Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 6, Issue, 02, pp.4851-4857, February, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

MICROBIOLOGICAL AND PHYSICOCHEMICAL ANALYSIS OF COASTAL WATERS IMPACTED BY POINT SOURCES POLLUTION IN SOUTHWESTERN MOROCCO

***[1]Laila Amar, [3]Bouchra Karraouan, [3]Hassan Nader, [2]Mustapha Lkhider, [1]Lahcen Wakrim, and [1]Naima Elmdaghri**

[1]Virology Laboratory, Pasteur Institute of Morocco, Casablanca, Morocco
[2]Department of Biology, Faculty of Science and Technology Mohammedia Hassan II, University Mohammedia, Mohammedia
[3]Bacteriology Laboratory of Product Safety, Food and Environment, Pasteur Institute of Morocco, Casablanca, Morocco

ARTICLE INFO

Article History:
Received 19th November, 2013
Received in revised form
04th December, 2013
Accepted 15th January, 2014
Published online 21st February, 2014

Key words:
Adenovirus,
Enterovirus,
HA,
Marine environment,
Norovirus,
polyomavirus JC,
Rotavirus A.

ABSTRACT

Viral and bacterial contamination of recreational coastal water is a rising public health concern. The aim of our study was to conduct a preliminary evaluation of the presence of indicator virus and pathogens in seawater, sand and mussels at recreational marine beaches in Southern Morocco. Forty six samples (mussels, sand and seawater) were collected from Dar Bouazza region and tested for the presence of enteric viruses by (qPCR) and viral viability by integrated cell culture-PCR (ICC-PCR) assay. The samples were collected from three beaches points and seawater was filtered by HA membrane for viruses concentration. Our results showed that 33% of seawater samples, 50% of mussels samples and 18% of sand samples were tested positive for enteroviruses. Human adenovirus was the most prevalent virus detected (33%) in seawater samples, followed by polyomavirus JC (11%). Mussels were positive for norovirus GI, Norovirus GII and rotavirus A in (36%), (11%) and (33%) of samples respectively. HAV was not present in any samples. The fecal indicator bacteria (FIB) levels exceeded regulatory thresholds in 26% of seawater samples for *Escherichia coli* and *Enterococcus* and 9% of them showed the presence of viruses. No correlation was demonstrated between bacterial contamination and virus presence. However, a possible correlation was showed between salinity and virus presence. This data reflected the viral contamination of recreational waters and suggested that human adenoviruses should be an index for human fecal pollution in conjunction with FIB. The presence of enteric viruses in marine samples may constitute a potential public health risk for shellfish consumers and swimmers. Therefore viral parameters should be adapted for monitoring the beach water quality and shellfish safety.

INTRODUCTION

Human enteric viruses are among microbial pathogens that enter the marine environment through point sources, such as sewage outfalls or non-point sources, such as storm water runoff, sand resuspension, animal fecal inputs, and human bather shedding (Abdelzaher *et al.*, 2011). It has been reported that the presence of viruses or the outbreaks of viral disease were related to seawater or shellfish, which meet legal standards based on bacteriological criteria (Pina *et al.*, 1998, Bosch *et al.*, 2006). Therefore, viral contamination of recreational coastal water is of particular importance and is a rising public health concern. Several studies showed that enteric viruses are able to persist for extended periods of time in the marine environment as compared to bacteria, increasing the probability of human exposure by recreational contact and ingestion of contaminated shellfish (Miagostovich *et al.*, 2008; Mesquita *et al.*, 2011). Additionally, gastrointestinal illness risks from viral exposures were generally orders of magnitude greater than bacterial exposures (Wong *et al.*, 2009) and may be the causative agent of nearly 50% of all acute waterborne gastrointestinal illnesses (Jiang *et al.*, 2007). Wong (2009) have studied the daily risk of acquiring a viral infection from swimming in polluted marine water and showed that it was greater for children (9-15/1000 swimmers) than adults (0.2 to 2.4/1000 swimmers). Beachgoers are also in contact with sand which the sanitary quality is not included within beach monitoring programs. In fact, sands and sediments provide a habitat of fecal viral populations (Halliday *et al.*, 2011) and about 5 MPN/g (about 1 MPN/g for pica) for enterovirus is sufficient to cause a risk of illness associated with exposure to marine beach sands (Shibata *et al.*, 2012). Currently, coastal recreational water quality standards and the sanitary quality of shellfish in Morocco (NM 03.7.200 and NM 08.0.147-2004)

Corresponding author: Laila Amar,
Virology Laboratory, Pasteur Institute of Morocco, Casablanca, Morocco.

and throughout most of the world are based on the fecal indicator bacteria (FIB) to control the spread of recreational waterborne illness. However, it is now recognized that there is no correlation between bacterial contamination and viral presence (Moresco *et al*., 2012, Myrmel *et al*., 2004). Dar Bouazza is one of the most popular bathing beaches in Casablanca with a coastline extending over 16 km. This region is located in the Atlantic coast of Morocco, at 25 Km of south of Casablanca center, and has an area of 13,500 hectares with a population of more than 400,000 people. It became a beach tourism area through three agglomerated beaches around which many residence complexes have been developed. However, Bay swimming continuously receives waste from the seaside residence or Merzegu River including wastewater and the overflow of combined sewer system in periods of heavy rain.

