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Transmission risk of hepatitis C virus in assisted reproductive techniques

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Medical assistance for procreation in a couple where one or both parents has hepatitis C viral infection (HCV) raises the issue of the transmission of the infection to the baby and/or of possible contamination of both the technicians and the gametes or embryos from virus-free parents in the laboratory. It becomes essential to assess transmission risk in assisted reproductive techniques in order to define clearly the management of couples according to their viral status. To define the HCV transmissibility risk in assisted reproduction related to the presence of virus in semen from infected infertile men, HCV RNA detection was performed in sera, and semen and sperm fractions obtained after Percoll gradient centrifugation. HCV RNA was detected in 5% (2/39) of the semen samples tested: in the raw semen, in the seminal fluid and in the cell pellet but never after Percoll selection. According to these results, we suggest a strategy for HCV-infected infertile men who need assistance for procreation.

Key words: assisted reproductive techniques/hepatitis C virus/viral screening

Introduction

The risk of viral transmissibility in assisted reproduction is still much debated, especially for hepatitis C virus (HCV) and in recent assisted fertilization techniques. Viral screening policies in assisted reproduction techniques differ in each country, and sometimes even between infertility centres (Abusheikha *et al*., 1999). Within the context of careful routine screening prior to each assisted reproduction attempt, the French bioethical law (Loi, 1994) makes provision for health security rules by announcing official guidelines that have only recently been published (Arrêté, 1999): assisted reproduction for HCV-infected male or female infertile patients is not authorized, except after agreement by two committees in a long and thorough procedure. Since the publication of these guidelines (Arrêté, 1999), assisted reproduction techniques for HCV-infected infertile patients have been stopped in French infertility centres.

HCV is the major causative agent for parenterally transmitted non-A, non-B hepatitis. HCV is a small RNA-encased linear virus, with ~10 000 nucleotides. Unlike human immunodeficiency virus (HIV), HCV has no reverse transcriptase activity, and, consequently, cannot succeed in DNA integration within infected cells. It is most important to note that no drug or preventive vaccine is presently fully effective against HCV.

In 1996, in France, the prevalence of anti-HCV antibodies was ~1%. About 550 000 persons are chronically infected with HCV: among them, 400 000 to 500 000 are viraemic for HCV (Roudot-Thoraval *et al*., 1993). In sera, the main genotypes are *1b* (50%, decreasing, observed mostly after contamination through transfusions), *1a* and *3a* (increasing, observed in drug-addicted patients).

Transmission of HCV occurs mainly through the parenteral route, up to 80% of persons exhibiting anti-HCV antibodies having a history of a parenteral exposure, and through the use of i.v. drugs or non-screened blood products. A French study (Roudot-Thoraval *et al*., 1996) analysed 6664 patients with chronic hepatitis C and concluded that 37% received contaminated blood products (before March 1990) or were haemophilic patients, 23.1% were i.v. drug abusers, 3.3% could be clearly attributed to professional transmission (medical context, nurses), 1.2% to sexual contact, and 14.9% to nosocomial transmission (haemodialysis), while the source of HCV was unknown in the remaining 23.8%.

Professional transmission also exists: 46% of professional hepatitis in 'Assistance Publique' hospitals in Paris in 1991 were attributed to HCV, and hepatitis C seroconversion has been demonstrated in hospital employees with needlestick injuries (Kiyosawa *et al*., 1991).

The estimated risk of vertical HCV transmission varies widely in the literature according to the study population and the tests used. In a prospective study, the authors concluded that there was a complete lack of vertical transmission of HCV in HIV seronegative women, even in the presence of active HCV replication (Roudot-Thoraval *et al*., 1993). In another study, none of the babies of the HCV RNA-negative women were positive for HCV RNA, but maternal transmission of HCV could be observed in babies born to mothers with high levels of viraemia ($>10^6$ copies/ml) (Ohto *et al*., 1994). The exact risk of mother-to-child transmission of HCV has not yet been quantified and the factors influencing the risk of this transmission remain to be evaluated (Gillet *et al*., 1996).

