International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-005, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

In search of enteroviruses in water media in Marrakech

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Accepted 7 February, 2019

Enteroviruses are among the most common viruses infecting the human intestine; they are very widespread in nature and resistant to external agents. They are eliminated in the faeces and contaminate water and food. These viruses cause various clinical syndromes and constitute a big public health problem. The aim of our study was to search for enteroviruses in the water samples. For this, a total of 225 L of tap water coming from the national network office for drinking water (ONEP) and 18 samples of waste water originating from Marrakech city were studied. These samples were concentrated, treated with polyethylene glycol 6000 and then analyzed by RT-PCR. Only two samples were found to be positive for enteroviruses by RT- PCR among the 18 waste water samples analyzed, which gave a rate of 11.11%. On the other hand, no positive samples were found in the tap water. This study made it possible on the one hand to apply for the first time RT-PCR for the detection of enteroviruses in water samples originating from Marrakech city, and on the other hand to show that tap water of this city does not present any risk of contamination by this type of germ.

Key words: Drinking water, waste water, enterovirus, RT-PCR, Marrakech.

INTRODUCTION

Water is essential to life, its availability in quantity and quality sufficient to meet basic needs is essential both for improving health and for sustainable development. Combating and reducing diseases linked to water is one of the important and urgent tasks which can be achieved only through enhanced cooperation at all levels.

Enteric viruses can be present naturally in aquatic environments. These viruses can be transported into the environment through groundwater, estuaries, sea water, rivers, aerosols, sewage, inadequately treated water, drinking water, wells etc. (Fong and Lipp, 2005). The risk of infection by enteroviruses in water is 10 to 10,000 times greater than bacterial infections (Bosch, 1998).

Enteroviruses were isolated from environmental samples for more than 60 years (Paul and Trask, 1941) and have subsequently been widely studied and qualified as a real threat to the quality of drinking water. The contamination rate allowed was set by the World Health Organization to be 3.2 x 10^{-5} particles per litre (WHO, 2006).

Viruses excreted in faeces are found in sewage, the viral concentration of waste water is variable depending on many factors, geographic, socio-economic, seasonal and especially health. Thus, the higher the proportion of children in a community, the lower the level of hygiene and the higher the quantity of virus found in waste water.

Enteroviruses are transmitted by the faecal-oral route, their effects may exceed gastroenteritis, because they pass from the intestinal tract to other organs. The enteric virus infections are primarily associated with diarrhoea in humans (Kocwa-Haluch, 2001). Thus, in the United States, cases of acute gastroenteritis recorded exceeded 50% of all waterborne diseases from 1946 until 1980 (Lippy and Waltrip, 1984) and, viruses were identified as the cause of 12% of the cases registered during this period.

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In Morocco, few studies have been devoted to sources of contamination of people by enteroviruses, which is why in the present work we focused on assessing the virological quality of water intended for public consumption as well as surface waste in the region of Marrakech. The aim of this study was to search for enteroviruses, by RT -PCR, in 225 L of tap water coming from the national network office for drinking water (ONEP) and in 18 samples of waste water originating from Marrakech city.

MATERIALS AND METHODS

Samples

Water faucet

Samples were taken directly from the tap (Régie autonome de distribution d'eau et d'électricité de Marrakech: RADEEMA) . 15 samples were taken during the months of July, September and August 2007 and October 2008 making a total volume of 225 L.

Wastewater

Samples were taken from the waste water treatment plant of Marrakech. Eighteen samples of 250 ml were taken, in June, July and October 2008, in clean glass containers. Eleven were taken from the raw water at the entrance to the station and seven from the treated waste water as it exited. These samples were taken to the Environment, Food and Health Laboratory of Marrakech for extraction and concentration of the virus. The last pellets after concentration were kept at -20°C for analysis by RT-PCR in the Diseases and Biologically Active Substances Laboratory of Monastir, Tunisia.

Water wells

During the months of June and July 2007, eight samples of one litre each were collected in eight wells located within a 2 km radius around the Oued Tensift located 6.3 km north of the city of Marrakech at 410 m altitude. The wells were located in clusters of habitats near Oued Tensift. This water is used by the people both for drinking and for the preparation of food without any treatment. These villages are not served by the national drinking water system.

Sample processing

Concentration of enteroviruses

The concentration of virus in samples of tap water and wells has been done by filtration through membranes of sterile cellulose nitrate with porosity of 0.22 microns. The filtration device was connected to a vacuum pump. Each filter is then placed in a moist chamber and stored at 4°C (Wallis et al., 1972).

