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Frequent and Abundant Merkel Cell Polyomavirus Detection in Urban Wastewaters in Italy

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Abstract Viruses strongly associated with human cancer have recently been detected in urban sewages and other water environments worldwide. The aim of the present study was to assess the presence of Merkel cell polyomavirus (MCPyV), a newly discovered, potentially oncogenic human virus, in urban sewage samples collected at wastewater treatment plants (WTPs) in Italy. A total of 131 raw sewage samples were collected from 21 WTPs in nine Italian regions and analyzed by both qualitative (PCR/nested) and quantitative (Real-Time qRT-PCR) methods. Of these, 66 samples (50.3 %) were positive for MCPyV by the qualitative assay. Quantitative data showed high viral loads in wastewaters (mean, $1.5E + 05$ genome copies/liter). High concentrations of MCPyV were found in all WTPs under study, suggesting a wide circulation of the virus and thus the need for further studies to assess possible waterborne MCPyV transmission.

Keywords Merkel cell polyomavirus · Wastewater · Surveillance · Waterborne

Introduction

Polyomaviruses (PyVs) are non-enveloped viruses with a circular double-stranded DNA genome of approximately 5 kb, belonging to a family of DNA tumor viruses (Johne et al. 2011). A marked escalation in the rate of discovery of new human PyVs has occurred over the past 5 years. To date, 12 PyVs have been discovered in humans: BK virus, JC virus, KI virus, WU virus, Merkel cell polyomavirus (MCPyV), human polyomavirus-6 and 7 (HPyV6 and HPyV7), trichodysplasia spinulosa-associated PyV, HPyV9, HPyV10, Saint Louis polyomavirus (STLPyV), and HPyV12. In addition, a non-human, primate PyV—simian virus 40 (SV40)—seems to circulate in human populations as well (Moens et al. 2014). Not all of these viruses have been causally linked to diseases with absolute certainty (Dalianis and Hirsch 2013). Nevertheless, PyVs are known to infect different tissues and organs, usually causing subclinical infections in immunocompetent individuals, and serious diseases in immunocompromised hosts (Dalianis and Hirsch 2013). Merkel cell PyV, first identified in 2008 from the skin lesions of a patient affected by Merkel cell carcinoma (MCC) (Feng et al. 2008), has been linked with this rare and aggressive human cancer, a disease with a case mortality rate exceeding 30 %. Approximately 80 % MCC harbor MCPyV, indicating its prominent role in the development of the disease. MCPyV is classified by IARC as ''probably carcinogenic to humans'' (Group 2A) (Bouvard et al. 2012). The annual incidence of MCC is 0.6 per 100,000 persons and has tripled over the past two decades, partly due to increased awareness and improved diagnostic techniques.

Seroprevalence data suggest that MCPyV is widespread in human populations and that exposure begins early in life (Chen et al. 2014; Viscidi et al. 2011). Infections with MCPyV are common in human skin but the virus can be detected in several anatomical sites. Although MCPyV is strongly associated with MCC, presence of the virus is not sufficient to induce cancer; sun exposure (especially for

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fair-skinned individuals) and immunosuppression play a significant role in carcinogenesis (Prieto, et al. 2013). Even though virus infection is common, MCC is a very rare cancer.

Recently, oncogenic MCPyV was shown to be present in urban wastewaters in Spain: the virus was detected in the vast majority (89 %) of sewage samples, and even in river waters (29 %) (Bofill-Mas et al. 2010). The same research group later detected MCPyV in 50 % of river samples from Barcelona and Rio de Janeiro (Calgua et al. 2013). These studies indicate that the MCPyV is prevalent in the population and may be disseminated through contaminated water (Calgua et al. 2013).

Whether MCPyV circulates in water environments in Italy is unknown. The aim of the present study was thus to assess the presence of this virus in urban sewage samples collected at wastewater treatment plants (WTPs), so as to improve our knowledge of the circulation of MCPyV in the Italian population and environment, as well as our understanding of its possible modes of transmission.