Although the parenteral mode of HCV transmission is well established, there remains a high proportion of patients without an identifiable source of infection. The potential infectivity of body fluids in HCV-infected patients has been identified by detection of HCV RNA in saliva, urine and ascites (Liou *et al*.,

1992; Numata *et al*., 1993; Young *et al*., 1993) but remains controversial in semen (Hsu *et al*., 1991; Fried *et al*., 1992). To study sexual transmission, epidemiological data were collected (Bresters *et al*., 1993) and semen of HCV-infected patients was analysed for the presence of HCV RNA (Fried *et al*., 1992; Fiore *et al*., 1995). Such studies yielded contradictory results (Table IV). McKee (1996) observed the disturbing presence of HCV RNA in different ejaculates of the same semen donor: HCV RNA detection became negative after a three-layer Percoll gradient. Particular attention must be paid to false-negative polymerase chain reaction (PCR) results due to the presence of *Taq* polymerase inhibitors in seminal fluid (Levy *et al*., 1997). Such inhibitors in seminal fluid were detected by Semprini *et al*. (Semprini *et al*., 1998) which is the reason why half of those results could not be interpreted. The authors emphasized the total absence of HCV RNA in seminal plasma, round cells and spermatozoa from HCV-infected fertile patients and gave reassuring advice to couples which was dangerously misleading.

We studied HCV viraemic infertile male patients. Performing assisted reproduction techniques in a patient with HCV infection and potentially HCV-infected gametes can lead to a contamination risk for (i) the couples' embryos, (ii) the other gametes and embryos originating from non-infected couples treated in the same period, (iii) technicians through infected cryopreserved semen storage and manipulation (Kiyosawa *et al*., 1991; Massey *et al*., 1996).

In this study, we looked for HCV RNA in the semen of HCV viraemic male patients. To understand which seminal fraction could serve as a reservoir for the virus and to test the sperm fraction used in IVF, we tested the ejaculates, the cell-free seminal plasma, cell pellet and spermatozoa.

Materials and methods

Study population

In all, 39 infertile patients (31–47 years old) who entered our IVF programme gave their fully informed written consent for using spare semen for research or diagnostic purposes. All were HIV-negative, hepatitis B antigen-negative but had anti-HCV antibodies and detectable serum HCV RNA. Blood plasma HCV RNA was quantified using the HCV-Monitor technique (Roche SA, Neuilly-sur-Seine, France) or the bDNA technique (Chiron Ciba Corning, Parc des Bellevues, France), according to the manufacturer's instructions. All subjects were asymptomatic with active chronic hepatitis.

HCV infection could be clearly attributed to blood products or haemophilia for five (13%) and three (7%) patients respectively; 12 (31%) were intravenous drug abusers. No HCV infection could be attributed to professional contamination or sexual contact. For three patients (7%), HCV infection could be related to nosocomial transmission (one through transplantation, one through endoscopy, one through haemodialysis). For 16 patients (38%), contamination circumstances remained unknown. All had increased alanine amino-transferase.

Semen parameters were evaluated following the criteria of the World Health Organization (WHO, 1992) and are given (Table I). Semen analysis was normal for 27 patients whereas 12 showed abnormalities: two oligozoospermia and 10 oligoasthenoteratozoospermia. The IVF indication was supported by male factor infertility in 30%, a female factor in 40% and by both male and female factors in 10% of the samples. Six couples had previously conceived children: the children have been tested negative for anti-HCV antibodies. None was taking any antiviral medication: however, three patients needed specific antiretroviral bitherapy (interferon alpha and ribavirin) but preferred to undergo the IVF procedure first because of the adverse effects of ribavirin.

