Use of the Minimum Spanning Tree Model for Molecular Epidemiological Investigation of a Nosocomial Outbreak of Hepatitis C Virus Infection

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The minimum spanning tree (MST) model was applied to identify the history of transmission of hepatitis C virus (HCV) infection in an outbreak involving five children attending a pediatric oncology-hematology outpatient ward between 1992 and 2000. We collected blood samples from all children attending since 1992, all household contacts, and one health care worker positive for antibody to HCV (anti-HCV). HCV RNA detection was performed with these samples and with smears of routinely collected bone marrow samples. For all isolates, we performed sequence analysis and phylogenetic tree analysis of hypervariable region 1 of the E2 gene. The MST model was applied to clinical-epidemiological and molecular data. No additional cases were detected. All children, but not the health care worker, showed genotype 3a. On six occasions, all but one child had shared the medication room with another patient who later seroconverted. HCV RNA detection in bone marrow smears revealed, in some cases, a delay of several months in anti-HCV responses. Sequence analysis and phylogenetic tree analysis revealed a high identity among the isolates. The MST model applied to molecular data, together with the clinical-epidemiological data, allowed us to identify the source of the outbreak and the most probable patient-to-patient chain of transmission. The management of central venous catheters was suspected to be the probable route of transmission. In conclusion, the MST model, supported by an exhaustive clinical-epidemiological investigation, appears to be a useful tool in tracing the history of transmission in outbreaks of HCV infection.

Since the introduction of blood donor screening through antibody testing, the risk of acquiring infection with hepatitis C virus (HCV) through the transfusion of blood or blood products has dramatically decreased in industrialized countries (8, 28, 34). Nonetheless, the nosocomial transmission of HCV continues to occur (21). Several recent studies provided evidence of patient-to-patient transmission in most of the cases of nosocomial transmission (2, 5, 6, 9, 13, 15–17, 19, 20, 29, 36), although some studies also reported transmission from health care workers to patients and vice versa (10, 26, 32).

In recent years, the use of molecular biology techniques has proven to be a powerful tool in the epidemiological investigation of HCV infection in health care facilities and other settings (2, 4, 6, 9, 10, 13, 15, 16, 19, 20, 24, 30, 32). In particular, phylogenetic tree analysis has often been used to identify the original source of infection. However, this technique does not allow a detailed history of transmission to be traced or the modes of transmission to be identified, except in cases of provider-to-patient transmission.

In an attempt to more completely describe nosocomial outbreaks of HCV infection, we adopted a parsimonious theoretical approach, referred to as the minimum spanning tree (MST) model (see Appendix). The MST approach, a concept of the graph theory, represents one of the most common problems of combinatorial optimization and is well known to mathematicians. The importance of the MST model lies in its capacity to provide an efficient solution to a variety of problems, provided that an appropriate data structure is available (12, 18, 25).

The objective of the present study was to trace the history of transmission of HCV in an outbreak involving five children attending a pediatric oncology-hematology outpatient ward by performing, together with an epidemiological investigation, a molecular analysis of virus isolates that consisted of constructing a phylogenetic tree and applying the MST model to the molecular data.

MATERIALS AND METHODS

Outbreak. The outbreak occurred in the pediatric oncology-hematology ward of a hospital in Italy. The ward consists of both an inpatient ward and an outpatient day treatment ward. Children discharged from the inpatient ward are usually monitored in the outpatient ward. The inpatient ward consists of five rooms with two beds each and can accommodate 10 children. The outpatient ward consists of three visitation rooms and one medication room for performing invasive procedures, and it can accommodate up to 15 children per day. All

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patients visiting the outpatient ward undergo clinical examination and blood sampling. On a given day, approximately two-thirds of the patients undergo invasive procedures, such as bone marrow aspiration, lumbar puncture, and management of a central venous catheter (CVC) for intravenous therapy.

Testing for antibody to HCV (anti-HCV) is conducted on all patients upon admission to the inpatient ward at the beginning of maintenance therapy and is repeated at the end of overall therapy. Patients with persistently high levels of serum alanine aminotransferase (ALT) or with symptoms of liver disease are also tested for anti-HCV during follow-up. Since the detection of HCV RNA by PCR is not routinely performed in the ward, the diagnosis of HCV infection is based exclusively on anti-HCV seroconversion.

