

Development and evaluation of quantitative-competitive PCR for quantitation of coxsackievirus B3 RNA in experimentally infected murine tissues

K. Nundita Reetoo, Shabina A. Osman, Shirin J. Illavia, Jangu E. Banatvala, Peter Muir *

Department of Virology, Guy's, King's & St. Thomas' School of Medicine, King's College London, St Thomas' Campus, Lambeth Palace Road, London SE1 7EH, UK

Received 30 April 1999; received in revised form 17 June 1999; accepted 17 June 1999

Abstract

A method is described for quantitation of enterovirus RNA in experimentally infected murine tissues. Viral RNA was extracted from tissue samples and amplified by reverse transcriptase PCR in the presence of an internal standard RNA. The ratio of PCR product derived from viral RNA and internal standard RNA was then determined using specific probes in a post-PCR electrochemiluminescent hybridization assay. This provided an estimate of the viral RNA copy number in the original sample, and detection of PCR product derived from internal standard RNA validated sample processing and amplification procedures. RNA copy number correlated with viral infectivity of cell culture-derived virus, and one tissue culture infective dose was found to contain approximately 10^3 genome equivalents. The ratio of RNA copy number to infectivity in myocardial tissue taken from mice during the acute phase of coxsackievirus B3 myocarditis was more variable ranging from 10^4 – 10^7 , and was dependent on the stage of infection, reflecting differential rates of clearance for viral RNA and viral infectivity. The assay is rapid, and could facilitate investigations which currently rely upon enterovirus quantitation by titration in cell culture. This would be useful for experimental studies of viral pathogenesis, prophylaxis and antiviral therapy. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Coxsackieviruses; Quantitative PCR; Viral myocarditis; Murine models

1. Introduction

Enteroviruses are common human pathogens which cause a wide spectrum of disease. Sixty-six serotypes which infect humans are recognised; these include the polioviruses, group A and B coxsackieviruses (CVA, CVB), echoviruses, and

* Corresponding author. Tel.: +44-171-922-8167; fax: +44-171-922-8387.

E-mail address: p.muir@umds.ac.uk (P. Muir)

additional numbered enterovirus serotypes. Enteroviruses are a common cause of acute myocarditis and aseptic meningitis (Melnick, 1996), and have been implicated in a number of chronic conditions, including dilated cardiomyopathy, insulin-dependent diabetes mellitus, and chronic fatigue syndrome, although experimental evidence for some of these associations is conflicting (reviewed by Muir and Archard, 1994; Melchers et al., 1994). To understand the pathogenesis of enterovirus infections, a number of investigators have developed murine models of enterovirus-induced myocarditis (Grodums and Dempster, 1959; Woodruff, 1970) diabetes (Webb et al., 1976; Yoon et al., 1978), poliomyelitis (Jubelt et al., 1980; Ren et al., 1990) and polymyositis (Strongwater et al., 1984). These models have been used to study the natural history of enterovirus infection and immunity, disease mechanisms, the influence of viral and host factors on pathogenesis, and potential therapies or immunization strategies.

Quantitation of virus in infected tissues and body fluids forms an important aspect of such experimental studies. This traditionally requires the use of laborious infectivity titrations. However molecular quantitation methods have recently been developed for a number of viruses; these assays have found application in clinical practice, and have also made significant contributions to our understanding of viral replication kinetics and viral pathogenesis (Ho et al., 1995; Wei et al., 1995; Cope et al., 1997; Gor et al., 1998; Neumann et al., 1998). As an aid to the study of CVB-induced murine myocarditis and other murine models of enterovirus infection we developed a quantitative-competitive PCR (EV qPCR) for quantitation of enterovirus RNA in infected murine tissues. In this assay an internal standard (IS) synthetic RNA transcript is added to test specimens prior to RNA extraction and PCR amplification. IS RNA is similar in sequence to the PCR target region of the enterovirus genome, and is co-purified with viral RNA during RNA extraction, and co-amplified during PCR. Amplification products derived from IS RNA are differentiated from those derived from enterovirus RNA present in the test

sample by virtue of a modified internal probe recognition sequence. We have characterized this assay using defined copy numbers of synthetic RNA transcripts corresponding in sequence to part of the CVB3 genome, as well as CVB3-infected cell culture material of defined infectivity. The EV qPCR was shown to quantify viral RNA load in CVB3-infected murine myocardium, thus demonstrating its potential for studies of viral pathogenesis.

2. Materials and methods

2.1. Virus stock

CVB3, Nancy strain, was obtained originally from R. Kandolf, (Tübingen) and was propagated in Vero cells. The 50% tissue culture infectious dose (TCID₅₀) was calculated by the method of Reed and Muench (1938).

2.2. CVB3-infected murine myocardial tissue

The murine model of enterovirus-induced myocarditis employed in this study has been described previously (Zhang et al., 1994). Sixteen 4-week-old SWR mice (Harlan UK Ltd., Oxon, UK) were infected intraperitoneally with 10⁶ TCID₅₀ of CVB3, and eight mice inoculated with 100 µl of normal saline served as uninfected controls. Myocardial tissue was collected from four infected and two uninfected mice on days 3, 5, 7 and 14 post infection (p.i.), and portions were snap frozen in liquid nitrogen and stored at –70°C.

2.3. Infectivity titration

Approximately 10 mg of frozen myocardial tissue from CVB 3-infected and uninfected mice was homogenised in 200 µl sterile PBS, filtered through a 0.45-µm pore size, then serially diluted in PBS. Aliquots of 100 µl were inoculated onto Vero cell monolayers. Development of cytopathic effect was observed over 7 days.