HCV detection in semen

Ejaculates were collected into sterile containers by masturbation after 3 days of sexual abstinence. After liquefaction at 37°C, samples were examined using routine semen analysis techniques (WHO, 1992). Remaining steps were performed at room temperature. One millilitre of the semen sample (fraction 1) was centrifuged at 800 *g* for 10 min and the seminal fluid (fraction 3) was separated from the cell pellet (fraction 2). Another millilitre was submitted to centrifugation through a three-layer (3 ml of 50%, 70% and 90% respectively) discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden), to separate motile spermatozoa from seminal plasma, round cells and immotile spermatozoa. Each layer was subsequently separated, washed and re-suspended with 500 µl of BM1 medium (BM1®; Ellios Bio Media, Paris, France). The ejaculates and the samples obtained after centrifugation (seminal fluid, cellular pellet) and after the Percoll gradient were then counted before storage. After centrifugation and counting, seminal plasma was stored in a sterile plastic tube (fraction 3) as were the cell pellet containing both round cells and spermatozoa (fraction 2). After Percoll treatment and counting, each of the four fractions obtained were carefully washed in 5 ml of BM1 and centrifuged at 400 *g* for 10 min and stored in a sterile tube: seminal plasma (fraction 4), 50% layer (fraction 5) containing round cells and immotile spermatozoa, 70% (fraction 6), an intermediate fraction and 90% layer (fraction 7) fraction used for assisted reproduction procedure, containing selected spermatozoa exhibiting a high proportion of motile and normal-shaped spermatozoa; all the samples were kept frozen at –80°C until tested later for HCV. The different steps of the procedure were carried out within 2 h of ejaculation. This is important to note because of the possible action of RNAses.

RNA extraction

RNA extraction was performed on seven semen fractions for each patient: ejaculate (sample 1), cell-free seminal plasma (sample 3), cell pellet (sample 2), and, after Percoll selection, seminal plasma (sample 4), 50% (sample 5), 70% (sample 6) and 90% motile spermatozoa (sample 7). RNA extraction was performed according to the Amplicor HCV Specimen Preparation Kit (Roche SA) which was not specific for semen.

HCV RNA tests

Semen samples were tested using a reverse transcription assay with a commercial kit for amplification and detection of HCV RNA (Amplicor HCV Amplification Kit and Detection Kit; Roche SA). dUTP-uracil-DNA-glycosylase (Boehringer Mannheim, Mannheim, Germany) was included for carry-over prevention of false-positive results. All samples were tested in triplicate together with negative control samples, including HCV-negative serum and reagent blanks, to exclude contamination. The sensitivity of this assay is 100 copies of HCV genomes/ml.

Test for PCR inhibitors

In enzyme-based amplification processes, such as PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimen. Inhibitors of PCR reaction in semen fractions have been reported, but their exact nature is still unknown. To validate the negative results for HCV RNA, we used the AMPLICOR Internal Control (IC) Detection Kit (Roche SA) in conjunction with the Amplicor Microwell Plate (MWP; Roche SA) which identifies processed specimens containing substances that may interfere with PCR amplification (Table II). In these tests, an IC is introduced into each

Table I. Description of sperm parameters of hepatitis C virus (HCV)-infected patients

	Ejaculate volume (ml)	Sperm concentration ($\times 10^6$/ml)	Sperm motility (type a + b) (%)	Abnormal morphology (%)	Leukocytes ($\times 10^6$/ml)
Mean (n = 39)	3.0	52.4	34.3	50.6	0.6
Range	0.3–8.5	1.80–346	5–55	12–86	0–6.4

Table II. Thresholds for Amplicor internal control detection kit

OD A_{450}	OD A_{450} + HCV IC	
<0.25	>0.6	Positive[a] result
<0.25	<0.6	*Taq* inhibitors

Amplicor hepatitis C virus (HCV) detection kit: optical density (OD) A_{450}, <0.25 = negative result; 0.25–0.6 = undetermined; >0.6 = positive result.
[a]i.e. RNA HCV detection positive

amplification reaction and co-amplified with target RNA in the clinical specimen. We considered that *Taq* inhibitors were present for the samples that, upon addition of the HCV IC, gave negative results (<0.6). When inhibitors were detected in semen fractions, PCR was controlled on two concentrations of the specimen (undiluted and 1:4), to avoid false-negative PCR due to *Taq* inhibitors.

Results

Of the 39 male infertile patients with HCV RNA-positive sera, two had HCV RNA detectable in semen (Tables II and III). For one of these patients (no. 35), no inhibitor was present in semen fractions after Percoll selection but HCV RNA could clearly be detected in the ejaculate, seminal fluid and cell pellet. He was contaminated by HCV in 1982, and was an intravenous drug abuser. The genotype was *3a*. Semen data showed a severe oligoasthenoteratozoospermia and 240 000 leukocytes were present in the ejaculate.

