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SHORT COMMUNICATION

Low prevalence of Gemycircularvirus DNA in immunocompetent and immunocompromised subjects

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SUMMARY

Gemycircularviruses (GemyCV) are a vast array of viruses belonging to the *Genomoviridae* family. Prevalence and pathogenesis in humans are still poorly understood. Different GemyCV species were investigated in 661 Italian subjects by species-specific PCRs. Only the GemyCV-C1c species was detected, with low prevalence and the highest rate in HIV immunosuppressed patients.

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Widespread groups of uncultured viruses discovered by viral metagenomics remain unclassified, particularly the circular replication-associated protein encoding single-stranded (CRESS) DNA viruses (Simmonds *et al.,* 2017). CRESS DNA viruses are abundant component of the human virome, but are often present in infected hosts at levels too low to prevent detection and metagenomic analysis. Therefore, their real prevalence needs to be confirmed using virus specific PCR protocols. *Genomoviridae,* an acronym name derived from *ge*minivirus-like, *no mo*vement protein, is a family of single-stranded DNA viruses with small genome (2.2-2.4 kilobases in length) encoding two opposite open-reading frames. These probably code for a capsid protein (CP) and a spliced rolling-circle replication-associated protein (Rep). In Rep-based phylogenies, genomoviruses and plant viruses of the family Geminiviridae form two sister clades (Krupovic *et al.,* 2016; Varsani *et al.,* 2017). Nine genera are currently recognized in this family. The genus Gemycircularvirus (GemyCV), which includes 73 species, stands for *Ge*mini-like *my*co-infecting circular virus from the prototype strain that was found in fungi in 2010. Members of this genus display 44% diversity (Yu *et al.,* 2010). *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1, the prototype species of the genus GemyCV, is the only isolate of the family cultivated so far (Krupovic *et al.,* 2016). The other genomoviruses have been discovered using metagenomics techniques (Varsani *et al.,* 2017).

Key words:
CRESS DNA virus; Gemycircularvirus; Genomoviridae; HIV; transplantation; healthy donors.

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Related viruses have been subsequently identified in animal blood and stools, raw and treated sewage, and insects and plant material, suggesting that GemyCV is a large group of viruses exhibiting considerable genetic diversity. The presence of these viruses was recently extended to humans after GemyCV sequences were identified in the serum and post-mortem brain of a multiple sclerosis patient in 2014 (Lamberto *et al.,* 2014), a French HIV-positive blood donor in 2015 (GemyCV-C1c) (Uch *et al.,* 2015), the cerebrospinal fluid (CSF) sample of a child with unexplainable encephalitis in 2015 (GemyCV-GeTz1) (Zhou *et al.,* 2015), feces from unexplained cases of diarrhea from Brazil (GemyCV-BZ1, and -BZ2) (Phan *et al.,* 2015), CSF of three Sri Lankan patients with unexplained encephalitis in 2016 (GemyCV-SL1, -SL2, and -SL3) (Phan *et al.,* 2015), and one fractionated plasma pool from healthy European donors (GemyCV-DB1 and -DB2) (Zhang *et al.,* 2016). In the last of these studies, the viral concentration estimated by digital PCR averaged 10^6 copies/mL, assuming that the virus was present in a single donor (Zhang *et al.,* 2016). Thus, from all these studies it was inferred that the prevalence of GemyCV is low. This conclusion is corroborated by another survey that found no GemyCV-C1c in 128 HIV-positive plasma samples and 256 HIV-negative healthy donors from southeastern France (Uch *et al.,* 2015). However, it is possible that other GemyCV-C1c-related sequences are too divergent to be detected by or are below the sensitivity threshold of current molecular assay. Thus, universal and highly sensitive GemyCV PCR systems are under development.

A total of 661 Italian subjects were examined (405 males, 256 females; mean age ± standard deviation: 41±20). The first group consisted of 290 unselected healthy donors whose plasma had been submitted to our laboratory to investigate their vaccination status. The other 371 patients were chosen on the basis of specific clinical diagnoses and tested on disease-specific biological samples,

Table 1 - *PCR oligonucleotides used for amplification of 9 human GemyCV species.*

