HCV genotype determination in monoinfected and HIV co-infected patients in Cuba

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ABSTRACT

With the aim to characterize the HCV genotype distribution in Cuba, sera were collected from two subgroups: HCV-monoinfected and HCV/HIV co-infected patients. A combination of reverse transcription-PCR using genotype-specific primers, restriction fragment length polymorphism and sequencing was used to determine the genotype of 84 samples. Seventy-nine (94%) showed single infections (10 [12%] were genotype 1a and 69 [82%] genotype 1b) and 5 (6%) samples corresponded to mixed infections (2 [2%] with genotypes 1a/3a and 1 sample [1%] each with 1b/3a, 1b/4a and 1a/1b/3a). HCV/HIV co-infected subjects had a higher frequency of mixed infections (p=0.08), infection with genotype 3a (p=0.18) and for the first time genotype 4a was found. There was no association of any demographic characteristics with any specific genotype although HCV/HIV co-infected patients showed a tendency to have mixed genotypes in those older than 45 years of age (p=0.11). Phylogenetic analysis showed that HCV isolates clustered with subtypes 1b (n=15, maximal genetic distance 2.51%) and 1a (n=2, maximal genetic distance 0.35%). This report presents the prevalence of HCV genotypes in monoinfected and HIV co-infected patients, mixed HCV infections in HCV/HIV co-infected men who have sex with men with high-risk sexual practices and for the first time identifies that the uncommon genotype 4a can be present in a patient co-infected with HIV.

1. Introduction

Hepatitis C virus (HCV) infection is a major global cause of chronic liver disease. The estimated number of HCV infected subjects worldwide is increasing, and now numbers approximately 170 million people.[1] In Cuba,

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the seroprevalence of HCV infection is low in the general population (0.6 to 1.9%)[2,3] and in blood donors (0.7 to 0.8%).[3,4] However, it is higher in risk groups such as people with hemophilia, plasmapheresis blood donors, patients with onco-hematological diseases and multi-transfused hemodialysis patients (59, 47.3, 19.5 and 95.6% respectively).[3,4] The Cuban HIV/AIDS epidemic has the lowest prevalence rate of the Caribbean region, and by the end of 2011, the island had 15 824 patients living with HIV-1 (National Registry of HIV/AIDS, Cuba). Studies in Cuba showed that in HIV-positive patients, 14.2% were positive for anti-HCV antibodies, and of them 77.7% were also positive for HCV-RNA.[5] Risk factors for parenteral exposure to both viruses clearly influence the risk of co-infection. In addition, the accumulated evidence suggests that HCV behaves like an opportunistic infection in people with HIV infection.[6]

The distribution of different HCV genotypes and subtypes varies geographically. Genotypes 1, 2 and 3 are prevalent in Europe, Japan, United States and Latin America; genotype 4 in Central and North Africa and in the Middle East; genotype 5 in South Africa and genotype 6 in Hong Kong and Viet Nam. Each of the six main genotypes is equally divergent from the others, differing at 31–34% of nucleotide positions on pairwise comparison of complete genome sequences and the variation among different subtypes is 15–30%. These differences result in approximately 30% amino acid sequence divergence in the encoded polyproteins. Patients chronically infected with HCV require significantly different durations of therapy and achieve substantially different sustained virologic response rates to therapies, depending on the HCV genotype with which they are infected.[7,8] This fact together with the possibility that different genotypes could affect diagnostics assays and the natural history of the disease has motivated a series of molecular epidemiology studies. However, prevalence rates of genotypes appear to be changing different populations with the emergence of different genotypes in diverse parts of the world, causing differences in the survival rate of the patients.[7–9]

Sequence variation is equally distributed throughout the HCV viral genome, apart from the highly conserved 5′ non-coding region (5′ NCR) and core, and highly variable HVR1 (hypervariable region 1) in E2. Because of its sequence conservation, the 5′ NCR is the target of most HCV detection and quantification assays. This 5′ NCR also contains genotypically variable sequence positions which permit discrimination of all of the major genotypes and many of the subtypes of HCV. Procedures based on 5′ NCR can be considered the most adequate for genotyping in clinical practice. The core region, although less conserved than the 5′NCR, is the most conserved region in the HCV genome open-reading frame and the most conserved protein in different genotypes.[10–12]

Little information regarding HCV genotype circulation and phylogenetic analysis of HCV strains is available from Cuba.[13–15] None of the studies have analyzed HCV infection within different populations and risk groups. Because each group has its own clinical and epidemiological characteristics, it is vital to know the distribution of HCV genotypes for the follow-up of chronic infections.

