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Molecular and Epidemiological Profiles of Hepatitis C Virus Genotype 4 in Denmark

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Keywords:	HCV, genotype 4, intravenous drug use , core/E1 region, phylogenetic analysis



TABLE I. Ethnicity and Route of Transmission for 72 patients with chronic HCV Genotype 4 Infection.

Characteristics	Patients, N (%)
All patients	72 (100)
Gender	
Male	50 (69.4)
Female	22 (30.6)
Age (years)	
Median (IQR*)	49.5 (40 – 59)
< 30	2 (2.8)
31 - 40	17 (23.6)
41 - 50	19 (26.4)
51 - 60	22 (30.6)
61 - 70	8 (11.1)
> 71	4 (5.5)
Ethnic origin	
Denmark	17 (23.6)
Southern Africa	17 (23.6)
Northern Africa	11 (15.3)
Egypt	8 (11.1)
Middle East	7 (9.7)
Pakistan	2 (2.9)
Southern Europe	5 (6.9)
Unknown origin (missing data)	5 (6.9)
Route of transmission	
Injecting drug use	16 (22.1)
Blood transfusion	4 (5.6)
Sexual contact	4 (5.6)
Needle injury	2 (2.8)
Vaccination	1 (1.4)
Unknown	36 (50)
Missing information	9 (12.5)
Subtypes	
4d	23 (31.9)
4a	21 (29.2)
4r	12 (16.6)
4UN	5 (6.9)
4k	4 (5.6)
4o	4 (5.6)
4h	1 (1.4)
4l	1 (1.4)
4n	1 (1.4)
Patients co-infected with HIV	9 (100)
Ethnic origin, route of transmission and subtype	
Denmark	
- Injecting drug use (4d)	3 (33.3)
- Sexual contact (4k)	1 (11.2)
Southern Africa	
- Unknown route of transmission (4k)	2 (22.2)
- Unknown route of transmission (4h)	1 (11.2)
- Unknown route of transmission (4UN)	1 (11.2)

* IQR – Interquartile range

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TABLE II. Characteristics of the Patients infected with subtype 4a, 4d and 4r

Characteristics	Subtype 4d (N, %)	Subtype 4a (N, %)	Subtype 4r (N, %)
All patients	23 (100)	21 (100)	12 (100)
Gender			
Male	17 (77.3)	15 (71.4)	10 (83.3)
Female	6 (22.7)	6 (28.6)	2 (16.7)
Age (Years)			
Median (IQR)	45 (41 – 51.5)	51 (38 – 58)	64 (58 – 71)
< 30	-	1 (4.8)	-
31 - 40	6 (26.1)	5 (23.7)	2 (16.7)
41 - 50	11 (47.8)	3 (14.3)	-
51 - 60	5 (21.7)	11 (52.4)	2 (16.7)
61 - 70	-	1 (4.8)	4 (33.3)
> 71	1 (4.4)	-	4 (33.3)
Route of Transmission in Relation to Ethnic origin			
Denmark	16 (100)	-	-
- IDU	15 (93.8)	-	-
- Unknown	1 (6.2)	-	-
Egypt	-	8 (100)	-
- Vaccination	-	1 (12.5)	-
- Unknown	-	7 (87.5)	-
Southern Europe	4 (100)	-	1 (100)
- IDU	1 (25)	-	-
- Sexual Transmission	2 (50)	-	-
- Missing Information	1 (25)	-	1 (100)
Middle East	1 (100)	4 (100)	-
- Blood Transfusion	-	2 (50)	-
- Unknown	1 (100)	2 (50)	-
Northern Africa	-	6 (100)	1 (100)
- Unknown	-	6 (100)	1 (100)
Pakistan	-	1 (100)	-
- Sexual Transmission	-	1 (100)	-
Southern Africa	-	-	10 (100)
- Unknown	-	-	9 (75)
- Missing Information	-	-	1 (25)
Missing Data	2	2	-

209x297mm (300 x 300 DPI)

