Detection and Characterization of Hepatitis C Virus RNA in Seminal Plasma and Spermatozoon Fractions of Semen from Patients Attempting Medically Assisted Conception

Thomas Bourlet,[1] Rachel Levy,[2] Anne Maertens,[1] Jean-Claude Tardy,[3] Florence Grattard,[1] Hélène Cordonier,[4] Jean-Louis Laurent,[2] Jean-François Guerin,[4] and Bruno Pozzetto[1]*

Laboratoire de Bactériologie-Virologie, GIMAP, Faculty of Medicine of Saint-Etienne,[1] and Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire de Saint-Etienne,[2] Saint-Etienne, and Laboratoire de Virologie[3] and Laboratoire de Biologie de la Reproduction,[4] Hôpital Edouard Herriot de Lyon, Lyon, France

To investigate the risk of transmission of hepatitis C virus (HCV) via semen in assisted reproduction techniques, semen samples from 32 men chronically infected with HCV attending a center for assisted procreation were tested for HCV RNA by a reverse transcription-PCR protocol by using a modified version of the Cobas AMPLICOR HCV assay (version 2.0; Roche Diagnostics). The sensitivity of the test was 40 copies/ml. Four of 32 seminal plasma samples (12.5%) were found to be positive for the presence of HCV RNA. The median HCV load in blood was significantly higher in patients who were found to be positive for the presence of HCV RNA in semen than in those who tested negative ($P = 0.02$). In one man, seven consecutive seminal plasma samples tested positive for HCV RNA, as did two consecutive motile spermatozoon fractions; the corresponding fractions obtained after migration of the spermatozoa remained negative. Despite the absence of the proven infectivity of virus in semen samples that test positive for HCV RNA, these findings highlight the fact that seminal fluid may exhibit prolonged HCV RNA excretion. The usefulness of HCV RNA detection in both seminal plasma and spermatozoon fractions before the start of a program of medically assisted reproduction in couples in whom the male partner is chronically infected with HCV would need to be evaluated prospectively with a larger population of subjects exhibiting HCV RNA in their semen.

The risk of transmission of hepatitis C virus (HCV) via seminal fluid is still much debated, especially in assisted reproductive techniques (ART). Even though the rate of HCV transmission by the sexual route has been found to be low (1, 9), the use of spermatozoa from men chronically infected with HCV in ART can lead to a theoretical risk of contamination for the female partner, for the technicians dealing with ART, and perhaps for the artificially conceived embryos of the couple or of other couples treated at the same time or stored in the same container. The management of HCV-infected men enrolled in programs of medically assisted reproduction is highly dependent upon the definition of standardized protocols of detection of HCV RNA in semen. Actually, although previous studies have reported the presence of HCV RNA in seminal plasma of men chronically infected with HCV and coinfected (7, 10, 15) or not coinfected (12, 13) with human immunodeficiency virus (HIV), other investigators have found the opposite pattern (6, 8, 16). Those contradictory results could be explained, at least in part, by the heterogeneity of the populations studied and by the diversity and the poor standardization of the techniques used for the extraction of RNA from semen and for the reverse transcription (RT)-PCR protocols. Moreover, the cellular fractions of semen, particularly the motile spermatozoa, have been poorly investigated for the presence of HCV.

We report herein on the detection of HCV RNA in seminal plasma and, for the first time, in the motile spermatozoon fractions of semen from men chronically infected with HCV and attempting medically assisted reproduction.

MATERIALS AND METHODS

Patients. Thirty-two male patients (mean age, 39 years; age range, 32 to 52 years) entering a program of ART were included in the study after they gave their fully informed written consent. All men tested positive for HCV RNA in blood by RT-PCR and were negative for HIV antibodies and hepatitis B surface antigen. The risk factors for HCV contamination were transfusion of blood products in 4 men (12.5%), hemophilia in 2 men (6.3%), intravenous drug addiction in 8 men (25%), and unidentified in 18 men (56.2%). No patient had received any antiviral drugs for chronic hepatitis C.

Sequential samples of semen were obtained from nine men: seven men each gave two samples (mean delay between collection of the two samples, 12.4 ± 8 months), one man gave three sequential samples within 8 months, and one man gave seven successive samples within 11 months.

