Molecular Confirmation of Hepatitis A Virus from Well Water: Epidemiology and Public Health Implications

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An outbreak of hepatitis A in a rural river-island community was found to be associated with consumption of contaminated well water. Specimens from case-patients, the implicated well, and a cesspool suspected to be the source of contamination were all positive for hepatitis A virus (HAV) RNA by immunocapture reverse-transcription polymerase chain reaction. All isolates were identical over about 400 bases from two capsid-encoding regions of the genome, identifying the chain of transmission. Other wells up to 60 m from the cesspool also contained HAV RNA. In addition, HAV RNA was detected in the contamination source well 6 months after the initial contamination, when fecal coliform bacteria were no longer present. These findings demonstrate the utility of viral detection techniques to evaluate contaminated ground water.

Domestic or non-community wells provide drinking water to a large proportion of rural populations in both Canada and the United States. Traditionally, ground water has been considered safe for human consumption without undergoing treatment; however, contamination may be frequent and unrecognized [1]. Contamination occurs in both shallow wells drawing from the phreatic aquifer and deep wells drawing from the aquifer under bedrock; microorganisms come from the soil surface or by seepage from septic tanks or cesspools [1, 2]. Regulations concerning the minimum distance between a well and contaminant source, such as a septic tank or cesspool, are not uniform in Canada or the United States and vary by locality.

In North America, water contaminated by sewage has been associated with hepatitis A outbreaks [3–8]. However, the long incubation period of hepatitis A virus (HAV) infection may obscure the relationship between illness and consumption of contaminated water, and the absence of an easily accessible test to detect HAV in water may make this source of transmission difficult to recognize. Although HAV has been detected in water samples or concentrates from several outbreaks [6–10], the same HAV isolate has not been demonstrated in the chain of transmission from the source to case-patients.

During the summer of 1995, a cluster of hepatitis A case-patients were reported to the Centre de Santé Publique de Québec (CSPQ). These case-patients lived in a rural community on Orleans Island in the St. Lawrence River. Domestic wells were the only source of water in the community, and sewage was disposed of in septic tanks or cesspools. An epidemiologic investigation was undertaken to identify the outbreak source. The implicated source was analyzed for contamination by coliform bacteria and HAV. Molecular epidemiology was done to investigate the chain of transmission.

Methods

Epidemiologic investigation. Data about case-patients were collected by the CSPQ through a standardized questionnaire. Information included the date of onset of symptoms, water consumption, personal contact with symptomatic case-patients, and other risk factors associated with hepatitis A (e.g., travel to endemic areas, shellfish consumption, homosexual activity, day care attendance, and drug use).

A case-patient was defined as a person with an illness compatible with hepatitis A and laboratory evidence of acute infection (i.e., IgM antibody to HAV in serum, HAV in feces). Immunity against HAV among persons living in the affected area was assessed by testing for total antibody to HAV (anti-HAV).

Laboratory investigations. Water from implicated wells was tested to detect total and fecal coliform bacteria by membrane filtration of 100 mL of water [11]. For detection of HAV, 150–1000 L of well water, depending upon turbidity, was filtered through cartridge filters (Virosorb 1MDS; CUNO, Meriden, CT) at ambient pH (7.0–7.2) with a flow of 10–12 L/min with a pressure reading no more than 0.09 km/cm^2. Adsorbed virus was sequentially eluted

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from filters with 800 mL of 0.5 *M* glycine, pH 9.5, and 800 mL of 3% beef extract, pH 9.5. For some filters, a third elution with 800 mL of beef extract was performed. The pH of each eluate was adjusted to 7.5, and 8% (wt/vol) polyethylene glycol (MW 8000) and 0.3 *M* NaCl were added for overnight precipitation at 4°C with stirring. Precipitates obtained by centrifugation at 10,000 *g* for 30 min were resuspended three times in 0.15 *M* Na_2HPO_4 at pH 9.0 to dissociate the virus, and supernatants were pooled in a final volume of 12–80 mL. After adjustment to pH 7.5, the virus concentrates were extracted with an equal volume of chloroform and kept at 4°C until testing.

