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Use of Versant™ TMA and bDNA 3.0 Assays to Detect and Quantify Hepatitis C Virus in Semen

Vyacheslav A. Pekler,[1] Wendie A. Robbins,[2]* Adeline Nyamathi,[3] Tatyana L. Yashina,[4] Barbara Leak,[3] and Terry A. Robins[5]

[1]*UCLA School of Public Health, University of California, Los Angeles, California*
[2]*UCLA Center for Occupational and Environmental Health, University of California, Los Angeles, California*
[3]*UCLA, School of Nursing, University of California, Los Angeles, California*
[4]*Specialty Laboratories, Inc., Santa Monica, CA*
[5]*Focus Technologies, Cypress, California*

Previous findings of hepatitis C virus (HCV) in human semen have been inconsistent. This study attempted to elucidate the presence of HCV in semen from 80 HCV RNA blood plasma positive homeless men using two novel non-PCR based techniques. Semen was frozen immediately upon ejaculation in order to preserve virus quantity. This study demonstrated that 36% of the study population had HCV in semen. Bayer's Versant™ HCV RNA Qualitative Assay (Bayer Diagnostics, Emeryville, CA) based on transcription mediated amplification (TMA) assay detected 29 positive semen samples and Versant™ HCV RNA 3.0 Assay (bDNA) (Bayer Diagnostics, Emeryville, CA) detected only six. This demonstrated that TMA was more sensitive than the bDNA in detecting HCV in semen ($P<0.002$). HCV blood plasma viral load was positively correlated with the presence of HCV in semen (Spearman's Rho=0.40, $P<0.0002$), while the presence of leukocytes in semen was not (Spearman's Rho=0.19, $P<0.12$). This supports the hypothesis that HCV is "leaked out" from the peripheral circulation into semen. Three semen samples had a viral load of $>5{,}000$ IU/mL. The presence of a high viral load in semen in certain men suggests that sexual transmission of the virus is possible. Laboratory capability to accurately detect HCV positive semen is an important step in establishing the risk of sexual transmission and in identifying strategies for protecting uninfected partners. J. Clin. Lab. Anal. 17:264–270, 2003. © 2003 Wiley-Liss, Inc.

Key words: hepatitis C virus; viral load; white blood cell count; semen; branched DNA assay; transcription mediated amplification

INTRODUCTION

Evidence for and against sexual transmission of hepatitis C virus (HCV) has previously been described (1–3). The subject remains controversial, in part, because of conflicting findings regarding the presence of HCV in semen. A number of researchers have found HCV in semen (4–10), while others have failed (11–16). Explanations for the inability to find HCV in various body fluids, including semen, have been summarized by Ackerman et al. (17). Specifically, in semen one, or a combination, of the following may explain discordant findings. 1) PCR inhibitors that are thought to be present in seminal fluid (9,15,16) could stop amplification producing false negative results. Therefore, the use of an internal standard (IS) is essential in the detection of PCR inhibitors in semen (9). 2) Seminal enzymes may degrade HCV RNA during storage and processing of semen (11,18,19). 3) The only study that quantified the amount of HCV present in semen found it to be below 200 copies/mL (8). These findings suggest that virus is present in small amounts in semen and highly sensitive methodology must be employed for its detection.

Several studies have successfully detected HCV in semen by employing a bead extraction method (8,10). Inhibitory material present in seminal plasma is removed

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*Correspondence to: Wendie A. Robbins, UCLA Center for Occupational & Environmental Health, Room 5-254 Factor Building, Mailcode 956919, UCLA, Los Angeles, CA 90095-6919.
E-mail: wrobbins@sonnet.ucla.edu

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by this method (20). The role of white blood cells (WBC) as carriers of HCV into semen remains unknown. The majority of WBCs in semen are monocytes/macrophages and neutrophils (21,22). Peripheral blood mononuclear cells (PBMC) are thought to be one of the HCV's extrahepatic replication sites (23) and have been implicated in vertical transmission of the virus (24). In addition, shedding of the virus has been significantly correlated with the presence of leukocytes in vaginal washings (25).