Samples. Plasma samples were separated from blood by centrifugation and frozen at −80°C until use. After 3 days of sexual abstinence, semen samples were obtained by masturbation into a sterile container and were processed within 2 h of ejaculation. One milliliter of the semen sample was centrifuged at 800 × g for 10 min, and the seminal plasma was separated from the cell pellet and stored at −80°C until further use (fraction 1). Another milliliter was submitted to centrifugation through a three-layer discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (3 ml of 50, 70, and 90% Percoll, respectively); the motile spermatozoa were recovered from the 90% fraction, observed by microscopic examination for number and to check for the absence of white blood cell contamination, and kept frozen at −80°C as aliquots of 500,000 cells until use (fraction 2). For one patient, 0.5 ml of sterile synthetic medium (BM1; Ellios Biotek Laboratories, Paris, France) used for in vitro cell culture was layered on the top of the 90% fraction obtained after Percoll selection; after incubation for 45 min at 37°C in a 5% CO_2 incubator, the upper 0.5 ml was carefully removed and the spermatozoa were counted. This "swim-up" fraction was kept frozen at −80°C as aliquots of 500,000 cells until use (fraction 3) (5).

* Corresponding author. Mailing address: Laboratoire de Bactériologie-Virologie, Hôpital Nord, CHU de Saint-Etienne, 42055 Saint-Etienne Cedex 02, France. Phone: 00 33 4 77 82 83 15. Fax: 00 33 4 77 82 84 60. E-mail: Bruno.Pozzetto@univ-st-etienne.fr.

TABLE 1. Characteristics of the four men whose seminal plasma was positive for HCV RNA

| Patient no. | Age (y) | Detection of HCV RNA in first set of samples tested[a] | | | Genotype of HCV in blood and semen |
		Viral load in blood (log no. of copies)	Viral load in seminal plasma (qualitative result/log copies viral load)	Result for Qualitative motile spermatozoa	
1	40	6.23	POS/3.40	NEG	1a
2	38	6.20	POS/2.88	NEG	3a
3	44	7.34	POS/<2	NEG[b]	3
4	52	6.32	POS/2.89	NEG	2

[a] POS, positive; NEG, negative.
[b] Further spermatozoon fractions from this patient were found to be positive (see text).

Detection of HCV RNA in blood plasma. The qualitative detection of HCV RNA in blood plasma was performed by the Cobas AMPLICOR HCV assay (version 2.0; Roche Diagnostics, Meylan, France), according to the instructions of the manufacturer. The quantification of HCV RNA was achieved by the Cobas AMPLICOR HCV Monitor assay (version 2.0; Roche Diagnostics).

Detection of HCV RNA in seminal plasma (fraction 1). The RNA extraction step was performed by a protocol modified from that of the QIAmp viral RNA kit (Qiagen, Courtaboeuf, France), as follows: 250 µl of thawed fraction 1 was diluted (vol/vol) in sterile water and centrifuged at $20,000 \times g$ for 1 h; the supernatant was removed, and the resulting pellet was resuspended in 600 µl of AVL lysis buffer (Qiagen) containing 6.4 µl of the internal control from the Roche Cobas AMPLICOR assay. After an incubation step of 10 min at room temperature, 600 µl of absolute ethanol was added and the whole mixture was centrifuged through a QIAmp column at $6,000 \times g$ for 1 min. After two successive washing steps with AW1 buffer for 1 min at $6,000 \times g$ and AW2 buffer for 3 min at $12,000 \times g$, the RNA extracts were removed from the column by addition of 200 µl of AVE elution buffer and were centrifuged at $6,000 \times g$ for 1 min. RT and qualitative PCR were done by the Cobas AMPLICOR HCV assay (version 2.0) according to the instructions of the manufacturer. The quantification of HCV RNA in seminal plasma was carried out as follows: the RNA extraction step was similar to that for the qualitative protocol, except that the volume of elution buffer was 400 µl instead of 1,000 µl. RT and quantitative PCR were done by the Cobas AMPLICOR Monitor HCV assay (version 2.0) as recommended by the manufacturer. The final result was expressed as the number of copies per milliliter with reference to the volume of 250 µl used in the extraction step. The sensitivities of the qualitative and quantitative assays were determined by using serial 2-fold dilutions (from 160 to 10 copies/ml) or 10-fold dilutions (from 10,000 to 10 copies/ml), respectively, in HCV-negative seminal plasma of blood plasma from an HCV-infected patient, which had been quantified previously by the Cobas AMPLICOR 4CV Monitor assay; then, each dilution was extracted and tested in six independent experiments.

