Epidemiological surveillance of enteric viruses in East Macedonia and Thrace region in Greece

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ABSTRACT

The enteric viruses (EVs) are the most common and widespread human viruses, as they can spread in the environment through the faecal excretion. These infectious agents may cause outbreaks throughout the year. The aim of this study was to investigate the environmental presence of EVs using molecular methods. A total of 144 wastewater samples were collected between December 2004 and November 2006 from six sewage treatment plants in Greece. The sampling was carried out once a month. Out of the 144 examined wastewater samples, 59.7%, 40.9%, 17.3%, 34%, 2% were positive to the detection of adenovirus, enterovirus, norovirus GI, norovirus GII and hepatitis A, respectively. Our results indicated the potential public health risk associated with transmission of human enteric viruses through environmental wastewater routes. This is the first time that enteric viruses have been isolated in the region of East Macedonia and Thrace in Greece.

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Abbreviations: EVs, enteric viruses; GI, genotype I; GII, genotype II

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Introduction

EVs are the most reason for gastroenteritis symptoms in both developed and developing countries as they are excreted in large numbers in feces \[1\]. EVs can engender outbreaks of diseases such as gastroenteritis and hepatitis A. Food and waterborne infections are of special importance, as these kind of outbreaks may involve a large number of people and wide geographical areas \[2,3\]. The traditional and classical technique for the detection and isolation of enteric viruses is cell culture, which is costly and time-consuming. Most important, some virus species like hepatitis A virus, adenovirus 40 and 41 and norovirus cannot be isolated efficiently via cell lines. Furthermore, environmental samples contain a large numbers of inhibitors and it is not able to avoid the co-isolation of inhibitors. For this reason, nucleic acid-based methods such as PCR, especially nested PCR are largely used.

Enteroviruses are significant concern for public health \[4\]. Respectable numbers of enteric viruses can be isolated from raw wastewater, faeces of humans as well as polluted waters \[5\]. Enteroviruses are small viruses that are constituted by single stranded RNA, they belong to the family of \textit{Picornaviridae} and the group includes the echoviruses, coxsackieviruses, polioviruses and other enteroviruses. Non-polio enteroviruses cause about 10 to 15 million infections in the United States each year \[6\]. They can also be isolated from saliva, spittle, rhinal mucus and in the excrements of patients. People are exposed to the risk of infection by person-to-person contact or through polluted surfaces, for example the mobile, a glass e.t.c.

Hepatitis A is the most important causative agent for acute hepatitis as it is primarily transmitted by the fecal-oral route \[7\]. Hepatitis A outbreaks can also occur from stool contamination of certain foods at their source. Each year there are two million cases of symptomatic hepatitis A. All high-income regions have very low incidence of illness, while in developing countries is very high.

The presence of Adenoviruses is considered an emergent issue, as these viruses are also pathogenic to humans and their presence in polluted waters may cause infections \[8\]. Adenoviruses are the only human enteric virus with double-stranded DNA. Their detection in contaminated waters and their role as a cause of gastroenteritis may have been underestimated. The detection of human adenoviruses by PCR method has caused the attention of scientists, in conjunction with the evaluation of the viral quality of environmental samples as their genome is quite distinct. In various environments like raw sewage and in any disinfection treatment (UV, chlorine) adenovirus is more stable in comparison with other EVs. Also, studies confirmed their predominant presence in shellfish,
compared to other EVs. Pina et al. proposed a molecular index for viral contamination of human origin using the detection of human adenoviruses \[^9\].