This study was a pilot study with the objective of determining the most sensitive laboratory assays and sample preparation techniques in order to detect HCV in semen. Versant™ HCV RNA Qualitative Assay (Bayer Diagnostics, Emeryville, CA) based on transcription mediated amplification (TMA) method and improved Versant™ HCV RNA 3.0 Assay (Bayer Diagnostics, Emeryville, CA) branched DNA (bDNA) assay were used to detect and quantify HCV in semen.

MATERIALS AND METHODS

Study Population

Study subjects were recruited in a free clinic from a large metropolitan city. Informed consent was obtained from all study participants and research was performed within the ethical guidelines and written approval of the UCLA Office of the Protection of Human Research Subjects. All persons eligible for the study were homeless men who had tested HCV sera positive and had returned for an educational session given by the clinic nurse (26). To be eligible, the men had to express interest in participating in this study and be: between the ages of 18 and 65; willing to complete a 15-min questionnaire detailing demographic, biologic, and behavioral risk factors; and willing to provide a semen specimen and have a blood sample collected on site. Exclusionary criteria included persons who were incoherent or actively hallucinating as determined by the study nurses, or persons who had a history of vasectomy. A total of 104 men met eligibility and were enrolled in the study.

Blood Processing

Blood and semen were collected, processed, and transported during the same visit. Blood was obtained by venipuncture. Plasma was separated and immediately frozen on dry ice. Prior to analysis, samples were thawed on wet ice and run on bDNA in triplicates. Negative samples were confirmed by TMA.

Semen Processing

Semen was collected by masturbation into sterile polypropylene containers and immediately frozen whole on dry ice. Blood plasma and semen specimens were transported together on dry ice to the University of California–Los Angeles within 4 hr and no specimens thawed during transportation. Specimens were then batched and transported to Specialty Laboratories, Inc. (SLI, Santa Monica, CA) on dry ice and stored at -70°C. At SLI, specimens were stored frozen at -70°C until further manipulation.

Prior to analysis, semen specimens were allowed to liquefy on wet ice. Liquefaction times ranged from 35–138 min (83 ± 23, mean $\pm$ SD and median of 80.5). Immediately upon liquefaction, semen was fully mixed by gently pipetting up and down 15–20 times. One aliquot of the sample was frozen immediately for the sperm cell (SPC) and WBC count. The rest was aliquotted into smaller tubes and spun for 3 min at 2,500 g at 4°C to separate the liquefied fraction from the coagulated layer containing gelatinous material and cells. If separation did not occur, specimens were further centrifuged at 5,000 or 12,000 g. If samples were completely unworkable, they were further incubated at room temperature until sufficient liquefaction occurred and seminal plasma could be separated. Samples were only thawed twice: once before aliquotting and a second time immediately before the HCV assay. Repeated freezing and thawing may degrade HCV RNA (27–29). In order to decrease chances of contamination, blood and semen were aliquotted at different times.

TMA

Bayer's Versant™ HCV RNA Qualitative Assay based on transcription mediated amplification (TMA) is a relatively new, highly sensitive, in vitro nucleic acid assay for the detection of HCV RNA in human plasma and serum. It is capable of detecting up to 5 IU/mL (30,31). The assay employs bead extraction capable of removing inhibitory material (20) and is based on the Boom et al. (32) methodology. The method utilizes an internal standard (IS), which is coextracted, amplified, and detected along with the target HCV RNA. The ratio between the IS signal and the target signal is used to determine the sample's positivity or negativity, which is automatically discriminated by the device's software. All procedures were done according to the manufacturer's instructions (30,33).

Both the seminal plasma and lower cellular/unliquefied layer were used to detect HCV. The TMA assay uses 500 μL of sample. Since this amount was not available for most of the semen samples, modifications were made for smaller amounts and/or the lower layer, i.e., cellular/unliquefied (coagulated) layer, was used (see Discussion).

bDNA

Versant™ HCV RNA 3.0 Assay (bDNA) is a kit-based assay with improved sensitivity and a range of 521 to 8.3×10^6 IU/mL (kit insert). It is based on a sandwich nucleic acid procedure for the quantification of HCV in human serum and plasma with high reproducibility. It uses 50 μL of sample, and procedures were done according to the manufacturers' instruction manual.

