Savey et al., 2005]. Several studies, by using molecular biology techniques, provided evidence of a nosocomial patient-to-patient mode of transmission in most of these HCV infection occurring in hemodialysis settings, despite rigorous preventive measures [Le Pogam et al., 1998; Delarocque-Astagneau et al., 2002; Kokubo et al., 2002; Savey et al., 2005]. Important risk factors for acquiring nosocomial HCV infection in patients on hemodialysis seems to be particularly a longer duration of hemodialysis, a high HCV prevalence in the unit and a low personnel/patient ratio (<1/3 or at least 1/4) [Petrosillo et al., 2001].

However, the exact mechanisms of the patient-to-patient transmission of HCV within hemodialysis units have not been clearly identified and they may be different in relation to the different policies followed in each hemodialysis unit for the management of patients [Petrosillo et al., 2001; Fabrizi et al., 2002]. Most authors currently believe that most cases of HCV patient-to-patients transmission can be attributed to lack of implementation of or breaks in infection control procedures [Le Pogam et al., 1998; Petrosillo et al., 2001; Delarocque-Astagneau et al., 2002; Fabrizi et al., 2002; Kokubo et al., 2002; Savey et al., 2005]. The possibility of HCV transmission between patients through the dialysis machines is controversial. However, this possibility cannot be entirely excluded especially in case of contamination of internal components of the machine not accessible to routine disinfection, and in the hemodialysis units in which the disinfection of the machines between treatments is not routinely performed or those in which dialysers and/or dialysis tubing sets are reused [Le Pogam et al., 1998; Delarocque-Astagneau et al., 2002; Fabrizi et al., 2002; Savey et al., 2005].

This study describes an outbreak of acute HCV type 2c infection involving four patients attending an outpatient hemodialysis unit in southern Italy. Molecular analysis of viral isolates in association with an epidemiological investigation was performed to trace the source and the possible routes of transmission of HCV during this outbreak.

PATIENTS AND METHODS

Hemodialysis Setting and Procedures

At the time of the outbreak, the unit consisted in a room with 8 dialysis machines in which 32 outpatients regularly underwent maintenance hemodialysis three times weekly (Monday-Wednesday-Friday or Tuesday-Thursday-Saturday) on 1 of the 2 shifts per day (either morning or evening shift). Thus, every machine was used by two persons per day. Normally patients were dialysed on the same shift, but not always on the same machine. No dedicated areas or machines or personnel were used for HCV infected patients. Hemodialysis was carried out using Hospal-INTEGRA® dialysis machine. The machines were disinfected with chlorine dioxide (ISTRUMET, Hospal®) between each shift and dialysers and tubing sets were disposable and were never reused. Two nurses took care of eight patients in each shift, but they could also move from patient to patient if needed. No multidose vials were used among patients.

HCV Infection Monitoring

HCV infection was monitored in all dialysis patients by performing testing for serum alanine aminotranserase (ALT) monthly and for anti-HCV upon admission and then every 6 months. Anti-HCV test was also performed in case of ALT elevation. Prior to the beginning of the outbreak, the prevalence of anti-HCV among the 32 patients attending the unit was 31.2% (10 patients).

Case Definition, Case Finding and Data Collection

During the routine screening for HCV infection conducted from April 2003 to October 2003 four incident cases of HCV infection were identified in the unit. That the four cases had occurred in a relatively brief period of time led to suspect a nosocomial outbreak. A potential outbreak case-patient was defined as any patient who had showed seroconversion between October 2002 and October 2003 and who had received dialysis in the unit at least 6 months before the detection of the first seroconversion case.

Since in the unit, at the time of the outbreak, the monitoring of HCV infection was based on the detection of anti-HCV only, to identify retrospectively other cases of new infections and the potential source of the outbreak on June 2004 blood samples for anti-HCV and HCV RNA testing were obtained from all the patients who had received dialysis in the unit since April 2002 and from all their household contacts. All healthcare workers employed in the unit underwent periodical testing for blood-borne viruses. A blood sample was also obtained from the one healthcare worker (a doctor) who was known to be anti-HCV positive.

