Human Reproduction Vol.17, No.12 pp. 3153–3156, 2002

A modified RT–PCR technique to screen for viral RNA in the semen of hepatitis C virus-positive men

N.G.Cassuto[1,6], C.Sifer[2], G.Feldmann[2], D.Bouret[1], F.Moret[3], J.L.Benifla[4], R.Porcher[5], M.Naouri[4], A.Neuraz[4], S.Alvarez[4], C.Poncelet[4], P.Madelenat[4] and A.Devaux[2]

[1]Laboratoire Drouot, 75009 Paris, [2]Service d'Histologie-Embryologie-Cytogénétique et Biologie Cellulaire, Hôpital Bichat-Claude Bernard, 75018 Paris, [3]Laboratoire Roche, 38240 Meylan, [4]Service de Gynécologie-Obstétrique, Hôpital Bichat–Claude Bernard, 75018 Paris, [5]Departement de Biostatistique et d'Informatique Médicale, CHU Saint-Louis, 75010 Paris, France

[6]To whom correspondence should be addressed at: Laboratoire Drouot, Unité AMP, 19, rue Drouot, 75009 Paris, France. E-mail: ninocassuto@aol.com

BACKGROUND: Our objective was to use an adapted RT–PCR technique to assess the presence of hepatitis C virus (HCV) in semen and also in different density gradient semen fractions collected from men with chronic viral hepatitis participating in an assisted reproduction programme. METHODS: This study included 50 semen samples from 35 HCV$^+$ men, with active viral replication assessed by RT–PCR, collected the day of oocyte retrieval and used for assisted reproduction. These samples were subjected to standard assisted reproduction sperm preparation conditions, using density-gradient centrifugation with 45 and 90% layers. Aliquots of semen, 45 and 90% fractions, and embryo culture media were frozen at –80°C for subsequent virological analyses. All aliquots were tested with a commercially available HCV RNA assay, adapted for use with semen after a number of technical changes. This assay yielded a sensitivity of 50–100 HCV RNA copies/ml and strongly diminished the effect of seminal amplification inhibitors. RESULTS: HCV RNA was detected in 7/50 (14%) semen samples tested, 5/35 (14.3%) men. HCV RNA was found in only 1/50 45% fractions but never in the 90% fraction or embryo culture media. Sera from 3/5 men contained 3.19–7.40$\times 10^5$ IU/ml, while the two others had 4.5 and 11.7$\times 10^6$ IU/ml. However, HCV RNA was quantified at <600 IU/ml in the HCV$^+$ semen of these five patients. The ongoing pregnancy rate was of 20% (10/50) with one delivery at the time of the present report. No anti-HCV antibody was found in any of the women or the newborn. CONCLUSIONS: Although HCV is present at low concentrations in the semen of a few HCV$^+$ patients, no purified sperm fraction (i.e. 90% fraction) used in assisted reproduction was HCV$^+$ and no seroconversion was observed in the women and the newborn, thereby suggesting a very low risk of virus transmission. Nevertheless, because the presence of HCV in semen implies a possible risk of nosocomial contamination, safety regulations must be strictly applied in assisted reproduction laboratories.

Key words: hepatitis C virus/IVF/purified sperm/sperm

Introduction

The presence of hepatitis C virus (HCV) RNA in the sperm of HCV$^+$ men is still being debated. Several technical problems could explain why some authors found HCV RNA in the semen of these patients (Liou *et al*., 1992; Liu *et al*., 1994; Tang *et al*., 1996; Leruez-Ville *et al*., 2000; Levy *et al*., 2000; Pasquier *et al*., 2000) whereas others reported that the virus was undetectable (Hsu *et al*., 1991; Fried *et al*., 1992; Terada *et al*., 1992; Caldwell *et al*., 1996; Semprini *et al*., 1998; Debono *et al*., 2000). Among them, the lack of sensitivity of the techniques and the presence of seminal amplification inhibitors are thought best able to explain the discrepancies (Semprini *et al*., 1998; Debono *et al*., 2000; Levy *et al*., 2000). Until now, because of the difficulty in detecting HCV RNA in semen, assisted reproductive technologies have not been authorized in France for HCV$^+$ men. However, since February 2000, a national research programme has begun to allow French assisted reproduction centres to include male HCV$^+$ partners of infertile couples. This protocol recommends that assisted reproduction centres establish a special organization to avoid any nosocomial viral risk. In particular, the search for HVC RNA in semen is obligatory before starting assisted reproduction.