HAV was concentrated from cesspool sludge by a standard method and kept at 4°C [12]. Stool samples from case-patients were stored at −20°C until converted to 10% suspensions that were stored at −20°C until further testing.

Concentrates from water and sewage and stool suspensions were tested for HAV RNA by nucleic acid amplification with the immunocapture reverse-transcription polymerase chain reaction (IC-RT-PCR) of two capsid regions [13]. Amplicons were generated from the VP3–VP1 junction of the capsid region with previously described primers [14] and reamplified with internal primers 5′-ATGTTACTACACAAGTTGGAGAT-3′ (forward) and 5′-GATCCTCAATTGTTGTGATAGCT-3′ (reverse) to generate a 197-bp fragment for sequence analysis. Amplicons from the VP1–P2A junction were generated with primers +2799 and −3375 [13] and internal primers 5′-CTATTCAGATTGCAAATTACAAT-3′ (forward) and 5′-AACTTCATTATTTACATGCTCCT-3′ (reverse) to generate a 374-bp fragment for sequence analysis. For this region, PCR product was generated by 45 cycles of denaturation at 95°C for 35 s, annealing at 45°C for 60 s, and extension at 72°C for 2 min. Second-round PCR was performed for 30 cycles by using the same conditions as the first-round PCR. HAV RNA detected in stool and environmental samples by IC-RT-PCR was confirmed by either dot blot hybridization, Southern hybridization, or nested PCR with the primers described above [15].

Amplified DNA was either purified by acrylamide gel electrophoresis extracted in SDS and phenol chloroform and precipitated in ethanol [14] or purified by QIAquick columns (Qiagen, Valencia, CA). Automated DNA sequencing of amplicons in forward and reverse directions was done (ABI 373 or 377 DNA sequencer; Applied Biosystems, Perkin-Elmer, Norwalk, CT) using the dideoxy terminator cycle method with rhodamine or dRhodamine Ready Reaction Kits (Applied Biosystems, Perkin-Elmer), with reaction products purified on columns (Centri-sep; Princeton Separations, Adelphis, NJ). Consensus sequence was obtained, and analysis was done by computer program (Pileup; Genetics Computer Group, University of Wisconsin, Madison).

Total anti-HAV was detected by microparticle EIA (IMx HAVAB; Abbott Laboratories, Abbott Park, IL) according to manufacturer's instructions. Anti-HAV IgM was detected by IMx HAVAB-M (Abbott Laboratories).

Results

Epidemiology. The index hepatitis A case-patient, with onset of icterus on 7 June 1995, was a 30-year-old woman who lived with her husband and 2 children in the rural community from May to October. Because this case was not reported to the CSPQ until 1 month after the onset of icterus, postexposure prophylaxis with immune globulin (IG) was not administered to her family contacts. She had no known hepatitis A–specific risk factors, and the source of her infection was not identified. At the beginning of August, her husband and daughter became ill (figure 1); however, her 14-month-old son never became ill. He did not attend day care and had no contact with others outside the home except for 2 grandparents, who were not ill. Because no serum specimen was obtained, it was impossible to determine if the child was infected.

Between 1 and 10 August, 6 more persons became ill (figure 1). All had consumed well water from residence 1 (R1), located adjacent to the index case-patient's home (R0; figure 2). Among these 6 additional case-patients, 2 were permanent residents of R1, 3 visited the house for several days between 24 June and 1 July, and 1 drank R1 well water brought to a construction site a few kilometers away. None of these 6 case-patients had direct contact with the index case-patient or her family; they did not share meals, visit, or have a social relationship. Between 29 August and 5 September, 6 more persons who visited R1 became ill. The latter could have been exposed to HAV from all case-patients from R0 who deposited virus into the cesspool. In contrast, those with onset in early August would only have been exposed to deposits from the index case-patient, since the incubation period for hepatitis A is 15–40 days. Among 20 persons who visited R1 between 24 June and 30 August, 12 (60%) became ill. All but 1 ill person had drunk water from the residence; the 8 persons who were not ill did not drink water. Neither common meals nor food suppliers linked the case-patients. Three case-patients who became ill between 29 August and 5 September had received IG but developed hepatitis within 12 days of its administration. The last case-patient, identified on 22 September, was the sister of a case-patient who became ill on 2 September (figure 1). Because this last case-patient did not drink water or go to R1, she is assumed to have been infected by person-to-person transmission.