Molecular and Epidemiological Profiles of Hepatitis C Virus Genotype 4 in Denmark

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Short title: HCV genotype 4 subtypes in Denmark

ABSTRACT

The prevalence of hepatitis C virus (HCV) genotype 4 has increased throughout Europe. This is an epidemiological study of patients infected chronically with HCV genotype 4 in Denmark. The HCV strains analyzed originated from patient samples collected between 1999 and 2007 as part of the national Danish hepatitis B and C network, DANHEP. Sequence analyses were based on the envelope 1 region of HCV. Results from a total of 72 patients indicated a high degree of genetic heterogeneity. Fifty-six patients (78%) were infected with one of the three dominating subtypes: 4d, 4a, or 4r. The remaining 16 patients (22%) were infected with subtypes 4h, 4k, 4l, 4n, 4o, or 4Unclassified. Three epidemiological profiles were identified: (1) Patients infected with HCV by intravenous drug use were infected solely with subtype 4d. They were all of European origin, and 15 of the 16 patients were ethnic Danes. No single transmission event could be confirmed, but the pairwise nucleotide identity within the patients of Danish origin was relatively high (~95%), suggesting a recent introduction into Denmark. (2) The 21 patients infected with subtype 4a all came from Northern Africa, Egypt, Pakistan, or the Middle East. (3) Patients from Southern Africa dominated among patients infected with subtype 4r (10 of 12 patients). This study demonstrates that HCV genotype 4d has been introduced in and spread among Danish intravenous drug users. The remaining subtypes show restricted distribution, infecting almost exclusively patients from geographical areas with a relatively high prevalence of HCV genotype 4 infections.

KEY WORDS

HCV; genotype 4; intravenous drug use; core/E1 region; phylogenetic analysis

INTRODUCTION

Hepatitis C virus (HCV) is currently one of the most widespread infectious viruses, with more than 170 million people infected worldwide [WHO, 1999]. About 65–85% of individuals with acute HCV infection develop a chronic infection, with risk increased significantly of developing liver cirrhosis and hepatocellular carcinoma [Hoofnagle, 1997; Seeff, 2002]. The HCV virion contains a single-stranded, positive-sense RNA genome and has been classified into the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome is very heterogeneous, as reflected both by genome length and by the identification of seven major genotypes and more than 67 subtypes with nucleotide divergences of 31–33% and 20–25%, respectively [Bukh et al., 1994; Simmonds et al., 2005; Kuiken et al., 2008; Kuiken and Simmonds, 2009]. Genotypes 1, 2, and 3 are found worldwide, particularly in Western countries. Genotypes 4 and 5 display a more restricted geographic pattern with high prevalences in the Middle East and Africa. Egypt has the highest prevalence of HCV; 20% of the Egyptian population is chronically infected with HCV, and among this group, almost 90% are infected with genotype 4 [Kamal and Nasser, 2008]. Genotype 6 is mainly found in Southeast Asia, and genotype 7 has been found in patients of African origin living in Canada and Belgium [Murphy et al., 2007].

Although infection with all HCV genotypes can progress to chronic liver disease, genotype-specific differences in the natural history of the disease may exist. For example, HCV genotypes 1 and 3 have been found to induce steatosis [Cross et al., 2010]. The efficacy of the standard treatment with ribavirin and pegylated interferon depends on the genotype, with infection with either genotype 1 or 4 leading generally to a poorer treatment response than infection with genotype 2 or 3 [Fried et al., 2000; Fabiani, 2007].