The aim of the present study was to report our first results regarding HCV RNA detection in the semen and sperm fractions of 35 HCV$^+$ men. To increase the sensitivity of the technique used to detect HVC RNA and to decrease the influence of seminal amplification inhibitors, several changes were made in the technique originally used to detect HCV RNA in serum. We also report herein the preliminary results regarding the follow-up of the women after assisted reproduction treatment and children conceived.

Materials and methods

Patients

Thirty-five couples, in which the men were HCV^+ with active viral replication, were enrolled in the study and attempted assisted reproduction 50 times with purified sperm from 50 semen samples. We measured the serum viral loads in all these men with a Cobas Amplicor HCV Monitor 2.0 Assay (Roche, Meylan, France) and ensured that none of them had received any antiviral treatment during the 7 months preceding their inclusion in our programme. Their viral loads in serum ranged between 600 IU/ml and 12×10^6 IU/ml. Their mean age was 41 years (range 33–58). Causes of HCV contamination were i.v. drug addiction for 16 patients (46%), blood transfusion for two patients, kidney transplantation for one, miscellaneous for three others, and were unknown for 13 patients (37%). No patient was positive for Hep B surface antigen (V2 AXSYM System; Abbott Laboratories, Chicago, IL, USA) and we did not find any human immunodeficiency virus type-1 or -2 (HIV-1/2) antibodies as tested with a specific Microparticle Enzymatic Immuno Assay (AXSYM System; Abbott Laboratories) and by ImmunoComb II HIV-1/2 BiSpot (PBS Orgenics, Courbevoie, France). Infertility of the 35 men or their partners was assessed by the clinical history, the absence of pregnancy or the failure to achieve a term of pregnancy for 1–11 years (mean: 5.5 years). All sperm were free of bacteria, including chlamydia and mycoplasma. The sperm count was (0.1–600) $\times10^6$/ml and the motility was between 1 and 50%. Motility and vitality were normal for 16 men. Causes of infertility were tubal diseases (five patients), dysovulation and ovarian disease (five patients), male factor (19 patients) with five severe and 14 moderate cases of oligoasthenozoospermia, undetermined infertility (one patient) and mixed (five patients). The decision to perform standard IVF (n = 26) or ICSI (n = 24) was made based on previously described clinical and biological parameters (Benifla *et al*., 2001). Ovarian stimulation was performed in all women according to the protocol used in our centre (Benifla *et al*., 2001). Briefly, ICSI was performed in our study in cases of oligoasthenoteratozoospermia when the total number of motile sperm selected by gradient-density centrifugation was $<5\times10^5$ or when previous standard IVF attempts failed completely to obtain oocyte fertilization. On the day of oocyte retrieval, aliquots of total sperm, 45 and 90% gradient-density fractions were obtained after centrifugation using Puresperm (Nidacon International, Gothenburg, Sweden) according to the manufacturer's recommendations, and were stored at –80°C until HCV RNA detection. In addition, aliquots of embryo culture medium B2 upgraded (CCD, Paris, France) or Universal IVF medium (MediCult, Lyon, France) were also frozen at the same temperature, and tested.

