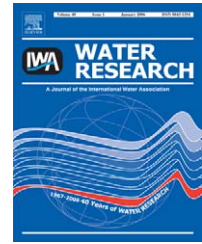


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Quantitation of hepatitis A virus and enterovirus levels in the lagoon canals and Lido beach of Venice, Italy, using real-time RT-PCR

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ABSTRACT

In order to assess the microbial water quality of the lagoon canals of Venice, Italy and nearby beach on Lido island, a study was conducted using real-time RT-PCR to enumerate levels of hepatitis A virus (HAV) and enteroviruses in these marine waters over a 3-year period from 2003 to 2005. A total of 17 sites (9 lagoon canal and 8 beach sites) were assayed. For the canals of the Venice Lagoon, 78% were positive for both HAV and enteroviruses, with levels ranging from 75 to 730 and 3 to 1614 genome copies/L, respectively. At Lido beach, HAV was never detected, but enteroviruses were detected in all Lido beach samples at levels ranging from 2 to 71 genome copies/L. There was a statistically significant correlation between thermotolerant coliform densities and HAV levels ($p = 0.0002$), but the relationship between thermotolerant coliform densities and enterovirus levels was not significant ($p > 0.05$). Despite the fact that enteroviruses were detected at low levels in the surfzone at Lido beach, the risk for enteroviral infection (calculated using the beta-Poisson model) for recreational exposure from swimming, was in the range of 1.9×10^{-3} – 6.1×10^{-2} , yielding a disease risk at or below the level (5% for gastroenteritis) deemed acceptable by European Guide standards.

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1. Introduction

The unique location of the city of Venice, Italy, built on a number of islands in the middle of the Venice Lagoon, has rendered it impossible to construct a sewage treatment infrastructure for the city and surrounding islands (Pavoni et al., 1990). The lagoon receives the untreated sewage from Venice with an organic and pathogen loading equivalent to more than 400,000 persons during the tourist season (Orlob et al., 1991). Although actual bathing is prohibited in the

lagoon itself, recreational beaches exist on the littoral strip of the Adriatic sea (e.g. Lido beach about 3km west of the northern Lagoon opening) which may be adversely impacted by contaminated lagoon waters flowing out on the outgoing tide. Additionally, Venice Lagoon is unusual insofar as there is potential to impact human health through combined exposure routes, some of which are not typical (Johnston et al., 1993). For example, transport in Venice is predominantly by boat. Disturbance of the contaminated water by motorized vessels may create aerosols which may pose an inhalation

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health hazard (Blanchard, 1989). Moreover, due to sea-level rise combined with soil subsidence, flooding events have become more frequent, occurring some 50 times a year in the past decade (Bernstein and Cecconi, 1996). Such floods which inundate streets and businesses increase the number of waterborne disease exposure scenarios. A project with an estimated cost of 4.5 billion US \$ has recently begun to construct movable tidal barriers to stop flooding of the city of Venice (the MOSE Project). However, a number of environmental concerns about this barrier project remain, particularly that the operation of these barriers could decrease the tidal flushing of the Lagoon and exacerbate an already serious sewage pollution problem.

There is currently a lack of microbiological and epidemiological studies that provide an assessment of the risk of human disease associated with sewage disposal into the Venice Lagoon (Aimo et al., 1999). Vazzoler and Stradella (1999) detected enteroviruses (by tissue culture techniques) in the canals of the city of Venice, but did not present quantitative results for virus levels that could be used in a risk assessment for bathing at Lido beaches or for other scenarios involving exposure to waters of the Venice Lagoon. The present study determined levels of both hepatitis A virus (HAV) and enteroviruses in the lagoon canals of Venice and the surfzone at nearby Lido beach by real-time RT-PCR, in order to better assess human disease risk from recreational exposure to these marine waters.

2. Methods

2.1. Sampling sites

A total of 17 water samples (Fig. 1 and Table 3) were collected, including seven samples from the Grand canal (at Rialto bridge), one sample from each of two interior canals, the Rio di San Marcuola and the Rio di Fuseri, and eight samples from a recreation beach at Lido (near Via Santa Maria Elisabetta (SME)), on the littoral strip of the Adriatic Sea beach about 3 km west of the northern Lagoon opening (Fig. 1 and Table 3).

Samples ranged from 2 to 12 L and were collected over three consecutive years in the summers of 2003, 2004, and 2005 (Table 3).

2.2. Virus concentration

Each sample was processed within 1–2 h of collection following a published protocol by Katayama et al. (2002). Seawater samples were filtered at a constant rate via a vacuum pump through a series of Whatman filters (of 11 and 2.5 μm pore size) to reduce particulate matter. Although it is well understood that viruses can adsorb to particles in the environment, removal of particulates is necessary for PCR assays. Samples were then applied to a type HA 0.45- μm negatively charged membrane (Millipore, Burlington, MA, USA). The negatively charged filter was washed with 200 mL of 0.5 mM H_2SO_4 to remove cations, and the virus was eluted from the filter with 10 mL of 1 mM NaOH, into a tube containing 0.1 mL of 50 mM H_2SO_4 and 0.1 mL of 100 \times TE buffer (Sigma-Aldrich, St. Louis, MO, USA). The filtrate was then concentrated to 450 μL volume by centrifuging the samples in a Centriprep Concentrator (YM-30, Millipore) at 1500g for 15, 10, and 5 min. Total RNA was extracted from the 450 μL filtrate using TRI ReagentTM (Molecular Research Center Inc., Cincinnati, OH, USA) and the RNA pellet was dissolved in 40 μL of TE buffer (pH 8.0).