Within HCV genotype 4, there is a widespread genetic heterogeneity, which is reflected by the presence of at least 19 subtypes (4a–h, 4k–4u) according to the Los Alamos HCV

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3 Database [Kuiken et al., 2005]. The overall prevalence of genotype 4 among the general
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6 population of patients infected with HCV in the United States and Europe is thought to be
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9 low. Two U.S. studies done 10 years apart reported a low prevalence (approximately 1%) of
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11 genotype 4 [Lau et al., 1996; Nainan et al., 2006]. A Slovenian study found a genotype 4
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13 prevalence of 1.4% among a large cohort of 1,504 patients infected with HCV [Seme et al.,
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15 2009]. A fourfold increase in the prevalence of genotype 4 during a five-year period was
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17 reported among older patients with sporadically acquired infection in Calabria, Southern Italy
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19 [Matera et al., 2002]. It is of note that the first identified subtype 4d strain was isolated from a
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21 Danish patient in 1993 [Bukh et al., 1993].
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25 Although HCV genotype 4 seems to constitute a rather low percentage of patients
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27 infected with HCV in the Western world, it has spread throughout Europe, especially among
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29 intravenous drug users. In Greece, genotype 4 is the second most frequent genotype among
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31 intravenous drug users [Savvas et al., 2005], and similar results were obtained in a study from
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33 Southern Spain [Sánchez-Quijano et al., 1997]. In Southwestern France, a genotype 4
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35 prevalence of 7.4% was found, with subtypes 4a and 4d identified almost exclusively among
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37 intravenous drug users (99%) [Nicot et al., 2005]. In the Netherlands, a study using a
38
39 molecular epidemiological approach identified genotype 4 in three different epidemiological
40
41 profiles. One group was found to be correlated to Egypt, one to intravenous drug users, and
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43 one with men who have sex with men [de Bruijne et al., 2009]. A European multi-center
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45 study of intravenous drug users co-infected with HCV and human immunodeficiency virus
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47 (HIV) found genotype 4 prevalences ranging from 6.9% in Northern Europe (Holland,
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49 Scotland) to 24.4% in Southern Europe (France, Spain), with subtype 4d dominating among
50
51 intravenous drug users [van Asten et al., 2004].
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58 An estimated 15,000 people in Denmark are infected chronically with HCV [Danish
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60 National Board of Health, 2002]. The prevalence of HCV in Denmark is approximately 0.2%

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3 and is similar to the prevalence found in the other Scandinavian countries [Esteban et al.,
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6 2008]. A 2003 study of 71 patients using phylogenetic analysis of C-E1 reported that the
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8 most common HCV genotypes found in Denmark are genotypes 1a (43%), 3a (39%), 1b
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10 (11%), and 2b (6%) [Corbet et al., 2003], but genotype 4 was not found. However, genotype
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12 4 has a prevalence of approximately 2% among patients with hepatitis C referred to hospital
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14 departments in Denmark [DANHEP, Annual Report 2004].
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16
17 The aim of the present study was to gain knowledge about patients infected with HCV
18
19 genotype 4 regarding subtypes, routes of transmission, ethnic origin, and co-infection with
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21 HIV. This information may make it possible to characterize the introduction and spread of
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23 HCV genotype 4 strains among subpopulations in Denmark, which could have important
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25 implications for preventing HCV transmission and treatment.
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MATERIALS AND METHODS

Study Population

The inclusion of patients into the study was based on initial HCV genotype 4 determinations carried out from 1999 to 2007 at the Department of Clinical Biochemistry, Unit for Molecular Diagnostics, Aalborg University Hospital, Denmark. Aalborg University Hospital is the primary site for HCV genotypic assay in Denmark, and no patients tested at Statens Serum Institut were found to harbor genotype 4. Patients were genotyped with genotype-specific primers located in the 5' HCV non-coding region, as described previously [Krarup et al., 1998; Krarup et al., 2000].

A total of 72 samples from patients infected chronically with HCV genotype 4 were available for analysis. Clinical data regarding the patients was received from the Danish nationwide viral hepatitis database (the Danish Database for Hepatitis B and C, DANHEP) covering all regions of Denmark.

The Danish Ethics Council and The Danish Data Protection Supervisory Authority approved this study.