HCV RNA tests

Cobas Amplicor HCV 2.0 Assay (Roche) was used for HCV RNA detection in semen after making four changes, mainly to the extraction of HCV RNA. First, an aliquot of 200 µl of semen was diluted in 400 µl phosphate-buffered saline (PBS) in order to dilute the amount of seminal inhibitory factors, since it has been reported that these factors are mainly present in seminal fluid (Semprini *et al*., 1998; Debono *et al*., 2000). Second, the cell suspension was centrifuged at 28 000 *g* for 1 h at 8°C in a Heraeus ultracentrifuge (Kendro, Courtabeuf, France). Third, after discarding the supernatant, the pellet was lysed with 600 µl (rather than 400 µl) lysis buffer (Roche). After these three steps, the standardized Cobas Amplicor HCV 2.0 technique was performed. Briefly, the lysate was put into a warm dry oven for 10 min at 60°C and vortexed. Isopropanol (600 µl) was added for 2 min at room temperature. The solution was centrifuged for 15 min at 13 600 *g* at 20°C. The supernatant was discarded and 1 ml of 70° ethanol was added to the pellet. The resuspended pellet was again centrifuged at 13 600 *g* for 5 min. The supernatant was discarded and 200 µl of HCV diluant (Roche) was added to the pellet. Finally, a last modification of the technique was made: the pellet was disrupted, mixed for 10 s and centrifuged again at 28 000 *g* at 8°C for 5 min. HCV RNA was amplified and detected according to the manufacturer's instructions.

Table I. Detection of HCV RNA in semen samples titrated at various viral loads using an adapted RT–PCR

Semen viral load[a] (copies/ml)	No. of HCV RNA detection tests	No. HCV^+ (%)
8000	9	9/9 (100)
1600	9	9/9 (100)
800	9	9/9 (100)
100	9	9/9 (100)
50	9	6/9 (66.6)
25	9	3/9 (33.3)

[a]Three semen samples of each viral load were prepared. Each sample from 8000 to 25 copies/ml was tested three times for HCV RNA detection, corresponding to a total test number of nine for each viral load. The calculated sensitivity of our adapted RT–PCR in semen was estimated to be between 50 and 100 copies/ml.

The following preliminary studies were conducted to validate the four changes. First, 100 µl of titrated serum containing 80 000 copies/ml of HCV RNA were added to 900 µl of pooled HCV^- semen from HCV^- men to obtain a sample titrated at 8000 copies/ml. This sample was diluted twice with the pooled HCV^- semen at 1/5 and 1/10 to obtain viral loads respectively of 1600 and 800 copies/ml. For each load, 200 µl samples were centrifuged in triplicate at 1500 *g* for 15 min. Under these conditions, following the manufacturer's procedures for the serum HCV detection, the virus was never detected in any specimen including internal controls. We hypothesized that this result might be caused by either the presence of seminal inhibitory factors of amplification or the insufficient speed of centrifugation. Using the same stock preparations, we diluted the 200 µl sample with 400 µl of PBS and then increased the speed of the initial centrifugation from 1500 to 16 000 *g* for 10 min. In the absence of inhibition (all the internal controls were positive), the samples initially titrated at 8000 and 1600 copies/ml were positive but the 800 copies/ml samples were negative. Because the official and registered sensitivity for the Cobas Amplicor HCV 2.0 Assay applied to serum is 100 copies/ml, a third test was undertaken with an initial centrifugation in PBS at 24 000 *g* for 1 h. The three viral loads (8000, 1600 and 800 copies/ml) were then positive. To calculate the sensitivity of our adapted procedure, we prepared samples with three low viral loads, 100, 50 and 25 copies/ml, as follows. The sample titrated at 800 copies/ml was diluted at 1/8 with the pooled HCV^- semen to obtain a viral load of 100 copies/ml which was diluted again at 1/2. The obtained sample titrated at 50 copies/ml was then diluted at 1/2 resulting in a viral load of 25 copies/ml. Each viral load from 8000 to 25 copies/ml was prepared in triplicate and each replica was tested three times for HCV RNA detection. The calculated sensitivity in semen was estimated to be between 50 and 100 copies/ml (Table I).

To prevent false-positive results due to cDNA contamination, dUTP-uracil-DNA-glycosylase was added to the samples.

Statistical analysis

Serum viral loads in HCV^+ semen and HCV^- semen groups are expressed as means ± SD and were compared using the Wilcoxon rank sum test with the level of significance set at 5%.

