

LOW LEVEL OF BASELINE RESISTANCE IN RECENTLY HCV INFECTED MEN WHO HAVE SEX WITH MEN WITH HIGH-RISK BEHAVIORS

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LOW LEVEL OF BASELINE RESISTANCE IN RECENTLY HCV INFECTED MEN WHO HAVE SEX WITH MEN WITH HIGH-RISK BEHAVIORS

Running head: Baseline HCV resistance in men having sex with men

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Highlights

- Uncommon NS3/NS5A RASs among recently HCV infected MSM with risky behavior
- High prevalence of subtype-specific polymorphisms in GT4d infections
- Reassurance of the clinical management of HCV infection in this population

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ABSTRACT

Background

The presence of baseline HCV resistance-associated substitutions (RASs) can impair treatment outcome by Direct-Acting Antivirals. We investigated the prevalence of pretreatment HCV resistance among recently HCV infected MSM with high risk behaviors, either HIV co-infected or at high risk of HIV acquisition and under Pre-exposure Prophylaxis (PrEP).

Methods

NS5A and NS3 fragments were deep sequenced on pretreatment samples of 72 subjects using Illumina Miseq paired-end sequencing technologies. UDS data was analyzed by Smartgene® platform. RASs mentioned in literature were analyzed and interpreted depending on genotypes (GT) at 10% cut-off.

Results

HCV genotyping showed 36 (50%) GT1a, 31 (43.1%) GT4d, and 5 (6.9%) GT3a infections. Fifty-five (76.4%) patients were co-infected with HIV and 15 (20.8%) received PrEP. In GT1a virus, NS3-RASs were found in 4/30 viruses (13.3 %: S122G/N, R155K, and I170V) and Q80K polymorphism was present in 14/30 (46.7%) viruses. No NS3-RASs were detected in GT4d and 3a viruses. NS5A-RASs were detected in 3/36 GT1a viruses (8.3%: Q30E/R, L31M, and H58L). NS5A subtype-specific polymorphisms L30R and T58P were found at high frequencies in 31/31 (100%) and 16/31 (51.6%) GT4d viruses, respectively. One RAS M31L was also observed along with the polymorphisms L30R and T58P. No NS5A-RASs were detected in GT3a viruses.

Conclusions

A low level of RASs to NS3 and NS5A inhibitors on pretreatment samples was detected in the study population. Our findings reassure the clinical management of HCV infection in this high-risk population.

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Keywords: Men who have sex with men; Deep sequencing; Hepatitis C virus; Resistance transmission; Pre-Exposure Prophylaxis.

I. INTRODUCTION

The introduction of direct-acting antivirals (DAAs) marked a new era of HCV infection treatment associated with outstanding sustained virological response (SVR) rates. However, some cases of HCV treatment failure still occur for multiples reasons and one of which is the selection of treatment resistance-associated substitutions (RASs).

Men who have sex with men (MSM) are considered as a key population vulnerable to HCV infection especially those with risky behaviors. Indeed, several outbreaks of HCV infection in MSM have been described since 2000 in urban centers in Europe, Australia, and the United States. A meta-analysis reported the incidence of HCV infection at 7.8/1000 PY in HIV-positive MSM, 19 times higher than in HIV-negative MSM [1]. However, HCV infection cases are seen not only in HIV-positive but also in HIV-negative MSM communities with high-risk behaviors. Indeed, a high incidence of HCV infection was reported in the population of MSM under pre-exposure prophylaxis (PrEP) for HIV prevention [2]. Furthermore, several studies have described a large European MSM-specific HCV transmission network among HIV-infected MSM and shared HCV transmission networks among HIV-positive and negative MSM, which signifies a dynamic transmission among these populations [3].

The selection of drug-specific RASs in patients experiencing DAAs failure together with the ongoing dynamic transmission might lead to more frequent transmission of RASs in these communities and might impair DAAs efficacy. Indeed, NS5A and NS3 RASs can be selected in patients experiencing failure with a NS5A or NS3 inhibitor-containing regimens, respectively. In contrast, RASs to NS5B inhibitors are rarely detected, even after failure to a DAA regimen containing a nucleotide inhibitor [4]. In case of transmission, the presence of pretreatment RASs has been shown to negatively impact the treatment outcome in some profiles of patients, especially with first-generation DAAs [5]. However, the second-generation pan-genotypic DAAs have a higher genetic barrier and associated sustained virologic

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response (SVR) rates justify the absence of recommended resistance testing before treatment. Nevertheless, some substitutions are still associated with a reduced sensitivity, even with the last generation of DAAs [6].