Table 1

Nucleotide sequences of primers used for the construction of IS RNA and ES RNA transcription templates, and of primers and probes employed in the EV qPCR assay

Primer designation		Primer reference	Nucleotide position ^a	Primer sequence (5' → 3')
PCR mutagenesis and sequencing primers	EVMutA ^b	This paper	563–589	<u>ATGCTGGTCCTGTTTGTGTTCA</u> TTT-TATTCCTATACTGGCTGCTT
	EVMutB ^b	This paper	544–524	<u>ACACAAACAGGACCAGCATGTCG</u> -GTTCCGCTGCAGAGTT
	EVMutC ^b	This paper	254–279	<u>ACGCTCAGACTCAGTCAAAACC</u> -TAGTAACACCGTGAAGTTGC
	EVMutD ^b	This paper	237–214	<u>GACTGAGTCGTGAGCGTATAAC</u> -GAACGCTTTCTCCTTCAACC
	EVU3	Nicholson et al., 1995	63–82	CGGTACCTTTGTGCGCCTGT
	EVD1 ^c	Nicholson et al., 1994	645–628	CACYGGRTGGCYAATCCA
	EVGU	This paper	1–24	TTAAAACAGCCTGTGGGTTGATCC
EV qPCR primers	SP6	–	NA ^d	GATTTAGGTGACACTATAG
	T7	–	NA ^d	TTATACGACTCACTATAGGG
	EVU2-B ^c	Muir et al., 1993	456–473	B-CCCCTGAATGCGGCTAAT
	EVD2	Muir et al., 1993	602–583	ATTGTCACCATAAGCAGCCA
EV qPCR probes	IS RNA probe ^f	This paper	NA ^g	Ru-CACAAACAGGATCAGCATG
	EV RNA probe ^f	Muir et al., 1993	562–544	Ru-AACACGGACACCCAAAGTA

^a Numbering of nucleotide positions of primer recognition sites is based on the sequence of CVB3 (8).

^b Underlined nucleotides represent modified probe recognition sequences incorporated in the IS RNA.

^c Redundant base codes: Y = C or T; R = A or G.

^d Not applicable: these primers hybridize to pGEM-T plasmid vector-derived sequences in plasmids pKNRI and pKNRII.

^e B, Biotin-labeled.

^f Ru, Ruthenium-labeled.

^g Not applicable: this probe hybridizes to a modified probe recognition sequence in the IS RNA.

2.4. Oligonucleotides

The nucleotide sequences of all oligonucleotides used for cloning procedures, nucleotide sequence analysis and for the EV qPCR assay are given in Table 1. All primers and probes except T7 and SP6 primers were designed using Oligo 4.0 (National Biosciences). Primers were synthesized by Pharmacia Biotech Laboratories, Milton Keynes, UK, R&D Systems, Abingdon, UK or Life Technologies, Paisley, UK. Ruthenium-labelled probes were synthesized by Baron Biotech., Millford, CT.

2.5. Construction of internal standard (IS) RNA

A synthetic RNA template was prepared for use as a competitive internal quantitation standard (IS RNA) in the EV qPCR assay. A DNA template for synthesis of IS RNA was prepared using the splicing by overlap extension (SOE) method (Ho et al., 1989) as shown in Fig. 1. This template corresponds to nucleotide positions 63–645 of the CVB3 nucleotide sequence (Klump et al., 1990), which includes the target sequences of most published enterovirus PCR assays, but contains two modified probe recognition sequences at

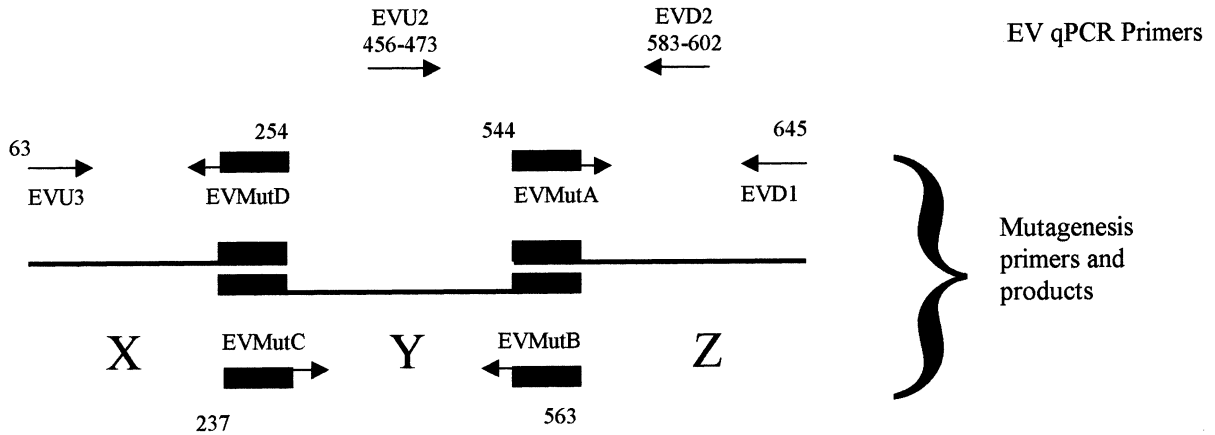


Fig. 1. Positions of mutagenesis primers (EVMutA–EVMutD) for the insertion of modified probe recognition sequences in the 5' NTR of the enterovirus genome. These primers were used to generate fragments X, Y, and Z, which were used to construct the IS RNA transcription template. The positions of primers used in the EV qPCR assay is also shown. Modified probe recognition sequences in the mutagenesis primers and fragments X, Y, and Z are represented as solid blocks. Numbering refers to nucleotide positions based on those of the CVB3 sequence (Klump et al., 1990).

