Epidemiol. Infect. (1994), **113**, 541–549

Exposure to enteroviruses and hepatitis A virus among divers in environmental waters in France, first biological and serological survey of a controlled cohort

D. GARIN[1], F. FUCHS[2]*, J. M. CRANCE[3], Y. ROUBY[1], J. C. CHAPALAIN[1], D. LAMARQUE[4], A. M. GOUNOT[5] AND M. AYMARD[2]

[1] *Biologie Médicale (Dr Bartoli) Hôpital d'Instruction des Armées Desgenettes, 108, Bd Pinel, 69275 Lyon Cedex 3 France*

[2] *Agence du Médicament, Unité de Virologie, 8, Avenue Rockefeller, 69373 Lyon Cedex 08 France*

[3] *Unité de Biologie Moléculaire (Dr Deloince) Centre de Recherche du Service de Santé des Armées, 24 Av. des Maquis du Grésivaudan BP 87 38702 La Tronche Cedex France*

[4] *Médecine des collectivités, Hôpital d'Instruction des Armées Robert Piqué, 33998 Bordeaux Armées France*

[5] *Université Claude Bernard, Bât. 405, 43 Bd 11 Novembre 1918, 69622 Villeurbanne Cedex France*

(*Accepted 28 June 1994*)

SUMMARY

An epidemiological study of hepatitis A and enteroviruses was conducted in a military diving training school, by evaluating the viral contamination of water using an ultrafiltration concentration technique, and assessing seroconversion and the presence of virus in stool specimens obtained from 109 divers and 48 controls. Three of 29 water specimens were positive for enterovirus by cell culture and 9 by molecular hybridization. There was little or no risk of virus infection during the training course (49 h exposure) because there was no significant difference between divers and controls for both viral isolation and seroconversion. However, a higher percentage of coxsackievirus B4 and B5 seropositive divers suggests that these were more exposed during previous water training. No hepatitis A virus (HAV) detection and no seroconversion to HAV was observed. The rate of HAV seropositive subjects was 17% in this 24·5-year-old population.

INTRODUCTION

Enteroviruses are common pathogens which cause a wide spectrum of systemic infections including myocarditis and meningitis [1, 2] and surveillance studies suggest that 10–15 million illnesses due to enteroviruses other than polioviruses occur each year [3]. Their ability to survive in environmental waters is well demonstrated by their isolation from swimming pools and surface waters [4].

* Reprints or correspondence: Florence Fuchs, Agence du Médicament, Direction des contrôles, 8, Avenue Rockefeller, 69373 Lyon Cedex 08 France.

Various concentrations of viruses have been detected in lakes, rivers, seawater and other water used for recreation [5–8]. Enteroviruses, hepatitis A virus (HAV) and rotaviruses can cause waterborne epidemics [9–13], but there is little quantitative information on the relation between water quality and disease attack rates after bathing and diving in contaminated water [14–16]. The detection of enteric viruses in large volumes of water presents major difficulties in collecting a representative sample and in concentrating the viruses for laboratory investigations. Also many enteroviruses can cause inapparent or silent infections that go unrecognized until secondary infections occur. However current developments in water concentration techniques [17–19], immunological assays [20] and in the use of nucleic acid probes for specific viral genes [21–24] make it possible to detect viruses which replicate poorly in cell cultures. We have used methods and conducted a prospective cohort study in a military diving training school in order to determine whether professional exposure when swimming or diving in rivers and lakes in France is hazardous to health. Clinical and/or biological monitoring of 109 divers during 2-month training courses, of their diving areas, and of 49 non-diver controls was performed over a period of one year.

MATERIALS AND METHODS

Participants

Healthy volunteers from French diving school trainees who took the elementary or first-level military diving certificate between September 1991 and August 1992 were followed up. Concerning the equipment of the divers, only wet suits are worn permanently. Most of the training is done without masks and breathing apparatus, and large quantities of water may be ingested, especially during long-distance swims (40 km). Data on age, food consumption, water activities and illnesses were kept each day by the school medical staff who were always present during the courses. Sera were collected before, during and one month after diving training. Faeces were collected before the course and every 15 days during the training course period. The training period involves spending a total of 96 h in the water. Non-diver controls from the same military unit, of identical age and rank as divers, were randomized during each training course. The ratio of exposed to non-exposed subjects was 2.