Materials and Methods

We analyzed 131 raw sewage samples in the framework of a WTP-based environmental network previously established for the surveillance of noroviruses and other enteric viruses (La Rosa et al. 2014; La Rosa et al. 2010; Muscillo et al. 2013). The study covered 9 Italian regions, with 21 WTPs. Samples were collected on a monthly basis from January to December 2013, but due to partial compliance with our sampling schedule, only 131 samples were available for analysis. Figure 1 is a geographic information system (GIS) map of the WTPs under study. The map was created using Quantum GIS (QGIS) version 2.0.1 Dufour, an open source GIS licensed under the GNU General Public License. Further details (European Environmental Agency WTP code, Population Equivalents served by each WTP), are shown in Table 1. Wastewater samples were collected, handled, and analyzed as previously described (Muscillo et al. 2013). Briefly, untreated wastewater samples were divided into 2 × 40 ml aliquots upon arrival, and stored at −20 °C before use. One aliquot was seeded with a known amount of Murine Norovirus 1 (MNV-1), added to the samples as a sample process control. An aliquot of 20 ml was treated with 2 ml of 2.5 M glycine pH 9.5 and incubated in ice for 30 min; the solution was then treated with 2.2 ml chloroform and centrifuged at 5,000 rpm for 10 min. Viral nucleic acids were extracted from 10 ml of chloroform-treated samples, using the NucliSENS easy-MAG (BioMerieux, Marcy l'Etoile, France) semi-automated extraction system with magnetic silica, according to the manufacturer's instructions. Eluates (100 µl each) were
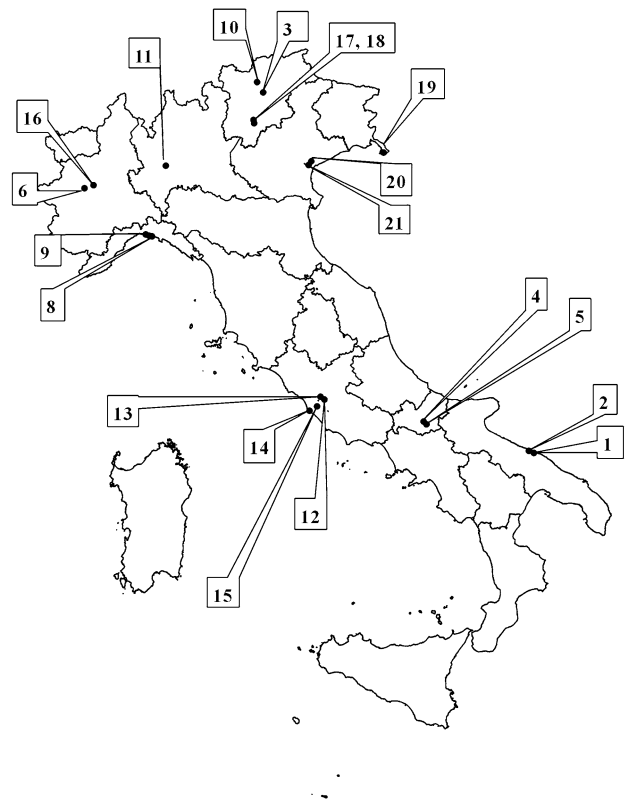

Fig. 1 GIS map of the WTPs under study

divided into small aliquots and subsequently frozen at −70 °C until analyzed.

For the detection of MCPyV, published primers targeting the small T-antigen coding sequence were used to amplify a 358-bp fragment in nested PCR (Sharp et al. 2009). PCR products were purified using a Montage PCRm96 Micro-well Filter Plate (Millipore, Billerica, Mass) and sequenced (Bio-Fab Research, Rome, Italy). Precautions were taken to prevent false-positive results: rooms for pre- and post-PCR procedures were physically separated with dedicated equipment, and reagents were prepared in large batches and stored in small aliquots.

DNA sequences were compared to the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database, using the NCBI Blast programme.

An initial screening by nested PCR with small T-antigen primer sets identified a strongly positive wastewater sample, which was used for the construction of the standard recombinant plasmid for absolute quantification. A 258-bp PCR product in the large T-antigen (LT) gene was amplified using newly designed primers (see Table 2) and cloned into pGEM-T vector (pGEM-T Vector System Promega) in order to construct a recombinant plasmid according to Goh et al. (2009). Plasmid DNA concentration was determined

