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ORIGINAL PAPER

First Detection of Human Papillomaviruses and Human Polyomaviruses in River Waters in Italy

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Abstract Waterborne exposure to human viruses is possible through contact with contaminated water environments and can result in infections associated with a wide range of illnesses, including gastrointestinal, respiratory, ear, ocular, and skin infections. Recently, the occurrence in water environments of two groups of human viruses—both known with oncogenic potential, human polyomaviruses (HPyVs) and papillomaviruses (HPVs)—has been reported worldwide. These viruses, responsible for highly prevalent infections worldwide, have recently been proposed as potentially emerging waterborne pathogens. The objective of the present study was to examine the occurrence of HPyVs and HPVs in surface waters, by monitoring two rivers in Northwestern Italy, by nested PCR assays and sequencing. HPyVs (JC, BK, and Merkel cell polyomavirus) were detected in 10/25 (40 %) samples. HPVs (HPV8, 17, 21, 25, 32, 80, 99, 105, and putative new HPVs) were identified in 14/25 (56 %) river samples. The number of HPV DNA copies in waters was measured by quantitative real-time PCR. To our knowledge, this is the first detection and quantification of HPVs in surface waters. The possibility that HPyVs and HPVs can be transmitted by the waterborne route deserves to be explored in future studies.

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Introduction

Human polyomaviruses (HPyVs) belong to a family of small, non-enveloped, DNA viruses, which infect different tissues and organs, causing infections that are usually subclinical in immunocompetent individuals but can be serious in immunocompromised hosts. Today the human polyomavirus family consists of 13 members, BK virus, JC virus, KI virus, WU virus, Merkel cell polyomavirus, HPyV6, HPyV7, trichodysplasia spinulosa virus, HPyV9, MWPyV, STLPyV, HPyV12, and NJPyV-2013. Serological studies suggest that HPyVs are ubiquitous in the general population with rates of infections ranging from 35 to 90 % (Dalianis and Hirsch 2013). Primary infection occurs during childhood through respiratory or oral transmission. The majority of individuals exhibit asymptomatic latent infection; immunocompromised persons are at risk for viral reactivation and disease progression, resulting in conditions such as progressive multifocal leukoencephalopathy, trichodysplasia spinulosa, Merkel cell carcinoma, and polyomavirus associated nephropathy (Wiedinger et al. 2014).

Human papillomaviruses (HPVs) are DNA viruses that infect basal epithelial cells inducing self-limiting benign tumors, which may progress to malignancy. They have strict tropism and infect either mucosa or skin. Most of the human viruses belong to α-, β-, and γ-papillomavirus genera. The majority of HPV infections do not cause symptoms or disease and resolve spontaneously. More than 200 HPV types have been identified, of which approximately 40 can infect the mucosa of the anogenital tract and

are classified based on their oncogenic potential as either low- or high-risk HPV types. The low-risk HPV type causes benign hyperproliferative lesions or genital warts, while the high-risk HPV type is strongly associated with premalignant and malignant lesions.

HPV infections are transmitted mainly through direct skin-to-skin or skin-to-mucosa contact, and the virus probably requires access to basal cells through micro-abrasions in the epithelium (IARC 2011). HPV is one of the most common causes of sexually transmitted diseases worldwide. Non-sexual transmission of the virus is debated but recent findings shows that HPV can survive well outside of its host to potentially be transmitted by non-sexual means (Ryndock and Meyers 2014).

Recently, HPyVs and HPVs have been detected in urban sewages worldwide (Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2000, 2013; Cantalupo 2011; La Rosa 2013; Symonds 2008). HPyVs have also been identified in other water environments: treated wastewater, fresh to marine water, and filtered drinking water (Albinana-Gimenez et al. 2009; Bofill-Mas et al. 2006, 2010). It is well known that sewage treatment can reduce viral contamination but cannot eliminate it (Okoh et al. 2010). As consequence, viral pathogens can find their way into fresh or marine waters when treated sewage effluent is discharged.

The objective of the present study was to examine the occurrence of HPyV and HPV in surface waters, by monitoring two rivers in Piedmont, Northwestern Italy.

Materials and Methods

During 2013, 25 rivers samples were collected from two rivers in Piedmont, Northwestern Italy, on quarterly basis: 17 samples from four sampling sites on the Po River (a, b, c, and d), and eight samples from two sampling sites on its tributary (a and b), the Dora Riparia River (see Fig. 1).