The objective of this study was to conduct a preliminary evaluation of the presence of indicator viruses and pathogens in the seawater, sand and mussels at recreational marine beaches in Dar Bouazza. The other parts of this study included the evaluation of fecal bacteria as microbial indicators of virus presence and the study of physicochemical parameters influencing the presence of enteric virus. We hypothesized that to evaluate the viral pollution in beaches and to further extend this study to different environmental matrices, would allow a better interpretation of water quality and human health risks at beaches impacted by known point source pollution.

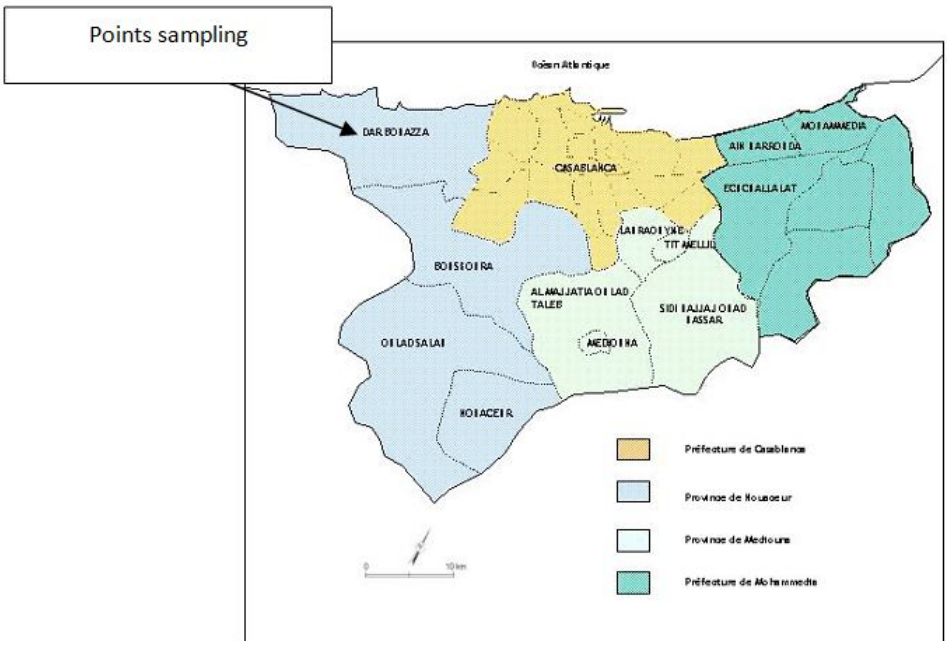

Figure 1. Points sampling of Dar Bouazza beaches

MATERIALS AND METHODS

Sample collection and sampling areas

Samples were collected from three recreational beaches (Beach house Bay, Mrissa bay and Merzegu bay) in Dar Bouazza costal between January and July 2012 (Fig.1). A total of 56 samples collected in the daytime at one site from each Bay and composed of seawater (n=23) and sediments (n=23). However mussels samples (n=10) were collected only from beach house Bay (Fig.1). The seawater was collected bimonthly in 2 bottles of 1 liter and 2 liters for virus and bacterial analyzes respectively. Sand was also collected at 100 g of sand. Mussels were collected monthly at 10 to 15 pieces and transported to the laboratory into clean plastic bottles and boxes (disinfected previously with chlorine and neutralized with sodium thiosulfate) at 4°C.

Chemical and microbial analysis

All the seawater samples were assayed for total coliforms, *E. Coli* and Enterococci according to the Standard Methods for the Examination of recreational Water in morocco as described in NM ISO 9308-1 and NM ISO 7899-2. Water samples were analyzed for temperature, pH, dissolved oxygen, salinity and conductivity with multi system 340 i/SET instrument (WTW).

Virus analysis

Virus concentration method from seawater

The beach waters from the three sampling sites were sequentially filtered through a HA membrane filter based on a concentration-elution approach previously described by katayama (2002) with minor modifications. Briefly, two liters of collected seawater were filtered through the HA membrane. A volume of 200 ml of 0.5 mM H_2SO_4 was passed through the membrane to rinse out the cations. Subsequently, the filter was eluted with 5 ml of 1 mM NaOH (pH 10.5 to 10.8) and recovered in a tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100mM TE buffer for neutralization. The eluate was purified by centrifugation at 10,000 × g for 10 min; supplemented with 100 U of penicillin, 100 µg of

streptomycin, and 0.25 µg of amphotericin B; brought to a neutral pH and prefiltered through a 0.22-µm filter (Millipore). The purified filtrates (PF) were stored at –80°C until analyzed by cell culture.