In the summer of 1991, a male child (referred to as patient A) was admitted to the inpatient ward with a malignant hematologic disorder. The child was anti-HCV negative upon admission. In January 1992, the child was found to have high levels of serum ALT and then was found to have seroconverted to anti-HCV. The child continues to visit the outpatient ward.

Four additional children, all of whom were anti-HCV negative upon admission to the ward, were found to have seroconverted to anti-HCV. Specifically, the first of these four children, a boy (referred to as patient B), was found to have seroconverted in April 1998, followed by a girl (patient C) who seroconverted in August 1999, another girl (patient D) who seroconverted in December 1999, and another girl (patient E) who seroconverted in January 2000.

In all five children, the infection was asymptomatic.

That the last four cases occurred in a relatively brief period of time (i.e., less than 2 years) led us to suspect a nosocomial outbreak of HCV infection. To identify other cases of HCV infection among patients and the possible source of infection, in July 2000 we collected blood samples for HCV testing from all children admitted to the ward since January 1992 ($n = 196$) and from all household contacts ($n = 21$) of children found to be infected. At least once a year, health care workers employed in the ward routinely undergo testing in the hospital for blood-borne viruses; we collected a blood sample from the one health care worker who, when the investigation began, was found to be anti-HCV positive.

Epidemiological investigation. The epidemiological investigation focused on determining when the infected patients had been in the inpatient and outpatient wards at the same time and, at these times, which invasive procedures had been performed, in addition to investigating other types of parenteral exposure. Considering the length of the period of time over which the children became infected, it is very unlikely that a single blood donor infected all of them. We thus decided that a retrospective analysis of blood donors would be performed only if HCV genotyping and phylogenetic analysis revealed that the patients were infected with different strains.

Virological assays. Serum samples collected in July 2000 were stored at $<-80°C$ until virological assays were performed. We assessed anti-HCV serum by using a third-generation microparticle enzyme immunoassay (AXSYM; Abbott Laboratories, North Chicago, Ill.). To confirm any positive samples, we used a second-generation immunoblot assay (Deciscan Pasteur; Sanofi Diagnostic Pasteur, Marne-la-Coquette, France).

To detect and quantify HCV RNA in the serum samples collected in July 2000, we used an Amplicor HCV Monitor kit (Roche Diagnostic Systems, Inc.) in accordance with the manufacturer's instructions. HCV genotyping was performed with a commercial probe hybridization assay (InnoLipa HCV; Innogenetics, Zwijndrecht, Belgium).

We also performed a retrospective detection of HCV RNA to ascertain which child was infected first, since seroconversion may be delayed for several months in immunocompromised persons with HCV infection (14). However, since no stored serum samples were available, we used smears of serially collected bone marrow samples, which are routinely obtained during treatment (from four to six bone marrow samples were available for each patient). This approach was chosen based on recent reports (1, 23) of the potential of reverse transcription (RT)-PCR to detect viral RNA in dried blood spots. We thus attempted to investigate the presence of HCV RNA in glass slide preparations of bone marrow aspirates which had been serially collected from the five children involved in the outbreak and which had been stored in sterile sealed bags at room temperature. The reliability of HCV RNA detection in bone marrow smears by RT-PCR was assessed through preliminary experiments with archival bone marrow samples from a patient with a known HCV RNA load in serum (HCV RNA in serum from 10,000 to 100,000 IU/ml) and with bone marrow samples from subjects positive for anti-HCV but negative for serum HCV RNA. We found complete consistency between positive or negative HCV RNA results found in bone marrow samples and those found in serum.