Surface waters (10 l each) were concentrated using the adsorption–elution procedure with 1MDS electropositive filters. A known amount of a murine norovirus (MNV-1) was spiked into the samples before concentration, as sample process control in order to calculate viral recovery efficiency (by quantitative PCR) and check for potential inhibitors (by qualitative PCR) as previously described (Muscillo et al. 2013). Briefly, water was passed through a standard filter apparatus containing a sterile electropositive filter, using a peristaltic pump and a flow rate of approximately 0.5 l/min. For virus elution (50 ml final volume), 1.5 % of beef extract pH 9.5 was recirculated through the filters for 20 min. The eluate was then neutralized and stored at −20 °C for future use.

Nucleic acids were extracted from 10 ml of concentrated waters, using the NucliSENS easyMAG (BioMerieux) semi-automated extraction system, according to the manufacturer's instructions. Eluates (100 µl) were stored in aliquots at −80 °C.

Three molecular nested PCRs for the detection of mucosal and cutaneous HPVs, and three assays for the detection of Merkel cell, JC polyomavirus, and BK Polyomavirus were used. Primers and PCRs used in this study are shown in Table 1, along with target regions, PCR amplicons, and references.

For each PCR reaction, 2 µl of the extracted DNA and 22 pmol of each primer were used in a final mixture of 25 µl, using the MyTaqTM Red Mix PCR Kit (Bioline). The products of the first round of amplification were then subjected to a second round using 1 µl of the first-step PCR as input. Positive controls were included in each PCR for the verification of negative amplification results, consisting of HPV and HPyV DNAs detected previously in wastewater samples (Di Bonito et al. 2015b; La Rosa 2013). All standards precautions were followed to prevent contaminations. Negative PCR controls containing all PCR components without template DNA were also included.

False-negative results due to PCR inhibitors were excluded by positive PCR signal for the murine norovirus (Muscillo et al. 2013). PCR products were purified using a Montage PCRm96 Micro-241-well filter plate (Millipore), and purified amplicons were directly sequenced with a capillary automatic sequencer (Bio-Fab Research, Rome, Italy), using the forward and reverse PCR primers. Sequences were submitted to BLAST analysis for genotyping. For HPVs, the genotype was assigned when the sequence showed a percentage of nucleotide identity higher than 90 % toward a known prototype (de Villiers et al. 2004).

In order to determine the quantity of HPVs present in river samples, we performed a SYBR Green q-PCR assay that targets the FAP region within the L1 capsid gene of the HPV genome. We extracted and purified a PCR product obtained with primers FAP6085/FAP6319 (sample ID 2005), to use as a standard curve for the q-PCR reaction. Standard curves were generated from tenfold serial dilutions of the purified PCR product, ranging from 5.50E+06 to 5.50E+01 copies/reaction. Genome copy numbers (GC) were calculated after measuring nucleic acid concentrations with a NanoDrop 2000 Spectrophotometer. DNA concentrations were then converted into copy numbers using the following equation: [GC/ml = C/MW * 6.02 × 10^{23}], where C represents DNA concentration (C = g/ml) and MW is molecular weight. The absolute quantification was achieved by comparing sample quantification cycle (C_q) values to the standard curve. Real-time q-PCR reactions were assembled in a 20-µl reaction volume, containing 1 µl of each forward and reverse primer (10 µM), 10 µl of 2× iQTM SYBR Green Supermix (Bio-Rad) plus

