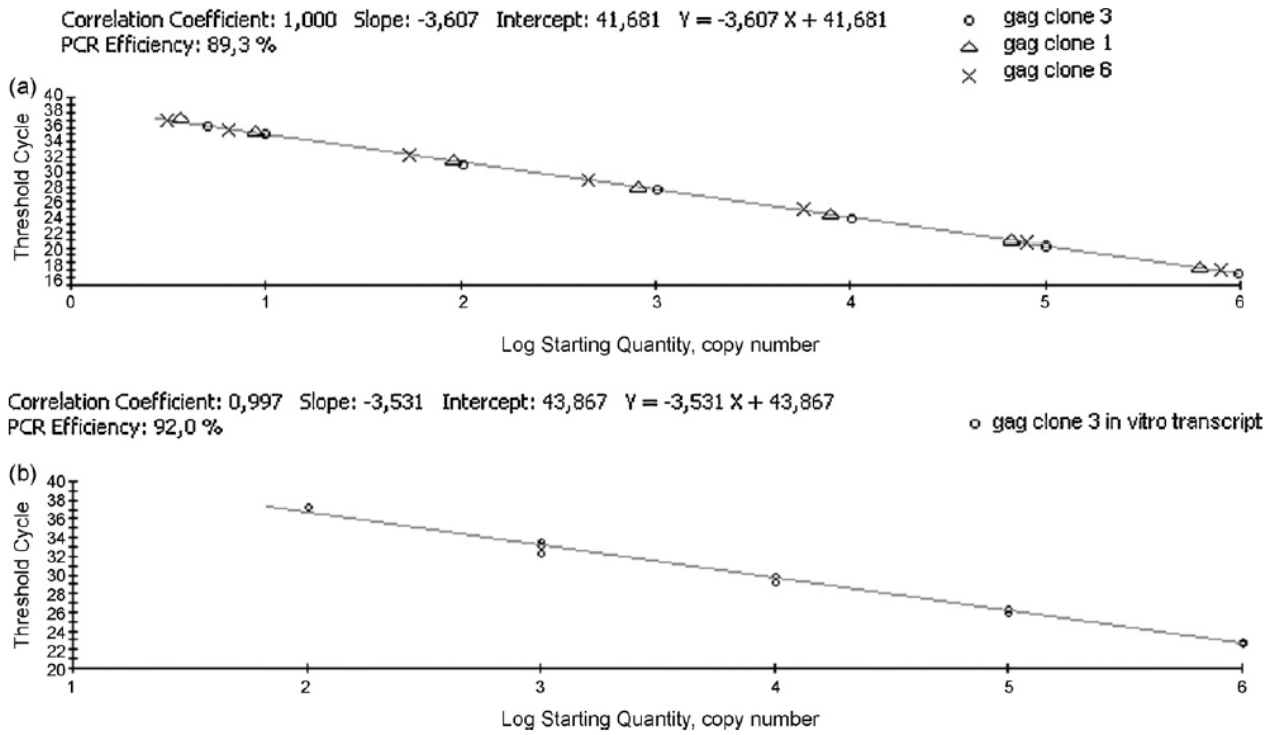

Fig. 2.

Linearity of the gag real-time PCR assay and RT PCR assay. (A) PCR assay standard curve: the standard curve was derived from the logarithmic input template quantity (copies per reaction) of consensus clone 3 (○) vs. the measured threshold cycle. Correlation coefficient of the curve 1.000, slope −3.607. The position of the Ct values for variant clone 1 (△) and 6 (×) are shown. The input template copy number of clone 1 and 6 were the same as those of clone 3. (B) RT PCR assay standard curve: the standard curve was derived from the logarithmic input template quantity (copies per reaction) of consensus clone 3 in vitro transcript vs. the measured threshold cycle. Correlation coefficient of the curve 0.997, slope −3.531.

3.1.2. Gag real-time PCR assay performance on variant clones

The ability of the real-time assay to detect variants within the viral stock was tested using serial dilutions of plasmids carrying either consensus or variant primer and probe sequences. The complete dilution series of all clones were amplified, with consensus clones 3 and 11 giving the same results (efficiency 89.3%). The amplification efficiency of variant clones 1 and 6 was somewhat lower (88.7% and 84.6% respectively) although the dynamic range was similar (Fig. 2A), which caused a large underestimation of the copy number of the viral variants especially at high copy numbers (Table 2).