From the medical records, kept constantly for all patients, data on medical and dialysis history, blood transfusion, recent surgical, or medical invasive procedures, intravenous drug use and other parenteral exposure, such as tattoos and piercing, were obtained. Furthermore, the dialysis schedule (day and shift) seating arrangements, type of vascular access, type of dialyser membrane, hemodialysis machine, bleeding episodes, nurse-patient assignment, dialysis equipment maintenance as well as infection control measures were all recorded.

Virological Analysis

The seroconversions of the patients involved in the outbreak were detected during the routine screening for anti-HCV performed in all patients attending the unit. In the unit, anti-HCV antibodies were detected by using a third generation enzyme immunoassay (Cobas Core Anti-HCV EIA II, Roche Diagnostic Systems, Basel,

Switzerland) and samples that resulted anti-HCV positive were then tested for HCV RNA (COBAS Amplicore, Roche Diagnostic Systems). An in-depth retrospective virological analysis was performed on serum samples obtained on June 2004 from all hemodialysis patients attending the unit, from the household contacts of the patients who had shown a seroconversion and from the only health care worker who was found to be anti-HCV positive.

All serum samples obtained were tested again for anti-HCV by a third generation enzyme immunoassay (Axsym HCV, version 3.0; Abbott Laboratories, Abbott Park, IL). The viral load in positive serum samples was determined by quantitative reverse transcription (RT)-PCR (HCV Amplicor Monitor, Roche Diagnostics, Milan, Italy), in accordance with the manufacturer's instructions. HCV genotyping was first performed with a commercial reverse-hybridization line probe assay (INNO-LiPA HCV II, Bayer Diagnostics, Milan, Italy) based on the 5'-noncoding region (NCR). The HCV RNA was extracted from serum samples collected in June 2004 using the QIAamp Viral RNA kit (QIAGEN, Hilden, Germany), underwent retrotranscription by random hexamer method and was used to perform molecular analysis. Two-strand direct sequencing was carried out on nested PCR products obtained from the NS5B region and from the hypervariable region 1 (HVR1) encompassing in the E2 gene, as previously reported [Faustini et al., 2005]. Sequencing was performed on ABI Prism 3100, using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK). Sequences of the NS5 regions obtained were aligned by using BLAST with the *National Center for Biotechnology Information* (NCBI) database (U.S. National Library of Medicine, Bethesda, MD, www.ncbi.nlm.nih.gov) and were able to attribute to the genotype 2c all the HCV from patients who seroconverted. For the amplification of the HVR1 region 2 type specific primers were chosen [Faustini et al., 2005]. The sequences were then aligned with CLUSTAL W software (version 1.5). The mean genetic distance between nucleotide sequences was calculated using a Kimura 2-parameter distance matrix with a transition/transversion ratio of 2.0. Phylogenetic trees were constructed using the neighbor-joining method, including NS5B and HVR-1 reference sequences (see figure legends) and local epidemiological unrelated HCV 1, 2b, and 2c strains. Bootstrap analysis with 1,000 replications was performed to assess the significance of the nodes; values >85% were considered to be significant. All of the algorithms used were included in MEGA software (version 2.1).

RESULTS

In October 2002, the anti-HCV seroprevalence in our HD unit was 31.2% (10/32). Between April 2003 and October 2003, four new HCV seroconversions were detected; the first (CT6) in April 2003, second and third (CT2 and CT4, respectively) in August 2003 and the fourth (CT11) in October 2003; thus, raising the total number of anti-HCV positive patients to 14 in the HD unit (Table I).

In all four patients who seroconverted the infection was asymptomatic. In the first patient who showed a seroconversion (CT6), ALT levels that were normal on the date of seroconversion and had always been normal previously, showed an important increase only in the following month. In one of the two patients (CT2) who were found to be anti-HCV on August 2003, ALT levels showed a moderate increase only at the time of seroconversion and they had always been normal previously. The other newly infected patient (CT4) who tested anti-HCV positive on August 2003 had shown an important ALT levels increase on May 2003, but he tested anti-HCV negative in that date; however, his ALT levels had always been normal until April 2003. In these two patients anti-HCV test was performed, and repeated at monthly interval for patient CT 4, as consequence of the detected ALT levels elevation. The last newly infected patient (CT11), who was found to be