For the other patient (no. 6), inhibitors were detected in fractions 1 (i.e. ejaculate) and 3 (i.e. seminal fluid). After dilution 1:4 of these semen fractions, we observed a positive result for a previously negative plasma seminal sample (fraction 3). Patient no. 6 was contaminated by HCV in 1970, and was also an intravenous drug abuser. Semen characteristics were normal with no leukocytes in the semen. The genotype was *1a*.

The test for PCR inhibitors revealed the presence of *Taq* polymerase inhibitors in all ejaculates (fraction 1) and seminal plasma fractions (fraction 3), except for six samples. Inhibitors were not detected either in cell pellet after centrifugation (fraction 2), or in semen fractions obtained after Percoll centrifugation (fractions 4, 5, 6 and 7). All motile sperm samples obtained after Percoll centrifugation were negative for HCV RNA detection (fraction 7).

For two patients (nos. 15 and 21) with previously negative samples, a second ejaculate was collected 1 month later. Both ejaculates were negative for HCV RNA detection. One patient had a retrograde ejaculation: urine was negative for HCV RNA. A second ejaculate was also collected for patient no. 6 and similar results were observed: inhibitors were detected in semen fractions no. 1 and 3; however, a positive result was clearly observed after dilution of a previously negative seminal fluid sample (fraction 3).

Quantification of HCV RNA in sera was performed for most of the infertile male patients (72%). The range was between 5331 copies per ml to 5×10^6 copies per ml. Both patients with semen-positive HCV RNA detection (nos. 6 and 35) had high amounts of HCV RNA in their sera: 1.7×10^6 and 1.6×10^6 copies/ml respectively.

Discussion

French recommendations strictly define prerequisite examinations, including an annual check for immune status for HIV antibodies, hepatitis B antigens, HCV antibodies and syphilis, that must be performed on both partners of infertile couples before their enrolment for assisted reproduction techniques. Positive anti-HCV antibody detection in patients' serum raises the medico-legal aspect which has resulted in most French IVF teams no longer carrying out the IVF procedure since the end of February 1999. However, data on antibody status does not provide a tool to differentiate between current, active infection, chronic infection and past, resolved infection. The aim of the present study was to investigate the detection of HCV RNA in semen and various semen fractions before assisted reproduction in infertile HCV-viraemic male patients. We were able to identify HCV RNA in two semen samples with a lowest detection limit of 100 copies of genome RNA per PCR assay. It is important to note that the tests for HCV carried out in this study do not preclude testing for other viruses such as cytomegalovirus (Levy *et al*., 1997) that might contaminate the semen.

Some recent reports have found a total absence of HCV RNA in semen (Hsu *et al*., 1991; Fried *et al*., 1992; Terada *et al*., 1992; Caldwell *et al*., 1996; Semprini *et al*., 1998), while other results suggest that HCV can be detected in the semen with a very low (Fiore *et al*., 1995) or a high prevalence (Liou *et al*., 1992; Kotwal *et al*., 1992; Kotwal, 1993; Liu *et al*., 1994; Tang *et al*., 1996) (Table V). These contradictory findings can be explained by differences in collection and/or storage of samples and in the sensitivity of the assays designed to detect HCV RNA or antigens. Since PCR can detect tiny amounts of viral RNA, stringent laboratory procedures must be employed to avoid false-positive results.

The occurrence of false-negative results due to the presence of PCR inhibitors in the ejaculates was an important concern in our study, and for this reason we tested each seminal fraction