2.3. Quantitation of HAV by SYBR Green real-time RT-PCR

Procedures for cDNA synthesis and SYBR Green real-time RT-PCR were performed as described by Brooks et al. (2005), except a BioRad iCycler real-time thermocycler was used instead of the Applied Biosystems GeneAmp 5700 Sequence Detection System for real-time RT-PCR. First strand cDNA (40 μL) was synthesized from 19.5 μL of RNA using random hexamers. Sample cDNAs were diluted 1:10 and 1:100 with DNase, RNase-free water containing sonicated herring sperm DNA (5 ng/mL) as carrier DNA (Leutenegger et al., 1999). The SYBR Green RT-PCR amplification was carried out in a 25 μL reaction volume that contained 7.1 μL of 2 \times SYBR Green

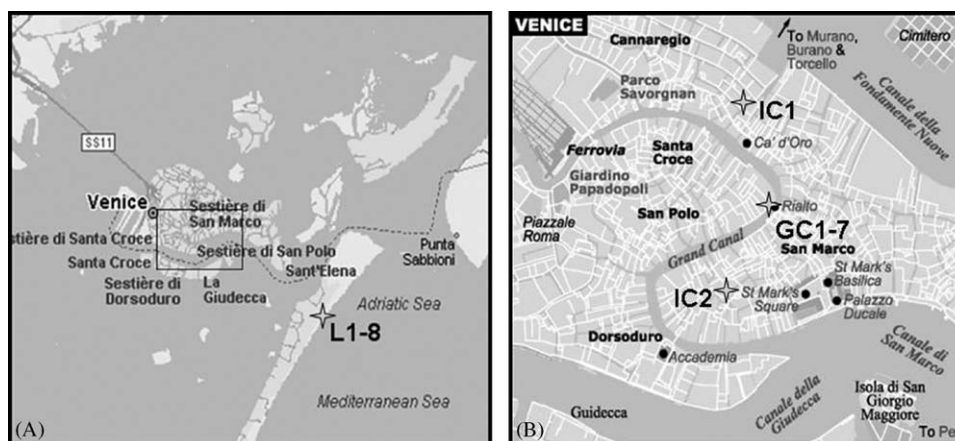


Fig. 1 – (A) Map of Venice Lagoon showing location of Lido beach sampling site. (B) Map of Venice canals showing location of sampling sites.

Table 1 – List of primers and probe used for HAV and enterovirus assays by conventional and real-time RT-PCR

RT-PCR	Primer name	Primer sequence (5'–3')	(%) GC	Amplicon size (bp)	References
Conventional HAV	HEPA1	Forward: GTT TTG CTC CTC TTT ATC ATG CTA TG	39	247	Brooks et al. (2005)
	HEPA2	Reverse: GGA AAT GTC TCA GGT ACT TTC TTT G	40		
Real-time HAV	HAV1FWD	Forward: TAC AGA GCA GAA TGT TCC TGA TCC	46	76	Brooks et al. (2005)
	HAV3RVS	Reverse: TCC CCT ATT GGC TTT CCC TT	50		
Enterovirus	EV1FWD	Forward: GGC CCC TGA ATG CGG CTA AT	40	151	MGB Alert™ Real-Time PCRKit (Xanogen)
	EV1RVS	Reverse: CAA TTG TCA CCA TAA GCA GCC A	55		
	Probe	MGB-EDQ-CTT TGG GTG TCC GTG T-Q14-FAM ^a	44		

^a MGB = minor groove binder, EDQ = eclipse dark quencher, FAM = 6-carboxy fluorescein.

Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM each of the forward and reverse primers (Table 1), and 1 µL of undiluted stock or diluted cDNA. Each sample had three replicates to ensure the reproducibility of results. The thermal profile for SYBR Green real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min.

2.4. Quantitation of enterovirus by molecular beacon real-time RT-PCR

Real-time PCR was accomplished using a One Step RT-PCR Kit (Qiagen, Valencia, CA, USA) and an MGB Alert™ Enterovirus Real-Time PCR Kit (Nanogen, San Diego, CA, USA). The enterovirus kit contained a 20 × primer mix as well as a 20 × MGB Eclipse Probe (Table 1) directed toward the 5' untranslated region (UTR) of enteroviruses (coxsackie A and B, echoviruses, polioviruses, and enteroviruses 68-71). The RNA samples were diluted 1:10 and 1:100 with DNase, RNase-free water containing sonicated herring sperm DNA (5 ng/mL) as carrier DNA (Leutenegger et al., 1999). Each 50 µL reaction mixture contained 17 µL of RNase-free water, 10 µL of 5 × Buffer, 10 µL of 5 × Q-Solution, 2.0 µL of dNTP Mix, 2.0 µL of Enzyme Mix (all components of the Qiagen kit), 2.5 µL of 20 × forward/reverse primer mix (Table 1), 2.5 µL of 20 × MGB Eclipse Probe (Table 1), and 4.0 µL of undiluted or diluted template RNA.

