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Short Communication

Uncommon Detection of Mixed HCV Genotype Infections in Recently Infected Men Who Have Sex with Men

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ABSTRACT

Introduction: Mixed hepatitis C virus (HCV) genotype (GT) infections are clinically important as different genotypes have varied sensitivities to direct-acting antivirals (DAAs). A high prevalence of mixed GT infections was observed in individuals who inject drugs and had multiple HCV exposures. The prevalence of mixed HCV GT infections in men who have sex with men (MSM) and high-risk behaviors was investigated by ultra-deep sequencing (UDS).

Methods: NS5B fragment was sequenced from viruses of patients with recent HCV infection: there were 50 HIV-positive and 18 HIV-negative patients, including 13 from the ANRS Pre-Exposure Prophylaxis (PrEP) IPERGAY study. UDS data were analysed using Geneious (version 10.3.2). Phylogenetic trees were constructed using FastTree (version 2.1).

Results: HCV sequencing showed GT1a (47.1%), GT4d (41.2%), GT3a (8.8%) and GT2k (2.9%). We detected three (4.4%) mixed GT infections: one between predominant GT4d and minority GT1a, one between predominant GT4d and minority GT1b, and one between predominant GT1a and minority GT4d virus. The rates of minority GT viral populations detected in viruses of the three patients with mixed GT infections were 0.32%, 10.7%, and 1.3%, respectively. The first two patients were HIV co-infected and the third was HIV-negative under PrEP. The anti-HCV treatment was successful in all three patients.

Conclusion: This work showed uncommon mixed HCV GT infections in MSM at high risk of multiple HCV exposures. The impact of these infections on treatment response has not been established but further studies on more patients are necessary. To prevent treatment failure in this population, regular monitoring of treatment response is needed, particularly when pan-genotypic treatment is not used.

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1. Introduction

Although current treatments of hepatitis C virus (HCV) infection, particularly pan-genotypic direct-acting antivirals (DAAs), are associated with a high rate of sustained virological response (SVR) [1], failures are still observed, e.g. in the case of HCV genotype (GT) 3 infection [2]. Mixed HCV GT infections (infection with two or more HCV GTs) [3] are a clinical concern as HCV of different GTs have varied sensitivities to current GT-specific DAAs. The observed prevalence of mixed HCV GT infections ranges from 14% to 39% in individuals who inject drugs depending on the sensitivity of methods used [3–6]. The prevalence is high in this population mostly because of their high-risk behaviors, such as ongoing injection and needle sharing. The prevalence of mixed HCV GT infections in men who have sex with men (MSM) at high risk of multiple HCV exposures is also likely to be high. However, few data about mixed HCV GT infections are available in this population. To the best of our knowledge, a few case reports of superinfection, defined as detection of different HCV strains after the persistent infection of primary HCV strains [3], were reported in HIV/HCV co-infected MSM via sexual transmission [7–9]. Further knowledge about the prevalence of mixed HCV GT infections in this community could help to establish an optimized strategy for surveillance, diagnostics, and treatment regimen. Ultra-deep sequencing (UDS) detects minority viral populations down to 1% and is suitable for an extensive analysis of complex viral populations. The aim of the current study was to investigate by UDS the prevalence of mixed HCV GT infections in MSM with high-risk behaviors who were recently diagnosed with HCV infection.

2. Materials and methods

2.1. Study design and patients

Pre-treatment plasma samples within the period defined as recent HCV infection (see definition below) were collected from 55 patients (50 HIV-positive and 5 HIV-negative) followed at the Pitié-Salpêtrière, Saint-Antoine and Tenon hospitals, Paris, France, and 13 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) [10,11]. The 55 patients followed at the three hospitals were previously enrolled in the recently published study that used Sanger sequencing technique and addressed HCV transmission and associated sexually transmitted infection issues in this population [12]. Overall, six patients were enrolled between July 2012 and December 2013 and 62 between March 2014 and May 2016.

The study was conducted in accordance with the Declaration of Helsinki. This work was a retrospective, non-interventional study with no addition to standard care procedures. Reclassification of biological remnants into research material after completion of the ordered virological tests was approved by the local interventional review board of Pitié-Salpêtrière hospital. According to the French Public Health Code (CSP Article L.1121-1.1), such protocols are exempt from individual informed consent.

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 GT. Furthermore, patients with a positive HCV VL with increased alanine aminotransferase (ALAT) $\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 GT were also enrolled and considered as possible recent HCV infections.