2. Materials and methods

2.1. Study population

Serum samples from 109 patients who were confirmed as anti-HCV positive by a third generation immunoassay were included. The samples were received in the National Reference Laboratory on Viral Hepatitis for HCV molecular diagnosis and were stored at −20 °C until use. For analysis purposes the population was divided into two main subgroups: HCV-monoinfected (n=63) and HCV/HIV co-infected (n=46) patients. The samples were collected from 2001 to 2005 in the first subgroup, and from 2007 to 2008 in the second. The demographics, epidemiological and clinical characteristics of the study population are summarized in Table 1. Patient data were collected from medical charts.

2.2. Laboratory assays

2.2.1. RNA extraction, reverse transcription, PCR reaction and genotyping by RFLP analysis (n=63)

Total RNA was extracted from 200 μL of serum with Trizol LS (Invitrogen Life Technologies, Carlsbad, CA, USA), followed by chloroform extraction and isopropanol precipitation. The RNA pellet was washed with 70% ethanol (vol/vol) and then briefly air dried and redissolved in 20 μL of RNAse/DNAse free water. The RNA was reverse-transcribed into cDNA using MMLV (200 U/μL) and random hexamer primers (50 μM) during 90 minutes at 37 °C. Following transcription, the cDNAs were amplified by nested-PCR targeting the 5′ NCR using primers 209, 939, 211 and 940.[16] Genotype and subtype determination of HCV strains was done by cleavage of the amplified 5′ NCR using restriction enzymes (*HAE III, RSA I, MVA I, HINF I, BSTU I* and *SCRF I*) that recognize sequence polymorphisms between six HCV genotypes: 1(a and b), 2 (a and b), 3 (a and b), 4, 5 and 6.[17]

2.2.2. PCR reaction and genotyping using specific primers targeting the core region (n=109)

After RNA extraction and cDNA synthesis, a nested PCR was performed using in the first PCR reaction primers 256 and 186 NTER and in the second reaction a mix of 10 (four sense and six antisense) primers specific for the amplification of four different HCV genotypes: 1 (a and b), 2, 3 (a) and 4. This primer combination produces PCR products of the following sizes: 125 bp for genotype 1a; 141 bp for 1b; 75 bp for 2; 87 bp for 3a and 336 bp for 4a.[11]

2.2.3. Amplification of the 5′ NCR and part of the core gene, PCR product purification, sequencing and phylogenetic analysis (n=48)

In addition, the cDNAs were amplified by nested-PCR directed to the 5′ NCR and part of the core gene of the HCV genome (spanning nucleotide positions of 107–452).[18] The amplified PCR fragments were purified using the QiaQuick PCR purification Kit (Qiagen, Valencia, CA, USA) and sequenced in both directions using the dideoxynucleotide terminator method, with the ABI Prism 3100 Genetic

Table 1
Characteristics of the 109 patients enrolled in the study, Cuba

Parameters	HCV-monoinfection, n=63 (58%)	HCV/HIV co-infection, n=46 (42%)
Gender		
Male	36 (57%)	37 (80%)
Female	27 (43%)	9 (20%)
Age, years		
Children	9–18 (median 14.2), 5 (7.9%)	0
Adults	26–70 (median 41.2), 58 (92.1%)	24–50 (median 35.2), 46 (100%)
Skin color		
White	39 (62%)	36 (78%)
Non-white	24 (38%)	10 (22%)
Place of Residence[a] (Province)		
Havana	50 (79%)	33 (77%)
Artemisa	12 (19%)	0
Villa Clara	0	5 (12%)
Other	1 (2%)	5 (12%)
Putative way of infection[a]	Multiple blood transfusions 10 (16%)	Sexually-transmitted
	Hemodialysis therapy 21 (33%)	Heterosexual 13 (30%)
	Liver transplantation 7 (11%)	MSM 26 (60%)
	Health-care workers 4 (6%)	MSM and exposure to tattooing 4 (9%)
	Frequent parenteral medication 1 (2%)	
HIV-1 infection stages[a]	No demonstrable or unknown 20 (32%)	
AIDS patients		37 (86%)
Non-AIDS patients		6 (14%)

MSM: Men who have sex with men.

