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Hepatitis C virus RNA detection in different semen fractions of HCV/HIV-1 co-infected men by nested PCR

V. Savasi [a,*], B. Parrilla [a], M. Ratti [a], M. Oneta [a], M. Clerici [b], E. Ferrazzi [c]

[a] Department of Obstetrics and Gynaecology, Sacco Clinical Sciences Institute, University of Milan Medical School, 20157 Milan, Italy
[b] Department of Immunology, DISP LITA Vialba, University of Milan Medical School, Milan, Italy
[c] Department of Obstetrics and Gynaecology, Childrens' Hospital Buzzi, University of Milan Medical School, Italy

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ABSTRACT

Objective: The aim was to evaluate, by nested PCR, the prevalence of hepatitis C virus (HCV) RNA in seminal plasma in different semen fractions of HCV/HIV-1 co-infected men.
Study design: This study enrolled 16 HCV/HIV-1 infected men. A total of 16 seminal samples and 16 blood samples were tested for the presence of HCV-RNA. HCV-RNA in blood plasma was quantified by Amplicor HCV Monitor Test version 2.0 and HCV-RNA detection in seminal plasma, non-spermatozoa cells (NSCs), spermatozoa pellet and swim-up was investigated by nested PCR.
Results: Thirteen blood plasma samples were positive for HCV-RNA. HCV-RNA was detectable in seminal plasma and in non-sperm cells, but not detectable in spermatozoa samples, neither before nor after swim-up. One of the two patients whose seminal plasma tested positive at nested PCR had undetectable HCV virus in blood plasma.
Conclusions: HCV-RNA can be found in seminal plasma and non-sperm cells but not in spermatozoa before and after swim-up. We observed HCV-RNA in the semen of an aviremic man. According to these findings we suggest that sperm washing should be performed for each semen sample of HCV patients before assisted reproduction techniques.

1. Introduction

Hepatitis C virus (HCV) transmission is known to occur essentially by the parenteral route [1]. Other potential ways of non-parenteral transmission in HCV-positive-infected patients are through body secretions as saliva, ascites, breast milk, urine and feces [2–4]. The studies that have analysed the HCV-RNA presence in semen have reported controversial results. Old studies failed to document the presence of HCV-RNA in seminal plasma [5–7] while some other old studies and all recent studies have indicated its presence [2,8–21].

The discrepancy in results on the presence of HCV-RNA in seminal plasma of men chronically infected by this agent is due to various factors, such as the molecular techniques used (Cobas amplicor monitor, nested PCR, real-time PCR, Amplicor HCV amplification kit and detection kit), the sensitivity of the assays designed to detect HCV-RNA, the wide range of protocols used for RNA extraction [9,22] and eventually the presence in semen of inhibitors of PCR, like lactoferrin, peroxides, and mostly zinc residues that might interfere with the action of *Taq* polymerases [23].

In order to standardize the methods of detection of HCV-RNA in semen, a multicentre quality control study was performed in 12 French laboratories [22]. All centres used RT-PCR and Amplicor HCV Cobas assay to evaluate the presence of HCV-RNA in semen samples. They concluded that the percentage of correct results ranged from 53.3 to 100 and that the poorest results were obtained when no centrifugation step preceded the Amplicor extraction protocol, due to the negative effects of inhibitors of RT-PCR in seminal plasma. In 2002 Meseguer et al. [12] demonstrated that semen samples tested HCV negative by use of commercial methods (Amplicor Monitor RT-PCR) for HIV/HCV detection, but proved positive at nested PCR examination-data confirmed by Garrido et al. in 2004 [16].

In order to determine whether discrepancies in reported findings on the presence of HCV-RNA in semen are the result of inadequate methodological approaches for the detection of HCV-RNA, or of different seminal fractions, we hypothesized that nested PCR applied to each one of the seminal components – seminal plasma, non-sperm cells (NSCs), washed spermatozoa before swim-up, and washed sperm after swim-up – could overcome these limitations and provide sufficient insight on this issue even in a small series of HCV infected patients.

* Corresponding author. Tel.: +39 0239042698; fax: +39 0239511.
E-mail address: valeria.savasi@unimi.it (V. Savasi).

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2. Materials and methods

2.1. Patients

We studied the semen of 16 HCV/HIV-1 infected clinically asymptomatic male patients (mean age 39 ± 3.7 years DS; age range 31–45 years). These patients were recruited among outpatient couples attending our Assisted Reproductive Technology (ART) Centre. Institutional Review Board (IRB)-approved personal informed consent was obtained from each patient before semen donation. The mean duration of HIV-1 infection in these patients was 14 years (range 4–20).

