Full Title

Quantitative genomic and anti-genomic human enterovirus RNA detection in explanted heart tissue samples of patients with end-stage idiopathic dilated cardiomyopathy

Running title

Genomic and anti-genomic enterovirus RNA in heart tissue.

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Abstract:
Standardized one-step real-time RT-PCR assay detected enterovirus RNA in cardiac biopsies from 4 out of 20 patients suffering from idiopathic dilated cardiomyopathy (IDCM). The median viral load was 287 copies per microgram of total extracted nucleic acids with positive- to negative-strand RNA ratios ranging from 2 to 20. These results demonstrate enterovirus persistence in the heart of IDCM patients characterized by low viral loads and low positive- to negative RNA ratios.

Key-words: enterovirus, real-time RT-PCR assay, RNA quantitation, viral persistence, idiopathic dilated cardiomyopathy.
Enteroviruses (EV), especially group B coxsackieviruses (CVB), are considered to be a common cause of acute myocarditis in children and young adults, a disease which is a precursor to 10-20% chronic myocarditis cases as well as dilated cardiomyopathy (prevalence = 7 cases / 100 000) characterised by an increase in both myocardial mass and volume compromising cardiac contractility and ultimately resulting in poor left ventricular function (8, 9, 12, 14). Dilated cardiomyopathy is considered to be the second leading cause of heart transplantation worldwide after ischemic heart disease. The concept that endomyocardial persistent HEV infection could be the aetiologic cause of a subset of idiopathic dilated cardiomyopathy (IDCM) cases is supported by the detection in up to 35% of explanted heart tissues from end-stage IDCM patients of HEV RNA and viral capsid protein VP1 in the absence of cultivable viruses by classical techniques (2, 3, 13). However, the viral molecular mechanisms involved in the progression of acute myocarditis to chronic myocarditis and subsequently to IDCM are currently poorly understood. In 2008, Chapman et al. (7) reported for the first time in human heart a CVB2 strain presenting with genomic 5’ terminal deletions (TD). These mutations induced significant slowing of viral replication and a lowering virus titer in cell culture models where an absence of classical cytopathic effects was associated with an abnormal positive- to negative-strand viral RNA ratio close to one rather than the high positive- to negative-strand ratios normally seen in wild-type virus infected cells (11). These findings demonstrated the existence of unexpected EV-TD genomic populations in clinical samples, thereby a mechanism by which EV can persist in heart long after the acute infection cycle.

In this context, the aim of the present study was to validate a sensitive and standardized one-step real-time RT-PCR assay capable of detecting and quantifying EV RNA (both positive- and negative-strands) of wild-type as well as TD EV strains in cardiac tissue samples obtained from IDCM patients. Reverse transcription and PCR were carried out using the superscript III platinum one-step qRT-PCR kit (Invitrogen, Life technologies, Saint-Aubin, France) containing 200nM of forward (5'-CCCTGAATGCGGCTAATCC-3' 456-474) and reverse primers (5'-ATTGTCACCATAAGCAGCCA-3' 582-601), and 100nM of probe (FAM 5'-AACCGACTACTTTGGGTGTCCGTGTTTC-3' TAMRA 539-566) (17). Reverse transcription was performed at 55°C for 45 min, the RT heat inactivated at 95°C for 2 min, then the cDNA amplified in 45 cycles as follows: denaturation at 94°C for 15 seconds, annealing at 63°C for 1 minute and an extension step at 68°C for 30 seconds. To validate
detection and quantitation of EV in cardiac biopsies, serial dilutions of positive- and negative-strand transcripts of wild-type and TD CVB3 clones kindly provided by N.M. Chapman (University of Nebraska Medical Center, Omaha NE USA) were tested. Both wild-type and TD positive- and negative-strand RNA molecules were detected in vitro by the one-step RT-PCR assay. The sensitivity and the reproducibility of the RT-PCR assay were then determined using serial 10-fold dilutions of the transcripts ranging from $3 \times 10^6$ to 30 copies diluted in DNA/RNA extracts of EV negative cardiac tissues with similar results in terms of standard curve regardless of the transcripts used for the experiments. The threshold of viral RNA detection was found repeatedly to be 30 copies per well for both wild-type and TD EV RNA transcripts for both positive and negative strand RNAs. This sensitivity of detection is crucial because TDs replicate slowly and to low titer (7, 10, 11). This will be important for future work and may explain past inability to detect RNA despite viral capsid protein VP1 detection (1).

Being able to quantitate both positive- and negative-strand viral RNA titers in clinical samples would permit the measurement of the positive- to negative-strand viral RNA ratio to facilitate an assessment of EV persistence mechanisms potentially associated with the development of IDCVM. To validate the specific negative-strand viral RNA isolation, a time course of CVB3 replication model was performed in MA-104 cells (rhesus monkey kidney cell line) infected at a multiplicity of infection of 1 TCID50 with CVB3 prototype strain Nancy and collected at 0, 2, 8, 10, 14 and 24h post-infection. Negative-strand RNA was isolated from total RNA molecules by annealing a biotinylated negative-strand specific primer (E3REV; 5'-GGAACCGACTACTTTGGGTGTCCGTG-3') and binding to streptavidine-labelled magnetic beads (Invitrogen, Life Technologies, Saint-Aubin, France) (7, 11). Purified negative-strand and total viral RNA molecules were quantified with the one-step real-time RT-PCR assay using serial dilutions of the transcripts for the generation of the standard curves. The positive- to negative-strand viral RNA ratio was then determined using the following calculation ($[\text{total EV RNA} - \text{negative-strand EV RNA}]$/negative-strand EV RNA). Positive/negative-strand viral RNA ratios assessed at 8h and 24h post-infection were 96 and 85, respectively, consistent with the high positive- to negative-ratios normally seen in wild-type virus infected cell models (Fig. 1).

