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Reconstruction of the evolutionary dynamics of hepatitis C virus subtypes in Montenegro and the Balkan region

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ABSTRACT

More than 20 million hepatitis C virus (HCV) carriers live in the countries of the Eastern Mediterranean. We determined HCV genotype distribution among chronically infected patients in Montenegro and investigated the phylodynamics and phylogeography of the most represented HCV subtypes. The HCV-NS5b sequences of the Montenegrin patients were compared with sequences isolated in different known localities of the Mediterranean area, Europe and Asia. A Bayesian approach was used in order to allow the simultaneous estimate of the evolutionary rate, time-scaled phylogeny, demography and ancestral spatial status.

The most frequent HCV subtypes among the Montenegrin patients, were 1b (34.7%) and 3a (24.7%), but there was also a significant prevalence of 1a and 4d (19.5%). Subtype 3a was significantly more frequent among younger patients and intravenous drug users (IDUs), whereas subtype 1b was more frequently associated with iatrogenic exposure and older ages.

The spatio-temporal analysis of the epidemic suggested that HCV-1b penetrated Europe at the beginning of the XX century, probably through Greece and Cyprus and in the 1920s reached Montenegro, where there was an exponential increase in the effective number of infections between the 1950s and 1970s. The phylogeographic and phylodynamic analysis of HCV 3a showed that its most probable origin was in the Indian sub-continent (Pakistan in our reconstruction) about 300 years ago. The evolutionary dynamics analysis showed that HCV-3a reached Montenegro more recently in the late 1970s and underwent multi-phasic growth still persisting.

Our data suggest multiple introduction of HCV subtypes in the area, supported by different causes of dispersion: adverse social conditions and unsafe medical practices for HCV-1b and i.v. drug use for HCV-3a.

1. Introduction

Hepatitis C virus (HCV) infects more than 170 million people and is a leading cause of liver disease throughout the world (Hoofnagle, 1997; Lauer and Walker, 2001).

Its genome is a positive single-stranded RNA of 9.6 Kb. At least six major genotypes and a number of subtypes with different ethno-geographical distributions have been identified. Phylogenetic studies indicate that genotypes 1, 2, 4 and 5 originated in sub-Saharan Africa, and genotypes 3 and 6 in South-east Asia (Simmonds et al., 2005). A new genotype, preliminarily assigned as 7a, has been identified in Canada, but it still remains unpublished and any one such genotype has been obtained by others until now (Nakano et al., 2012). Most of the HCV infections worldwide are caused by a small subset of "epidemic" HCV subtypes, including subtypes 1a, 1b, 2b, 3a and 4a (Pybus et al., 2001). It is widely agreed that these subtypes spread rapidly throughout the world during the 20th century, mainly as a result of transfusions, blood products utilization, unsafe medical injections and injection drug use (Hauri et al., 2004; Pybus et al., 2007).

According to World Health Organisation (WHO) estimates, at least 21.3 million people living in the countries of the Eastern Mediterranean are HCV carriers (http://www.who.int/en/), but little is

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known about the prevalence of HCV infection in Serbia, Montenegro or the Balkans in general. The only available information relates to intravenous drug users (IDU), and shows that HCV positivity increased from 22% to 53.7% between 2005 and 2008 among Montegrin IDUs, and that there is a high prevalence of HCV (63%) among the IDUs in Belgrade, Serbia (Bacak et al., 2012; Judd et al., 2009).

Even less is known about the distribution of HCV genotypes in the area, although two papers published more than five years ago reported that the most prevalent HCV sub/genotypes in Serbia and Montenegro were 1b and 3a (Stamenkovic et al., 2000; Svirtlih et al., 2007).

A Bayesian statistical inference framework that allows the simultaneous reconstruction of the temporal and spatial history of an epidemic on the basis of isolates randomly sampled at known times in different places has recently been developed (Lemey et al., 2009), and used to reconstruct the epidemiological history of some highly variable viruses (Ciccozzi et al., 2011a; Zehender et al., 2011a).

The aim of this study was to investigate HCV sub/genotype distribution in Montenegro and estimate the phylodynamics and phylogeography of the most prevalent subtypes in order to reconstruct the origin and diffusion of the virus on a local and regional scale.