Virus concentration and RNA extraction in mussels and marine sediments

The outer surface of mussels was cleaned with water and ethanol (96%), dissected and digestive tissues (DT) were recovered. 50 grams of sediment samples were collected and placed into sterilized tubes. The viruses were concentrated and RNA was extracted from DT mussels and marine sediments by The Melt Total Nucleic Acid isolation system kit (Ambion) according to the manufacturer's instructions. The RNA was eluted in 40 µl of tampon elution and conserved in -80°C.

Cell culture of viruses

Human epidermis carcinoma cells of the larynx (HEp-2 cells) and rabdomyosarcome (RD) were used to isolate the enterovirus in concentrated samples. Cells were grown in flasks containing MEM medium (PAN) supplemented with 10% (growth medium) or 2% (maintenance medium) heat inactivated fetal bovine serum (Eurobio), 1% of L glutamine and 100 units mL−1 penicillin, 10mg mL−1 streptomycin. The fifty suspensions of seawater PF were inoculated in plates 6 wells of Hep2C and RD cells with negative control cell and incubated for 45 min at 37°C in an atmosphere of 5% CO2. The plates were rocking every 15 min for efficient adsorption of viral particles then the sample inoculums was removed and the cells were washed twice with 10 ml PBS and re-fed with maintenance medium. The plates were incubated for 7 days at 37°C in a 5% CO2 and observed daily under microscopy inverted for a cytopathic effect (CPE). The cells plates were frozen and thawed twice and the previous supernatants with and without CPE were determined by using integrated cell culture RT-PCR (ICC/RTPCR) for human enteroviruses (HEV).

Nucleic acids extraction

Aliquots of 140µL of cell supernatants and filtrate water were used for nucleic acid (NA) extraction using high viral nucleic acid kit (Roche) following the manufacturer's instructions. NA was eluted with sterilized water in a final volume of 60µL.

Detection of Human Enteroviruses by ICC/qRTPCR

RT-PCR amplification was carried out with supernatant cell culture using the one step Access quick RTPCR kit (Promega) with the pan- enterovirus primer set (Ent-up-2 and Ent-down-1) developed by De Leon (1990). Primers were selected from the 5'-untranslated region (UTR) representing high conserved region of HEV genomes. The RT-PCR was performed in a final volume of 25 µl containing (20µM) for each primer, 12.5 µl of mix buffer, and 0.5 µl of reverse transcriptase enzyme. Five microliters of nucleic acid extract or control were added per tube. Thermal cycling was performed on an ABI 2720 system (Applied Biosystems). RT-PCR was started with an initial 45-min RT step at 45°C, and 15 min at 95°C followed by 40 amplification cycles of 95°C for 30 s, 58°C for 1min and 72°C for 30 s. A final extension was done at 72°C for 7 min. The RT-PCR amplicons were resolved by electrophoresis in 1.6% agarose gels containing ethidium bromide, followed by UV light illumination (Gel-Doc system; Bio-Rad Laboratories).

Detection of Human Adenoviruses and JC polyomaviruses

Primers used for PCR amplification of HAdV and HPyV JC were described previously by Hernroth (2002) and Pal (2006). Amplification reaction mixtures were performed with the Platinum PCR superscript III System with Rox (Invitrogen) in a final volume of 25 µl contained 5 µl of DNA template, 12.5 µl of TaqMan Master Mix (Invitrogen), 250 nmol l^{-1} of each primer and 150 nmol l^{-1} of each probe, 0.5 µl of enzyme and 1 µl/reaction of Rox reference dye. The thermal cycling protocols for both viruses were as follows: 15 min at 95°C for initial denaturation, followed by 45 cycles of two steps consisting of 10 s at 95°C, 30 s at 55°C. Raw data were analyzed using the ABI 7000 sequence detection system (Applied Biosystems) to calculate the CT values.

Detection of Rotaviruses and Noroviruses GI/GII

One-step real-time RT-PCR assays were performed using the Platinum RT-PCR superscript III System with Rox (Invitrogen) and primers and probes previously described by Jothikumar (2009; 2005). Viral RNA (5µl) was added to 12.5 µl of 2x Mastermix, 0.5 µl reverse transcriptase-Taq polymerase mix, 1 µl/reaction of ROX reference dye and 20U RNase inhibitor (RNaseOUT; Invitrogen), using primers and probes in a final volume of 25 µl. The RT-PCR for NoV comprised an initial 60 min RT step at 42°C, 95°C for 5 min, and then 45 cycles of PCR, with 1 cycle consisting of 95°C for 15 s, 58°C for 1 min, and 60°C for 1 min. The thermal cycling conditions for RoV consisted of RT for 30 min at 50°C, followed by denaturation at 94°C for 15min, amplification for 45 cycles, followed by denaturation at 94°C for 10 s, and annealing-extension at 55°C for 30 s. Raw data were analyzed using ABI 7000 sequence detection system (Applied Biosystem).