Study sites

The Rhône and Saône rivers (20 km north of Lyon), the 'Rudigoz' pond (30 km north-east of Lyon), Lake Bourget (100 km east of Lyon), and swimming-pools of the diving school were chosen because a preliminary study had shown that these areas represented 88% of all swimming practice house in 1990.

Water sampling

About 60 l samples were collected in 2 h during a training course from the actual depths at which diving was performed (0·5–7 m). We used an ISCO 3700 sampler pump (Lincoln, USA) on a rubber dinghy, a prefiltration housing containing a prefilter (5 μm) manufactured by Millipore Corporation, Bedford, Mass. (Polycard®, cat. no. CR05010006) and polypropylene tanks.

Bacteriological analysis

Total flora, total and faecal coliforms were counted using a standard membrane filter method [25].

Water concentration

The first concentration step was performed with two polysulphonate membranes of Pellicon® cassette system of 10^5 nominal molecular weight limit (NMWL) (Millipore PTHK cat. 000C5) corresponding to 1 m^2 of surface. We worked with a 7 psi inlet pressure, a 3:1 retentate to filtrate ratio and a 100 l per h total pump discharge. After clamping off both filtrate lines, membranes were slowly eluted with 150 ml of beef extract (3% in borate buffer, pH 9·5 (bioMérieux cat. no. 53201). At the end of each run, the membranes were washed with tap water, and cleaned with 2 l of 0·1 N-NaOH before storage. The eluate was immediately neutralized and filtered with the concentrate through 0·22 μm membranes to eliminate bacterial and fungal contamination. The specimen obtained was further concentrated by polyethylene glycol 6000 precipitation (PEG 6000, Merck Corporation cat. 807491): after the addition of 10% PEG 6000 and 2·3% NaCl, the sample was kept overnight at 4 °C. Precipitated viruses were collected by centrifugation at 10000 g for 30 min at 2 °C and suspended in 2 ml phosphate buffer saline (PBS, pH 7·4).

Faecal sample preparation

The samples were diluted in sterile Eagle's BME medium treated with antibiotics during 1 h at room temperature and clarified by centrifugation (2500 g for 1 h at +4 °C) [26]. The crude stool extract called 'p0' was dot-blotted without extraction for hybridization and passed in parallel and successively once (p1), twice (p2) or more times in mono-layer cultures.

Isolation and identification of virus in concentrate water and stool samples

The water and stool samples were cultured in BGMK cells, HEP2 cells and MRC5 cells propagated in Eagle MEM (Flow Laboratories, France) supplemented with 2% foetal calf serum, 1% (v/v) amino acids, glutamine (Whittaker, France) and antibiotics. Lim–Benyesh–Melnick antiserum pools were used for identification of virus isolates by virus neutralization tests [27].

Enterovirus serology

A microneutralization test was used to detect the presence of antibodies to 16 most frequently occurring enteroviruses. Neutralizing titres were expressed as the highest dilutions of sera (ranging from 10 to 12800) in a volume of 0·05 ml that inhibited the cytopathic effect of 50% tissue culture infective doses (TCID 50) of the virus. We accepted as evidence of infection either seroconversion or a four-fold increase in antibody titres.

Enterovirus probes and hybridization

Riboprobes with negative polarity corresponding to the 5′ non-coding region (*n* 221–670) or to the VP1 region (*n* 3064–3417) were used as previously described [24]. In summary, stool samples incubated with proteinase K and diluted in a 3:2

(v:v) 20 × SSC-37% formaldehyde mixture were spotted on nylon membranes and baked at 120 °C for 30 min. Hybridization was performed for 1 h at 42 °C and followed by the hybridization step with the digoxigenin-labelled probe at a 300 ng/ml concentration. The digoxigenin-labelled hybrids were revealed by means of an enzyme reaction or a chemiluminescent detection technique.