The mean age of case-patients was 17 years (range, 5–41).

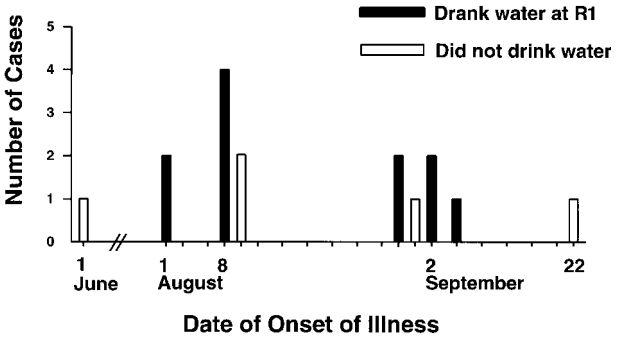

Figure 1. Dates of onset of 1995 hepatitis A outbreak, Orleans Island, Canada. R1 = residence 1.

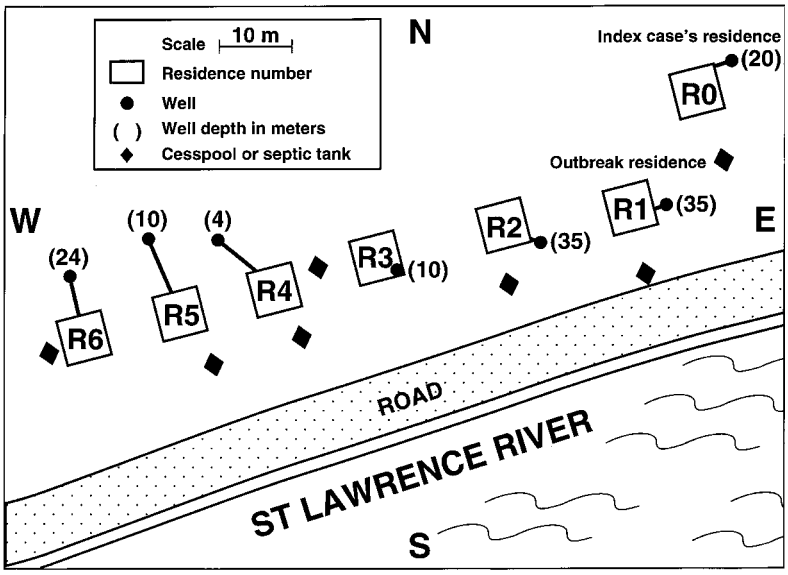

Figure 2. Location of residences, wells, septic tanks, and cesspools in area of hepatitis A outbreak

All had fever, nausea, and fatigue, and 94% were icteric. Fifteen of the 16 case-patients were confirmed serologically; the other had HAV detected by IC-RT-PCR in her stool (see below). One case-patient was hospitalized for 1 day. All recovered without sequelae.

Persons living in residences with no reported illness (R2–R6; figure 2) were evaluated for symptoms attributable to hepatitis A, consumption of well water, and serologic evidence of previous HAV infection. None of the 15 residents reported symptoms and, among the 8 who agreed to have serologic testing, 5 had evidence of prior infection (total anti-HAV positive and IgM anti-HAV negative) and 3 were susceptible. Two of the serologically susceptible persons lived in R5, where no HAV was found in the well water (see Results), and 1 lived in R3, where HAV was found in well water, but this person reported drinking only commercially bottled water. These 3 susceptible persons subsequently received hepatitis A vaccine. Residents of these houses were not aware of any symptoms consistent with hepatitis A among their guests. The 2 senior residents of R2 did not have visitors during the summer. The 2 visitors to R3 most likely drank bottled water, as did the residents.

