No evidence of occult hepatitis C infection among long-term Tunisian hemodialysis patients: A single-center study

Aucune infection occulte par le VHC détectée chez des hémodialysés chroniques tunisiens : Résultats d'une étude monocentrique

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ABSTRACT **Introduction**: Occult hepatitis C infection (OCI) is a new concept, characterized by the presence of hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells (PBMC), hepatocytes, or ultra-centrifuged serum, with no RNA detectable in plasma. This study aimed to investigate occult hepatitis C infection in a vulnerable Tunisian population: chronic hemodialysis patients.
Methods: A retrospective study, including chronic hemodialysis patients of the Hemodialysis Unit of the University Hospital Fattouma Bourguiba of Monastir. Viral load was assessed by real-time RT-PCR on plasma and peripheral blood mononuclear cells, and chemiluminescent assay was used for HCV antibodies detection.
Results: A total of 53 chronic hemodialysis patients were enrolled. Three patients (5.7%) had a history of HCV infection with a sustained virological response. Multivariate analysis revealed that these patients had a longer median duration of hemodialysis (OR=1.33; [IC 95%=1.071-1.666]; p=0.01). HCV RNA was undetectable in both blood and peripheral blood mononuclear cells in all cases. Thus, no OCI was identified in any of our patients.
Conclusion: A low seroprevalence of HCV was observed in our study population and no OCI were detected. However, considering the restricted number of patients enrolled, this result should be confirmed in a larger population cohort.

Key words: HCV, Occult infection, Peripheral blood cells, Hemodialysis, Prevalence

RÉSUMÉ **Introduction** : L'infection occulte par le virus de l'hépatite C (OCI) est une nouvelle entité, caractérisée par la présence de l'ARN du virus de l'hépatite C (VHC) dans les cellules mononuclées du sang (PBMC), les hépatocytes ou le sérum ultra-centrifugé, en l'absence d'ARN détectable dans le plasma. Cette étude avait pour but de rechercher l'OCI chez les hémodialysés chroniques (HDC).
Méthodes: Etude transversale, ayant inclus les HDC suivis à l'unité d'hémodialyse du CHU Fattouma Bourguiba de Monastir. La charge virale a été évaluée par RT-PCR en temps réel sur plasma et PBMC, et la technique de chimiluminescence a été utilisée pour la détection des anticorps anti-VHC.
Résultats : Un total de 53 HDC ont été inclus parmi lesquels 3 (5,7 %) présentaient un antécédent d'infection par le VHC avec une réponse virologique soutenue. L'analyse multivariée a révélé une durée médiane d'hémodialyse plus longue chez ces patients (OR=1,33; [IC95%=1,071-1,666];p=0,01). L'ARN du VHC était indétectable dans le sang et les PBMC chez tous les patients. Ainsi, aucune OCI n'a été identifiée.
Conclusion : Une faible séroprévalence du VHC chez les HDC était observée et aucune OCI n'a été détectée. Cependant, ce résultat devrait être confirmé dans une cohorte de population plus importante.

Mots-clés : Virus de l'hépatite C, infection occulte, cellules sanguines périphériques, hémodialyse, prévalence

BACKGROUND

The Tunisian general population has a low endemicity for HCV (less than 1%) [1]. However, some groups present a high risk of infection, including chronic hemodialysis patients (HDP) (7.3%) [2]. Tunisia, like many countries around the world, has followed a National Hepatitis C Elimination Program since 2016 [3]. The use of direct-acting antivirals (DAA) has substantially transformed the prognosis of HCV-infected patients, with sustained virological response rates exceeding 90% [4].

Occult hepatitis C infection (OCI) is a new concept, based on the detection of RNA in hepatocytes, PBMC, or ultra-centrifuged sera in patients with a negative plasmatic viral load (VL) [5,6]. PBMC has been recognized as an extra-hepatic reservoir of HCV replication since 1985, and confirmed by several subsequent studies [7–9]. In 1994, viral RNA was isolated from PBMC of patients with histological damages suggesting chronic hepatitis C infection (CH-C), and with undetectable VL in plasma [10]. It was only a decade later that occult HCV infection (OCI) was first described by Castillo et al [5].

Two types of OCI are currently acknowledged: seropositive OCI (patients with positive anti-HCV antibodies) and seronegative OCI (patients who have negative anti-HCV antibodies) [11].

