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ORIGINAL ARTICLE

Hepatitis E virus (HEV) in sewage from treatment plants of Messina University Hospital and of Messina city council

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Key words

HEV • Sewage treatment plants

Summary

Samples of sewage from treatment plants at the "G. Martino" University Hospital of Messina (AOU) and that of Messina City Council were analysed to detect the hepatits E virus. Samples were taken on sewage entering and exiting the treatment plants on a monthly basis over a one-year period from both the hospital plant (24 samples) and the municipal plant (22 samples).

All sewage samples were pretreated by ultrafiltraton and concentration and finally processed by the PCR method to amplify gene material. A total of three samples tested positive: two (8.33%) entering the AOU treatment plant and one (4.5%) entering the municipal plant while no cases of HEV were detected in samples of treated sewage. These findings confirm the presence of the virus in the city of Messina and showed that the two treatment plants to be working efficiently when tested.

Introduction

HEV is an RNA virus that was first recognised in 1983 as an etiological agent of hepatits E, an acute disease that is often icteric but which does not tend to become chronic. It is generally self-limiting, with an incubation period ranging from 15 to 60 days. The main characteristic of this infection is the high frequency of fulminant clinical forms (1-12%) which takes a particularly severe course in pregnant women, especially in the third trimester, with mortality rates reaching as high as 40% [1-16].
Although the epidemiology of HEV is still not fully understood, it is certain that the mode of transmission is fecal-oral, in particular via water contaminated with sewage and this therefore constitutes a useful investigatory tool for epidemiological studies [2].
The spread of this disease is greater in geographical areas with inadequate standards of health and hygiene where both sporadic cases and epidemics may appear. Epidemics have recently been recorded in India, Burma, Iran, Bangladesh, Ethiopia, Nepal, Algeria, Libya, Somalia, Indonesia, Mexico, China, Pakistan, in former Soviet Central Asian Republics [3-6]. In countries with high standards of health and hygiene, the majority of cases are people returning from travels to countries at risk (imported cases) while to a lesser extent they are due to autoctonic strains of HEV [1].
In Italy, a study by Zanetti and Dawson showed 10% of hepatitis cases (not A-C) to be caused by HEV, often found in persons of other nationalities. Moreover, the same research isolated for the first time a strain of HEV in a patient who had neither travelled nor been in contact with anyone returning from endemic areas thus demonstrating the existence of actoctonic HEV strains [7-9].

Aim

To contribute to the definition of the epidemiological framework of hepatitis E by means of a study of HEV in sewage taken from two wastewater treatment plants in the city of Messina: one municipal plant and the other of the University hospital (AOU).
In particular, the analysis of the wastewater from the latter plant focuses the field of research on a specific sector of the population.

Materials and methods

In this study we studied samples of sewage entering and exiting the activated sludge sewage treatment plant, serving the municipality of Messina, located in Mili in the area of Barone, and the treatment plant for Messina University hospital situated onsite. The samples, 22 for the council plant and 24 for the hospital plant, were collected monthly over nearly a year from 14 April 2006 to 31 March 2007.
The samples of untreated and treated sewage (2 litre) were stored at -80°C until they were tested, after being defrosted and left to settle for two hours.
Samples were concentrated by tangential-flow ultrafiltration.

Tangential-flow ultrafiltration

This method uses a peristaltic pump fitted with a cellulose membrane with a mesh size of 10000 Dalton Millipore. The membrane was pretreated (in order to saturate the sites present on it) by allowing a 0.3% meat extract solution at pH 7 to circulate in the column. After ultrafiltration, the samples underwent filtration using 40ml

of a 3% meat extract solution concentration at pH 9, the solution was left to act for 10 minutes to enable any viral particles stuck to the membrane to be recovered.
This treatment yielded 15-20 ml of concentrated sample, which was then used to detect HEV using the molecular biological technique Reverse transcription polymerase chain reaction (RT-PCR) method ("HEV RNA presence" kit, Symbiosis, Asti, Italy). In brief, this method consists of three fundamental phases: extraction of the RNA, reverse transcriptation and amplification with specific primers, and colorimetric detection of the amplified products on plates.

RNA EXTRACTION

The RNA of HEV was extracted from each sample using the "Qiagen" method (Qiagen, Milan, Italy) starting with aliquots of 140 µL of concentrated sample. After various rinse phases lo eliminate the aspecific impurities bound to the silica gel, the RNA was collected by eluting it from the fixing solution using a specific buffer.