[a] Data available for 43 HCV/HIV co-infected patients.

Analyser (Applied Biosystems, Foster City, CA, USA). Sequence assembly was done using DNASTAR (DNASTAR, Inc, Madison, WI, USA). The new sequences were compared with a set of references and with sequences obtained by BLAST (HCV-1a (**EU781753** and **EU255993**), HCV-1b (**JN120912**, **EU482849**, **HM638188**, **AB523065**, **FJ390397**, **EU155370**, **EF407459**, **EU155376**, **AF356827**, **EU081424** and **AB523141**), HCV-1c (**AY051292** and **D14853**), HCV-2a (**AB047640**) and HCV-2b (**D10988**).

The sequences were aligned using the CLUSTAL W program (www.clustal.org).[19] Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (www.megasoftware.net)[20] and a phylogenetic tree was created by the neighbour-joining method applied to the distance matrix obtained under the Kimura two-parameter model. As a measure of the robustness of each node, we utilized the bootstrap method (1000 pseudo-replicas).

Seventeen Cuban HCV sequences characterized in the present study and selected based on their unique characteristics have been submitted to EMBL Nucleotide Sequence Database under the indicated accession numbers: isolate CUBA1 (**FR872799**), CUBA7 (**FR872800**), CUBA13 (**FR872801**), CUBA10 (**FR872802**), CUBA16 (**FR872803**), CUBA19 (**FR872804**), CUBA24 (**FR872805**), CUBA25 (**FR872806**), CUBA26 (**FR872807**), CUBA36 (**FR872808**), CUBA37 (**FR872809**), CUBA39 (**FR872810**), CUBA42 (**FR872811**), CUBA43 (**FR872812**), CUBA45 (**FR872813**), CUBA47 (**FR872814**) and CUBA51 (**FR872815**).

2.3. Statistical analysis

Statistical analysis was carried out using the χ^2 test and Fisher's exact test.

3. Results

3.1. Distribution of HCV genotypes according to laboratory assay and study population

Of the 109 anti-HCV positive samples, 84 (77%) tested positive for HCV-RNA (48 and 36 samples of HCV-monoinfected and HCV/HIV co-infected patients respectively). Of these, samples belonging to the first subgroup were genotyped by RFLP, with genotype-specific primers and confirmed by sequence analysis: 8 (17%) belonged to genotype 1a and 39 (81%) to genotype 1b. All techniques yielded similar results, except for one sample in which a mixed genotype 1b/3a was obtained with genotype-specific primers, while RFLP and sequencing only detected genotype 1b.

For the 36 samples belonging to HCV/HIV co-infected patients an analysis based on genotype-specific primers only was done: three samples (56%) belonged to genotype 1a, 30 (83%) to genotype 1b, 2 (6%) to a mixed infection with genotypes 1a/3a, one (3%) to a mixed infection with genotypes 1b/4a and one (3%) to a triple infection with genotypes 1a/1b/3a.

Overall, all 84 HCV-RNA positive samples were genotyped using one or more techniques. Of these, 79 (94%) were infected with a single virus genotype. Genotype 1b was the most frequent (69/84, 82%, p=0.04) followed by genotype 1a (10/84, 12%). Mixed genotypes were found in five samples, with genotype 1b present in three (60%; Table 2).