Each smear was scraped with a sterile razor blade in the presence of 200 μl of lysis buffer containing tRNA carrier (Invitrogen-Life Technologies, Paisley, Scot-

land). To avoid cross-contamination, each sample was physically isolated during RNA extraction, and gloves were changed prior to beginning extraction. Three-fourths of each smear lysate was subjected to RNA extraction with a QIAamp viral RNA kit (Qiagen, Hilden, Germany). RNA was reverse transcribed and amplified with *Taq* Gold polymerase and primers directed against the 5′ untranslated region of the HCV genome. The first round of PCR was performed for 35 cycles (95°C for 1 min, with an additional 14 min in the first cycle; 45°C for 30 s; and 72°C for 1 min) with primers B1 (sense, 5′-AAC TAC TGT CTT CAC GCA GAA-3′) and A1 (antisense, 5′-GAT GCA CGG TCT ACG AGA CCT C-3′). The second round of amplification was carried out for 30 cycles (95°C for 1 min, with an additional 14 min in the first cycle; 50°C for 30 s; and 72°C for 1 min) in the presence of inner primers B2 (sense, 5′-ATG GCG TTA GTA TGA GTG-3′) and A2 (antisense, 5′-GCG ACC CAA CAC TAC TCG GCT-3′). Under these conditions, we were able to detect 100 HCV RNA copies/RT-PCR tube.

To confirm the HCV genotype, HCV RNA extracted from each bone marrow smear was subjected to genotyping with the same commercial probe hybridization assay as that used for blood samples.

Molecular analysis. The HCV RNA extracted from serum samples collected in July 2000 was used to perform molecular analysis. Since all patients were infected with genotype 3a, previously described primers specific for HCV genotype 3a, chosen according to the most conserved representative sequences present in GenBank (10, 22), were used to amplify the HCV genomic region encompassing the E1 and E2 genes, including hypervariable region 1 (HVR1). PCR products from each patient were purified and cloned. Both strands of plasmid DNA were sequenced by using an automated sequencer (ABI model 373 DNA sequencer; Applied Biosystems, Foster City, Calif.) in accordance with the manufacturer's instructions.

The sequence analysis was carried out with a 188-nucleotide fragment encompassing the E1-E2 region, including HVR1 (10). Three to five clones for each patient were analyzed. As a control, we used 27 unrelated genotype 3a and 1b sequences from GenBank for the phylogenetic analysis. A neighbor-joining unrooted tree (27) of all of the aligned sequences was produced in order to show an eventual close clustering of viral sequences isolated from all patients.

MST analysis. The application of the MST model is briefly described in the Appendix. The model was applied to the molecular data and, together with the available clinical-epidemiological data, was used to identify the root of transmission of the outbreak and the most probable patient-to-patient chain of transmission. The basic concept of the model is that the outbreak and the most probable patient-to-patient chain of transmission are considered as a special graph connecting all patients with the minimum viral genetic distances among them.

Nucleotide sequence accession numbers. All sequences obtained in the present study have been submitted to GenBank under accession numbers AF548545 to AF548564.

RESULTS

The virological analysis conducted in July 2000 did not reveal any additional cases of infection beyond the five outbreak cases. All five of the children who had seroconverted to anti-HCV were infected with genotype 3a. None of the household contacts were found to be anti-HCV positive. Since the one anti-HCV-positive health care worker harbored genotype 2a/2c, we excluded the hypothesis of personnel-to-patient transmission. One patient was already anti-HCV positive when admitted to the ward, but this patient was infected with genotype 1b. All but one of the five infected children had high HCV RNA levels in sera drawn in July 2000 (median, 219,000 IU/ml; range, 30,000 to 1,060,000 IU/ml).

