Original Article

Frequency of HEV contamination in sewerage waters in Pakistan

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Abstract

Introduction: Enteric viruses, including Hepatitis E virus (HEV), are able to persist under environmental conditions and may cause public health problems by contaminating natural and drinking water resources. Routine procedures for monitoring viruses in water samples have not been established for the water microbiology screening panel.

Methodology: Eighty-six raw sewerage samples were collected from the different regions of Islamabad and Rawalpindi, the twin cities of Pakistan. Samples were concentrated for HEV, using a polyethylene glycol-based method followed by viral RNA extraction using a commercial kit-based method. Reverse transcription polymerase chain reaction (RT-PCR) with HEV specific primers was used for the detection of HEV.

Results: The present investigation focused on 86 raw sewerage water samples taken from different locations of drainage outlets of Islamabad and Rawalpindi. After careful experimentation, 35 samples were found to be RT-PCR positive. Nineteen (44.7%) out of 47 samples from Rawalpindi city were HEV positive while 16 (41.02%) out of 39 samples from Islamabad were HEV positive. All positive samples were found in the highly congested areas.

Conclusions: The high detection rate of HEV in this study shows that HEV circulates at a relatively high frequency in the sewerage waters in Pakistan. This study is the first report on detection of HEV from sewerage waste water from Pakistan and suggests that HEV might be a potent indicator of viral pollution in environmental specimens.

Key words: HEV; PCR; detection method; sewerage water

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Introduction

Hepatitis E virus (HEV) is a non-enveloped virus approximately 27-34 nm in diameter. It was previously classified into the family of Caliciviridae, but is now classified as Hepevirus genus under the separate family of Hepeviridea [1]. The HEV is a positive-sense, single-stranded RNA virus of approximately 7.2 kb containing three open reading frames (ORFs). ORF1, located at the 5' end of the genome, is about 5 kb in length and encodes nonstructural polyprotein that contains motifs characteristic for methyltransferase, protease, RNA helicase, and RNA-dependent RNA polymerase. ORF2 is about 2 kb in length and encodes for the structural protein(s). The small ORF3 of only 369 nucleotides overlaps ORF1 and ORF2 and encodes for a protein of unknown function [2].

HEV is the leading cause of enterically transmitted non-A, non-B hepatitis. It is responsible for the major outbreaks of acute hepatitis in developing countries, especially in the tropical and subtropical regions of the world where outbreaks are usually associated with fecal contamination of drinking water [3,4].

Hepatitis E occurs primarily in Africa, Mexico, Central Asia and some South Asian countries [5,6]. It affects mainly young adults in the 15-40 year age group, and runs a sub-clinical infection among children [7]. Being faeco-orally transmitted infection, HEV is often associated with poor standards of sanitation and hygiene. The highest concentration of virus is found in stools during the incubation and early symptomatic phase of the disease. Infections are self-limited [8], but severe complications with a high mortality of 20-30% have been reported during epidemics, particularly in the third trimester of pregnancy [9, 10]. The fatality rate of HEV is 15-20% among pregnant women. HEV is also associated with hepatocytes necrosis, cholestatic jaundice, miscarriages or premature delivery. [10]. Unlike

hepatitis A virus (HAV), HEV is primarily transmitted through contaminated drinking water [9], but rarely through person-to-person contact [11]. Recently, swine have been implicated as the reservoir of human infection [12].

Most of the enterically shed viruses that cause gastrointestinal infections, hepatitis or neurological diseases are excreted in large titres in human faeces [13], resulting in their accumulation in community sewerage. Viruses in sewerage may contaminate drinking water resources, recreational waters, and natural waters (lakes and rivers), potentially causing a high public health risk. Sewerage treatment and disinfection procedures for wastewater have only a limited effect on a number of viruses, and therefore viruses are able to contaminate environmental waters thus preserving a long-term capacity for infection [14]. Many viruses, including enteroviruses hepatitis A and E viruses, noroviruses, rotaviruses and enteric adenoviruses [14] have been detected in different waters in the past few decades. However, the number of reports of astrovirus detection is relatively low [15]. Hence this study was conducted and the aim of this study was to detect human HEV in raw waste water samples.

Methodology

Sewerage water sample collection

Raw sewerage samples (30 ml) were collected from different outlets of Islamabad and Rawalpindi, areas with total population of 3.5 million people.

Sample concentration

Polyethylene glycol 6000 (PEG 6000) precipitation was employed which has been reported to enhance the chances for detecting human virus pathogens in environmental samples [16]. Sewerage samples were divided into aliquots of 10 ml, and 10% (w/v) PEG 6000 was added to each sample along with sodium chloride to the final concentration of 0.4M. This mix was stirred over night at 4˚C and then centrifuged at 10,000xg for 90minutes. The resulting pellet was suspended in 4mL of 0.15M phosphate buffer pH 9.0 and stored at -20˚C.

Extraction of Nucleic Acid

Viral nucleic acid was extracted by using a QIAamp RNA extraction kit Qiagen, Germany, Hamburg) [17]. Samples were stored at -20ºC.

Specific primers

PCR amplification was conducted by a set of primers, which amplified a 375 bp region of ORF-1 of HEV. The sequences of the primers were, 5'- CCT GGC ATC ACT ACT GCT ATT GAG CAG – 3' (sense) and 5' – ACC TCA GGC GGG AGG TGG AGG – 3' (antisense).

Primer design

The gene bank accession numbers of HEV isolates used for the primer designing are listed below.

M94177, X98292, D10330, AF076239, AY230202, AF459438, AF444002, AF444003, NC001434, AF051830, D11093, D11092, M74506, AY115488, AB248520, AB246676, AB089824, AB189070, AF455784, AF082843, AY575859, AB074920, AB222182, AB108537, DQ279091, AB220978, AB074917, AB097811, AY723745, AY535004.