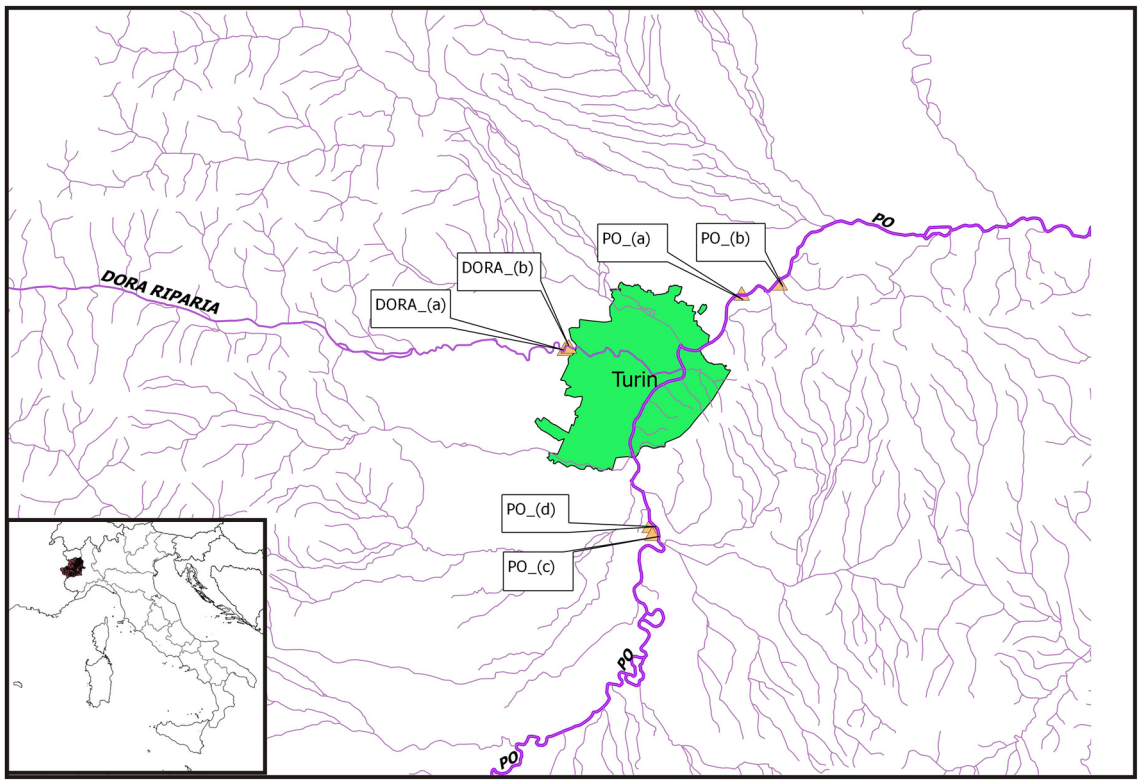

Fig. 1 GIS map of collection sites along the rivers

5 µl of DNA. The PCR cycling programme consisted of 15 s at 95 °C followed by 40 s at 60 °C repeated for 40 cycles and included a heating step (10 min at 95 °C) at the beginning of each run. A melting curve was recorded at the end of each run to assess the amplification product specificity. The real-time PCRs were carried out in a Bio-Rad Miniopticon Real-Time PCR System on a triplicate set using 5 µl of the extracted genome. Quantification data were analyzed with CFX Manager software and exported into a Microsoft Excel file for subsequent statistical analysis.

Results

Viral recovery rates associated with the adsorption–elution method used to concentrate the viruses, calculated based on the MNV-1, ranged from 31 to 52 %. Combining the results obtained from the 6 different nested PCRs for HPyVs and HPVs, 17/25 (68 %) samples were positive. Inhibition in negative samples was ruled out using the sample process control (positive PCR signal for MNV1). HPyVs were detected in 10/25 (40 %) samples, while HPVs were identified in 14/25 (56 %) samples. Seven samples were positive for both HPV and HPyV. PCR results are shown in Table 2, which includes samples positive at least to one assay.

Among polyomaviruses, 3 Merkel cell polyomavirus, 7 JC, and 5 BK strains were detected. Merkel sequences were all identical to each other, and showed 100 % identity with the EurCauC1 (a.n. KF266963) strain. JC sequences were also identical to each other. On the other hand, we found four different BK variants, sharing 99 % nucleotide identity.

Eight known papillomavirus genotypes were detected, belonging to alphapapillomavirus 1 (HPV32), betapapillomavirus 1 (HPV8, HPV21, HPV25, HPV99, and HPV105), and betapapillomavirus 2 (HPV17 and HPV80) species. Putative new genotypes, showing less than 90 % nucleotide identity with known prototypes, were also detected. These samples showed 95–100 % nt identity with GeneBank unclassified beta papillomaviruses detected in the skin of non-melanoma skin cancers and solar keratosis (a.n AY170668 and HPU85660), and in normal skin of immunocompetent hosts (a.n. AJ001059). Mixed electropherograms suggestive of the presence of two or more HPV types were identified in the remaining samples.

