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Hepatitis C virus in a hemodialysis unit: Molecular evidence for nosocomial transmission

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Hepatitis C virus in a hemodialysis unit: Molecular evidence for nosocomial transmission. Between February 1991 and January 1992, elevated alanine aminotransferase (ALT) levels were observed in several hemodialysis patients in a dialysis center in Dendermonde, Belgium. By the end of 1992, 25 out of 68 patients had seroconverted for HCV antibodies. The HCV strains from 23 of these seroconverters were genotyped and classified as genotype 1b. Sequence analysis of the HCV Core region was carried out in 12 patients, 9 of whom were infected with a strain bearing a unique sequence motif as compared with the currently known HCV 1b strains. A new 5′ UR/Core line probe assay was designed to screen for such variations. Twenty patients tested positively for this special sequence motif, while the other 3 showed the regular subtype 1b sequence. Phylogenetic analysis of the Core sequences further revealed that the latter three were neither related to the main special strain of the infection, nor to each other. These three strains could be traced to two patients already infected at the time of residence in other dialysis units and to one patient who already showed ALT elevations in 1989. Epidemiological studies revealed no traceable source for this outbreak. In conclusion, molecular analysis demonstrates nosocomial transmissions by a peculiar genotype 1b strain in a dialysis center. Three other genotype 1b strains were also present in the unit, but were not responsible for the outbreak.

Hemodialysis (HD) patients are at high risk of infection by several blood-borne pathogens. About two decades ago, hepatitis B virus (HBV) infection became a major concern in HD units resulting in the strict isolation of HBV-positive patients to reduce viral spread [1]. Introduction of a vaccination policy for HBV has generally resolved the problem of HBV infection in HD units, but left non-A, non-B hepatitis (NANBH) as a major remaining threat [2].

Until 1989, the causative agent of most NANBH cases was unknown. After the molecular cloning of the hepatitis C virus (HCV) in 1989 [3], HCV was shown to be the major cause of NANBH. Screening and confirmatory assays for circulating antibodies to HCV became available soon thereafter. The first generation assay only included the nonstructural protein 4 (NS4) antigens. This assay was hampered by low sensitivity, especially in the screening of immunocompromised patients such as HD patients [4, 5]. The sensitivity problems were largely resolved by the introduction of second and third generations assays which were complemented with antigens from the Core, NS3, and NS5 regions [6]. The presence of HCV in HD patients has been reviewed recently [7].

In addition to the serological assays, direct detection of HCV-RNA in serum by means of the polymerase chain reaction (PCR) has been introduced [8]. Several methods for HCV genotyping were also developed, based on either the type-specific amplification of the Core [9], or NS5B regions [10, 11], or on restriction fragment length polymorphism (RFLP) analysis of the 5′ untranslated region (5′ UR) [12] or the NS5B region [13]. We previously described a genotyping protocol targeting the 5′ UR based on the reversed hybridization line probe assay (LiPA) procedure [14]. The combination of different diagnostic and molecular biology tools allowed screening of HCV infections and provided evidence for patient-to-patient transmission of HCV in dialysis units [15–17].

In this study, we describe an HCV outbreak in a Belgian HD unit between 1990 and 1992. Screening for anti-HCV antibodies was started in June 1990. All patients attending the center were tested. With the first generation screening assay, no convincing evidence for HCV infections could be obtained. However, upon introduction of second and third generation screening assays, up to 25 of the 68 (37%) patients were found to be anti-HCV seropositive. Molecular analysis of the infecting viral strains provided unequivocal evidence for nosocomial transmission of HCV within the HD unit.

Methods

Patients

Sixty-eight patients (17 males and 51 females; mean age 66 years) were dialyzed in the same HD unit. The majority of the patients under dialysis were females and were suffering from analgetic nephropathy, a disease status which is uncommon in the Belgium male population. Alanine aminotransferase (ALT) elevations were retrospectively studied from January 1989 to June 1992. ALT levels above 30 IU were considered as elevated. From June 1992 onwards, all patients were followed prospectively for ALT (monthly), anti-HCV antibodies (monthly), and HCV-RNA (bi-annually).