HCV detection was performed on seminal plasma that was free of cellular or coagulated material. Each sample was run in triplicate. If the sample quantity permitted, positive semen samples were repeated in triplicate. Thus, all data from bDNA runs are presented as a mean of triplicates, unless stated otherwise. Negative bDNA results are shown as <521 IU/mL.

White Blood Cell Count and Sperm Cell Count

Sperm concentration per milliliter was estimated following WHO protocols for hemocytometer counts (34). WBCs were estimated using phase microscopy and quantified as a percentage of sperm cell count, and then were converted to cells per milliliter of semen.

Statistical Analysis

Spearman rank and Pearson (on normal transformed data) correlations were used to detect relationships between blood plasma viral load (BPVL), semen volume (SV), liquefaction time (LT), WBCs, and SPCs in seminal fluid. For statistical testing, data was then divided into two groups: 1) men positive for HCV in semen (HCV+, n = 29) detected by TMA, inclusive of six who were also quantifiable by bDNA (bDNA+, n = 6); and 2) men negative for HCV in semen (HCV–, n = 51) determined negative by TMA and/or bDNA assays. For the normally distributed variables of liquefaction time (LT) and semen volume (SV), Student's *t*-test was used to compare means between both groups. Fisher's exact test was used to test for differences between the presence of HCV in semen in men who did not have HCV RNA in blood plasma vs. men who did, and comparison of TMA and bDNA for the ability to detect HCV in semen. Significance between percentage of TMA positive and negative results in the liquefied and unliquefied seminal layer was determined using a Chi-square test. Data was analyzed using SAS (SAS Institute Inc., Cary, NC) and/or STATA (Stata Corporation, College Station, TX) statistical software. For the one case where BPVL could not be detected by bDNA, but was positive on TMA, the sample was treated as if it would have 521 IU/mL. The significance level for statistical testing was set at $P < 0.05$.

RESULTS

Subjects Excluded From Analysis

Out of 104 subjects from whom paired blood plasma and semen were collected, 14 (13.5%) were excluded from analysis for to the following reasons: four blood samples (3.8%) had clotted; six samples (5.8%) had coagulated semen that could not be pippetted out of the collection cup due to viscosity; and four semen samples (3.8%) were considered indeterminate by the TMA assay due to the failure of the IS coamplification.

HCV Detection in Blood and Semen

Ten out of the remaining ninety anti-HCV positive subjects (11%) were RNA negative in blood plasma and were also found negative in semen by both TMA and bDNA (Table 1). HCV RNA was found in the blood of the remaining eighty men, and further results are shown only for these subjects (Table 2). Three men had blood viral load above the quantifiable upper limit of the

TABLE 1. Presence of HCV in paired blood and semen samples from 90 men using bDNA and/or TMA assays

	Blood		
Semen	HCV+	HCV–	Total
HCV+	36	0	36
HCV–	44	10	54
Total	80	10	90

TABLE 2. Selected characteristics of the semen positive and negative groups

Group	Number of cases (%)	BPVL[a] $\times 10^6$ (IU/mL)	WBC[b] $\times 10^6$	SPC[c] $\times 10^6$
HCV+[d]				
Mean ± SD		2.89 ± 2.58	1.36 ± 1.37	138 ± 162
Median	29 (36.3)	1.8	0.80	82.4
Range		0.25–10.6	0–4.43	0–701
HCV+[e]				
Mean ± SD		1.66 ± 2.88	0.82 ± 0.84	81.9 ± 83.0
Median	51 (63.7)	0.78	0.41	56.4
Range		521×10^{-6}–15.6	0–3.59	0–296
Statistics[f]		$P < 0.0004$	NS[g]	NS[g]

[a]Blood plasma viral load.
[b]White blood cell count per mL^{-1} in semen.
[c]Sperm count per mL^{-1} in semen.
[d]Combined subjects where HCV could be detected by TMA (n = 23) and quantified by bDNA (n = 6).
[e]Subjects (n = 51) negative in semen by TMA and/or bDNA assays.
[f]Difference between groups determined by Wilcoxon rank-sum test.
[g]Not significant.