nucleotide positions 237–254, and positions 544–563. The template was synthesized in three fragments, designated X, Y and Z, into which the desired modified sequences were incorporated using the mutagenesis primers EVMutA, EVMutB, EVMutC and EVMutD (Fig. 1). These fragments were then spliced together to form the complete template (X–Y–Z). Fragments X, Y and Z were generated by reverse transcriptase PCR as follows. CVB3 RNA was extracted from 10^7 TCID₅₀ of CVB3 (Nancy) using RNAzol B, resuspended in 20 μ l water, and cDNA generated by reverse transcription in three separate 20- μ l volume reactions containing First Strand buffer (Life Technologies), 200 U of Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Life Technologies), 0.7 U PRIME RNase Inhibitor (5 Prime \rightarrow 3 Prime, Inc., Boulder, CO), 15 pmol either primer EVD1, primer EVMutB, or primer EVMutD (Table 1), each deoxynucleotide triphosphate (dNTP) at a concentration of 1 mM, and 2 μ l of CVB3 RNA. Reverse transcription reactions were carried out at 37°C for 90 min then at 99°C for 5 min using an Omnigene thermal cycler (Hybaid, Teddington, UK). PCR products X, Y and Z were then generated using the proof-reading enzyme *Pfu* DNA polymerase (Stratagene Ltd, Cambridge, UK). One hundred and thirty microlitres of PCR mix were

added to each completed reverse transcription reaction to give a *Pfu* reaction mix with final concentrations of 20 mM Tris–HCl (pH 8.75), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 1.8 mM MgSO₄, 200 μ M each dNTP, 2.5 U *Pfu* polymerase, and primers as indicated in Table 2. Thermal cycling conditions are given in Table 2.

By virtue of the complementary sequences incorporated into terminal 5' sequences of primers EVMutC and EVMutD, and of primers EVMutA and EVMutB, PCR products X, Y and Z were spliced in sequential SOE reactions. SOE reactions consisted of five thermal cycles to allow annealing and extension of overlapping sequences, followed by 35 cycles to amplify the spliced DNA template. Initially, PCR products X and Y were spliced using *Pfu* DNA polymerase with primers EVU3 and EVMutB, and 0.5 μ l of each of product X and Y, using cycling parameters indicated in Table 2. In a second reaction, products X–Y and Z, were spliced together using *Taq* DNA polymerase (Promega UK), which generates 3' terminal overhanging A residues to facilitate cloning. This reaction was carried out in Thermo buffer (Promega UK) containing 200 μ M dNTPs, 1.8 mM MgCl₂, 2.5 U *Taq* polymerase and primers EVU3 and EVD1 using cycle conditions given in Table 2.

Table 2

Reaction conditions for PCR reactions employed in the construction of IS RNA and ES RNA transcription templates^a

Primers ^b	Template for reaction	DNA polymerase	Optimal [Mg ²⁺] (mM) ^c	Number of cycles	Optimal annealing temperature (°C) ^d	Reaction product ^e
EVU3 and EV-MutD	EVMutD-primed cDNA ^f	<i>Pfu</i>	1.8	30	55	X
EVMutB and EVMutC	EVMutC-primed cDNA ^f	<i>Pfu</i>	1.8	30	58	Y
EVMutA and EVD1	EVD1-primed cDNA ^f	<i>Pfu</i>	1.8	30	49	Z
EVU3 and EV-MutB	X and Y	<i>Pfu</i>	1.8	5 ^g 35	32 ^g 38	X–Y
EVU3 and EVD1	X–Y and Z	<i>Taq</i>	1.8	5 ^g 35	38 ^g 45	X–Y–Z
EVGU and EVD1	EVD1-primed cDNA ^f	AmpliTaq Gold	1.5	40	57	CVB3 5' NTR
SP6 and T7	pKNRI or pKN-RII plasmids	<i>Taq</i>	2.0	30	52	IS RNA and ES RNA transcription templates

^a Thermal cycling reactions were performed using an Omnigene thermal cycler. Denaturation steps were 15 s at 95°C (when using *Pfu* DNA polymerase) or 94°C (*Taq* and AmpliTaq Gold DNA polymerase). Primer annealing steps were 15 s, and primer extension steps were 30 s at 75°C (*Pfu* DNA polymerase) or 72°C (*Taq* and AmpliTaq Gold DNA polymerase). Thermal cycling was preceded by an initial denaturation of 5 min (*Pfu* or *Taq* DNA polymerase) or 10 min (AmpliTaq Gold DNA polymerase) at 95°C, and was followed by a final primer extension of 5 min at 75°C (*Pfu* DNA polymerase) or 72°C (*Taq* and AmpliTaq Gold DNA polymerase). Primer concentrations were 0.15 pmol/μl. Composition of buffers is described in the text.

^b Primer sequences are given in Table 1.

^c Optimum magnesium ion concentrations were determined empirically by titration of magnesium salt concentration.

^d Optimum annealing temperatures were determined using Oligo 4.0.

^e The positions of reaction products within the CVB3 5' NTR are shown in Fig. 1.

^f cDNA, complementary DNA, produced by reverse transcription of CVB3 RNA as described in the text.

^g In SOE reactions, the initial five cycles were designed to allow extension of overlapping sequences, as explained in the text.

The resulting amplicon X–Y–Z was ligated into pGEM-T vector (Promega UK) and transformed into competent JM109 cells (Promega UK) according to manufacturer's instructions. Use of the pGEM-T vector allows the synthesis of RNA transcripts of either polarity corresponding to the inserted sequence by virtue of T7 and SP6 RNA polymerase promoter sequences which flank the cloning site. The nucleotide sequence and orientation of inserts in recombinant clones was determined by di-deoxy sequencing in both directions using T7 and SP6 sequencing primers (Table 1) with an ALF DNA sequencer (Pharmacia Biotech, UK). A recombinant clone containing the desired insert was selected and designated pKNR I.