Detection of hepatitis A virus antigen

A solid phase radioimmunoassay based on that described by Purcell and colleagues [20] with some modifications [28] was performed. In summary, 50 μl of test sample were incubated for 2 h at 45 °C in a well of a polyvinyl microtitration plate (Costar, Cambridge, Mass.) previously coated for 4 days at 4 °C with IgG to HAV obtained from a convalescent human. After washing, 50 μl of I^{125}-IgG to HAV (150000 cpm) labelled by the chloramine-T method [29] were added. The plate was incubated overnight at room temperature. After repeated washings, the bound radioactivity was counted with a gamma counter. A sample value to negative control value ratio $\geqslant 2{\cdot}1$ was considered positive.

Hepatitis A virus antibody detection by enzyme-linked immunosorbent assay

Detection of anti-HAV IgM and IgG was performed using the IM®X system (HAVAB®, Abbot Laboratories, Chicago).

Statistical analysis

Statistical analyses were done on Apple microcomputers with the program Statis® (O. Mericq, Toulouse, France). Means were compared by Student's t test, percentages were compared with $\chi 2$ test. All statements of significance are made at $P < 0{\cdot}05$.

RESULTS

Features of the studied population

A total of 109 divers and 49 controls was studied. Age means in the two groups showed no significant difference (divers $24{\cdot}5 \pm 3{\cdot}3$ years; controls $24{\cdot}5 \pm 2{\cdot}7$ years). Diving and swimming time exposures were accurately calculated for each diver (about 29·5 h in environmental waters and 19 h in swimming pools) and are summarized in Table 1.

Microbiological water sample results

During one year, 29 samples were taken in the different diving areas corresponding to a final volume of 1830 l. Enumeration of bacterial content, enterovirus and HAV detection were performed and results are shown in Table 2. Viral isolation allowed the identification of two non-polio enteroviruses, and interestingly of one poliovirus type 3 Sabin strain. Molecular hybridization assays were performed in parallel and 9 samples could be identified as containing enterovirus genome. Among the 9 positive samples, 5 were found in the Rhône river. All the specimens positive in cell culture were also positive in hybridization technique. No positive detection of HAV antigen was observed.

https://doi.org/10.1017/S0950268800068564 Published online by Cambridge University Press

Table 1. *Diving and swimming time exposures in the different diving areas*

	Sum of duration of diving (109 divers)	Maximum of duration of diving for one diver	Mean of duration of diving (109 divers)
Swimming pools	2135 h 28 min	55 h 00 min	19 h 35 min
Environmental water			
Rhône river	1480 h 57 min	28 h 10 min	13 h 35 min
Rudigoz pond	238 h 48 min	8 h 00 min	2 h 11 min
Bourget lake	815 h 49 min	12 h 25 min	7 h 29 min
Other environmental water	655 h 40 min	40 h 00 min	6 h 00 min
Sub-total environmental water	3191 h 14 min	47 h 40 min	29 h 16 min (±8 min)
Total	5326 h 42 min	55 h 00 min	48 h 52 min (±18 h 35 min)

Table 2. *Results of bacterial and virological water sampling*

	No. of samples	Vol. (litres)	Temp (°C)*	Total bacterial flora†	Total coliform†	Faecal coliform†	Viral isolation	Molecular detection of enterovirus	HAV antigen detection (RIA)
Swimming pools	7	455	19±2	6±6	0	0	0	1	0
Rhône river	13	785	11±4	3163±1700	64±41	9±5	1echo11	5	0
Saône river	1	60	8	1200	37	26	0	0	0
Rudigoz pond	4	240	15±7	120±30	21±6	5±1	1echo11	2	0
Bourget lake	4	290	13±5	1075±925	29±2	3±2	1polio3 (Sabin)	1	0
Total or mean	29	1830	14±2	1513±1819	34±37	4±5	3	9	0

* Mean (±S.D.) of water temperature.

† Mean (±S.D) of colonies numerated on membrane filtre per ml of water.

Table 3. *Results of virological investigations in participant faeces*

	Enterovirus isolation		Enterovirus hybridation		Adenovirus isolation		Rotavirus ELISA		HAVRIA antigen	
	Number	%	Number	%	Number	%	Number	%	Number	%
Divers	1*	1	7	6	9	8	4	4	0	0
Control	0	0	1	2	4	8	3	6	0	0
Total	1	1	8	5	13	8	7	4	0	0
χ^2 =	ND†		Yates‡ = 0·592: NS		Yates‡ = 0·085: NS		Yates‡ = 0·075 NS		ND†	

* Coxsackievirus B2.
† Not determined.
‡ Yates correction of the χ^2 test.

