Epidemiological surveillance of human enteric viruses by monitoring of different environmental matrices

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Abstract In the aim of studying possible relations between viruses detected in clinical specimens and the ones found in different environmental matrices, in the period May 2004 to April 2005, the collection of faecal samples from gastroenteritis cases and the monthly monitoring of raw and treated wastewater, river water, seawater and mussels were carried out. The viruses considered for environmental monitoring were adenovirus, rotavirus, enterovirus, norovirus, hepatitis A virus (HAV) and Torque teno virus (TTV): they were searched for with PCR and RT-PCR and confirmed by gene sequencing. Faecal coliforms and somatic coliphages' counts were also determined. The surveillance of case detected 45 positive faecal samples out of 255 (17.6%) while 35 of 56 environmental samples (62.5%) resulted positive for at least one of the considered viruses. The detection of the same viral strain in the faeces of gastroenteritis cases and in water was possible for adenovirus and rotavirus, which were also predominant in environmental matrices; thus they could be considered as a reference for risk assessment.

Keywords Adenovirus; enterovirus; epidemiological surveillance; norovirus; rotavirus; water

Introduction

Enteric viruses can be transmitted not only by direct contact, but also by indirect routes, such as the consumption of contaminated food and water. In particular, the different uses of water, such as drinking, irrigating, bathing and growing food (i.e. shellfish) can frequently expose people to enteric viral infections (Lopman *et al.*, 2003). The evaluation of environmental viral hazards is an emerging problem, which deserves particular attention because of the peculiar characteristics that differentiate viruses from other microorganisms. Viruses, in fact, require lower infectious doses than most bacteria, can withstand environmental factors and purification treatment, even for very long periods of time, and present multiple transmission routes (WHO, 2004). In recent years progress in virological assays (mostly biomolecular tests) has increased knowledge, but many questions remain still unsolved: the standardization of detection methods, the choice of the most significant agents for risk assessment, and the selection of a reliable indicator for viral contamination (Carducci, 2005). For this last problem, a new possible agent can be proposed: it is the TTV (Torque teno virus) (Nishizawa *et al.*, 1997), a virus without a specific associated pathology, but causing a persistent infection in the large majority of healthy people and present in blood, faeces, respiratory secretions and other body fluids.

Environmental monitoring through effective, standardised virus detection techniques combined with clinical surveillance could promote continuous, rapid exchange of information on the spread and distribution of the main enteric viral agents and the incidence of correlated pathologies. For this reason we planned a study aimed to analyse the human enteric viruses environmental spread and its relations with virological diagnosis of gastroenteritis, to identify the most frequent viral pathogens in different types of water and to evaluate the possible correlations between pathogenic enteric viruses and commonly used

faecal indicators. The search for TTV was also performed to evaluate its diffusion in water environment.

Materials and methods

Since May 2004 an epidemiological surveillance of viral gastroenteritis diagnosed on faecal samples in the Clinical Virology Laboratory of the Department of Experimental Pathology of the University of Pisa was carried out, simultaneously with an environmental monthly monitoring of raw and treated wastewater by a treatment plant of the city of Pisa (samples of 1 and 10 L respectively), of water from the river receiving this effluent (10 L), and from the sea at this river outfall (10 L). Samples of mussels (20–30 individuals for each sample) were also collected in this area.

Faecal samples analysed with immunological techniques and resulted positive were retested with PCR and the detected nucleic acids were sequenced (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) to compare strains coming from clinical specimens with the ones found in the environment.

Water monitoring and mussel analysis concerned enteric viruses (enterovirus, adenovirus, rotavirus, norovirus genotypes I and II, HAV) bacterial (*Escherichia coli*) and viral (somatic coliphages and TTV) indicators. *E. coli* was determined by membrane filtration technique on TBX agar (ISO 9308-1: 2000). For virological examination water samples were concentrated using two stage tangential-flow ultrafiltration (Carducci *et al.*, 2003). Mussels were eviscerated and hepatopancreas from the same sampling were pooled. The pool was divided in aliquots of 0.03 g, then nucleic acids directly extracted by Total Quick RNA cells and tissues (Talent kit) and QIAamp DNA kit (Qiagen-Germany) (Carducci *et al.*, 2004).