2.2. Extraction, amplification, and deep-sequencing

HCV RNA were extracted from 1 mL plasma using NucliSENS® easyMAG® (bioMérieux Clinical Diagnostics) and the NS5B fragment of 388 bp (8256 to 8644) was reverse-transcribed and amplified by polymerase chain reaction (PCR) in a one-step process (Superscript III One-step RT-PCR with platinum Taq kit; Invitrogen, USA) according to the manufacturers' protocol by 2 pan-genotypic primers, Forward: 5′-ATATGAYACCCGCTGYTTTGACTC-3′ and Reverse: 5′-GCNGARTAYCTVGTCATAGCCTC-3′. Multiplexed samples were pooled and subjected to standard Illumina Miseq paired-end sequencing at 2×250 bp.

2.3. UDS data analysis

UDS data were analysed using Geneious software (version 10.3.2, http://www.geneious.com) [13]. Paired reads were merged, primer-removed and quality-trimmed. Sequences of good quality were error-corrected by BBNorm from the BBtools package included in Geneious. Corrected reads of each sample were clustered by *de novo* assembly approach at 90% of similarity where almost all reads were assembled. All contigs and unassembled reads were aligned to a reference sequence corresponding to the predominant subtype, with maximum mismatches allowed per reads depending on the intra-GT variability (according to the literature, 17% of maximum mismatches for GT1, 18% for GT2, 20% for GT3 and 16% for GT4) [14]. Sequences that could not be mapped to the reference were put aside and their subtypes were verified by Geno2Pheno (available at https://www.geno2pheno.org/) [15]. When their subtypes differed from the predominant subtype, these sequences were considered either mixed infections or contaminations. Suspected contaminations were detected by building phylogenetic trees using FastTree [16] (General Time Reversible model, available at http://www.microbesonline.org/fasttree/#Install) with viral sequences of the other samples in the same experiment. If the genetic distance between them was superior to 3%, these sequences were considered to be mixed infections, otherwise, contamination was suspected.

3. Results

3.1. Sequencing results and patient characteristics

A median of 2389 sequences (interquartile range [IQR]: 1851-2960) per sample was obtained after the quality trimming step. The median age of patients was 38.5 years (IQR: 30.5-46.0); the median HCV VL was 5.9 log IU/mL (IQR: 5.3-6.6); and the median value of ALAT was 320.0 IU/L (IQR: 146.5-535.5). Most of the patients were MSM (85.3%) and the remaining patients were of unknown sexual orientation. HCV genotyping by Sanger sequencing showed GT1a, GT4d, GT3a, and GT2k infection in 47.1%, 41.2%, 8.8%, and 2.9% of patients, respectively. Fifteen patients (22.1%) experienced HCV reinfections and three (4.4%) were possible recent HCV infections. HIV-coinfection was found in 50 patients (75.3%) with a median of 673 CD4 cells/mm^3 (IQR: 531-873, available data on 25 patients). Five of these patients had a detectable HIV-RNA level ($>$ 50 copies/mL) because of antiretroviral therapy (ART) absence (n=1), loss of follow-up (n=1), resistance to the received tenofovir/emtricitabine/raltegravir (n=1), viral blips (n=1), and no resistance to the received ART but suppression of the replication after treatment intensification (n=1). Sexually transmitted infections were detected in 15 patients (22.1%) $\leq$ 1 month before recent HCV infection diagnosis (seven *Chlamydia trachomatis,* eight *Treponema pallidum*, two *Neisseria gonorrhoeae*). HCV infection mainly occurred in the context of high-risk sexual behaviors (unprotected