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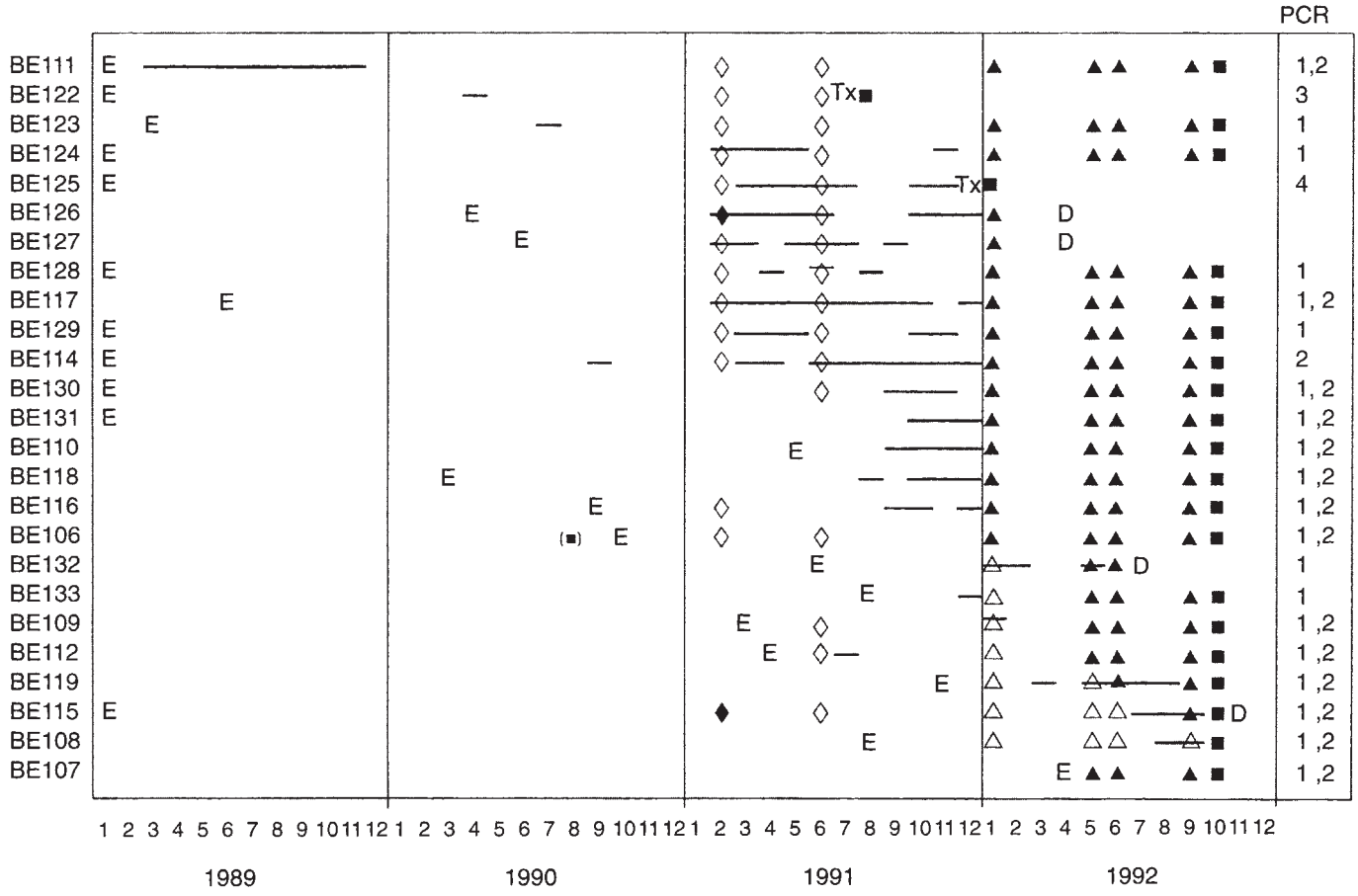

Fig. 1. *Clinical and serological profile of 25 HCV-infected HD patients.* Dashed lines indicate periods of ALT elevation. Abbreviations are: E, entry date of first dialysis; D, death; Tx, date of kidney transplantation and serum sample available. Symbols are: (◇) negative result in first generation screening assay; (◆) positive result in first generation screening assay, but negative in confirmation assay; (△) negative result in second generation screening assays; (▲) positive result in second generation screening assay; (■) positive in third generation screening assay. Serum samples tested on LiPA are indicated in the PCR column: (1) June 1992; (2) September 1992; (3) August 1991; (4) December 1991.