The concentrated samples were tested for coliphage analysis by plaque assay according to the double agar layer method (using *E. coli C*, ATTC 13706, as host strain) (ISO, 1999). For enteric viruses the concentrated samples were decontaminated with chloroform, the nucleic acids extracted using QIAmp Viral RNA and DNA kits (Qiagen-Germany) and the biomolecular tests (PCR, RT-PCR) performed using primers reported in Table 1. The positivity was confirmed and the strains identified with gene sequencing. Sequence analysis was carried out with NCBI Genebank.

Statistical analysis aimed to search correlations between *E. coli* and coliphages, and the association between viral presence and indicators counts was performed by Excel, Microsoft (Pearson correlation).

Results and discussion

In the period from May 2004 to March 2005 the surveillance of cases revealed 45 (17.6%) positive faecal samples out of 255, of which 17/255 (6.6%) were for rotavirus, 5/255 (1.9%) for adenovirus, 6/255 (2.3%) for astrovirus, 7/246 (2.8%) for norovirus genotype I and 10/246 (4.1%) for norovirus genotype II, without particular epidemic peaks, but with only small clusters of rotavirus infections in May 2004 and March to April 2005 (Figure 1).

The study of *E. coli* and somatic coliphage counts showed a slight decrease due to the wastewater treatment (on average from 1×10^7 to 2.4×10^5 CFU/100 mL for *E.coli* and from 2.9×10^6 to 2×10^4 PFU/100 mL for the somatic coliphages), followed by a new increase in the river (7.9×10^5 CFU/100 mL and 3.4×10^5 PFU/100 mL) probably due to further pollution (Figure 2).

At the river mouth *E. coli* was found only once and coliphage counts were highly variable, probably due to the seawater dilution. No correlation was found between *E. coli* and somatic coliphage concentrations. The coliphage concentration in mussels followed

Table 1 Primers used in RT-PCR and PCR assays

Virus and oligonucleotide	Region	Sequences 5′–3′	References
Enterovirus			
EV03	5′ UTR	ATT GTC ACC ATA AGC AGC CA	Gilgen et al., 1997
EV05		CAC GGA CAC CCA AAG TA	
EV06		CAA GCA CTT CTG TTT CCC CGG	
Norovirus gen. I			
SRI-1	Capside	CCA ACC CAR CCA TTR TAC AT	Gilgen et al., 1997
SRI-2		AAA TGA TGA TGG CGT CTA	
SRI-3		AAA AYR TCA CCG GGK GTA T	
Norovirus gen. II			
SRII-1	RNA pol.	CGC CAT CTT CAT TCA CAA A	Gilgen et al., 1997
SRII-2		TWC TCY TTY TAT GGT GAT GAT GA	
SRII-3		TTW CCA AAC CAA CCW GCT G	
Rotavirus			
RV1	Gene VP7	GTC ACA TCA TAC AATTCT AAT CTA AG	Gilgen et al., 1997
RV2		CTT TAA AAG AGA GAA TTT CCG TCT G	
RV3		TGT ATG GTA TTG AAT ATA CCA C	
RV4		ACT GAT CCT GTT GGC CAW CC	
HAV			
HAV 1	2A-2B	ATG CTT GGA TTG TCT GGA GT	Divizia et al., 1989
HAV 2		GAA CAA ATA TCT CTT AAC CA	
HAV 3		ATG ATG TTT GGA TTT CAT CAT	
HAV 4		CTG GAG TCC ATT TGC CAA TT	
Adenovirus			
ADDEG 1	E1-A/B	GCC SCA RTG GKC WTA CAT GCA CAT C	Allard et al., 1992
ADDEG 2		CAG CAC SCC ICG RAT GTC AAA	
ADDEG 3		GCC CGY GCM ACI GAI ACS TAC TTC	
ADDEG 4		CCY ACR GCC AGI GTR WAI CGM RCY TTG TA	
TTVirus			
NG 132	UTR	AGC CCG AAT TGC CCC TTG AC	Okamoto et al., 1999
NG 133		GTA AGT GCA CTT CCG AAT GGC TGA G	
NG 134		AGT TTT CCA CGC CCG TCC GCA GC	
NG 147		GCC AGT CCC GAG CCC GAA TTG CC	

the ones found in the seawater confirming the role of mussels as bioindicators of water microbial pollution (Carducci et al., 1998).

