Viral genome detection and quantitation in explanted heart tissues of end-stage dilated cardiomyopathy adult patients using broad-range PCR amplification coupled with mass spectrometry analysis.

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Y. N'GUYEN[1*], F. RENOIS[1], N. LEVEQUE[1], M. PICARD-MOREAU[2], P. BRUNEVAL[3], P. FORNES[4], L. ANDREOLETTI[1]

* ynguyen@chu-reims.fr

[1]Clinical and Molecular Virology Unit, CHU and EA-4684 Medicine Faculty of Reims, France;

[2]Abbott GmbH & Co. KG, Wiesbaden, Germany;

[3] Pathology Department, HEGP university hospital and Paris VI University, Paris, France;

[4]Pathology Department, University Hospital and EA-4684 Cardiovir SFR-CAP sante, Faculty of Medicine, Reims, France.

Introduction and purpose

- Several human viruses are suspected to be etiological causes of idiopathic dilated cardiomyopathy (DCM) [1]. The pathophysiological importance of common viruses detected in cardiac samples remains to be explored [2]. To gain insight into the pathogenesis of virus-associated DCM, detection of a broad panel of virus species combined with the measurement of the viral load in heart tissue is required to distinguish active from chronic or persistent cardiac viral infections [3].
- We identified cardiac viral infections and we assessed the viral load levels in DCM patients, using a new technology that couples PCR amplification to electrospray ionization/time-of-flight mass spectrometry analysis (PCR-MS) [4].

Patients & Controls

▪**Patients**: Explanted heart tissues samples (n=67) were retrospectively obtained from 31 adult patients (M/F= 23/8; mean age= 44.3 years (SD=12.2)) with idiopathic DCM according to the classification of the European Society of Cardiology [5]. For each patient, a mean number of 2.1 (SD=1.3, range = 1-7) large heart tissue samples were fixed in 10% neutral buffered formalin and paraffin-embedded.

▪**Healthy heart controls:** For comparison, 14 adult patients who had died accidentally or by suicide and who were autopsied between 2002 and 2009 (Reims, France) and not exhibiting any known cardiac pathology were selected (not shown). For each of these controls, two ventricular biopsies sampled at the time of necropsy were analyzed.

▪ **Viral strains:** Coxsackievirus B1, B3-B6 (CVB) strains (ATCC numbers: VR-1032, VR-1034, VR-1035, VR-1036 and VR-1037) were used as positive control for PCR-MS genotyping identification.

Methods

•**PCR and high-resolution mass spectrometry analyses (PCR-MS**) were carried out using the Ibis T5000 Biosensor (Ibis Biosciences®, Carlsbad, CA, USA) at the Athogen® laboratory (Irvine, CA, USA) on total nucleic acids extracts. The viral assay used for analysis was the Ibis Sterile Fluids Viral Surveillance Kit (Ibis Biosciences) consisting of primer pairs targeted to Adenoviruses, *Alphavirus*, *Herpes* viruses (with the notable exception of HHV-6), Parvovirus B19 (PVB19), *Flavivirus* and Enterovirus (EV) (Table1).The assay-specific database contains more than 80 viral species. Semi-quantitation was obtained by quantifying the total number of amplicons against an internal calibrant that is included in every well with a known copy number.

•Results were compared to those obtained by classical real time quantitative PCR ((RT)-qPCR)assays and expressed as the number of genomic DNA or RNA copies by µg of total extracted nucleic acids [6-8].

•A mixed infection was defined as the presence of both EV and PVB19 in a same sample or the presence of an EV positive sample and a PVB19 positive sample taken from the same patient.

•**Statistical analyses:** The Spearman's rank correlation was used to evaluate linear associations between viral load values obtained with (RT)-qPCR assays and the PCR-MS system. Fischer's test, Mann-Whitney and Kappa tests were carried out with SAS software, version 8.2 (SAS Institute, Cary, NC, USA). Results were considered as statistically significant for two-sided *P* values <0.05.

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Results

•PCR-MS identified single or mixed EV and PVB19 infections in 27 (40.2%) of 67 samples, whereas (RT)-qPCR assays identified viral infections in 26 (38.8%) samples. Mixed EV-PVB19 infection was evidenced by PCR-MS and (RT)-qPCR assays in 4 and 2 samples respectively (Fig.1A). No viral species other than PVB19 and EV was identified using the new PCR-MS technology in analyzed samples. PCR-MS results correlated well with EV and PVB19 detection by (RT)-qPCR assays, kappa tests= 0.85 [0.72–1.00; 95%] and 0.82 [0.66–0.99; 95%], respectively.