Table 2.

Gag real-time PCR reaction efficiency for consensus and variant templates.

Gag clone	Consensus clone 3		Clone 1		Clone 6	
Starting copy number	Ct	Copies/reaction	Ct	Calculated copies/reaction	Ct	Calculated copies/reaction
10^6	20.1	1,000,000	20.5	725,000	20.7	668,000
10^5	23.5	100,000	24.2	68,600	24.1	77,600
10^4	27.3	10,000	27.8	7060	28.1	5700
10^3	30.9	1000	31.3	732	32.2	426
10^2	34.5	100	34.7	86	35.7	45
10	38.1	10	38.1	10	39.2	5
5	39.1	5	39.7	4	39.9	3
Reaction efficiency	89.3%		88.7%		84.6%	

The same quantities of input template were used for all plasmid clones. The copies per reaction values for variant clones 1 and 6 were calculated from consensus clone 3 standard curve.

3.1.3. Linear range of amplification of gag real-time RT PCR assay

To determine the linear range of amplification for the RT PCR assay a 10 fold serial dilution of gag transcript ranging from 1 × 106 to 10 copies/reaction was examined. The amplification was linear over five orders of magnitude (Fig. 2B) with a lower detection limit of 100 RNA molecules per reaction.

3.1.4. Gag real-time PCR vs. real-time RT PCR assay performance

To evaluate the performance of the real-time PCR in comparison with the real-time RT PCR assay, DNA and RNA were extracted from aliquots of an EV1 infected ovine fibroblast cell culture, quantified and serially diluted to 10−6. The highest dilutions of DNA and RNA still yielding a positive signal in at least one replica sample were 1 × 10−6 and 2 × 10−5, corresponding to 0.1 pg of DNA and 0.7 pg of RNA, carrying 4 copies of proviral DNA and 139 copies of viral RNA respectively (mean values).

3.2. Pol LUX real-time PCR assay

Primer sequences selected with LUX Designer software were highly conserved: only 1 out of 11 clones had multiple mutations, within the forward primer (ntd 3403, 3411, 3413 and 3415). Amplicon length was 77 bp. The assay was performed on serial dilutions of a consensus plasmid clone, ranging from 1 × 106 to 3 copies/reaction. The sensitivity of this assay was unsatisfactory with templates of less than 100 copies because no good correlation was found between viral copy number and threshold cycle. Templates with 100, 50 and 10 copies had Ct values of 44.06, 45.66 and 45.36 (mean values of six experiments).

3.3. Pol real-time primers and probe

The consensus sequence of pol clones amplified from fibroblasts infected with EV1 viral stock shared 94% identity with the published EV1 sequence. The real-time primer probe combination selected by best fit with

the consensus sequence (Table 1), displayed higher interclonal sequence heterogeneity than gag, within the probe and both primers (Fig. 1A and B). Amplicon length was 146 bp. When the primers ability to amplify the viral variants was tested by conventional PCR, 3 out of 11 clones (1, 5 and 9), all carrying the same three mismatches, within the reverse primer, escaped detection.

3.3.1. Pol dual labelled real-time PCR assay

The real-time pol assay amplified the complete dilution series of consensus plasmid clone 3 (1 × 106 to 5 copies/reaction, reaction efficiency 88.5%, slope −3.632), as well as all variant clones, with a detection limit of 10 copies. However, templates carrying variant primer and probe sequences were not efficiently amplified: as a result the number of template molecules was underestimated in variant clones (data not shown).