Table 1 List of WTPs under study

ID	ID _EEA	Name and location	Capacity (P.E.)	Positive samples/analyzed samples
1	IT160000000065	Bari—Bari Est	389.000	4/7
2	IT160000000066	Bari—Bari Ovest	242.235	2/5
3	IT21000000000013	Bolzano	374.000	5/6
4	IT14Q90000002470	Campobasso—San Pietro	50.000	2/6
5	IT14Q90000002472	Campobasso—Scarafone	40.000	2/5
6	IT01000000000078	Collegno	267.000	8/10
7	IT070000000035	Genova—Quinto	68.000	0/1
8	IT070000000046	Genova—Valpocevera	125.000	3/3
9	IT070000000034	Genova—Punta Vagno	250.000	1/2
10	IT21000000000008	Merano	364.000	7/8
11	IT03160128000464	Milano—Peschiera B.	316.000	6/11
12	IT12000000000311	Roma—Nord	780.000	1/4
13	IT12000000000321	Roma—Ostia	350.000	1/2
14	IT12000000000304	Roma—Roma Est	920.000	5/7
15	IT12000000000317	Roma—Roma Sud	1.160.000	2/6
16	IT01000000000043	Torino—Castiglione T.	3.839.940	7/11
17	IT220000000064	Trento—Trento Nord	120.000	2/5
18	IT220000000065	Trento—Trento Sud	100.000	3/5
19	IT06000000000072	Trieste—Zaule	66.700	1/3
20	IT05000000000212	Venezia—Campalto	110.000	1/12
21	IT05000000000213	Venezia—Fusina	400.000	3/12
Total				66/131

by measuring the optical density at 260 nm. The DNA content in micrograms was converted to genomic copies using Avogadro's number and the number of nucleotide pairs in the plasmid. Using a TaqMan assay targeting the LT gene, we then performed an absolute quantification of the MCPyV copies contained in the samples, using an external calibration curve generated through 10-fold serial dilutions of the standard. Reactions were performed in triplicate in a 25 μL mixture, containing 12.5 μl of 2 × SensiMixT II Probe No-ROX Kit (Bioline), 400 nM (each) of forward and reverse primers, 100 nM of fluorescence-labeled probe, and 5 μL of DNA template. Cycling conditions were 95 °C for 10 min, 45 cycles at 95 °C for 15 s, and 60 °C for 1 min. Real-time PCRs were carried out in a MiniOpticon Real-Time PCR System (Bio Rad) with CFX Manager software control. Run acceptability was defined as a correlation coefficient ($R2$) >0.98 and a slope between −3.6 and −3.1, corresponding to reaction efficiencies between 90 and 110 %, according to the equation: Efficiency = 10(−1/slope)−1.

The sample process control (MNV-1), added in order to monitor nucleic acid extraction and the presence of potential inhibitors, was detected using previously published primers (Muscillo et al. 2013).

Results

A total of 131 sewage samples were tested for MCPyV by qualitative nested PCR assay using primers targeting the small T-antigen coding sequence. Positive samples were subsequently quantified using a quantitative TaqMan assay in the LT gene. Sixty-six of 131 samples (50.3 %) were found to be positive for MCPyV by the qualitative nested PCR assay described above, all confirmed by sequencing analysis. In order to exclude inefficient extraction and PCR inhibition, some negative samples were tested for the presence of MNV-1, added as process control (Muscillo et al. 2013). The process control procedure revealed no false negative results.

The proportion of positive samples by WTP is reported in Table 1. Positive samples were detected in all Italian regions under study, all year round, with no evident seasonality.

To confirm the presence of MCPyV-specific DNA in PCR-amplified products, these were subjected to sequencing analysis and the resulting sequences compared with public nucleotide sequence databases. With the exception of a single nucleotide substitution in one sample (not affecting the composition of the amino acid),

Table 2 Primers and PCRs used in this study

Primer ID	Sequence 5′-3′	PCR-ID/cycle	PCR product (bp)	References
1940-fw	CCACCAGTCAAAACTTTCCCAAGTAGG	802/1°cycle	435	(Sharp et al. 2009)
1939-rev	GGCAACATCCCTCTGATGAAAGC			
1942-fw	AAACCAAAGAATAAAGCACTGATAGCA	803/nested	358	
1941-rev	CTTAAAGCATCACCCTGATAAAGG			
1954-fw	AAGGCATACGAATATGGG	807/1°cycle	299	This study
1955-rev	GAATTTCTAGGTACACTGG			
1956-fw	CAATCCACACGGGGCCAACT	808/nested	258	
1957-rev	CCATTGGGTGTGCTGGATTC			
1951-fw	CCACAGCCAGAGCTCTTCCT	806/Real-Time	146	(Goh et al. 2009; Sadeghi et al. 2012)
1952-rev	TGGTGGTCTCCTCTCTGCTACTG			
1953-fw	FAM-TCCTTCTCAGCGTCCCAGGCTTCA-TAMRA			

all sequences detected were identical to each other and showed 100 % identity with the strain EurCauC1 (a.n. KF266963).

A portion of the samples (one third) was randomly selected and further analyzed in order to obtain quantitative data on viral loads in wastewater samples. Viral load quantification ranged from 3.96E + 04 to 4.79E + 05 genome copies/liter, with a mean value of 1.5E + 05 genome copies/liter.