Elution

Each filter was cut into very fine pieces separately and put in 20 ml of a solution of meat extract pH 9.5 and centrifuged at 10, 000 rounds/min for 35 min. The float was recovered and its pH adjusted to 7.2 with 5M HCl. One milliliter of the mixture of antibiotics and antifungal (penicillin, streptomycin and fungizone) was added and the final mixture was placed at 4°C overnight and stored at -20°C.

Extraction and concentration from waste water

We adapted the protocol described by Gerba et al. (1979), based on the adsorption of virus to acidic pH and elution at basic pH. Thus, 100 ml of sample were homogenized by magnetic stirring. A solution of $AlCl_3$ was added until a final concentration of 0.0005 M and the pH was adjusted to 3.5 with 5N HCl. The mixture was stirred vigorously for 30 min then centrifuged at 2500 g for 15 min at 4°C and the pellet was taken in 100 ml of 10% beef extract of at pH 9. The mixture was stirred for 30 min and centrifuged at 10 000 g for 30 min at 4°C and the supernatant was collected in a sterile container. PEG 6000 was added until a final concentration of 10% and the mixture was homogenized and incubated overnight at 4°C. The next day, after a centrifugation step at 10 000 g for 45 min at 4°C, the pellet was resuspended in 10 ml phosphate buffer pH 7.2 and kept at -20°C.

Extraction of viral RNA

A volume of 120 µl of each sample was put in 500 l of Tri-reagent (Sigma) and vortexed for 15 s and left in contact 5 min at room temperature. Then, 200 l of chloroform were added to the mixture to separate the nucleic acids of proteins to which they are associated. The mixture was vortexed, left to react at room temperature for 15 min and then centrifuged at 13 500 rpm for 15 min at 4°C. The aqueous phase was transferred to a new tube, and mixed with 500 l of isopropanol. The mixture was allowed to react for 15 min at -20°C and then centrifuged at 13500 rounds/min for 10 min at 4°C to remove the cell debris. The pellet underwent two successive washes with 75% ethanol followed by centrifugation at 7500 rounds/min at 4°C for 15 min for the first wash and 5 min for the second. The pellet was left to dry at room temperature for 15 min. To standardize the RNA in the solution, the pellet was put in 30 l of Diethylpyrocarbonate treated water.

Detection of the viral genome

We used RT-PCR, a method considered sensitive in the detection of enteroviruses in both environmental and clinical samples (Zoll et al., 1992; Rutjeset al., 2005; Soule et al., 2000) using reverse transcriptase (Moloney-Murine Leukemia Virus) and two primers from the conserved non-coding region of the viral genome. The product of amplification gives a 154 bp band.

Choice of primers: In the case of enteroviruses, a sequence which is stable and common to all serotypes must be identified. Thus, several sequences have been described by Zoll et al. (1992) . The primers used in this work are positioned at 445 - 464 and 599 - 580 on the genome of Coxackievirus B3 and their nucleotide sequences are 5 'TCCTCCGGCCCCTGAATGCG 3' and 3 'ATTGTCACCATAAGCAGCCA 5' (Invitrogen), respectively.

Preparation of complementary DNA (cDNA): For the preparation of cDNA, we mixed 2.5 l of of reverse transcriptase buffer (Promega), 1 l of dithiothreitol (Promega), 0.5 l of NTPS (Boehring Mannheim), 0.5 l of antisense primer (25 pmole) and 0.5 l of the enzyme reverse transcriptase (Promega). To this mixture, we added 5 l of extracted RNA and incubated for 30 min at 42°C and then amplified.

Table 1. Presence of enteroviruses in waste water samples of Marrakech.

Samples	Date	Results
RW1	02/06/2008	-
TW1		-
RW 2	18/06/2008	-
TW2		-
RW 3	20/06/2008	-
TW3		-
RW 4	25/06/2008	-
TW4		-
RW 5	27/06/2008	-
TW5		-
RW 6	01/07/2008	-
RW 7	02/07/2008	-
TW7		-
RW 8	07/07/2008	+
RW 9	08/07/2008	+
TW9		-
RW 10	25/10/2008	-
RW 11	26/10/2008	-

RW: Raw water; TW: Treated water.