Table 1
Patient characteristics

Characteristics	Total (n=68)	HIV-positive patients (n=50)	HIV-negative patients (n=18)
Age (years), median (IQR)	38.5 (30.5-46.0)	42.5 (34.5-46.0)	32.0 (27.5-35.8)
Men who have sex with men, n (%)	58 (85.3)	43 (86.0)	15 (83.3)
Unknown sexual orientation, n (%)	10 (14.7)	7 (14.0)	3 (16.7)
HCV viral load, log IU/mL, median (IQR)	5.9 (5.3-6.7)	5.9 (5.3-6.9)	5.5 (5.3-5.6)
HCV genotype			
➢ Genotype 1a, n (%)	32 (47.1)	24 (48.0)	8 (44.4)
➢ Genotype 4d, n (%)	28 (41.2)	20 (40.0)	8 (44.4)
➢ Genotype 3a, n (%)	6 (8.8)	5 (10.0)	1 (5.6)
➢ Genotype 2k, n (%)	2 (2.9)	1 (2.0)	1 (5.6)
ALAT (IU/L), median (IQR)	320.0 (146.5-535.5)	315.0 (144.8-480.8)	467.0 (234.0-647.0)
HIV co-infection (%)	50 (73.5)	50 (100.0)	0 (0.0)
Number of patients with detectable HIV-RNA, n (%)	N/A	5 (10.0)	N/A
CD4 count (cells/mm^3), median (IQR)	N/A	673.0 (531.0-873.0)	N/A
Number of patients with STIs*, n (%)	15 (22.1)	10 (18.2)	5 (27.8)
HCV reinfection (%)	15 (22.1)	14 (28.0)	1 (5.6)

ALAT: ALanine AminoTransferase, HCV: hepatitis C virus, HIV: human immunodeficiency virus, IQR: interquartile range, RNA: ribonucleic acid, *: sexually transmitted infections detected less than 1 month before recent hepatitis C diagnosis, N/A: not applicable

anal sex) and was frequently associated with recreational drug use. Patient characteristics are presented in Table 1.

3.2. Mixed HCV GT infections

After eliminating suspected contaminations (as described in the methods section), three (4.4%) mixed GT infections in three patients were detected. All three patients were infected with HCV for the first time. Two of the three patients were co-infected with HIV and the other patient was HIV-negative and enrolled in the ANRS IPERGAY trial.

A mixed HCV GT infection between predominant GT4d (at frequency of 99.68%) and minority GT1a (at frequency of 0.32%) was detected in the viral population of one HIV-positive patient. The patient was treated for 6 months with peginterferon alfa-2a/ribavirine in 2013 and obtained undetectable HCV VL after one month. His HCV viral load remained undetectable during the 5 years of follow-up.

In the viral population of the second patient co-infected with HIV, another mixed infection between predominant GT4d (at frequency of 89.3%) and minority GT1b (at frequency of 10.7%) was identified. This patient was treated later with 12 weeks of sofosbuvir and ledipasvir. The HCV VL was undetectable 9 months after the end of treatment.

The third mixed infection between predominant GT1a (at frequency of 98.7%) and minority GT4d (at frequency of 1.3%) was detected in the viral population of the HIV-negative patient under PrEP. Interestingly, a switch of virus from GT1a to GT4d was observed by Sanger sequencing in this patient 2 years later. The comparison between anterior minority GT4d sequences obtained from UDS with posterior GT4d sequence obtained from Sanger sequencing showed a 2% minimum genetic distance between these sequences. At the time of HCV GT4d infection diagnosis, the patient was treated for 12 weeks with sofosbuvir and ledipasvir and obtained an undetectable HCV VL after 2 months. However, the patient did not continue his follow-up in the hospital, so we could not obtain more details about the SVR post-treatment.

An example phylogenetic tree constructed from viral sequences of a mixed infection between predominant GT4d and minority GT1b virus (in the second patient) is shown in Fig. 1. This patient was possibly infected with multiple minority transmitted GT1b viruses.