OCI prevalence varies (from 1.2% to 57%) according to the considered populations, and is higher in some populations at high risk of hepatitis C, such as HDP, in whom it may reach 45% [12]. In patients with sustained virological response, it could reach 11.3%, which could represent an obstacle to the national virus elimination program [13]. In fact, OCI could be associated with a higher risk of developing chronic liver damage and hepatocellular carcinoma [14], and could potentially lead to viral spread [15].

One of the most high-risk populations for developing OCI would be HDP. Indeed, considering the great risk of viral transmission in hemodialysis units, this population would also be at risk of OCI and viral diffusion in this population [16]. Therefore, our study aimed to estimate the prevalence of OCI in Tunisian HDP.

METHODS

Study population and design

A descriptive cross-sectional study, conducted over 6 months, including HDP attending the Hemodialysis Unit of the Nephrology Department of the University Hospital Fattouma Bourguiba of Monastir. All participants were over 18 years old, undergoing hemodialysis for more than 2 months, and consenting to take part in the study. Patients with a history of other chronic liver diseases or positive VL in plasma were excluded.

Virological analyses were performed at the Microbiology Laboratory of the same hospital.

Sample Size Determination

The sample size was estimated using the Cochran's formula [17]:

$$[n = z^2 \times p \times (1-p) / d^2]$$

Where:

- -n: Calculated final sample size
- - z: Confidence interval (for a confidence interval of 95%, z = 1.96)
- - p: estimated proportion of the population presenting the condition
- - d: desired precision

To study OCI, which has a prevalence of 3% in HDP patients in Iran, a country that has a prevalence of HCV close to our national level [18] the sample size should be: ($n= 1.96^2 \times 0.03 \times 0.97 / 0.05^2 = 45$) patients.

Data Collection

Demographic, clinical, and biological data were collected by interviewing patients and referring to their clinical records using a pre-established standard questionnaire.

Serological tests

The detection of anti-HCV antibodies was performed on serum using chemiluminescent microparticle immunoassay assay Architect (Abbott Diagnostics, USA) which has a sensitivity and a specificity of 99.6% and 99.1% respectively.

PBMC and plasma isolation and preservation

For each patient, two 5-ml blood samples were gathered in EDTA sterile tubes for viral RNA detection in plasma and PBMC.

The cell fraction was extracted from the first tube, using Ficoll-paque density gradients (Sigma-Aldrich) immediately once received. After diluting the blood with Phosphate-buffered saline (PBS) (1/2), the diluted blood was transferred to a conical tube above the Ficoll-Paque layer (1/3 Ficoll and 2/3 diluted blood). After centrifugation (400xg at room temperature: 18-25°C) for 30 minutes with brake off, the PBMC layer, at the interface between the Ficoll-Paque and the diluted plasma was obtained. The PBMC was then washed 3 times with PBS and cryopreserved at -80°C, in a solution of DMSO (dimethyl sulfoxide; 20%), RPMI (Roswell Park Memorial Institute Medium; 60%), and Fetal Calf Serum (20%), until further use. The second sample was centrifuged at 800xg for 20 minutes, and the resulting plasma was stored at -80°C.

A positive control (sample from a patient with CH-C) was included and was processed in the same way as the other samples.

After thawing plasma and PBMC in a 37°C water bath, viral RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions [19]. Real-time RT PCR was performed on Rotor-Gene using the kit (Artus HCV QS-RGQ kit). Four standards were included defining a linearity range of 15-108 IU/ml and a

sensitivity of 15 IU/ml. An internal control was added at the extraction step to detect any PCR inhibition.

Ethical Considerations

The project was approved by the Ethics Committee of the University of Medicine of Monastir on July 15, 2021, (IORG 0009738 N°106 / OMB 0990-0279). Each patient provided written informed consent.

Statistical Analysis

Statistical analyses were conducted by SPSS 20.0 (Statistical Package for the Social Sciences). Categorical variables were compared using the chi2-test and Fisher-test with an error risk α of 0.05. Student's t-test was used to compare the means of quantitative variables. A comparison of medians was performed using the Mann-Whitney test.