TABLE I. Anti-HCV Test Results, Genotype Determination and First Detection of Elevated Alanine Aminotransferase Levels and of HCV RNA in HCV Infected Patients Receiving Dialysis in the Unit (April 2002–October 2003)

Pt	April 2002	October 2002	April 2003	May 2003	June 2003	July 2003	August 2003	September 2003	October 2003
CT 7	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 14	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 1	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 10	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 13	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 8	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 3	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 5	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 9	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 12	Pos	Pos	Pos	NT	NT	NT	NT	NT	Pos
CT 6	Neg	Neg	**Pos**	* NT ↑	NT	NT	NT	NT	Pos
CT 2	Neg	Neg	Neg	Neg	Neg	Neg	**Pos** ↑	* NT	Pos
CT 4	Neg	Neg	Neg	Neg ↑	Neg	Neg	**Pos**	* NT	Pos
CT 11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	* **Pos**

Pos, anti-HCV test positive; Neg, anti-HCV test negative; NT, not tested for anti-HCV; ↑ denotes the first detection of elevated ALT levels; * denotes the first HCV RNA detection. Pos in bold denotes the first anti-HCV positive test.

anti-HCV positive on October 2003 (on the occasion of the anti-HCV six-monthly screening), had always shown ALT normal values before seroconversion at the monthly screening routinely performed in the unit (Table I). All patients newly infected showed a positive HCV RNA test after the seroconversion.

No further cases of new HCV infection were detected when the serum samples drawn on June 2004 from all the patients receiving hemodialysis in the unit were tested for anti-HCV and HCV RNA. Genotyping performed by INNO-LiPA revealed that all the patients who had shown a new seroconversion were infected with HCV genotype 2c. Six out of the 10 dialysis patient with chronic HCV infection were infected with genotype 1b, 2 with genotype 2c (CT1 and CT3), and in 2 patients it was not possible to determine the infecting genotype (one refused to give a blood sample and another one deceased before June 2004). Serum HCV RNA levels ranged from 7,000 to 488,000 IU/ml for the four newly infected patients and from 43,000 to >500,000 IU/ml for patients with chronic HCV infection, for which a serum sample was available. The two chronically infected patients harboring genotype 2c had serum HCV RNA titres of 171,000 UI/ml (CT1) and >500,000 IU/ml (CT3). Since in June 2004 all newly infected patients still resulted HCV RNA positive, that is more than 6 months after the detection of their seroconversion, they must be considered as having all developed a persistent HCV infection. These patients continued to be viremic while attending the unit, although their ALT levels were constantly normal, and none of them underwent antiviral therapy.

Figure 1 shows the phylogenetic tree analysis of the NS5B region sequences isolated from all HCV RNA positive patients receiving hemodialysis in the unit (newly infected and chronically infected patients). All four newly infected patients harbored very closely related viral isolates that clustered together with the 2c isolate found in one of the two 2c chronically infected patients, which is consistent with the hypothesis that the outbreak had a single epidemiologic origin (patient CT3). Phylogenetic analysis also revealed that the other patient with chronic infection harboring genotype 2c (CT1) was not associated with the outbreak. The results of the phylogenetic analysis in the HVR1 region, using only genotype 2 sequences, with the majority being 2c collected from GenBank, were consistent with the findings in the NS5B region (Fig. 2).

The results of the epidemiological investigation were also consistent with a patient-to-patient mode of transmission of the infection during the outbreak. The four newly infected patients had never received transfusion of blood or blood products. Two of them (CT2 and CT6) had no exposure to surgical or medical invasive procedures outside the hemodialysis unit, while patient CT4 had undergone dental extraction 2 months before his seroconversion, and patient CT11 had dialysed outside the unit on summer holidays (July and August). None of the newly infected patients were known to have used intravenous drug and none of their household