TABLE 3. Parameters of the three highest blood plasma viral load subjects

		Parameters in semen			
Subject number	BPVL[a] $\times 10^6$ (IU/mL)	TMA result (seminal layer)	bDNA result (IU/mL)	WBC[b] $\times 10^6$	SPC[c] $\times 10^6$
53	12.6	NR(LL)[d]	<521	1.60	61.6
74	15.6	NR[e] (LL)[d]	<521	1.20	27.6
87	10.6	R[f] (UL)[g]	<521	0.80	158.4

[a]Blood plasma viral load estimated by dilution.
[b]White blood cells per mL^{-1} in semen.
[c]Sperm cells per mL^{-1} in semen.
[d]Liquefied layer.
[e]NR, TMA non reactive.
[f]R, TMA reactive.
[g]Unliquefied layer.

TABLE 4. Comparison of TMA and bDNA assays in detection of HCV RNA in semen from HCV RNA blood positive men

Methods	Number tested	Number positive (%)	Number negative (%)
TMA	80	29(36)	51(64)
bDNA[a]	77	6(8)	71(92)

[a]Three samples could not be tested by the bDNA method because semen could not be liquefied or not enough sample volume.

bDNA assay and specimens were diluted in order to estimate true viral load (Table 3).

HCV in semen was detected by TMA in 29 out of 80 (36%) semen specimens. Except in the indeterminate cases noted above, no inhibition of the reaction in both positive and negative cases was observed as evidenced by coamplification of IS. HCV could be quantified by bDNA in six out of 77 samples (8%) (Table 4). Semen from three subjects was not tested by bDNA because it failed to liquefy or did not have adequate volume. Summary characteristics of the six samples where HCV could be quantified by the bDNA method in semen are shown in Table 5.

DISCUSSION

This is the first study, to our knowledge, that used the highly sensitive TMA assay to detect HCV in semen and also used an improved version of the bDNA technique to quantify HCV in semen. In addition, a representative number of participants was evaluated; thus statistical comparisons could be done.

In this study, HCV was found in 29 out of 80 (36%) semen samples in men who had detectable viral load in blood, but none was found in 10 subjects without detectable HCV RNA in blood (Table 1, Fisher's exact test, $P=0.027$). This is in agreement with Liou et al. (4) and corresponds with the statement that men with nondetectable HCV RNA in blood do not shed virus into semen and may not be infectious. Possible reasons for failing to detect HCV RNA in anti-HCV positive subjects may be: false positivity of antibody techniques (35,36); clearance of the infection; or inability of TMA to detect low viral load of the virus (37) that may still be present in the liver (38).

Men with HCV positive semen had significantly higher BPVL than men in the HCV-negative semen group (Table 2, Wilcoxon rank sum test, $P<0.004$). This supports the hypothesis by Hsu et al. (11) and Liou et al. (4) that the virus escaped from peripheral circulation into the semen (4) and that a certain threshold must exist before this takes place (11). In addition, present findings are in agreement with Hisada et al. (39) where higher blood viral load associated with sexual transmission of the virus. Interestingly, out of the three samples where HCV viral load was found highest in blood (Table 3), virus was detectable by TMA in semen in only one person. This suggests that, in some men, factors other then viral load are associated with presence of HCV in semen.

Seminal WBC and SPC did not differ significantly between the semen HCV+ and HCV– groups in this study. Although men with HCV+ semen had higher numbers of both WBC and SPC (Table 2), both groups contained a few men with high values and the difference was not statistically significant (Wilcoxon rank-sum test, $P<0.12$ and $P<0.11$, respectively). Two explanations can be given: 1) examination of semen for WBC without staining may not accurately detect WBC (40); and 2) immediate freezing to preserve virus, resulting in subsequent partial liquefaction of specimens, prevented homogeneous mixing, thus affecting the ability to achieve accurate counts. HCV+ and HCV– semen did not differ in regard to liquefaction time and seminal volume (Student's t-test, $P<0.09$ and $P<0.42$, respectively). BPVL was not correlated with WBC and SPC