Detection of HAV

The qRT-PCR was performed with primers (SH PolyA /SH Poly-1) and probe (SH Poly Q) as previously described by Houde (2007). Amplification was performed with RNA Ultrasens quantitative RT-PCR One-step system (Invitrogen) using procedures recommended by the manufacturer in a 25 µl reaction mixture at final concentration of 0.4µM and 0.2µM for primers and probes respectively. The RT-PCR conditions for the amplification were as follows; 30 min at 45 ◦C for the reverse transcription step, 95 ◦C for 15 min for the initial denaturation step, followed by 45 cycles with 15 s at 95◦C for denaturation and 1 min at 60 ◦C for annealing. PCR amplification was carried out in ABI 7000 sequence detection system (Applied Biosystem).

Quality control of the amplification methods

To reduce the probability of sample contamination by amplified DNA molecules, separate areas of the laboratory

were used for mix and manipulation of extracted nucleic acids. All assays included negative and positive controls. Undiluted and a 10-fold dilution of the nucleic acid extract of all samples were analyzed in order to avoid false negatives because of inhibition of the reactions.

Statistical analysis

To investigate the use of standard bacterial as microbial indicators of viruses and to explore the relationship between those waterborne viruses and physical-chemical environmental factors, the STATA version 11.0 was used to implement the Fisher exact test. P-values below 0.05 were considered statistically significant.

RESULTS

Detection of human enteric viruses from seawater

HEV were detected in 9 out of 27 (33%) seawater samples by ICC/RT-PCR and two out of 27 (11%) showed apparent cytopathic effect (CPE) in BGM cells. Six seawater samples (22%) determined as negative by cell culture were positive by ICC–PCR. HEV infectious were the most frequent in Mrissa beach and Merzegu beach (Table1). HAdV were more frequent (33%) than polyomavirus JC(11%) in seawater samples collected between February and March. These viruses were present with similar frequency in Mrissa and Merzegu beach. HPyV JC was detected only in Merzgu beach in March but neither HAV nor NoV and RoV A were detected in seawater during the sample collection period. House beach was the least contaminated site throughout the study period, with only one HEV positive sample in February. However, Merzegu beach was the most contaminated site with at least one virus detected in 11% of samples (1/9). Human enteric viruses were most frequently detected at Merzegu beach followed by Mrissa beach and house beach (Table. 1). HEV and HAdV were detected concurrently from 6 out of 27 (22%) seawater samples. For Mrissa beach, HEV and HAdV were each detected in 3 out of 27 seawater samples (11%).

Detection of enteric viruses from sand and mussels

Samples evaluated from marine sand exhibited CPE in 7% of cases (2/27) collected in March and April and infectious HEV particles were detected in 18% (5/27) of samples. All samples were negative for NoV, RoV and HAV. Sand samples from house beach and Mrissa beach were most frequently contaminated than Merzegu beach (Tab.2). It was noted that all of the CPE-negative but ICC–PCR positive samples were determined as enterovirus positive in six of the 12 mussels samples (50%). Mussels showed a high contamination with all groups of viruses except HAV, which was no detected in none of samples analyzed. This study indicated that infectious enteroviruses were the most frequently detected in mussels followed by norovirus GI, RoV A and norovirus GII (Tab.2).
One of the 12 mussels samples (9%) was contaminated by at least one of the three studied viruses (NoV, RoV and EV) while simultaneous presence of 2 or 3 viruses in a sample was detected in 1 (9%) and 2 (18%) of the samples, respectively. Co-contamination with NoV/RoV, NoV/EV and EV/RoV, was observed in 18%, 27% and 18%, of samples respectively.

Analysis of bacterial indicators in seawater

Microbial indicators of fecal contamination were present, with concentrations consistently low than the recommended recreational-contact standard by the NM 03.7.200 except for one and two sampling from Mrissa and Merzegu respectively (Fig.2). According to Moroccan recreational water quality criteria, the *E. coli* and *Enterococcus* concentrations were higher than mandatory quality values of 2000 and 400 CFU/100ml respectively, allowing a routine classification of beaches as class C on the basis of the NM 03.7.200 classification. Accordingly; it was deemed that beaches were momentarily polluted in April coinciding with a period of high precipitation and July, the summer bathing period. The statistical analysis showed that *E. coli* and *Enterococcus* concentrations were not correlated with viruses presence ($p< 0.05$).