2. Materials and methods

2.1. Patients

One hundred and twenty-three patients with chronic HCV infection living in Montenegro (37 women [30.1%] and 86 men [69.9%]; with a median age of 45 years; range 24–77) were consecutively enrolled between 2007 and 2010 at the Institute of Public Health of Podgorica, and the serum samples of the 98 patients who were positive for HCV RNA at PCR were further characterised by NS5b gene sequencing. Demographic and epidemiological data were available for all patients. The presumptive mode of trasmission of HCV infection was unknown in the case of 34 subjects (34.7%); 34 (34.7%) were IDUs; and 30 (30.6%) were infected by iatrogenic exposure, including surgery, blood transfusions and dialysis.

2.2. Sample processing and HCV RNA sequencing

Viral RNA was extracted from 400 μl of serum using the QIAmp viral RNA extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Serum samples from healthy subjects were used as negative controls. The RNA was reverse-transcribed using the SuperScript II reverse transcriptase protocol (Invitrogen, Life Technologies, Carlsbad, CA), and the cDNA was amplified by means of nested PCR using the FastStart High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany). The primers for the first and second rounds of amplification have been previously described (Lu et al., 2005).

The nested PCR product encompassed the NS5b gene (from nucleotide 8256 to nucleotide 8632) of the HCV genome (Accession No. NC_004102). The PCR conditions for both rounds were 94 °C for 2 min, followed by 28 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s, and a last extension step at 72 °C for 7 min. The nested PCR product was analysed on 1.5% agarose gel stained with ethidium bromide.

The PCR product was purified using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions, and both strands were sequenced using the GenomeLab DTCS Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA). The sequencing reactions were run on an automated DNA sequencer (Beckman Coulter, Inc., Fullerton, CA).

2.3. Genotype characterization

All of the Montenegrin sequences were aligned with 75 representative sequences of the main genotypes/subtypes circulating in Europe that were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The genotype/subtype was characterised by means of phylogenetic analysis of the NS5b gene sequences using two methods: a neighbour-joining (NJ) distance method implemented in version 5 of the MEGA program (Tamura et al., 2011), and a maximum likelihood (ML) approach with a new hill-climbing algorithm implemented in Phyml v.3.0 server (http://www.atgc-montpellier.fr/phyml/) (Guindon et al., 2005). The trees were displayed using Figtree software v 1.3.1, which is freely available on the web (http://tree.bio.ed.ac.uk/software/figtree/). The nucleotide substitution model (GTR+G+I) was selected as previously described (Ciccozzi et al., 2011a).

The reliability of the observed clades was established on the basis of internal node bootstrap support values of more than 0.90 (after 1000 replicates in NJ, or 200 in Phyml).

2.4. Phylogeography and phylodynamics

2.4.1. HCV dataset

In order to estimate the phylodynamics and phylogeography of the HCV subtypes more frequently found among the Montenegrin patients, two separate datasets were prepared that included the HCV-NS5b sequences of HCV subtypes 1b and 3a (318 nucleotides in length). All of the NS5b sequences of subtypes 1b and 3a isolated in different Mediterranean and Asian countries and available at the Los Alamos HCV sequence database (http://hcv.lanl.gov/content/index) were downloaded, cropped and aligned with the Montenegrin isolates of the same subtype. The reference sequences were selected on the basis of the following inclusion criteria: (1) they had to have been already published in peer-reviewed journals (except for the new sequences described before); (2) there had to be no uncertainty about the subtype assignment of each sequence, and all were classified as non-recombinant; (3) the city/state of origin were known and clearly established in the original publication. Globally, we obtained two data sets that respectively included 188 HCV-1b and 236 HCV-3a sequences.

The data sets were aligned using ClustalX software (Thompson et al., 1997), and manually edited using Bioedit software. The JModelTest (Posada and Buckley, 2004) was used to select the simplest evolutionary model fitting the data which resulted the GTR+G+I model of nucleotide substitution (Lanave et al., 1984). The sampling locations of the isolates included in the different datasets are shown in Table 1S.

2.4.2. Likelihood mapping analysis

In order to obtain an overall impression of the phylogenetic signal present in the NS5b genotype 1b and 3a sequences, we made a likelihood-mapping analysis of 10,000 random quartets generated using TreePuzzle (Schmidt et al., 2002). A likelihood map consists of an equilateral triangle: each dot within the triangle represents the likelihoods of the three possible unrooted trees for a set of four sequences (quartets) randomly selected from the dataset. The dots close to the corners or at the sides respectively represent tree-like (fully resolved phylogenies in which one tree is clearly better than the others) or network-like phylogenetic signals (three regions for which it is not possible to decide between two topologies); the central area of the map represents a star-like signal (the region where the star tree is the optimal tree).