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Presence of enteroviruses and reoviruses in the waters of the Italian coast of the Adriatic Sea

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SUMMARY

EEC directive 76/160 requires member states to apply microbiological and chemico-physical standards for the quality of recreational waters. In observation of this regulation, in the present study 144 samples of seawater were taken over a 12-month period and tested to determine viral contamination. The samples were collected from the coastal waters of the Italian town of Pesaro, which is located on the Adriatic Sea. Using cell culture techniques, 32·6% of the seawater samples were found to be contaminated with enteroviruses. Isolation of these viruses was most frequent in the summer months. Thus, our results indicate the need to increase the frequency of monitoring of these waters and to eliminate the sources of contamination.

INTRODUCTION

Numerous studies have reported the contamination of water with human enteric viruses which cause gastrointestinal disorders [1–4].

The main source of contamination of surface waters, including seawater, is the discharge of treated and untreated sewage from urban and rural areas. In water, viral particles undergo a considerable decrease in concentration due to their dilution and the action of deactivating factors. Some viruses are, however, stable in water (e.g. enteroviruses) and these pathogens excreted by infected members of the population are able to survive for long periods of time, especially when bound to organic material of faecal origin [5–9].

Enteric viruses are transmitted by the faecal-oral route through ingestion of contaminated water or the consumption of contaminated seafood, such as molluscs. Enteroviruses are enteric viruses which cause a variety of illnesses ranging from gastroenteritis to pericarditis, myocarditis, cardiomyopathy, aseptic meningitis and encephalitis [10–13]. Although episodes of gastroenteritis have been linked to bathing in seawater [14], particularly in children [15–17], there is a paucity of data relating to the role of seawater in the transmission of viral infections [14]. In fact, viral diseases associated with bathing in seawater are difficult to document for many reasons, including the wide spectrum of syndromes, the presence of numerous different viral strains and the high frequency of subclinical infections, etc [10, 18, 19]. Furthermore, the difficulties encountered in using the available diagnostic techniques have limited the detection of enteroviruses in both clinical and environmental samples, and thus these pathogens have frequently not been identified as the agents of water-borne diseases.

The range of pathologies caused by another class of enteric viruses, the reoviruses, is still not clear. In fact, several types would appear to be responsible for biliary atresia in newborns, while other studies have

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linked these viruses to a range of human diseases including mild cases of gastroenteritis, pre- and perinatal infections, hepatitis, etc. [20–22]. Some authors [23–29] have reported the frequent isolation of reoviruses from contaminated waters. Epidemiological and ecological studies suggest that the main source of reoviruses in polluted waters may be excretion from animals [24], and above all from humans [23, 24].

Given the potential importance of gastroenteric viral infections transmitted by water, Italian legislation (law decree No. 155 of 14-5-1988(30)), in accordance with EEC directive 76/160 regarding sea bathing waters, now plans to monitor for enteroviruses as an indicator of human faecal contamination. Unfortunately this requirement is often ignored, and whether or not testing is carried out depends on the good will and judgement of the competent authorities.

Thus, given the significant public health risk that high levels of contamination would pose, the main aim of the present study was to evaluate the presence of enteroviruses and reoviruses in the coastal waters of the Adriatic Sea off the seaside resort town of Pesaro and to evaluate the possible relationship with faecal indicators. Pesaro is an important seaside resort town, with 96000 inhabitants and a noteworthy increase in population during the tourist season.

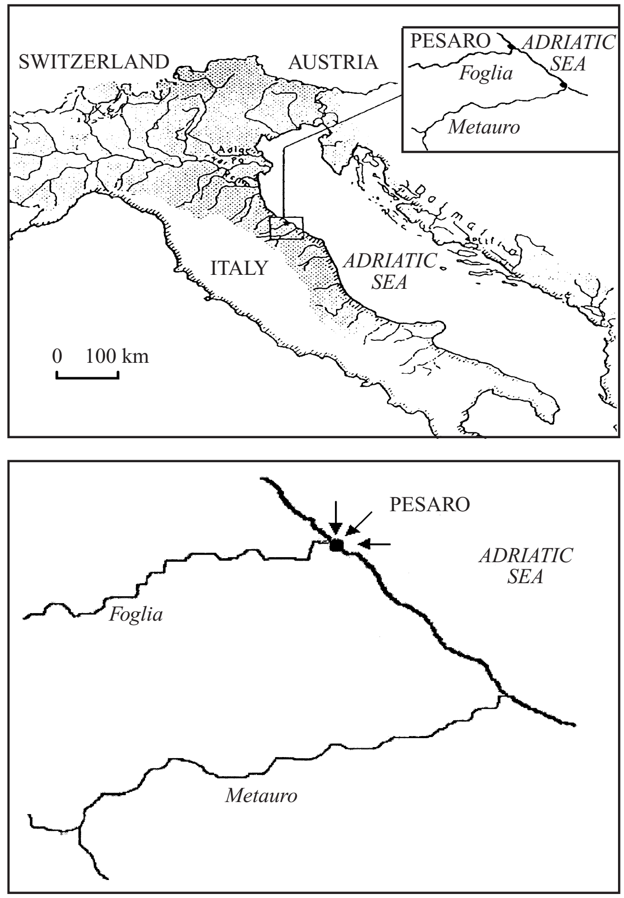

Fig. 1. Map of the area from which the samples described in this study were obtained.

