Hepatitis C Virus Detection in the Semen of Infected Patients

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Though HCV infection is a serious public health problem, some aspects of its biology are still not well understood, such as its transmission through seminal fluid and sexual transmission. We looked for HCV in the semen of infected patients. Thirteen patients were included. Semen fractions (seminal plasma, leukocytes and spermatozoa) were separated with 45% and 90% Percoll gradients. The HCV-RNA in blood and semen fractions was extracted using the same protocol (AMPLICOR Roche) and was detected using the qualitative Roche Amplicor test and by agarose gel electrophoresis, with ethidium bromide staining. The mean age of the patients was 40.7 years. Risk factors for the acquisition of HCV included injectable and inhaled drug use in six (42.8%), blood transfusion in four (28.6%), and no risk factors in four (28.6%) patients. Genotype 1 was detected in 62% of the patients, followed by genotype 3 in 23% and genotype 2 in 15%. All blood samples were positive, regardless of the technique used for detection. All semen samples identified by Roche Amplicor and analyzed by agarose gel electrophoresis were negative. Among the 52 semen samples (total and fractions) identified by the Roche Amplicor method, 45 (87%) were inhibited. A negative result was recorded for one (1.9%) total semen sample, one (1.9%) leukocyte and four (7.7%) seminal plasma fractions. Only one (1.9%) sample of the spermatozoon fraction was positive. The results obtained suggested false-negative reactions for the semen samples.
Key-Words: HCV, PCR, sexual transmission, semen.

Various studies suggest that the risk of sexual transmission of hepatitis C virus (HCV) is minimal or even inexistent, with its incidence ranging from 0 to 3% [1,2]. The first report discussing the sexual transmission of HCV, in which multiple sex partners were considered to be a risk factor, was published by Alter et al. in 1989 [3].

As is the case for sexual transmission, the risk of HCV transmission through seminal fluid is also controversial. Risk factors that may increase the probability of transmission are the type of relationship, since monogamic couples tend to present lower transmission rates than individuals reporting sex with multiple partners, sexual relations that involve trauma, co-infection with acquired immunodeficiency virus (HIV), partners using drugs, associated sexually-transmitted diseases, paid sex, and a long-term relationship (>10 years) with an HCV-positive partner [4-6].

The direction of sexual transmission of HCV from men to women has been reported by Rooney and Gilson [7], who showed that the estimated risk of HCV infection is 3.7 times higher in women with HCV-positive partners. Cavalheiro et al. [8] studied a series of 24 couples with a diagnosis of hepatitis C; there was an average viral similarity of 98.3% for 22/24 (91.7%) couples. In that study, the NS5b-HCV region was chosen for phylogenetic analysis. Nine couples attracted attention because the women did not report any risk factor for acquisition of the virus, whereas all nine men reported one or more risk factors. In this case, the average genomic similarity was 98%. That study supports the hypothesis of infection from men to women.

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Studies analyzing semen by molecular biology techniques have reported difficulty in eliminating natural inhibitors present in the sample, which frequently leads to false-negative results [9]. Cassuto et al. [10], in a study of semen from 35 men, reported difficulty in removing inhibitors during HCV-PCR, with only five men testing positive, suggesting false-negative results. Various investigators have been able to detect HCV in semen, while others could not. The difficulty in isolating this virus from body fluids, especially semen, is probably due to the presence of inhibitors and the lack of standardized techniques and protocols for RNA extraction and reverse transcription and polymerase amplification (RT-PCR); these factors may contribute to false-negative results [9,11].

Our objective was to investigate whether HCV is present in semen samples from infected patients.

Material and Methods

Patient Selection

Between June and December 2004, male patients with a clinical and laboratory diagnosis of HCV infection were recruited from the Hepatitis Outpatient Clinic of the Infectious Diseases Department. All recruited patients signed an informed consent agreement.