RESULTS

A total of 53 HDP patients regularly hemodialyzed at the Hemodialysis Unit of University Hospital Fattouma Bourguiba were included. The average age of our patients was 47.9±14 years. Our population consisted of 37 men and 16 women, giving a sex ratio M/F of 2.3. The background disease of patients was dominated by chronic glomerular nephropathy, observed in almost half the population (N=25; 47.2%), represented mainly by chronic glomerulonephritis (N=7; 13.2%) and diabetic nephropathy (N=7; 13.2%). The median duration of hemodialysis for our patients was 4 years IQ [2-7.7], and 22 patients (41.5%) were dialyzed for more than 5 years. All patients had normal levels of liver enzymes (AST/ALT), bilirubin, and Gamma-glutamyl transferase (GGT), and 8 (16%) had elevated alkaline phosphatase levels. They all had negative human immunodeficiency virus (HIV) serology and most of them had protective anti-HBs levels (70%). No one was co-infected with Hepatitis B virus (HBV).

Three patients had a history of HCV infection: The first patient had a spontaneously resolved hepatitis C. The 2 other patients were treated with DAA with a sustained virological response of 3 years. Both had genotype Ib.

In univariate analysis, significant factors associated with a history of CH-C were dyslipidemia (p=0.021), higher ALT levels (p=0.042), and a longer median duration of hemodialysis (p=0.005) (Table 1,2). In multivariate analysis, a longer median duration of hemodialysis was the only factor associated with HCV infection (OR=1.33; [IC 95%=1.071-1.666]; p=0.01).

All patients tested negative for HCV RNA in blood and PBMC by real-time RT-PCR. Thus, no cases of OCI were found in our study population.

Table 1. Biological markers associated with a history of HCV infection

Parameters		*Anti-HCV*		*p-value*
		Negative	**Positive**	
White blood cells	Mean	6168.9	6350	0.870*
	SD	1 772.3	3087.7	
Hemoglobin	Mean	8.5	10.3	0.114*
	SD	1.8	1.7	
Platelets	Mean	181 608.9	194 666.7	0.707*
	SD	58 800	35 809	
Alkaline phosphatases	Mean	147.85	64.6	0.211*
	SD	110	14	
Total bilirubin	Mean	4.93	3.5	0.605*
	SD	1.7	0.7	
AST	Mean	12	15.33	0.266*
	SD	4.9	5.6	
ALT	Median	11	18.3	**0.042****
	IQR	7	6	

*: Means comparison by Student's T test, **: Median comparison by Mann-Whitney test; **ALT**: alanine transaminase; **AST**: aspartate transaminase; **IQR**: interquartile range; **SD**: standard deviation

DISCUSSION

OCI is a recently established entity, which has not yet been investigated in Tunisia. Since the prevalence of CH-C is low in the Tunisian general population (less than 1%)[1], we would expect a low prevalence of OCI in this population. However, high-risk HCV populations would be at greater risk of OCI. We therefore aimed in the present study to estimate the prevalence of OCI in a high-risk group, HDP.

HDP are 60 to 100 times more at risk of developing CH-C [20]. Nevertheless, this prevalence has decreased considerably in recent decades, thanks to periodic viral screening of patients, systematic screening of blood donations, and dialyzing infected patients on separate machines [2].

According to our study, three patients (5.6%) had positive HCV serology. In a national screening survey carried out on 11,653 Tunisian HDP (2021-2022), the overall HCV seroprevalence was 3.2%, from which 2 8.1% of patients had positive VL [21]. The prevalence of this disease among HDP varies around the world. According to a meta-analysis published in 2022 by Greeviroj et al, it was about 21%, with higher levels in Africa (28%) and in low-income countries (48.5%) [22].

Table 2. Demographic and clinical characteristics of patients according to their hepatitis C serology

Demographic / Clinical data	*Variables*	*Univariate analysis*			*Multivariate- analysis*		
		anti-HCV		p-value	p-value	OR	IC 95%
		positive	Negative				
Male	n (%)	2 (5.4%)	35 (94.6%)	1.000 *	-	-	-
Age >50 years	n (%)	1 (4.8%)	20 (95.2%)	1.000 *	-	-	-
Diabetes	n (%)	0	9 (100%)	1.000 *	-	-	-
Dyslipidemia	n (%)	1 (100%)	0%	0.021 *	-	-	-
Hypertension	n (%)	0	28 (100%)	0.098 *	-	-	-
Surgical history (other than the confection of an arteriovenous fistula)	n (%)	3 (14.3%)	18 (85.7%)	0.05 *	-	-	-
High-risk sexual behavior	n (%)	0	0	-	-	-	-
History of peritoneal dialysis	n (%)	0	0	1.000 *	-	-	-
Blood transfusion	n (%)	2 (10.5%)	17 (89.5%)	0.290 *	-	-	-
Tattooing/scarification	n (%)	1 (33.3%)	2 (66.7%)	0.163 *	-	-	-
Digestive endoscopy	n (%)	0	7 (100%)	1.000 *	-	-	-
History of drug addiction	n (%)	0	1 (100%)	0.500 *	-	-	-
Duration of hemodialysis (months)	Median (IQR)	24 (17-24)	4 (2 -7.7)	0.005 **	0.01	1.33	1.071-1.666