Samples were run in duplicate on a BioRad iCycler real-time PCR system. The real-time PCR conditions were as follows: reverse transcription for 30 min at 50 °C, polymerase activation for 15 min at 95 °C, 50 cycles of denaturation for 10 s at 95 °C followed by annealing/detection for 30 s at 56 °C and extension for 30 s at 76 °C, and a final extension step for 10 min at 76 °C.

2.5. Cloning and sequencing of HAV and enterovirus cDNA

Samples found positive for HAV and enterovirus by real-time RT-PCR were cloned and sequenced. A 247 bp HAV cDNA was amplified by conventional RT-PCR following a published protocol (Brooks et al., 2005). The primers for HAV amplification are given in Table 1. Amplified cDNAs were separated by electrophoresis in a 2% agarose gel and eluted from the gel using a Qiagen gel extraction kit (Qiagen, Inc., Valencia, CA, USA). In order to clone the enterovirus cDNA, real-time RT-PCR amplified cDNAs were run in a 2% agarose gel, and gel-purified using a Qiagen QIAquick gel extraction kit. The enterovirus and HAV gel-purified cDNAs were cloned into a TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated from recombinant clones and three to five clones were sequenced for each sample using the vector-derived T7 primer.

2.6. Sequence alignment and phylogenetic analysis

Nucleotide sequences of HAV and enterovirus clones were BLAST searched and identified based on similarity to GenBank database entries. Multiple alignments and phylogenetic analyses were performed using MEGA version 3.0 by Kumar et al. (2004). Kimura's two-parameter distance was calculated using transitions and transversions and a neighbor-joining tree was built. The confidence of reconstructed clusters was tested by bootstrapping with 1000 replicates.

2.7. Generation of HAV and enterovirus standard curves by real-time RT-PCR

An HAV standard curve was generated using plasmid DNA (8×10^7 copies/µL) with inserted cDNA from HAV strain HM

175 (VR-2089; ATCC, Manassas, VA, USA). Serial dilutions (from 8×10^4 to 8×10^0 copies/ μ L) were prepared in TE buffer (Sigma-Aldrich). Plasmid containing enterovirus cDNA (1×10^7 copies/ μ L) was obtained from Nanogen and a dilution series (10^1 – 10^4 copies/ μ L) was prepared in TE buffer. Real-time RT-PCR was performed in triplicate for each dilution of HAV and enterovirus plasmids. Standard curves were created by plotting the log of the number of HAV and enterovirus genome copies versus their corresponding C_T values and creating a best-fit line through these points (Fig. 2). The cycle threshold (C_T) is defined as the PCR cycle at which an increase in the fluorescence above the baseline signal is first detected. The C_T value is inversely related to the genome copy number. Using the standard curves, HAV and enterovirus levels in the canal and beach samples were calculated with the following equations. Concentrations were calculated assuming that no viral genomes were lost during the synthesis of cDNA (Haramoto et al., 2005; Deffernez et al., 2004; Mohamed et al., 2004),

$$\begin{aligned} & \{\text{HAV genome copies/L}\} \\ &= \frac{\{(1 \times 10^{[(C_T - 36.8)/-3.6]})(\text{dilution factor})(40)(40/19.5)\}}{\{\text{Liters of seawater}\}}, \end{aligned} \quad (1)$$

$$\begin{aligned} & \{\text{Enterovirus genome copies/L}\} \\ &= \frac{\{(1 \times 10^{[(C_T - 38.2)/-3.4]})(\text{dilution factor})(10)\}}{\{\text{Liters of seawater}\}}. \end{aligned} \quad (2)$$

2.8. Calculation of HAV and enterovirus recovery efficiencies

Two 1L seawater samples were seeded with known titers of virus, one with poliovirus 2 (VR-301, W-2 strain; ATCC, Manassas, VA, USA) and the other with HAV (VR-2089, Strain HM 175, clone 1; ATCC) prior to filtration (Table 2). The same amount of each virus was also spiked directly into a paired concentrated seawater sample, following filtration, but before RNA extraction. Real-time PCR was performed and copy numbers were determined using the standard curves (Table 2). The recovery assay was performed twice for each virus and the HAV and enterovirus recoveries were calculated by dividing the number of virus genome copies in the filtered samples by the number of copies in the unfiltered samples (Table 2).