Recent studies have shown that noroviruses are recognized as a worldwide cause of nonbacterial gastroenteritis. Numerous molecular epidemiological studies have committed a global distribution of these viruses. In Europe, a study on viral gastroenteritis showed that noroviruses were responsible for >85% of all nonbacterial outbreaks of gastroenteritis reported from 1995-2000 \[^10\]-\[^12\]. In the United States, infections caused by noroviruses are estimated to be responsible for 23 million cases of gastrointestinal illness per year \[^13\]. Noroviruses which have been previously named Norwalk-like viruses or small round structured viruses belong to the \textit{Norovirus} genus of the \textit{Caliciviridae} family, which also contains the \textit{Sapovirus} genus. Noroviruses can be classified into 5 genogroups, GI-V; three genogroups containing viruses that have been found in humans GI, GII, and GIV, and can be divided into 15 genetic clusters \[^14\]. Noroviruses are the viruses most commonly associated with food- and waterborne outbreaks of gastroenteritis. Illness takes place in people of all ages, is characterized by nausea, vomiting and diarrhea. Noroviruses are transmitted mostly through the fecal-oral route but may also be transmitted through person to person contact \[^15\]. There have been reported many outbreaks due to norovirus contaminated foods, like salads, oysters, fishes and to noroviruses contamination of water. Low infectious dose, resistance to disinfection, multiple routes of transmission and strain diversity give reasons for the high prevalence and their persistence. Rapid and secured methods have been developed to detect noroviruses in contaminated food and water since noroviruses cannot be propagated in cell culture.

In the present study, enteroviruses, adenoviruses, noroviruses and hepatitis A were detected in raw sewage. Sewage samples were collected from inlet effluents of six biological treatment plants in the region of North-Eastern Greece. Detection was performed only at the inlet of wastewater treatment plants, because in the present study it was only examined the prevalence of these viruses in the community. Raw sewage samples (144) were analyzed for the presence of these viruses during the period December 2004 to November 2006.

\textbf{Materials and methods}

1. Virus concentration: Each wastewater sample was collected in a sterile 500mL polyethylene container, kept at 4°C for less than 8 h until processed. Then, sewage (100 ml) was processed by adding 100 ml 6% (w/v) beef extract (BBL) solution pH 9.5 to 100 ml of
sample, shaking vigorously for 30 min at room temperature and then centrifuging at 7000g at 4°C for 30 min to remove any debris. The supernatant was flocculated at pH 3.5 and centrifuged again. The floc was deposited by centrifugation at 7000g at 4°C and was dissolved in phosphate buffer saline buffer (Fluka), pH 7 to a total volume of 1 ml, and stored at -20°C [16-17].

2. RNA extraction and PCR: Viral RNA was extracted from 140 μl aliquots of concentrate using QIAmp Viral RNA Mini Kit (Qiagen) according to manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) and nested PCR techniques have been used, according to previously published protocols. The sequence, specificity and sensitivity of the oligonucleotide primers used were described previously [18-20]. In Table 1, a summary of all primers characteristics is presented. All oligonucleotide primers have been tested for primer–dimmer formation using the PubMed NCBI Blast software.

3. Quality control: Adenovirus type 41, Enterovirus echo 11, Hepatitis A MRC5 and Norovirus GI & GII were used in-run positive extraction controls for quality molecular assays in RT-PCR. (Source: The Centre for Environment, Fisheries & Aquaculture Science, Weymouth, Dorset DT4 8UB UK).

4. Statistical analysis: Statistical analysis was performed using SPSS software pack (version 15). P value < 0.05 was considered for statistic significance.

Results

1. Prevalence of EVs

Adenoviruses were found in 86 samples (86/144, 59.7%). Sequencing analysis of the positive sewage samples showed the presence of type 41. Enteroviruses were detected in 59 samples (59/144, 40.9%) and sequencing analysis of the positive sewage samples reported the presence of several types such as (a) coxsackievirus types (b) echovirus (c) poliovirus. Noroviruses were found in only 25 samples (25/144, 17.3%) for GI strains and in 49 (49/144, 34%) samples for GII strains (Table 2). The most prevalent norovirus type in sewage was closely related to the GGII variant. Finally the presence of hepatitis A was virtually nil since the positive samples were only 3 (3/144, 2%).