All patients were former injecting drug users. None of the 16 men was on antiviral therapy for HCV infection at inclusion, and none was previously treated for HCV infection. Fifteen patients were on antiretroviral therapy, on average for 10 years. Median CD4 cell count was 510 ± 323 cells/ml (range, 35–1093). Eight patients were HIV aviremic and on HAART, while in the other eight men the range of HIV RNA load was between 80 and 90,093 copies/ml. One semen and one blood sample were collected from each patient during the same visit.

2.2. Samples

Semen samples were produced by masturbation after 4–7 days of sexual abstinence and collected into sterile jars. Blood samples were collected into EDTA vacutainers on the same day. All samples could be processed within 2 h. Blood plasma was separated from other blood components by centrifugation and frozen in aliquots at −80 °C.

Semen samples were processed using a 40–80% density gradient (Pureception kit, Sage, USA) to separate motile spermatozoa from non-sperm cells (NSCs), immotile spermatozoa and seminal plasma. The ejaculate was layered over the gradient and centrifuged at 400 × *g* for 30 min. After centrifugation, the seminal plasma was filtered using a 0.20 μm filter (Sterile Syringe Filter, Corning Incorporated, Germany) and stored at −80 °C. The NSCs from the layer between seminal plasma and 40% PureSperm solution were washed twice in phosphate-buffered saline (PBS) and stored at −80 °C as a dry pellet. The sperm pellet was recovered and resuspended in 3 ml of fresh medium (sperm washing medium, Sage, USA). A washing at 400 × *g* for 10 min was performed, and the supernatant was discarded, 1 ml of medium was subsequently gently layered on the pellet, and the tube was incubated at 37 °C for 1 h to allow spontaneous migration of spermatozoa to the surface of the culture medium. Motile spermatozoa at the upper layer of the culture medium were recovered by pipetting and stored at −80 °C. The remaining spermatozoa were washed twice in PBS and stored at −80 °C as a dry pellet.

2.3. HCV-RNA detection in blood plasma

HCV-RNA in blood plasma was quantified by Amplicor HCV Monitor Test version 2.0 (Roche Diagnostic Systems, Meylan, France) using the ultrasensitive protocol with a lower detection limit of 50 copies/ml. Each sample was tested with the addition of an internal control to validate the extraction and the quantification steps. The internal control was made by both a positive control, to detect the presence of transcription or amplification inhibitors, and a negative control, to detect the presence of amplicon contamination. In order to confirm the absence of viremia in patient number 12, we tested this blood sample also by nested PCR with reported methodology.

2.4. HCV-RNA detection in seminal plasma, non-spermatozoa cells (NSCs), spermatozoa pellet and swim-up.

2.4.1. Extraction

RNA extraction was performed according to the Isolation Reagents Nuclisens Kit procedure (Biomerieux, Lyon, France). This procedure used silica particles to remove seminal inhibitory factors, since it has been reported that these factors are mainly present in seminal fluid [8]. Moreover, aliquot of semen was diluted 1:10, 1:5, and 1:1 in μl phosphate-buffered saline (PBS) to dilute the amount of seminal inhibitory factors. Each sample was tested with the addition of negative and positive controls included to validate the extraction. To avoid false-positive results, we applied the method to prevent contamination described by Kwok and Higuchi [24].

2.4.2. Retrotranscription and nested PCR

A volume of ≤1 μg of RNA solution was converted to cDNA with 2.5 U/μl of MuLV-Reverse Transcriptase (Promega, USA), in a 20 μl reaction mixture containing 1× PCR Buffer (PerkinElmer, Norwalk, CT, USA), 5 mM $MgCl_2$, 1 mM dNTPs, 2.5 μM random primer (PerkinElmer) and 1 U/μl of RNAsin (Promega). The reaction mixture was incubated at ambient temperature for 10 min, at 42 °C for 15 min and the reverse transcriptase was inactivated by heating the reaction at 99 °C for 5 min, finally at 5 °C for 5 min.

Table 1
Viral copies in blood plasma and nested PCR results in each fraction semen.