2.9. Detection of thermotolerant coliforms in Venice canal and Lido beach samples

To determine the thermotolerant coliform levels, 100mL water samples were collected and processed within 2 h of collection. The membrane filter procedure (MF) was used to enumerate thermotolerant coliform concentrations (American Public Health Association (APHA), 1992). Up to three 10-fold serial dilutions of each water sample were applied to cellulose acetate filters and coliforms were grown on M-FC media for 24 h at 44.5 °C. Thermotolerant coliform colonies

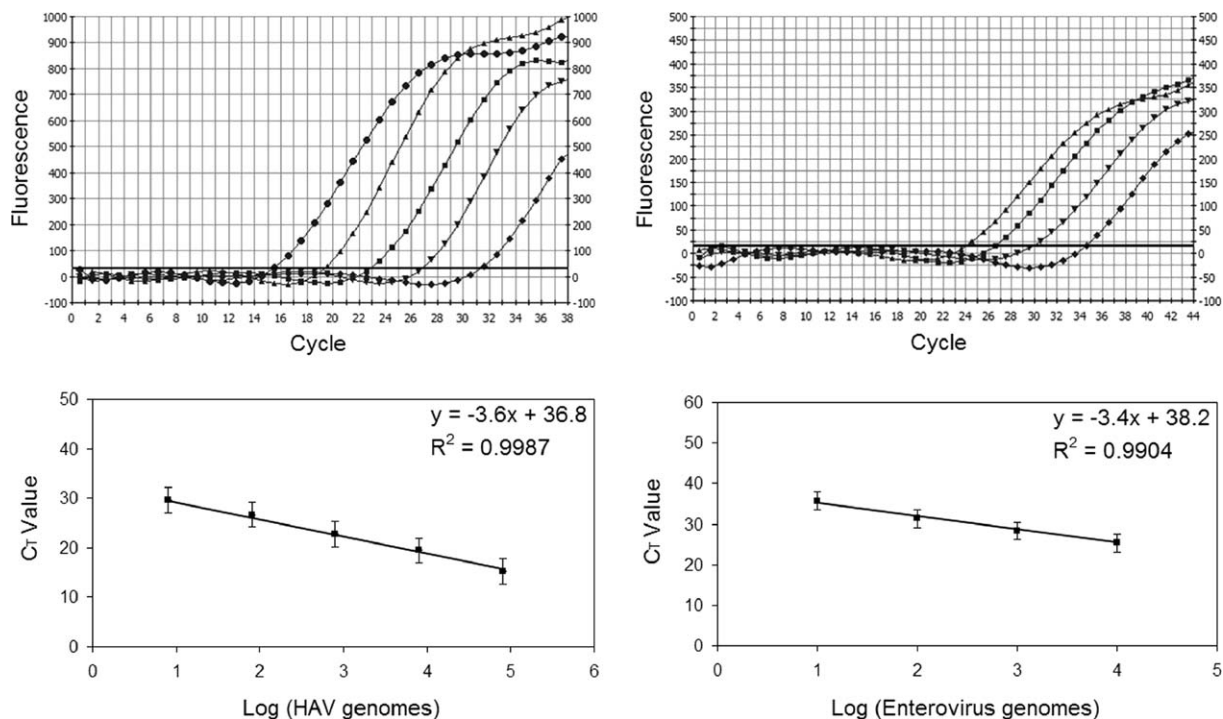


Fig. 2 – Standard curves of the real-time RT-PCR assays: (A) detection of serial dilutions of HAV inserted plasmid at 8×10^4 (●), 8×10^3 (▲), 8×10^2 (■), 8×10^1 (▼), 8×10^0 (◆) genome copies. (B) Detection of serial dilutions of enterovirus inserted plasmid at 10^4 (▲), 10^3 (■), 10^2 (▼), 10^1 (◆) genome copies. HAV (C) and enterovirus (D) standard curves were generated by plotting the C_T value versus the log of the number of viral genome copies. Error bars indicate the standard error of triplicate measurements.

were counted and densities then calculated without confirmation. The detection limit for this method is 1 CFP/100 mL.

3. Results

3.1. Recovery of spiked HAV and poliovirus from seawater

In order to determine the efficiency of our virus extraction and concentration protocol, seawater samples were seeded with known amounts of HAV or poliovirus on two occasions and virus levels were quantified using the real-time standard curves (Fig. 2). The mean percent recovery was 12% for HAV and 71% for poliovirus (Table 2).

3.2. Detection and quantitation of HAV and enterovirus levels in Venice canal and Lido beach samples

Real-time RT-PCR was performed to detect and quantitate levels of HAV and enterovirus in 17 samples from the Venice

Lagoon canals and Lido beach (Table 3). Samples that were positive for either virus using real-time RT-PCR, were further confirmed by sequencing and were then quantitated using the standard curves (Fig. 2). Concentrations were calculated assuming that no viral genomes were lost during the synthesis of cDNA (Haramoto et al., 2005; Deffernez et al., 2004; Mohamed et al., 2004). For each sample, the value from the dilution which exhibited the highest number of genome copies (i.e. showed the least inhibition) was used in Table 3.

HAV was successfully detected in five of seven Grand canal samples, and in both interior canal samples, at levels (uncorrected for recovery efficiency) ranging from 75 to 730 genome copies/L, but was never detected in samples from the beach at Lido (Table 3). Enterovirus was also successfully detected in five of seven Grand canal samples, and in both of the interior canal samples. However, unlike HAV, enterovirus was also detected in all eight Lido beach water samples (Table 3). Enterovirus levels (uncorrected for recovery efficiency) ranged from 3 to 1614 genome copies/L in the Venice Lagoon canal samples and from 2 to 71 genome copies/L in the seawater samples from Lido beach (Table 3).