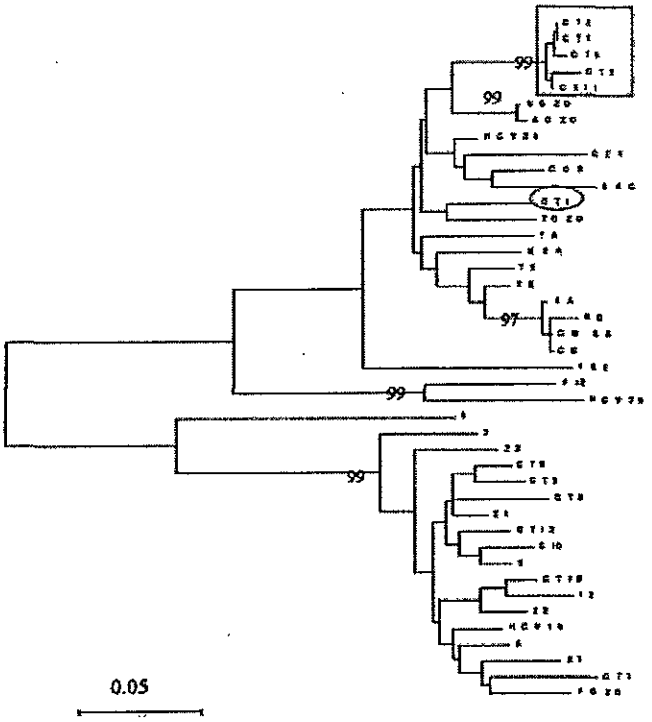

Fig. 1. Phylogenetic tree of NS5B region. The patients involved in the outbreak were indicated with the initials CT and numbered as in the table. Reference genotypes 2b, 2c, and 1b sequences from GenBank are indicated with their accession numbers (for HCV2c AJ291280; for HCV2b AB030907; for HCV 1b AY257435). Local nonrelated patient sequences, including patients with genotypes 2c, 2b, and 1b, are also included. Genetic distance is indicated by a horizontal bar. The numbers at the nodes indicate the frequency with which the node occurred in 1,000 bootstrap replicates; values greater than 95% are indicated.

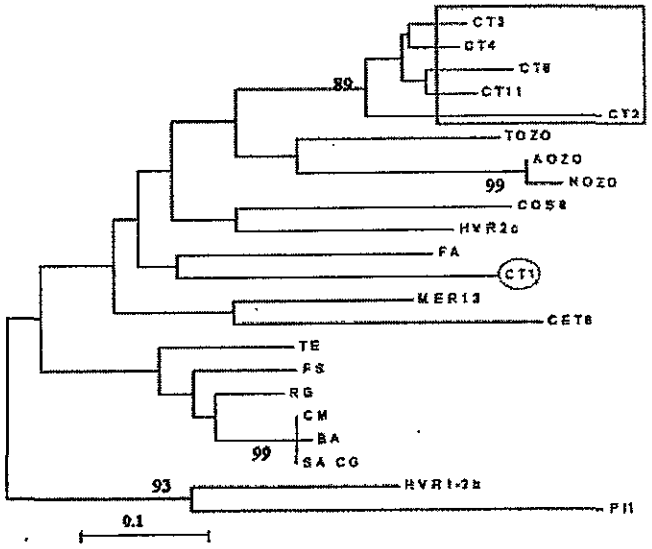

Fig. 2. Phylogenetic tree of HVR1/E2 region. Patients are indicated as in Figure 1. Reference genotypes 2c and 2b GenBank accession numbers are AF 237649 and AB030907, respectively. Local nonrelated patient sequences with genotypes 2c and 2b are also included. Genetic distance is indicated by a horizontal bar. The numbers at the nodes indicate the frequency with which the node occurred in 1,000 bootstrap replicates; values greater than 85% are indicated.

contacts were found to be anti-HCV positive. The only healthcare worker who was anti-HCV positive resulted negative when tested for HCV RNA. All patients infected by closely related genotype 2c isolates (new and old infections: patients CT2, CT3, CT4, CT6, and CT11) received dialysis on the same days (Monday-Wednesday-Friday). Patients CT2, CT3, CT4, and CT6 received dialysis on the morning shift (shift 1) and used different machines. Patients CT11 received dialysis on afternoon shifts but he had shared in several occasions the same machine with patient CT2.

The healthcare workers employed in the unit denied any violation of the standard infection control procedures. After the implementation of the infection control procedures and the use of dedicated machines for anti-HCV positive patients (but not dedicated rooms or personnel), no additional cases of new HCV infection were observed in the unit.