Amplification: The amplification mixture consisted of 2.5 l Taq polymerase buffer, 0.5 l of NTPs: dATP, dCTP, dGTP and dTTP, 0.5 l antisense primer (25pmole), 0.5 l of sense primer 25 pmole: oligo (dt) 12 - 18 (pharmacia Biotech), 0.15 l of Taq polymerase 1.25 l (Invitrogen), 0.75 l of $MgCl_2$ and 18 l of sterile double-distilled water.

The positive control (RNA Coxackievirus Nancy B) and negative control (sterile distilled water) were treated in the same conditions as the samples studied. The amplification was performed in an Eppendorf thermal cycler with a denaturing RNA-cDNA for 5 min at 94°C and a series of thirty cycles of the following: 30 s at 94°C, hybridization of primers for a minute at 42°C, elongation 2 min at 72°C and an extension of 10 min at 72°C, the final stretch.

Revelation: After amplification, electrophoretic migration of the PCR product was performed on 2% agarose gel. Ten milliliters of each sample of PCR product mixed with 5 l of migration blue were deposited on the gel. Migration was done in TBE buffer (1x) at 90 V and lasted for approximately 45 min. The gel was then soaked in a solution of ethidium bromide (Sigma) at 10 mg/ml. Visualization of the specific band was conducted by transillumination under ultraviolet light (UV) against a size marker of 100 bp (Pharmacia Biotech).

RESULTS AND DISCUSSION

During the study, a total of 41 samples were collected including 18 samples of waste water during the months of May, June, July and October 2008, 15 samples of tap water during the months of July, September and August 2007 and October 2008 and 8 samples of water wells during the months of June and July 2007.

Using RT-PCR enabled the detection of enterovirus RNA in 2 samples of waste water; this gives a rate of 11.1% (2/18). These two positive samples were collected in July, known as the driest month of the year 2008 in the city of Marrakech. However, no positive result was obtained in the treated waste water, tap water or water wells (Table 1 and Figure 1).

We have little data on the detection of enteroviruses in the environment in Morocco because there are few studies that have been done. This allows us to emphasize that our present work is original. Our results are compared to those recorded by researchers in other countries.

Several studies have shown that the increase in enteroviruses is seasonal Summer- Fall (Troudi et al., 1997), while other studies claim that the rainy season is the perfect time when the frequency of enterovirus reaches its peak (Bini et al., 2006). A more prolonged follow-up of our study throughout the year can confirm either study. We also noted that no positive sample of treated waste water coming out of the treatment plant has been detected. This can be explained by the fact that much of the suspended matter is removed during the treatment; where the viruses are adsorbed on this material under the influence of physical surface properties. Some authors argue that the setting of virus particles suspended in the water environment is linked not only to the species and serotype but also to the strain itself (Nestor and Brisou, 1986). For more sensitivity, it will be interesting to use nested-PCR for low levels of enteroviruses (Lee and Lee, 2008; Zhang et al., 2008). Indeed, RT-PCR is a sensitive technique but is influenced by the presence of inhibitors which are rich environmental samples (Sdiri et al., 2006), a factor that limits its effectiveness. However, many precautions must be taken both in the implementation of the RT-PCR (including choosing the method of virus concentration and extraction technique of RNA). Thus the large amount of genetic material from environmental samples makes possible a non-specific amplification of sequences unrelated to the virus searched.

Regarding the tap water, 225 L of water were analyzed without any positive result. This could be explained bythe efficiency of the water decontaminating treatment process by the treatment plant. Our results are consistent with those found in Korea (Kyung and Jeong, 2004) where no enterovirus was detected in treated water for consumption. However they are different from the study of Coin (1966) which was the first to have isolated viruses in water intended for consumption in Paris, and those of