Water analysis. Two residents of R1 reported the well water had a foul smell and was brownish after heavy rains. Bacteriologic analysis of samples obtained in August 1995 yielded a population density of 57 cfu/100 mL total coliform bacteria from residence R0 and >80 cfu/100 mL from residences R1 and R2 (table 1). No fecal coliform bacteria were found at R0, while water from R1 and R2 had a count of >60 cfu/100 mL. In September, water from wells with fecal coliform counts (i.e., R1, R2) was filter-concentrated for HAV detection, and although well R1 was treated with chlorine a few days prior to the sample, HAV was detected (table 1). In October, wells R1–R4 and R6 were sampled, coliform bacteria were detected in all of them, and HAV was present in R1, R2, and R3 (table 1).

During October 1995, the cesspool at the index case-patient's residence was emptied of sludge but not removed, since no one lived in the residence between October 1995 and June 1996. Well water samples from R1, R3, R5, and R6 were tested for coliform bacteria and HAV in December or January (table 1). The owner of R2 refused further testing after October. Water from R3 did not contain HAV in December, although total coliform bacteria exceeded 80 cfu/100 mL. HAV was detected in water from R1 in January, when no fecal coliform bacteria

Table 1. Bacteriologic and virologic analysis of wells according to sampling date.

	Coliform bacteria[a]		
Residence, date of sampling	Total	Fecal	HAV
R0, 23 August 1995	57	0	Not tested
5 December 1995	20	0	Not tested
R1, 23 August 1995	>80	>60	Not tested
6 September 1995[b]	0	0	+
11 October 1995	>80	>60	+
30 January 1996	30	0	+
R2, 28 August 1995	>80	>60	+
11 October 1995[c]	>80	>60	+
R3, 10 October 1995	58	20	+
4 December 1995	>80	0	−
R4, 10 October 1995	>80	0	−
R5, 4 December 1995	12	0	−
R6, 31 October 1995	>80	0	Not tested
6 December 1995	16	0	−

NOTE. +, positive; −, negative; HAV, hepatitis A virus.
[a] Data are expressed as cfu/100 mL of water.
[b] Well was treated with chlorine several days before sampling.
[c] Further testing was refused.

were detected. It was not possible to conduct a dye study at this location.

Hydrogeology. The houses implicated in the outbreak were located on Orleans Island in the St. Lawrence River (figure 2). In this area, there is a thin clay overburden, the bedrock is composed of schists, sandstone, and conglomerate, and the bedrock dip is abrupt and directed toward the river. Ground water, which is thought to flow parallel to the cleavages in the bedrock and fractures in the major regional fault system, generally flows to the St. Lawrence River, although the general southeast direction can be modified by the rock structure. The hydraulic conductivity in the area is estimated at 10 cm per day, and the ground water temperature at 5–8°C year-round. Wells at R1 and R2 are 35 m deep and extend into the bedrock; at R3, the well is only 10 m deep, does not extend into the bedrock, and is limited to the phreatic aquifer. Wells at R1, R2, and R3 are 3–5 m lower in elevation than the well at R0. The distance between the index case-patient's cesspool and wells at R1, R2, and R3 is 10, 35, and 60 m, respectively (figure 2). Isolation of HAV from wells located at variance with the general direction of the ground water flow for the area (R2 and R3) suggests that local hydrology may differ from the general movement of water flow.

Nucleic acid sequence analysis. The sequences of HAV cDNA from well water at R1, R2, and R3, the cesspool sludge, and stools of 4 case-patients (daughter of index case-patient, R1 owner, an employee, and a friend) were identical over 424 bases encompassing both capsid regions (designated Orleans sequence; submitted to GenBank). An isolate from the husband of the index case-patient could only be amplified in the VP3–VP1 junction region, and this sequence was identical to the other Orleans isolates. The nucleic acid sequence of HAV isolated from well water at R1 6 months after the initial contamination was identical to sequences of HAV at all earlier time points listed (table 1).