The lowest viral concentrations we detected in our seawater samples via real-time RT-PCR and confirmed by sequencing were 1.9 genome copies of HAV and 1.2 genome copies of enterovirus per PCR reaction. Due to differences in the volumes of water collected and the amounts of RNA used in the PCR reactions, these numbers correspond to lowest detection limits of 13.6–77.9 genomes/L for HAV and 1.2–7.0 genomes/L for enterovirus.

Table 2 – Recovery efficiencies of HAV and poliovirus 2 (seeded into 1 liter of natural seawater) by negatively charged membrane followed by centrifugal ultrafiltration

Virus	Spiked virus (genomes)	Recovered virus (genomes)	Mean recovery (%)
HAV	12,568–32,271	1760–3240	12
poliovirus 2	591–775	451–516	71

Table 3 – Levels of HAV and enterovirus as determined by real-time RT-PCR, as well as thermotolerant coliform bacterial densities, in Venice Lagoon canals and the beach at Lido

Sample ID	Location	Date	Sample size (L)	Thermotolerant coliforms (CFP/L)	HAV concentration (genomes/L)	Enterovirus concentration (genomes/L)
GC1	Grand canal (Rialto)	5/12/2003	3.0	40,000	ND ^a	3
GC2	Grand canal (Rialto)	5/13/2003	3.0	30,000	75	ND
GC3	Grand canal (Rialto)	5/14/2003	3.0	8000	270	4
GC4	Grand canal (Rialto)	5/15/2003	3.0	27,000	ND	ND
GC5	Grand canal (Rialto)	5/25/2004	4.0	5000	128	1614
GC6	Grand canal (Rialto)	5/27/2004	4.0	87,220	94	234
GC7	Grand canal (Rialto)	5/31/2004	2.0	91,670	108	35
IC1	Interior canal (Marcuola)	5/27/2004	2.0	540,000	730	164
IC2	Interior canal (Fuseri)	6/1/2004	2.0	9110	128	51
LI	Lido beach	5/26/2004	8.0	7	ND	71
L2	Lido beach	5/31/2004	8.0	60	ND	19
L3	Lido beach	6/1/2004	4.0	ND	ND	15
L4	Lido beach	5/24/2005	11.5	155	ND	2
L5	Lido beach	5/25/2005	10.5	26	ND	2
L6	Lido beach	5/26/2005	9.0	7	ND	2
L7	Lido beach	5/27/2005	7.5	24	ND	12
L8	Lido beach	5/30/2005	6.0	13	ND	3

^a ND = Non-detectable.

Table 4 – Enteroviral types and the designations used to create the neighbor-joining tree in Fig. 3

Viral type	Designation	Genbank accession number
Human echovirus 13 isolate BE00-51 5' UTR, partial sequence	Echovirus13	AF521464
Human enterovirus 90 genomic RNA, complete genome	Enterovirus90	AB192877
Human enterovirus B strain EV30_18733_02 5' untranslated region	EnterovirusB	AY271469
Human poliovirus 2 genomic RNA, complete sequence	Poliovirus2	POL2CG1
Human echovirus 11 strain Pz 87 5' non-translated region, partial sequence	Echovirus11	AF447476
Human poliovirus 1 isolate CHN-Jiangxi 89-1, complete genome	Poliovirus1	AF111984
Coxsackievirus A16 G-10, complete genome	CoxsackievirusA16	CAU05876

3.3. Cloning and sequencing of HAV and enterovirus cDNA from Venice Lagoon and Lido beach water samples

Successful HAV amplification was obtained for 7 out of 17 samples. Multiple alignments of the HAV sequences showed 100% similarity among the isolates. A BLAST (Altschul et al., 1997) search showed 100% similarity with the VP1-VP3 gene of HAV strains (accession #AY441443). Of the 17 samples, 15 provided successful enterovirus amplification by real-time RT-PCR. The length of the amplicon was 151 bases. Three to five clones were sequenced for each sample with a total of 60 clones. A BLAST search using the 151 nucleotide sequence showed that all the clones had a similarity to the 5'-untranslated region (UTR) of enteroviruses in the database entries. Seven types of enteroviruses were identified among the clones sequenced (Table 4). A neighbor-joining tree constructed from an alignment of the 151 bases nucleotide sequence of 5'-UTR revealed two major clusters (Fig. 3). The larger clade contained poliovirus 1 and 2 and enterovirus 90 while the smaller clade contained echovirus 11 and 13, enterovirus B, and coxsackievirus A16 (Fig. 3). The large cluster could be further divided into two groups with one group comprising poliovirus 2 only and the other group containing enterovirus 90 and poliovirus 1. The most prevalent enterovirus was poliovirus 2 which was isolated from 11 out of 15 samples. Two samples were positive for enterovirus 90 and three were positive for poliovirus 1.