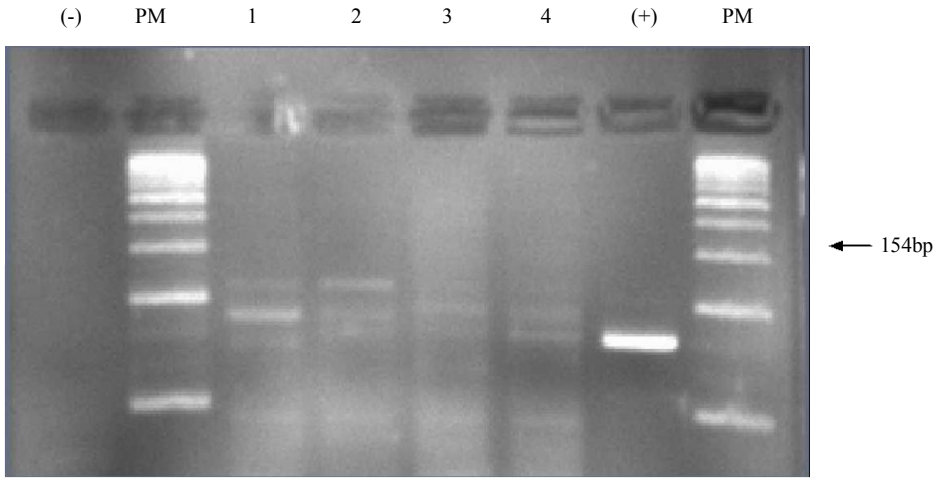

Figure 1. Results of electrophoresis of amplification products by RT-PCR of gene 5'NC enteroviruses from samples of waste water.
(-): negative control; (+): positive control (Coxackievirus B Nancy); 1, 4: positive samples; 2, 3: negative samples; PM**:** Size Marker 100 bp.

other studies that were conducted in Romagne (France) (Nestor and Costin, 1976) and Russia (Rabshko, 1974).

The authors had explained the presence of the virus by inadequate treatment which did not allow effective disinfection. In addition, other studies have shown high contamination of drinking water by enteroviruses in Korea (Lee and Kim, 2002), South Africa (Ehlers et al., 2005) and Egypt (Ali et al., 2004) with positivity rates of 47.8, 18.7% and 17.5 PFU/L respectively. These high rates have been explained by the insufficiency of the treatment used and the possible contamination of water pipes.

As the methods used in environmental virology are limited by the low concentration of virus in drinking water, the techniques of adsorption/elution using electropositive filters or electronegative methods are most recommended by APHA (1985) and Karim et al. (2009). Nevertheless, our study has demonstrated the effectiveness of the treatment technologies for water consumption, the technique used and the volume of samples analyzed to meet international quality standards (EPA, 1999b).

Regarding groundwater, we have made samples in 8 wells and none was found to be positive. These wells which are not deep (between 6 and 10 m) are located near agricultural fields irrigated with waste water. Study should be conducted using wider areas and larger volumes of water. In other studies, conducted between 1971 and 1982, groundwater accounted for 51% of waterborne diseases (Craun, 1986). Hepatitis viruses and rotaviruses are considered the main causes of these diseases (Gerba et al., 1985). These infections are mainly due to infiltration of waste water through the pits as well as due to the spread of waste water and sewage sludge, domestic discharge, oxidation ponds etc (Borchardt et al., 2007) . With their small size, viruses are able to travel hundreds of meters through soil to reach groundwater (Gerba and Bitton, 1984) this movement depends mainly on the type of virus, the type of soil and climate.

The present work allowed us to detect for the first time, enteroviruses in waste water of the region of Marrakech. This encourages us to continue the search for these viruses in a larger number of water and human biological samples.

REFERENCES

Ali MA, Al-Herrawy AZ, El-Hawaary SE (2004). Detection of enteric viruses, Giardia and Cryptosporidium in two different types of drinking water treatment facilities. Water Res., 38: 3931-3939.

American Public Health Association (APHA) (1985). Standard Methods for the examination of water and wastewater, 16th ed. Washington, D.C.

Bini JC, Ekaza E, Gnagne T, Borget AMY, Veh KA, Akran AV, Coulibaly ND, Faye-Kette H, Sess ED, Dosso M (2006). Apport de la RT-PCR pour la détection des entérovirus dans les eaux usées à Abidjan (Côte d'ivoire). Cah. Santé Publique, 5: 55-65.

Borchardt MA, Bradbury KR, Gotkowitz MB, Cherry JA, Parker BL (2007). Human enteric viruses in groundwater from a confined bedrock aquifer. Environ. Sci. Technol., 15: 6606-6612.

Bosch A (1998). Human enteric viruses in the water environment: a minireview. Microbiol., 1: 191-196.

Coin L (1966). Modern microbioligical and virological aspects of water pollution. In Jaag, O (eds) Advances in water Pollution Research, Pergamon Pres, London, pp. 1-10.

Craun GF (1986). ed. Waterborne diseases in the United States. Boca Raton: CRC Press, p.13.

Ehlers MM, Grabow WOK, Pavlov DN (2005). Detection of enteroviruses in untreated and treated drinking water supplies in South Africa. Water Res., 39: 2253-2258.