Detection of HCV RNA in spermatozoon fractions. Twenty-five fractions 2 (motile spermatozoa) from 14 patients and four fractions 3 (swim-up spermatozoa) from 1 patient were tested for the presence of HCV RNA. The RNA extraction was performed by combining a lysis step with proteinase K (50 mg/ml) by use of the RNAeasy Mini kit (Qiagen). With reference to the standard Qiagen protocol, an additional step of elution of the column was performed with the first eluate. The internal control of the Cobas AMPLICOR HCV kit was diluted 1:16 in 50 µl of the column eluate. RT and qualitative PCR were done according to the instructions of the manufacturer. In addition, a test with an extraction control was performed by RT-PCR amplification of an intron-containing region of the protamin 2 gene, as described previously (14). Use of a threshold of 500,000 cells in the PCR experiments was justified by the reproductive amplification of the control gene at this cell concentration but not at a lower one.

HCV genotyping. The genotypes of the HCV strains in blood and seminal samples were determined by sequencing the 5′ noncoding region by the TruGene HCV assay (Visible Genetics, Espinay sur Orge, France).

Statistical analysis. Quantitative and qualitative values were compared by Student's t test and the chi-square test, respectively. The threshold for a significant value was 0.05.

RESULTS

Detection of HCV RNA in blood and seminal plasma. All 32 patients included in the study were positive for the presence of HCV RNA in blood plasma. Their mean viral load was $5.97 \pm$ 0.51 log copies/ml (range, 4.97 to 7.34 log copies/ml). The genotypes of the HCV strains detected in the blood samples of 24 subjects were distributed as follows: 1 (two patients), 1a (seven patients), 1b (four patients), 2 (three patients), 3 (one patient), 3a (six patients), and 4 (one patient).

PCR inhibitors were detected in only 1 of 32 (3.3%) seminal plasma specimens, but they were not detected after the seminal sample was diluted 1:2 in RNase-free water. The sensitivity of the qualitative assay, determined in six independent experiments as the lowest dilution found to be positive in all the experiments, was 40 copies/ml (1.6 log copies/ml). By using the same algorithm, the sensitivity of the quantitative assay was 100 copies/ml (2 log copies/ml).

Seminal plasma samples from 4 of 32 patients (12.5%) were found to be positive for HCV RNA by the qualitative test. The four patients had been infected through intravenous drug abuse. The seminal viral loads of these four patients are listed in Table 1. The difference in viral loads between blood and semen ranged from 2.83 to more than 5.34 log copies/ml. The mean blood viral load was significantly higher in patients positive for HCV RNA in semen than in those negative for HCV RNA in semen (6.52 ± 0.55 versus 5.88 ± 0.46 log copies/ml [$P = 0.02$]) (Table 2). For the four patients positive for HCV RNA in semen, the same HCV genotype was found in both blood and seminal plasma (Table 1).

Analysis of sequential seminal samples. Successive seminal specimens were obtained from nine patients. Of the seven subjects who each provided two seminal samples, the detection of HCV RNA in seminal plasma was found to be unchanged over time in six patients (negative in five patients and positive in one patient); in the remaining patient, HCV RNA was detected only in the first seminal sample. For one man, three sequential samples, collected within 8 months, tested negative for HCV RNA. For another man (patient 3 in Table 1) who provided seven successive samples within 11 months, all the seminal plasma fractions tested positive for HCV RNA (with the quantities ranging from <100 to 860 copies/ml).

