Molecular epidemiology of hepatitis C virus
van de Laar, T.J.W.

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CHAPTER 1.1

Hepatitis C Virus: a general introduction

Modified from:

Hepatitis C Virus: a general Introduction

Hepatitis C Virus (HCV) was identified in 1989 as a major causative agent of post transfusion non-A, non-B hepatitis [1], and is primarily transmitted by exposure to infected blood [2]. Although symptoms in the acute phase of infection are usually absent or mild, the majority of those infected develop chronic infection which, over decades, frequently results in progressive liver disease [3,4]. Recommended HCV treatment currently consists of the combined administration of pegylated interferon and ribavirin for a period of 24-48 weeks, but results in complete viral eradication in only 40-80% of patients, depending on e.g. patient characteristics and HCV genotype [5]. Among those coinfected with HIV-1, only 30%-40% of patients resolve HCV after an HCV treatment of 48 weeks or more [6,7]. HCV related end stage liver disease now is the main indication for liver transplantation in the Unites States [8]. Worldwide, HCV affects an estimated 180 million people, causing an additional 3 – 4 million new cases each year [2,9]. No vaccine is available to prevent infection in those at risk.

Epidemiology

Global prevalence

The World Health Organisation estimates that approximately 3% of the world population, or about 180 million people, may be infected with HCV [2,9]. Although HCV is endemic worldwide, reported prevalence rates vary greatly per country (Figure 1). Areas with an estimated low HCV prevalence (<1%), include north-western Europe, Canada and Australia. In Africa, South Asia and the Eastern Mediterranean the reported prevalence of HCV generally is much higher. In Egypt, HCV has affected 15-20% of the general population as a result of intravenous administered treatment against schistosomiasis [10]. From 1920 till 1980 multiple dose injections were administered in mass settings without sufficient sterilisation of re-used injection materials. It must be kept in mind, however, that the description of HCV global epidemiology relies heavily upon HCV seroprevalence studies that are typically done in selected populations which usually are not representative of the region as a whole [9].

Risk factors – developing countries

HCV is transmitted primarily by exposure to infected blood [2,11]. In developing countries, the use of unscreened blood and blood products is still widespread, sterilisation of medical instruments and supplies of sterile syringes may be inadequate or non-existent, non-
professionals often give injections outside the medical setting, and therapeutic injections are often given to deliver medications that could otherwise be administered orally [7]. Worldwide, unsafe injection practices alone may still lead to an estimated 3 million new HCV infections every year [12-14]. Hence, the use of blood or blood products from unscreened donors, unsafe therapeutic injections and other healthcare related procedures still account for the bulk of HCV transmission, and might serve as a bridge to the general population [15]. Outside the medical setting, other activities that under poor hygienic conditions may cause blood-blood contact such as tattooing, cultural/religious practices (e.g. circumcision, scarification, acupuncture) and barber shop shaving might play an important role in the spread of HCV in these countries [11]. The HCV prevalence among household contact of chronic HCV carriers varies from 0-15%, and might be partly explained by the sharing of e.g. razors and dental appliances [16,17]. Children born to HCV infected mothers, are infected with a rate of about 4%, which increases to 20% in maternal HCV/HIV co-infection [18].

Figure 1: Estimated anti-HCV seroprevalence by country, source WHO 1999 [19]. Low (<1%), intermediate (1%-2.5%), high (2.5-5%) and very high (>5%).

Risk factors – developed countries
The discovery of HCV in 1989 [1] and the development of the first serological assay for the detection of HCV antibodies in 1991, drastically reduced the incidence of transfusion acquired HCV in countries that introduced screening of donor blood [20-23]. Accidental health-care related HCV transmission does still occur, but the contribution to the overall HCV incidence in developed countries is assumed to be very low [24,25]. In developed countries, the majority of new HCV infections is caused by the sharing of injection equipment among injecting drug users [26]. However, in a large proportion of HCV prevalent cases no recognisable HCV risk factor can be identified [27-29]. Other activities that may
cause blood-blood contact have been described as routes for HCV infection, including tattooing/piercing, cultural/religious practices (e.g. scarification, circumcision, acupuncture), the shared use of straws and non-injection equipment among drug users, barber shop shaving and beauty treatments, but results are inconsistent and it remains uncertain whether these risk factors make any measurable contribution to overall HCV transmission [11]. Although HCV RNA has been detected in body fluids other than blood (e.g. semen, vaginal secretions and saliva) [30-32], HCV-RNA discordant partners in long-term monogamous heterosexual relationships show only slightly higher rates of HCV infection than the general population [33-35]. Even in the presence of HIV coinfection, HCV is rarely transmitted by heterosexual intercourse [36]. Since 2000, however, outbreaks of sexually transmitted HCV have been reported among men who have sex with men (MSM) in Europe [37-39], Australia [40] and the United States [41,42]. Longitudinal cohort studies have confirmed a recent rise in the incidence of HCV among HIV-positive MSM in England [43] and the Netherlands [44]. Although the exact determinants still need to be elucidated, sexual transmission of HCV among MSM has been associated with HIV, concurrent ulcerative sexually transmitted infections (STI), rough sexual practices, an increasing number of sex partners and sex under the influence of drugs [36-38,44].