METHODS

Sampling sites

The north-western coast of the Adriatic Sea is characterized by shallow water (depth 4–5 m), artificial cliffs and bottom attritions which decrease the speed of marine currents and obstruct the natural dilution and dispersion of coastal river waters and illegal sewage discharge. Three sampling stations in the Pesaro area were set up in this study (hereafter referred to as bay, pier and port) (Fig. 1). The bay and pier stations are located in an area of the coast in which sea bathing is permitted. Bathing is not allowed in the waters of the port area, which coincides with the mouth of the Foglia River. The Foglia River is a typical floodstream with a low flow, running through small urban areas used mainly for agriculture and livestock breeding. The principal source of pollution for these rivers is waste-water from urban and industrial areas and livestock farms, from which almost 40 % of the sewage flows directly into the river [31]. Most of the sewage from the city of Pesaro is instead treated in the municipal wastewater treatment plant located approximately 5 km from the mouth of the river before being discharged again into the river, although a small portion of the sewage is only treated with chlorine before being discharged into the river. Examination of these waters allows us to highlight the presence of viral pathogens in the population.

Water specimens

Sampling was carried out 48 times (4 times a month) from January through December 1998. A total of 144 samples was obtained. In accordance with the guidelines given in the Italian legislation (14 May 1988, No. 155), samples for use in isolating the viruses were taken in a sterilized 10-litre bottle and samples taken for the determination of indicators of faecal contamination and salmonellae were taken in a sterilized 2-litre bottle, at a depth of approximately 30 cm below the water surface and at a distance from the

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water's edge sufficient to assure a depth of 80–120 cm. These samples were carried to the laboratory in refrigerated containers (4 °C).

For virus isolation, samples at natural pH (7·8–8·2) were concentrated by filtration through electropositive membranes (Ø 142 mm) (Virosorb 1MDS, AMF Corp., Cuno Division, Meridian, USA). The filters were used in double layers for maximum virus retention and were eluted very slowly three times with 30–40 ml of 3% beef extract (Oxoid) solution at pH 9–9·5. Eluates were neutralized with 1·0 mol/l HCl, then decontaminated with 1:10 chloroform and shaken for 30 min in order to eliminate the microbial flora. The samples were centrifuged at 1200 ***g*** for 15–20 min. The supernatants were aired for 3 h under a vacuum hood with agitation to eliminate the chloroform.

Virus isolation

The BGM (Buffalo Green Monkey) cell line was used to isolate the enteroviruses. The cell monolayers were prepared by culturing the cells in flasks containing Eagle MEM plus Earle's salts with 10% foetal calf serum (FCS). Samples were inoculated onto cell monolayers in maintenance medium supplemented with 2% FCS. Cell monolayers were incubated at 37 °C in 5% CO_2 atmosphere and examined every 2 days over a 15-day period to detect cytopathic effects (CPE). Subsequent passages were performed after freeze-thawing.

The MA104 (rhesus monkey) continuous cell line was used for reovirus isolation. The concentrated samples were inoculated into cells with maintenance medium (Eagle MEM plus Earle's salts supplemented with 2% FCS plus 1 mg/ml trypsin). Cell monolayers were incubated at 37 °C in 5% CO_2 atmosphere and examined every 2 days over a 15-day period to detect CPE. Subsequent passages were performed after freeze-thawing.

To improve the isolation of the various viral strains, each positive sample was submitted to the plaque cytolysis technique. The material taken from each plaque was again seeded in cell monolayers and incubated at 37 °C until cytopathic effect was obtained.

Enteroviruses were typed by serum neutralization tests using pools of antisera obtained from the World Health Organization – Collaborating Centre for Virus Reference and Research, Copenhagen (Ornithosis Department, Statens Seruminstitut, DK 2300 Copenhagen S, Denmark).

Reoviruses were typed using CPE neutralization antisera obtained from the Istituto Zooprofilattico della Lombardia e dell'Emilia 'Bruno Umbertini' (Brescia, Italy).

Bacteriological assay

In accordance with Italian legislation [30], assays of total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS) were performed employing the multiple tube fermentation technique, and the assay for *Salmonella* spp. was performed employing filtration membrane methods [32].