Ninety-nine paraffin-embedded cardiac biopsies collected by the department of pathology of the European Hospital Georges Pompidou (Paris, France) were investigated. These samples were explanted heart tissue samples collected between the years 2000-2009 from a population of 20 adult patients demonstrating histological findings compatible with
IDCM (4). As control samples, heart tissue was selected from 10 adult patients who had died accidentally or by suicide (CHU, Reims France). EV RNA was detected in 6 (9%) of the 99 samples analysed corresponding to 4 (20%) of the 20 IDCM patients whereas no viral RNA was detected in the heart tissue samples taken from the 10 control subjects. These results confirm those published by our group and others that have reported EV RNA detection up to 35% of cardiac explants in subjects with IDCM (2, 5, 9, 13). The median viral load assessed was 287 copies/μg of total extracted nucleic acids ranging from 10 to 3368 (Table 1). These results are in agreement with those obtained from murine heart tissues chronically infected with CVB3 (3, 15). By contrast, this median viral load was 500 times lower than that obtained in our model of MA-104 cells (1.83x10^5 copies) infected with the wild-type CVB3 Nancy strain which produce an acute and lytic viral infection (Fig. 1).

Negative-strand RNA molecules were detected in the 4 EV positive IDCM patients. The ratios observed in explanted heart tissues samples ranged from 2 to 20, indicating several levels of viral genome replication activities, though all were consistent with a chronic viral infection (Table 1). These ratios were closer to those reported (4/1) in a persistent CVB infection of muscle in mice afflicted with chronic inflammatory myopathy (16). Moreover, they are also consistent with CVB-TD strains infection previously reported at present low levels of viral replication with positive- to negative-strand viral RNA ratios of 2 to 3 in chronically infected mice and humans (7, 11). In contrast, the experimental curve obtained in the MA-104 cell infection model revealed an average positive/negative-strand viral RNA ratio of 47/1 in accordance with ratios previously observed (40/1 to 75/1) in similar cellular models of active EV infections (11, 16).

In summary, a standardised one-step real-time RT-PCR assay was validated for wild-type and TD EV strains detection in human heart tissues. This reliable analysis method detected EV RNA in up to 20% of IDCM patients, in agreement with previously published studies. Moreover, this quantitative molecular tool provided information fundamental to the diagnosis and understanding of EV cardiac infections by demonstrating low viral loads and low positive- to negative-strand RNA ratios in the human heart samples analysed, which were clearly consistent with chronic/persistent viral infection (2, 6, 13). Further experiments of molecular cloning and sequencing of the 5’NTR of the EV strains detected in IDCM patients are underway and will help identify and characterise the potential presence of EV strains presenting with terminal deletions in the 5’ NTR.
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Fig. 1. Generation of total viral RNA, negative-strand viral RNA and the positive- to negative-strand viral RNA ratios during the first 24h of CVB3 replication in MA-104 cells. Copy numbers of normalised total and negative-strand CVB3 RNA depicted with the histograms were plotted on a logarithmic scale on the left Y-axis while the positive- to negative-strand viral RNA ratios depicted by black squares are indicated on the right Y-axis.
Fig. 1

HEV RNA copy per µg of total extracted DNA/RNA

<table>
<thead>
<tr>
<th>Time (hours post-infection)</th>
<th>Total RNA</th>
<th>Negative-strand RNA</th>
<th>Positive-to-negative-ratio of viral RNA</th>
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<tbody>
<tr>
<td>0</td>
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<td></td>
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<tr>
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</tbody>
</table>

Postive-to-negative-strand viral RNA ratio
Table 1. Total and negative-strand EV RNA loads and the deduced positive- to negative-strand viral RNA ratios assessed in the 4 EV positive IDCM patients.

<table>
<thead>
<tr>
<th>Patient Nb</th>
<th>Cardiac anatomic location</th>
<th>Total EV RNA load (copies/µg of total extracted RNA)</th>
<th>Negative-strand EV RNA load (copies/µg of total extracted RNA)</th>
<th>Positive- to negative-ratio of viral RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Septum</td>
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<td>40</td>
<td>2.2</td>
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<tr>
<td>2</td>
<td>Septum</td>
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<td>120</td>
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<td>3</td>
<td>Right ventricle</td>
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<td>2.5</td>
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<tr>
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<td>Left ventricle</td>
<td>2108</td>
<td>347</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>3368</td>
<td>160</td>
<td>20</td>
</tr>
</tbody>
</table>

 Nb = number