Table 4. *Serological survey of the divers and control population: a microneutralization test was used to detect the presence of antibodies to 16 most frequently occurring enteroviruses*

	Number of seropositive participants			Number of seroconversions	
Virus	Divers	Controls	χ^2 test	Divers	Controls
Cox A7	23 (21%)	12 (24%)	NS*	0	0
Cox A9	75 (69%)	37 (76%)	NS	1	1
Cox B1	31 (28%)	15 (31%)	NS	0	0
Cox B2	68 (62%)	27 (55%)	NS	1	1
Cox B3	62 (57%)	30 (61%)	NS	0	1
Cox B4	86 (79%)	31 (63%)	$P < 0{\cdot}05$	2	0
Cox B5	68 (62%)	21 (43%)	$P < 0{\cdot}05$	0	0
Cox B6	14 (13%)	1 (2%)	NS	1	0
Echo 3	42 (39%)	24 (49%)	NS	0	0
Echo 4	68 (62%)	28 (57%)	NS	1	0
Echo 6	57 (52%)	20 (41%)	NS	0	0
Echo 7	42 (39%)	18 (37%)	NS	3	0
Echo 9	32 (29%)	17 (35%)	NS	3	1
Echo 11	50 (46%)	21 (43%)	NS	1	1
Echo 30	41 (38%)	23 (47%)	NS	3	0
Echo 33	11 (10%)	2 (4%)	NS	0	0
HAV	18 (17%)	9 (18%)	NS	0	0

* Not significant.

Virological analysis of faecal samples

A total of 578 samples was treated with antibiotics and both inoculated to cell culture and further investigated for rotavirus detection by ELISA, for enterovirus detection by molecular hybridization and for HAV detection by RIA. Results are summarized in Table 3. Enteric viruses could be detected by viral isolation: 13 adenoviruses were identified and only one enterovirus could be identified as a coxsackievirus B2. Molecular hybridization allowed the detection of 5% positive participants without significant difference between divers and the control groups. The incidence rate of rotavirus positive participants was 4%.

https://doi.org/10.1017/S0950268800068564 Published online by Cambridge University Press

Serological survey

A follow-up of each diver and control included in the study was performed by blood sampling on the day of arrival at the school, at the end of the training course and one month after departure. Specific antibodies to the most commonly occurring enteroviruses were tested by seroneutralization and for HAV by ELISA. Results are presented in Table 4. More than 400 sera were studied: 21 subjects showed at least 1 seroconversion, and sometimes up to 3 seroconversions. The percentage of participants who seroconverted (divers 15% and controls 10%), was not significantly different. At the beginning of the training course, only the rates of seropositive subjects to coxsackie B4 and B5 are significantly higher in the divers. No seroconversion to HAV was observed and actually the percentage of seropositive subjects was equivalent to both populations (17 versus 18%).

DISCUSSION

The human enteric viruses, notably the HAV virus and the agents of gastroenteritis, are virtually the only viruses transmitted by water [15]. Because enteroviruses frequently circulate in natural water, we have developed a strategy to evaluate the risk associated with swimming or diving in controlled environmental areas and, if necessary, to modify training conditions. We have previously described and validated an ultrafiltration water concentration technique which is suitable for large volumes of water and usable in the field [19]. We decided to perform virological investigations by using conventional isolation, serological methods and hybridization techniques which exhibit a higher sensitivity [25]. This is the first report of a virological and serological survey of a population exposed to waterborne viral contamination. Most of the studies in this field are retrospective [30] and describe the epidemiological feature without information about the viral hydric contamination [13]. The very few prospective studies carried out [16, 31] so far merely report clinical data after interviewing the participants. We did not include clinical information because the aetiology of gastroenteritis and respiratory symptoms is difficult to establish precisely, and because diver trainees do not report their diseases to avoid medical exemption from exercise. For the same reason we worked with systematic periodic stool and serum samples.