The presence of pretreatment HCV RASs to DAAs has not been widely studied in the population of MSM with high risk behaviors. Then, we investigated in this work the prevalence of pretreatment RASs to NS3 and NS5A inhibitors in recently HCV-infected MSM, either co-infected with HIV or at high risk of HIV acquisition.

II. MATERIALS and METHODS

2.1. Study design and patient's biological data

This work was a retrospective non-interventional study. Fifty-eight male patients with recent HCV infection (55 HIV-positive and 3 HIV-negative), followed at the Pitié-Salpêtrière, Saint-Antoine and Tenon hospitals, Paris, France and 14 HIV-negative patients from the ANRS IPERGAY study (Intervention for prevention of HIV acquisition by antiretroviral therapy for PrEP among gay men at high risk of HIV-1 infection) [2] were studied. Overall, seven patients were enrolled between July 2012 and December 2013 and 65 between March 2014 and May 2016.

Recent HCV infection was defined as a positive serology test and/or a positive HCV viral load (VL) associated with a negative HCV serology within the previous 12 months, or a positive HCV VL beyond 24 weeks of a successful treatment or spontaneous clearance with modification of genotype. Furthermore, patients with a positive HCV VL with increase of alanine aminotransferase (ALT) $\geq$10 upper limit of normal without any other etiology of hepatitis, or a positive HCV VL beyond 24 weeks of a successful treatment or spontaneous clearance without modification of genotype were also enrolled and considered as possible recent HCV infections.

Patients demographic (age and sexual orientation) and biological data (HCV, HIV status, HCV viral load, HCV genotype and ALT levels) were extracted from electronic database and medical records designed for virological and medical follow-up at hospitals. Patients were informed by an information note that their demographic, virological and clinical data could be used for retrospective studies. This

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work was performed on biological remnants with no additional care procedures and was carried out in accordance with the Declaration of Helsinki.

2.2. RNA extraction, amplification and ultra-deep sequencing (UDS)

Eighty microliters of HCV RNAs were extracted from 1 ml of plasma using® easyMAG® (bioMérieux Clinical Diagnostics, Marcy-l'Étoile, France). Extracted RNAs were reverse transcribed in complementary DNAs, and NS5A fragment (482 bp in size, nucleotide positions 6279-6761, amino acid 1-170, compared to H77-AF009606 NS5A genome) and NS3 fragment (492 bp in size, nucleotide positions 3477-3969, amino acid 19-189 compared to H77-AF009606 NS3 genome) were amplified by PCR in a one-step process (Superscript III One-step RT-PCR with platinum Taq kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol. Primer sequences for NS5A PCR are Forward primer: 5'-TGGYTAHGKGASATCTGGGACTGG-3' and Reverse primer 5'-TCCCKCAGCAWGGGCTTGCA-3' and for NS3 PCR are Forward primer: 5'-AGCYTSACYGGCMGAGACA-3'and Reverse primer 5'- GGRGAKGARTTGTCYGAGAA -3. PCR products were purified using Agencourt AMPure XP magnetic beads (Agencourt Bioscience Corp., Beckman Coulter, Beverly, MA, USA). Library construction from purified PCR products (NS5A and NS3 amplicons) and 2x300 bp Illumina Miseq paired-end sequencing (Miseq Reagent Kit v3, 600 cycles) were performed.

For UDS data analysis, FASTQ files were uploaded to the commercial web-based ASP-IDNS®-5 analysis software (SmartGene, Zug, Switzerland). Analysis was performed using the "HCV Pipeline" version 2.0.6_HCV_v0.1, which applied the following workflow: briefly, once the original FASTQ files produced by the sequencer were uploaded, paired-end reads were processed and merged with resolution of ambiguities based on the quality score. The generated sequences were trimmed for low quality using a sliding window method: regions are removed towards the 3' using a quality cut-off of 23 corresponding to a probability of 0.005 of a false base call.

The processed reads were mapped against genotype-specific HCV profiles for NS3 and NS5A regions. Subsequently, relevant events such as mutations were detected and reported in a quantitative manner

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(percentage of aligned reads) using a ≥ 10% cut-off, as recommended to detect clinically relevant mutations [7]. A 5% cut-off was also used to detect mutations between 5 and 10%. Mutations representing less than 20% of the viral population were referred to as "minority mutations", by definition.

