A new analytical tool to assess health risks associated with the virological quality of drinking water (EMIRA study)


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Abstract This work assessed the risks associated with the virological quality of tapwater using a molecular analytical tool manageable in a field survey. It combined a daily epidemiological follow-up of digestive morbidity among a panel of volunteers and a microbiological surveillance of drinking water. RT-PCR was used for detection of enterovirus, rotavirus and astrovirus. 712 cases of acute digestive conditions occurred in the 544 volunteers. 38% (9/24) raw water and 23% (10/44) tap water samples were positive for at least one virus marker with 9/10 positive tap water samples complying with bacterial criteria. No statistically significant association was found between the presence of viral markers and observed incidence of digestive morbidity. However, when an outbreak occurred, enterovirus and rotavirus RNA was detected in the corresponding stored tap water samples. Sequencing of the amplified fragments showed that the rotavirus detected was of bovine origin. This work demonstrated that enteric virus markers were common in tapwater of the study communities (characterised by a vulnerable raw water) despite absence of bacterial indicators. Tangential ultrafiltration coupled to RT-PCR allowed a simultaneous and fast detection of the study viruses from environmental samples. This process is a promising tool usable for virological water surveillance, in as much the corresponding know-how is transferred to the field professionals.

Keywords Drinking water; epidemiological study; enterovirus; rotavirus; astrovirus; RT-PCR

Introduction Diarrhoea is a worldwide public health problem, especially affecting young children and the elderly, with viruses being implicated in 30–40% of cases (Blackbow and Greenberg, 1991). Even in developed countries, consumption of contaminated drinking water has been documented by recent epidemiological studies as a significant source of gastroenteritis outbreaks (Nicand et al., 1998). One route of transmission for rotaviruses, Norwalk viruses, enteroviruses, adenoviruses and hepatitis A virus is person-to-person via the faecal-oral route. However, several studies have shown the importance of waterborne viral transmission, especially through drinking water and shellfish (Gantzer et al., 1998). Virus resistance to environmental factors, such as physical and biochemical inactivation, contribute to the spread of infection and non-enveloped enteric viruses are resistant to many disinfection processes. Persistence in the environment is enhanced by organic substances (as faeces or suspended matter) and virus adsorption makes them less sensitive to inactivation agents. Some viruses are also characterised by their stability at pH 3–4 and their resistance to thermal agents (Glass et al., 1996).

Rotavirus is the virus most often involved in gastroenteritis outbreaks (Blackbow and Greenberg, 1991) with infections occurring in winter because of a high level of inter-human faecal–oral transmission. Rotavirus is believed to have been spread by water in many outbreaks with several studies showing its presence in drinking water associated with
the occurrence of epidemics. Hence, in parallel with inter-human contamination, drinking water might also play a role in the occurrence of sporadic episodes.

Astrovirus is frequently associated with gastroenteritis in humans and animals. It is a cause of diarrhoea worldwide with reported detection rates of 5–9% among young children. Recent evidence has suggested that astroviruses are an important cause of acute non-bacterial gastroenteritis in children, adults and the elderly (Abad et al., 1997). Large outbreaks of astrovirus diarrhoea are being described with increasing frequency but the role of astrovirus in gastroenteritis in healthy young adults remains unclear (Kurtz, et al. 1979; Belliot et al., 1997). Advances in methods for detecting human astrovirus has led to an increased understanding of their importance as a cause of gastroenteritis in children and immunocompromised patients. Astrovirus is difficult to grow in cell culture needing molecular techniques for identification. Data on astrovirus survival in drinking water with chlorine disinfection scenarios suggest a better persistence at 4°C than 20°C but, in general, few data on the persistence of astrovirus in environmental samples exist at the present time (Abad et al., 1997).