2. Seasonal distribution

The distribution of Enteroviruses in summer and winter months was analyzed (Figure 1). Approximately, 22.2% more cases were reported in summer than during the winter. In the summer months the positive samples were detected in 52.8%, while in winter the rate fell to 30.6%. Statistical analysis showed this difference to be statistically significant (p = 0.007). On the contrary, in the case of Adenoviruses, the correlation between winter and summer months gave no statistically significant results (p> 0.05).
Table 1: The sequence and the specificity of the oligonucleotide primers

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type</th>
<th>Primer’s name</th>
<th>Sequence</th>
<th>Position</th>
<th>PCR product</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus</td>
<td>RT a - primer</td>
<td>ENT2</td>
<td>ATTGTCAACCATAAGCAGCCA</td>
<td>603-584</td>
<td>540</td>
<td>5’NTR&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>ENT1</td>
<td>CGGTACCCTTTGATCGGCTG</td>
<td>63-82</td>
<td>540</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>neENT1</td>
<td>TCCGCCCCCTGAAATGCGGCTA</td>
<td>449-469</td>
<td>123</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>neENT2</td>
<td>GAAACAGGACACCCAAATG</td>
<td>568-548</td>
<td>123</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>RT - primer</td>
<td>HAR</td>
<td>CTGAGTACCTCAGAGGCAAC</td>
<td>700-680</td>
<td>368</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>HAL</td>
<td>TTGGAACGTCACCTTGACGTA</td>
<td>332-352</td>
<td>368</td>
<td>5’NTR</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>neHAL</td>
<td>GAAAGTCCAGCTGCAATGG</td>
<td>661-641</td>
<td>290</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>neHAL</td>
<td>ATCTCTTTTATCCTCCAAC</td>
<td>371-391</td>
<td>290</td>
<td>5’NTR</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>HEXA</td>
<td>GCCGCAGTGGTCTTACATGCACATC</td>
<td>18858-18882</td>
<td>308</td>
<td>Hexon gene</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Nested Primer</td>
<td>NHEXA</td>
<td>GCCACCGAGACGTACTTCAAGCCTG</td>
<td>18937-18960</td>
<td>143</td>
<td>Hexon gene</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>NHEXB</td>
<td>TTGTACGAGTACCGGTCATCTCGCGTC</td>
<td>19079-19051</td>
<td>143</td>
<td>Hexon gene</td>
</tr>
<tr>
<td>Norovirus GI</td>
<td>RT - primer</td>
<td>SM31</td>
<td>CGATTTCATCATCACATACA</td>
<td>4871-4853</td>
<td>190</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>GI</td>
<td>TCAGAAATGATTTGG</td>
<td>4679-4696</td>
<td>190</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td>Norovirus GII</td>
<td>Nested Primer</td>
<td>ANDO(GA)</td>
<td>GTGACGACATGAAACAGGCCATGTG</td>
<td>4762-4782</td>
<td>112</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>E3</td>
<td>ATCTTCATCATCACCATA</td>
<td>4869-4853</td>
<td>112</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; PCR</td>
<td>SM31</td>
<td>CGATTTCATCATCACCATA</td>
<td>4871-4853</td>
<td>270</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>Ni</td>
<td>AGCCATAGAAATAATGCTG</td>
<td>4338-4355</td>
<td>270</td>
<td>Rna polymerase gene</td>
</tr>
<tr>
<td></td>
<td>Nested Primer</td>
<td>E3</td>
<td>ATCTTCATCATCACCATA</td>
<td>4869-4853</td>
<td>113</td>
<td>Rna polymerase gene</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reverse transcription  <sup>b</sup>NTR: 5’-non-translated region.
The same results was observed for Noroviruses for both types I and II (p = 0.194 and p = 0.724, respectively). The low number of Hepatitis A samples was insufficient for statistical analysis (Figure 1).

**Discussion**

Sewage treatment commonly applied in depuration plants, including biological and physicochemical processes, has significantly reduced the incidence of diseases among the population, especially those etiologically related to bacteria. However, viruses are more resistant than bacteria to most treatments [21]. Water quality may be affected by the attendance of these pathogenic enteric viruses derived from wastewater discharged to the aquatic environment [22]. Untreated wastewater was found contaminated by different types of EVs that cause the majority of gastroenteritis. Therefore, it is necessary to use the most efficient wastewater treatment measures in sewage treatment plants [21-25].