Table III. HCV detection in semen fractions

No.	Semen fractions without *Taq* inhibitors	Semen fractions with *Taq* inhibitors, before and after dilution	Blood plasma HCV RNA quantification[a]
1	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	194 831 copies /ml
2	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	4.9 mEq/ml
3	2, 5, 6, 7 negative	1, 3, 4 (negative after dilution)	Not done
4	1, 2, 4, 5, 6, 7 negative	3 (negative after dilution)	Not done
5	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	3.8×10^4 copies/ml
6	2, 4, 5, 6, 7 negative	1,3 (3 positive after dilution)	1.7×10^6 copies/ml
7	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	0.7 mEq/ml
8	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
9	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
10	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	0.7 mEq/ml
11[b]	2 negative	1, 3 (negative after dilution)	5331 copies/ml
12	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
13	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	0.7 mEq/ml
14	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
15	2, 3, 4, 5, 6, 7 negative (no tube 1)	Absence of inhibitors	Not done
16	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
17	1, 2, 3,4,5, 6, 7 negative	Absence of inhibitors	Not done
18	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	3.1×10^5 copies/ml
19	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.5×10^6 copies/ml
20[b]	2, 3 negative	Absence of inhibitors	1.1×10^5 copies/ml
21	1, 2, 3, 4, 5, 6, 7 negative	Absence of inhibitors	1.7×10^5 copies/ml
22	1, 2, 4, 5, 6, 7 negative	3 (negative after dilution)	1.7×10^6 copies/ml
23	2, 4, 5, 7 negative (no tube 6)	1, 3 (negative after dilution)	1.3 mEq/ml
24	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	2.0×10^6 copies/ml
25	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.1 mEq/ml
26	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.15×10^6 copies/ml
27	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1 mEq/ml
28[b]	2 negative	1, 3 (negative after dilution)	5.4 mEq/ml
29	1, 4, 6, 7 negative (no tube 2, 3, 5)	Absence of inhibitors	0.6 mEq/ml
30	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.1×10^5 copies/ml
31	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	33.9 mEq/ml
32	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	0.5×10^6 copies/ml
33	2, 4, 5, 6, 7 negative (no tube 1)	3 (negative after dilution)	5.0×10^6 copies/ml
34	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.0×10^6 copies/ml
35	1, 2, 3, 4 positive 5, 6, 7 negative	Absence of inhibitors	1.6 mEq/ml
36	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	Not done
37	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	5.9×10^5 copies/ml
38	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.1×10^6 copies/ml
39	2, 4, 5, 6, 7 negative	1, 3 (negative after dilution)	1.0×10^6 copies/ml

[a]Blood plasma HCV RNA quantification was determined either by the HCV-Monitor technique (Roche SA, Neuilly-sur-Seine, France), or by the bDNA technique (Chiron Ciba Corning, parc des Bellevues, France): results were expressed as copies of HCV RNA/ml and bDNA mEq/ml respectively.
[b]Semen volume not sufficient for full analysis.

individually with a positive control in each assay. We consider that previous findings reporting the absence of HCV RNA in semen could be due to the technical difficulties in testing unfractionated raw semen and that the presence of RNAses or lipoperoxidases could have interfered with the amplification of nucleic acid and could explain the lack of HCV RNA detection in semen. We demonstrated the presence of *Taq* polymerase inhibitors and/or damaging enzymes in >80% of our unfractionated semen (fraction 1) and/or seminal plasma (fraction 3) samples (only six semen samples (15.4%) had no inhibitors). In contrast, no inhibitor was detected in fractions obtained after Percoll centrifugation (where sufficient semen was available for the full analysis). These data led us to test two concentrations of each semen sample with inhibitors: undiluted and 1:4. We consider that the detection of PCR inhibitors is essential but must not represent the end-point of the analysis. To complete the analysis, a dilution (e.g. 1:4) may be used to suppress or dilute these PCR inhibitors and thus allow a conclusion to be drawn for all patients. Without suppression or dilution of the *Taq* inhibitors in semen fractions, no result can be validated. Dilution of the samples can be criticized because of the risk of being under the detection limit, so we are now testing RNA extraction protocols using silica to eliminate or decrease *Taq* inhibitors (Tachet *et al.*, 1999).

HCV RNA could be clearly identified in semen for two patients. For one patient (no. 6), HCV RNA was only identified in seminal plasma after dilution 1:4. For the other patient (no.