Detection of HCV RNA in spermatozoon fractions. The corresponding motile spermatozoa (fraction 2) collected from the four patients whose seminal plasma was HCV RNA positive were tested for HCV RNA. In addition, 15 fractions 2 belonging to 10 patients whose seminal plasma was negative for HCV RNA were tested. All fractions 2 tested remained negative for HCV RNA with the exception of two of five samples collected from patient 3, in which HCV RNA was persistently detected in seminal plasma from July 2000 to May 2001. Fraction 2 of

TABLE 2. Characteristics of patients whose blood was chronically infected with HCV according to presence or absence of HCV RNA in seminal fluid

Criterion	Patients with HCV RNA in seminal plasma	Patients without HCV RNA in seminal plasma	P value
Mean (SD[a]) age (yr)	43.5 y (6.2) (n^b = 4)	38.5 y (3.9) (n = 28)	NS[c]
Mean (SD) HCV load in blood (log no. of copies/ml)	6.52 (0.55) (n = 4)	5.88 (0.46) (n = 24)	0.02
% Non-type 1 HCV genotypes	75 (n = 4)	40 (n = 20)	NS
Mean (SD) vol of semen (ml)	3.08 (0.30) (n = 4)	3.20 (1.77) (n = 28)	NS
Mean (SD) no. of motile spermatozoa	33.9×10^6 (36.2×10^6) (n = 4)	19.7×10^6 (22.3×10^6) (n = 28)	NS

[a] SD, standard deviation.
[b] n is the number of patients tested.
[c] NS, not significant.

patient 3 was positive for HCV RNA only for a short time corresponding to the first 2 weeks of April 2001; moreover, the four samples of fraction 3 (swim-up spermatozoa) from patient 3 tested negative during the course of the study.

The efficiency of the extraction step with the spermatozoon fractions was evaluated by the detection of protamin 2 gene RNA, and all specimens tested were found to be positive. In addition, cell samples were found to be positive for the internal control, demonstrating the absence of PCR inhibitors in these fractions.

Assays after assisted reproduction. Eleven attempts at medically assisted reproduction were performed for couples for whom the male partner tested negative for the presence of HCV RNA in seminal plasma. Five women became pregnant, with the occurrence of two singleton births and with one set of triplets and two sets of twins being born. The blood of the nine babies tested negative for HCV RNA at birth, and the babies remained uninfected at least 6 months later.

For patient 3, given that HCV RNA was detected in all seminal plasma samples tested and in two motile spermatozoon fractions, the in vitro fertilization program with fresh semen was discontinued. When the motile spermatozoon fractions became negative, they were frozen in specific highly secure straws for future ART. For the three other patients whose seminal plasma was positive for HCV RNA, the ART were delayed.

DISCUSSION

The overall results of this study show that the seminal plasma of 4 of 32 (12.5%) subjects chronically infected with HCV were found to be positive for HCV RNA by the RT-PCR technique. Previous studies on the same topic gave conflicting results: three failed to detect any HCV-positive seminal sample, and two reported anecdotal cases of HCV-positive semen. In addition, two reported that relatively high proportions of patients were positive for HCV RNA in this compartment: 8 of 21 (38%) patients and 4 of 20 (20%) patients in the studies of Leruez-Ville et al. (10) and Pasquier et al. (15), respectively. The HIV serological status is an important point to be addressed since HIV has been shown to be a factor that dramatically enhances the pathogenicity of HCV in the livers of patients infected with both viruses (3, 17). Altogether, 35 of the 41 patients tested in the two studies cited above were HIV positive (10, 15), and 10 of them exhibited HCV RNA in their semen. In comparison, only six HIV-negative subjects (two of

whom were positive for HCV RNA in their semen) were tested in the first study and no HIV-negative subjects were tested in the second one. These small numbers of patients do not allow statistical comparison of the prevalence of HCV RNA in the semen of HIV-negative subjects, especially since no data concerning the selection of patients were available. Conversely, the 12.5% rate of HCV RNA positivity for the semen of 32 consecutive HIV-negative subjects in the present study seems more representative of the actual rate of positivity for this compartment.

The threshold of the assay reached 40 copies/ml, with a very low rate of false-negative results due to the presence of PCR inhibitors, as demonstrated by the use of a standardized commercial technique that included an internal control. This high rate of sensitivity compared to that of the protocol used in the standard AMPLICOR assay with blood samples can be explained by the addition of a step consisting of high-speed centrifugation of the sample. It was close to the one reported for seminal specimens by Leruez-Ville et al. (10), who used the same PCR kit used in the present study but a silica-based extraction technique.