The sequence comparisons of HVR1 of the E2 gene of HCV showed a high identity among the isolates from all of the patients. Figure 1 shows a phylogenetic tree that illustrates the positions of the clones from the infected patients and in which a large number of HCV3a and HCV1b sequences were included. The sequences from all of the outbreak patients seem to form distinct nonoverlapping clusters with short distances between the isolates from each patient. The viral genetic distances between the patients are also much shorter than those for the outgroup sequences. These results indicate that the five
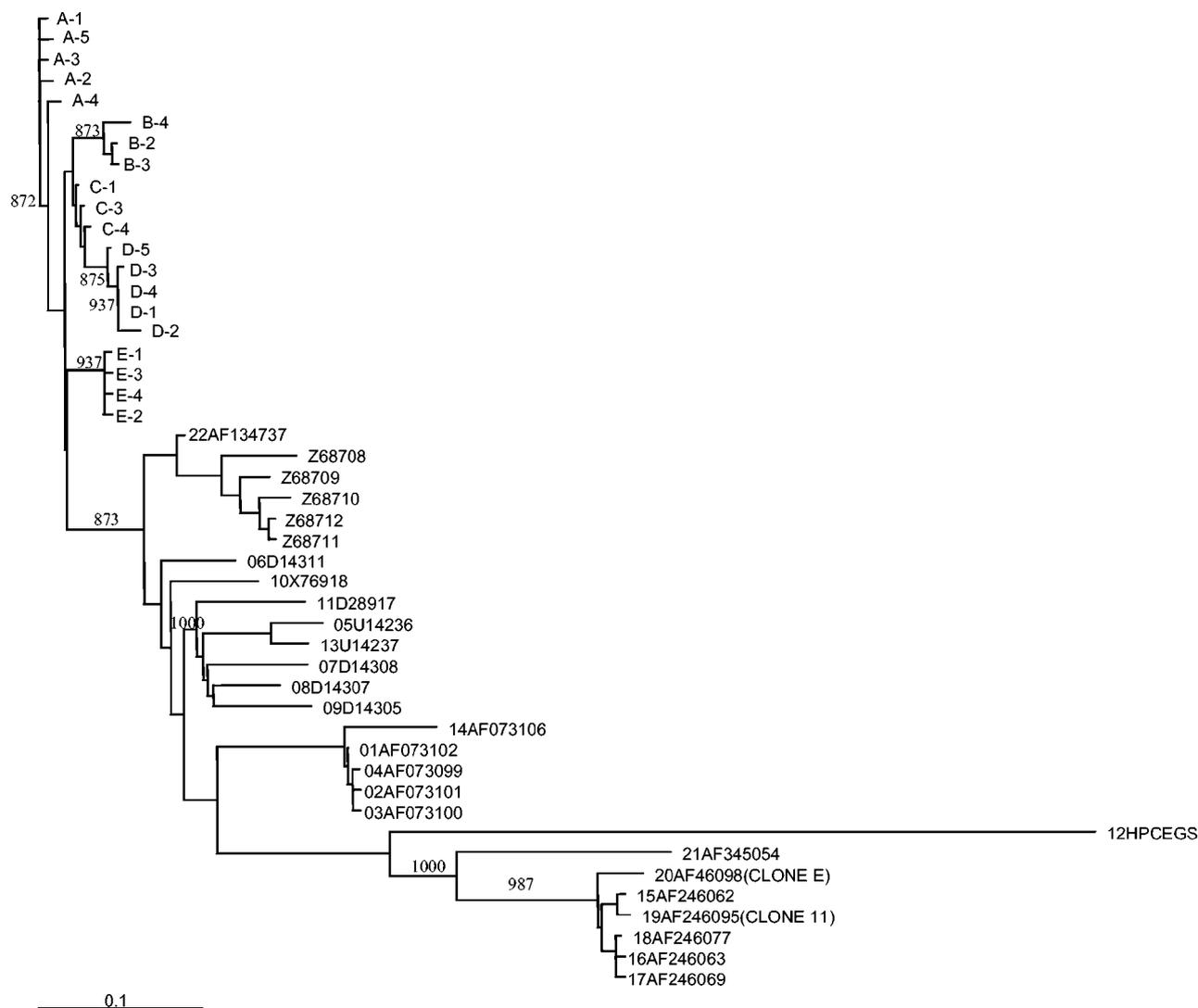
FIG. 1. Phylogenetic tree of the E1-E2 region, including HVR1. Nucleotide sequences were constructed by using the neighbor-joining method with at least three clones for each patient. The clones are designated with a letter indicating the patient (i.e., A, B, C, D, and E). Numbers from 1 to 22 represent HCV genotype 3a and 1b sequences from diagnostic samples not related to the outbreak. To confirm the reliability of the phylogenetic tree, a bootstrap test was performed with 1,000 resamplings of the sequences representing every branch of the tree. The numbers given at the branch points indicate the numbers of bootstrap resamplings among 1,000 resamplings, supporting an observed phylogeny restricted to values of 75% or greater. The scale is measured in nucleotide changes per site.

patients involved in the outbreak were infected by very closely related isolates, a notion which is consistent with the hypothesis that the outbreak had a single epidemiological origin (patient A).