HCV Reverse Transcription, Genotyping, and Phylogenetic Analysis

The envelope 1 (E1) gene of HCV was chosen because this assay is used at the Statens Serum Institut for routine patient samples for HCV genotyping. To amplify and sequence part of the E1 gene, HCV RNA was isolated from 280 μ l plasma using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer guidelines. This was followed by a one-step RT-PCR with 60 pmol primers (Outer C-E1 F and Outer C-E1 R) using the Qiagen OneStep RT-PCR Kit according to manufacturer guidelines (Qiagen, Hilden, Germany). The cycling conditions were as follows: (1): 50°C (30 minutes), (2): 95°C (15 minutes), (3): 94°C

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3 (30 seconds), (4): 55°C (30 seconds), and (5): 72°C (30 seconds); steps 3–5 were repeated 39
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5
6 times. Final extension was carried out at 72°C for 10 minutes followed by 30 minutes at 4°C.
7

8 PCR products were sequenced with the primers “Inner C-E1 F” and “Inner C-E1 R”
9
10 using the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI 3130x sequencer
11
12 (Applied Biosystems, Foster City, CA, USA). Primers used were from a study published
13
14 previously [Ray et al., 2000] and have been shown previously to have equal sensitivity
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16 against isolates of the different HCV genotypes [Corbet et al., 2003]. Sequences were aligned
17
18 and edited manually using the SeqScape Software v2.6 (Applied Biosystems, Foster City,
19
20 CA, USA) and BioEdit Software [Hall, 1999]. Multiple alignments of the patient and
21
22 reference sequences were carried out with ClustalX software v. 1.8 [Thompson et al., 1997].
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27 To ensure an equal length of all included sequences, the phylogenetic analysis was
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29 based on a subdomain of the E1 region from nucleotides 923–1202, with nucleotide positions
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31 relative to the HCV H77, genotype 1a reference strain [Kolykhalov et al., 1997]. The
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33 phylogenetic tree was inferred by the neighbor-joining (NJ) method using the Kimura 2-
34
35 parameter correction distances with bootstrap values based on 1,000 replicates (Fig. 1). To
36
37 ensure the strength of the phylogenetic analysis, a multiple sequence comparison by log-
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39 expectation (MUSCLE) alignment with a maximum number of iterations equal to 2 was
40
41 carried out [Edgar, 2004]. A maximum likelihood tree was built with PHYML using the
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43 HKY85 substitution model and bootstrapped with 100 replicates [Guindon and Gascuel,
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45 2003]. The reference strains included in the phylogenetic tree are named with
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49 genotype/subtype and country of origin when possible, otherwise with sampling country and
50
51 the GenBank accession numbers (Fig. 1). Reference sequences were included for genotype 1
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53 [Kolykhalov et al., 1997], genotype 2 [Kato et al., 2001], genotype 3 [Sakamoto et al., 1994],
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55 and genotype 4 [Bukh et al., 1993; Stuyver et al., 1994; van Doorn, 1994; Fujimura et al.,
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57 1996; Ray et al., 2000; Morice et al., 2001; Lavillette et al., 2005; Brown et al., 2007; Franco
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3 et al., 2007; Hmaied et al., 2007; Murphy et al., 2007; Timm et al., 2007; Kuntzen et al.,
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6 2008; Demetriou et al., 2009; Khan et al., 2009].

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8 The patients were defined as being infected with a particular subtype when included in
9
10 a defined cluster with given reference strains and with bootstrap values > 90. Subtypes were
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12 defined as unclassified (UN) if sequences clustered with reference sequences of unknown
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14 subtype and a BLAST search did not reveal similarity with reference sequences of known
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16 subtypes.
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19 20 21 22 **Statistical Analysis**

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24 For statistical analysis, the Fisher's exact test and the Student's *t*-test were used for
25
26 comparing age distribution of the patients and for comparing the HIV prevalence of the
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28 DANHEP participants and HIV prevalence among the patients in this study. A Bonferroni
29
30 adjustment was used to correlate for multiple comparisons. *P* values of < 0.05 were
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32 considered significant.
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36 37 38 **RESULTS**