3.4. Concentrations of thermotolerant coliforms in Venice Lagoon and beach samples and their relationship to levels of HAV and enterovirus

Thermotolerant coliform levels ranged from 5000 to 540,000 CFP/L (colony forming particles per liter) in the Venice canals and from <1 to 155 CFP/L at Lido beach (Table 3). There was a statistically significant correlation ($R^2 = 0.62$, $p = 0.0002$) between thermotolerant coliform densities and HAV levels, but the relationship between thermotolerant coliforms and enterovirus was not significant ($R^2 = 0.08$, $p = 0.2572$) (Fig. 4).

4. Discussion

Indicators for assessing water quality have been a subject of some controversy for over 50 years. Waterborne marine

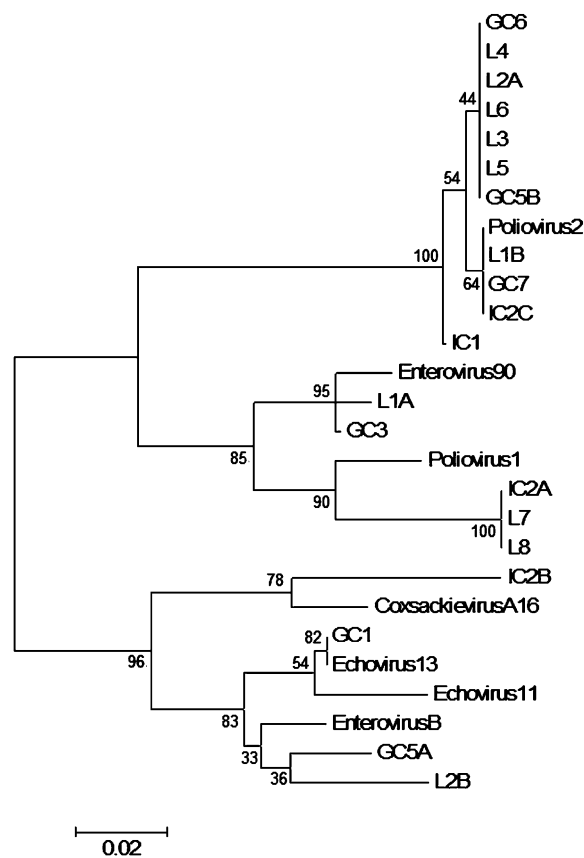


Fig. 3 – A neighbor-joining tree derived from an alignment of 151 base pairs of the 5' UTR from enteroviral standards and our specimens from Venice, Italy. Kimura's two-parameter distance was calculated using transitions and transversions and the confidence of reconstructed clusters was tested by bootstrapping with 1000 replicates.

illnesses are most often associated with viruses rather than bacteria (Griffin et al., 2003). However, current bathing water quality requirements in European Union countries are based on levels of fecal bacteria indicators (thermotolerant coliforms) rather than virus. Thermotolerant coliform indicators have been shown to die off more quickly in seawater than many viruses, and therefore may not be found in contaminated water where viruses can still persist (Fattal et al., 1983). Therefore, in order to assess the human health risk asso-

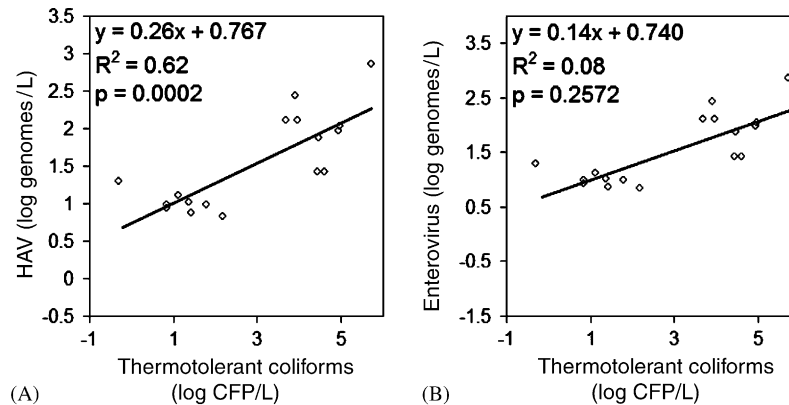


Fig. 4 – Regression analysis of thermotolerant coliform densities as a function of (A) HAV and (B) enterovirus concentrations in seawater, collected from the Venice Lagoon canals and Lido beach in Venice, Italy. Non-detectable (ND) levels of bacteria and virus were assigned the value of one-half of the limit of detection.

ciated with exposure to sewage-contaminated waters of the Venice Lagoon, we used real time RT-PCR to measure levels of HAV and enteroviruses directly, and to better define the relationship between these viruses and thermotolerant coliforms.

To date, there have been few studies performed to evaluate the viral water quality of beaches and coastal waters along the Adriatic coastline of Italy. Muscillo et al. (1999) used RT-PCR to detect poliovirus 3 in estuarine waters of the Foglia River and along the beaches of Pesaro along the Adriatic coast of Italy. Another study by a different group of researchers in the same area of the Adriatic coast, detected the presence of enterovirus by cell culture in 32% of 144 samples (Pianetti et al., 2000). These authors concluded that viral pollution originating from regional public and resort wastewater disposal systems could negatively impact regional beach water quality. In another survey of water quality in the Adriatic Sea near Fano, Italy, Muscillo et al. (2001) used RT-PCR to identify reoviruses in 30% of 72 seawater samples; however, no enteroviruses were detected. The fact that Muscillo et al. (2001) did not detect enteroviruses in their study while we did in most of our samples may have been due to better quality water samples in their study or to the generally superior detection sensitivity of real-time RT-PCR versus conventional RT-PCR.