The epidemiological investigation revealed that none of the patients had stayed in the inpatient ward at the same time. However, on six different occasions, all but one of the patients (i.e., patient C) had shared the medication room of the outpatient ward with at least one other patient who later became infected with the same genotype (Fig. 2). At no time were all five patients simultaneously present in the outpatient ward, and for patient C, we failed to find any documentation of contact with the other infected patients. Although patients C and D had visited the outpatient ward during the same period, they were never present on the same day. During the overlap-

ping days in the medication room of the outpatient ward, patients had undergone chemotherapy, invasive procedures, or both, and management of a CVC had been performed for at least one of the patients. Multidose vials of saline, heparin, and other solutions were routinely used in the outpatient ward. According to the staff, which consisted of specifically trained personnel who were aware of the risk of blood-borne infections for immunocompromised patients, none of the standard procedures for infection control had been violated. Since the results of the phylogenetic analysis showed that the five children were infected with the same strain, we did not perform a retrospective analysis of blood donors.

Based on the detection of HCV RNA in smears of bone marrow samples from all patients except patient A, we determined that the time of diagnosis of HCV infection predated
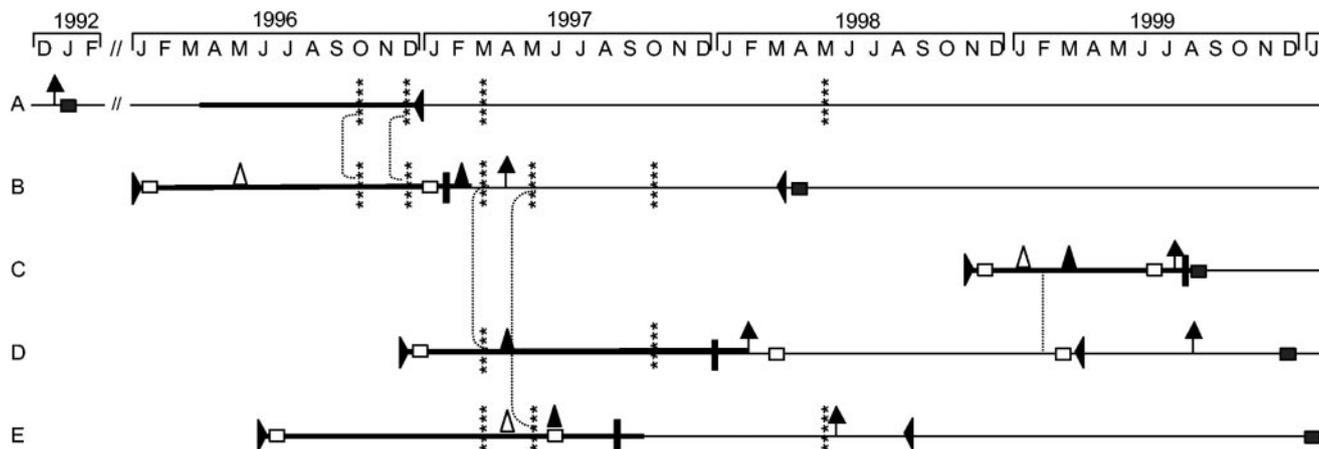
FIG. 2. Period of each patient's stay in the outpatient ward during the outbreak of HCV infection (genotype 3a). Patients are designated with letters A to E according to the time of serodiagnosis. The horizontal lines show the treatment periods in the outpatient ward; the bold portions of these lines denote the periods in which a CVC was used. The white and black boxes represent negative and positive anti-HCV tests, respectively. A white triangle denotes the last negative HCV RNA test; a black triangle denotes the first positive HCV RNA test. The beginning and the end of therapy are indicated by arrowheads pointing to the right and to the left, respectively. Vertical bold lines indicate the beginning of maintenance therapy. The arrows pointing up denote the time at which the ALT level peaked. Vertical lines of asterisks indicate the times at which patients shared the medication room. Vertical lines of dots indicate the time and the direction of patient-to-patient transmission.

the time of diagnosis based on the seroconversion data. For at least two patients (patients B and D), there was certainly a lead time of several months. For all of the patients, the bone marrow smears (four to six smears for each patient) were consistently positive after the first positive HCV RNA detection. Based on HCV RNA detection, the possible chain of transmission was modified, and these data were used to define possible and impossible pairs of infection when the MST model was applied.