Enteroviruses are not considered as potentially involved in waterborne gastroenteritis (Codex Alimentarius, 1999). Although these viruses could be responsible for various symptoms, they are mostly considered as indicators of viral water quality rather than strict gastroenteric pathogens. Gastrointestinal illnesses occur in developed countries even when drinking water has complied with microbial standards (Payment et al., 1997) and, during some outbreaks, enteric viruses may have played an important role (Lisle and Rose, 1995; Goldstein et al., 1996). However, monitoring of microbial quality of tapwater is still based on the bacterial indicators of faecal contamination which are recognised as poor viral (and parasitological) contamination predictors (WHO, 1993; Nicand et al., 1998). Hence, infectious risks associated with viral transmission through drinking water are not well evaluated and the actual viral transmission by water may have been underestimated (Soule et al., 2000). One important reason is the technical difficulties of enteric virus detection in environmental samples that do not allow, to date, an efficient surveillance of viral water quality. This study (part of the EMIRA, Epidemiology and Microbial Risk Assessment study), aimed to assess the risks associated with virological quality of tap water using a molecular analytical tool manageable in a field survey.

Materials and methods
The EMIRA study was carried out between October 1998 and June 1999 in the French Alps (Isère and Savoie departments, south-east France). One year earlier, a pilot study had been performed to test the protocol feasibility. The EMIRA study combined a daily epidemiological follow-up of digestive morbidity among a panel of volunteers and a microbiological surveillance of drinking water.

Panel follow-up
Volunteers were recruited (through the media, schools, town councils and community water systems files) among communities supplied by four public water systems chosen for their raw water vulnerability: a “pristine” groundwater (1) located in a quarstic environment, a vulnerable groundwater in a quarstic watershed (2), a vulnerable groundwater in an unprotected watershed (3) exposed to livestock and community sewage and (4) a surface water (a lake surrounded by human activities). All finished waters usually complied with current EU bacterial standards and, except in the first group which is untreated, waters were disinfected by chlorine only. Each family had to complete a self-administered daily questionnaire whereby all health problems were to be registered. Each weekday, 20% of the families were telephone interviewed to retrieve the data thus collecting information on the
cases that occurred the same day and every day since the previous week’s call. This surveillance scheme allowed a continuous description of digestive morbidity incidence and outbreak detection. An “alert” threshold was based on the pilot study data defined as the occurrence of two cases of acute digestive conditions in the same community during 48h. Acute digestive conditions (ADC) were defined as episodes of abdominal pain, nausea, vomiting and/or diarrhoea; a diarrhoeic episode (DE) was diarrhoea with at least another digestive condition; and gastroenteritis (GE) was an episode of diarrhoea with at least another objective sign e.g. fever, vomiting.

**Microbiological surveillance – water sample collection**

In parallel with the epidemiological surveillance, tapwater (4.5 L) was sampled daily in each city by a local sentinel and stored at 4°C for 3–5 d. All samples were collected in sterilised glass flasks (1.5 L) with sodium thiosulphate to neutralise residual chlorine. The time of storage varied because telephone calls were carried out only on work days; thus, bottles collected on Thursdays and Fridays were kept a little longer in order to be able to analyse the water in case an outbreak occurred during a weekend. This scheme allowed the time coupling between the health and microbial surveillance systems. When the alert threshold was exceeded in a community, stored samples were collected and submitted for analysis. Hence, these tap water samples represented the water quality the day of the suspected outbreak and the two days before; this period encompassed the usual lag for viral infections and diseases. This scheme could not hold for bacteria (because of microbial development or, conversely, of competitive inhibition), nor for protozoans (because the amount of water that must be sampled did not allow its storage by the local sentinel). Thus, bacterial water quality was assessed on the waters sampled by the local sentinel the evening the outbreak was suspected (he was warned to do so immediately with 500 mL samples). Another aspect of the EMIRA study dealt with the parasitic water quality (assessed after filtration of 100 L through a polyethersulfone cartridge, Gelman, 1 µm, at the sentinel’s home. Besides microbial quality assessment during outbreaks, tap and raw water samples were collected and analysed monthly.