Serological assays

From June 1990 to June 1991, first generation screening assays were used for HCV antibody detection (Ortho HCV ELISA Test System, Ortho Diagnostic Systems, Raritan, NJ, USA). Confirmations were performed with the Neutralization Assay (Abbott, Delckenheim, Germany). From January 1992 onwards, three second generation assays were compared (Ortho HCV, Ortho Diagnostic Systems; Abbott HCV; Monolisa anti-HCV, Sanofi Pasteur, Marnes la Coquette, France). The sera of July 1992 and October 1992 were also tested with a third generation screening assay (Ortho HCV). Serological confirmation and further characterization of the antibody response were performed with the Supplemental Assay (Abbott) and three immunoblot assays (RIBA 2nd and 3rd generation, Chiron, Emeryville, CA, USA; INNO LIA HCV III, Innogenetics, Antwerp, Belgium).

RT-PCR, genotyping, and sequence analysis

Detection of HCV-RNA was performed by reverse transcription PCR (RT/PCR), using nested biotinylated 5′ UR primers as previously described [14, 18]. HCV genotyping was performed by reverse hybridization of the biotinylated PCR product on INNO-LiPA HCV strips (INNO-LiPA HCV, Innogenetics, Belgium). Primers and probes, necessary for the development of the 5′ UR/Core LiPA, were tested for specificity [21]. Further characterization of these PCR fragments was obtained by direct sequencing as detailed elsewhere [19]. Phylogenetic analysis was performed with the PHYLIP program 3.5c [20]. The sequences will appear in the EMBL/DDBJ/Genbank with the accession numbers L38344 (BE111), L44598 (BE107), and L44599 (BE114).

Results

ALT and serological analysis

As of July 1991, all patients undergoing dialysis were screened systematically for anti-HCV antibodies. The number of patients varied on a monthly basis as a consequence of transplantation, death, or new admission. A total of 68 patients were dialyzed within the period 1990 to 1992. In February 1991, two patients showed weak positivity in the first generation HCV screening assay (BE126 and BE115; Fig. 1), but these reactivities could not be confirmed. Upon the introduction of the second generation screening assays, 17 anti-HCV-positive patients were detected in

January 1992. From January 1992 to October 1992, a total of 25 anti-HCV positives were discovered.

Figure 1 shows the clinical and serological history of all confirmed anti-HCV seropositive patients. Patients are listed from top to bottom according to the appearance of the first ALT elevations, but it should be stressed that the sequence of transmission cannot be deduced from Figure 1. Seventeen patients (including BE122 and BE125 from the retrospective study) were HCV-antibody positive in January 1992. One of these (BE111) already showed an ALT elevation from February to October 1989. The reason for this elevation is unclear, but may be attributed to the HCV infection detected only in 1992. Two patients (BE122 and BE123) showed a single ALT elevation in 1990. Retrospectively, and after inspection of the serum bank at the transplantation unit of Gent University, one serum sample from patient BE122, collected in August 1991, and another serum from patient BE125, collected in December 1991, were seropositive in the INNO-LIA HCV Ab III confirmation assay. Patient BE106 never showed an ALT elevation, but retrospectively tested anti-HCV positive in third generation assays on a serum sample from mid-1990, stored at another dialysis center.

After a second general screening (May 1992), four additional patients (BE132, BE133, BE109, BE112) had become seropositive, all with preceding ALT elevations. A fifth patient (BE107) entered the HD unit on April 13, 1992 and was HCV Ab positive upon admission. The third general screening one month later (June 1992), which included testing for HCV-RNA, diagnosed an additional positive patient (BE119), and two patients with serum samples which were HCV-PCR positive but enzyme immunoassay (EIA) negative (BE115 and BE108, data not shown). Both patients subsequently seroconverted: BE115 in September 1992, BE108 in October 1992. From October 1992 until now, no additional seroconversions or HCV-PCR positive serum samples were detected.

Analysis of the HCV RNA genome in the 5′ UR and Core regions

Sixteen serum samples collected in September 1992 were analyzed for the presence of genotypic variations. All serum samples were tested in the prototype HCV genotyping test [14] and could be classified as genotype 1b. Sequence analysis of 12 5′ UR/Core PCR fragments (from patients BE111, BE106, BE107, and 9 other randomly selected patients), covering 238 bp of the 5′ UR and 223 bp of the aminoterminal Core region, revealed a rare but specific motif in the Core region of 9 sera between nucleotide positions 176 to 190 [special motif, 176-GCGGAAGGCGACAGC-190 (HCPr298) vs. subtype 1b consensus, 176-GTGGAAGGCGACAAC-190 (HCPr235)]. These Core sequences were further compared with published sequences, by means of the Phylip program [20]. The phylogenetic tree confirmed the LiPA subtype 1b classification, but also showed that these nine isolates clustered closely together, while the three others (BE106, BE107, BE111) were each found on separated branches (Fig. 2).