Blood and Semen Collection and Preparation

Blood was collected from patients into 10mL dry vacuum tubes, after an 8 to 12-h fast. Semen samples were obtained by self-masturbation, after a period of sexual abstinence of at least three days. Blood and semen samples were stored at -80°C until the time of use.

In addition to an aliquot of total semen, fractions were isolated on 90 and 45% Percoll gradients. The samples were centrifuged for 30 min. at 3,000 rpm, and the following three phases were obtained: seminal plasma, leukocytes and spermatozoa.

Serum

All serum samples were analyzed with the qualitative Amplicor HCV test, version 2.0 (Roche Diagnostics Corp., Indianapolis, IN, USA). If positive, the blood samples were genotyped with INNO-LiPA HCV II kit (INNO-LIPA HCV, Versant Bayer, Tarrytown, NY, USA).

Semen

All semen samples were analyzed with the qualitative Amplicor HCV test, version 2.0 (Roche Diagnostics Corp., Indianapolis, IN, USA). After the end of the PCR-HCV reaction, the presence of HCV in semen samples was revealed by two methods: i) electrophoresis in agarose gel and ii) Roche Amplicor test by colorimetric determination.

Electrophoresis Agarose Gel Detection

The PCR-HCV products were detected by 2% Agarose Gel electrophoresis, stained with ethidium bromide and observed under ultraviolet light [13-15].

Amplicor HCV Test, v 2.0

The Amplicor HCV Test, v 2.0, is an RT-PCR in a manual, microwell format that amplifies a 244-nucleotide segment of the 5'-UTR of the HCV genome. The test was performed at all sites according to manufacturer's instructions, as previously described. HCV RNA optical density (OD) values were interpreted as follows: <0.3, negative; ≥0.3 and <1.0, equivocal; and ≥1.0, positive. An OD value of 0.3 was used as the cutoff for the internal control RNA. Any specimen with an OD value <0.3 for both HCV and internal control wells was considered to be inhibited [12].

HCV Genotyping by the Line Probe Assay (LiPA)

RNA was isolated from serum and 5'-UTR genotyping was performed with the INNO-LiPA HCV II kit (INNO-LIPA HCV, Versant Bayer, Tarrytown, NY, USA), according to manufacturer's instructions. Briefly, the 5'-UTR region was amplified with biotinylated primers. The biotin-labeled PCR products were then reverse hybridized to specific probes attached to nitrocellulose strips, and the results were developed as a purple precipitate that formed a positive line on the strip. The HCV type was deduced on the basis of the patterns of hybridizing bands using the LiPA interpretation chart [16].

Results

The mean age of the 13 patients was 40.8 years (range: 28-50, median: 45 years). The mean time since the diagnosis of HCV infection was 7.15 years (range: 2-14, median: 7 years).

Serum

Analysis was made of the same HCV-PCR product by the Roche Amplicor method and agarose gel electrophoresis with ethidium bromide staining; all serum samples tested positive by these two techniques, i.e., all patients presented viremia.

Semen

The products analyzed with the Amplicor HCV test and revealed by agarose electrophoresis gel detection were negative for all samples of total semen and fractions (spermatozoa, leukocytes and seminal plasma).

The same semen and seminal fractions (52 samples) analyzed by optical density (Roche Amplicor method) revealed 45 (86.5%) of the results to be inhibited. Negative results were detected for one (1.9%) total semen sample, one (1.9%) leukocyte and 4 (7.7%) seminal plasma fraction. Only one (1.9%) sample of the spermatozoon fraction was positive.

Discussion

The inhibition of HCV-PCR in semen samples was clearly and unquestionably demonstrated, with 45 (87%) of the 52 reactions involving total semen and fractions, spermatozoa, leukocytes and seminal plasma presenting inhibited results (Table 3). Amplicor HCV qualitative tests have an internal control, permitting the evaluation of the efficiency of the tests. In contrast, when the products of PCR (Amplicor HCV qualitative tests, v. 2.0 (Roche Diagnostics) were examined on the agarose gel with ethidium bromide staining, all samples tested negative. False-negative results were obtained with the in-house nested HCV-PCR method used in this preliminary study (data not shown). The presence of HCV-PCR inhibitors in semen masks the results and impairs their interpretation, a fact explaining the wide disparity in HCV detection rates reported in the international literature, which vary from 0 to 36% [10,11,17-25].