**Microbiological analyses**

Analyses were performed by the regional water testing laboratory to control compliance of drinking water to microbial quality regulation; *Giardia* and *Cryptosporidium* were analysed by IFA and microscopy. Three categories of enteric viruses were selected based on a review of the literature and on technical feasibility: enterovirus, rotavirus and astrovirus. Sample concentration and virus detection followed the procedures of the Laboratory of Medical Virology, Grenoble Hospital (Soule et al., 2000). Tangential ultrafiltration (Minitan, Millipore) was performed within 24 h of sampling with 4.5 L being concentrated to 15 mL and then to 0.5–1 mL. The concentrates were frozen at –80°C before molecular detection. RNA extraction was carried out with the Boehringer Kit High pure viral DNA. RNA amplification (400 bp in the 5’ non-coding region) allowed detection of all human enteroviruses. For rotaviruses (animal and human) RT-PCR analysis was carried out using Beg9 and RTB primers to amplify a 392 bp fragment on the gene coding for the VP7 capsid protein and a semi-nested PCR with the RTA/RTB primers to amplify a 341 bp (Gratacap-Cavallier et al., in press). For astroviruses, RNA amplification was performed on 289 bp in the protease region (ORF1a). Negative and positive control samples were included in all experiments. In our study, the detection limit was 1 TCID50/L. At the time of this study, given that quantification was not routinely feasible for these viruses in environmental samples, results were expressed semi-quantitatively thanks to external standards (a plasmid of cloned PCR product for each virus) allowing the building of a control range.
Results

The study population

The epidemiological follow-up of digestive morbidity involved 544 volunteers (176 households), with 27.9% of 0–14 years old subjects, distributed across the four water groups: 122 in the untreated water group (1), 99 persons in the quarstic water group (2), 100 persons in the unprotected watershed group (3) and 223 persons in the surface water group (4). As expected, in this kind of study based on a volunteer call, our panel was not representative of the general local population as to sex ratio, with most being female. The socioeconomic status of volunteers included mostly white collar workers with fewer farmers and blue collar workers. The panel had a greater fraction of children, a feature that was wanted.

Digestive morbidity

A total of 712 cases of acute digestive conditions, 105 diarrhoeic episodes and 46 cases of gastroenteritis were observed. The average incidence rate was 2.8 cases/person-year for ADC, 0.4 for DE, and 0.2 for GE (Table 1).

On 20 February 1999, the telephone survey detected a significant increase of ADC in the third group around 17 February. As the complete retrospective follow-up confirmed an outbreak, microbiological analyses were performed on daily tapwater samples taken 17–20 February. Figure 1 shows this episode along with the daily incidence of ADC observed the week before and after the peak shown. The 17 volunteers (19 cases) involved in this outbreak did not differ from the other 83 volunteers in the same water group as to age (p = 0.4), gender (p = 0.9), SES (p = 0.9), tapwater intake (p = 0.4) or tapwater consumption outside their community (p = 0.8). However, they did show a greater background incidence rate of ADC than the other panelists: 6.7 person-year (49 incident cases/2,667 person-days) versus 4.3 (124/10574) (p = 0.007). The background incidence rate of ADC was defined as the incidence rate of ADC during the whole study but during the epidemic period (i.e. out of 10

Further molecular investigations

Four positive samples (February and March in the third study group) were sequenced to assess the origin (animal or human) of the rotavirus RNA detected during this critical period. Sequencing concerned one of the three hypervariable regions of VP7, the region A (45bp) determining serotype.

Statistical analysis

Analysis was performed using Excel, Epi-info and SPSS software. To evaluate the difference between characteristics of volunteers involved in the February outbreak and those not involved in this outbreak, ANOVA was used to compare their age, gender, socio-economical status (SES) and tapwater consumption. Non-parametric Mann–Whitney and $\chi^2$ tests were used to compare their background incidence and their background incidence rate of, respectively, ADC, DE and GE. Incidence rates of digestive morbidity corresponding to positive and negative viral exposure were also compared using a $\chi^2$ test and a multiple linear regression model. The seasonal trend, the difference between the four groups and the water virological quality variations were taken into account to model the daily incidence rate of ADC. Multiple linear regression used the following predictors: a monthly dummy variable, a group dummy variable and a viral quality dummy variable (virus above the detection level = 1) and interaction terms concerning groups and viral quality.
to 24/02/99). Given the small sample size, this difference also held true for DE (1.0 versus 0.5 person-year) and GE (0.4 versus 0.1) (p = 0.17 and p = 0.07 respectively).

Water quality
Eight routine sampling runs were performed between November 1998 and June 1999 with four epidemic alerts being declared (one alert in each group) although three were not confirmed as true outbreaks. Raw water (24) and tapwater (32) samples were collected as routine with an additional 12 tapwaters being analysed during the four suspected alerts.