Previously, a research LiPA was established combining probes for detection of the 5′ UR type 1 motifs with subtype 1a and 1b specific Core motifs [21]. Figure 3 shows the position of some relevant type- and subtype-specific probes, and examples of the two different observed reactivities. Because Core probes HCPr235 and HCPr298 target the same nucleotide positions, reactivities with these two probes are mutually exclusive (Fig. 3, strip 1 vs. strip 2). Thirty-seven serum samples [PCR column in Fig. 1; one from 08/91 (BE122); one from 12/91 (BE125); 20 from 06/92; and 15 from 09/92], obtained from 23 patients, were found to be RT-PCR positive for this 5′ UR/Core region. Serum samples from patients BE126 and BE127 were no longer available. All PCR fragments strongly hybridized with the 5′UR typing probes HCPr124 and HCPr125, 5′UR subtype 1b probe HCPr188, and Core subtype 1b probes HCPr273 and HCPr250 (Fig. 3). However, PCR fragments from only three different patients (BE106, BE107, BE111) were reactive with the ordinary subtype 1b probe HCPr235 (Fig. 3, strip 1), while PCR products from the 20 other patients all reacted with probe HCPr298 (Fig. 3, strip 2). Typings performed on the September 1992 samples further indicated that patient BE106 and BE109 had become co-infected. While BE106 was uniquely reactive with HCPr235 in the June 1992 serum (Fig. 3, strip 3), this serum also recognized the HCPr298 motif in September 1992 (Fig. 3, strip 4). BE109 was only reactive with HCPr298 in the June 1992 serum (Fig. 3, strip 5), but the September 1992 serum clearly hybridized with both Core probes (Fig. 3, strip 6). To rule out the possibility of PCR contamination, this experiment was repeated twice, with inclusion of the appropriate controls. These observations indicated that BE106 became co-infected with the strain of the major outbreak, while BE109 became co-infected with an ordinary type 1b strain, inside or outside the HD unit.

Epidemiology of the outbreak

We tried to find the route of contamination in our HD unit by statistical analysis of all available host-, viral-, treatment-, and biochemically-related factors. Since we had to compare the group of patients infected with the special strain of the outbreak with patients infected with other strains or HCV negative patients, the following parameters were excluded as playing a role in the virus transmission: age of patients, presence of other markers for viral hepatitis such as HBsAg, anti-HBs and anti-HBc antibodies, treatment by an HCV-infected doctor or nurse (no infected staff members), and risk behavior such as intravenous drug abuse or multiple sexual partners (no such patients). Cases (patients infected with the strain of the outbreak) and control subjects (other patients) were grouped into four different cohorts: dialysis started before 1989, during 1989, during 1990, and during 1991 (Table 1). Patients entering the HD unit in 1992 ($N = 5$) were not included, since none of them seroconverted for HCV antibodies. From the latter observation, we concluded that the period of possible infection was most likely limited to 1990 and 1991, or at maximum 104 weeks of risk exposure (average weeks on dialysis with range, Table 1). Rather than analyzing biological compounds (which were no longer available), we evaluated the amount of manipulations and nursing each patient required during that two-year period, by determining the consumption of the following products (for each group, the minimum and maximum values are given): stabilized solution of plasma proteins (SOPP, Belgian Red Cross), physiological solution (physio), use of fistula clamps (an indication for the general condition of the patient), erythropoietin (EPO), administration of packed red blood cells (blood), heparin, and xylocaine (Table 1). Additional investigations were also included such as electromyography (EMG), and gastroscopy. Finally, the reuse of the artificial kidney for some of the patients was considered. Table 1 also shows the ALT elevations for each cohort. A first multiple linear regression analysis was carried out (Table 1) to find out which one of the above parameters (gender,