Sequence comparison for primer designing was done in CLC workbench software (Karinebjerg, Denmark).

Complementary DNA (cDNA) synthesis

Extracted RNA served as the template for the cDNA synthesis. The reaction mixture for reverse transcription had a volume of 20ul, contained 13ul of RNA, 1 ul dNTPs (10mM), 4ul MMulv Buffer, 1 ul M.Mulv enzyme (Fermentas Canada) and 1ul specific antisence primer was used. The reaction conditions for reverse transcription were: incubation at 37°C for 90 min, denaturing at 96ºC for 5 min then quick chilling on ice.

PCR

PCR reaction was performed by using 5 ul of cDNA as template, 1 ul each of sense and antisense primer, 2 ul of DNTPs (2mM), 2.5 ul of Dream Taq buffer (Fermentas Canada), 13 ul of nuclease free water, and 1.5 unit of DreamTaq Enzyme (Fermentas Canada). The cycling conditions were as follows: Initial denaturation step at 94˚C for 3 minutes, 35 cycles for 1 minute at 94˚C, 1 minute at 58˚C for annealing, 1 minute at 72°C for extension, and a final extension at 72˚C for 7 minutes. Nuclease-free water was used in all experiments as a negative control. PCR products (5ul) were mixed with 2 ul of 6x loading dye and analyzed on 1.5% agarose gel. Fragment sizes were compared using a commercially available size standard 50bp DNA ladder (Fermentas Canada).

Results

Eighty-six sewerage water samples were taken for the diagnosis of HEV, out of which 35 (40.7%) samples were HEV positive [Figure 1]. Out of 47 samples from Rawalpindi city 19 (44.7%) were HEV positive while 16 (41.02%) out of 39 samples from Islamabad were HEV positive.

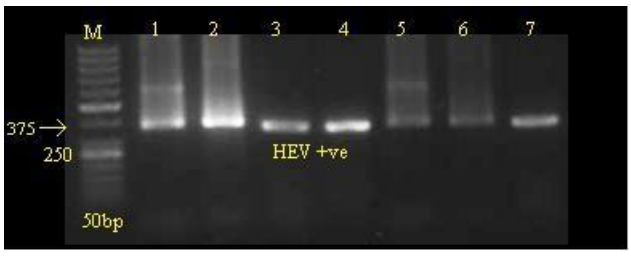
Virus incidence in municipal waste water

The present investigation focused on 86 sewerage water samples taken from different location of drainage outlets of Islamabad and Rawalpindi. After careful experimentation, 35 (40.7%) samples were found to be RT-PCR positive. There were 47 samples from Rawalpindi city out of which 19 (44.7%) were HEV positive, while 16 (41.02%) out of 39 samples from Islamabad were HEV positive. All positive samples were found in the highly congested areas.

Public health indicators

This study reports the widespread presence of viral RNA in the sewerage water of Islamabad and Rawalpindi, Pakistan. The virus was detected by an RT-PCR-based method. HEV infection is not notifiable in Islamabad and Rawalpindi cities of Pakistan and we are not aware of any documented cases of HEV infection here. However, HEV infection may be undiagnosed and unreported. The risk factors for HEV infection are related to poor sanitation in large areas of the world. Person-to-person transmission is uncommon. There is no evidence of sexual transmission or of transmission by transfusion. In this perspective, water has to be purified and disinfected to make it safe to drink to prevent health hazards.

Figure 1. This is a representative gel picture in which the displayed bands (lane 1 to lane 7) indicate detection of hepatitis E virus from the sewerage water. The marker used is 50bp and the specific bands are of approximately 375bp



Discussion

In Pakistan urban people live in congested colonies and thickly populated wards. Open drains, the dumping of garbage at public places, poor maintenance of the sewerage disposal system, and the close proximity of drinking water supply lines adjacent to or through the sewerage disposal system result in the high risk of the drinking water supplies becoming contaminated due to leakage. We examined the procedure of HEV in such open source places that could pose a serious threat for infants and pregnant women. This assessment is critical to contain the viral spread.

The present investigation focused on 86 sewerage water samples taken from different locations of drainage outlets of Islamabad and Rawalpindi. After careful experimentation, 35 (40.7%) samples were found to be RT-PCR positive. There were 47 samples from Rawalpindi city out of which 19 (44.7%) were HEV positive, while out of 39 samples from Islamabad 16 (41.02%) were HEV positive. All positive samples were found in the highly congested areas. Nowadays molecular techniques are widely used in various environmental procedures because they are very specific and sensitive for the detection of viruses and bacteria from a wide range of sample sources.

Pakistan is a developing country with low health and educational standards. According to the human development index of the United Nations, Pakistan was ranked 134 out 174 countries [18]. Proper sanitation conditions are lacking, drinking water pipelines lay adjacent to sewerage water lines, and in most of the cases there is mixing of sewerage water with drinking water.

HEV can be detected from the stools of HEV positive patients with duration of fecal shedding ranging from 9-12 days [19]. There are several reports regarding HEV detection from sewerage water. Reported from India disclosed that HEV prevalence was very high (41%) throughout the year in sewerage water [20]. A separate study reported that 57% of sewerage workers from India were positive for IgG anti-HEV [21]. Turkish farmers who used untreated water for agriculture were highly seropositive for HEV [22]. These studies support the presence of high HEV RNA from sewerage water samples. The frequency of HEV infection was very high because 40.6 % of sewerage water samples were positive for RT-PCR.

In conclusion, the present study demonstrates that high proportions of sewerage water samples can contain HEV particles. This is a clear indication of a high prevalence of HEV among the population of Pakistan.

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