•Using PCR-MS, viral genomes were detected in heart tissues of 15 (48.3%) of the 31 IDCM patients, whereas (RT)-qPCR assays identified viral infections in 16 (51.6%) of the study patients (Fig.1B)

Table 1. Primer pairs used for the viral detection using the PCR –MS analysis.

Primerpair	Target	Fwd Primer	Rev Primer
Adenovirus 1 943	HEX	TTGCAAGATGGCCACCCCATC GAT	TGTGGCGCGGGCGAACTGCA
Adenovirus 2 769	HEX	TCACCAACACCTACGAGTACAT GAA	TGGTTGAAGGGATTTACGTTG TCCAT
Alphavirus 1 966	NC	TCCATGCTAATGCTAGAGCGTT TTCGCA	TGGCGCACTTCCAATGTCCAG GAT
Alphavirus 2 2499	NC	TGCCAGCIACAITGTGIGAICAIA TGAC	TGACGACTATICGCTGGTTIAG CCCIAC
Enterovirus 1 3760	5' UTR	TGGCTGCGTTGGCGGCC	TAGCCGCATTCAGGGGCCGG A
Enterovirus 2 3758	5' UTR	TTCCTCCGGCCCCTGAATG	TGAAACACGGGCACCGAAAGT AGT
Flavivirus 1 2215	NC	TAGCCGAGCCATCTGGTACAT GTGG	TCTCTGGAAAGCCAGTGGTCT TCATT
Flavivirus 2 2217	NC	TGTGTCTACAACATGATGGGAA AGAGAGA	TGCTCCCAGCCACATGTACCA
Alphaherpesvirus 1 3398	DNA-Pol	TCTGGAGTTTGACAGCGAATT CGAG	TGTTGTAACCGGTGGCGAACT CGGG
Alphaherpesvirus 2 3399	DNA-Pol	TCTGGAGTTTGACAGTGAATTC GAG	TAACTCGGGGGCGTACTGTTT
Betaherpesvirus 1 3379	DNA-Pol	TCGCGCGCAGGTGGGC	TGGCCCCCGCCTCGTAGTG
Betaherpesvirus 2 3377	DNA-Pol	TGACAAGGAGCAGCTGGCACT CAA	TAGCATGTCGCGACCTATGCG GGT
Gammaherpesvirus 1 3407	DNA-Pol	TCGTCCCCATCGACATGTAC	TACTGTGTCCAGCTTGTAGTC TGA
Gammaherpesvirus 2 3405	DNA-Pol	TAAGCAGCAGCTGGCCATCAA	TGCCACCCCCGTGAAGCCGT A
Parvovirus 1 3110	NS1	TGGGCCGCCAAGTACTGGAAA AAC	TGTTTTCATTATTCCAGTTAAC CATGCCATA
Parvovirus 2 3118	VP1	TTACACAAGCCTGGGCAAGTT AGC	TCCTGAATCCTTGCAGCACTG TC

A- positive samples

	(RT)-qPCR +	(RT)-qPCR -
EV		
PCR-MS +	13	1
PCR-MS -	2	51
PVB19		
PCR-MS +	13	4
PCR-MS -	0	50

B- positive cases

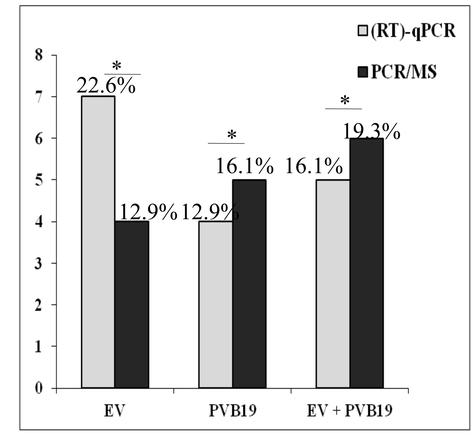

Figure 1. Viral findings obtained in 67 heart samples taken from 31 patients with DCM using classical (RT)-qPCR and PCR-MS analysis. * *P*>0.05 according to Fischer 's exact test.

- Only two of 14 healthy heart control subjects were positive for the detection of viral DNA genome that was identified as PVB19, with viral loads of 90 and 120 genomes per microgram of extracted nucleic acids (not shown).
- As depicted in Fig 2A and 2B, the estimated levels of EV and PVB19 viral loads were not significantly different between the two molecular techniques (median values of 550 [178–3200] vs. 385 [50–2108] EV copies/mg: *P*= 0.26; 486 [80–1157] vs. 504 [186–2730] PVB19 copies/mg; *P*=0.34). These moderate median viral load values obtained for EV and PVB19 were compatible with persistent cardiac infections in DCM patients.
- Viral loads obtained by PCR-MS correlated well with those obtained by (RT)-qPCR assays (Fig.2C and 2D).