With the MST model, the viral genetic distances for all pairs of patients were determined, and the number of initial roots of infection transmission was determined to be 1. Figure 3A shows the viral genetic distances between the possible pairs of infection. Using these distances, we identified the MST for the set of all possible single connected graphs (see Appendix).

After an exhaustive search of all spanning trees and based on the knowledge, provided by the clinical records, that patient A was the root of the outbreak, the most likely chain of transmission was determined to be as follows (Fig. 3B): patient A infected patient B, patient B infected patients D and E, and patient D infected patient C.

Based on this chain of transmission and the epidemiological data, we concluded that, with the exception of patient C, transmission probably occurred on one of four specific days in a time period that spanned less than 8 months (i.e., from October 1996 to May 1997) (Fig. 2). For patients D and E, who were infected by patient B, we identified a single contact in which transmission would have been able to occur (i.e., in March 1997 for patient D and in May 1997 for patient E), whereas for patient B, who was infected by patient A, we documented two possible dates of contact (October and December 1996). Although no contact was documented between patients D and C, transmission probably could have occurred, directly or indirectly, during the period from January to March 1999. In all cases, the patient to whom the virus had been transmitted had undergone management of a CVC on the implicated day. In all cases except for that of transmission from patient A to patient B, the patient who was implicated as the source of infection had not undergone management of a CVC.

DISCUSSION

Immunocompromised adults and children with malignant diseases, who undergo various percutaneous procedures, have been involved in nosocomial outbreaks of HCV infection on a number of occasions (2, 7, 9, 17, 29, 31, 36). Among these patients, both disease-associated and iatrogenic immunosuppression may facilitate HCV transmission, even when contamination is minimal. In most of these nosocomial outbreaks, the epidemiological investigation failed to identify with certainty the source or mode of HCV transmission (2, 9, 17, 29, 36). This failure occurred mainly because the onset of infection is frequently asymptomatic, increases in ALT levels during chemotherapy are not necessarily attributable to hepatic infection, and the appearance of anti-HCV in immunocompromised individuals is delayed (14).

The results of the present study indicate that the use of the

A   **B**

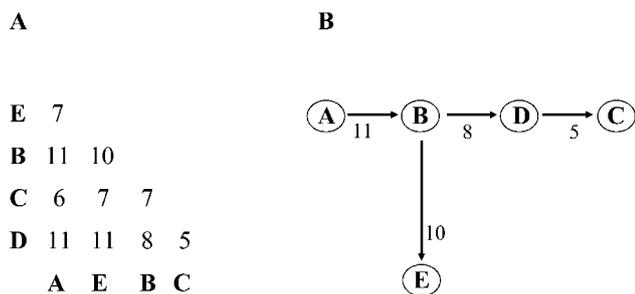|				
E	7			
B	11	10		
C	6	7	7	
D	11	11	8	5
	A	E	B	C

FIG. 3. (A) Matrix of pairwise viral genetic distances among the individuals. (B) Direct graph of the MST for the patients and the viral genetic distances among them. Infection pairs not compatible with the clinical history of the patients were excluded from the analysis.

MST model can contribute significantly to tracing the history of HCV transmission with a certain degree of accuracy. However, in order for this model to work well, an appropriate data structure must be available. In our study, this data structure was obtained through an exhaustive clinical-epidemiological investigation, which allowed us to exclude other sources and modes of transmission, to determine the actual time that patients could have been considered to be infected, to define the sequence of contacts among patients likely to provide the basis for cross-contamination, and to define possible and impossible pairs of infection.

Our investigation allowed us to establish that the outbreak was due to patient-to-patient transmission, to identify patient A as the source of infection, and to describe the probable chain of transmission among patients. However, with regard to the chain of transmission, several considerations need to be addressed. Considering that HCV RNA usually becomes detectable in serum from 7 to 21 days after exposure (11, 33), we rejected the hypothesis of transmission from patient A to patient E because patient E was still HCV RNA negative nearly 1 month after contact with patient A. For the same reason, we excluded transmission from patient D to patient E and transmission from patient B to patient E in March 1997.