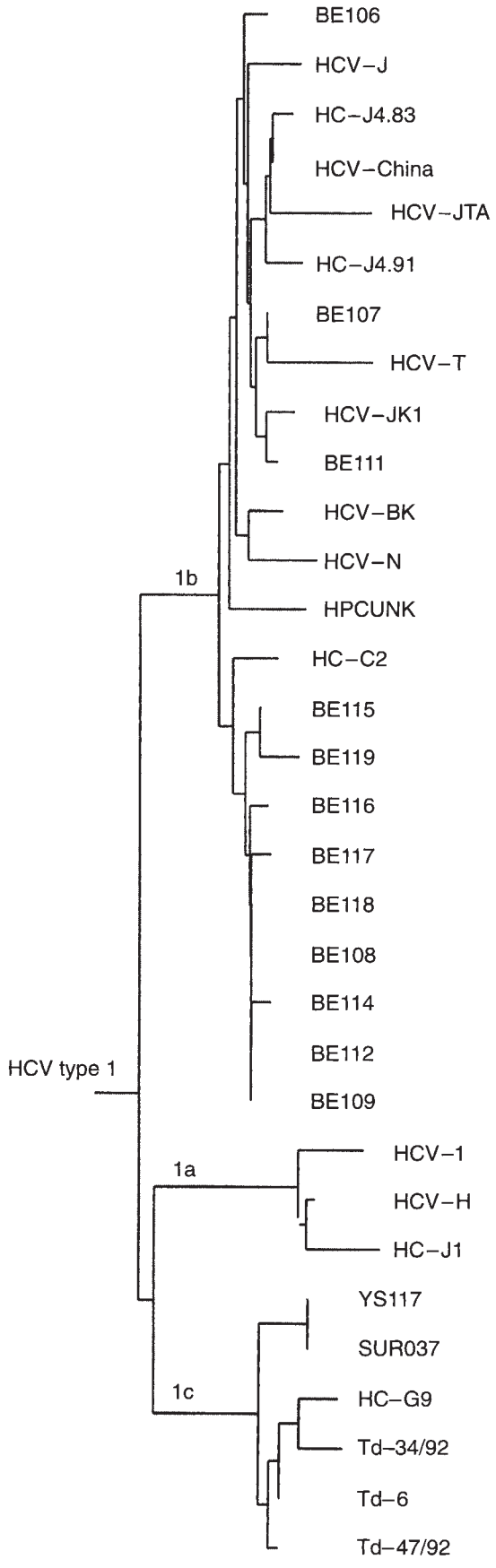

Fig. 2. *Phylogenetic tree of the HCV type 1 Core region (nucleotide position 1 - 223).* Accession numbers of the sequences used to create this tree are detailed elsewhere [19].

weeks, SOPP, etc.) was responsible for the transmission. Only the administration of SOPP ($P = 0.098$) and the reuse of the artificial kidney ($P = 0.028$) showed any statistical significance. ALT elevations were found to be directly related to the infection ($P = 0.0001$). This multiple linear regression analysis was than repeated with SOPP, reuse, and ALT as the only parameters. Again, the administration of SOPP tended towards significance ($P = 0.083$), but reuse of the artificial kidney was not significantly related to virus transmission ($P = 0.349$).

Discussion

We have described and traced the occurrence of 25 documented HCV infections among 68 patients who regularly attended a Belgian HD unit between January 1989 and September 1992. In most of these patients, detection of seropositivity coincided with the introduction of second generation serological assays. These findings could at first have been interpreted in terms of enhanced sensitivity of these new assays. Indeed, in a number of studies, first and second generation serological assays have been compared, and most reports found an increased number of seropositives with the latter assays [22]. However, in the present study, several observations need to be considered independently: (1) there is the exceptionally high number of seropositives found in January 1992; (2) most patients showed a monophasic ALT elevation during the preceding year; and (3) from all patients entering the HD unit between March 1991 and November 1991, six seroconverted after January 1992, while a seventh (BE110) was already seropositive in January 1992. Taken together, these data strongly suggest that most patients experienced *de novo* infection, and that a virus was spreading within the unit. Similar series of seroconversions in HD units were recently reported [16, 17, 23]. Detailed studies by Allander et al [16] and Sampietro et al [17] proved nosocomial transmission of HCV in HD units by molecular biology techniques. By viral fingerprinting of the HVR E2 region, Allander et al showed that infections were not transfusion-associated or due to shared dialysis equipment, but rather were related to the fact that HCV infected and non-infected HD patients were dialyzed in the same room at the same time. Sampietro et al [17] used the single-stranded conformation polymorphism (SSCP) technique to prove that the viral strain in the HD unit (mainly genotype 4) was different from that in a control group of environmentally unrelated patients. However, none of those authors was able to ascertain the real contaminating source in their respective clinical settings.

