Original Paper

Detection of Hepatitis C Virus (HCV) in Body Fluids from HCV Monoinfected and HCV/HIV Coinfected Patients

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ABSTRACT

Background/Aims: The possibility of the nonparenteral Hepatitis C Virus (HCV) transmission is supported by the demonstration that the actual virus is present in several body fluids. In this study, we investigated the relationship between the detection of HCV RNA in body fluids (saliva, cervical smears, seminal fluid and peripheral blood mononuclear cells) from chronically HCV-infected patients and several viral and host factors.

Methodology: This study comprised 16 HIV/HCV coinfected and 21 HCV monoinfected patients with a median age of 38 and 45 years, respectively. HCV-RNA was detected in serum and fluids samples by reverse transcription-nested polymerase chain reaction. Genotypes were determined by using RFLP and direct nucleotide sequencing of the PCR products and plasma viral loads by using NASBA HCV-QT.

Results: When compared on the basis of the results of the detection of HCV-RNA in fluids, patients did not differ significantly in relation to viral load, genotype, HCV/HIV coinfection, and epidemiological host factors.

Conclusions: Our data suggest that HCV can be detected in body fluids of chronically HCV-infected patients independent of these cofactors, including circulating HCV load and HCV/HIV coinfection. Studies on HCV dynamics are needed to gain insights into nonparenteral transmission of HCV.

KEY WORDS: Hepatitis C (HCV); HCV/HIV co infec–tion; HCV viral load; Body flui

ABBREVIATIONS: Hepatitis C Virus (HCV); Human Im–muno–Deficiency Virus (HIV); Cervi–cal Smears (CS); Seminal Fluid (SF); Saliva (SA); Peripheral Blood Mononuclear Cells (PBMCs); Reverse Transcription Polymerase Chain Reaction (RT Nested–PCR); Restriction Length Polymorphism Analysis (RFLP); Ribonucleic Acid (RNA); 5' Non–Coding Region (5'NCR); Nonstructural Region 5B (NS5B); Non Significant (NS); Assisted Reproduction Technology (ART)

INTRODUCTION

Infections by hepatitis C virus (HCV) represent a major public health problem worldwide nowadays. HCV belongs to the *Flaviviridae* family, genus *Hepacivirus*. Epidemiological studies have demonstrated that the most efficient routes for HCV transmission are parenteral, transfusion of blood and blood products, intravenous drug use, occupational needle-stick injuries, hemodialysis and organ transplantation (1). However, 15–40% of HCV infected patients have no obvious parenteral risk factors. In these patients, perinatal, intrafamilial and/or sexual transmission is suspected. It is assumed that viral transmission in these cases occurs through various body fluids. The study of human secretions searching for the presence of HCV-genome has yielded conflicting results. Sexual transmission or other close human contacts may play a role in sporadic and community-acquired infections (2). Several studies have reported detection of HCV RNA in semen (3-5) and cervical smears (CS) (6, 7). The detection of HCV in seminal fluid (SF) and CS would provide further evidence for the biological plausibility of sexual transmission of HCV. On the other hand, other authors have demonstrated the presence of HCV RNA in saliva (SA) of HCV infected patients, by RT-nested PCR. These studies report varying results, with detection rates ranging from 0 to 100% (8).

Although HCV is prevalently a hepatotropic virus, the presence of extra hepatic replication is still under debate. Several studies on peripheral blood mononuclear cells (PBMCs) isolated from chronically infected patients suggest that these cells also support HCV replication. Furthermore, several studies suggest that extra hepatic replication of HCV may be an important predictor of HCV treatment outcome (9).

HCV and human immunodeficiency virus (HIV) share routes of transmission, although the mechanism of interaction between both viruses remains unclear. HCV viremia is particularly high in HCV/HIV co infected patients, which would favor the presence of HCV in other fluids (10). Many studies have been conducted to demonstrate the presence

of HCV RNA in different body fluids, but only a few refer to various body fluids of the same patient simultaneously (2,11).

The aim of this study was to investigate the presence of HCV RNA in plasma, saliva, seminal fluid, cervical smears and PBMCs of HCV monoinfected and HCV/HIV co infected patients and establish the relationship between the detection of HCV in body fluids and several viral and host factors.

METHODOLOGY

Patients and specimen collection

Body fluids from 37 patients (21 males/16 females) were prospectively studied. Sixteen of them were co-infected with HIV. All the subjects had HCV RNA detectable in serum by RT-Nested PCR and were not receiving antiviral therapy. Serum samples were retested to confirm HCV RNA at the time of body fluid sampling. The samples were collected and frozen at -70ºC until detection of viral RNA.