IC: Confidence interval; **IQR**: interquartile range; **OR**: odds ratio; *: Data analysis done by khi-2/ Fisher exact test; **: Median comparison by Mann-Whitney test

Since 2004, OCI, a new form of HCV infection has been identified, characterized by the presence of HCV RNA in hepatocytes, PBMC, or ultra-centrifuged serum, without any RNA detectable in plasma [8,9]. The consequences of this infection were reported by Wang et al [8], who described persistent hepatic inflammation and a more severe fibrosis score in patients with OCI when compared to patients without OCI. Furthermore, an association between hepatocellular carcinoma of undetermined etiology and OCI has been established [14]. Moreover, concerns were raised since the discovery of this infection, related to transmission risks, particularly due to fluctuations in the levels of viremia detected by ultracentrifugation in these patients [6]. Roque-Cuellar et al reported a large prevalence of OCI among seronegative heterosexual partners of patients with CH-C (13% of partners)[23]. In 2009, a study carried out on family members of patients with OCI and with CH-C found similar seroprevalence between the 2 groups. Phylogenetic analysis of viral sequences in patients and their families revealed a common source of infection within each family [15] which suggests a risk of transmission of this new form of HCV infection.

Our study is the first to investigate OCI among Tunisian HDP. No cases of OCI were detected among the 53 patients included despite the use of techniques of similar sensitivity as in international studies

In 1995, long before the first description of OCI in 2004, viral RNA had already been detected in PBMC, among 1.7% of Australian HDP [24]. This condition was subsequently investigated and its prevalence varied between 0 and 45% in the literature depending on the countries and populations considered (table 3) [12,25–33]. Two studies carried out in Iran did not detect any OCI [26,27], whereas in Spain, a 45% rate was reported [12]. This significant difference between studies could be attributed to several reasons, including the different HCV prevalence in general populations, and the different technical procedures [12]. Moreover, in the study conducted in Spain, the main selection criteria for HDP patients was hepatic cytolysis. This patient selection strategy could explain the disparity in results.

Regarding methodology, there are still no precise VL thresholds or consensual methodology for OCI diagnosis [9], making it difficult to draw definitive conclusions about the true prevalence of this infection. RNA detection in hepatocytes remains the gold standard for OCI, however, less invasive alternatives can be used, particularly after proving viral replication in PBMC [6]. Several studies have confirmed the possibility of RNA detection in PBMC or ultra-centrifuged serum [6,34] as an alternative to liver biopsy (LB), which represents an invasive procedure. In the study conducted by Castillo et al, on 57 patients with OCI diagnosed by LB, HCV RNA was detected in 70% of cases also in PBMC. Another alternative to LB would be RNA detection in ultra-centrifuged sera. Indeed, Bartolomé et al measured the VL in 106 patients with abnormal liver function tests of undetermined etiology, from ultra-centrifuged serum and PBMC and did not find a significant difference between the 2 techniques since an OCI was found in 58.5% of patients in ultra-centrifuged serum, vs 65% from PBMC [34]. However, the effectiveness of these techniques could be improved if they are associated, increasing their sensitivity [34].

It also has been shown that viral RNA levels can fluctuate in PBMC and plasma [35]. Therefore, it has been suggested to get multiple repetitive PBMC and serum samples [23]. On the other hand, extraction and amplification methods (PCR, in situ hybridization) have a direct influence on OCI detection rate. Indeed, VL quantification closely depends

on the quality and quantity of the extracted RNA. Therefore, the choice of RNA extraction kit is crucial. In our study, we used the RNeasy Mini Kit (Qiagen), which is validated for RNA extraction from PBMC using use silica columns [36]. Other kits are used for RNA extraction from PBMC, based on phenol-chloroform extraction, such as the RiboPure RNA Purification Kit-blood (Thermo Fisher Scientific) and the TRI Reagent (Sigma-Aldrich). Other extractions are based on the use of magnetic silica particles which capture nucleic acids, such as the NucliSENS easyMAG platform (bioMérieux) [37]. Several studies have compared the performance of these kits in the extraction of RNA from PBMC and concluded that they were comparable overall with a slight superiority to protocols using phenol-chloroform (RNeasy and RiboPure) [37].