TABLE 5. Parameters of six subjects where HCV could be quantified in semen by bDNA*

		bDNA in semen (IU/mL)			
Subject number	BPVL[a] (IU $\times 10^6$ mL)	1st run	2nd run	WBC[b] $\times 10^6$mL	SPC[c] $\times 10^6$ mL
12	3.42	11958	9423	3.84	64.0
14	1.51	32280	60929	0.00	0.00
21	8.87	5065	5849	3.99	700.8
52	2.01	1890	ND[d]	1.20	292.4
86	3.14	570	ND[d]	1.20	98.4
103	5.20	1237	3767	3.19	207.2
Mean $\pm$ SD	4.03 $\pm$ 2.77			2.24 $\pm$ 1.66	227 $\pm$ 254

*All bDNA numbers shown is a mean of triplicates except subject #52 where first two samples were done on 50 µL and the third on <50 µL, which was disregarded.
[a]Blood plasma viral load.
[b]White blood cells in semen.
[c]Sperm cells in semen.
[d]ND, not done, due to small amount of sample material or uncooperative sample.

TABLE 6. Percent positive and negative HCV results as determined by TMA in liquefied and unliquefied seminal layers

	TMA result		
Seminal fraction	Number positive (%)	Number negative (%)	Total
Liquefied layer	19 (35)	35 (65)	54
Unliquefied layer	10 (38.5)	16 (61.5)	26
Total	29	51	80

(Spearman's Rho = 0.19, $P<0.09$ and Spearman's Rho = 0.20, $P<0.06$, respectively). Therefore, further studies must be done using methods that overcome the limitations of this study. However, these findings suggest that, in some men, factors other than the presence of leukocytes are associated with HCV in semen. Twenty-nine cases where semen was positive for HCV RNA could be detected by TMA and only six of these could be quantified by bDNA. The sensitivity of the TMA assay (5 IU/mL) was much higher then the bDNA (521 IU/mL) (Fisher's exact test, $P=0.002$). Out of all HCV positive samples detected in semen, 79% (23/29) were below 521 IU/mL, which is in agreement with Leruez-Ville et al. (8) where all HCV positive results in semen (38% or 8/21) were below 200 copies/mL.

To our knowledge, this is the first study that was able to detect high HCV viral load in semen from six viremic men (Table 5), of whom three were higher than 5,000 IU/mL. These findings emphasize HCV can be present in semen in certain men in high viral numbers and these men could be potentially infectious. Currently, barrier precautions are not recommended in a stable monogamous relationship where a partner is infected with HCV (1,3,41,42). In light of the findings of this study, it seems better to agree with Rooney and Gilson (2) who recommend condom use in all at-risk couples, especially with dually HIV and HCV infected males.

The limitations of this study included the lack of published validation of the methodology employed for use with semen. In addition, the current population of homeless men may have a higher percentage of HCV in semen due to generally poorer health and living conditions. Therefore, these results must be interpreted cautiously.

For some of the TMA, 100 µL seminal specimen was used instead of the 500 µL required. Hypothetically, if 50 IU/mL is present in semen, 100 µL (the lowest amount used in this study) should contain only 5 IU, which can be detected with 95% sensitivity (30,31). Therefore, all TMA negative results in semen were, at least, below 50 IU/mL.

Identification of the seminal fraction that may contain the virus was not pursued in the current investigation due to the technical difficulties of working with fresh-frozen, partially coagulated semen. Only liquefied seminal fluid was used in the bDNA assay, while both liquefied and/or unliquefied layers were used in TMA. This may partially explain the difference between detection rates by both assays. However, the percent positive vs. negative specimens did not differ significantly for the TMA assay based on use of the liquefied vs. unliquefied fraction (Table 6, Chi-square test $P<0.1$).

In conclusion, 36% of the men with blood positive for HCV demonstrated HCV in semen. Many studies suggest that sexual transmission of HCV is minimal, but the specific population of men that can transmit virus has not been identified (43). Additionally, 8% of the men (6/77) had viral load higher than 521 IU/mL. An approximately similar fraction of cases is attributed to HCV transmission (1).

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