Finally, a report was generated, as a PDF and as an XML-file (which included the consensus sequences and quantitative residue frequencies).

2.3. Resistance interpretation

Amino acid positions implicated in resistance to DAAs therapies according to the European Association for the Study of the Liver (EASL) guidelines 2018 and Sorbo *et al.* review [6] were analyzed and considered as RASs, or resistance-associated polymorphisms (RAPs) if known to be naturally present among the subtype, even if we can't be sure about the natural character of it. In this case, the frequency is very high among the viral population by definition.

The amino acid positions studied are presented in Table 1.

III. RESULTS

3.1. Patients characteristics and sequencing results

The median age of patients was 40.5 years (interquartile range (IQR): 33.0-46.0). The median HCV viral load (HCV-RNA) was 5.9 $\log_{10}$ IU/mL (IQR 5.1-6.6), and ALT value was 320.0 IU/L (IQR 139.5-490.0). Most of them were MSM (88.9%) and the others were reported with unknown sexual orientation. HCV genotyping showed GT1a, GT4d, and GT3 infections in 36 (50.0 %), 31 (43.1 %), and 5 (6.9 %) patients, respectively. Fourteen (19.4%) patients experienced HCV reinfections. Fifty-five (76.4%) patients were co-infected with HIV and 15 (20.8%) patients received PrEP including 14 from the ANRS IPERGAY trial. Patients characteristics are presented in table 2.

NS5A fragment was successfully amplified for samples of 36/36 HCV GT1a, 31/31 GT4d, and 4/5 GT3a-infected patients and successful NS3 amplification was achieved for samples of 30/36 GT1a,

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27/31 GT4d, and 4/5 GT3a-infected patients. A median (IQR) of 32596 reads (26345-44005) per sample was obtained after quality trimming step.

3.2 NS3 resistance-associated substitutions and polymorphisms

The presence of NS3 and NS5A RASs across the different HCV GT infections is recapitulated in table 3.

Overall, NS3-RASs were found in 4/61 viruses (6.6%) at the 10% cut-off. If considering the presence of polymorphisms, the prevalence of NS3 resistance-associated variants (RASs and polymorphisms) was 18/61 (29.5%).

In detail, the NS3 Q80K polymorphism was the most dominant in GT1a viruses and present at high frequencies (>95%) among 14/30 viral populations (46.7%). RASs S122G (n=1), R155K (n=1), and I170V (n=1) were also separately observed at high frequencies (>95%). Additionally, RASs S122N was detected among another viral population (12.1%; mutational load of 0.56 $\log_{10}$ IU/mL)).

In GT4d and GT3a viruses, NS3-RASs or polymorphisms were detected neither in majority (frequency >20%) nor in minority (from 10 to 20%).

No significant difference in the prevalence of NS3-RASs detected at 10% cut-off was observed in GT1a viruses of HIV-negative *versus* HIV co-infected patients (18.2% vs 0.0%, *p* value = 0.195).

3.3 NS5A resistance-associated substitutions and polymorphisms

The presence of NS5A-RASs across the different HCV GT infections is recapitulated in table 3.

Overall, NS5A-RASs were detected in 5/71 (7.0%) viruses at the 10% cut-off in the study population. If considering the presence of polymorphisms, NS5A resistance-associated variants (RASs and polymorphisms) were detected in 34/71 (47.9%) viruses.

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In detail, NS5A-RASs were detected in 3/36 (8.3%) at high frequencies > 68% in GT1a viruses. Indeed, one RAS Q30E (100%) and one RAS Q30R (68.3%; mutational load of 2.90 log_{10} IU/mL) associated with a RAS L31M (68.2%; mutational load of 2.89 log_{10} IU/mL) were detected, as well as one H58L (n=1; 95.3%). No linkage of RASs and/or polymorphisms was observed for GT1a viral population among NS3 and NS5A fragments.

In GT4d viruses, NS5A subtype-specific polymorphisms L30R and T58P were found at high frequencies (>95%) in all (31/31; 100%) and in 16/31 (51.6%) samples, respectively. One additional T58P was detected at frequencies of 13.4% (mutational load of 0.49 log_{10} IU/mL). One RAS M31L (at 19.1%; mutational load of 1.1 log_{10} IU/mL) was also observed along with the polymorphisms L30R and T58P.