39
40 Seventy-two Danish patients with genotype 4 were included in the phylogenetic and
41
42 epidemiological analyses. Two patients were excluded because of negative PCR results in the
43
44 C-E1 assay, and an additional two patients were excluded because of uncertainty about their
45
46 identity. The consensus E1 partial sequence (nucleotides 923–1202, according to the H77
47
48 reference strain) was the same length without insertions or deletions in all 72 genotype 4
49
50 isolates, and each sequence encoded 59 amino acids. All patients except two were infected
51
52 with unique HCV strains. Pairwise identity values at the nucleotide and amino acid levels
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54 were 76.6% (range 64–99%) and 81.8% (range 68–100%).
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Epidemiology of the Patients with Genotype 4

Almost 70% of patients with chronic infection with HCV genotype 4 were males (Table I). The median age was 49.5 years (interquartile range (IQR) 40–59 years) (Table I). The phylogenetic N-J tree of the partial E1 sequences (Fig. 1) defined accurately the subtypes of the 72 patients infected with HCV genotype 4 and was reproducible (maximum likelihood tree, not shown). The partial E1 region (nucleotides 924–1202) was sufficient to define and distinguish both the different HCV genotypes and the genotype 4 subtypes, as indicated by bootstrap values > 90. The subtypes detected most frequently were 4d, 4a, and 4r, infecting 23 (31.9%), 21 (29.2%), and 12 (16.7%) of 72 patients, respectively. Thus, subtypes 4a, 4d, and 4r accounted for 77.8% of the genotype 4 infections in Denmark. The infections of the remaining 16 patients with genotype 4 (22.2%) represented subtypes 4h, 4k, 4l, 4n, 4o, and unclassified subtypes (4UN). The majority of patients were ethnic Danes (17 of 72 patients; 23.6%) and Africans (28 of 72 patients; 38.9%), but the group included also patients from Southern Europe, Egypt, the Middle East, and Pakistan (Table I).

The main route identified for transmission among patients with genotype 4 was intravenous drug use, which accounted for transmission in 16 of the 72 patients (22.2%); for the majority (36 patients; 50%), however, the transmission routes were unknown. Other transmission routes included blood transfusion, sexual contact, needle injury, and vaccination (Table I). The three major subtypes (4d, 4a, and 4r) were associated with different routes of transmission. Of the 23 patients with subtype 4d, 16 were infected via intravenous drug use (69.5%, Table II). Of these patients infected with subtype 4d via intravenous drug use, 15 patients (93.8%) were ethnic Danes; the remaining patient was from Southern Europe. The phylogenetic tree indicated two monophyletic clusters with seven and eight Danish subtype 4d patients, plus one patient of unknown origin; these clusters were not supported, however, by significant bootstrap values (Fig.1). For patients infected with other subtypes, the route of

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3 transmission was stated mostly as unknown (Table I and II). The patients infected with
4 subtype 4a all came from Africa, Pakistan, or the Middle East. Subtype 4r infected 10
5 patients from Southern Africa (83.3%), one patient from Northern Africa (8.3%), and a
6 Southern European patient (8.3%). The 4r patients were significantly older (median age: 64
7 years; IQR: 58–71 years, Table II) than subtype 4d ($P = 0.0044$) and subtype 4a ($P = 0.013$)
8 patients. The median ages in the latter two groups were comparable (4d, median: 45 years,
9 IQR: 41–51.5 years; 4a, median: 51 years, IQR: 37.75–58 years, $P = 0.588$).

22 **Pairwise Identity and Similarity of Patient Sequences with Published Sequences**

23 The similarity between the patient nucleotide sequences (subtypes 4a, 4d, 4r, and 4UN) and
24 published nucleotide sequences was investigated using BLAST, and each closest match for
25 4a, 4d, 4r, and 4UN was retrieved. All of the subtype 4d patient strains were most similar to a
26 published genotype 4d sequence isolated from an African patient (FJ462437). The average
27 pairwise nucleotide identity within the entire group of subtype 4d patient strains was 94.2%
28 (64–99%); within the Danish patient strains, it was 94.9% (91–99%). The different subtype
29 4a patient strains were most similar to various sequences: an isolate from a Pakistani patient
30 (AB444559), an isolate from a Yemeni patient (D43681), an isolate from an Egyptian patient
31 (DQ988079), an isolate published from the United States (DQ418787), and an isolate
32 published from Japan (D45193).