The results showed that the rate of detection of enteroviruses, hepatitis A, adenoviruses, noroviruses genogroup I and noroviruses genogroup II was 40.9%, 2%, 59.7%, 17.3% and 34% respectively.

Enteroviruses isolated by nested PCR method in percentage 40.9%. Many studies have been carried out in different countries, estimating the large proportion of presence of enteroviruses. In a total of 188 samples collected between 2005 and late 2008, in the town of Parma in Italy, enteroviruses were detected in 78.7%. Of the 148 positive samples, only one standardized as polio-virus while the others were identified as coxsackie viruses and echo-viruses [26].

The persistence of the hepatitis A virus in Greece and generally in developed countries is quite low. During the 1980s and 1990s the incidence of hepatitis A showed a significant decrease. The national policy implemented by vaccination at an early age acted to the extent that any cases are regarded sporadically. The 150 to 200 positive cases

<table>
<thead>
<tr>
<th>Enteric Virus</th>
<th>Positive samples</th>
<th>Negative samples</th>
<th>Percentage of positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>86</td>
<td>58</td>
<td>59.7</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>59</td>
<td>85</td>
<td>40.9</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>3</td>
<td>141</td>
<td>2</td>
</tr>
<tr>
<td>Norovirus GI</td>
<td>25</td>
<td>119</td>
<td>17.3</td>
</tr>
<tr>
<td>Norovirus GII</td>
<td>49</td>
<td>95</td>
<td>34</td>
</tr>
</tbody>
</table>
each year nationwide giving an annual incidence of approximately 1.3-1.8 cases in 100000 [27]. The only three positive samples for the virus of hepatitis A are expressed with the rate of 2% is not possible to draw conclusions. The presence of the virus in a small proportion in our study is in agreement with a recent study done in Brazil [28]. In the case of the experiments conducted Arraj and colleagues in France in sewage treatment plant is also not isolated hepatitis A virus [29].

Adenoviruses identified at a greater rate than other enteric viruses. The results agree with those of previous studies about the incidence of adenoviruses [30]. With the application of molecular techniques, the detection rate reached 59.7%. La Rosa et al. isolated in large quantities adenoviruses both at the entrance and the exit of wastewater treatment plant in up to 96% and 76% respectively [31]. A previous study in Japan in effluent samples, indicating the universal presence of adenoviruses as 99% (71/72) of samples were positive [32]. Other studies have also show that adenoviruses survive more than other enteric viruses and bacteria of fecal origin. Their strength even to ultraviolet radiation is typical and has been labeled previously [33].

Norovirus has identified the main cause of gastroenteritis in adults over the last two decades. The presence proportion of norovirus genotype I (GI) and II (GII) amounted to 17.3% and 34% respectively. The genogroup II detected in the untreated wastewater at higher rate, as confirmed in

Figure 1: Seasonal distribution of EVs during December 2004 until November 2006
the literature by other researchers. The genotype II of norovirus is the most prevalent in humans followed by groups I and IV. In 2006 a new strain of type 2 caused a multitude of epidemics of gastroenteritis worldwide [34]. For several years the strains of genotype II, especially strain GII4, have shown worldwide and a vast geographical distribution due to contamination from person to person during epidemics [35-37]. A previous study reported that the genome of genotype II detected hundred times more than the genotype I, in people who are infected with this type of virus [38]. Strains of type I most often transmitted through food [39].

The untreated sewage is the main route of contamination of groundwater and surface water. The surveillance of enteric viruses strains, with molecular methods and techniques, the standardizing and the comparison between environmental and clinical isolated, could be an important tool for the protection of public health. In general, direct and specific epidemiological and environmental studies must unmask the root of an outbreak. In the near future, detailed sequence data is required; following the molecular detection, in the investigation for correlation among environmental causative agents and clinical samples from patients.

References


[6]. Centers for Disease Control and Prevention. National Center for Immunization and Respiratory Diseases, Division of Viral Diseases 2010.