REVERSE TRANSCRIPTATION AND AMPLIFICATION

The reverse transcriptase-PCR "RT- PCR "technique realised in two step: in the first "reverse transciption"10µl of RNA extracted from each sample was added up to the final volume of 30µl, with 1.5µl "antisense"primer (5'-CCTCGAAGCAGTAAGTGCGGTC-3'), at the temperature of 37°C for 30 minutes and at 95°C for 5 minutes, in the second step "amplification phase", 20µl of the reverse transcribed samples product of the first step was added up to a final volume of 50 µl, with 1 ul of "sense" primer (5'-GGCTCCTGGCATCACTACTG-3'), 0.3 ul of Taq, it was then placed in a thermal cycler 1 cycle at 95°C for 5 minutes and 5 times at a cycle of 95°C for 50 seconds, at 57°C for 30 seconds and at 72°C for 1 minutes, and 35 times at a cycle of 95°C for 30 seconds, at 57°C for 30 seconds; at 72°C for 45seconds; lastly to 1 cycle of 10 minutes at 72°C.

COLORIMETRIC DETECTION

Five µl of the amplification products and of the controls are first hybridised in microtubes with 120 µl of a specific probe at the temperature of 95°C for 5 minutes, and at 37°C for 10 minutes. The hybridisation process consists in annealing a molecule of fluorescein to the biotin-labelled 5' end of the amplified DNA, 45 µl of the hybridised product was placed in the wells of a microplate and incubated for 1 hour at 37°C to allow it to fix to the walls of the wells. After rinsing to eliminate the unfixed hybridised product in excess, 100 µl of conjugated-HRP solution (antibody anti-fluorescein horseradish peroxidase), was added to each well. The microplate was then placed on an agitator at room temperature for 30 minutes. After further rinsing for the elimination of the conjugated product in excess, 100µl of chromogenic substrate (orthophenylenediamine -OPD) were added to each well and the microplate incubated at room temperature for 30 minutes. If the antigen-conjugated complex was present, colour developed on the plate. Its intensity was proportional to the concentration of the fixed complex. After 30 minutes, the reaction was blocked with a solution of hydrochloric acid, and the microplate was transferred to a spectrophotometer for densitometry at 450 nm and 630 nm. The cut-off value beyond which the sample was considered positive for the presence of HEV-RNA, was given by doubling the average of the optical density values of the negative controls.

Results

Three of the 46 sewage samples examined (15.33%) tested positive for HEV by RT-PCR. Two of these three samples came from the untreated sewage entering the University hospital treatment plant and one was raw sewage entering the municipal plant at Mili. No trace of HEV was found in any of the samples taken of the treated wastewater exiting the two plants. The presence of the virus in the sewage sample taken from the hospital plant (8.33%) was found in samples collected during spring months, in May and June, while the sample containing the virus (4.5%) in the municipal plant was collected in September.

Discussion and conclusions

The results obtained confirm the presence of the hepatitis E virus in the city of Messina, as previously found in a seroprevalance study conducted by Squeri et. al. in 1996 in which the rate was 3.93% [10]. Other studies carried out in Italy have reported a seroprevalance rate of 2.9% in the total population of the city of Lecce, 4.3% in hemodialysis patients and in 15.3% of immigrants [11]. The seroprevalence found for the general population of Italy has been reported in the range of 1% and 5%, a fairly high percentage considering that reports of the disease are quite rare with a higher rate of anti-HEV antibodies (1%-6%) being found in hemodialysis patients, drug addicts and subjects found positive for markers of post-transfusion viral hepatitis [7, 8, 12].
Several seroepidemiological studies have also shown high rates of anti-HEV antibodies in a significant proportion of healthy individuals (5-20%) in industrialised countries, including regular blood donors. This suggests that the disease is present, albeit at a subclinical level [13].
The risk of contracting the infection is greater in Sicily which is located at the centre of the Mediterranean where HEV is present in numerous countries and which has a significant number of non European residents, a higher incidence of pathologies transmitted by fecal-oral means and a tradition of eating raw shellfish.
The higher presence of the virus found in the sewage entering the hospital plant may be attributed to hospital patients who are receiving dialysis, drug addicts and non Europeans [14, 15].
In conclusion, the results confirm the HEV virus to be in circulation in Messina, although to a modest extent. It may be favoured by the presence of numerous non Eu-

ropeans coming from countries in which the disease is endemic or where there are poor standards of health and hygiene, or by people returning from visits to countries at risk [14].
Our study also showed the two treatment plants tested to be working effectively, insofar as HEV was identified solely in samples entering the plants.

Our study underlines the need for greater epidemiological surveillance regarding this disease since prevention of HEV, in the current absence of a vaccine, depends on general measures to control enterically transmitted infections, this means improving not only health and hygiene conditions but also tightening controls on water.

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