Table II. Different characteristics of the five men (seven semen samples) whose semen was HCV RNA$^+$

Characteristic	Semen samples						
	A	B		C		D	E
	1	1	2	1	2	1	1
Age (years)	36	36	36.5	40	40.5	34	36
Duration of infertility (years)	4	4	4.5	4	4.5	7	2
Mode of HCV contamination	surg	ivdu	ivdu	?	?	surg	ivdu
Sperm							
Count ($\times 10^6$/ml)	24.8	1.5	1.5	15	45	30	95
Progressive motility (%)	40	20	10	10	10	20	50
Assisted reproduction							
Technique	IVF	ICSI	ICSI	ICSI	ICSI	ICSI	IVF
Outcome	No P	P (Mis)	No P	No P	P (On)	No P	P (Mis)
HCV RNA detection							
Semen	+	+	+	+	+	+	+
45% fraction	–	+	–	–	–	–	–
90% fraction	–	–	–	–	–	–	–
Embryo media	–	–	–	–	–	–	–
Viral load							
Serum ($\times 10^6$ IU/ml)	4.5	0.319	0.198	11.7	10.5	0.708	0.740
Semen (IU/ml)	<600	<600	<600	<600	<600	<600	<600

P = pregnancy; No P = no pregnancy; Mis = miscarriage; On = ongoing; surg = surgery; ivdu = intravenous drug use; ? = unknown.

Results

HCV RNA detection and quantification

Among the 50 semen samples from 35 HCV$^+$ men, seven (14%) were HCV RNA$^+$ and had been collected from five (14.3%) men, two of whom participated in a second assisted reproduction attempt after a first failure (Table II). Their sperm, obtained 4 months and 9 months after the first positive sample, were again HCV$^+$. One of the five patients also had HCV RNA in the 45% density-gradient fraction, but no patient's 90% fraction contained HVC RNA. Serum viral loads in HCV$^-$ semen (n = 43) and HCV$^+$ semen (n = 7) groups were respectively 1498 ± 2693 and 3587 ± 5149$\times 10^6$ IU/ml and no statistically significant difference was found between these two groups. The amount of HCV RNA was <600 IU/ml in HCV$^+$ semen. HCV RNA was never detected in the embryo culture media (Table II).

Assisted reproduction and follow-up of women and children

IVF led to pregnancy in four women but two suffered miscarriage. One child has been born and another is almost at term. ICSI gave rise to 10 pregnancies, with two miscarriages and eight are ongoing. All the women and the baby born from the assisted reproduction attempts have remained negative for HVC RNA.

Discussion

The results of this study demonstrated the ability of our modified RT–PCR technique to efficiently decrease factors inhibiting Taq polymerase, known to be frequently present in the seminal fluid (Semprini *et al*., 1998; Debono *et al*., 2000; Leruez-Ville *et al*., 2000; Levy *et al*., 2000). Associated with high sensitivity, our modified RT–PCR technique seems to be an efficient one-step method, overcoming: (i) false-negative results which could explain the lack of HCV RNA detection in semen or seminal fluid from infected men, as reported in previous studies (Hsu *et al*., 1991; Fried *et al*., 1992; Terrada *et al*., 1992; Caldwell *et al*., 1996; Semprini *et al*., 1998; Debono *et al*., 2000); and (ii) the necessity, stated in other studies, to test two or more dilutions of the semen samples (Semprini *et al*., 1998; Levy *et al*., 2000).

The presence of HVC RNA in semen has been investigated for 10 years with various results. Thus, the frequency of HCV RNA detection in semen of infected men ranged from 0% (Hsu *et al*., 1991; Fried *et al*., 1992; Terada *et al*., 1992; Caldwell *et al*., 1996; Semprini *et al*., 1998; Debono *et al*., 2000), to 5% (2/39) (Levy *et al*., 2000), 24% (4/17) (Liou *et al*., 1992), 33% (2/6) (Leruez-Ville *et al*., 2000), 57% (4/7) (Tang *et al*., 1996) and even 100% (3/3) (Liu *et al*., 1994). We detected HCV RNA in 14% of the 50 tested sperm. Our findings confirm that the presence of HCV in sperm from infected men is variable, and, even though we have never documented virus transmission to the woman or child, the possibility of a contamination by HCV$^+$ sperm cannot be excluded. However, the HCV loads in the HCV RNA$^+$ semen samples were below the quantification threshold. This result is in agreement with those of Leruez-Ville *et al*. who showed that semen HCV loads were low (Leruez-Ville *et al*., 2000) and suggests that the risk of HCV sexual transmission is also probably very low, a point which is consistent with epidemiological data (Dienstag, 1997). We also observed that the virus persists in semen, since two patients whose semen samples were again collected and tested after an interval of 4 or 9 months remained HCV$^+$. This latter observation needs to be confirmed to determine whether the presence of HCV in