In the analysis of the HCV genotype distribution according to the subgroups HCV-monoinfected (n=48) and HIV/HCV co-infected patients (n=36), a predominance of genotype 1b was observed in both subgroups,

Table 2
HCV genotype distribution in monoinfected and HIV co-infected patients, Cuba

Groups	RNA positive (%)	Genotype (%)					
		1a	1b	Mixed			
				1a/3a	1b/3a	1b/4a	1a/1b/3a
HCV-monoinfected patients, n=63	48 (76)	8 (17)	39 (81)	0	1 (2)	0	0
HCV/HIV co-infected patients, n=46	36 (78)	2 (6)	30 (83)	2 (6)	0	1 (3)	1 (3)
Total, n=109	84 (77.1)	10 (11.9)	69 (82.1)	2 (2.4)	1 (1.2)	1 (1.2)	1 (1.2)

corresponding to 39 (81%) and 30 (83%) patients respectively, while genotype 1a was present in 8 (17%) and 2 (6%) samples respectively (Table 2).

HCV/HIV co-infected patients had a higher frequency of mixed infections (4, 11%) than HCV-monoinfected patients (1, 2%; p=0.08). Genotype 3a was more frequent in the HCV/HIV co-infected patients than in the HCV-monoinfected patients, corresponding to 3 (8%) and 1 (2%) of the samples respectively (p=0.18). Genotype 4a was found only in the HCV/HIV co-infected subgroup.

There was no association between genotype and demographic variables when analysis was performed for HCV genotype distribution in general and for mixed infections in particular. In HCV/HIV co-infected patients mixed infections of HCV (two or more genotypes) showed a tendency to be more frequent (p=0.11) in patients older than 45 years of age and a higher frequency of genotype 1b in those with white skin (p=0.06).

According to the place of residence of the enrolled patients, mixed infections were found in patients from Havana (n=4) and from Artemisa (n=1), a province located very close to Havana. Analyzing HCV genotype distribution according to sexual orientation and putative way of HIV infection in the HCV/HIV co-infected patients showed that subtype 1a was found only in men who have sex with men (MSM) whereas subtype 1b was found in MSM (16/30, 53%), in the heterosexual group (10/30, 33%) and in MSM with exposure to tattooing (1/30, 3%). Interestingly, mixed infections were present only in MSM (one of them with exposure to tattooing) and all of them were patients with AIDS.

3.2. Phylogenetic analysis

Figure 1 shows the phylogenetic relatedness based on 286 nt of the 5′ NCR and part of the core of 17 unique strains derived from HCV-monoinfected patients. HCV isolates from Cuba clustered in genotype 1 with high bootstrap values. Two isolates clustered with sequences of subtype 1a and 15 clustered with sequences of subtype 1b. All the viruses were isolated from patients living in Havana (n=12), Artemisa (n=4) and other province (n=1).

Cuban strains of subtype 1a (13 and 16) isolated from patients living in Havana and of subtype 1b (39 and 47) isolated from patients living in Artemisa, clustered together and distinct from the other strains from Cuba. Additionally, these strains were isolated from patients with common putative way of HCV infection.

The maximal genetic distance calculated for the sequences from Cuba was 2.51% among the genotype 1b strains and 0.35% among the 1a strains. The strains from

Cuba were similar or even identical to viruses from Asia, Europe and North America collected around the same time (Figure 1).

4. Discussion

In the present study we characterized the HCV genotype distribution in Cuba and performed a phylogenetic analysis of the strains based on 286 nt of the 5′ NCR and part of the core of the HCV genome recovered from monoinfected patients. Previous reports of HCV genotyping in Cuba point to genotype 1b as the most prevalent. Roca et al.[13] used a PCR-based genotyping method and reported the presence of 1b in 93.3% of cases of single, dual or multiple infections. Other genotypes included 1a, 2b and 3a, but always in combination with 1b. Padron et al.[14] found that 92.9% of the patients included in their study had genotype 1b. In this study HCV genotyping was performed using a nested PCR procedure with specific primers for each described genotype. They also reported a high rate of multiple genotypes. A recent study using sequencing and phylogenetic analysis derived from the HCV core region also revealed the presence of genotype 1 in all samples tested, with a predominance of subtype 1b.[15] None of the previous studies reported genotype 4 in the Cuban population. It seems that in Cuba the most prevalent genotype is 1b, followed by 1a, with a minor circulation of 2b, 3a and 4a. Genotypes 1a, 1b and 3a, have become very widely distributed as a result of transmission through blood transfusion over the past 30–70 years and now represent the vast majority of infections in Western countries.[21] These are the genotypes that are encountered most commonly in the clinical setting and for which much information on treatments has been collected.