Table IV. Semen HCV RNA data of the infertile patients (HCV–Amplicor procedure)

Samples	Fraction no.	OD A_{450} without HCV IC	OD A_{450} with HCV IC	OD A_{450} without HCV IC after dilution 1:4	OD A_{450} with HCV IC after dilution 1:4
Negative control[a]		0.06	0.91		
Positive control[b]		0.84	0.92		
Patient no. 1[c]					
Semen	1	0.06	0.05*	0.07	>3
Cell pellet	2	0.06	0.91		
Seminal plasma	3	0.05	0.05*	0.08	>3
Seminal plasma after Percoll	4	0.06	0.89		
50% after Percoll	5	0.06	0.84		
70% after Percoll	6	0.06	0.83		
90% after Percoll	7	0.06	0.85		
Patient no. 35[d]					
Semen	1	2.71	>3		
Cell pellet	2	2.86	2.96		
Seminal plasma	3	1.99	2.79		
Seminal plasma after Percoll	4	1.56	>3		
50% after Percoll	5	0.09	2.76		
70% after Percoll	6	0.08	>3		
90% after Percoll	7	0.08	2.91		
Patient no. 6					
Semen	1	0.07	0.10*	0.10	2.77
Cell pellet	2	0.72	>3		
Seminal plasma	3	0.08	0.06*	2.74	2.92
Seminal plasma after Percoll	4	0.07	2.80		
50% after Percoll	5	0.08	2.85		
70% after Percoll	6	0.08	2.54		
90% after Percoll	7	0.08	2.74		

[a,b]Provided by Roche SA, Neuilly-sur-Seine, France; they consist of buffer solution containing poly RNA EDTA or non-infectious RNA transcript with HCV sequences poly RNA EDTA respectively, plus 0.05% sodium azide.
[c]Additional negative control: typical pattern of analysis, i.e. presence of *Taq* inhibitors in semen (fraction 1) and seminal plasma (fraction 3) as shown, unlike the manufacturer's negative control (a).
[d]As no *Taq* inhibitors were detected, dilution was not necessary in order to complete the analysis.
*Presence of *Taq* inhibitors.
HCV-Amplicor procedure was carried out for seven samples: the semen sample, the seminal fluid, the cell pellet and for seminal fluid and the three fractions (90%, 70%, 50%) obtained after Percoll centrifugation.
OD = optical density; IC = internal control.

Table V. Hepatitis C virus detection in semen

	Assay	Results in semen	Population
Semprini *et al.* (1998)	RT–PCR	Negative round cells, seminal fluids and spermatozoa	90 drug addicted infected males 56 viraemic patients, 27 co-infected with HIV
Caldwell *et al.* (1996)	RT–PCR	Negative	12 viraemic patients
Tang *et al.* (1996)	RT–PCR	Positive (57%) 7 viraemic patients	
McKee *et al.* (1996)	RT–PCR	Positive	Donor semen No detection after Percoll treatment
Fiore *et al.* (1995)	RT–PCR	Positive	
Liu *et al.* (1994)	RT–PCR	Positive	3 viraemic patients
Liou *et al.* (1992)	RT–PCR nested PCR	Positive (24%)	34 viraemic patients
Fried *et al.* (1992)	RT–PCR nested PCR	Negative	14 viraemic patients
Hsu *et al.* (1991)	RT–PCR nested PCR	Negative	19 viraemic patients
Terada *et al.* (1991)	RT–PCR nested PCR	Negative	6 viraemic patients

RT = reverse transcription; PCR = polymerase chain reaction; HIV = human immunodeficiency virus.

35), HCV RNA was detected in the ejaculate, the seminal plasma and even in the cell pellet obtained after centrifugation (fractions 1, 2 and 3) which contains leukocytes, spermatozoa and round cells. In both cases, all tests were negative after a three-layer Percoll gradient, which used to be performed routinely (see below) in most infertility centres when preparing the sperm suspension used for IVF or artificial insemination (fraction 7). Our data are in agreement with McKee *et al*. (1996), who noted the elimination of HCV RNA after Percoll centrifugation of an HCV-infected cryopreserved semen specimen (McKee *et al*., 1996).

The different steps of semen preparation (selection of motile spermatozoa with a discontinuous gradient, elimination of leukocytes, immotile spermatozoa and round cells, then subsequent washing of each fraction) can explain the elimination of HCV RNA after a Percoll gradient centrifugation. Even if the Percoll selection step can be omitted in sperm preparation for intracytoplasmic sperm injection (ICSI) without an adverse effect on fertilization and embryo cleavage (De Vos *et al*., 1997), we consider that a discontinuous gradient is an easy procedure to select motile spermatozoa. It efficiently eliminates (or decreases) infectious agents such as cytomegalovirus (Levy *et al*., 1997), *Chlamydia trachomatis* (Levy *et al*., 1999) or HCV RNA in infected ejaculates as indicated by the present study, and is neither expensive nor too time-consuming. It is important to note that Percoll has now been withdrawn worldwide for use in sperm preparation for assisted reproduction procedures: in the current study, Percoll was used to test the semen samples for HCV RNA detection, not for assisted reproduction procedures. Alternative products which can be used for assisted reproduction techniques include PureSperm (Nidacon International AB, Göteborg, Sweden).