In our study, two lines of evidence are strongly indicative that a strain carrying a special Core sequence (covered by probe HCPr298) was involved in the outbreak: (i) isolates from 9 of 12 patients clustered very closely together in the phylogenetic tree (Fig. 2), and (ii) isolates from 20 of 23 patients showed reactivity with probe HCPr298 and, therefore, contained the special Core motif (Fig. 3). The EMBL/DDBJ/GSDB was screened for this special motif. Only one sequence Td-47/92 (HPCCP2, Acc. Nb. D26384) shared the same sequence motif at the position of probe

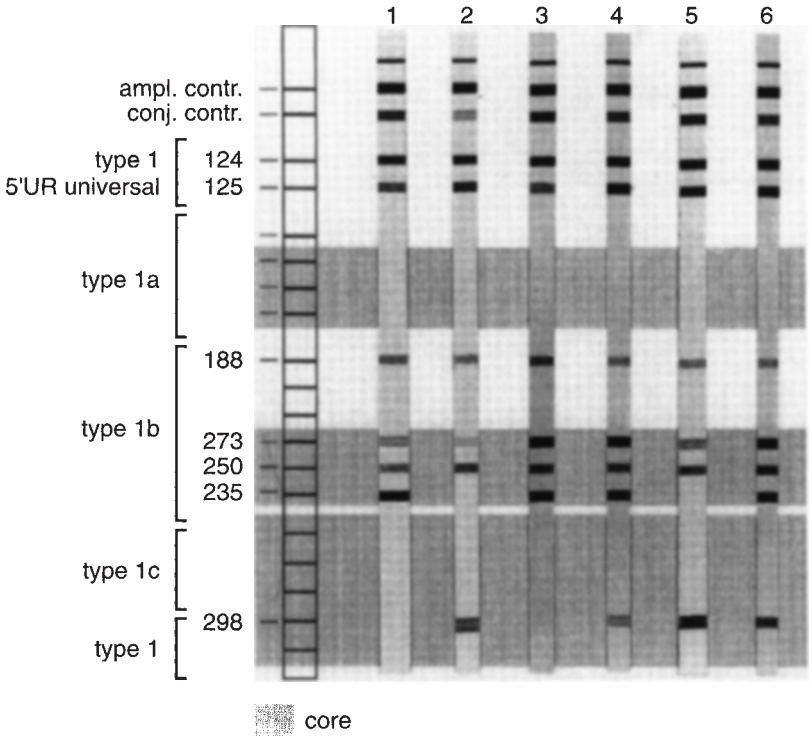

Fig. 3. *HCV type 1 5'UR/Core prototype LiPA.* The scheme on the left depicts positions of the relevant probes. Strips were incubated with PCR fragments of the following serum samples: (1) BE111; (2) BE122; (3) June 1992 serum of BE106; (4) September 1992 serum of BE106; (5) June 1992 serum of BE109; (6) September 1992 serum of BE109.

Table 1. Epidemiology of the outbreak

		Gender male/	Weeks on	Manipulations							Investigations			
	N	female	dialysis	SOPP	Physio	Clamps	EPO	Blood	Heparin	Xylocaine	EMG	Gastro	Re-use	ALT
Started before 1989														
Control	5	3/2	104	0–2	6–23	0	117–263	1–5	1–4	0–4	0–4	0–1	1	1
Case	7	5/2	104	0–2	6–100	5	63–204	1–20	0–3	0–2	1–3	0–5	4	7
Started in 1989														
Control	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Case	3	0/3	104	4–6	78–127	2	136–210	6–13	1–22	6–22	0–1	0–1	2	2
Started in 1990														
Control	4	2/2	72–95	1–3	8–69	1	21–135	0–13	0–2	1–7	0–1	0	3	1
Case	2	0/2	63–65	3	27–38	2	104–105	7–18	0–2	2–3	1–2	0–2	1	1
Started in 1991														
Control	14	5/9	1–48	0–3	0–90	8	0–131	0–10	0–6	0–5	0–1	0–1	2	0
Case	8	1/7	8–42	0–6	3–43	8	9–203	1–12	0–7	0–4	0	0–1	4	4
First analysis														
regression constants		−0.18	−0.03	0.077	0.0002	−0.038	−0.0002	0.0071	0.013	−0.014	0.11	0.057	0.24	0.8
P =		0.19	0.422	0.098	0.995	0.85	0.9	0.56	0.44	0.49	0.29	0.43	0.028	0.0001
A = 0.018, P = 0.926		F value = 9.91		R^2 value = 0.883		R^2 adjusted = 0.794								
Second analysis														
regression constants				0.044									0.083	0.792
P =				0.083									0.349	<0.0001
A = 0.021, P = 0.777		F value = 34.3		R^2 value = 0.75		R^2 adjusted = 0.72								

Abbreviation n.a. is not available.