Bacterial results
The three communities with chlorinated tapwater complied with the study inclusion criteria: while their resources were always contaminated with faecal indicators, tapwaters were free of such bacteria most of the time. Surprisingly, the untreated tapwater group, chosen initially as a control, showed faecal indicators in two monthly water samples. Results showed 22% non-compliance in the first and the second groups and 11% in the third and the fourth groups. Of six non-compliances, three were associated with the presence of 1 thermotolerant coliform, one with 2 sulphite-reducing clostridial spores, one with 1 faecal streptococci and the last with 90 thermotolerant coliform and 102 faecal streptococci (run 6 of the

Table 1 Incidence (I) and incidence rate (IR in person-year) of acute digestive conditions, diarrhoeic episodes and gastro-enteritis, for the four study groups followed over 210 d (16/10/98 to 18/06/99).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>ADC</th>
<th>DE</th>
<th>GE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I (95% CI)</td>
<td>I (95% CI)</td>
<td>I (95% CI)</td>
</tr>
<tr>
<td>Group 1</td>
<td>19 391 p–d</td>
<td>122</td>
<td>167 (2.7–3.6)</td>
<td>26 (0.3–0.7)</td>
</tr>
<tr>
<td>Group 2</td>
<td>15 310 p–d</td>
<td>99</td>
<td>83 (1.6–2.4)</td>
<td>14 (0.2–0.5)</td>
</tr>
<tr>
<td>Group 3</td>
<td>14 961 p–d</td>
<td>100</td>
<td>191 (4.0–5.3)</td>
<td>30 (0.5–1.0)</td>
</tr>
<tr>
<td>Group 4</td>
<td>42 830 p–d</td>
<td>223</td>
<td>271 (2.0–2.6)</td>
<td>35 (0.2–0.4)</td>
</tr>
<tr>
<td>Total</td>
<td>92 186 p–d</td>
<td>544</td>
<td>712 (2.6–3.0)</td>
<td>105 (0.3–0.5)</td>
</tr>
</tbody>
</table>

Figure 1 Daily incidence of ADC in the third group around the outbreak peak (10–24 February 1999)
During the third group outbreak in February 1999, tapwater complied with bacterial criteria whereas viruses and protozoans were found. Viral RNA was routinely found in the samples of raw and tap water. 38% (9/24) of raw water samples and 23% (10/44) of tapwater samples were positive for at least one virus marker. No enteric virus RNA was detected in group 1 which was occasionally contaminated by faecal bacterial indicators. In the other groups, the proportion of positive samples for viruses was respectively 11%, 37% and 53%. Of virus positive tapwater samples, 9/10 complied with bacterial criteria. Enterovirus, rotavirus and astrovirus were found in 13%, 15% and 12% of the samples respectively (raw and tap water samples confounded) (Table 2). Raw water of the second group was rarely contaminated by viruses while raw water of the third group. During the third group outbreak in February 1999, tapwater complied with bacterial criteria whereas viruses and protozoans were found.

### Virological results

Viral RNA was routinely found in the samples of raw and tap water. 38% (9/24) of raw water samples and 23% (10/44) of tapwater samples were positive for at least one virus marker. No enteric virus RNA was detected in group 1 which was occasionally contaminated by faecal bacterial indicators. In the other groups, the proportion of positive samples for viruses was respectively 11%, 37% and 53%. Of virus positive tapwater samples, 9/10 complied with bacterial criteria. Enterovirus, rotavirus and astrovirus were found in 13%, 15% and 12% of the samples respectively (raw and tap water samples confounded) (Table 2). Raw water of the second group was rarely contaminated by viruses while raw water of the third group. During the third group outbreak in February 1999, tapwater complied with bacterial criteria whereas viruses and protozoans were found.