What the MST analysis added to the clinical-epidemiological investigation was that it indirectly confirmed the data and clarified several uncertainties regarding the chain of transmission. Specifically, patient B, and not patient A, transmitted the infection to patient D. Moreover, patient D probably had transmitted the virus to patient C, who had no documented contact with the other infected patients. Patient C, according to the matrix of viral genetic distances, was closer than any other patient and in all probability was not infected by an external source. That patient D was indeed the probable source of infection was supported by the finding that she was the only patient who had visited the outpatient ward in the same period as patient C, although never on the same day. Nonetheless, we cannot exclude the possibility that there was another patient involved in the outbreak, acting as a link between patients C and D.

We also evaluated the involvement of external sources of infection. The possibility that the patients were infected through blood transfusion is extremely unlikely, in that they were infected with the same strain over a period of at least 8 years. For this reason, we did not perform a retrospective investigation to search for blood donors who could have acted as a continuing common source of infection. We cannot completely rule out the possibility that the index case (i.e., patient A), who began to be treated in the ward in July 1991 and who received the last transfusion in February 1995, acquired the infection through a blood transfusion. However, even if a donor in the window phase of HCV infection had transmitted the infection to patient A in 1992, it is very unlikely that he or she could have transmitted the infection to other patients years later, since seroconversion would have occurred and the infection would have been detected at the time of successive donations. Furthermore, for the types of blood components received by the outbreak patients, the maximum duration of storage allowed by Italian law is shorter than the time elapsed between the infection of patient A and the infection of the other patients. We also excluded the possibility of personnel-

to-patient transmission. The one health care worker found to be anti-HCV positive harbored genotype 2a/2c. Furthermore, since health care workers employed in the ward undergo screening for the detection of blood-borne infections at least once a year, we excluded the possibility that another health care worker could have been a continuous common source of HCV infection.

Once the source of infection was identified and the history of transmission was traced, we searched for the most probable route of transmission. With regard to specific health care procedures, the staff members steadfastly affirmed that they had not violated any infection control procedures. In hematology-oncology settings, contaminated multidose vials used for the flushing of intravenous catheters and treatment have been reported to be the most important suspected risk factor (9, 36). However, the use of contaminated vials would result in a large outbreak occurring in a restricted period of time rather than an outbreak consisting of relatively few cases occurring over months or even years, as witnessed in our study (2, 29). If the contamination of these vials had been intermittent, then, once again, larger outbreaks would have occurred. The most likely route of infection seemed to be the management of a permanent CVC, which was present on the estimated date of transmission in the patient to whom the virus had been transmitted, including patient C but not the index case, for whom no documentation was available. Furthermore, the patient who was implicated as the source of infection did not have a CVC on that day of transmission, with the exception of transmission from patient A to patient B, both of whom had a CVC on the day on which patient B was infected. Frequently performed percutaneous procedures, even when carried out by trained and experienced personnel, may easily provide opportunities for the contamination of instruments and surfaces with small amounts of HCV-infected blood, especially during periods of heavy workloads. In this context, the management of a CVC, which consists of opening the catheter, flushing it, and using it for all injections, infusions, and blood sampling, may represent an insidious and favorable route of HCV transmission, especially in immunocompromised persons. We did not perform a case-control study to identify particular nosocomial procedures that could have increased the risk of acquiring HCV infection among children involved in the outbreak because this study would have provided little significant information, given the very small number of individuals found to be infected and the fact that almost all of the patients admitted to the ward underwent the same kind of medical assistance and treatment, including the installation of a CVC.

Our results show that transmission seems to have occurred in the medication room of the outpatient ward. This finding stresses the need for the use of more than one medication room for invasive procedures, particularly when intravenous therapy and the management of a CVC in immunosuppressed individuals are required. In this way, health care personnel could dispose of materials, disinfect the environment, and prepare a sterile tray for the next patient while another patient is being treated in the other medication room. The use of multidose vials should also be restricted, and rigorous infection control procedures should be observed (e.g., changing disposable gloves for every percutaneous procedure performed; paying particular attention to the catheter entry site, including

coverage; and cleaning and disinfecting surfaces and nondisposable tools). All of these measures were adopted to prevent additional infections in the ward when the results of the epidemiological and virological investigations led to the suspicion of patient-to-patient transmission. From July 2000 to date, no additional cases of de novo HCV infection have been observed among patients in this ward.