A template for *in vitro* transcription was generated from plasmid pKNRI by amplifying the inserted sequence and flanking RNA polymerase promoter sequences from 0.1 µg of plasmid DNA using SP6 and T7 primers (Table 1) with reaction conditions given in Table 2. Two microlitres of the resulting product were used for *in vitro* transcription using an SP6 MEGAScript RNA transcription kit (Ambion, Austin, TX) to synthesize IS RNA. Transcription was carried out for 3–5 h at 37°C, then 2 U of RQ1-DNase (Life Technologies) was added and incubation continued for 2 h to remove the DNA template. IS RNA transcripts were extracted using RNazol B, resuspended in 20 µl formamide (Chomczynski, 1992), visualized by agarose gel electrophoresis in a 2% 3-(*N*-morpholino)propanesulfonic acid (MOPS)-formaldehyde gel (Sambrook et al., 1989) to confirm size homogeneity, quantified by UV-spectrophotometry and stored at –70°C.

2.6. Preparation of external standard RNA transcripts

To evaluate the performance of the EV qPCR, and to allow construction of standard curves, a second synthetic RNA template was prepared for use as an external standard (ES RNA) which was identical to the first 645 bases of the CVB3 genome. To generate a DNA template for ES RNA, the first 645 nucleotides of the CVB3 genome was amplified by reverse transcriptase-

PCR using primers EVD1 and EVGU (Table 1). A cDNA template of CVB3 (Nancy) was generated using primer EVD1 as described above except that 200 U Superscript II (Life Technologies) was used as reverse transcriptase. The reaction was incubated at 42°C for 1 h then 5 min at 99°C. Eighty microlitres of PCR mix in PCR Buffer II (Perkin Elmer, Warrington, UK) were then added to give final concentrations of 200 µM each dNTP, 1.5 mM MgCl₂, 15 pmol EVGU primer and 2.5 U AmpliTaq Gold DNA polymerase (Perkin Elmer). Thermal cycling parameters are given in Table 2. The PCR product was cloned, sequenced and used to generate synthetic RNA as described above, except that a T7 Megascript RNA transcription kit (Ambion) was used, because the insert in the selected clone (designated pKNR II) was of the opposite orientation to that in pKNR I.

2.7. EV qPCR

To quantify enterovirus RNA in test samples, a fixed amount of IS RNA was co-extracted and co-amplified with test sample RNA. IS RNA (10⁴ copies) was added to 100 µl sample prior to RNA extraction. RNA was then extracted using RNazol B (Biogenesis, Poole, UK) and precipitated with isopropanol using 50 µg of linear polyacrylamide as co-precipitant (Gaillard and Strauss, 1990) as described previously (Muir et al., 1993). When testing frozen tissue samples, pre-weighed portions of approximately 100 µg cryopreserved tissue were ground to a fine powder in liquid nitrogen and homogenized in RNazol B. IS RNA was added after homogenization, but before RNA extraction and precipitation. Extraction blanks containing 10⁴ copies of IS RNA only (IS RNA control), or no RNA, were processed in parallel with test samples. Extracted RNA was resuspended in 20 µl of water, heated at 80°C for 2 min, then chilled on ice prior to reverse transcription and PCR amplification. Reverse transcription was performed in 20 µl of First Strand buffer containing 200 U MMLV reverse transcriptase, 0.7 U PRIME RNase Inhibitor, 10 µM tetramethyl ammonium chloride (Sigma, Poole, UK), 15 pmol of primer EVD2 (Table 1) and 2 µl of

test or control RNA sample at 37°C for 90 min then 99°C for 5 min. Quantitation controls containing 10^3 copies of IS RNA and varying amounts of ES RNA were processed in parallel. ES RNA controls, containing 10^8 copies of ES RNA but no IS RNA were also included. After reverse transcription, 80 μ l of PCR mix was added, which contained PCR buffer II, 15 pmol of primer EVU2-B (Table 1), $MgCl_2$ to give a final concentration of 1.8 mM and 2.5 U AmpliTaq Gold DNA polymerase. Thermal cycling consisted of 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 53.5°C for 15 s and 72°C for 15 s, followed by a final incubation at 72°C for 5 min.

PCR products were quantified by electrochemiluminescence using the QPCR 5000 (Perkin Elmer) and readings were expressed in relative light units (RLU). Three microlitres of PCR product were mixed with 5 pmol of either IS RNA probe or EV RNA probe (Table 1) in 100 μ l Thermo buffer (Promega UK), and incubated at 94°C for 1 min to denature PCR products, then 40°C for 10 min to allow probe hybridization. Hybrids were transferred to assay tubes containing 300 μ l QPCR assay buffer (Perkin Elmer) and 1 μ l (10^5 beads) of streptavidin coated M-280 magnetic beads (Dyna, UK). The tubes were held at room temperature for 10 min with intermittent agitation to allow capture of hybrids to magnetic beads. Beads were automatically washed by the instrument prior to quantitation of bound probe by electrochemiluminescence. To estimate the number of enterovirus RNA copies in the original sample an EV RNA/IS RNA quotient ($Q_{EV\ RNA/IS\ RNA}$) was determined using the formula:

$$Q_{EV\ RNA/IS\ RNA} = \frac{\text{Test RLU}_{(EV\ RNA)} - \text{IS RNA control RLU}_{(EV\ RNA)}}{\text{Test RLU}_{(IS\ RNA)} - \text{ES RNA control RLU}_{(IS\ RNA)}} \times \text{IS RNA}_{\text{COPY NO.}} \times f$$

where Test $RLU_{(EV\ RNA)}$ is the mean RLU reading of duplicate EV qPCR reactions performed on the test sample using the EV RNA probe; IS RNA control $RLU_{(EV-RNA)}$ is the mean background RLU reading of at least two EV qPCR reactions performed on the IS RNA control using the EV RNA probe; Test $RLU_{(IS\ RNA)}$ is the mean

RLU reading of two duplicate EV qPCR reactions performed on the test sample using the IS RNA probe; ES RNA control $RLU_{(IS\ RNA)}$ is the mean background RLU reading of at least two EV qPCR reactions performed on the ES RNA control using the IS RNA probe; $IS\ RNA_{\text{COPY NO.}}$ is the number of IS RNA copies added to the test sample prior to RNA extraction (for quantitation standards $IS\ RNA_{\text{COPY NO.}}$ is the number of IS RNA copies added to the EV qPCR reaction); and f is the fraction of the original RNA extract used for EV qPCR (for quantitation standards, $f = 1$).