Table 1. Results of occurring viruses from seawater

Sampling points	HEV		HAdV	HPyV JC	NoV		RoVA	HAV
	BGM Culture	ICC/RTPCR			GI	GII		
House Beach	1/9 (11%)	1/9 (11%)	1/9(11%)	0/9	0/9	0/9	0/9	0/9
Mrissa Beach	0/9	4/9 (44%)	4/9(44%)	1/9(11%)	0/9	0/9	0/9	0/9
Merzegu Beach	1/9 (11%)	4/9 (44%)	4/9(44%)	2/9 (22%)	0/9	0/9	0/9	0/9
Total	2/27(7.4%)	927(33%)	9/27(33%)	3/27(11%)	0/27	0/27	0/27	0/27

Table 2. Results of naturally occurring virus detection from sand and mussels

Sampling points	BGM Culture	ICC/RTPCR HEV	NoV		RoV A	HAV
			GI	GII		
Sand House beach	2/ 9(22%)	2/9(22%)	0/9	0/9	0/9	0/9
Sand Mrissa beach	0/9	2/9(22%)	0/9	0/9	0/9	0/9
Sand Merzegu Beach	0/9	1/9(11%)	0/9	0/9	0/9	0/9
Mussels	0/12	6/12 (50%)	4/11(36%)	1/11 (9%)	2/11 (18%)	0/11

Table 3. Results of mean Physical and chemical parameters related to seawater quality

Sampling points	pH	T°C	DO2 mg/l	Conductivity mS/cm	Salinity mg/l
House beach	8,16	20,2	3,22	53,13	34,41
Merzegu beach	8,13	20,2	3,02	49,08	28,42
Mrissa beach	8,2	23,4	3,76	47,64	29,22

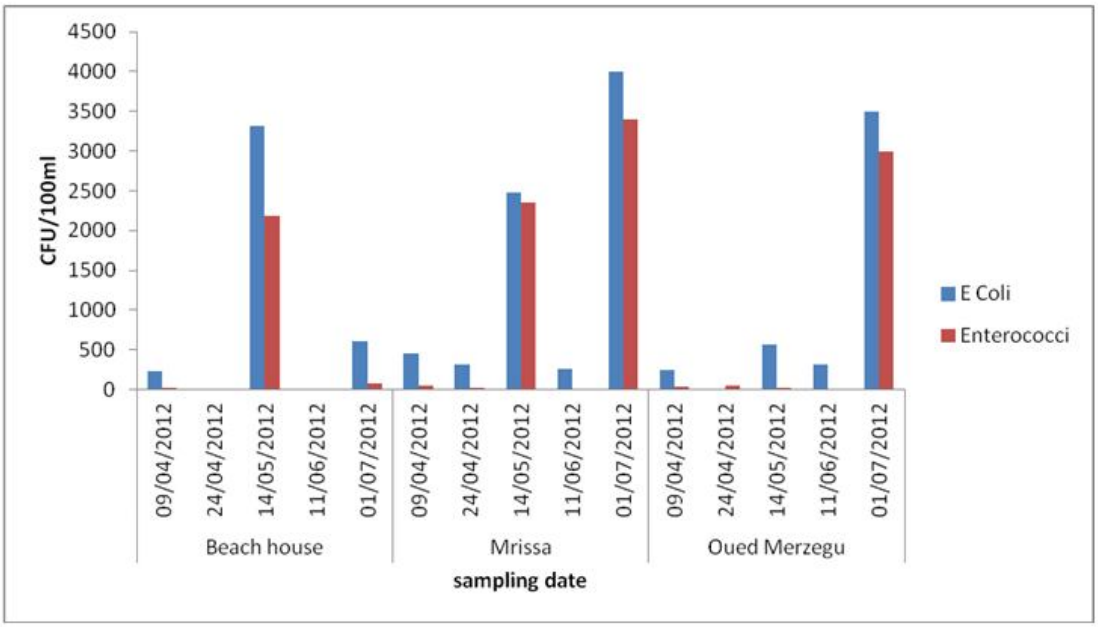

Figure 2. Results of bacterial indicators profile related to seawater quality: 2000 and 400 CFU/100ml values are the mandatory quality of recreational water in NM 07.3.200

Physicochemical parameters related to seawater quality

Water pH ranged between 7.2 and 8.8 (Table. 3). Average water temperature were similar for each points means ranged between 17-19°C (February-March) and increased significantly as summer approached 19-24°C. Water temperature varied between the sampling sites and the date of sampling. The highest temperature was noted in Mrissa beach which influenced by the warm waters released from the traditional bathroom. The ranged dissolved oxygen levels varied with an average of 2 mg liter-1 and 4 mg liter-1 with 6.58 mg liter-1 as highest levels noted in Mrissa beach after heavy rains. However, based on statistical analysis, there was no a significant correlation between virus and any physicochemical parameter ($P > 0.05$). Salinity levels showed significant variation with sampling dates at all sites and ranged between 30 and 34 mg liter-l. Mrissa beach consistently had the lowest salinity level (mean, 30mg liter-1) and were moderately influenced by increased stream flow. Total monthly precipitations were higher in February, March and April. However, stream flow was highest in February with levels salinity at 11 mg liter-l and 15.4 mg liter-l reported in Merzegu beach and Mrissa beach respectively (Table. 3). The salinity levels were significantly decreased with low conductivity after heavy rains during February in Merzgu beach and Mrissa beach. The conductivity levels varied between 50 and 53 mS cm-1 in all points' beaches except for Merzegu beach where the lowest level (18 mS cm-1) was reported after heavy rains. In addition, two lowest levels (25 and 47 mS cm-1) were reported in Mrissa beach at March and April.