Enteric viruses were mostly detected in raw sewage (nine positive samples out of 12 examined, 75%): two of them were positive for norovirus (genotype I in July, genotype II in June), three for enterovirus in July 2004 (poliovirus 1-AY017242), February (enterovirus 74 AY556057.1) and March 2005 (enterovirus 74 AY556057.1). Seven samples
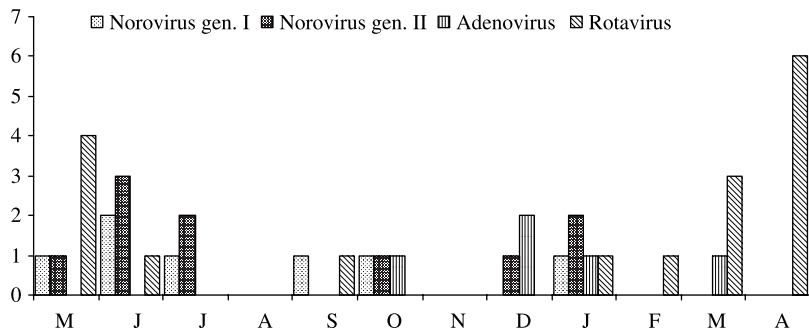

Figure 1 Monthly number of the observed gastroenteritis cases according to the detected virus, specified by X and Y content in the Figure (X: Month; Y: Number gastroenteritis cases)
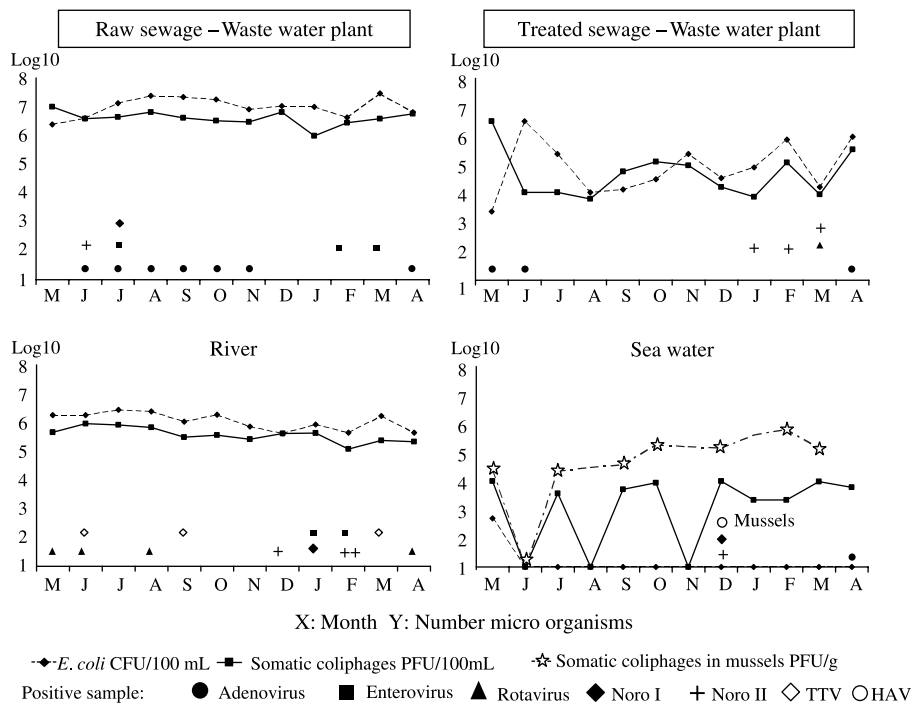
Figure 2 Results of microbiological analysis in raw sewage, treated sewage, river water and seawater

resulted positive for adenovirus in June, July and August 2004 (uncultured adenovirus AY747675), in September (human adenovirus type 31 AY220987), October and November 2004 (human adenovirus type 31 AF161576.1) and in April 2005 (human adenovirus type 41 AY220986.1).