No significant difference in the prevalence of NS5A-RASs detected at 10% cut-off was observed in GT1a and GT4d viruses of HIV-negative *versus* HIV co-infected patients (10.7% vs 0.0%, *p* value = 0.334 for GT1a infections and 8.7% vs 0.0%, *p*-value = 0.389 for GT4d infections).

In GT3a viruses, NS5A-RASs or polymorphisms were detected neither in majority (frequency >20%) nor in minority (from 10 to 20%).

3.4 Additional minority drug-resistance mutations with a 5% cut-off

- One additional NS3 RASs S122G was detected among another G1a viral population (6.7%; mutational load of 0.40 log_{10} IU/mL) and two NS3 Q80R were detected among G4d viral populations (6.8% and 7.6%; mutational loads of 0.46 and 0.40 log_{10} IU/mL respectively).
- One additional NS5A-RAS Q30R was detected (6.1%; mutational load of 0.39 log_{10} IU/mL), associated with the RAS K24R (6.2%; mutational load of 0.40 log_{10} IU/mL) among another G1a viral population. Lastly, one additional NS5A-RAS M31V was detected among a G4d viral population (7.4%; mutational load of 0.38 log_{10} IU/mL), associated with the polymorphisms L30R and T58P.

DISCUSSION

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We studied the presence of pretreatment RASs to NS3 and NS5A inhibitors in a population of MSM who were either co-infected with HIV or at high risk of HIV acquisition and under PrEP. A dynamic HCV transmission, including between HIV-negative and HIV-positive patients, and high rates of HCV reinfections were previously observed in this population [8].

Our results showed a high prevalence of subtype-specific polymorphisms in GT4d infections and a low level of RASs to NS5A and NS3 inhibitors compared to previous studies, even with the 5% cut-off of detection [9,10].

In GT1a infection, the NS3 Q80K polymorphism was highly prevalent and found in 46.7% viruses. The Q80K variant is well-known for conferring lower response rates to simeprevir. Regarding recent NS3 inhibitors, voxilaprevir could be slightly impacted by the polymorphism 80K, as suggested *in vitro* and *in vivo* during 8 weeks-treatment with sofosbuvir and velpatasvir [6,11], but no impact was seen with a 12 weeks-treatment of this regimen among DAA-experienced patients [12]. Other NS3 RASs including S122G, R155K, and I170V were individually detected at high frequencies in GT1a viruses of HIV-coinfected patients. None of them are known for impacting last-generation DAAs alone. The S122G/N RASs have been showed to impact voxilaprevir efficacy *in vitro* among GT1a viruses, but only in association with other RASs at positions 36, 54, 55, 155, 168 or 170 [6].

NS5A RASs were detected in 8.3% of GT1a viruses at 10% cut-off, which is consistent with the literature (from 6 to 15.6%) [5,9]. The RASs Q30E/R have an impact on previous-generation DAA ledipasvir, with *in vitro* fold-changes >100 among GT1a viruses [13]. When combined with the RAS L31M, the velpatasvir is then also deeply affected *in vitro* (fold-change of 198) [14]. *In vivo*, this combination of substitutions was retrieved at baseline in one of the 2 virological failures observed among the 624 patients treated in ASTRAL-1 [15], but it globally has a marginal impact.

In GT4d infection, in agreement with results from other studies [16], a high prevalence of GT4d-specific polymorphisms L30R and T58P was observed on NS5A fragment. The presence of subtype-specific polymorphisms in GT4d virus has not been associated with an impact on DAAs response and high SVR rates are frequently observed in GT4d-infected patients treated by DAAs [17]. However, it should be

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noted that the emergence of substitutions at a signature resistance position (codon 28) accompanied by baseline polymorphisms L30R and T58P has been described in several GT4-infected patients (GT4a and GT4d) failing DAAs regimens [18].

The lack of data on treatment outcome, one of the study limitations, restrains an adequate evaluation of RASs and their clinical impact on DAAs response. Moreover, minority mutations at a frequency < 5% of the viral population were not analyzed in this study [7]. Indeed, although several studies showed the emergence of minority baseline RASs in several patients failing DAAs [19], last generation DAAs-based therapies have showed extremely high genetic barrier and SVR rates, even in real-life experiences [15,20].