Four samples positive with the FAP6085/6319 assay could be quantified using the SYBR Green q-PCR assay. The quantification of HPVs detected 1.08E+02 to

Table 1 Primers and PCRs used in this study

Primer name	Sequence 5′–3′	PCR	Amplicon (bp)	Target region	References	Target	Virus
MCPyV_OAS	CCACCAGTCAAAACTTTCCCAAGTAGG	1st cycle	435	T-small antigen	(Sharp et al. 2009)	Merkell cell	Human Polyomavirus
MCPyV_OS	GGCAACATCCCTCTGATGAAAGC						
MCPyV_IAS	AAACCAAAGAATAAAGCACTGATAGCA	Nested	358				
MCPyV_IS	CTTAAAGCATCACCCTGATAAAGG						
PM1+	TCYTCTGGNNTAAARTCATGTTCC	1st cycle	551	T-large antigen	(Polo et al. 2004)	JC and BK	
PM1−	TTWTAGRTKCCAACCTATGGAAC						
JC+	ATATTATGACCMCCAAAACCATG	Nested	189			JC	
PM2−	GGTAGAAGACCCYAARGACTTTCC						
BK+	GAATGCTTTCTTCTATAGTATGGTATG	Nested	353			BK	
PM2−	GGTAGAAGACCCYAARGACTTTCC						
MY11	GCMCAGGGWCATAAYAATGG	1st cycle	452	L1 region	(de Roda Husman et al. 1995; Manos et al. 1989)	Broad range HPV	Human Papillomavirus
MY09	CGTCCMARRGGAWACTGATC						
GP5+	TTTGTACTGTGGTAGATACTAC	Nested	141				
GP6+	GAAAAATAAACTGTAAATCATATTC						
FAP59	TAACWGTIGGICAYCCWTATT	1st cycle	484		(Forslund et al. 1999, 2003)		
FAP64	CCWATATCWVHCATITCICCATC						
FAP6085	CCWGATCCHAATMRRTTTGC	Nested	238				
FAP6319	ACATTTGIAITTGTTTDGGRTCAA						
CP65	CARGGTCAYAAYAATGGYAT	1st cycle	455		(Berkhout et al. 1995)		
CP70	AAYTTTCGTCCYARAGRAWATTGRTC						
CP66	AATCARMTGTTTRTTACWGT	Nested	377				
CP69	GWTAGATCWACATYCCARAA						

Table 2 PCR and sequencing results

ID sample	Collection site	Date of collection	GP5/GP6	Fap6085/6319	CP66/69	MCPyV_IAS/IS	JC+/PM2−	BK+/PM2−
2000	Po (d)	06 Mar 2013	–	HPV21	Unclassified	–	–	–
2001	Po (c)	06 Mar 2013	–	HPV8	–	–	–	–
2006	Po (b)	06 Mar 2013	–	HPV21	–	–	+	+
2022	Po (d)	04 Jun 2013	–	HPV99	–	–	–	–
2023	Po (c)	04 Jun 2013	–	Nd*	–	–	–	–
2029	Po (b)	05 Jun 2013	–	–	Nd*	–	–	–
2040	Po (c)	17 Sep 2013	–	Unclassified	–	–	–	–
2048	Po (b)	18 Sep2013	HPV32	–	–	+	–	–
2065	Po (c)	03 Dec 2013	–	–	–	+	–	–
2071	Po (a)	04 Dec 2013	–	–	Unclassified	–	+	+
2073	Po (b)	04 Dec 2013	–	–	–	–	+	–
2005	Dora (b)	06 Mar 2013	–	HPV80	–	–	+	+
2007	Dora (a)	06 Mar 2013	–	Nd*	HPV25	–	+	–
2028	Dora (b)	05 Jun 2013	–	–	HPV25	–	–	–
2030	Dora (a)	05 Jun 2013	–	Unclassified	HPV17	–	+	–
2070	Dora (a)	04 Dec 2013	–	–	–	+	–	+
2072	Dora (b)	04 Dec 2013	–	–	HPV105	–	+	+

Nd* Genotype not determined because of mixed electropherograms

3.70E+03 GC per reaction (5 µl), corresponding to 1.08E+04 to 3.70E+05 GC/10 l.

Discussion

Polyomavirus infections are common, ranging from 60 to 100 % depending on the virus (Signorini et al. 2014). JC and BK are among the first human PyVs discovered. BKPyV was isolated from the urine sample of a renal transplant patient, and JCPyV virus was isolated from the brain tissue of a patient with progressive multifocal leukoencephalopathy. They can be transmitted via various routes including: fecal–oral, respiratory, through blood transfusions, organ transplantation, transplacentally, and through seminal fluid (Pinto and Dobson 2013). Both BKPyV and JCPyV have been shown to be important causes of morbidity and mortality in a variety of distinct cohorts of immunocompromised patients (De Gascun and Carr 2013). Merkel cell polyomavirus (MCPyV) is present in approximately 80 % of Merkel cell carcinoma tumors (MCC), which is a primary neuroendocrine tumor of the skin. Despite the rarity of MCC, MCPyV is a common skin commensal, found in healthy individuals. It has also been detected in urine, in immunosuppressed organ transplant recipients and non-immunosuppressed subjects (Husseiny et al. 2010; Signorini et al. 2014).