DISCUSSION

Swimming-related illnesses are attributed predominantly to exposure to microbial pathogens. More than 120 million cases of gastrointestinal disease and 50 million cases of severe respiratory diseases are caused by swimming and bathing in wastewater-polluted coastal waters (Abdelzaher *et al*., 2011). The increasing pollution of the coastal waters from source points requires monitoring of bivalve mollusks and recreational water to ensure health safety standards. Guidance for Water Quality of Morocco Ocean recommended criteria for marine waters solely based on *E. coli* and Enterococci. However, our results showed the presence of viruses in samples meeting FIB regulatory standards and suggest that the current coastal recreational water quality standards are inadequate to reflect the viral quality of the water. Indeed, several studies confirmed that there is no correlation between viral presence and bacterial contamination of sewage discharge into coastal waters (Moresco *et al*., 2012, McQuaig *et al*., 2009). Moreover, it has been reported that outbreaks of viral diseases have occurred after the consumption of mollusks with accepted values of coliform standards (Lowther *et al*., 2012). Our results showed that human enteric viruses were detected in sediment samples next to the shoreline. The resuspension of this contaminated sediments may be a risk factor for swimmers and for children playing with sand (Abdelzaher *et al*., 2011). The presence of enteroviruses and adenoviruses with high frequencies in seawater at three beaches sites demonstrated the fecal viral pollution and suggested that adenovirus may be a good indicator of viral contamination of bathing water as reported by several studies (Aslan *et al*., 2011; Wyn-Jones *et al*., 2011). According to virological and bacteriological results, the studied beaches received human fecal waste in very different proportions. The Merzegu beach receives discharges from the river Merzegu in winter, yet it is disconnected in the summer. However, the Mrissa beach is more contaminated as compared to other beaches by several sources originating from domestic residences located in the metropolitan region, popular baths and marina fishing activities. The House beach was the least

polluted point receiving a low concentration of virus in discharges from coastal residences of this area. In fact, this explanation was supported by the presence of norovirus and rotavirus in mussels and not in seawater collected from the same area. The microbial data are possibly caused by a diversity of fecal pathogens introduced into the aquatic environment by point sources such as wastewater facilities and combined sewer overflows, or by diffuse nonpoint sources stemming from fish market, leaky septic tanks, urban runoff, agricultural runoff, discharge from boats, from bathers themselves, and from local animal populations. Mrissa beach received several sources of contamination than other beaches and that may be related to presence of fish market adjacent. On the other hand, the differences in the virus detection between beaches may be related to differences in PCR-inhibiting environmental compounds at each location (Rodríguez *et al*., 2012). Remarkably, all positive samples may occur during heavy rain periods. These factors also probably account for why the majority of documented outbreaks of waterborne disease outbreaks increase after periods of above normal rainfall (Bosch *et al*., 2005).

High prevalence of NoV GI compared to NoV GII was observed in mussels, despite the fact that most NoV strains circulating in humans are GII and such concordance was reported by several studies (de Roda Husman *et al*., 2007; Jothikumar *et al*., 2005). The occurrence of rotavirus in mussels reflected low rotavirus vaccine coverage and supports the use of enteric viral detection in mussels as a useful tool to measure the impact of specific interventions. Contamination of bivalve shellfish with norovirus and rotavirus from human fecal sources is recognized as a major human health risk and has been linked to many outbreaks (Lowther *et al*., 2012, le guyader *et al*., 2008). Therefore, the presence of rotavirus and norovirus in natural mussels might present a potential health risk of bivalves for the consumer and support the need for appropriate controls of viral contamination. However, the absence of noroviruses and rotaviruses in the seawater samples could be explained by less viral adsorption to the filters during the isolation step as reported by several studies (Keuckelaere *et al*., 2013; Victoria *et al.*, 2009; Fumian *et al*., 2010). Our results showed a possible correlation between salinity and virus presence as demonstrated in other studies (Victoria *et al*., 2010; Griffin *et al*., 2003). It has been reported that seawater and shellfish may contain diverse PCR inhibitors (Rodríguez *et al*., 2012; Kittigul *et al*., 2008). In our study, inhibitory problems were noticed during the real-time PCR runs in all matrices and diluting of the lake extracts could successfully overcome the inhibitory problems as demonstrated previously (Rigotto *et al*., 2009; Xagoraraki *et al*., 2007). In conclusion, viral contamination of seawater and shellfish samples is increasingly found in outbreak investigations, epidemiological surveys, and sample analysis. Contamination of these systems can represent high risks to human health and significant economic losses due to closure of beaches and shellfish harvesting areas. This study provided meaningful data for human water and foodborne viral risk assessment. Simple standardized diagnostic procedures for selected pathogens are needed to establish specific virological guidelines in recreational water and food products, notably shellfish. Furthermore, exposure to viral risk can also be reduced by avoiding swimming after heavy rainfall at sites affected by runoff to reduce exposure to any increase in contaminants, and by not swimming near storm drains or pipes that might release contamination into water bodies.