#### Table 2  Virological results of raw and tap water (one sample for each run, three samples for an alert)

<table>
<thead>
<tr>
<th>Run</th>
<th>Group 1 Tap (untreated)</th>
<th>Group 2 Raw</th>
<th>Group 2 Tap</th>
<th>Group 3 Raw</th>
<th>Group 3 Tap</th>
<th>Group 4 Raw</th>
<th>Group 4 Tap</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
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<tr>
<td>5</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
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<tr>
<td>6</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
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<tr>
<td>7</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
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<tr>
<td>8</td>
<td>Entero &lt; Rota &lt; Astro &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td>&lt; + + &lt; &lt;</td>
<td></td>
</tr>
<tr>
<td>Alert 1</td>
<td>Entero &lt;&lt;&lt; Rota &lt;&lt;&lt; Astro &lt;&lt;&lt;</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Alert 2</td>
<td>Entero / Rota / Astro /</td>
<td></td>
<td></td>
<td></td>
<td>&lt;&lt;&lt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alert 3*</td>
<td>Entero / Rota +++ Astro &lt;&lt;&lt;</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Alert 4</td>
<td>Entero / Rota &lt;&lt;&lt; Astro &lt;&lt;&lt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

< = below detection limit 1 TCID<sub>50</sub>/L; + = presence of viral RNA; *this alert was the only true epidemic
the third and the fourth groups was often contaminated (respectively 2/8 and 6/8). As to tap-water, the first group did not show viral contamination and the second was rarely contaminated. The third group exhibited a high viral contamination during February and March 1999. The tapwater for the fourth group, whose source often showed astrovirus genetic material, was regularly contaminated by enteroviruses but surprisingly not by astroviruses.

The February outbreak in the third group
In February 1999, an outbreak occurred in the third group. Enterovirus and rotavirus RNA were detected in the 3 stored tap water samples, while no faecal bacteria was found. *Giardia* was also found in tap water the first day (10 cysts per 100 L). Further molecular investigations were not performed on the enterovirus because it is not considered to be directly involved in acute digestive symptoms associated with drinking water consumption. Sequencing of the amplified fragments was performed to evaluate the origin (animal or human) of the rotavirus RNA detected during this episode and two bovine strains were found (Table 3).

Association between digestive morbidity and enteric viruses exposure
The health indicator chosen for statistical analysis was the incidence of ADC; while less specific, it was more sensitive than the other two. The results of the multiple linear regression showed a seasonal trend with decreasing incidence of ADC from November to April followed by a slight increase in May and June. Superimposed over this seasonal trend, was the evidence of the February epidemic in the third group. The morbidity rate was quite similar in groups 1, 2 and 4 (p = 0.42 for group 2 with reference to group 1; p = 0.27 for group 4 in reference to groups 1 and 2 and p = 0.08 with group 3). When the interaction terms between viral presence in water and the study groups was included, although non-significant, it suggested a slightly better association between markers of rotavirus (p = 0.39) than between markers of any study viruses (p = 0.51).

Discussion
The main results of this study were: (1) the analytical tool was usable to find viral markers from environmental water samples; (2) a frequent occurrence of enteric viruses RNA was observed – on average 38% of raw and 23% of tap samples being positive for at least one virus marker with a significant difference between the four communities (p = 0.0035) according to their degree of raw vulnerability – this occurrence not being associated with the presence of bacterial indicators; (3) the average incidence rates of ADC, DE and GE observed among the volunteer panel were respectively 2.8, 0.4 and 0.2 cases/person-year; (4) sequencing of rotavirus RNA detected during the outbreak showed the bovine origin of this contamination; (5) no statistical association was found between the presence of viral markers in drinking water and the observed incidence rate of ADC.

Table 3  Rotavirus sequencing results during February and March 1999 (ND = not detected; AA = amino acid)

<table>
<thead>
<tr>
<th>Date (sample number)</th>
<th>RT-PCR</th>
<th>RT-PCR</th>
<th>Sequencing results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>AA 21 26 39 41 45 47 69 86 103 104</td>
</tr>
<tr>
<td>17/02 (33) February routine tap</td>
<td>ND</td>
<td>+</td>
<td>Lack of material</td>
</tr>
<tr>
<td>17/02 (34) February routine raw</td>
<td>+</td>
<td>ND</td>
<td>I M V T A T N V Q L</td>
</tr>
<tr>
<td>18/02 (35) February epidemic tap</td>
<td>ND</td>
<td>+</td>
<td>I T V T A T N V</td>
</tr>
<tr>
<td>19/02 (36) February epidemic tap</td>
<td>+</td>
<td>Lack of material</td>
<td>Lack of material</td>
</tr>
<tr>
<td>20/02 (37) February epidemic tap</td>
<td>+</td>
<td>+</td>
<td>M T I V T I D V Q L</td>
</tr>
<tr>
<td>17/03 (43) March routine raw</td>
<td>+</td>
<td>+</td>
<td>M T I V T I D V Q L</td>
</tr>
</tbody>
</table>

45
One should keep in mind that water samples analysed during this study came from four public water systems chosen for their raw water vulnerability; hence they are not representative of the average drinking water quality in France. In our study communities, increased sampling showed that tapwater did not comply perfectly with bacterial standards. Additionally, in reference to WHO recommended treatment criteria to produce water with negligible virus risk, the water systems of the studied communities water systems received inadequate disinfection treatment (WHO, 1993).