Using the $Q_{EV\ RNA/IS\ RNA}$ value, the enterovirus RNA copy number was determined by reference to a standard curve generated using values obtained by EV qPCR analysis of quantitation standards containing 10^1 – 10^8 copies of ES RNA. The detection limit of the assay was determined as the ES RNA copy number which consistently gave $Q_{EV\ RNA/IS\ RNA}$ values greater than the mean value of at least three replicate IS RNA control reactions plus three standard deviations.

At high enterovirus RNA or ES RNA copy numbers, amplification of the IS RNA was competitively inhibited, precluding accurate quantitation. In this situation, test samples were re-extracted using 10^5 or 10^7 copies of IS RNA instead of 10^4 copies, and EV qPCR was repeated using a one-tenth volume of RNA extract. The $Q_{EV\ RNA/IS\ RNA}$ value was re-calculated and the RNA copy number estimated using a standard curve in which ES RNA dilutions were co-amplified with 10^4 or 10^6 copies of IS RNA.

Where test failure occurred, identified by failure to amplify either enterovirus RNA or IS RNA,

RNA extracts were re-precipitated with 0.1 vol. 5 M NaCl and 2.5 vol. absolute ethanol, and washed three times with 70% ethanol in an attempt to remove residual enzymatic inhibitors. RNA samples were then air-dried and re-dissolved prior to repeating EV qPCR.

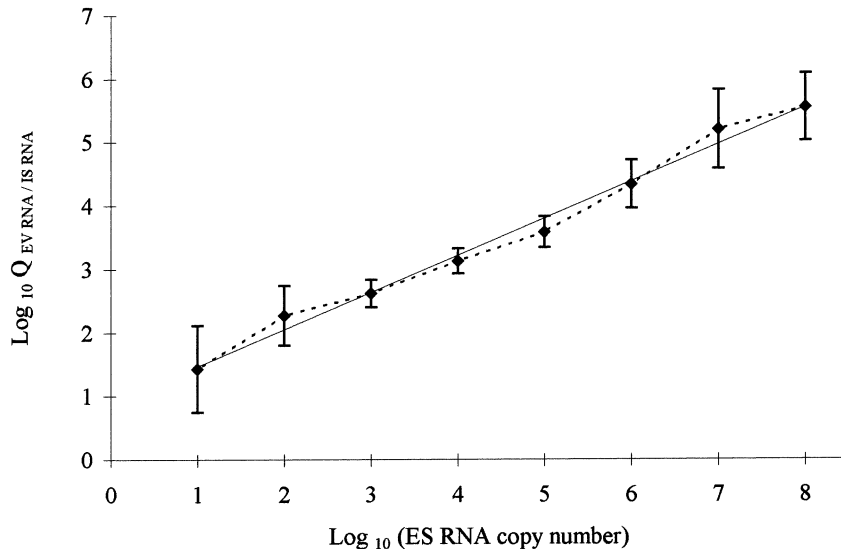


Fig. 2. Standard curve for the determination of enterovirus RNA copy number, generated by EV qPCR analysis of quantitation standards. Results are based on the mean of three separate experiments. Error bars represent 95% confidence intervals. The solid line represents the best fit, and was used to determine enterovirus copy numbers in test samples. The sensitivity cut-off was determined as the mean $Q_{(EV\ RNA/IS\ RNA)}$ value of eight IS RNA controls plus three standard deviations.

3. Results

3.1. Characterization of the EV qPCR assay

The EV qPCR assay was characterized using quantitation standards in which 10^1 – 10^8 copies of ES RNA were co-amplified with 10^3 copies of IS RNA per reaction. $Q_{EV\ RNA/IS\ RNA}$ values were determined as described in Section 2, and plotted against the input number of ES RNA copies (Fig. 2). As little as 10^2 ES RNA copies could be detected consistently, and quantitation was accurate to approximately ± 1 \log_{10} value over a range of 10^2 – 10^6 ES RNA copies. Although a proportion of samples containing 10 ES RNA copies gave $Q_{EV\ RNA/IS\ RNA}$ values above the sensitivity cut-off, quantitation was not reliable, probably due to sampling errors. At ES RNA copy numbers of $> 10^6$, amplification of IS RNA was competitively inhibited, precluding accurate quantitation. Quantitation standards containing $> 10^6$ copies of ES RNA per reaction were therefore re-analysed in the presence of 10^5 and 10^6 copies of IS RNA, which allowed accurate quantitation of up to 10^7 and 10^8 copies per reaction respectively (not shown).

3.2. Relationship between enterovirus RNA copy number and cell culture infectivity

CVB3 of known tissue culture infectivity was serially diluted and analysed by EV qPCR. Quantitation was achieved with RNA derived from as little as 1 TCID₅₀, which was found to contain approximately 1.7×10^3 copies of viral RNA (Fig. 3).

3.3. Quantitation of enterovirus RNA and virus infectivity in murine myocardium

Myocardial tissue from CVB3-infected and uninfected mice was analysed by EV qPCR, and virus infectivity was determined by titration in cell culture. EV qPCR test failure persisted after reprecipitation in two samples collected from infected mice on day 3 p.i., and these samples were excluded from analysis. Results for the remaining samples are shown in Fig. 4. Viral RNA levels were 10^4 – 10^7 times higher than infectivity titres in samples collected between 3 and 7 days p.i., and the ratio of viral RNA copy number to infectivity titre was greater in tissues collected 7 days p.i.