This study was approved by the Ethics Committee of the Medical School of National University of Córdoba. Written informed consent was obtained from all patients included in this study.

Serum and body fluid processing

TABLE 1 Characteristics of the Study Population and Genotyping of 37 HCVRNA Infected Patients of Cordoba, Argentina

Variables	HCV mono infected	HCV/HIV co-infected
Age at enrollment (mean)	45	38
Gender		
Male	10	11
Female	11	5
Total	21	16
Parental Risks		
Blood transfusion	6	2
IVDU	3	13
Surgery	13	5
Uncertain Risk*	6	2
HCV Genotypes		
Genotype 1	10	13
Genotype 2	9	1
Genotype 3	2	2
Plasma HCV RNA level (mean) log	4.6	5.6
Receipt ART	No	No

* History of dental care, unsafe injections, household transmission (sharing razors, toothbrushes and combs), tattoo, sexual, multiple sexual partners, Men Sex Men (MSM) or different sexual habits.

In all patients, blood was collected in sterile tubes and the serum was separated by centrifugation and stored at -20ºC.

Non-stimulated saliva samples were collected by spitting into a sterile falcon tube. Saliva was recovered by centrifugation and visually checked for the presence of blood cells. None of the samples contained blood at the macroscopical exam.

After three days of sexual abstinence, semen samples were obtained by masturbation into a sterile container and processed within the next 2 hours after ejaculation. The samples were diluted and centrifuged 1200g for 10 min and the SF was separated from the cell pellet and stored at -70ºC until used.

CSs were collected by swabbing the vaginal canal (sterile dracon swabs; Baxter Scientific, McGaw Park, IL) during pelvic examination. The swab was dipped into 3 ml of PBS (pH=7.5). The probes were vortexed within the 3 hours after retrieval and the resulting material was centrifugated 15 min at 1800 rpm.

PBMCs were isolated using Ficoll-Hypaque density gradient; after three washes in PBS, the cell pellet was resuspended in RPMI medium; the number of cells was limited to 1x106cell/ml. All washes were negative for HCV RNA by PCR.

RNA extraction was performed using a commercially available reagent Trizol LS (Invitrogen, Life technologies, Rockville, MD) for serum and body fluid with the QIAamp Viral RNA Kit (QIAGEN GmBH, Germany) according to the manufacturer's instructions.

RT-PCR and genotyping

The presence of HCV RNA was determined by RT Nested-PCR using 5' non-coding region (5'NCR) primers, as previously described by Ré *et al.* (12).

HCV genotyping was carried out using the restriction length polymorphism analysis (RFLP) of the 5´NCR. This RFLP assay properly allowed distinguishing between genotypes 1, 2 and 3 in previous HCV isolates from Argentina (15).

NS5B sequencing

Amplification of the nonstructural region 5B (NS5B) and direct sequencing were performed as previously described by Chen and Weck, (13); in some cases to confirm the 5'NCR genotyping.

HCV RNA quantification in serum

HCV RNA in serum was quantified with the NASBA HCV QT (Organon Teknika, Boxtel, The Netherlands).

Statistical analysis

Statistical analysis was performed using SPSS for Windows and p values lower than 0.05 were considered significant. Student's test was used to compare continuous variables and Chi-square or Fisher's exact test to compare proportions.

TABLE 2 Detection of HCV RNA in Different Body Fluids of 37 Patients

Variables (*)	Saliva		Seminal Plasma		Cervical Smears		PBMCs	
Genotypes	N	%	N	%	N	%	N	%
1	5	63	4	67	2	40	18	62
2	2	25	0	--	2	40	7	24
3	1	12	2	33	1	20	4	14
Age								
> 40	5	63	2	33	3	60	16	55
< 40	3	37	4	67	2	40	13	45
IVDU								
Yes	5	63	4	67	2	40	13	45
No	3	37	2	33	3	60	16	55
HCV viral load								
> 5 log	7	88	6	100	5	100	19	66
< 5 log	1	12	0	--	0	--	10	34
HIV coinfection								
Yes	5	63	4	67	2	40	12	41
No	3	37	2	33	3	60	17	59
Surgery								
Yes	3	37	2	33	3	60	13	45
No	5	63	4	67	2	40	16	55
Transfusion								
Yes	3	37	0	--	3	60	8	28
No	5	63	0	--	2	40	21	72
Uncertain Risk								
Yes	2	25	1	17	0	--	6	21
No	6	75	5	83	0	--	23	79