33 The pairwise nucleotide identity within the entire group of subtype 4a patients was 87%
34 (67–93%); within the Egyptian patient group, it was 85.4% (69–93%). The pairwise
35 nucleotide identity within the subtype 4r patient strains was 91.3% (86–96%), and 11 of the
36 12 patients were most similar to two Yemeni isolates (D43681, D43678). In the five cases of
37 unclassified subtype (4UN), two of the patient strains were most similar to sequences from
38 Canada (EF115910) and Cyprus (EU684694), one was related to a strain from Canada
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3 (EF115886), one was situated between subtypes 4a and 4n, and one was situated between 4e
4 and 4q. Neither the BLAST investigation nor comparison against the HCV Los Alamos
5 Database yielded further subtype information.
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10 11 12 **HCV/HIV Co-infection**

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15 Of the 47 patients who were tested for HIV, 9 were HIV positive (19.2%) and 38 (80.8%)
16 were HIV negative (Table I). Of the 72 patients, 25 (34.7%) had not been tested.
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20 Among patients co-infected with HIV, the known routes of HCV transmission were
21 intravenous drug use and sexual contact. One patient co-infected with HCV/HIV was a man
22 having sex with men and self-reported to have been infected by sexual contact with a former
23 partner from Germany. No single HCV genotype 4 subtype dominated among patients co-
24 infected with HCV and HIV (Table II).
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34 **DISCUSSION**