In addition to previous studies on the same topic, this report provides several new findings: (i) the amount of viral RNA in seminal plasma was larger than 500 copies/ml in three of the four positive patients and was up to 3,000 copies/ml in one patient; (ii) in all four of these patients, the genotypes of the strains in the blood and semen of the same patient were found to be identical; and (iii) a positive correlation between the viral loads in seminal plasma and blood was noted. None of these data describe the compartmentalization of HCV RNA between seminal plasma and blood. They suggest that the semen of patients who are chronically infected with HCV for a long time and who exhibit high viral loads are more likely to be positive for HCV RNA; similar findings have been shown for HIV-infected patients (4, 18).

Another finding original to this study was the detection of HCV RNA not only in seminal plasma but also in spermatozoon fractions used in ART. Relevant controls were used to validate the extraction and amplification steps performed on these cells: for all samples tested the RNA of the protamin 2 gene was amplified and the internal control of the PCR assay was positive. In one patient whose blood and seminal plasma were both chronically infected with HCV, two successive samples of the motile spermatozoon fraction were found to be positive for HCV RNA, whereas the same test performed with

spermatozoa corresponding to the swim-up fractions was negative. This observation, which to our knowledge has never been described previously, needs discussion; although the possibility that spermatozoa support HCV replication cannot be definitively excluded, the more valuable explanation relies on the passive adsorption to the cell membrane of HCV RNA or virions present in seminal plasma, as suggested by the intermittent detection of HCV RNA in spermatozoa and by the absence of RNA in the corresponding swim-up fraction. In fact, the adsorption of viral particles to cell membranes could reduce the mobility of the spermatozoa, as described previously for cytomegalovirus (11).

Even though the detection of HCV RNA in fractions of semen does not necessarily imply the presence of replicative virions and the infectivity of spermatozoa via medically assisted reproduction, our results plead for the reinforcement of precaution measures for men whose blood is chronically infected with HCV and who are candidates for ART. It is suggested that seminal plasma be systematically evaluated for the absence of viral RNA in order to ensure that ART are performed with samples free of HCV RNA. In those subjects found to be positive, it is proposed that motile spermatozoa be investigated for the presence of HCV RNA and that only spermatozoon fractions that test negative be used for ART. French authorities have recently recommended a similar algorithm (2). Since HCV loads can vary dramatically within a few days both in seminal plasma and in spermatozoa, the safest approach to ART for patients exhibiting HCV RNA in seminal plasma would consist of the use of cryopreserved cells that tested negative at the time of sampling. Although the uneventful deliveries of infants to the cohort evaluated in the present study are encouraging, the validation of this guideline would need to be assessed with a larger population of chronically infected individuals.