REFERENCES

Abdelzaher AM, Wright, M.E., Ortega, C., Hasan, A.R., Shibata, T., Solo-Gabriele, H.M., Kish, J., Withum, K., - Abdelzaher AM, Wright, M.E., Ortega, C., Hasan, A.R., Shibata, T., Solo-Gabriele, H.M., Kish, J., Withum, K., He, G., Elmir, S.M., Bonilla, J.A., Bonilla, T.D., Palmer, C.J., Scott, T.M., Lukasik, J., Harwood, V.J., McQuaig, S., Sinigalliano, C.D., Gidley, M.L., Wanless, D., Plano, L.R., Garza, A.C., Zhu, X., Stewart, J.R., Dickerson, J.W., Yampara-Iquise, H., Carson, C., Fleisher, J.M. and Fleming, L.E. 2011. Daily measures of microbes and human health at a non-point source marine beach. Water Health 9 (3):443-57.

Aslan A, Xagoraraki, I., Simmons, F. J., Rose, J. B. and Dorevitch, S. 2011. Occurrence of adenovirus and other enteric viruses in limited-contact freshwater recreational areas and bathing waters. *Journal of applied microbiology*., 111(5): 1250–61.

Bosch A, Abad, F. X. and Pint, R. M. 2005. Human Pathogenic Viruses in the Marine Environment. Oceans and Health: Pathogens in the Marine Environment. Edited by Belkin and Colwell, Springer, New York.

Bosch A, Pintó, R.M. and ABAD, F.X. 2006. Survival and transport of enteric viruses in the environmental. Universitat de Barcelona. p. 151-188. Available from: <http://www.ub.edu/ microbiologia/viruse/papers/GOY6. pdf>.

De Keuckelaere A, Baert, L., Duarte, A., Stals, A. and Uyttendaele.M. 2013. Evaluation of Viral Concentration Methods from Irrigation and Processing Water. *Journal of Virological Methods*., 187(2) : 294–303.

De Leon R, Shieh, C., Baric, R.S. and Sobsey, M.D. 1990. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p. 833–853. Proceedings of the American *Water Works Association Water Quality and Technology Conference*, Den- ver, Co.

De Roda Husman AM, Lodder-Verschoor, F., vanden Berg, H.H., Le Guyader, F.S., van Pelt, H., van der Poel, W.H. and Rutjes, S.A. 2007. Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks. *J. Food Prot.*, 70: 967–974.

Fumian TM, Leite, J.P.G., Castello, A.A., Gaggero, A., de Caillou, M.S.L. and Miagostovich, M.P. 2010. Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. *J. Virol. Methods*., 170: 42–46.

Griffin DW, Donaldson, K. A., Paul, J. H. and Rose, J. B. 2003. Pathogenic Human Viruses in Coastal Waters. clinical microbiology reviews., 16(1):129–143.

Halliday, E. and Gast, R. J. 2011. Bacteria in beach sands: an emerging challenge in protecting coastal water quality and bather health. *Environmental science and technology*., 45(2) : 370–9.

Hernroth BE, Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R. and Allard, A.K. 2002.Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel. Mytilus edulis: the first Scandinavian report. *Appl Environ Microbiol*., 68(9): 4523-33.

Houde A, Guévremont, E., Poitras, E., Leblanc, D., Ward, P., Simard, C. and Trottier, Y.L. 2007. Comparative evaluation of new TaqMan real-time assays for the detection of hepatitis A virus. *Journal of virological methods*., 140(1-2): 80–9.

Jiang SC, Chu, W. and He, J.W. 2007. Seasonal detection of human viruses and coliphage in Newport Bay, California. *Applied and environmental microbiology*., 73(20) : 6468–74.

Jothikumar N, Cromeans, T.L., Sobsey, M.D. and Robertson, B.H. 2005.Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. *Appl Environ Microbiol*., 71: 3359–3363.

Jothikumar N, Kang, G. and Hill, V.R. 2009.Broadly reactive TaqMan assay for real- time RT-PCR detection of rotavirus in clinical and environmental samples. *J. Virol. Methods*., 155: 126–131

Katayama H, Shimasaki, A. and Ohgaki, S. 2002. Development of a Virus Concentration Method and Its Application to Detection of Enterovirus and Norwalk Virus from Coastal Seawater. *Applied and environmental microbiology*., 68(3) : 1033–1039.