GemyCV species detected (Oligonucleotide)	*Sequence (5'-3')*	*Gene localization*	*Position (nt) a*	*PCR product length*
GemyCV-C1c				
(C1cS1)	GTT CCG ATT TCC ACC GAT TTC CA	CP	281-303	
(C1cR1)	TGC AAC TCA ACA GTT TCT TGG AG	CP	464-486	
(C1cS2)	GATTTCCAACAACAGAATCCTGG	CP	296-318	
(C1cR2)	CTTGGAGGCCTTTGACGAAACAC	CP	448-470	175 bp
GemyCV-GeTz1				
(Gmv435FO)	GGACGGTAGCGATGCTCGGC	Rep	1371-1390	
(Gmv435RO)	TCGCGATGGCGGAATTCACCT	Rep	1711-1731	
(Gmv435FI)	TGCTCGGCATTGTGTGAAGG	Rep	1395-1414	
(Gmv435RI)	ACACCATCCGAACACCAGCC	Rep	1625-1644	250 bp
GemyCV-DB1				
(Gemy1F1)	TGATCCCGGTTGGGGACTCGT	Rep	1418-1438	
(Gemy1R1)	GCAGAGGTTGCATTGTCGCACG	Rep	1831-1852	
(Gemy1F2)	CGGATCCAGCGCCTTGCATGT	Rep	1512-1532	
(Gemy1R2)	CGCACGAGAGTCACACCTGGA	Rep	1816-1836	325 bp
GemyCV-DB2				
(Gemy2F1)	CCCCAACGCTCGAGACACGC	CP	261-280	
(Gemy2R1)	GGCATCCACCCTCGGTGGTCT	CP	479-499	
(Gemy2F2)	TCGAGACACGCGCACCCCTA	CP	270-289	
(Gemy2R2)	GGCAGGAACGTTGCCGGAGTT	CP	445-465	196 bp
GemyCV-SL1, -SL2, -SL3, -BZ1, -BZ2				
(SL Gemy_F1)	GTGGTAATGGTCGTCGGTATT	CP	170-190	
(SL Gemy_R1)	CCTCATCATTCGTAGTACGCAATCTCA	CP	788-814	
(SL Gemy_F2)	AGTCCTGAATGTTTCCACTCG	CP	240-260	
(SL Gemy_R2)	CAAGCGTTCCCTCGAAAATGAC	CP	694-715	476 bp

[a]Nt positions are according to isolate KP987887, KT363839, KP974693, KP974694, KP133075, for GemyCV-C1c, -GeTz1, -DB1,- DB2 and -SL1, respectively.

e.g., 100 plasma samples from HIV-positive patients, 65 plasma samples from hematopoietic stem cell transplant (HSCT) recipients, 35 CSF samples from patients with meningitis/encephalitis disorders, 121 fecal samples from patients with gastroenteritis symptoms, and 50 nasopharyngeal aspirates from patients with acute respiratory diseases. All samples had been obtained 1 week to 2 years prior to GemyCV DNA testing and stored frozen at -80°C until use. All research was conducted under institutional review board-approved protocols (CEAVNO, Pisa University Hospital). Viral DNA was extracted from 200 ul of plasma, liquor, nasopharyngeal aspirates, and purified fecal extracts by using QIAamp DNA Mini kit (QIAGEN, Chatsworth, CA) manually or associated with QIAsymphony SP/AS instrument (QIAGEN, Chatsworth, CA). A domain of the CP or Rep genes of GemyCV genome was amplified by different nested PCR protocols, each of them employing primers specific for amplification of each of 9 human GemyCV species (C1c, GeTz1, SL1, SL2, SL3, BZ1, BZ2, DB1, and DB2) (Uch *et al.,* 2015; Zhou *et al.,* 2015; Phan *et al.,* 2015; Zhang *et al.,* 2016) (*Table 1* and *Figure 1*). To minimize sample handling contamination risk, DNA extraction, PCR amplification, and electrophoresis analysis were conducted in separated rooms. Appropriate negative controls were added during DNA extraction and PCR amplification. To assure reproducibility of the results obtained excluding the presence of environmental and endogenous DNA contamination, samples found to be positive were tested again starting from a second round of DNA extraction. GemyCV-C1c was the only species detected. *Table*