Three enteroviruses were isolated under the same circumstances: at the end of the summer and after a period of stormy weather when the water was very turbid. Molecular hybridization techniques allowed the detection of 6 other positive samples and finally, 36% of environmental water samples were positive, and 1 of 7 swimming pool samples was positive. This swimming pool is used exclusively for military purposes. A higher positive percentage has been reported for public swimming pools which are much visited by children [24].

Evidence of infection with enteroviruses was based on two criteria: viral detection in stool samples and specific seroconversion. We did not find evidence of increased contamination in divers. We conclude that there was no significant viral risk during the training course, (which involves a total of 96 h in the water), because there was no significant difference between divers and controls as regards

both viral isolation and seroconversion. However, a higher percentage of coxsackievirus B4 and B5-seropositive subjects among divers suggests that these were more exposed during previous water training. Unfortunately, this study was conducted between September 1991 and August 1992 and during this period few enteroviruses were isolated in hospitalized patients according to the National Reference of Enteroviruses in France. It would be of interest to conduct such a study during an epidemic.

No HAV detection and no seroconversion to HAV were observed, and we confirmed the low virus circulation in the country. The HAV seropositive rate was 17% for a 24·5-year-old population and is comparable with 21·35% observed in a 20-year-old French military recruit population in 1990 [32]. In military personnel assigned to US Navy ships, the percentage of seroprotected subjects is lower, about 10% [33]. These observations point out the necessity for military personnel to be vaccinated when travelling to developing countries [34].

ACKNOWLEDGEMENTS

This work was supported by a grant from: 'Mission Innovation', Ministère Français de la Défense.

We are thankful for the help received from Generals Lesbres and all the personnel of 'CEPAT', particularly Colonel Augarde, Cdt Bilhère, Dr Lablanche, Dr DeCamaret and Dr Dombey for excellent technical assistance, and Messrs Sierzputowski, Todesco and Variot for help with serological analysis. We thank Dr Deloince for valuable discussions and critical reading of the manuscript.

REFERENCES

1. Rueckert RR, Wimmer E. Systematic nomenclature of picornavirus proteins. J Virol 1984; **50**: 957–9.
2. Melnick JL. Enteroviruses – polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Fields BN, Knipe DM eds. Virology. New York: Raven Press, 1990: 549–605.
3. Kogon A, Spigland I, Frothingham TE, et al. The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VII. Observations on viral excretion, seroimmunity, intrafamilial spread and illness association in coxsackie and echovirus infections. Am J Epidemiol 1969; **89**: 51–61.
4. Rao CV, Metcalf TG, Melnick JL. Human viruses in sediments, sludges and soils. Bull World Health Org 1986; **64**: 1–14.
5. Keswick BH, Gerba CP, Goyal SM. Occurrence of enteroviruses in community swimming pools. Am J Pub Hlth 1981; **71**: 1026–30.
6. Hovi T, Huovilainen A, Kuronen T, et al. Outbreak of paralytic poliomyelitis in Finland – widespread circulation of antigenically altered poliovirus type 3 in a vaccinated population. Lancet 1986; ii: 1427–32.
7. Panà A, Divizia M, DeFilippis P, DiNapoli A. Isolation of hepatitis A virus from polluted river water on Frp/3 cells. Lancet 1987; ii: 1328.
8. Lawson HW, Braun MM, Glass RIM, et al. Waterborne outbreak of Norwalk virus gastroenteritis at a southwest US resort: role of geological formations in contamination of well water. Lancet 1991; **337**: 1200–4.
9. Deetz TR, Smith EM, Goyal SM, et al. Occurrence of rota- and enteroviruses in drinking and environmental water in a developing nation. Water Res 1984; **18**: 567–71.
10. Cliver DO. Vehicular transmission of hepatitis. Pub Hlth Rev 1985; **13**: 235–92.
11. Ansari SA, Springthorpe VS, Sattar SA. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. Rev Infect Dis 1990; **13**: 448–61.