Kittigul L, Pombubpa, K., Rattanatham, T., Diraphat, P., Utrarachkij, F., Pungchitton, S., Khamrin, P. and Ushijima, H. 2008. Development of a method for concentrating and detecting rotavirus in oysters, *International journal of food microbiology*., 122: 204–210.

Le Guyader FS, Le Saux, J.C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., Giraudon, H., Delmas, G., Pommepuy, M., Pothier, P. and Atmar,R.L. 2008. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis out- break. *J. Clin. Microbiol*., 46:4011–4017

Lowther JA, Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. 2012. Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Applied and environmental microbiology*., 78(16): 5812–7.

McQuaig S, Griffith, J. and Harwood, V. J. 2012. Association of fecal indicator bacteria with human viruses and microbial source tracking markers at coastal beaches impacted by nonpoint source pollution. *Applied and environmental microbiology*., 78(18): 6423–32.

Mesquita JR, Vaz ,L., Cerqueira, S., Castilho, F., Santos, R., Monteiro, S., Manso, C.F., Romalde, J.L. and Nascimento, M.S.J. 2011. Norovirus, hepatitis A virus and enterovirus presence in shellfish from high quality harvesting areas in Portugal. *Food microbiology*., 28(5): 936–41.

Miagostovich MP, Ferreira, F.F.M., Guimaraes, F.R., Fumian, T.M., Diniz-Mendes, L., Luz, S.L.B., Silva, L.A. and Leite, J.P.G. 2008. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of manaus Central Amazonia, Brazil. *Appl. Environ. Microbiol*., 74 : 375–382

Moresco V, Viancelli, A., Nascimento, M.A., Souza, D.S., Ramos, A.P., Garcia, L.A., Simões, C.M. and Barardi, C.R. 2012. Microbiological and physicochemical analysis of the coastal waters of southern Brazil. *Mar Pollut Bull*., 64(1):40-8.

Myrmel M, Berg, E. M. M., Rimstad, E. and Grinde, B. 2004. Detection of Enteric Viruses in Shellfish from the Norwegian Coast. *Applied and environmental microbiology*., 70(5): 2678–2684.

Pal A, Sirota, L., Maudru, T., Peden, K. and Lewis, Jr. A.M. 2006.Real-time, quantitative PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. *J Virol Methods*., 5(1):32-42.

Pina S, Lucena, F., Jofre, J. and Girones, R. 1998. Viral Pollution in the Environment and in Shellfish : Human Adenovirus Detection by PCR as an Index of Human Viruses. *Applied and environmental microbiology*., 64(9) : 3376–3382.

Rigotto C, Kolesnikovas, C. K., Moresco, V., Simões, C. M. O. and Barardi, C. R. M. 2009. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepatitis A virus in different water matrices. *Memórias do Instituto Oswaldo Cruz*., 104(7): 970–4.

Rodríguez RA, Thie, L., Gibbons, C.D. and Sobsey, M.D. 2012.Reducing the Effects of Environmental Inhibition in Quantitative Real-time PCR Detection of Adenovirus and Norovirus in Recreational Seawaters. *Journal of Virological Methods*., 181(1): 43–50.

Shibata, T. and Solo-Gabriele, H.M. 2012.Quantitative Microbial Risk Assessment of Human Illness from Exposure to Marine Beach Sand. *Environmental Science and Technology*., 46 (5): 2799–2805.

Victoria M, Guimarães, F.R., Fumian, T.M., Ferreira, F.F., Vieira, C.B., Shu- bo, T., Leite, J.P. and Miagostovich, M.P. 2010.One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil. *J Water Health*., 8: 158-165.

Victoria M,Guimarães, F., Fumian,T., Ferreira,F., Vieira,C., Leite, J.P. and Miagostovich, M. 2009.Evaluation of an Adsorption–elution Method for Detection of Astrovirus and Norovirus in Environmental Waters. *Journal of Virological Methods*., 156: 73–76.

Wong M, Kumar, L., Jenkins, T. M., Xagoraraki, I., Phanikumar, M. S. and Rose, J. B. 2009. Evaluation of public health risks at recreational beaches in Lake Michigan via detection of enteric viruses and a human-specific bacteriological marker. *Water research*., 43(4): 1137–49.

Wyn-Jones AP, Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Höller, C., de Roda Husman, A.M., Kay, D., Kozyra, I., López-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R. and Wyer, M. 2011. Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research*., 45:1025-1038.

Xagoraraki I, Kuo, D.H-W., Wong, K.., Wong, M. and Rose, J.B.2007.Occurrence of human adenoviruses at two recreational beaches of the great lakes. *Applied and environmental microbiology*., 73(24):7874–81.

****