In the plant effluent positive samples were less frequent (6/12, 50%), one for rotavirus in March 2005 (human rotavirus A G3-AY900173.1) and three for adenovirus in May (uncultured adenovirus AY747675), June 2004 (uncultured adenovirus AY747672.1) and April 2005 (human adenovirus type 41 AY220986.1). Moreover, three noroviruses genotype II were found in January, February and March.

River water resulted contaminated in 9/12 (75%) samples, four were positive for rotavirus: in May (human rotavirus A G4 AF373896.1), June (human rotavirus A G1 AF260950.1), August 2004 (human rotavirus A G2 AY660563.1) and April 2005 (human rotavirus A G1 AB081799.1). Two positive samples for enterovirus were found in January (enterovirus 90 AY773285.1) and in February 2005 (human Coxsackie virus A1, AF499635.1). Moreover, one strain of norovirus genotype I was found in January 2005 and three strains of genotype 2 in December 2004, February and March 2005. TT virus was found in June (strain TCHN-G2), September (strain TCHN-A) and March 2005 (strain TCHN-E).

In seawater (two positive samples of 12, 16.7%) were found human adenovirus type 2 AY293903.1 in April and two strains of norovirus genotype I and genotype II, both in December. Only one of the eight samples of shellfish analysed (12,5%) was positive in December for HAV (AF485328.1). This was the only case of HAV detection, but the virus presence in mussels indicates a possible food related risk. No significant association was found between the enteric viral presence and *E. coli* or coliphage counts.

The typing by gene sequencing of the detected viruses resulted in identification of some link between adenovirus and rotavirus strains isolated from faeces and the ones

found in raw and treated sewage, in river and seawater. Such comparisons also showed the same strain in different environmental matrices and periods.

Human adenovirus type 2 AJ293902 was found both in gastroenteritis cases in December 2004 and January 2005 and in seawater in April 2005; in June 2004 the strain AY747675 was detected in raw and treated wastewater, indicating a possible high load of the virus in this month, but also an insufficient efficiency of the plant in virus removal. In the raw sewage other adenovirus strains were also found, the same (type 31 AF161576.1) in October and November 2004.

The phylogenetic analysis of adenovirus strains proved that they belong to the same superfamily A,F,C1 according to the VA RNA gene tree (Ma and Mathews, 1996). The presence of the same strain of rotavirus (human rotavirus G3 AY900173.1) was revealed in faeces in December 2004 and in treated wastewater in March 2005 showing a continuous circulation of this strain. The phylogenetic distance among all the rotavirus detected strains was great on a gene tree built on a 500-nucleotide sequence (Kostouros *et al.*, 2003).

TT virus strains were all different, they were found only in the river, and not in the raw sewage. This apparent discrepancy may be due to the possible inhibition of PCR by the concentrated wastewater.

Conclusions

Clinical virological data did not demonstrate any epidemic peak, but a continuous circulation of enteric viruses, mainly adenovirus, norovirus and rotavirus. Environmental virological analysis frequently resulted positive for adenovirus, enterovirus, norovirus, rotavirus and TTV. *E. coli* and coliphages counts indicated a scarce reduction of microbial pollution in the wastewater plant, a new increase in the river and a strong dilution in the sea. No correlation was found between indicators and enteric virus presence. The detection of the same viral strain in faeces of gastroenteritis cases and in water was possible for adenovirus and rotavirus that were also predominant in environmental matrices; thus they could be considered as a reference for risk assessment. Regarding a possible indicator for viral pollution, the lack of correlation between somatic coliphages and coliforms and the scarce representativity of both for the viral presence confirm the uselessness of these parameters for virological risk assessment. The number of positive samples for TT virus is still insufficient to allow conclusions about its use as an indicator.

Acknowledgements

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