EPA (1999b). Drinking water criteria document for enteroviruses and hepatitis a: an addendum, p. 173.

Fong TT, Lipp EK (2005). Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools. Microbiol. Mol. Biol. R, 69: 357-371.

Gerba CP, Bitton G (1984). Microbial pollutants: their survival and

transport pattern to groundwater. In Bitton G, Gerba CP (eds) Groundwater pollution microbiology. Wiley, New York, pp. 39-54.
Gerba CP, Goyal SM, Labelle RL, Cech I, Bodgan GF (1979). Failure of indicatorbacteria to reflect the occurrence of enteroviruses in marine water. A. J. Pub. Health, 69: 1116-1119.
Gerba CP, Sing SN, Rose JB (1985). Waterborne viral gastroenteritis and hepatitis. CRC Crit. Rev. Environ. Contr., 15: 213-216.
Karim M, Rhodes E, Brinkman N, Wymer L, Fout GA (2009). New Electropositive Filter for Concentrating Enterovirus and Norovirus from Large Volumes of Water. Appl. Environ. Microbiol., 75: 2393-2399.
Kocwa-Haluch R (2001). Waterborne Enteroviruses as a Hazard for Human. Pol. J. Environ. Stud., 10: 485-487.
Kyung Lee H, Jeong YS (2004). Comparison of Total Culturable Virus Assay and Multiplex Integrated Cell Culture-PCR for Reliability of Waterborne Virus Detection. Appl. Environ. Microbiol., 70: 3632-3636.
Lee G, Lee C (2008). Molecular detection and characterization of human enteroviruses in Korean surface water. J. Microbiol., 46: 319-324.
Lee S, Kim S (2002). Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. Water Res., 36: 248-256.
Lippy EC, Waltrip SC (1984). Waterborne disease outbreaks-1946-1980: a thirty-five –year perspective. J. Am. Water Works Assn, 76: 60-67.
Nestor I, Costin L (1976). Presence of certain enteroviruses (coxsakie) in sewage effluents and river waters of Romania. J. Hyg. Epidemiol. Microbiol. Immunol., 21: 137-149.
Nestor J, Brisou J (1986). Incidence sanitaires de l'adsorption sur les sédiments marins et fluviaux. Rev. Epidemiol. Sante Publique, 34: 181-190.
Paul JR, Trask JD (1941). The virus of poliomyelitis in stools and sewage. J. Am. Med. Ass., 116: 493-498.
Rabshko EV (1974). Certain problems of circulation of enteroviruses in the environemental objects. Gig. Sanit. 39: 105-106.
Rutjes SA, Italiaander R, van den Berg HHJL, Lodder WJ, de Roda Husman AM (2005). Isolation and Detection of Enterovirus RNA from Large-Volume Water Samples by Using the NucliSens miniMAG System and Real-Time Nucleic Acid Sequence-Based Amplification. Appl. Environ. Microbiol., 71: 3734-3740.
Sdiri K, Khelifi H, Belghith K, Aouni M (2006). Comparaison de la culture cellulaire et de la RT–PCR pour la détection des entérovirus dans les eaux usées et les coquillages en Tunisie. Pathol. Biol., 54: 280-284.
Soule H, Genoulaz O, Gratacap-Cavallier B, Chevallier P, Liu J, Seigneurin J (2000). Ultrafiltration and reverse transcription-polymerase chain reaction: an efficient process for poliovirus, rotavirus and hepatitis A virus detection in water. Water. Res., 34: 1063-1067.
Troudi M, Aouni M, Poozzetto B, Dhidah M (1997). Surveillance de la circulation des entérovirus dans la région du Sahel. Tunis Med., 75: 23-26.
Wallis C, Henderson M, Melnick J (1972). Enterovirus concentration on cellulose membranes. Appl. Environ. Microbiol., 19: 476-480.
World Health Organization (2006). Guidelines for Drinking-water Quality. First *Addendum* To Third Edition Recommendations. – 3rd ed. 1: 121-144c.
Zhang CM, Wang XC, Liu YJ, Xue XP (2008). Characteristics of bacterial and viral contamination of urban waters: a case study in Xi'an, China. Water Sci. Technol., 58: 653-560.
Zoll GJ, Melchers WJG, Kopecka H, Jambroes G, van der Poel HJ, Galama JM (1992). General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistant infections. J. Clin. Microbiol., 30: 160-165.