12. Halliday ML, Kang LY, Zhou TK, et al. An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China. J Infect Dis 1991; **164**: 852–9.
13. Mahoney FJ, Farley TA, Kelso KY, Wilson SA, Horan JM, McFarland LM. An outbreak of hepatitis A associated with swimming in a public pool. J Infect Dis 1992; **165**: 613–18.
14. Cabelli VJ, Dufour AP, McCabe LJ, Levin MA. Swimming-associated gastroenteritis and water quality. Am J Epidemiol 1982; **115**: 606–16.
15. Cliver DO. Significance of water and environment in the transmission of virus disease. In: Melnick JL ed. Enteric viruses in water. Monog Virol 1984; **15**: 30–42.
16. Fewtrell L, Godfree AF, Jones F, Kay D, Salmon R, Wyer MD. Health effects of white-water canoeing. Lancet 1992; **339**: 1587–9.
17. Biziagos E, Passagot J, Crance JM, Deloince R. Hepatitis A virus concentration from experimentally contaminated distilled, tap, waste and seawater. Wat Sci Tech 1989; **21**: 255–8.
18. Divizia M, Santi AL, Panà A. Ultrafiltration: an efficient second step for hepatitis A virus and poliovirus concentration. J Virol Methods 1989; **23**: 55–62.
19. Garin D, Fuchs F, Crance JM, Deloince R, Aymard M, Bartoli M. Validation of an ultrafiltration process to concentrate viruses from large volumes of water. Environ Technol 1993; **14**: 397–400.
20. Purcell RH, Wong DC, Moritsugu Y, Dienstag JL, Routenberg JA, Boggs JD. A microtiter solid phase radio-immunoassay for hepatitis A antigen and antibody. J Immunol 1976; **116**: 349–56.
21. Metcalf TG, Jiang X. Detection of hepatitis A virus in estuarine samples by gene probe assay. Microbiol Sci 1988; **5**: 296–300.
22. Ticehurst JR, Feinstone SM, Chestnut T, Tassopoulos NC, Popper H, Purcell RH. Detection of hepatitis A virus by extraction of viral RNA and molecular hybridization. J Clin Microbiol 1987; **25**: 1822–9.
23. Gerba P, Margolin B, Trumper E. Enterovirus detection in water with gene probes. Z Gesamte Hyg 1988; **34**: 518–19.
24. Fuchs F, Lepare I, Kopecka H, Garin D, Aymard M. Use of cRNA digoxigenin-labelled probes for detection of enteroviruses in humans and in the environment. J Virol Methods 1993; **42**: 217–26.
25. American Public Health Association. Standard methods for the examination of water and wastewater. Washington, DC: American Public Health Association, 1976.
26. Melnick JL, Rennick V. Infectivity titers of enterovirus as found in human stools. J Med Virol 1980; **5**: 205–20.
27. Lim KA, Benyesh-Melnick M. Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (coxsackie and ECHO). J Immunol 1960; **84**: 309–17.
28. Biziagos E, Passagot J, Crance JM, Deloince R. Long-term survival of hepatitis A virus and poliovirus type 1 in mineral water. App Environ Microbiol 1988; **54**: 2705–10.
29. Hunter WM, Greenwood FC. Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature 1962; **194**: 495–6.
30. Lenaway DD, Brockman R, Dolan GJ, Cruz-Uribe F. An outbreak of an enterovirus-like illness at a community wading pool: implications for public health inspection programs. Am J Pub Hlth 1989; **79**: 889–90.
31. Jones F, Kay D, Stanwell-Smith R, Wyer MD. Results of the first pilot-scale controlled cohort epidemiological investigation into the possible health effects of bathing in seawater at Langland Bay, Swansea. J Inst Water Environ Management 1990; **5**: 91–8.
32. Joussemet M, Bourin P, Deloince R, Fabre G, Buisson Y. Evolution du taux de prévalence des anticorps anti VHA chez les jeunes recrues. Méd Armées 1991; **19**: 579–83.
33. Hawkins RE, Malone JD, Cloninger LA, et al. Risk of viral hepatitis among military personnel assigned to US Navy ships. J Infect Dis 1992; **165**: 716–19.
34. Flehmig B, Heinricy U, Pfisterer M. Immunogenicity of a killed hepatitis A vaccine in seronegative volunteers. Lancet 1989; i: 1039–41.