Statistical analysis

The possible relationships between the virus isolation from different sites was evaluated using the χ^2 test.

RESULTS

Forty-seven (32·6%) of 144 samples tested positive for enteroviruses, whereas 71 (49·3%) were found positive for reovirus. In 20·1% of the samples, both reoviruses and enteroviruses were found in the same lysate (Table 1). For enteroviruses, the overall isolation frequency was found to be higher in the two areas in which bathing was allowed (37·5% for the bay and 35·4% for the pier) than in the port area (25·0%), a difference which was not significant ($P >$ 0·05). In all of the areas examined, the highest isolation rate was found during the summer months, with a peak of 100% in the month of July in the bay area, in August in the pier area and a peak of 50% in the port area in the months of July and August (Table 1).

The enteroviruses most frequently isolated were coxsackieviruses B (31/47, 65·9%) (41·9% B4; 29·0% B5; 25·5% B3 and 12·9% B1) followed by polioviruses (11/47, 23·4%) (45·4% polio 2; 27·2% both polio 1 and polio 3) and echoviruses (5/47, 10·6%) (all of which were Echo 11). In 6·4% (3/47) of the positive samples, there was the simultaneous presence of polio, coxsackie and echo viruses, while in 12·7% (6/47) both polio and coxsackie viruses were present. The viral species most frequently isolated were reoviruses, with isolation frequencies almost identical to those found in the two areas in which bathing was authorized (43·7% and 47·9% respectively), and only

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Table 1. *Isolation of enteroviruses from the coastal waters of the Adriatic sea in different months of the year*

	Enteroviruses		Reoviruses		Enteroviruses and reoviruses in same lysate	
	N	%	N	%	N	%
Bay*						
Jan.	0	—	0	—	0	—
Feb.	0	—	1	25·0	0	—
Mar.	1	25·0	1	25·0	0	—
Apr.	1	25·0	2	50·0	0	—
May	2	50·0	2	50·0	1	25·0
June	3	75·0	2	50·0	1	25·0
July	4	100·0	3	75·0	3	75·0
Aug.	3	75·0	4	100·0	3	75·0
Sept.	2	50·0	3	75·0	1	25·0
Oct.	2	50·0	2	50·0	1	25·0
Nov.	0	—	1	25·0	0	—
Dec.	0	—	0	—	0	—
Total	18	37·5	21	43·7	10	20·8
Pier						
Jan.	0	—	0	—	0	—
Feb.	0	—	2	50·0	0	—
Mar.	1	25·0	2	50·0	0	—
Apr.	1	25·0	3	75·0	1	25·0
May	2	50·0	2	50·0	1	25·0
June	3	75·0	2	50·0	1	25·0
July	3	75·0	4	100·0	3	75·0
Aug.	4	100·0	3	75·0	3	75·0
Sept.	2	50·0	3	75·0	1	25·0
Oct.	1	25·0	1	25·0	0	—
Nov.	0	—	1	25·0	0	—
Dec.	0	—	0	—	0	—
Total	17	35·4	23	47·9	10	20·8
Port						
Jan.	0	—	1	25·0	0	—
Feb.	1	25·0	1	25·0	0	—
Mar.	1	25·0	2	50·0	0	—
Apr.	1	25·0	3	75·0	1	25·0
May	1	25·0	2	50·0	0	—
June	1	25·0	3	75·0	1	25·0
July	2	50·0	4	100·0	2	50·0
Aug.	2	50·0	4	100·0	3	75·0
Sept.	1	25·0	2	50·0	1	25·0
Oct.	1	25·0	3	75·0	0	—
Nov.	1	25·0	2	50·0	1	25·0
Dec.	0	—	0	—	0	—
Total	12	25·0	27	56·2	9	18·7
Total						
Jan.	0	—	1	8·3	0	—
Feb.	1	8·3	4	33·4	0	—
Mar.	3	25·0	5	41·7	0	—
Apr.	3	25·0	8	66·7	2	16·7
May	5	41·7	6	50·0	2	16·7
June	7	58·4	7	58·4	3	25·0

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Table 1. (*cont*.)

	Enteroviruses		Reoviruses		Enteroviruses and reoviruses in same lysate	
	N	%	N	%	N	%
July	9	75·0	11	91·7	8	66·7
Aug.	9	75·0	11	91·7	9	75·0
Sept.	5	41·7	8	66·7	3	25·0
Oct.	4	33·4	6	50·0	1	8·3
Nov.	1	8·3	4	33·4	1	8·0
Dec.	0	—	0	—	0	—
Total	47	32·6	71	49·3	29	20·1

* For Bay, Pier and Port, 12 samples (4 each) were collected for each month (Jan.–Dec.), totalling 144.

slightly higher in the port area (56·2%). Also for these viruses, the greatest isolation frequency was found in the summer months, with the highest values found in July and August.