Although the development of real-time RT-PCR methods now makes it possible to quantitate levels of human viruses in other impacted coastal waters like the Venice Lagoon, such molecular detection techniques cannot distinguish between infectious and non-infectious particles. On the other hand, since HAV detection and quantification by conventional cell culture assay is often difficult and time-consuming, there is little data that has been published to date on HAV levels in impacted marine waters. Moreover, despite its limitations in recognizing infectivity, real-time RT-PCR can still be valuable as an indicator of recent viral contamination (Gantzer et al., 1999). Using real-time PCR, Brooks et al. (2005) detected HAV in all eight samples taken during rain events from either the mouth of the Tijuana River (near the US–Mexico border) or the nearby surfzone at Imperial Beach, CA, at levels ranging from 90 to 3523 and 347 to 2656 copies/L, respectively. These

relatively high levels of HAV measured during wet weather were attributed to the inadequate sewage collection infrastructure in the region of Tijuana, Mexico.

In the present study, both HAV and enteroviruses were detected in 78% of the canal samples analyzed, with levels (uncorrected for recovery efficiency) ranging from 75 to 730 and 3 to 1614 copies/L, respectively (Table 3). It is important to note here that Venice hosts as many as 14 million tourists per year from all parts of the world. This, coupled with the inadequate sewage infrastructure in the city of Venice, suggest that the relatively high levels of these viruses that we measured may not be characteristic of other urban coastal waters in Europe. On the other hand, the occurrence of enteroviruses in European coastal marine waters has been previously reported for the Mediterranean Sea off Italy (Muscillo et al., 1999, 1994; Pianetti et al., 2000), along the beaches of southwest Greece (Vantarakis and Papapetropoulou, 1998), and the coastal waters of Northern Ireland (Hughes et al., 1992).

Extensive studies of the Florida Keys have shown widespread bacterial and viral contamination of nearshore surface waters often associated with septic systems for sewage disposal. In a total of 17 canal sites and 2 nearshore water sites, 79% of the samples sites were positive for enterovirus, 63% positive for HAV, and 11% positive for Norwalk viruses when samples were assayed by RT-PCR (Griffin et al., 1999). In southern California coastal waters near the US–Mexico border, Jiang et al. (2001) found 33% (4 of 12) of marine samples were positive for adenoviruses as determined by nested PCR. Interestingly, at these marine sites located outside of river discharge points, Jiang et al. (2001) noted that bacteria indicators did not correlate with the presence of viruses. In our study, concentrations of HAV at the beach on Lido island were always below the level of detection (Table 3). However, enteroviruses were detected in all Lido samples at relatively low levels (uncorrected for recovery efficiency) ranging from 2 to 71 copies/L (Table 3).

Our seawater samples were processed following a published virus concentration method (using negatively charged filters) for enteroviruses and noroviruses where the recovery was reported to be 60–70% (Katayama et al., 2002). However, in

a more recent study by Fuhrman et al. (2005), using the Katayama et al. (2002) protocol but modified for subsequent RNA extraction of the concentrate, recovery efficiencies were only in the range of 12.3–22.6% for seeded poliovirus in seawater. In our study, the recovery efficiency of poliovirus seeded into natural seawater was found to be relatively high (71%), more comparable to the original results of Katayama et al. (2002) (Table 2). On the other hand, although the recovery efficiency of HAV from seawater by the Katayama et al. (2002) protocol has not been previously reported, we found it to be rather low (12%) (Table 2). Since the levels of HAV and enteroviruses in the Venice canals were more or less comparable, this suggests that the poorer HAV recovery efficiency might explain why enteroviruses were sometimes detected at Lido beach, while HAV was not.

The sequence data from all the HAV clones isolated for both years 2003 and 2004 showed 100% similarity indicating the prevalence of predominantly a single isolate of HAV in the sampled region of the Venice Lagoon. Ticehurst et al. (1988) reported that different human HAV strains of diverse geographic origin were remarkably closely related. This isolate was almost identical (98%) to an isolate from Mexico (accession #AY441441) and only slightly less similar to isolates from southern Italy (97%; accession #AJ505803), Argentina (96%; accession #AF452067), and Japan (95%; accession #AB020569).

A BLAST search of enterovirus sequences identified seven different enterovirus types. This suggests that although the primer sequences in the 5'-UTR are highly conserved among enteroviruses, the sequences flanking these primers are variable enough to distinguish different enterovirus types. However, the short length of the amplicon (151bp) did not allow for discrimination between enteroviral strains (e.g., wild type and vaccine strain polioviruses). Among the clones sequenced, poliovirus 2 was most prevalent followed by poliovirus 1 and enterovirus 90. A neighbor-joining tree grouped the enterovirus isolates into two major clades. One clade contained poliovirus 1 and 2, and enterovirus 90 whereas the second clade contained coxsackievirus A16, enterovirus B, echovirus 11, and echovirus 13. This is in general agreement with previously published enterovirus phylogeny (Muir et al., 1998).