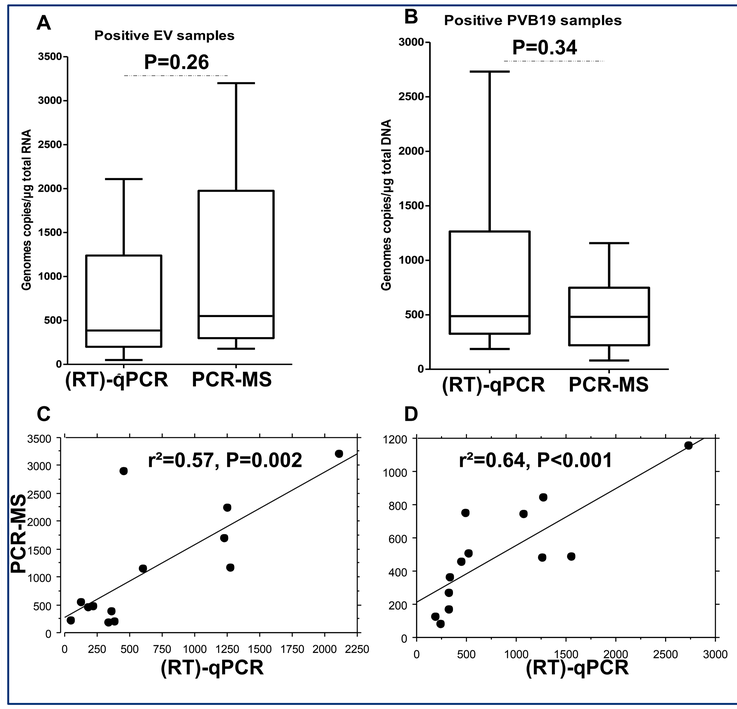

Figure 2. Viral load levels in cardiac samples obtained by the new PCR-MS analysis comparatively to those obtained by classical (RT)-qPCR assays.

Panels A and B: Box plots demonstrating the distribution (median and range values) of the viral loads levels (Enteroviruses (EV); (panel A); and Parvovirus B19 (PVB19) (panel B)) measured by (RT)-q PCR assays and by the new PCR-MS system.

Panels C and D: Correlation curves obtained by the comparison of the estimated viral load levels obtained by (RT)-qPCR assays and by the new PCR-MS system for EV (panel C) and PVB19 (panel D) strains in heart tissue samples.

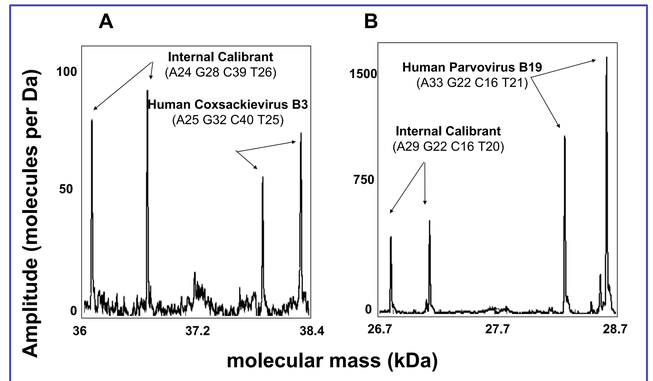

Figure 3. Examples of mass spectra obtained from the PCR-MS analysis for one Enterovirus positive heart sample (Panel A) and for one Parvovirus B19 positive heart sample (Panel B).

- Using the PCR-MS, a mass spectra was obtained from amplified viral DNA/RNA fragments in 27 (40.2%) of the 67 heart samples allowing identification of 31 single or multiple viral heart infections by comparative analyses of the base-composition signatures. Figure 3 shows examples of mass spectra of single infections in two distinct heart samples. Positive genotyping CVB strain controls provided by ATCC were included in each serial assay analysis; all were successfully identified as EV genus and Coxsackieviruses B. Only the reference CVB5 strain was misidentified as CVB3 strain (data not shown).

Conclusions

▪ We identified single or mixed EV and PVB19 cardiac infections as leading potential causes of DCM. The low viral load levels were compatible with chronic persistent cardiac infections.

▪The PCR-MS analysis appeared to be a valuable tool to rapidly detect and semi-quantify common virus in cardiac tissues and may be of major interest to better understand the role of viruses in unexplained cardiomyopathies .

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