With regard to bacterial faecal contamination, the levels of these indicators in the bay and pier areas were always within the limits stipulated by Italian law (total bacterial count $\leqslant$ 2000; faecal coliforms $\leqslant$ 100; faecal streptococci $\leqslant$ 100 in 100 ml), while for the port area these limits were consistently exceeded for all of the indices. *Salmonella* spp. were never found in the pier and bay areas, but were detected in the summer months from June to September (mean frequency 25%) in the port area.

DISCUSSION

We have shown that in Pesaro, despite the law in force relating to the microbiological safety of bathing water [30], which requires the testing of a ten-litre water sample for the absence of enteroviruses before the necessary declaration of conformity can be given, viruses have been isolated in coastal waters in which bathing is authorized. Similar findings have been shown in other studies [27, 33–35]. Our study revealed a significant presence of enteroviruses (32·6%) in the waters of three areas along the Pesaro coastline. For two of these areas, authorization had been given for bathing and in one bathing was not allowed. The overall frequency of isolation was found to be similar in the two swimming areas (37·5 and 35·4%) but higher than that found in the area in which bathing was not permitted (25·0%), although there was not a significant statistical difference. Other studies performed in recent years on recreational waters along other areas of the Italian coast revealed frequencies of 12·7–47·8% [28, 35, 36]. This variability was also found at the mouth of various rivers and in recreational waters, with values of 8·3–77·8% [35–38].

With regard to the seasonal pattern of isolates, an increase was observed in the summer for all three of the areas considered (in June, July and August for the bay and pier areas and in July and August for the port). The same trend was found for the indices of bacterial faecal contamination. Furthermore, salmonellae were isolated during the summer in the port area. This finding was not surprising, in that in previous studies of samples taken along the course of the Foglia River and at its mouth these agents [39] and other emerging bacterial pathogens had already been isolated [40, 41]. The greater isolation frequency observed in the summer could be due to the fact that Pesaro is a seaside resort town, with a considerably higher population during the summer tourist season during which period there is an increase in the volume of sewage discharged.

The viruses detected in waters approved for bathing (tested and found acceptable on routine bacteriological monitoring) confirm what has been reported by other authors [3, 19, 34–36, 42–44] that the traditional bacterial indicators of faecal contamination do not always give a valid correlation with the presence of enteric viruses. In fact, their resistance to environmental factors is known to be greater than that of bacteria, and thus they are frequently isolated in water environments which are acceptable from a bacteriologic standpoint.

The lower frequency with which viral agents were found in the waters of the port area could be explained by the fact that, when the microbiological load is of faecal origin, there is competition between the viruses and bacteria, perhaps due to their utilization of the

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proteins from the viral capsid or to the production of metabolites with an antiviral action [45]. In any case, water with even small loads of viruses can play an important role in the development of human disease. In fact, these viruses are able to infect human beings even at very low doses (1–10 viral particles are sufficient) [19, 46, 47].

With regard to the enteroviruses, the typing experiments revealed that coxsackieviruses were the predominant enteroviruses isolated (65·9%) compared with polioviruses (23·4%) and echoviruses (10·6%). These viral serotypes were isolated in other studies carried out in Italy on surface waters and wastewater [27, 37, 48–50] although with different frequencies. The different prevalence of various serotypes, as well as the different incidence from year to year and area to area may depend on various factors such as hygiene, population density, prevalence of infection in the community and the season [51].

With regard to the reoviruses, various studies have reported their isolation from surface waters [23–29]. The percentages found in our study reflect an intermediate position with respect to the data reported by other researchers in Italy, who have reported prevalences of 8·7–80·0% for areas where bathing is permitted [28, 35, 36] and 8·7–58·6% for areas in which bathing is not permitted or at the mouths of rivers [28, 35, 36]. Testing for these viruses is not usually included in standard water monitoring procedures, although numerous authors have indicated that they are ideal candidates for water contamination linked to domestic wastewater due to their wide distribution and great stability in the environment [24, 28, 35, 52], and their frequent isolation in surface water [23–29].

The viruses identified in our study are potential pathogens and therefore they represent a potential public health risk. The source of the polioviruses is probably immunized children and individuals coming into contact with them. Some authors believe that strains used for vaccination are genetically unstable and can revert to neurovirulence during the passage through the human intestine [53–55]. The group B coxsackieviruses are associated with a wide spectrum of diseases including aseptic meningitis, encephalitis, muscle pain and myocarditis [3].

In conclusion, given the results reported it is to be hoped that the public authorities will give greater attention to the problem, requiring routine monitoring, an increase in the frequency of testing and measures to reduce or remove sources of faecal contamination in order to protect the population from these health hazards linked to recreational waters.

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