Since we found a predominant type of HAV and enterovirus (poliovirus 2, the same type as our positive control), one might argue that this resulted from a contamination event in the laboratory. However, this is unlikely for several reasons. First, negative controls run in parallel with positive samples were consistently negative by both PCR and sequencing. In addition, four of the water samples positive for poliovirus 2 also contained at least one other type of enterovirus. Finally, levels of HAV and poliovirus 2 were highly variable among the water samples.

Despite our detection of low levels of enteroviruses in all the Lido beach samples, it is important to note here that in these same samples, thermotolerant coliform levels ranged from 0 to 155 CFP/L at Lido beach and never exceeded the criteria under Italian national law (Presidential Decree no. 470 of 1982 which acknowledges European Council Directive 76/160/EEC on Bathing Water Quality) of an upper limit (imperative value) for thermotolerant coliforms of 2000 CFP/

100 mL. Indeed, only a single sample taken on 25 May 2005 exceeded the guideline value of 100 CFP/100 mL. Moreover, while our analysis showed there was a statistically significant ($p = 0.0002$) relationship between densities of thermotolerant coliform bacteria and HAV levels for all the samples pooled (Fig. 4), there was no significant relationship ($p = 0.2572$) evident between thermotolerant coliform indicator densities and enterovirus levels. This latter result supports the need for the development of both rapid and sensitive methods to quantitate human pathogens directly rather than relying on the conventional bacterial indicators to assess human health risk in recreational marine waters.

We attempted to interpret enteroviral levels in terms of a quantitative risk assessment for swimming at Lido beach, by relating the PCR-quantified viral densities to infectivity. Donaldson et al. (2002) concluded from data of a side-by-side comparison of cell culture and real-time RT-PCR for enteroviruses, that 55 viral particles in a sample equates to one infectious particle. Using this infectivity relationship for enterovirus would equate to 0.04–1.3 infectious particles/L for Lido beach. Assuming an incidental ingestion of 100 mL of seawater during swimming, then the risk of infection may then be calculated by using the beta-Poisson model (Regli et al., 1991):

$$P_i = 1 - \left(1 + \frac{\mu V}{\beta}\right)^{-\alpha} \quad (3)$$

where P_i is the probability of infection resulting from ingestion of a single volume V of water containing an average of μ organisms per unit volume, and α and β are model parameters that characterize the dose-response curve exposure. Using the best-fit model parameters of $\alpha = 0.409$ and $\beta = 0.788$ for poliovirus III (Regli et al., 1991) as being most conservatively representative of the infectivity of enteroviruses, the daily risk of enteroviral infection for exposure at Lido beach can be calculated to range from 1.9×10^{-3} to 6.1×10^{-2} with the lowest risk detectable by our method at 1.1×10^{-3} . It is noted here that the risk estimates do not take into account our recovery efficiency of enteroviruses, but since the efficiency was relatively high (71%) (Table 2), the risk outcomes would not be significantly changed by such a correction. A sensitivity analysis of the risk outcomes using the beta-Poisson model showed that over a range of ± 1 order of magnitude from the base values, the model was nearly equally sensitive to changes in each model parameter (though in an inverse way for β).

Since the risk of symptomatic disease may range from 1 percent for poliovirus to more than 75 percent for some of the coxsackie B viruses (Cherry, 1981), then the daily risk of disease may be assumed to be no higher than about 4.5×10^{-2} even at the highest enterovirus level we measured. Such a conclusion suggests that bathers at Venice's Lido beach are at or below the disease risk (5% for gastroenteritis) that is deemed acceptable by complying with the standards of the European Directive (Commission of the European Communities, 2002). It should be noted here, however, that enteroviruses can cause a variety of other and more serious disease symptoms besides (or in addition to) gastroenteritis including poliomyelitis, aseptic meningitis and myocarditis, but these

diseases are not subsumed within the risk outcomes of the European Union Directive.

Real-time RT-PCR is relatively rapid with respect to current water quality monitoring procedures, with an entire processing time of less than 12 h. In addition, this method has the potential to offer greater sensitivity and quantitative ability than any single method currently offers. With further optimization of viral concentration procedures, the applicability of this method to high throughput reproducible assays could be developed for routine detection of human pathogens in marine recreational waters impacted by sewage contamination such as the Venice Lagoon.

5. Conclusions

Venice canal samples were often contaminated with high levels of both HAV and enteroviral genomes, reflecting the high degree of sewage contamination of these waters. At the beach on Lido island, concentrations of HAV were always below the level of detection, and enteroviruses (when detected), were always present at relatively low levels.

The risk for enteroviral infection (calculated using the beta-Poisson model) for recreational exposure from swimming at Lido beach was in the range of 1.9×10^{-3} – 6.1×10^{-2} , yielding a disease risk at or below the level deemed acceptable by European Guide standards.

There was a statistically significant correlation between thermotolerant coliform densities and HAV levels, but not between thermotolerant coliforms and enterovirus levels, supporting the need for methods to quantitate human viruses directly rather than relying on the conventional bacterial indicators.

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