**Microbial water quality**

Our results confirmed that bacterial standards cannot be considered good predictors of potential viral risk through drinking water: enteric virus markers were common in tapwater of communities with vulnerable raw water, in spite of the absence of bacterial indicators (probably due to effective removal by chlorine disinfection). The presence of RNA viruses in water complying with bacterial standards has been reported previously (Gratacap-Cavallier et al., in press). Current microbial standards used as safety criteria for water may not always be indicative of viruses; therefore additional indicators of the virological quality of water are needed (Soule et al., 2000). The WHO emphasized that, because enteroviruses and the resting stages of *Cryptosporidium*, *Giardia* and other parasites are more resistant to disinfection than *E. coli* and faecal streptococci, absence of the latter does not necessarily indicate absence of the former. Although, virological, epidemiological and risk analysis studies accumulate evidence in favour of this statement, direct virological water criteria still cannot be recommended because of the complexity of virological analyses (WHO, 1993). Three options are proposed in the literature to monitor virological quality of drinking water. The first consists of isolating the infectious enteric viruses in cell cultures. This is the ideal solution but is inapplicable for routine use as it is long, complicated and expensive. Furthermore, some enteric viruses of health significance do not (or only poorly) grow in cell culture. The second option, currently used for water quality surveillance, is based on classical bacterial indicators. It is now widely recognized, as our results confirm, that they are mediocre viral contamination indicators. The third option rests on specific virus indicators. Two such possibilities have been reported: (a) detection of enteric virus genomes using RT-PCR (presence of viral molecular sequences leads to the assumption that water may convey viruses) and (b) isolation of enteric bacteriophages amongst which the best candidates appear to be: (i) RNA F-specific bacteriophages (which are good indicators of animal and human faecal contamination) and (ii) *Bacteroïdes fragilis* phages (which offer a very good human specificity) (Gantzer et al., 1998). Virological surveillance, based on specific virus indicators, would be more relevant for health impact assessment than the current bacterial parameters. Hence, research on faecal virus predictors in drinking water represents an important challenge for microbial risk assessment and management. However, technical tools are rather scanty and still under-developed for field surveillance.

**Improvement of virological surveillance**

Given the low viral density, sampling volumes have to be sufficient whilst extraction and concentration steps have to precede direct virus detection (Nicand et al., 1998). We used 4.5 L samples as a compromise between the average human daily consumption (about 2 L) and the volume required for analysis. The analytical process, tangential ultrafiltration concentration and RT-PCR, showed a good recovery and a simultaneous, fast and reproducible detection of the study viruses from environmental samples (Gantzer et al., 1998; Soule et al., 2000). Ultrafiltration has the advantage of concentrating viruses from waters without any additional steps for their recovery. Coupled with RT-PCR, it simultaneously detects several viruses. The procedure may be applied to the great majority of viruses because it
does not need chemical reactives for their recovery, which can be toxic for some. As technically this is easily feasible as a routine method, it may prove a useful tool in the control of water quality for public health protection (Soule et al., 2000). However, in order to be used for virological water surveillance when needed, it requires that the corresponding know-how be transferred to the field professionals.