REFERENCES

1. **Ackerman, Z., E. Ackerman, and O. Paltiel.** 2000. Intrafamilial transmission of hepatitis C virus: a systematic review. J. Viral Hepatitis **7:**93–103.
2. **Anonymous.** 2001. Arrêté du 10 Mai modifiant l'arrêté du 12 Janvier 1999 relatif aux règles de bonnes pratiques cliniques et biologiques en assistance médicale à la procréation, p. 7735–7736. Journal Officiel de la République Française, Paris, France.
3. **Bonacini, M., S. Govindarajan, L. M. Blatt, P. Schmid, A. Conrad, and K. L. Lindsay.** 1999. Patients co-infected with human immunodeficiency virus and hepatitis C virus demonstrate higher levels of hepatic HCV RNA. J. Viral Hepatitis **6:**203–208.
4. **Bourlet, T., C. Cazorla, P. Berthelot, F. Grattard, F. Cognasse, A. Fresard, C. Defontaine, F. R. Lucht, C. Genin, and B. Pozzetto.** 2001. Compartmentalization of HIV-1 according to antiretroviral therapy: viral loads are correlated in blood and semen but poorly in blood and saliva. AIDS **15:**284–285.
5. **Carrell, D. T., P. H. Kuneck, C. N. Peterson, H. H. Hatasaka, K. P. Jones, and B. F. Campbell.** 1998. A randomized, prospective analysis of five sperm preparation techniques before intrauterine insemination of husband sperm. Fertil. Steril. **6:**122–126.
6. **Debono, E., P. Halfon, M. Bourliere, V. Gerolami-Santandrea, M. Gastaldi, P. Castellani, G. Cartouzou, D. Botta-Frislund, P. Cau, and A. Gauthier.** 2000. Absence of hepatitis C genome in semen of infected men by polymerase chain reaction, branched DNA and in situ hybridization. Liver **20:**257–261.
7. **Fiore, R. J., D. Potenza, L. Monno, A. Apice, M. Distephano, A. Gianelli, L. Lagrasta, C. Romanelli, C. Dibari, and G. Pastore.** 1995. Detection of HCV RNA in serum and seminal fluid from HIV-1 co-infected intravenous drug addicts. J. Med. Virol. **46:**364–367.
8. **Fried, M. W., M. Shindo, T. L. Fong, P.C. Fox, J. H. Hoofnagle, and A. M. Di Bisceglie.** 1992. Absence of hepatitis C viral RNA from saliva and semen of patients with chronic hepatitis C. Gastroenterology **102:**1306–1308.
9. **Halfon, P., H. Riflet, C. Renou, Y. Quentin, and P. Cacoub.** 2001. Molecular evidence of male-to-female sexual transmission of hepatitis C virus after vaginal and anal intercourse. J. Clin. Microbiol. **39:**1204–1206.
10. **Leruez-Ville, M., J. M. Kunstmann, M. De Almeida, C. Rouzioux, and M. L. Chaix.** 2000. Detection of hepatitis C virus in the semen of infected men. Lancet **356:**42–43.
11. **Levy, R., F. Najioullah, B. Keppi, D. Thouvenot, S. Bosshard, J. Lornage, B. Lina, J. F. Guerin, and M. Aymard.** 1997. Detection of cytomegalovirus in semen from a population of men seeking infertility evaluation. Fertil. Steril. **68:**820–826.
12. **Levy, R., J. C. Tardy, T. Bourlet, H. Cordonier, F. Mion, J. Lornage, and J. F. Guerin.** 2000. Transmission risk of hepatitis C virus in assisted reproductive techniques. Hum. Reprod. **15:**1083–1085.
13. **McKee, T. A., S. Avery, A. Majid, and P. R. Brinsden.** 1996. Risks for transmission of hepatitis C virus during artificial insemination. Fertil. Steril. **66:**161–163.
14. **Miller, D., P. Z. Tang, C. Skiner, and R. Lilford.** 1994. Differential RNA fingerprinting as a tool in the analysis of spermatozoal gene expression. Hum. Reprod. **9:**864–869.
15. **Pasquier, C., M. Daudin, L. Righi, L. Berges, L. Thauvin, A. Berrebi, P. Massip, J. Puel, L. Bujan, and J. Izopet.** 2000. Sperm washing and virus nucleic acid detection to reduce HIV and hepatitis C virus transmission in serodiscordant couples wishing to have children. AIDS **14:**2093–2099.
16. **Semprini, A. E., T. Persico, V. Thiers, M. Oneta, R. Tuveri, P. Serafini, A. Boschini, S. Giuntelli, G. Pardi, and C. Brechot.** 1998. Absence of hepatitis C virus and detection of hepatitis G virus/GB virus C RNA sequences in the semen of infected men. J. Infect. Dis. **177:**848–854.
17. **Soriano, V., J. Garcia-Samaniego, R. Rodriguez-Rosado, J. Gonzalez, and J. Pedreira.** 1999. Hepatitis C and HIV infection: biological, clinical, and therapeutic implications. J. Hepatol. **31:**121–123.
18. **Tachet, A., E. Dulioust, D. Salmon, M. De Almeida, S. Rivalland, L. Finkielsztejn, I. Heard, P. Jouannet, R. Sicard, and C. Rouzioux.** 1999. Detection and quantification of HIV-1 in semen: identification of a sub-population of men at high potential risk of viral sexual transmission. AIDS **13:**823–831.