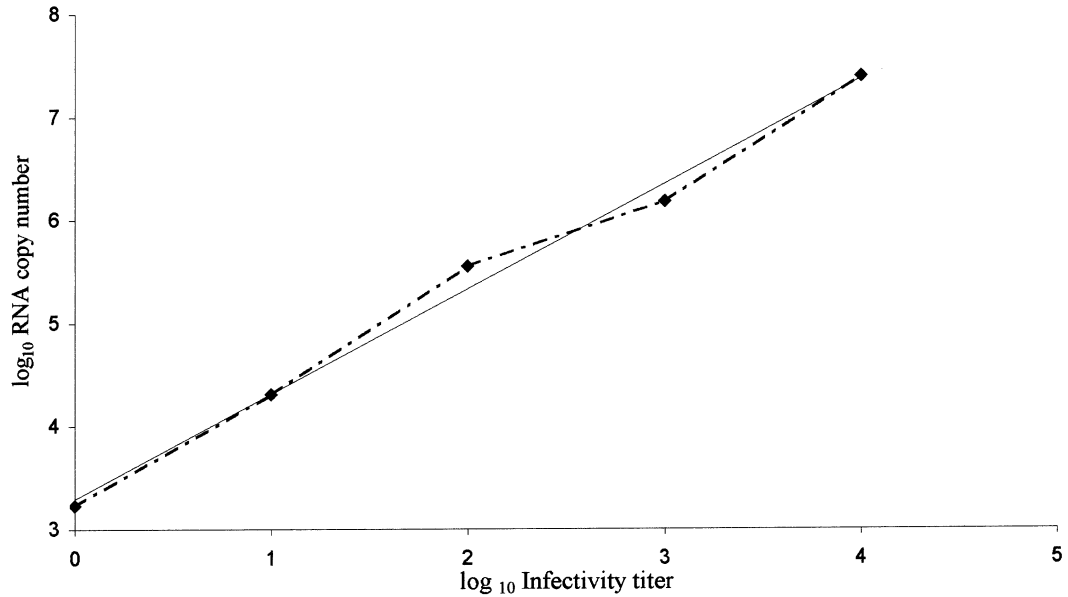


Fig. 3. Relationship between cell culture infectivity and viral RNA copy number of cell culture-derived CVB3. Results of individual determinations are shown as a dashed line. The solid line represents the best.

than in tissues collected at earlier stages of infection ($P = 0.009$, Student's *t*-test). Although there was a mean 400-fold decline in infectivity titres between days 5 and 7 p.i. ($P = 0.0005$), viral RNA levels declined only by a factor of 11 during this time ($P = 0.049$). A greater than 6000-fold mean reduction in viral RNA levels was observed between days 7 and 14 p.i. ($P = 0.0001$), during which time infectious virus was eliminated.

4. Discussion

Viral quantitation using culture-based methods requires testing of serial dilutions of test samples in plaque assays or replicate cultures to determine the infectivity titre in plaque-forming units or TCID₅₀ respectively (Khatib et al., 1980). This is labor-intensive, and becomes impractical when testing large sample numbers. The EV qPCR assay may thus be useful for experimental studies of viral pathogenesis, *in vitro* and *in vivo* evaluation of candidate antiviral agents, and functional analysis of enterovirus variants or mutants.

Accurate quantitation of 10^2 – 10^6 RNA copies

was achieved using a single amplification reaction and two probe hybridization reactions, and the assay was at least as sensitive as viral culture. If quantitation of RNA copy numbers above this range was required, the assay could be adjusted to allow this by increasing the IS RNA copy number added during RNA extraction. The assay also allowed false negative results due to failure of RNA extraction or presence of enzymatic inhibitors in RNA extracts to be identified by failure to amplify either enterovirus RNA or IS RNA.

Although we have employed primers which detect a wide range of enterovirus serotypes (Rotbart, 1990; Muir et al., 1993), it is possible that RNA of different enterovirus types may be amplified at different efficiencies, giving rise to incorrect quantitation values. This problem has been observed with other viral quantitation assays (Alaeus et al., 1997; Hawkins et al., 1997). This may affect the accuracy of the EV qPCR assay when studying enteroviruses other than CVB3. We plan to use synthetic RNA transcripts derived from representative enterovirus serotypes to inves-

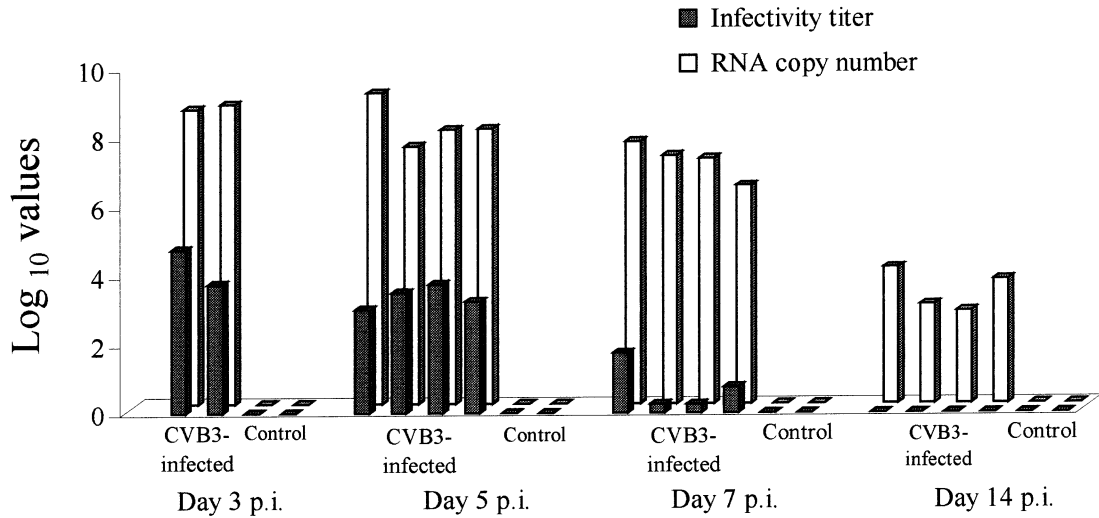


Fig. 4. Viral RNA copy number and infectivity per milligram of myocardial tissue collected from CVB3-infected and uninfected mice at days 3–14 post infection.

tigate the ability of the EV qPCR assay to quantify enterovirus RNA of different serotypes accurately.