The relatedness of the HAV isolates obtained from this outbreak was compared to HAV isolates obtained from other Province of Quebec patients obtained about the time of the outbreak. In Quebec Province, the mean incidence of hepatitis A is 5 case-patients/100,000 persons per year, and 25%–45% of all case-patients are linked to foreign travel [16]. Stool samples were available from 2 of 3 waste water workers from Quebec

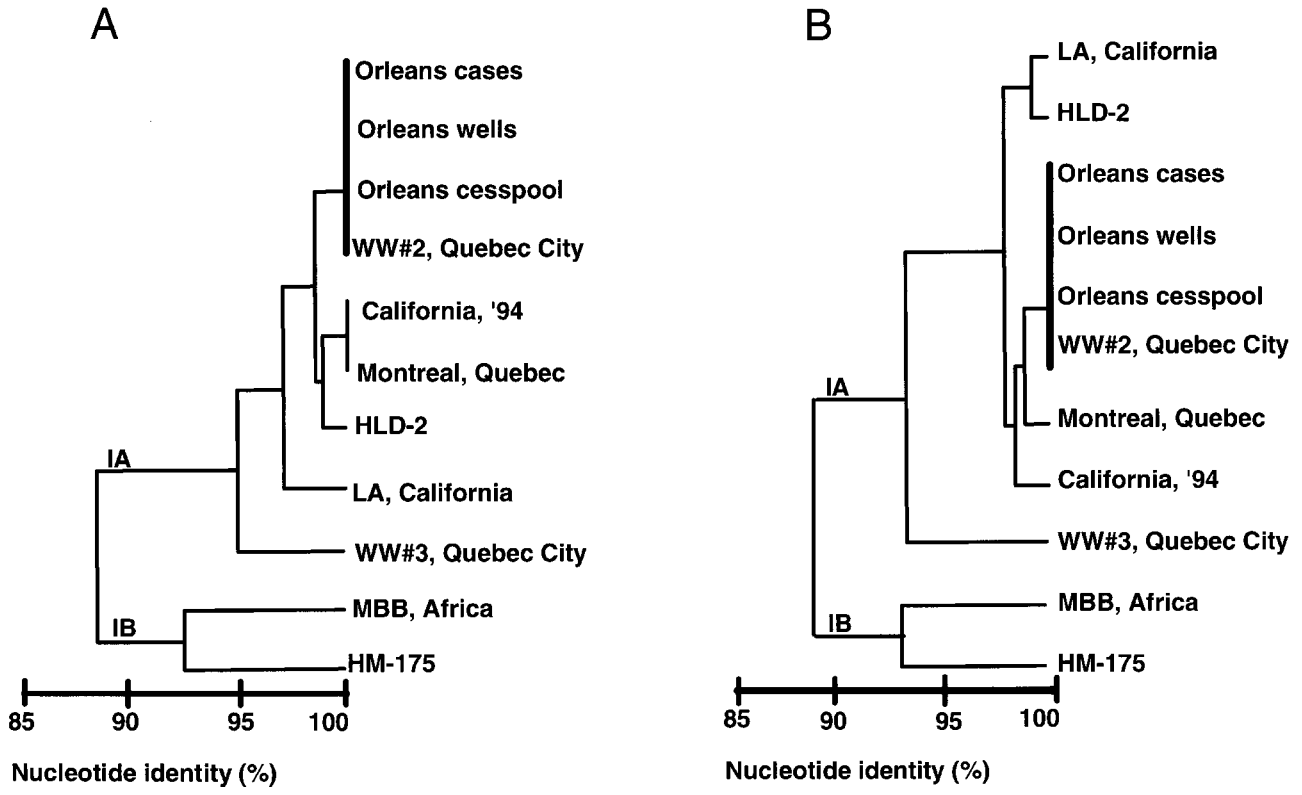

Figure 3. Genetic relationship of Orleans isolates to other HAV isolates and strains. *A,* Pairwise comparative analysis of VP3–VP1 region (nt 2231–2331, numbered according to wild-type sequence HM-175 [17]). Isolates are from Orleans case-patients, well water samples, and cesspool sludge (Orleans sequence, submitted to GenBank); case-patients from Province of Quebec: Montreal and waste water workers 2 and 3 (WW#2, WW#3); Los Angeles [13]; California '94 (isolated from communitywide outbreak); MBB, originally from Africa [13]; and laboratory strains HLD-2 [18] and HM-175 [19, 20]. Subgenotypes are designated IA and IB. *B,* Pairwise comparative analysis of 324 bases in VP1-P2A region (nt 2925–3249) of same HAV isolates and strains as in *A.*