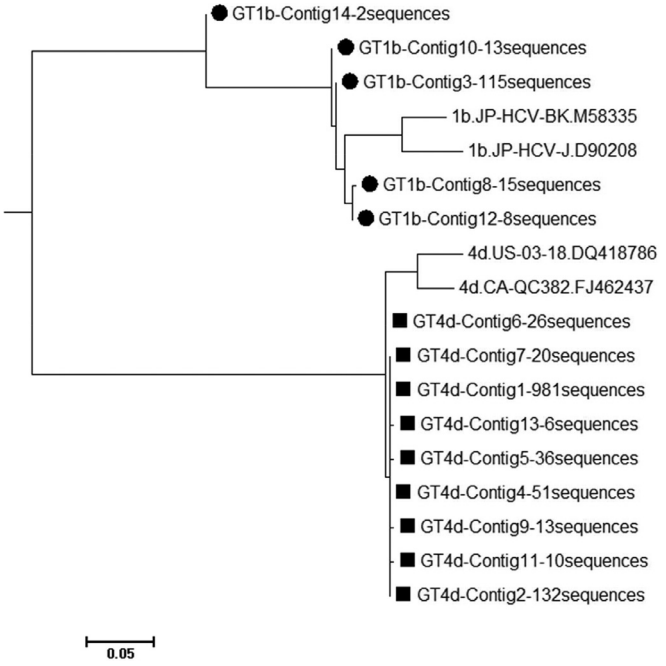

Fig. 1. Phylogenetic tree constructed from UDS contig sequences of individual with mixed hepatitis C virus (HCV) genotype (GT) infection between predominant GT4d and minority GT1b and reference sequences of GT4d and GT1b virus from Los Alamos HCV database (accession number in their names). Viral sequences of patients are marked with shape (black square for GT4d and black circle for GT1b virus). Number of sequences assembled in each contig is also presented in the taxon's name.

4. Discussion

In our study, a low prevalence (4.4%) of mixed HCV GT infections was observed in a population of MSM with high-risk behaviors who were recently diagnosed with HCV infection. The prevalence of mixed HCV GT infections varies depending on the study population and technique sensitivity. Indeed, a study using UDS showed a low prevalence of 1.7% mixed HCV GT infections in 76 seronegative, HCV-RNA-positive blood donors, whereas a higher prevalence of 14-39% of mixed HCV GT infections was reported

in individuals who inject drugs and have both chronic and acute hepatitis C [4,17]. In the current study, the prevalence of mixed HCV GT infections was investigated using UDS in a population of MSM patients at high risk of multiple HCV exposures, HIV+ and HIV- at high risk of HIV acquisition. Among 68 patients enrolled, only three (4.4%) were infected with HCV of mixed GTs involving GT4 and GT1, with frequencies of minority viral populations ranging from 0.32% to 10.7%. Interestingly, a switch from GT1a to GT4d virus (based on Sanger sequencing) after 2 years was observed in a patient previously infected by predominant GT1a and minority GT4d virus (based on UDS). However, the actual 2% minimum genetic distance between the previous minority GT4d sequences obtained from UDS and the later GT4d sequence from Sanger sequencing could not distinguish if the same virus emerged, or a different virus was contracted.

Of note, two of the three patients were HIV co-infected and the other was HIV-negative and included in a PrEP program (the IPERGAY trial). A concurrent mixed HCV GT infection is associated with faster immunological and clinical progression in patients with HIV if they are not treated effectively with antiretrovirals [5]. Moreover, mixed HCV GT infections possibly impact the treatment outcome of GT-specific DAAs [18,19]. However, in this study, the three patients with mixed infections obtained virological success under anti-HCV treatment. This success is not surprising because the minority GT and the predominant GT viral populations involving GT1 (GT1a and GT1b) and GT4 virus have equivalent susceptibility to anti-HCV treatment (either by sofosbuvir/ledipasvir or peginterferon alfa-2a/ribavirine). Indeed, a study on 335 patients co-infected with HIV-1 and HCV GT1 (GT1a and GT1b) or GT4 who received sofosbuvir/ledipasvir showed similar SVRs across different HCV GTs [20]. This probably explains why there was no deleterious impact on treatment response in the three cases of mixed GT infections in this study.

A strict cut-off of 3% of genetic distance was used in this study to eliminate contamination from PCR or sequencing steps. Only sequences of a sample with genetic distance greater than 3% compared to sequences of other samples in the same experiment were considered as mixed infections. This cut-off is quite strict and may underestimate the mixed GT infection rate in our study. Indeed, another mixed GT infection between predominant GT3a and minority GT1a virus was detected using a less strict cut-off of 1% of genetic distance. In this study, we identified only mixed infections of different GT viruses; however, mixed infections of different subtype viruses in the same GT are possible. Further studies using different analysis approaches are needed to address this.

In conclusion, we observed a low prevalence (4.4%) of mixed HCV GT infections in a population of MSM with high-risk behaviors and recent HCV infection. Determining HCV GT becomes less clinically significant with the introduction of pan-genotypic DAAs. However, these treatments are still not globally available and affordable, particularly in resource-limited countries. The impact of mixed HCV GT infections has not been established in this study. The study population involved a small group of MSM in a specific area (Paris) and treatment success of patients with mixed HCV GT infections was limited to only three patients. From a public health perspective, the MSM population engaging in high-risk behaviors still requires special attention in terms of mixed infections compared with the general HCV-infected population with a regular monitoring of anti-HCV treatment response, particularly when pan-genotypic treatment is not used.

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Declaration of Competing Interest

No.

Ethical Approval

Not required.

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