Discussion

This is the first study to report the presence of MCPyV DNA in urban wastewater systems in Italy. The virus was found to be widely and abundantly present in Italian urban wastewaters.

MCPyV can find its way into sewage through the washing of skin or by urine/fecal shedding. The exact route of MCPyV transmission has not been established yet, but several possible modes have been suggested. The virus can be detected in several anatomical sites, most frequently on the skin, as it is part of the normal skin microflora, and is regularly shed from the skin's surface (Spurgeon and Lambert 2013). The virus has also been detected in tonsillar tissue, nasopharyngeal aspirates and nasal swabs and thus could spread by the respiratory route (Viscidi et al. 2011). Fecal/oral transmission is also possible, since the virus has been detected in the gastrointestinal tract (Campello et al. 2011; Feng et al. 2008; Loyo et al., 2010), in urine (Bofill-Mas et al. 2010; Husseiny et al. 2010) and in saliva (Baez et al. 2013; Loyo et al. 2010). Results from a recent study, showing the presence of MCPyV DNA on laboratory bench tops, public places, and private houses (Foulongne et al. 2011), suggest that transmission by fomites cannot be ruled out.

Positive samples were detected in all the regions analyzed, throughout the year, confirming the presence of a countrywide circulation of the virus, with no seasonality trends. A detailed comparative analysis of the distribution of positive samples by WTP and month of collection was unfortunately not feasible, since the number of available wastewater samples varied significantly from one WTP to another, with only two WTPs fully complying with our sample collection schedule (12 samples/year).

All sequences, with the exception of one, were identical to each other and to the MCPyV EurCauC1, GenBank sequence KF266963. Recent studies have suggested the existence of geographically-related variants with five major MCPyV genotypes: Europe/North America, Africa (Sub-Saharan), Oceania, South America, and Asia/Japan, based on the analysis of a 1,284-bp long fragment of MCPyV (Martel-Jantin et al. 2014). The complete identity of our sequence with sequences of all five groups suggests that the small T-antigen gene fragment amplified is highly conserved and may therefore not be useful for phylogenetic analysis. We thus decided to explore the possibility of variability between the samples in the LT. For this purpose, positive samples were amplified using the primers previously employed to clone PCR products for the purposes of quantitative analysis. PCR amplicons were subsequently sequenced (data not shown). Here too, sequences were all identical to each other as well as to MCPyV strain EurCauC1. Indeed, sequence diversity of known MCPyV isolates is very low, with nucleotide identity across the viral genome being greater than 98.5 % (http://monographs.iarc.fr/ENG/Monographs/vol104/mono104-005.pdf).

To our knowledge, only one other study on MCPyV in urban sewages has been published so far. It was based on a small number of samples (eight sewage samples from one WTP), and documented the presence of MCPyV in 89 % of the samples tested (Bofill-Mas et al. 2010). The present

study —a year-long monitoring of 21 WTPs throughout Italy (131 samples)—represents a larger and more in-depth molecular-epidemiological investigation.

The implications of the abundant presence of MCPyV in urban wastewaters are unknown. It is well known that the discharge of untreated or even treated sewage into the aquatic environment is the main cause of fecal pollution in water. Conventional wastewater treatment methods do not guarantee the complete removal of viral pathogens that can therefore end up in water environments. Indeed MCPyVs have also been detected in rivers in Spain and Brazil (Calgua et al. 2013). It is important to note that the presence of MCPyV DNA in wastewater samples does not necessarily imply that these samples were infectious. Unfortunately, the lack of straightforward cell culture models able to support MCPyV replication prevents infectivity studies on this virus (Spurgeon and Lambert 2013). Our findings support the hypothesis that MCPyV can be transmitted by the fecal-oral route through contaminated waters. Other oncogenic viruses, such as papillomaviruses, were detected in urban wastewaters in the United States (Symonds 2008) and in Italy, as described in a recent paper by our group (La Rosa et al. 2013). The possibility that oncogenic viruses could be transmitted by the waterborne route, initially suggested 40 years ago (Berg 1973a, 1973b), was hypothesized once again in two recent reviews (Fratini et al. 2013; Reynolds 2012), and deserves to be explored in future studies.

In conclusion, our results indicate that MCPyV is frequently and abundantly detected in urban sewages, suggesting that water may be an important transmission route for MCPyV. Further studies on the prevalence, excretion pattern, and genetic variability of MCPyV in environmental matrices are needed.

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