City who became ill between August and December 1995 and from case-patients from a restaurant-associated HAV outbreak in Montreal in late 1996. The nucleotide sequence from the waste water worker who became ill in September (WW#2) was identical to that of the case-patients in the outbreak. The nucleotide sequence from the waste water worker who became ill in late December (WW#3) differed from the outbreak sequence in 5 of 100 bases in the VP1–VP3 junction region and in 18 of 324 bases in the VP1–P2A junction region (figure 3). Comparison of the sequences from the Montreal case-patients showed a difference in 1 of 100 bases in the VP1–VP3 junction region (figure 3A) and in 4 of 324 bases in the VP1–P2A junction region (figure 3B).

When compared with genotype I HAV strains, the Orleans isolate was most similar to isolates from Montreal and the western United States and was a subgenotype 1A. The Orleans isolate is distinct from our laboratory strains (i.e., HM-175 or HLD-2; figure 3).

Discussion

In this outbreak, the epidemiologic investigation implicated consumption of well water as the source of infection. The environmental investigation strengthened this hypothesis and was confirmed by the nucleic acid sequence analysis of HAV isolates from case-patients and environmental samples. HAV has been detected in water or environmental samples during other outbreaks [6–10]. An epidemiologically implicated source has not been linked to case-patients through the genetic relatedness of HAV isolates, although such a linkage was recently reported for an outbreak of diarrhea from a small round-structured virus [21]. In addition, the nucleic acid analysis of isolates from other case-patients in the Province of Quebec indicated they were generally not related to the outbreak.

The identity between the Orleans Island isolates and that of the Quebec City waste water worker who became ill in September suggested either that the Orleans strain may have been circulating in Quebec City or that outbreak case-patients introduced HAV into sewage water when they went to the city. The source of the infection could not be ascertained for the index case-patient or for the waste workers. Although a temporal relationship suggested an association between the waste water worker (WW#3) who became ill after sludge from the index case-patient cesspool was disposed of at the waste water plant [22], the nucleic acid sequence of the isolate from the worker who became ill in December did not match those of the Orleans Island case-patients.

The sequence and epidemiologic comparisons between the Montreal case-patients, the waste water workers, and the outbreak case-patients suggest varied sources of HAV in the Province of Quebec. All of the Canadian isolates were classified as subgenotype IA (figure 3), which includes most HAV circulating in North and South America and other regions of the world [13]. Analysis of the VP1–P2A (324 bases) region was required to distinguish Montreal and California '94 isolates, while only the VP1–VP3 (100 bases) determined the lack of identity of WW3 with other Quebec isolates.

Detection of fecal coliform bacteria at the beginning of the investigation in well water from R1 was the incentive to proceed with the detection of HAV. Although fecal coliform bacteria were detected in well water from all of the HAV-contaminated wells in the first few months of the investigation (except when chlorine was added to R1 in September), no fecal coliform bacteria were found when R1 was positive for HAV in January.