Viral infections might contribute to male infertility either by direct toxic effects on male cells in the male reproductive tract or indirectly by causing local inflammatory or immunological reactions (Keck *et al*., 1998). The impact of HCV infection on male fertility is still debated.

The question arises as to whether a doctor (or a lawyer) has the right to refuse infertility treatment to a well-informed couple where one of the partners is HCV-viraemic. We do propose a strategy for infertile infected couples who need artificial insemination or IVF. Concerning HCV male infection, our local strategy is to complete the classic preliminary work-up (HCV antibody screening with the enzyme-linked immunosorbent assay) with HCV RNA detection in serum. If negative, we propose that IVF can be performed without further delay. If positive, the procedure is stopped and complementary data are collected. Semen is submitted to selection by centrifugation in a discontinuous gradient, washing in BM1, followed by the swim-up technique. The motile spermatozoa are divided into two parts. One half is frozen. The other half is used to detect HCV RNA. If negative, the frozen, safe, 'non-infected' cryopreserved semen fraction could be used for assisted reproduction with minimal risk of viral contamination.

In conclusion, HCV can be detected in semen from a population of infertile men. These data provide direct evidence that a contamination risk may actually exist not only through sexual transmission, but also through assisted reproduction. Even if the occurrence of HCV in semen is low, and although HCV infection does not seem to play a significant role in infertility, these data must be considered with respect to the risk of HCV transmission not only to the mother and the child but also through laboratory contamination of other non-infected couples' gametes and of technicians, and even through infected cryopreserved semen storage and manipulation.

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References

Abusheikha, N., Akagbosu, F., Marcus, S. *et al*. (1999). Viral screening and Assisted Conception Treatment — The Bourn Hall Experience. *J. Assist. Reprod. Genet*., **16**, 337–339.

Arrêté du 12-1-1999, Journal Officiel du 28-2-1999 relatif aux règles de Bonnes Pratiques cliniques et biologiques en Assistance Médicale a la Procréation.

Bresters, D., Mauser-Bunschoten, E.P. and Reesink H.W. (1993) Sexual transmission of hepatitis C virus. *Lancet*, **342**, 210–211.

Caldwell, S.H., Sue, M., Bowden, J.H. *et al*. (1996) Hepatitis C virus in body fluids after liver transplantation. *Liver Transpl. Surg*., **2**, 124–129.

De Vos, A., Nagy, Z.P., Van de Velde, H. *et al*. (1997) Percoll gradient centrifugation can be omitted in sperm preparation for intracytoplasmic sperm injection. *Hum. Reprod*., **12**, 1980–1984.

Fiore, R.J., Potenza, D., Monno, L. *et al*. (1995) Detection of HCV RNA in serum and seminal fluid from HIV-1 co-infected intravenous drug addicts. *J. Med. Virol*., **46**, 364–367.

Fried, M.W., Shindo, M., Fong, T. *et al*. (1992) Absence of hepatitis C viral RNA from saliva and semen of patients with chronic hepatitis C. *Gastroenterology*, **102**, 1306–1308.

Gillet, P., Hallam, N. and Mok, J. (1996) Vertical transmission of hepatitis C virus infection. *Scand. J. Infect. Dis*., **28**, 549–552.

Hsu, H.H., Wright, T.L., Luba, D. *et al*. (1991) Failure to detect hepatitis C virus genome in human secretions with the polymerase chain reaction. *Hepatology*, **14**, 763–767.

Keck, C., Gerber-Schäfer, C., Clad, A. *et al*. (1998) Seminal tract infections: impact on male fertility and treatment options. *Hum. Reprod. Update*, **4**, 891–903.

Kiyosawa, K., Sodeyama, T. and Tanaka, E. (1991) Hepatitis C in hospital employees with needlestick injuries. *Ann. Intern. Med*., **115**, 367–369.