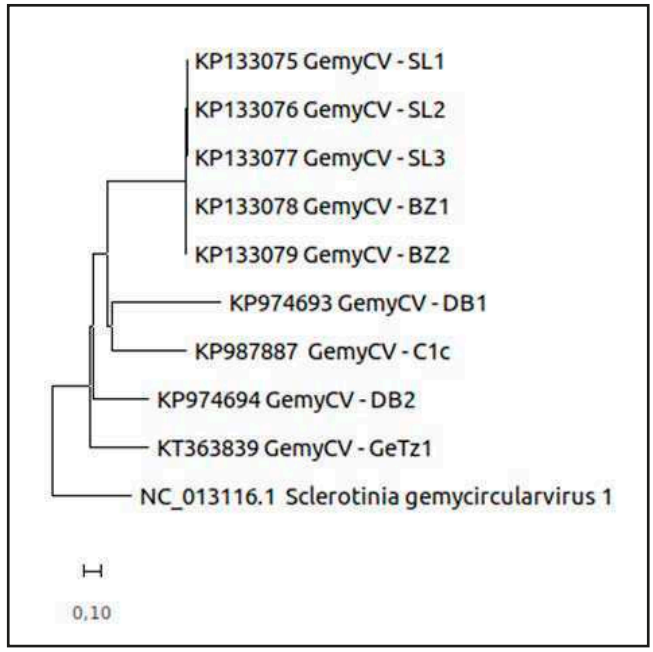

Figure 1 - *Phylogenetic analysis of human GemyCV isolates based on full-length nucleotide sequence of the viral genome. The evolutionary history was inferred using the Neighbor-Joining method. Isolate NC_013116.1 was used as an outgroup. The evolutionary distances were computed using the Maximum Composite Likelihood method, and the bar indicates the number of nucleotide substitutions per site. Evolutionary analyses were conducted in MEGA X.*

Table 2 - *Prevalence of GemyCV-C1c DNA in groups of subjects.*

Group	*No. examined*	*Age (years ± SD)*	*Male/ Female*	*Sample type*	*GemyCV-C1c DNA positive (%)*
Healthy donors	290	42 ± 20	172 / 118	Plasma	1 (0.3)
Patients with:					
HIV infection	100	45±18	56/44	Plasma	4 (4)
HSCT	65	43±25	40/25	Plasma	1 (1.5)
Meningitis/encephalitis	35	50±17	19/16	Liquor	0 (0)
Gastroenteritis	121	33±23	86/35	Faeces	0 (0)
Acute respiratory disease	50	35±16	32/18	Nasopharyngeal aspirates	1 (2)
Total	*661*	*41±20*	*405/256*	/	*7 (1)*

SD, standard deviation.
HSCT, hematopoietic stem cell transplant.

2 summarizes the results obtained. Sequence analysis of the amplified PCR segments (175 nucleotides) exhibited 100% nucleotide homology with GemyCV-C1c (GenBank accession no. KP987887).

Overall, the results showed that positivity of GemyCV-C1c DNA was 1% with the virus detectable in plasma samples and nasopharyngeal aspirates, thus demonstrating that this GemyCV species, unlike the other species, circulates in the Italian population and can be found in different biological specimens of an infected host. This latter finding could come out on the side of genuine GemyCV replication in human cells; however, a different origin of the virus (i.e., from fungal infection) cannot be completely excluded. No indications were found in the study subjects that GemyCV-C1c prevalence was especially frequent among individuals belonging to a given sex or age group. Of 7 GemyCV-C1c positive samples, 5 were from males, and 2 from females (mean age ± standard deviation: 52±19 years). However, because the study population was gender unbalanced (405 males versus 256 females, *Table 2*), further studies are needed to more clearly exclude the existence of an association between GemyCV prevalence and gender. More interestingly, although the overall prevalence of GemyCV-C1c viremia was very low, the virus was less frequently detected in healthy donors than in patients with a specific disease. This latter group of people was selected on the basis of either clinical conditions indicating that they had a dysfunction of their immune system (patients with HIV infection or receiving HSCT) or the suspected viral nature of their diseases (meningitides/encephalitis, gastroenteritis, and acute respiratory illness). As shown in *Table 2*, GemyCV-C1c DNA carriers were more frequent among HIV positive patients (4%), thus suggesting that, as for other similar viruses (Maggi *et al.*, 2018), the immune system status of the infected host could influence the size of viral replica. However, GemyCV-C1c presence didn't correlate with HIV RNA load (data not shown), and only 1 of 65 HSCT recipients was positive for GemyCV-C1c DNA. Such findings prompt further investigations aimed at exploring GemyCV-C1c prevalence in immunocompromised hosts and to assess the role, if any, of the immune system in influencing GemyCV-C1c replication in humans. Further studies carried out using different PCR protocols will also be needed to confirm the prevalence of the other GemyCV species and to exclude an impact on their detection of the PCR approach used in this study.

Conflicts of interest

All authors declare that they have no conflicts of interest related to this manuscript.

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