DISCUSSION

In this study, molecular analysis together with epidemiological investigation provided strong evidence for nosocomial patient-to-patient HCV transmission during an outbreak in a hemodialysis unit. Two potential routes of transmission were identified: horizontal transmission via healthcare workers and/or environmental contamination allowed by breaks in infection control procedures; vertical transmission via the dialysis apparatus. The occurrence of all but one cases of infection with closely related subtype 2c strains in patients who had received dialysis on the same days and the same shift (CT2, CT3, CT4, and CT6) suggests horizontal transmission during care due to breaks in infection control procedures. Several types of breaks in infection control procedures able to facilitate HCV transmission in hemodialysis setting have been suggested, such as absence of hand washing or inconstant glove use [Niu et al., 1992], failure to change gloves between patients [Fabrizi et al., 2002], sharing of multidose vials between patients [Kokubo et al., 2002], and lack of environmental disinfection (surfaces and instruments) [Delarocque-Astagneau et al., 2002; Savey et al., 2005]. Although the healthcare workers employed in the unit denied any violation of the standard infection control procedures, occasional or inadvertent mistakes might have occurred particularly during busy periods or an emergency with a patient. In an environment in which frequently performed percutaneous procedures may contribute through blood spillage to HCV contamination of surfaces and instruments [Caramelo et al., 1999], occasional mistakes could be enough to transmit the virus between patients, particularly if they are immunocompromised. Indeed, patients CT2, CT3, CT4, and CT6 had not always used the same machine, but in several occasions, two of them had shared a given machine with 36 hr of difference. However, vertical transmission via dialysis machines in this group of patients has to be considered very unlikely for two principal reasons: first, after these patients had received dialysis, each machine regularly underwent two complete cycles of disinfection during the following 36 hr (after the fist and second shift, respectively); second, none of patients receiving dialysis in the first shift on the following day had resulted to have acquired a new infection with genotype 2c or other genotypes. On the contrary, vertical transmission via dialysis apparatus between patient CT2 and CT11 could not be quite excluded. These two patients had never received dialysis on the same shift, but in several occasions have shared the same machine in the same day. Apart from patient CT2, none of the other patients infected with closely related 2c strains had had any kind of contact with patient CT11 (dialysis on the same shift or use of the same machines). Thus, even if patient CT11 had received dialysis in another unit on vacation, he had undoubtedly acquired HCV infection in the unit where the outbreak occurred, and with high probability patient CT2 had been the source of his infection. HCV transmission via the dialysis apparatus has been suggested to occur in case of dialysers reuse [dos Santos et al., 1996; Fabrizi et al., 2002] or by dialysate [Sampietro et al., 1994], when the dialysis fluid circuit was not disinfected after each session [Le Pogam et al., 1998]. Transmission has also been suggested to occur in case of potential contamination of internal components of the dialysis machine not accessible for routine disinfection. Some authors [Niu et al., 1992; Delarocque-Astagneau et al., 2002; Savey et al., 2005] have reported wetting of arterial and venous filters due to accidental blood backflow creating a potential contamination of the internal pressure sensing port, which is not accessible for routine disinfection. Thus, two successive episodes of blood backflow in the filters can contribute to the transmission of HCV to another patient. However there has been controversy in literature about the potential role of hemodialysis machines in HCV transmission: several other studies concluded that this route probably is a rare occurrence, playing a minor role or no role at all in the transmission of HCV in hemodialysis settings [Jadoul et al., 1998; Fabrizi et al., 2002; Barril and Traver, 2003]. In the unit where the outbreak occurred, dialysers and tubing sets were disposable and were never reused and disinfection of dialysis machines were performed after each shift. Furthermore episodes of accidental blood backflow into external filter were not reported on patient CT2 medical charts in the days in which he had shared the machine with patient CT11. However, it cannot be excluded that some incidents could have not been registered in the case that the nurses had considered the backflow not sufficient for concern. Alternatively, a break in infection control procedures can be supposed, such as an unsatisfactory environmental cleaning and disinfection between the first and second shift that had resulted in HCV transmission from CT2 to CT11 via contaminated environmental surfaces. A recent study suggests that HCV in dried plasma can cause infection in experimental animals when left at room temperature for $\geq$16 hr but not longer than 4 days [Krawczynski et al., 2003].

Since this investigation was retrospective it had some limitations. It was not possible to directly observe the