3.4. Evaluation of specificity and sensitivity of gag and pol dual labelled probe PCR assays: analysis of PBMC samples

A panel of 204 DNAs extracted from PBMCs of 68 sheep infected experimentally and 222 DNAs from PBMCs of 74 uninfected sheep (Niesalla et al., 2009) was analyzed with both dual labelled probe real-time PCR assays. A set volume of 5 µl of DNA was used as template irrespective of DNA concentration of the samples. In some cases the amount of DNA per reaction exceeded the maximum suggested by the manufacturer (500 ng). However, experiments with increasing amounts of DNA (200 ng to 2.5 µg) showed no adverse effect on assay performance (data not shown). Neither gag nor pol PCRs generated any false positive results with the known uninfected samples, showing 100% specificity. The average viral loads detected with gag assay ranged from 1 to 1009 copies per microgram of DNA, and with pol assay from 1 to 432. The majority of samples had loads below 100 copies. In most cases, the pol assay gave results showing lower numbers of viral molecules than the gag assay and generated some false negative results, when the number of viral genomes was low (Fig. 3) The gag assay detected 185 and the pol assay 172 out of 204 infected samples (Table 3). The sensitivity values of the gag and pol real-time PCRs were 91% and 84% respectively.

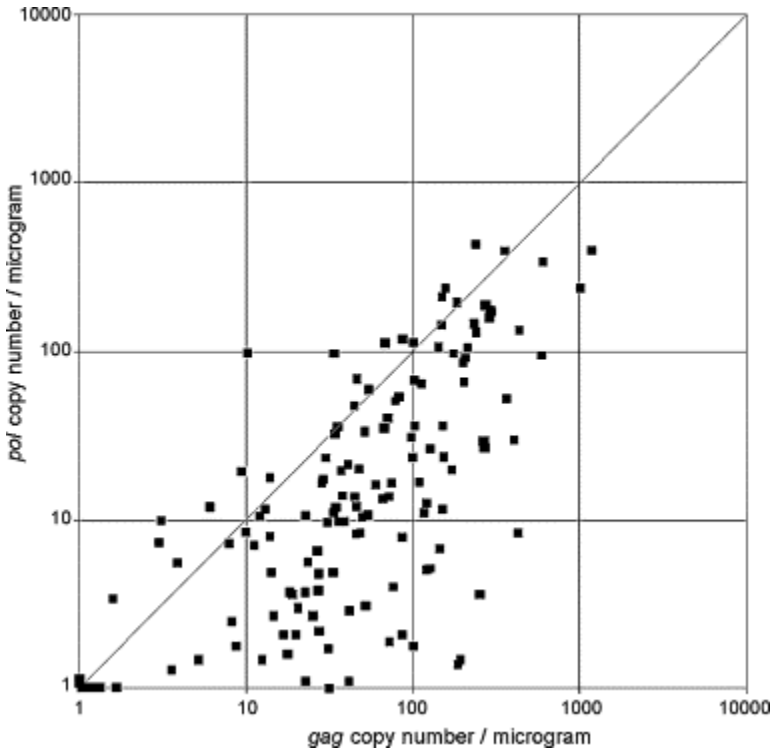

Fig. 3.

Dot plot comparison of gag and pol input DNA (copy number/microgram). A total of 222 DNA samples from 68 sheep experimentally infected with EV1 were analysed.

Table 3.

Analysis of gag and pol real-time PCR assay sensitivity and specificity on PBMC samples.

	Infected	Uninfected	Total
(A)			
gag +	185	0	185
gag −	19	222	241
Total	204	222	426
(B)			
pol +	172	0	172
pol −	32	222	254
Total	204	222	426

	pol +	pol −	Total
(C)			
gag +	169	16	185
gag −	3	238	241
Total	172	254	426

(A) *gag* assay sensitivity: 91%; *gag* assay specificity: 100%. (B) *pol* assay sensitivity 84%; *pol* assay specificity 100%. (C) comparison of *gag* vs. *pol* results in infected sheep.

4. Discussion

Real-time PCR technology represents the tool of choice to detect and quantify viral genomes or transcripts. Recently, real-time PCR and real-time RT PCR assays have been developed for SRLVs, for diverse purposes, such as the analysis of viral transcription pattern in infected cell cultures (Gudmundsson et al., 2003), the study of viral persistence in long term infected goats (Ravazzolo et al., 2006) and the characterization of the growth properties of viral strains (Barros et al., 2004). In these experiments, either a single viral isolate or a SRLV molecular clone was used for the infections. Primers and probes were designed based on the sequence of the isolate or the molecular clone utilized throughout the study, to amplify env (Ravazzolo et al., 2006), gag (Barros et al., 2004) or each of the viral transcripts (Gudmundsson et al., 2003). This approach resulted in robust PCRs linear over 6 or 7 orders of magnitude which were able to detect templates carrying respectively less than 10 copies and 60 copies of viral molecules (Gudmundsson et al., 2003 and Ravazzolo et al., 2006). Few reports describe real-time protocols designed for diagnostic use. Given the high degree of genetic heterogeneity which characterizes the SRLVs (Zanoni, 1998), diagnostic assays were based on conserved sequences of the LTR, gag and env regions (Brinkhof et al., 2008,