Our results support the hypothesis of the presence of HCV in semen samples from infected patients and also indicate the need for more sensitive tests and/or laboratory measures that remove HCV-PCR inhibitors so that reliable results can be obtained. Analysis of the HCV-PCR products by electrophoresis of an agarose gel and ethidium bromide staining apparently yielded false-negative results for all the semen samples. The commercial Roche Amplicor test demonstrated interference from inhibitors present in the semen samples, because it uses an internal control that guarantees the efficacy of the reaction; but it was unable to eliminate the inhibitors.

According to Abou-Setta [26] the contradictory results of HCV-PCR in semen can be explained by i) the different techniques used, ii) different HCV-PCR sensitivities, and iii) presence of inhibitors in semen that lead to false-negative results. Furthermore, this author reported that viral concentrations in semen samples go through rapid variations and indicated that inhibition might be due to the action of RNases or lipoperoxidase. The enzyme Taq polymerase may be inhibited by seminal fluid enzymes, lactoferrin, peroxidase or zinc residues [26].

The two main adaptations needed for testing semen are elimination of PCR inhibitors, especially frequent in seminal plasma, and the use of small sample volumes. The choice of the extraction protocol is critical [9].

Table 1. Distribution of the patients according to risk factors for the acquisition of HCV.

Epidemiology	No. of patients	%
Use of injectable and/or inhaled drugs	6	42.8%
Blood transfusion	4	28.6%
Unknown	4	28.6%

Table 2. Distribution of the patients according to HCV subtype.

Genotype	No. of patients (%)
1a	5 (38.5%)
1b	1 (7.7%)
1a/1b	2 (15.4%)
2b	2 (15.4%)
3a	3 (23.0%)
Total	13 (100%)

Information regarding the quantity of circulating virus is important; it helps increase the probability of HCV detection in semen and demonstrates the potential of sexual transmission. Nyamaty et al. [27] showed that the quantity of virus in blood is correlated with the virus in semen. The distribution of HCV viral types was compatible with the prevalence that was previously reported for Brazil, with most of the (infected) population being infected with HCV genotype 1, followed by genotypes 3 and 2 (Table 2) [28]. On average, 40 to 50% of patients infected with HCV indicate no specific source of acquisition of the virus [26]. We had 28.6% unknown risk factors (Table 1).

This preliminary study of semen samples by HCV-PCR indicates the need for new research protocols in order to obtain more reliable results. Other laboratory resources or even new techniques for viral detection should be tested for the evaluation of semen samples so that the results better correspond to reality.

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Table 3. Results of HCV-PCR with the Roche Amplicor test.

Patient	Serum	Total semen	Spermatozoa	Leukocytes	Seminal plasma
1	Positive	Inhibition	Inhibition	Inhibition	Inhibition
2	Positive	Inhibition	Inhibition	Inhibition	Inhibition
3	Positive	Inhibition	Inhibition	Inhibition	Negative
4	Positive	Negative	Positive	Negative	Negative
5	Positive	Inhibition	Inhibition	Inhibition	Inhibition
6	Positive	Inhibition	Inhibition	Inhibition	Inhibition
7	Positive	Inhibition	Inhibition	Inhibition	Inhibition
8	Positive	Inhibition	Inhibition	Inhibition	Inhibition
9	Positive	Inhibition	Inhibition	Inhibition	Negative
10	Positive	Inhibition	Inhibition	Inhibition	Inhibition
11	Positive	Inhibition	Inhibition	Inhibition	Negative
12	Positive	Inhibition	Inhibition	Inhibition	Inhibition
13	Positive	Inhibition	Inhibition	Inhibition	Inhibition

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