Kotwal, G.J., Rustgi, V.K. and Baroudy, B.M. (1992) Detection of hepatitis C virus specific antigens in semen from non A, non B hepatitis patients. *Dig. Dis. Sci*., **35**, 641–644.

Kotwal, G.J. (1993) Routine laboratory diagnosis of hepatitis C virus infection. *J. Hepatol*., **17** (Suppl. 3), S83–S89.

Levy, R., Najioullah, F., Keppi, B. *et al*. (1997) Detection of cytomegalovirus in semen from a population of men seeking infertility evaluation. *Fertil. Steril*., **68**, 820–826.

Levy, R., Layani-Milon, M.P., Giscard d'Estaing, S. *et al*. (1999) Screening for Chlamydia trachomatis and Ureaplasma urealyticum infection in semen from asymptomatic male partners of infertile couples prior to *in vitro* fertilization. *Int. J. Androl*., **22**, 113–118.

Liou, T., Chang, T.T., Young, K.C. *et al*. (1992) Detection of HCV RNA in saliva, urine, seminal fluid and ascites. *J. Med. Virol*., **37**, 197–202.

Liu, F.H., Tian, G.S. and Fu, X.X. (1994) Detection of plus and minus strand hepatitis C virus RNA in peripheral blood mononuclear cells and spermatid. (Abstract.) *Chinese Med. J*., **74**, 284–286.

Loi no. 94-654, Journal Officiel du 30-7-1994, pp 11059 relative au Don et a l'utilisation des éléments et produits du corps humain, à l'Assistance Médicale à la Procréation et au Diagnostic Prenatal.

Massey, E.J., Dasani, H., Jones, P. and Saleem, A.K.N. (1996) Storage facility exists for sperm from patients positive for antibody to hepatitis C virus. *Br. Med. J*., **313**, 1078.

McKee, T.A., Avery, S., Majid, A. and Brinsden, P.R. (1996) Risks for

transmission of hepatitis C virus during artificial insemination. *Fertil. Steril*., **66**, 161–163.

Numata, N., Ohori, H., Hayakawa, Y. *et al*. (1993) Demonstration of hepatitis C virus genome in saliva and urine of patients with type C hepatitis: usefulness of the single round polymerase chain reaction method for detection of the HCV genome. *J. Med. Virol*., **41**, 120–128.

Ohto, H., Terazawa, S., Sasaki, N. *et al*. (1994) Transmission of hepatitis C virus from mothers to infants. *New Engl. J. Med*., **330**, 744–750.

Roudot-Thoraval, F., Pawlotsky, J.M., Thiers, V. *et al*. (1993) Lack of transmission of hepatitis C virus in human immunodeficiency virus seronegative women: a prospective study with hepatitis C virus RNA testing. *Hepatology*, **17**, 772–777.

Roudot-Thoraval, F., Pawlotsky, J.M., Dhumeaux, D. et le Groupe d'Etude de la Prêvalence et de l'Epidémiologie de l'hepatite C (1996) Étude de 6664 malades atteints d'hépatite chronique C. *Bull. Epidemiol. Hebd*., **4**, 20–21.

Semprini, A.E., Persico, T., Thiers, V. *et al*. (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.

Tachet, A., Dulioust, E., Salmon, D. *et al*. (1999) Detection and quantification of HIV-1 in semen: identification of a subpopulation of men at high potential risk of viral sexual transmission. *AIDS*, **7**, 823–831.

Tang, Z., Yang, D., Hao, L. *et al*. (1996) Detection and significance of HCV RNA in saliva, seminal fluid and vaginal discharge in patients with hepatitis C. *J. Tongji Med. Univ*., **16**, 11–13.

Terada, S., Kawanishi, K. and Katayama, K. (1992) Minimal hepatitis C infectivity in semen. *Annals Intern. Med*., **117**, 171–172.

World Health Organization (1992) *Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*. Cambridge University Press, Cambridge.

Young, K., Chang, T., Liou, T. and Wu, H. (1993) Detection of hepatitis C virus RNA in peripheral blood mononuclear cells and in saliva. *J. Med. Virol*., **41**, 55–60.

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