In conclusion, considering the risky behaviors with an onward dynamic transmission and the context of PrEP, implementation of effective infection screening, early initiation of DAAs, and regular monitoring of treatment response/reinfection are necessary in this population. Nevertheless, our findings showed a low RAS prevalence and reassure the clinical management of HCV infection treatments in this high-risk population.

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Competing interests

None declared

Ethical approval

Not required

Contributions

Thuy NGUYEN: Methodology, Investigation, Formal analysis, Writing - Original Draft.

Marc-Antoine VALANTIN and Nesrine DAY: Conceptualization, Resources, Writing - Review & Editing.

Emmanuelle NETZER, Thomas L'YAVANC, Michel OHAYON and Nadia VALIN: Resources, Writing - Review & Editing.

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Corinne AMIEL : Formal analysis, Writing - Review & Editing.

Constance DELAUGERRE, Georges KREPLAK, Gilles PIALOUX, Vincent CALVEZ, Jean-Michel MOLINA and Anne-Geneviève MARCELIN: Conceptualization, Writing - Review & Editing

Eve TODESCO: Conceptualization, Methodology, Supervision, Writing - Original Draft.

All authors have approved the manuscript version submitted.

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Table 1. Amino acid positions analyzed for resistance to DAAs therapies

Table 2: Patients' characteristics

	Genotype 1a	**Genotype 3a**	**Genotype 4d**
NS3	V36A/C/G/L/M	Q41K	Q41R
	Q41R	Y56H/N	Y56H
	F43S	Q80K/R	Q80R
	T54A/S	A156G/P/T/V	R155C/K
	V55A/I	S166T	A156G/H/K/L/S/T/V
	Y56F/H	Q168K/L/R/T	D168A/E/H/T/V
	Q80H/K/L/R	L175M	
	S122G/N/R		
	R155G/I/K/M/N/Q/S/T/W		
	A156G/L/M/P/S/T/V		
	V158A/I		
	D168any		
	I/V170F/T/V		
NS5A	K24G/N/R	S24F	L28I/M/S/T/V
	K26E	M28G/I/K/T	L30F/G/H/R/S
	M28A/G/S/T/V/del	A30G/H/K/S/V	M/L31I/L/V
	Q30C/D/E/G/H/I/K/L/N/Q/R/S/T/Y	L31F/I/M/P/V	T58D/L/P/S
	L31F/I/M/P/V	P58G/T	Y93C/H/N/R/S/W
	P32L/S	A62L	
	S38F	E92K	
	H58D/L/R	Y93H/N/R	
	A92K/T		
	Y93C/F/H/L/N/R/S/T/W		

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Characteristics	Total (n=72)
Age (years), median (IQR)	40 (33-46)
Men having sex with men, n (%)	64 (88.9)
Unknown sexual orientation, n (%)	8 (11.1)
HCV viral load, log_{10} IU/mL, median (IQR)	5.9 (5.1-6.6)
HCV genotype	
➢ **Genotype 1a, n (%)**	36 (50.0)
➢ **Genotype 4d, n (%)**	31 (43.1)
➢ **Genotype 3a, n (%)**	5 (6.9)
ALT (IU/L), median (IQR)	320.0 (139.5-490.0)
HIV co-infection (%)	55 (76.4)
Patients under PrEP, n (%)	15 (20.8)
Patients with HCV reinfection, n (%)	14 (19.4)

ALT: Alanine aminotransferase, IQR: Interquartile range

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Table 3: Presence of RASs/RAPs detected at 10% cut-off

Genes	RASs/RAPs (n, frequencies)	
NS3	**GT1a (N=30)**	**GT4d (N=27)**
	Q80K (14)	
	S122G (1)	
	S122N (1; 12.1%)	/
	R155K (1)	
	I170V (1)	
NS5A	**GT1a (N=36)**	**GT4d (N=31)**
	Q30E/R (2)	L30R (31)
	L31M (1)	*M31L (1 ; 19.1%)*
	H58L (1)	T58P (16)
		T58P (1; 13.4%)

RASs/RAPs: resistance-associated substitutions/resistance-associated polymorphisms. N: total number of patients with successful viral amplification, n: number of patients harboring virus with RASs/RAPs. Minority mutations (representing less than 20% of the viral population) are in italic with their frequency in brackets.

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