We compared enterovirus RNA levels with tissue culture infectivity using murine myocardial tissue collected during the acute phase of CVB3 infection. Viral RNA copy numbers exceeded infectivity titres by several orders of magnitude, and this ratio was greater at day 7 p.i., when most infectious virus had been eliminated from heart tissue, than at earlier stages of infection. While infectious virus could not be detected at 14 days p.i., viral RNA was still present. These observations suggest that viral infectivity is cleared more rapidly than viral RNA following the peak of viral replication in myocardium. This is consistent with previous studies of enterovirus-induced murine myocarditis, in which viral RNA was detected in myocardium beyond the clearance of infectious virus using *in situ* hybridization (Klingel et al., 1992) or qualitative reverse transcriptase PCR (Leparc et al., 1993; Ouyang et al., 1995). There is considerable interest in the possibility that persistence of enterovirus RNA in myocardium may play a role in the evolution of chronic dilated cardiomyopathy (Wessley et al., 1998), which may be mediated by viral protease-induced cleavage of cytoskeletal proteins (Badorff

et al., 1999). EV qPCR may therefore be useful in studying viral RNA kinetics during the post-infectious or persistent phase of infection. Indeed we have recently found, using this assay, that low levels of viral RNA can be detected as late as 90 days p.i. (manuscript in preparation).

Competitive PCR assays described previously for quantitation of enterovirus RNA have required densitometric or autoradiographic analysis of electrophoretically resolved PCR products, with or without prior restriction endonuclease digestion (Martino et al., 1993; Arola et al., 1996; Le Guyader et al., 1997). The electrochemiluminescent probe hybridization assay used in this study is more straightforward and faster (results of 20 samples are available less than 90 min after completion of PCR amplification). It is not affected by PCR product heterodimer formation (Martino et al., 1993), and is more amenable to standardization. The principle of differential probe hybridization is used in commercially available competitive amplification assays for quantitation of Hepatitis C virus and Human Immunodeficiency Virus RNA. The probe hybridization assay can be adapted to a microplate format, several of which have been described (Rotbart et al., 1994; Bosch et al., 1996), if electrochemiluminescent detection is not available.

Although the QPCR 5000 used in this study is no longer available, equivalent equipment and reagents can be obtained from other suppliers. We have designed an IS RNA molecule with two modified probe recognition sequences in different genomic regions. This allows the possibility, if desired, of employing a second confirmatory PCR assay employing primers which flank the second modified probe recognition sequence.

Acknowledgements

This study was funded by the Chronic Fatigue Syndrome Research Foundation. K.N. Reetoo is the recipient of a scholarship from the Association of Commonwealth Universities. S.J. Illavia is supported by the Philip Fleming Trust.

References

- Alaeus, A., Lidman, K., Sönerborg, A., Albert, J., 1997. Subtype-specific problems with quantification of plasma HIV-1 RNA. *AIDS* 11, 859–865.
- Arola, A., Santti, J., Ruuskanen, O., Halonen, P., Hyypiä, T., 1996. Identification of enteroviruses in clinical specimens by competitive PCR followed by genetic typing using sequence analysis. *J. Clin. Microbiol.* 34, 313–318.
- Badorff, C., Lee, G.-H., Lamphear, B.J., Martone, M.E., Campbell, K.P., Rhoads, R.E., Knowlton, K.U., 1999. Enteroviral protease 2A cleaves dystrophin: Evidence of cytoskeletal disruption in an acquired cardiomyopathy. *Nat. Med.* 5, 320–326.
- Bosch, A., Gajardo, R., Diez, J.M., Pinto, R.M., 1996. Non isotopic automatable molecular procedures for the detection of enteroviruses. *Mol. Cell. Probes* 10, 81–89.
- Chomczynski, P., 1992. Solubilization in formamide protects RNA from degradation. *Nucleic Acids Res.* 20, 3791–3792.
- Cope, A.V., Sabin, C., Burroughs, A., Rolles, K., Griffiths, P.D., Emery, V.C., 1997. Interrelationships among quantity of human cytomegalovirus (HCMV) DNA in blood, donor-recipient serostatus, and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation. *J. Infect. Dis.* 176, 1484–1490.
- Gaillard, C., Strauss, F., 1990. Ethanol precipitation of DNA with linear polyacrylamide as carrier. *Nucleic Acids Res.* 18, 378.
- Gor, D., Sabin, C., Prentice, H.G., Vyas, N., Man, S., Griffiths, P.D., Emery, V.C., 1998. Longitudinal fluctuations in cytomegalovirus load in bone marrow transplant patients: relationship between peak virus load, donor/recipient serostatus, acute GVHD and CMV disease. *Bone Marrow Transplant.* 21, 597–605.
- Grodums, E.I., Dempster, G., 1959. The age factor in experimental coxsackie B3 infection. *Can. J. Microbiol.* 5, 595–603.
- Hawkins, A., Davidson, F., Simmonds, P., 1997. Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by Quantiplex HCV RNA Assay Versions 1 and 2, Roche Monitor Assay, and an in-house limiting dilution method. *J. Clin. Microbiol.* 35, 187–192.
- Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M., Markowitz, M., 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373, 123–126.
- Ho, N.S., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site directed mutagenesis by overlap extension using polymerase chain reaction. *Gene* 77, 51–59.
- Jubelt, B., Gallez-Hawkins, B., Narayan, O., Johnson, R.T., 1980. Pathogenesis of human poliovirus infection in mice. I. Clinical and pathological studies. *J. Neuropathol. Exp. Neurol.* 39, 149–158.
- Khatib, R., Chason, J.L., Silberberg, B.K., Lerner, A.M., 1980. Age-dependent pathogenicity of group B coxsackieviruses in Swiss-Webster mice: infectivity for myocardium and pancreas. *J. Infect. Dis.* 141, 394–403.
- Klingel, K., Hohenadl, C., Canu, A., Albrecht, M., Seemann, M., Mall, G., Kandolf, R., 1992. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: Quantitative analysis of virus replication, tissue damage, and inflammation. *Proc. Natl. Acad. Sci. USA* 89, 314–318.
- Klump, W.M., Bergmann, I., Müller, B.C., Ameis, M.D., Kandolf, R., 1990. Complete nucleotide sequence of infectious Coxsackievirus B3 cDNA: Two initial 5' uridine residues are regained during plus strand synthesis. *J. Virol.* 64, 1573–1583.
- Le Guyader, F., Menard, D., Dubois, E., Haugarreau, L., Kopecka, H., Pommepuy, M., 1997. Use of an RT-PCR internal control to evaluate viral removal. *Water Sci. Technol.* 35, 461–465.
- Leparc, I., Fuchs, F., Kopecka, H., Aymard, M., 1993. Use of the polymerase chain reaction with a murine model of picornavirus-induced myocarditis. *J. Clin. Microbiol.* 31, 2890–2894.
- Martino, T.A., Sole, M.J., Penn, L.Z., Liew, C.-C., Lui, P., 1993. Quantitation of enteroviral RNA by competitive polymerase chain reaction. *J. Clin. Microbiol.* 31, 2634–2640.
- Melchers, W., Zoll, J., van Kuppeveld, F., Swanink, C., Galama, J., 1994. There is no evidence for persistent enterovirus infections in chronic medical conditions in humans. *Rev. Med. Virol.* 4, 235–243.