Herrmann-Hoesing et al., 2007 and Peterson et al., 2008). Samples were simply classified as positive or negative by Brinkhof and by Peterson, who did not report quantitative data. Herrmann Hoesing and colleagues described identification of samples with proviral loads ranging from 1 to 6 × 104 copies per microgram of DNA in sheep infected naturally. However, the efficiency of the assay was not reported, and the copy number of the reference gene, glyceraldehyde 3-phosphate dehydrogenase (gapdh), showed tenfold variations among samples. The present report describes the development of real-time PCR assays to measure the viral load of the British VMV EV1 strain. This strain has been and is currently the object of extensive investigations focusing on diverse aspects of lentiviral infections. Several in vivo and in vitro models of infection with EV1 have been established over the years, where the viral sequences were detected mostly by nested PCR, allowing only approximate quantitative evaluations (Bird et al., 1993, Niesalla et al., 2008 and Ryan et al., 2000). A QC PCR was developed to determine accurately and compare the EV1 viral load in peripheral blood monocytes and in alveolar macrophages of infected sheep (Zhang et al., 2000). However this technique is quite laborious and not suited for the analysis of large numbers of samples. In the present study, real-time assays were developed for two genes, gag and pol, to compare and confirm the results obtained with each individual gene. Based on the observation that the genetic variability of lentiviruses occurs not only among isolates but also within a single isolate during in vitro cultivation or during the course of the infection (Balfe et al., 1990, Pisoni et al., 2007, Sargan et al., 1991 and Sargan et al., 1995), real-time amplicons were designed based on the consensus sequences of several gag and pol clones representative of EV1 stock variants. Both gag and pol dual labelled probe PCRs had a sensitivity sufficient to amplify the most diluted standard templates (3 copies/reaction). The presence of mutations within primers and probe sequences reduced the sensitivity of the PCR assays, as demonstrated when individual clonal variants were used as templates. Although the efficiency values with mutant plasmids did not differ dramatically from the efficiency of the consensus plasmid used to generate the standard curve, the resulting copy numbers of mutant templates were greatly underestimated, especially for high copy samples. In vivo, however, the situation is more complex because infected samples are likely to contain a heterogeneous population of viral variants represented differentially and the overall efficiency of the assay will reflect this genetic complexity. The performances of the two PCRs in vivo were evaluated and compared on a large panel of DNAs extracted from PBMCs of sheep infected experimentally with EV1, at increasing time intervals until 3 months p.i. The results showed that the pol assay detected in most cases lower numbers of viral molecules than gag and failed to detect some infected samples which were low copy positive with the gag assay, probably due to the higher sequence heterogeneity of pol. The viral loads detected with gag and with pol assays were in the same range as those detected with env assays in PBMCs of long term experimentally infected goats (Ravazzolo et al., 2006) and long term naturally infected sheep (Herrmann-Hoesing et al., 2007). The gag real-time RT PCR showed linearity over 5 orders of magnitude (106 to 100 copies of template) and the gag real-time PCR over 6 orders (106 to 10 copies of template), when gag transcript or plasmid DNA were used as templates. This difference was not expected to result in a lower performance of the RT PCR compared to the PCR in cells and tissues permissive for virus replication, which are likely to contain higher numbers of viral transcripts than proviral genomes. This was shown by Ravazzolo et al. (2006) who quantified mRNA and proviral DNA in several goat tissues and is confirmed in this study with the quantitation of RNA and DNA extracted from the same cell culture infected in vitro with EV1 stock. In conclusion, the real-time assays developed in this study, particularly the gag assay, provide a sensitive tool which can be used to quantify the viral load in experimental infections. Given the evidence that mutations within primer and probe sequences affect to some extent the performance of the assays, and depending on the nature of the investigation, the use of a molecular clone of the virus could be beneficial in reducing loss of efficiency.

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