While detection of viral genomes in water has been advocated as an interesting alternative to assess potential viral contamination by several authors (Gantzer et al., 1998; Soule et al., 2000), with biomolecular techniques being rather quick and their cost steadily decreasing, it only allows the conclusion that the analysed medium is or was probably contaminated by viruses. The qualitative analysis (absence or presence) provides partial information; quantitative analysis is more relevant for health interpretation (Gantzer et al., 1998). The advent of molecular biology has prompted the development of procedures for the detection of fastidious enteric viruses; however, molecular techniques fail to distinguish between infectious and non-infectious particles which may be of critical relevance in environmental virology. Molecular procedures are not adequate to monitor the presence of infectious viruses after disinfection (Abad et al., 1997). Many authors suggest that viral genome detection might be considered as a viral contamination marker: detection of viral nucleic acid by RT-PCR is fast, cheap, very sensitive and routinely feasible. A drawback, however, is that the viral genome in water is detectable for a longer period (twice as long) than infectious viruses, particularly when particulate matter is present. As RNA persistence in water is 2d maximum, it is plausible that the detected RNA is encapsidated, these capsids not being able to induce cell infection (Gantzer et al., 1998). Moreover, chemical alteration of the nucleocapsids by chlorine also produces non-infectious virions which, however, encapsidate a detectable RNA (Abad et al., 1997). As a consequence, detection of viral genome in water cannot be considered, to date, as a perfect indicator of infectious viruses.

Rotavirus sequencing
Sequencing of the amplified fragments (341bp) was performed to evaluate the origin of the rotavirus RNA detected during February and March 1999. A similar bovine strain was found in the first two samples (17 and 18 February) with another bovine strain being found on 20 February and 17 March. The bovine origin of the detected rotavirus did not allow the conclusion of its implication in the detected outbreak although it has been suggested that interspecies crossing is possible with rotavirus (Gratacap-Cavallier et al., in press). What can be said, however, is that rotavirus RNA occurrence in drinking water showed that viruses had been present in the drinking water supply not long before this episode and that contamination by pathogenic rotavirus remains possible. It should be noted that Giardia and enteroviruses were also found in some February tapwater samples. The Codex Alimentarius (1999) does not classify enteroviruses as waterborne pathogens responsible for gastroenteritis but Giardia could also have been a candidate cause of this outbreak.

Statistical analysis
The time coupling between health and microbiological surveillance offered the opportunity to investigate the association between viral molecular markers and digestive morbidity, including when the outbreak occurred. The outbreak seen in the third group with consistent presence of rotavirus RNA did not show, however, a significant association with the incidence rate of ADC. We hypothesize that this “negative” result may be due to lack of statistical power: this study group was only 100 subjects, an outbreak occurred only once during the study period and rotavirus RNA was detected in tapwater only during the epidemic period. Also, the choice of ADC as the health indicator may be not be pertinent for this analysis. Further statistical analysis is ongoing, based on individual data rather than aggregated data as
used in this paper, using different predictors (e.g. individual tapwater intake) and also testing the incidence rate of diarrhoeic episodes and gastroenteritis as digestive morbidity indicators.

A previous prospective study of the circulation of rotaviruses in water had been carried out in the Isère department by the Virology Laboratory of Grenoble Hospital during winter 1996. Samples of tapwater were collected from several towns while cases of acute gastroenteritis were interviewed as to location and type of water consumption. In this study also, the risk of acute gastroenteritis caused by rotavirus and requiring hospitalisation was not significantly increased among children drinking tapwater in comparison with those consuming bottled water. However, an excess risk of acute gastroenteritis caused by rotaviruses was observed among children living in a locality where the bacteriological quality of tapwater was not good (OR = 2.7; p = <0.02) regardless of whether or not they drank water (Soule et al., 1999). That suggested that even if bacterial indicators were poorly correlated with viruses in drinking water, they continued to play an important role for public health protection. Hence, the question remains about the actual role (direct or indirect) of drinking water in the transmission of rotavirus in particular and enteric viruses in general. Another question concerns the immunity induced or not by contact with enteric viruses transmitted by the faecal-oral route. To date, very few data are available on human immunity against the enteric viruses that were considered in our study. Some authors suggest that, even if this immunity exists, it is very short and limited to the infective genotype. It is also considered that in developed countries, where there is a good level of hygiene and a low circulation of enteric viruses, the probability of immunising contacts is lower and, consequently, receptivity to infections may be higher than in developing countries (Nicand et al., 1998).

Conclusions
This work showed that further studies are warranted for a better understanding of the health impact of enteric viruses conveyed by drinking water; such studies can rest on molecular analytical tools that were efficient for water surveillance in field surveys; the health significance of viral genome identification remains to be assessed, however.

References
Codex Alimentarius (1999).