- Melnick, J.L., 1996. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), *Virology*, 3rd edn. Lippincott-Raven, Philadelphia, PA, pp. 655–712.
- Muir, P., Archard, L.C., 1994. There is evidence for persistent enterovirus infections in chronic medical conditions in humans. *Rev. Med. Virol.* 4, 245–250.
- Muir, P., Nicholson, F., Jhetam, M., Neogi, S., Banatvala, J.E., 1993. Rapid diagnosis of enterovirus infection by magnetic bead extraction and polymerase chain reaction detection of enterovirus RNA in clinical specimens. *J. Clin. Microbiol.* 31, 31–38.
- Neumann, A.U., Lam, N.P., Dahari, H., Gretch, D.R., Wiley, T.E., Layden, T.J., Perelson, A.S., 1998. Hepatitis C viral dynamics in vivo and the antiviral effect of interferon- α therapy. *Science* 282, 103–107.
- Nicholson, F., Meeto, G., Aiyar, S., Banatvala, J.E., Muir, P., 1994. Detection of enterovirus RNA in clinical samples by nested polymerase chain reaction for rapid diagnosis of enterovirus infection. *J. Virol. Methods* 48, 155–166.
- Nicholson, F., Ajetunmobi, J.F., Li, M., Shackleton, E.A., Starkey, W.G., Illavia, S.J., Muir, P., Banatvala, J.E., 1995. Molecular detection and serotypic analysis of enterovirus RNA in archival specimens from patients with acute myocarditis. *Br. Heart J.* 74, 522–527.
- Ouyang, X., Zhang, H., Bayston, T.A., Archard, L.C., 1995. Detection of coxsackievirus B3 RNA in mouse myocarditis by nested polymerase chain reaction. *Clin. Diagn. Virol.* 3, 233–245.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Ren, R., Constantini, F.C., Gorgacz, E.J., Lee, J.J., Racaniello, V.R., 1990. Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. *Cell* 63, 353–362.
- Rotbart, H.A., 1990. Enzymatic RNA amplification of the enteroviruses. *J. Clin. Microbiol.* 28, 438–442.
- Rotbart, H.A., Sawyer, M.H., Fast, S., Lewinski, C., Murphy, N., Keyser, E.F., Spadaro, J., Kao, S.-Y., Loeffelholz, M., 1994. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. *J. Clin. Microbiol.* 32, 2590–2592.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Sambrook, J., Fritsch, E.F., Maniatis, T. (Eds.), *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 7-1–7-87.
- Strongwater, S.L., Dorivini-Zis, K., Ball, R.D., Schnitzer, T.J., 1984. A murine model of polymyositis induced by coxsackievirus B1 (Tucson strain). *Arthritis Rheum.* 27, 422–433.
- Webb, S.R., Loria, R.M., Madge, G.F., Kibrick, S., 1976. Susceptibility of mice to group B coxsackievirus is influenced by the diabetic gene. *J. Exp. Med.* 143, 1239–1248.
- Wei, X., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H., Saag, M.S., Shaw, G.M., 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373, 117–122.
- Wessley, R., Klingel, K., Santana, L.F., Dalton, N., Hongo, M., Lederer, W.J., Kandolf, R., Knowlton, K.U., 1998. Transgenic expression of replication-restricted enteroviral genomes in heart muscle induces defective excitation–contraction coupling and dilated cardiomyopathy. *J. Clin. Invest.* 102, 1444–1453.
- Woodruff, J.F., 1970. The influence of post-weaning undernutrition on coxsackievirus B-3 infection of adult mice. I. Viral persistence and increase in severity of lesions. *J. Infect. Dis.* 121, 137–163.
- Yoon, J.-W., Onodera, T., Notkins, A.L., 1978. Virus-induced diabetes mellitus: XV. Beta cell damage and insulin-dependent hyperglycemia in mice infected with coxsackie virus B4. *J. Exp. Med.* 148, 1068–1080.
- Zhang, H., Yousef, G.E., Ouyang, X., Archard, L.C., 1994. Characterization of a murine model of myocarditis induced by a reactivated coxsackie B3. *Int. J. Exp. Pathol.* 75, 99–110.