Patient	Peripheral blood (copies/ml)	Seminal plasma	Non-sperm cells	Positive washed, separated Spz before swim-up	Positive washed, separated Spz after Swim-up
1	377,604	–	–	–	–
2	8720	–	–	–	–
3	10,761,689	–	*	–	–
4	Undetectable	–	–	–	–
5	2259	–	Positive	–	–
6	4,554,860	–	–	–	–
7	29,697	–	–	–	–
8	2,139,801	–	–	–	–
9	29,603	–	–	–	–
10	Undetectable	–	–	–	–
11	7830	–	Positive	–	–
12	Undetectable	Positive	–	–	–
13	2,239,969	–	–	–	–
14	4,745,047	Positive	*	–	–
15	4,227,170	–	–	–	–
16	700,000	–	Positive	–	–

–, Negative samples at nested PCR; *, test not performed.

Table 2

Author	No patient	No samples	positive blood plasma (%)	Positive whole SEMEN (%)	Positive seminal plasma (%)	Positive non-sperm Cells (%)	Positive washed, separated Spz before swim-up (%)	Positive washed, separated Spz after swim-up (%)	Technique
Semprini Infect Dis 1998	90	90	62.2	–	0	0	0	0	Nested PCR + southern blot
Leruez-Ville Lancet 2000	21	21	86	–	38	–	–	–	Cobas amplicor monitor
Pasquier AIDS 2000	20	20	80	–	20	0	0	0	Cobas amplicor monitor
Levy HumReprod 2000	39	39	100	5	5	2,5	0	–	Amplicor HCV amplification kit and detection kit
Bourlet J clin Microbiol 2002	32	32	100	–	12.5	–	3*	0	Cobas amplicor monitor
Levy HumReprod 2002	1	12	50	–	75	–	17*	0	Cobas amplicor monitor
Cassuto HumReprod 2002	35	50	100	14	–	–	0	0	Cobas amplicor monitor
Bourlet Clin Microbiol 2003	16	192	0	68	–	–	–	–	Nuclisense Qiagen RNA amplicor
Garrido HumReprod 2004	73	108	48	–	–	–	–	10	Nested PCR
Briat AIDS 2005	120	196	100	–	31.6	6	0	–	Real-time PCR
Halfon AIDS 2006	170	191	100	–	11	–	0	–	Cobas amplicor monitor
Present study	16	16	81	–	12.5	19	0	0	Nested PCR

–, Samples not tested; *, samples from the same patient.

About 1 μg of cDNA was used for the nested PCR. The reaction mixture (50 μl) contained 1× PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U/reaction of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.2 μM of each primer. The two primer pairs used for nested PCR were complementary to noncoding region of the HCV-RNA genome, which has been shown to be the most conserved region among different HCV strains.

The first round of PCR was performed with primers antisense primer (HCVOA 59-TGCACGGTCTACGAGACCTC-39 [nucleotides {nt} 320–339] sense primer [HCVOS, 59-GCCATG GCGTTAGTAT-GAGT-39 {nt 82–101}] [25]. The reaction mixture was incubated at 95 °C for 10 min, at 95 °C for 30 s, at 60 °C for 30 s, at 72 °C for 1 min, for 35 cycles; at 72 °C for 10 min. First PCR product was used in the second round of PCR using primers antisense [HCVIA], 59-GGGCACTCGCAAGCACCCTAT-39 [nt 296–316]; sense [HCVIS], 59-GTGCAGCCTCCAGGACCCCC-39 [nt 105–124]) [25]. The amplification was carried out at 95 °C for 10 min, at 95 °C for 30 s, at 60 °C 30 s, at 72 °C 1 min, for 25 cycles; at 72 °C for 10 min. The final PCR products were electrophoresed in 1.5% agarose gel. Each experiment was carried out twice to confirm our results. Furthermore, negative and positive controls were included in each PCR assay. Nested PCR (nPCR) has been demonstrated to be the most sensitive technique, since it allows the detection of a single viral RNA or DNA particle.

3. Results

HCV-RNA was detectable in five semen samples (31.5%), two seminal plasma (12.5%) and three non-sperm cells (19%). Table 1 summarizes Viral HCV copies in blood plasma and nested PCR results in seminal plasma, non-sperm cells and spermatozoa before and after swim-up.

Blood plasma samples were found to be positive for HCV-RNA in 13 of 16 patients (81.3%). One of the two patients whose seminal plasma tested positive at nested PCR had undetectable HCV virus in blood plasma test also by nested PCR.

All separated spermatozoa samples before and after swim-up were negative for HCV-RNA.

Table 2 compares our present findings with selected papers published from 1998 to 2007.