For VL evaluation method, we used the Artus HCV QS-RGQ Kit®, the sensitivity of which is within national and international recommendations (sensitivity threshold ≤15 IU/ml) [38] and which complies with statistical requirements. The majority of studies investigating OCI used nested RT-PCR or real-time RT-PCR [27]. Other more sensitive techniques have been developed with significantly lower detection limits (sensitivity threshold <15 IU/ml), such as NAH (Nucleic Acid Hybridization) or in situ hybridization, particularly used for the detection of HCV in liver tissue [39].

In our study, no cases of OCI were observed in HDP. Several hypotheses could explain the difference between our results and those found in the literature. First of all, the difference in HCV prevalence between the countries could justify the huge difference between our results and those observed in Egypt for instance (Table III). Indeed, countries that had high rates of OCI among HDP were often those with high endemicity of HCV infection. Furthermore, the HCV genotypes that have been implicated in this condition (genotypes 1a, 2a, 2b, 3b, 4, and 6a) are rare in Tunisia where genotype 1b predominates [1,40]. Moreover, diagnosis of OCI on PBMC only could underestimate the detection of viral RNA compared to the gold standard which is on hepatocytes. Another plausible explanation would be the intermittent nature of viral RNA detection in PBMC during OCI. Thus, repeated analysis would have a better yield. Finally, the small size of our sample and its monocentric nature could also explain the absence of OCI in our series. Larger-scale multicenter studies, as well as targeting research to patients presenting risk factors for OCI (such as HDP with hepatic cytolysis, patients with a history of CH-C, intravenous drug users, etc.)[12,16], could result in higher rates of OCI.

Our study is the first to investigate OCI in the Tunisian population and this result comforts us about the absence of this disease, which would have been a real obstacle to the national HCV elimination program, with an increased concern for HDP.

Being in the midst of the HCV elimination program in our country, OCI investigation remains a challenge, but an essential step to ensure viral eradication.

CONCLUSION

HDP patients are particularly at risk of viral hepatitis, especially CH-C. In our study population, a low seroprevalence of HCV was observed and was correlated with the median duration of hemodialysis. Our study does not support the presence of OCI infection among Tunisian HDP despite the use of valid techniques already tested in several previous works. However, this result should be confirmed by a larger cohort, covering more dialysis units with a better representation of HCV risk factors; considering the risks of viral transmission and chronic histological consequences that OCI may have on this vulnerable population.

Table 3. Prevalence of occult hepatitis C in hemodialysis patients in different studies

Author/country	Year	Population	Serology	Sample type	Technique	OCI	
						N	%
Barril et al/ Spain [12]	2008	109 HDP with cytolysis	Negative	PBMC	Real-time PCR and hybridization in situ	49	45%
Baid-Agrawal et al/ Germany [25]	2014	407 HDP	Negative	PBMC	Versant HCV RNA qualitative TMA assay	1	0,25%
Ramezani et al [26]/ Iran	2014	30 HDP avec cytolysis	Negative	PBMC	Nested RT- PCR	0	0
Eslamifar et al/ Iran [27]	2015	70 HDP	Negative	PBMC	Nested RT- PCR	0	0
Abdelrahim et al/ Egypt [28]	2016	81 HDP	Negative	PBMC	Real-time PCR: Artus HCV-RG RT-PCR Kit	3	3,7%
Naghdi et al/ Iran[29]	2017	198 HDP	Negative	PBMC	Real-time PCR: High Pure Viral Nucleic Acid Kit	6	3,03%
Ali et al/ Egypt [30]	2018	39 HDP	Negative	PBMC	Real-time PCR: TaqMan PCR Master Mix Reagents Kit	9	23%
Alduraywish et al/Saudi Arabia[32]	2020	98 HDP	Negative	PBMC	Real time PCR: Artus HCV-RG RT-PCR Kit	8	8,16%
A Serwah et al / Egypt [33]	2021	204 HDP	Negative	PBMC	Real time PCR	14	7%
Our study / Tunisia	2022	53 HDP	50 Negative/3 positive	PBMC	Real-time PCR	0	0%

HDP: chronic hemodialysis patients, **PBMC**: peripheral blood mononuclear cells; **OCI**: occult hepatitis

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