semen is persistent or intermittent and if any such persistence increases with time the risk of contamination in assisted reproduction treatment or sexual transmission.

When the viral status of sperm fractions, obtained after gradient-density centrifugation followed (Pasquier *et al*., 2000) or not (McKee *et al*., 1996; Levy *et al*., 2000) by a swim-up step, were studied, no HCV RNA was found in the purified 90% fraction of sperm, which is the one used for assisted reproduction. That finding was confirmed in our series, since HCV RNA was found only in the 45% fraction of 1/7 HCV^+ semen samples but never in the 90% fraction of purified sperm. The absence of virus in the latter could be explained by the association of low-speed centrifugation, routinely used to select sperm for assisted reproduction which does not concentrate HCV in the 90% fraction, and the very low HCV load in seminal fluid.

However, the risk of contamination does potentially exist both for the embryos resulting from an assisted reproduction performed in an HCV^- couple but incubated in the same incubator as those resulting from an HCV infected couple, and for the biologists and technicians performing the different steps of assisted reproduction procedures. That is why, until February 2000 in France, inclusion in an assisted reproduction programme of infertile couples, in which one member was HCV^+ with active viral replication, was prevented by the law. However, since February 2000, a multicentric study allowed assisted reproduction centres to include infertile couples with one or both HCV^+ partners who wanted to attempt assisted reproduction treatment to procreate. Thus, it will be possible to evaluate whether assisted reproduction raises the risk of HCV contamination for newborns, compared with HCV^+ women who become pregnant spontaneously. More recently, French law has again been modified, and since May 2001 all centres can include HCV^+ couples with active viral replication in assisted reproduction programmes. These centres must separate—in time or space—the inclusion of infected and uninfected HCV couples and, in the case of HCV^+ men, must freeze and test the semen or purified sperm before performing treatment, which can proceed only if HCV is not detected. Even though no HCV was detected in the purified fraction of sperm (McKee *et al*., 1996; Levy *et al*., 2000; Pasquier *et al*., 2000), the principle of precaution predominates in France since the HCV and HIV-1/2 blood contamination affair of the 1990s.

To the best of our knowledge, our preliminary results showed for the first time that standard IVF or ICSI techniques, including oocyte washing and changing embryo culture media, do not increase the risk of contamination for couples. Indeed, none of the tested embryo culture media inseminated with the 90% fractions of the seven HCV^+ semen samples was HCV RNA^+. Furthermore, none of the women nor the only newborn were infected after these assisted reproduction attempts. Therefore, these preliminary data strongly suggest that the HCV contamination risk is very low for infertile couples with HCV-infected men included in assisted reproduction programmes. Should this conclusion prove true for a larger population, freezing sperm from HCV infected men could become unnecessary. It is known that freezing sperm results in decreased motility, vitality and number of sperm cells (Donnelly *et al*., 2001) with, as an eventual consequence, poor oocyte fertilization and pregnancy rates during assisted reproduction treatment. Consequently, it is not obvious that freezing sperm is necessary in HCV^+ men because we observed, like all the other previous studies (McKee *et al*., 1996; Levy *et al*., 2000; Pasquier *et al*., 2000), that routine technical means can eliminate the virus. However, the possible presence of HCV in semen implies a potential risk of nosocomial contamination. Thus, to prevent this risk, safety rules must be strictly respected (Steyaert *et al*., 2000) in all assisted reproduction laboratories that include infertile couples with HCV^+ men.

Acknowledgements

The authors thank J.Jacobson for reviewing the English text.

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Submitted on March 1, 2002; resubmitted on June 13, 2002; accepted on August 25, 2002