HCPr298 but, according to the phylogenetic analysis, this sequence belonged to the genotype 1c cluster (Fig. 2). Although found 20 times among the patients in this study, the sequence motif between nucleotide position 176 to 190 seemed to be a rare feature for genotype 1b. Taking into account that the mutation rate for the Core region was estimated to be 1.06×10^{-3} per nucleotide position per year [24] (only about 1 nucleotide substitution in 2 years is expected for the complete Core region), it is impossible that identical variations occurred at identical positions within the period of the outbreak in all 20 patients. We conclude that the 20 patients were infected with the same strain of HCV. Apart from this major infection, a sporadic transmission of another strain of type 1b virus may also have occurred. This is illustrated by the co-infection detected in the September 1992

serum sample of patient BE109 (Fig. 3). If this took place inside the HD unit, we can also not exclude the possibility that some patients were infected multiple times with the special strain of the outbreak. In fact, none of our attempts to trace for the route of contamination was successful, except perhaps for a weak indication that during nursing and/or the manipulations required for the administration of SOPP, the virus was transmitted. Another factor that could not be analyzed was the reuse or occasional sharing of contaminated disposables or solutions. Finally, it remains a mystery why co-infections had not occurred more frequently given that: (i) three different strains of HCV were present in the HD unit during the study period, and (ii) the extremely efficient spread of one of these strains in 20 patients over a two year period. Because no other biological materials have been stored from the period before June 1992, the origin of the HCV strain with the special sequence motif or the route of transmission of the infection remains unknown.

How did the four different strains of HCV enter the HD unit? This is only clear for the two strains from patients BE106 and BE107, who were already positive upon transfer to this unit. Initially this was not known for patient BE106, but retrospective analysis of a serum sample from mid-1990 showed anti-HCV positivity when tested on third generation assays. The manner in which patient BE111 acquired a HCV infection, and how the fourth strain infected a patient, which led to infection of another 19 patients, is as yet unknown. Interestingly, the serum samples from BE122 (August 1991) and BE125 (December 1991) both showed reactivity with HCPr298. As a consequence, at least 11 months before the first general PCR screening (June 1992), this remarkable strain of HCV type 1b was already present in, or even transmitted to, some of the patients. Moreover, all patients were dialyzed in three different subunits. The ALT elevations in the beginning of 1991 were observed mainly in subunit 1 (BE122, BE124, BE125, BE126, BE127) and in subunit 3 (BE117, BE129, BE114). Occasionally some patients had to be dialyzed in another subunit, and this may explain the transmission of the virus from one subunit to the other.

In February 1992, measures were taken to prevent further spread of the virus. Those measures included more stringent nursing rules, which were apparently sufficient to arrest the spread of the virus in other patients, and from June 1992 onwards HCV-seropositive patients were separately dialyzed. The separate treatment of HCV-infected HD patients has recently become a moot point. Since HCV shows a lower rate of infectivity when compared to HBV, it might well be possible to treat infected- and non-infected patients together without new seroconversions provided that some general precautions are strictly followed, such as cleaning and disinfection of instruments, machines, and environmental surfaces, frequent hand washing, and systematic use of gloves [25]. Nevertheless, since HCV very often leads to chronic infection with very serious sequelae, it is our opinion that HCV-infected HD patients should be treated separately whenever possible. Moreover, to enhance the identification of infected patients, we recommend that regular RT/PCR analyses be carried out on apparently non-infected patients within the HD unit to narrow the window between infection and seroconversion, especially because dialyzed patients often show low anti-HCV titers [26]. As the risk of patient-to-patient infection is real, RT/PCR analysis upon admission of new patients in an existing cohort should also be considered. Finally, to establish the origin of infection, subtyping of the HCV strains can be used as an additional epidemiological tool. In this study, it was genotyping alone which provided significant data related to the epidemiology of the outbreak.

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