This study found five samples (6%) with mixed genotype infection; but always in combination with genotype 1, similar to previous reports from Cuba.[13,14] The gold standard methodology for genotyping HCV in serum samples is sequencing. However, in many diagnostic virology laboratories with no sequencing facilities, other methods are required, with RFLP being the most preferable methodology.[22] In our experience, PCR and genotyping using specific primers[11] is faster, less expensive than other genotyping methods and primers used for the PCR showed high homology when compared with consensus sequences of the respective genotype.

HCV co-infection has emerged as a significant factor influencing the survival of HIV patients. Patients with HCV/HIV co-infection have a faster rate of fibrosis progression resulting in more frequent occurrences of cirrhosis,
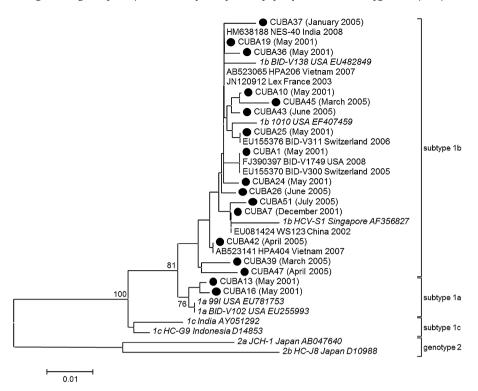
Figure 1. Phylogenetic analysis of 17 unique HCV strains from Cuba (marked with a black dot) compared to references sequences of genotypes 1 and 2 and sequences obtained by BLAST search. The Cuban strains in the tree are shown by their names and the collection time is indicated behind in parentheses. Each reference strain is identified by genotype and subgenotype, strain name, location and GenBank accession number (Italics). The sequences obtained by BLAST search are labeled with their GenBank accession number, strain name, location and year of collection. The tree was constructed using the neighbor-joining method and the Kimura 2-parameter model and is based on 286 nt of the 5′ NCR and part of the core of HCV Numbers at the nodes show bootstrap percentages over 1000 replicates (only values ≥ 70 are shown). The bar indicates genetic distance.

end-stage liver disease, and hepatocellular carcinoma.[9] In this scenario, particularly HCV genotype 4 has become increasingly prevalent in European countries and Australia in the last years.[23–26] HCV genotype 4 may spread quickly among the general population once it is introduced in a given region.[25] In the Americas genotypes 1 to 4 have been reported in HCV/HIV co-infected patients, with genotype 1 as the most frequently found followed by genotype 3 and mixed infections.[27–29] Epidemiological data demonstrate the recent introduction of HCV 4a and 4d subtypes into the Caribbean. In 2004, Martial et al.[27] reported genotype 4 in this region (2.8% and 5.9% samples from HCV-monoinfected and HCV/HIV co-infected patients from Martinique Island, respectively).

This is the first report of HCV genotype 4a in Cuba, specifically in an HIV positive patient. In the clinical setting, the emergence of HCV genotype 4 in the Caribbean and its presence in Latin America should be considered because it is difficult to treat and has a sustained virological response rate of only about 60%.[30]

HCV transmission is mainly via parenteral routes, with differences in developed and developing countries. In the developing world, unsafe therapeutic injections and transfusions are likely to be the major modes of transmission.[6] Patients with known risk factors such as multiple transfusions, multiple percutaneus procedures, immunosuppression and frequent contact with blood and blood products place these individuals at risk to HCV infection.