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36 This nationwide study of patients infected with genotype 4 identified three epidemiological
37 profiles. HCV subtype 4d was found in an ethnic Danish patient for the first time in 1993
38 [Bukh et al., 1993] and seems to have spread, dominating completely now among patients
39 who are ethnic Danish intravenous drug users infected with genotype 4. Genotype 4 has been
40 reported throughout Europe, and subtype 4d has been linked particularly in epidemiological
41 studies to intravenous drug use in the Netherlands, Poland, and Southern Europe [van Asten
42 et al., 2004; Nicot et al., 2005; Chlabicz et al., 2008; de Bruijne et al., 2009]. Our results do
43 not support a hypothetical single-transmission introduction of subtype 4d in Danish
44 intravenous drug users. This hypothesis would require a distinct, single monophyletic cluster
45 containing intravenous drug users, supported by significant bootstrap values [de Oliveira et
46 al., 2006]. Immigration of intravenous drug users from regions or countries with a high
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3 prevalence of subtype 4d among intravenous drug users [van Asten et al., 2004; Nicot et al.,
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6 2005] could indicate a possible route of introduction of subtype 4d in the population of ethnic
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8 Danish intravenous drug users. In all, HCV genotype 4 has been introduced into the European
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10 intravenous drug-use milieu including Denmark, and there is an increasing prevalence of
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12 HCV genotype 4 throughout Europe [Sánchez-Ouijano et al., 1997; Savvas et al., 2005].
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15 The presence of only subtype 4d among Danish intravenous drug users might reflect an
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17 effect of preventive measures such as needle-exchange and substitution programs, leading to
18
19 a decrease in the frequency of HCV exposure. However, the overall prevalence of hepatitis C
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21 among intravenous drug users was not lower than in other European countries [EMCDDA;
22
23 annual report, 2009]. Missing information on transmission route weakens the conclusion
24
25 about introduction of subtypes other than subtype 4d in Danish intravenous drug users. The
26
27 high degree of nucleotide identity among patients infected with subtype 4d who were ethnic
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29 Danish could indicate a recent introduction of subtype 4d among Danish intravenous drug
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31 users. De Bruijne et al. [2009] showed introduction of 4d among intravenous drug users in
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33 the Netherlands. The introduction and spread of subtype 4a among European intravenous
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35 drug users has been reported also [Schröter et al., 2002; Murphy et al., 2007], but according
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37 to our results, subtype 4a has not been introduced in Danish intravenous drug users. In
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39 addition, in this study, genotype 4 has not been established among men who have sex with
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41 men, as has been seen the Netherlands and in other European countries [de Bruijne et al.,
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43 2009; van de Laar et al., 2009].
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50 A second epidemiological profile comprised patients infected with subtype 4a who
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52 originated from restricted geographic areas like Northern Africa, Egypt, Pakistan, and the
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54 Middle East, where subtype 4a is prevalent [Djebbi et al., 2003]. As expected, all of the eight
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56 Egyptians included in the study (Table II) were infected with subtype 4a [Elkady et al.,
57
58 2009]. The 4a sequences from these patients did not cluster, and the nucleotide divergence
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3 ranged widely, explained possibly by the early introduction and exponential spread of this
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5 subtype in Egypt [Tanaka et al., 2004].
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8 The third epidemiological profile was found for patients with subtype 4r. This subtype
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10 was found mostly among Southern African patients, and these patients were significantly
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12 older than patients infected with subtypes 4d and 4a (64 years versus 45 and 51 years,
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14 respectively, Table II). Though not supported by significant bootstrap values, nine Southern
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16 African patients and one Southern European patient along with the GenBank reference strains
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18 D43681, D43679, and D43678 seemed to form a monophyletic cluster. Subtype 4r has been
19
20 described previously in patients of Central African origin: A Canadian study found a
21
22 prevalence of 10.2% of subtype 4r among patients mostly of Central African origin [Murphy
23
24 et al., 2007; Ndong-Atome et al., 2008]. The nucleotide variation within subtype 4r patient
25
26 strains is between that of subtypes 4d and 4a. However, the molecular epidemiology of HCV
27
28 in Africa is documented poorly, and the number of African 4r sequences available publicly is
29
30 small [Ndong-Atome et al., 2008]. A high prevalence of HCV genotype 4 has been found in
31
32 the Central African Republic, but the prevalence of subtype 4r was low (5%) [Njouom et al.,
33
34 2009].
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40
41 Five African patients were infected with unclassified subtypes (4UN). Two of these
42
43 patients were men of Northern African origin and clustered with GenBank reference strains
44
45 of unclassified subtypes, EU684694, and a Canadian strain, EF115910, isolated from a
46
47 patient originating from Rwanda [Murphy et al., 2007] (Fig. 1). Unclassifiable genotype 4
48
49 strains have been described in African patients [Ndong-Atome et al., 2008]. These new
50
51 sequences could form a new subtype, genetically closely related to subtypes 4q, 4e, and 4l,
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53 but to determine this, more sequence data are needed. In addition, the unclassified strains
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55 were sequenced in NS5B and analyzed phylogenetically, with results showing a similar
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3 distribution of the sequences as found for E1 (data not shown). BLAST analysis of the
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5 remaining 4UN patient strains did not identify a subtype.
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8 The subtypes infecting the patients co-infected with HCV and HIV varied, and no
9
10 particular genotype 4 subtypes dominated (Table I). The HCV subtype 4d sequences from co-
11
12 infected patients did not cluster together, and there was no indication of a connection between
13
14 these patients. In addition, there were no significant differences between the prevalence of
15
16 HCV/HIV co-infection among the genotype 4 patients (19.2%) in this study and the
17
18 prevalence of HCV/HIV co-infection among the entire group of DANHEP participants,
19
20 which included patients of all genotypes (17.9%), ($P = 0.4153$).
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24 In conclusion, this population from Denmark seemed to display a subtype distribution
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26 pattern similar to that found earlier in other Western countries, but with a more restricted
27
28 distribution of subtypes in relation to ethnic origin [Nicot et al., 2005; Cantaloube et al.,
29
30 2008]. Genotype 4 has been established and spread among men who have sex with men in
31
32 Europe, an epidemiological profile not found in Denmark. The subtypes 4d, 4a, and 4r
33
34 dominate among patients who are infected chronically with HCV and living in Denmark.
35
36 This study has shown that subtype 4d as a single subtype has been introduced in and spread
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38 among Danish intravenous drug users with chronic HCV. In general, the strains among
39
40 Danish patients infected with chronic HCV genotype 4 are very heterogeneous genetically.
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42 Numerous genotype 4 subtypes were found, with a distribution resembling findings from
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44 other Western countries [Nicot et al., 2005; Murphy et al., 2007; Cantaloube et al., 2008].
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17 **Appendix**