Recently, the occurrence of HPyVs and HPVs in water environments has been reported worldwide. HPVs have been detected in the vast majority of raw sewages tested, with a wide range of genotypes, including oncogenic high- and low-risk HPVs (La Rosa 2013). Recent data from our group showed that HPVs are excreted in the feces of patients with diarrhea, suggesting that epitheliotropic HPV can find their way into sewage not only through the washing of skin and mucous membranes, but also by fecal shedding (Di Bonito et al. 2015a). These data pave the way for investigations on HPV transmission through contaminated water.

HPyVs are shed in the urine and feces of infected individuals. Among HPyVs, BK, JC, and the newly described oncogenic Merkel cell PyV have been detected in sewage and in fresh waters from various geographical areas (Bofill-Mas et al. 2013). Both HPyVs and HPVs have recently been proposed as potentially emerging waterborne pathogens (Fratini et al. 2013), but their potential for waterborne transmission has not yet been investigated.

This study reports the first detection of JC, BK, and Merkel cell Polyomavirus in rivers in Italy. We detected JCPyV in 28 % of samples and BKPyV in 20 % of samples. JCPyV and BKPyV have been previously detected in surface waters in different countries worldwide, including Spain, Germany, United States, Australia, Japan, and Brazil, in percentages ranging from 11.1 to 100 % (Ahmed et al. 2009; Bofill-Mas et al. 2000; Calgua et al. 2013; Fongaro et al. 2012; Hamza et al. 2009; Haramoto et al. 2010; McQuaig et al. 2009; Rusinol et al. 2014). MCPyV was detected in 12 % of river samples in this study. This is the first detection of MCPyV in rivers receiving wastewater effluents in Italy, thus indicating the possibility of contamination of surface waters through sewage discharges. MCPyV has been previously identified only in river samples from Barcelona (from 28 to 50 % of tested samples) and Rio de Janeiro (50 %) (Bofill-Mas et al. 2010; Calgua et al. 2013). In a previous study, we detected MCPyV in about 50 % of sewages in Italy, with high concentrations found in all the wastewater treatment plants under study (Di Bonito et al. 2015b), suggesting a wide circulation of the virus.

HPVs are transmitted by direct physical contact between infected individual and susceptible host: anogenital viruses are transmitted through sexual activity, while cutaneous HPVs by skin-to-skin contact. However, indirect modes of transmission, such fomites or contaminated waters, cannot entirely be ruled out (Czegledy 2001; Ryndock and Meyers 2014). In the present study, HPVs have been detected in rivers samples. We detected both alpha and beta HPV species. HPV32 is the only alpha genotype detected in this study. It is the most common HPV found in the oral cavity and is often found in the warts of HIV positive individuals; it is also one of the etiological agents of the focal epithelial hyperplasia, a rare and benign papillomatosis disease of the oral cavity (Herrel et al. 2009). HPV8 is associated with skin cancer and is thus considered possibly carcinogenic. Data for this association are strong for patients with epidermodysplasia verruciformis (EV), a rare, inherited disorder that predisposes patients to widespread HPV infection and cutaneous squamous cell carcinoma.

HPV17, 21, 25, 80, and 105 are frequently found in cutaneous lesions and commonly associated with lesions in EV or immunosuppressed patients (Bernat-Garcia et al. 2014). HPV99 has been isolated in normal skin. Of the genotypes detected, HPV25 and HPV80 were also detected previously by our group in sewages in Italy in 2011 (La Rosa 2013), while HPV8 was detected in fecal specimens from patients with diarrhea (Di Bonito et al. 2015a).

In conclusion, we detected both HPVs and HPyVs in two Italian rivers, suggesting that surface water can be a potential source for exposure to these viruses. To our knowledge, this is the first detection and quantification of HPVs in surface waters. Whether HPV and HPyV infections might be transmitted by the waterborne route is unknown, but this possibility cannot be excluded as recently raised by some authors (Fratini et al. 2013; La Rosa et al. 2012; Reynolds 2012). Future studies are needed to determine the occurrence and quantity of these

viruses in different water matrices, from raw sewage, through treated sewage, to downstream rivers and other superficial and recreational water bodies, in order to shed light on the fate of these viruses in water environments and their potential for waterborne transmission. Research efforts focusing on their resistance to inactivation and stability in water environments are crucial.

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Conflict of interest The authors declare that they have no conflict of interest.

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