In HIV-positive cohorts with a single predominant risk factor for acquiring HIV, the proportion of patients co-infected with HCV depends largely on the primary HIV risk factor.[6] In addition, it is well known that: HCV sexual transmission is enhanced by high-risk sexual behaviour such as multiple casual partners and anal intercourse, the use of recreational drugs and HIV co-infection; the HCV-RNA has been found with higher levels in semen of HCV/HIV co-infected patients than in HCV-monoinfected patients; and the incidence of HCV infection is increasing in HIV-infected MSM and phylogenetic analysis has revealed a large international network of HCV transmission among this population.[26,31,32]

Routes of transmission of HIV infection in Cuba are different in some aspects from that in other countries as the transmission by intravenous drug use and infection by blood and blood derivatives is extremely rare. Instead, 99% of the cases contracted the infection through sexual relations, 77% of the HIV positive adults are men, most (85%) of the detected HIV positive men were reported as MSM, and most of the HIV positive women reported having had sex with MSM.[33,34] Taking into account the results of the present study and the epidemiological characteristics of the HIV epidemic in Cuba, the possibility of the spread of genotype 4a in this population or in the general population

must be an alert to the health authorities and clinicians have to be aware of this circumstance.

Mixed HCV infection was found in a patient with history of frequent parenteral medication for severe chronic disease and in four samples from MSM. This mixed infection is possibly due to multiple exposures around the time of primary infection, taking into account that ongoing or chronic HCV infection does not protect from reinfection, at least experimentally.[35] Another possibility is simultaneous infection with several genotypes. The presence of genotype 1 in all of these samples, reinforces the data reported here and previously in Cuba of the high prevalence of genotype 1. Of interest is the presence of genotype 3 in four samples, three of them (75%) being HCV/HIV co-infected persons. Similar results have been reported in Brazil and in Martinica.[27,36]

The lack of association between a specific HCV genotype and demographic characteristics was not unexpected due to the predominance of genotype 1b in 82% of the global population and also in both subgroups included in this study. The tendency of HCV/HIV patients older than 45 years of age to have mixed infections could be due to the life style of this group with high-risk sexual practices associated with HCV transmission. Mendes-Correa and co-workers reported that age older than 30 years was strongly associated with hepatitis C transmission in HIV-infected patients in Brazil,[36] but they did not report detection of HCV mixed infections in a series where most patients reported more than two risk factors for HCV transmission,[29] which is possibly related to the genotyping method or the risk factors associated with HIV infection in Brazil. Similar to our findings, Marcial et al. reported the association between HCV subtype 1b and old age in the HCV/HIV-coinfected group.[27]

Studies in the Caribbean Islands[27] and in the Continental region of the Americas[37,38] report the existence of the six genotypes of HCV with the predominance of genotype 1 (subtypes a and b) with some countries having a geographic distribution pattern of different HCV genotypes. The molecular epidemiology and natural history of HCV shows a wide range of genotypes circulating among the main risk groups for HCV infection and in populations with no apparent risk for HCV infection around the world. In Cuba, prevalence of HCV genotypes in the two population groups studied is consistent with the HCV genotype distribution reported for the Latin America region,[39] although further investigations will be performed to confirm the detection and spread of genotype 4a in HCV/HIV co-infected patients and in the general population.

Cuban HCV sequences were identical, or very similar to, strains from Asia, Europe and North America (Figure 1). These findings were not entirely unexpected, because of the long history of communication between these regions and Cuba. Information regarding HCV phylogenetic analysis is limited in Cuba, but recently Gonzalez-Horta and collaborators[15] studying an HCV core fragment reported that Cuban HCV sequences showed highest similarity with isolates from the US and Europe, very similar to our findings. Blood transfusions and medical invasive procedures were the main potential modes of HCV infection in the population analyzed by Gonzalez-Horta, also similar to

the HCV-monoinfected population shown in the present study.

The analysis showed that some Cuban strains cluster together and are distinct from the other strains from Cuba. This is important to consider because these strains were isolated from patients with a common place of residence, year of collection and putative route of infection. More studies with different HCV genome regions are needed in order to add data regarding the genetic diversity of HCV in Cuba. Also, further studies may provide important evidence for understanding the origin and spread of HCV infection in the Caribbean region, a place with a mixed population from Europe, Africa and Asia, but also geographically close to the American continent.

In conclusion, this report presents the prevalence of HCV genotypes in monoinfected and HIV co-infected patients and for the first time the detection of subtype 4a in a patient co-infected with HIV in Cuba. Additionally HIV-infected MSM with high-risk sexual practices should be aware of sexually transmitted HCV superinfection and reinforce the necessity of take appropriate preventive measures.

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