*Non significant (NS), $p>0.05$

RESULTS

Patient's population, descriptive data on age, gender, risk factors, HCV viral load and genotypes in serum were recorded (**Table 1**).Genotype distribution among HCV- monoinfected patients was as follows: 48% (10/21) genotype 1, 43% (9/21) genotype 2 and 9% genotype 3 (2/21). For patients coinfected with HIV, genotype distribution was 76% (13/17), 6% (1/17) and 18% (3/17), respectively (**Table 1**). The genotypes found in body fluids and in corresponding plasma sample was the same. The prevalence of HCV RNA in body fluids was: 31% (5/16) in cervical smears, 18.7% (8/37) in saliva, 29% (6/21) in seminal fluid, and 78% (29/37) in PBMCs. The HCV RNA detected in PBMCs was not due to contamination from plasma, since no viral sequences could be detected in the third washing of PBMCs (data not show). When compared on the basis of the results of the detection of HCV-RNA in fluids, patients did not differ significantly in relation to viral load and genotype, HCV/HIV co infection, and epidemiological host factors (**Table 1**). Even though statistical association was not found ($p>0.05$), a higher percentage of detection in different fluids was noted when viral load was higher than 5 Log (**Table 2**).

DISCUSSION

Nonparenteral transmission of HCV through body fluids different than blood, including horizontal heterosexual and vertical mother-to-child transmission may occur, although infrequently (2,

14, 15). The biological plausibility of HCV transmission by nonparenteral routes is provided by the demonstration of HCV in body fluids such as saliva, semen, or cervico-vaginal secretions (16). However, the low efficiency of nonparenteral routes of HCV transmission is yet poorly understood. Our data suggest that HCV can be detected in body fluids of chronically HCV-infected patients independent of circulating HCV viral load, genotype, HCV/HIV coinfection and epidemiological host factors.

From a review of the literature many investigators have found the presence of HCV-RNA in saliva, however, widely contrasting results emerge, with detection rates ranging from 0-100%. Some authors have demonstrated an association between high viral load and the detection of HCV in saliva (10, 17, 18); however, others have been unable to support such association (19, 20). The frequency of HCV RNA found in saliva samples of this study was 29%. Even though HCV RNA has been found in leukocytes and in oral epithelial cells (21) from HCV infected patients, the saliva samples of this study were free of such cells. Specific tests to investigate occult blood were not performed in this study, but some authors (8, 11, 20) have demonstrated that among HCV infected individuals, the presence of blood in saliva samples does not correlate with the presence of HCV RNA in the same samples. A possible explanation for such finding is that HCV could replicate in oral epithelial cells in patients without HCV in blood, as demonstrated by Arrieta *et al.* (21).

The detection of HCV RNA in saliva may have several implications in medical and dental care.

Even though HCV is a prevalently hepatotropic virus, convincing evidence of HCV extrahepatic reservoirs has been demonstrated (12, 22, 25). In accordance with several studies, (22, 24, 25) we detected HCV RNA in PBMCs in high prevalence (78%). This fact has important implications for transmission, disease progression and effective treatment.

The presence of HCV-RNA in semen and CS are a strong argument in favor of HCV sexual transmission. In this study, we detected considerable prevalence of HCV RNA in SF and CS (29% and 31%, respectively). Our results suggest that the recommendation of protect sex for HCV infected individuals should be reinforced. Moreover, considering that the Centers for medically assisted reproduction of Argentina do not perform routine tests for HCV and HIV detection, these results should be considered in the broader perspective of safety in laboratories and for counseling and management of HCV sero-different couples who intend to embark on assisted reproduction technology (ART). In several European countries, ART is under strict legal control, in case of patients with HIV and HCV together or separately (26-31).

Even though the detection of HCV RNA in fractions of semen does not necessarily imply the presence of replicative virions and the infectivity of spermatozoa by medically assisted reproduction, our results plead for the reinforcement of precaution measures for men whose blood is chronically infected with HCV and who are candidates for this procedure.

We applied a sensitive non commercialized nested PCR and demonstrated the presence of HCV RNA in different body fluids obtained of the same patient simultaneously. The rates of RNA HCV prevalence in different body fluids obtained in this study are in accordance with others worldwide reports but discrepant with other ones. An explanation of this fact is the lack of standardized protocols of sampling, centrifugation, manipulation, storage conditions, freezing methods and RNA extraction used that may have affected subsequent HCV RNA detection and contributed to these differences.

Although the occurrence of the viral salivary and genital secretions shedding does not necessarily mean that HCV transmission occurs by this fluids. Studies on HCV dynamics are needed to provide new insights into nonparenteral transmission of HCV.

ACKNOWLEDGMENTS

This study was supported in part by grants from SECYT (Secretaría de Ciencias, Tecnología e Innovación Productiva, Argentina) and SIP-UCC (Secretaría de Investigación Universidad Católica de Córdoba).

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