In interpreting the results of this study, certain potential limitations need to be mentioned. Specifically, HCV RNA PCR is probably less sensitive when used on bone marrow samples rather than on properly frozen and stored serum samples; this situation could have resulted in false-negative results for the samples with extremely low virus titers and consequently delayed diagnosis. However, when considering the results for the contacts among patients, apart from patient C, only in the case of patient E could the retrospective diagnosis have been delayed, although an earlier diagnosis in this patient would not have affected the estimated direction of the chain of transmission. Moreover, in all patients, once HCV RNA was detected in bone marrow smears, the smears remained positive for HCV RNA, indicating that transmission occurred after the date of the last negative sample. The phylogenetic analysis was performed at the beginning of the investigation with sera obtained in July 2000, when we were still not sure whether we would have detected bone marrow samples that were positive for HCV RNA. Taking this into account, it may seem surprising that sequences of the same strain isolated from patients who, in some cases, had acquired the infection years after the infecting patient had been infected (e.g., patient C, with respect to patients B, D, E and, particularly, A) showed such a low degree of genetic variability. In interpreting these results, it should be considered that these children were immmunocompromised; host immune pressure represents the most important factor in the induction of viral genetic variability (3, 35). Given that the genetic variability was low when we analyzed HVR1, we did not perform analyses of other genomic regions generally used for analysis of distant events of transmission (e.g., NS5b and E1) (30).

In conclusion, the MST model, when supported by an exhaustive clinical-epidemiological investigation, appears to be a useful tool for tracing the history of transmission in outbreaks of HCV infection. This model could be applied not only to nosocomial outbreaks of other infectious diseases but also to outbreaks occurring in other settings.

APPENDIX

Any outbreak can be described by using a spanning tree (a graph without cycles) of the involved individuals. Assuming a parsimonious evolution of the virus during the outbreak, we use the MST model to clarify the entire outbreak scenario.

Viral genetic distance estimator. The viral genetic distance $d(x, y)$ between patients x and y is defined as the number of nucleotide differences between the consensus sequences of the quasispecies sequences of x and y.

Transmission graph. The transmission graph is a weighted directed graph $G = (V, E)$, where V is the vertex set and E is the edge set of G. The elements of V are points indicating the patients, and the elements of E are arrows denoted by ordered pairs (x, y) indicating that y was infected by x. For each arrow connecting patient x to patient y, we assign a weight $d(x, y)$ of the viral genetic distance between the patients.

Root of transmission. The root of the transmission graph is the patient from whom the outbreak began. This patient was infected by an outside source. It is possible to have more than one root in an outbreak. The number of transmission graphs needed to describe the outbreak is equal to the number of roots (k).

Pairs of infections. For a group of n infected patients, $n(n − 1)$ pairs of infection are possible, and only $n − k$ of these pairs occur in the graphic representation of an outbreak with k roots. However, some of the possible pairs are not compatible with the clinical history of the patients and are excluded from the analysis.

MST. Theoretically, for n individuals, there are $(n + 1)(n − 1)$ scenarios describing the possible modes of transmission of the outbreak. We accept as the most probable scenario the scenario described by the MST, which is defined as the spanning tree with the minimum sum of the weights of the edges (viral genetic distances).

Remarks. The method for estimating roots of transmission is the subject of additional research (J. Sourdis et al., unpublished data). Briefly, applying this method, we constructed the distributions of the viral genetic distances among two, three, four, and five random subgroups of patients by bootstrap testing of the sites of the viral sequences. Based on these distributions, and assuming all possible initial sources of infection, we examined the viral genetic distances among the patients both within and between the derived groups, and we failed to detect any significant values for a specific case of $k > 1$. We thus concluded that there was a single original root of transmission, which was also easily detected on the basis of the clinical records. Once this root (the first infected patient) was identified, the undirected graph of the MST was transformed into a directed one.

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