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20 Members of the DANHEP group:

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24 Health Sciences, University of Copenhagen, Denmark; Karin Grønbaek, Department of
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3 Figure Legend
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8 Fig. 1
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10 Phylogenetic tree based on E1 sequences (nucleotides 923–1202, according to the H77 Los
11 Alamos reference sequence) from 72 patients infected with HCV genotype 4. Colors show
12 the ethnic origin of the patients as indicated. Reference sequences were retrieved from
13 GenBank and are indicated by their accession number and if possible, country of origin,
14 otherwise by country of sampling. The neighbor-joining tree was based on the Kimura 2-
15 parameter correction distances with bootstrap values based on 1,000 replicates. Bootstrap
16 values > 90 are indicated with a dot.
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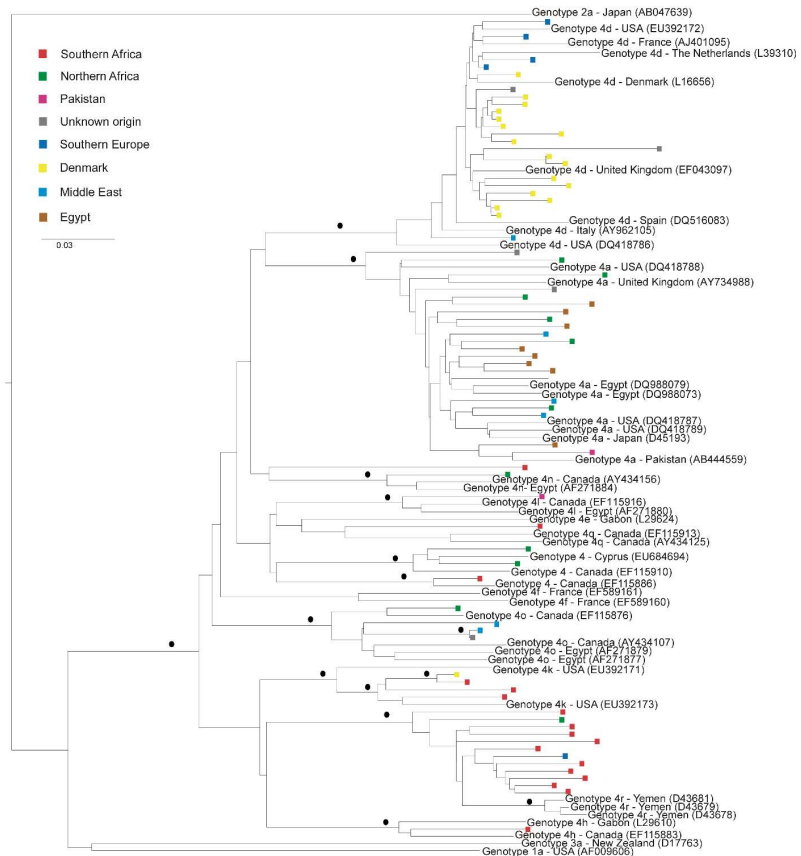


Fig. 1
Phylogenetic tree based on E1 sequences (nucleotides 923–1202, according to the H77 Los Alamos reference sequence) from 72 patients infected with HCV genotype 4. Colors show the ethnic origin of the patients as indicated. Reference sequences were retrieved from GenBank and are indicated by their accession number and if possible, country of origin, otherwise by country of sampling. The neighbor-joining tree was based on the Kimura 2-parameter correction distances with bootstrap values based on 1,000 replicates. Bootstrap values > 90 are indicated with a dot.

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