3.1. Comments

To the best of our knowledge this is the first study to apply nested PCR to all the different fractions of human semen-seminal plasma, non-sperm cells (NSCs), washed spermatozoa before swim-up, and washed sperm after swim-up. Nowadays nested PCR is considered the best technique to research HCV-RNA in semen, since it allows the detection of a single viral RNA or DNA particle. Although the number of subjects in our study is limited we consider these data a significant contribution to the still controversial issue of HCV presence in semen.

This study was performed on 16 HCV-HIV co-infected patients. Among these, five were positive for HCV-RNA in semen: two were positive for HCV-RNA in seminal plasma and three in non-sperm cells. All samples of spermatozoa before swim-up (washed only spermatozoa) and after swim-up were HCV-RNA negative.

This result is comparable with other papers that report a prevalence of HCV-RNA in semen between 11% [21] and 31.6% [20], and negative findings in samples of washed separated spermatozoa [11,20,21], and negative findings after swim-up [6,9–11]. However different combinations of findings were reported on washed and swim-up spermatozoa. Levy et al. [9] and Bourlet et al. [10] reported positive findings in washed spermatozoa (17% and 3% of the samples respectively) from one patient. However, these authors did not use nested PCR. Among all authors, only Garrido et al. [16] reported HCV-RNA in spermatozoa after swim-up in 10% of the samples. A possible explanation is that these authors used a different gradient and less time of centrifugation compared to the sperm washing technique published by Persico et al. in 2006 [26].

An additional point of interest in our results is the case of an aviremic patient in whom seminal plasma proved positive at

nested PCR for HCV, confirmed by double internal controls for each patient. We tested the blood sample of this patient also by nested PCR in order to be sure of this result. This could be explained by the fact that the correlation between HCV viral load in blood plasma and in seminal plasma is unpredictable and inconstant, and in addition to this HCV-RNA in semen shows dramatic and rapid variations through time [9]. Briat et al. [20] found that some men with low blood viral load had detectable HCV-RNA in their semen and conversely some men with high blood viral load had no detectable HCV-RNA in semen. These authors explained such discrepancies either by local replication in male genital tract leukocytes, as reported in blood lymphocytes and monocytes, or by a selective passage of some HCV variants, which might exhibit a preferential tropism for genital tract.

The third point of interest is the presence of virus in non-sperm cells when the viral load was low, and seminal plasma was even negative. The presence of HCV-RNA in non-sperm cells has been investigated in four papers with a different range of findings from 0% [6,9] to 2.5% [2] and 6% [20].

The only paper which reported negative nested PCR results in all of the seminal components was published by Semprini et al. in 1998 [6]. In that paper Semprini used a viral RNA extraction based on acid phenol-guanidium thiocyanate-modified method (RNAzol), followed by isopronanol precipitation. We choose to adopt a different viral extraction technique based on the NucliSens RNA extraction, that is considered the RNA extraction technique less influenced by inhibitors of PCR [22,27].

A limitation of the present cohort is that all patients were HIV-1 co-infected. Pasquier et al. [14] and Halfon et al. [21] reported that the HIV-1 positive status does not influence the positivity of HCV-RNA in semen, while Briat et al. [20] found that HCV-RNA was more frequently found in semen of HIV-1/HCV co-infected men (37.8%), than in semen of non-co-infected men (18.4%). Moreover, some recent work suggests an unequivocal increase of HCV sexual transmission among HIV-1 infected men who have sex with men [28]. These data could suggest that in HIV-1/HCV-positive semen there are more leukocytes than in only HCV-positive semen and that HCV could replicate in male genital tract leukocytes, as reported in blood lymphocytes and monocytes [29]. On the contrary Halfon et al. [21] consider that HCV sexual transmission is more frequent in HIV-1/HCV co-infected patients, and it may be due to infected blood passed during intercourse via abrasions of mucosa rather than through HCV infected semen.

These findings obtained on a small group of patients studied by nested PCR after NucliSens RNA extraction confirm data already reported by a less specific methodology: HCV-RNA can be found seminal plasma and non-sperm cells but not in spermatozoa before and after swim-up.

This work was designed to exploit the best of available methodologies so far reported; this allowed us to track the presence of HCV-RNA in different fractions of semen even in a case of undetectable viremia. According to these findings we suggest that sperm washing should be performed for each semen sample of HCV patients before ART. In our opinion these findings suggest a revision of old certainties in order to reach an up-to-date consensus for semen preparation and provision of safety for all parties involved.

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