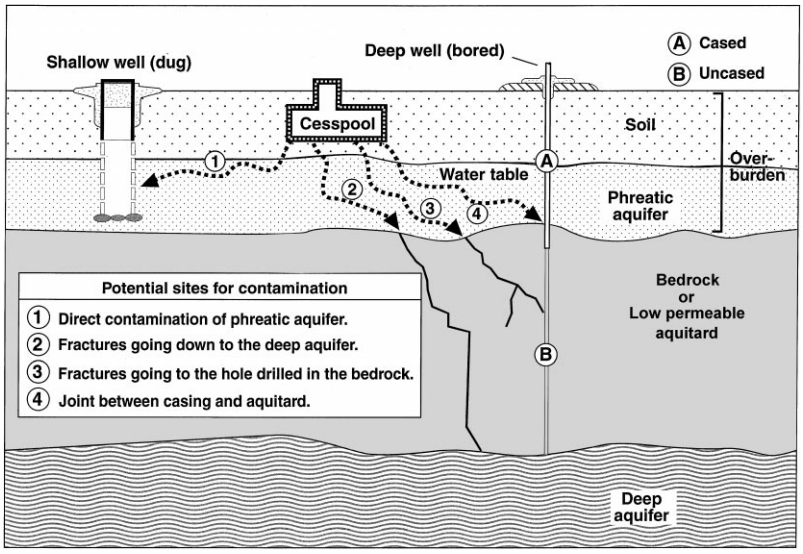

Figure 4. Schema of shallow (dug) and deep (bored) wells and potential sites of HAV contamination

This may be because HAV survives longer than fecal coliform bacteria in this environment or because the movement of contaminants from the cesspool into the surrounding ground water was reduced because the house was empty for 5 months preceding the sampling. Investigation of other waterborne outbreaks of viral disease have also shown that bacteriologic indicators of fecal contamination may be inadequate to detect viral contamination [23, 24].

Although PCR amplification is sensitive and has allowed detection of HAV RNA in well water [9], detection of HAV RNA does not establish infectivity. Detection of HAV RNA by IC-RT-PCR suggests possible infectivity, since the technique relies on capture of immunoreactive viral structural antigen associated with the RNA. Other viral surface antigens, such as those required for interaction with cell receptors to initiate infection, may also be undamaged and reactive. Cell culture detection of HAV in environmental samples is relatively insensitive, lengthy, and difficult, and nucleic acid or antigen detection assays are necessary to verify viral replication. Potential viability of the HAV in the investigated environment is supported by the long-term stability of HAV at 4°C in various humid or aqueous environments [25–28]. For these reasons, it was decided that long-term protection was needed for vulnerable residents and that vaccination was more advisable than IG administration. Recovery of HAV >6 months after the probable date of contamination of the well (June 1995) confirmed our assumptions. Although this modest intervention left unaddressed the basic problem of safe water supply and appropriate disposal of waste water at this location, the increased awareness of the problem in the community may lead to future improvements.

In this outbreak, the distance between the cesspool of the index case-patient and the source well for case-patients who drank contaminated water (R1) was less than the 30 m required by provincial regulations. However, the regulation would not have protected wells R2 and R3, located 35 and 60 m, respectively, from the cesspool. The multiple fractures in the bedrock of the area are one explanation for the contamination, although other mechanisms may have contributed (figure 4). Viruses can migrate 1000–1600 m in limestone, and studies by the US Environmental Protection Agency (EPA) have shown that distances of 210–325 m from a septic tank may be necessary to achieve a reduction of 11 logs in virus concentration [1]. In addition to the longer distance migration potential of viruses, inactivation proceeds at a much slower rate than with bacteria: polio virus has been detected by PCR 3 months after application to soils [29]. In laboratory studies, >90% of HAV survived ⩾12 weeks in ground water, waste water, and soil suspensions at 5°C [30]. In the United States, the EPA recommends that hydrogeology be considered when states establish regulations concerning the setback of wells from potential contaminating sources. However, state laws allow wells to be located 0–151 m from septic tanks or privies.

This outbreak demonstrated the importance of rapidly investigating hepatitis A cases that occur in settings where well water may become contaminated. When hepatitis A cases occur in an area supplied by domestic wells, it is important to evaluate the adequacy of sewage disposal and the likelihood of well water contamination (e.g., geology, location of neighboring wells, and bacteriologic and virologic water quality). When HAV is detected in well water, viral monitoring may be an important adjunct to assess the duration of contamination. With the availability of PCR methodology, the detection of HAV in ground water should become a routine capability of public health laboratories.

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