The Netherlands

In the Netherlands, little is known about the HCV seroprevalence in the general population [45]. In a large seroprevalence survey in 1995, 0.08% of Dutch inhabitants tested positive for HCV-antibodies [46]. As those born abroad, and citizens without a Dutch nationality were undersampled, true seroprevalence is estimated to be higher, approximately 0.1-0.4% [47,48]. This would correspond to 16,000 – 65,000 HCV infected individuals living in the Netherlands, of whom an estimated 50-70% does not yet know that they are infected [49]. Six risk groups have been identified with varying HCV prevalences [42]; (i) injecting drug users (47-79%) [50,51], (ii) recipients of blood and blood products before 1991 (0.17%) [49], especially haemophiliacs (55%) [52], (iii) haemodialysis patients (2-3.5%) [53], (iv) first generation immigrants and individuals who underwent medical procedures abroad (prevalence depends on country of origin) (v) sexual and household partners of chronic HCV carriers (unknown) and (vi) children born to HCV infected mothers (4-20%, dependent on HIV-status of the mother). Since 2000, however, a seventh risk group has been identified. In Amsterdam, HCV-incidence among HIV-positive MSM rose tenfold in the period 2000-2003, compared with previous years 1985-1999 [44]. Significantly, 15-20% of HIV-positive MSM attending the Amsterdam STI clinic during three anonymous surveys in 2007 and 2008, tested HCV-RNA and/or anti-HCV positive [54].
Virology

Hepatitis C virus

HCV is a positive sense single stranded RNA virus, its host range is confined to humans and closely related primates. Due to its similarities in genome organisation, structure and replication to a large group of vector borne diseases (e.g. Yellow fever) as well as animal pestiviruses, HCV has been added as the sole member of the genus hepacivirus into the family of Flaviviridae [55-57]. HCV primarily targets the hepatocytes, but infection and viral replication also may occur in non-hepatic cells such as white blood cells [58,59]. The viral genome codes for one single polyprotein of about 3000 amino acids, flanked by highly conserved 5' and 3' termini that contain signals for translation and replication (Figure 2). The polyprotein is processed by viral and cellular proteases into 3 structural core and envelope proteins, and 7 additional non-structural proteins that code for the viral enzymes [60,61]. The high viral turnover and the error prone replication process, result in rapid evolution of HCV within an infected host [62]. The swarm of highly similar viral variants that develops within one host is called quasispecies, and probably provides one of the mechanisms by which HCV evades host immunity and establishes and maintains chronic infection [63].

Genetic variability

The high genetic variability of the HCV genome has led to the classification of the virus in seven major genotypes (1 to 7) that except for genotypes 5 and 7, are each further divided into a total of more than 70 distinct related subtypes (a,b,c,...) [55,64]. Genotypes have 65-70% similarity on the nucleotide level, subtypes 70-85% and within a subtype sequence variability is below 15% [65]. New HCV variants that can not be assigned to a previously recognised genotype or subtype are still being discovered [64]. Some HCV subtypes are found globally, due to a swift spread in the 20th century through needle sharing among injecting drug users (types 1a and 3a) or contaminated blood products (types 1b, 2a and 2b). These genotypes represent the majority of infections in Europe and Northern America. In contrast, the presence of numerous and highly divergent subtypes in western/central Africa and the Middle East (genotypes 1,2 and 4) as well as South-East Asia (genotype 3 and 6) suggest that these genotypes originate from these areas where they have been endemic for a long time [65,66].

Diagnostics

Standard HCV testing includes screening for HCV antibodies using an anti-HCV EIA confirmed by immunoblot. In a clinical setting, however, a positive HCV antibody test will be followed directly by a qualitative and, if RNA-positive, a quantitative HCV RNA test to establish the presence of ongoing HCV infection. In the Netherlands, as in most developed
countries, in addition to HCV serologic screening, all donor blood is tested for HCV RNA using nucleic acid testing (NAT) minipool screening [20]. The time between infection and the appearance of HCV antibodies is estimated to be approximately 60 days [67]. Hence, the presence of HCV RNA in HCV antibody negative individuals indicates acute infection. Prolonged seroconversion, or complete failure to mount or maintain HCV antibodies have been observed among immunosuppressed individuals, e.g. those coinfected with HIV [68]. Once HCV antibody positive, current lab-techniques are unable to provide information on the moment HCV was acquired. Loss of HCV antibodies (HCV seroreversion) has been described, but mainly among individuals that were able to clear the virus [69]. Presence of HCV RNA in the blood confirms active HCV infection. In order to diagnose the widest span of viral variants, HCV RNA tests are generally based on the highly conserved 5' NTR of the HCV genome [70]. Genotyping on the other hand requires viral heterogeneity, reference regions globally used for genotyping are the structural proteins core/E1 and the non-structural NS5B protein that codes for the RNA polymerase [55].

Figure 2: HCV model structure and genome organisation. The HCV polyprotein is cleaved into 3 structural proteins (C=core, E1=Envelope glycoprotein 1, E2=Envelope glycoprotein 2) and 7 non structural (NS) proteins that code for the different viral enzymes. Reproduced with permission from the author [61]
**HCV reinfection, coinfection and superinfection**

Molecular typing techniques have confirmed that both HCV reinfection and superinfection with a homologous or heterologous HCV strain can occur [71,72]. Neither viral clearance nor ongoing HCV chronic infection provides full protection against new HCV infections [73-75]. Evidence for partial HCV immunity is derived largely from chimpanzee studies. Chimpanzees that previously cleared HCV and were rechallenged with homologous HCV strains, generally show lower levels of HCV viremia and self-limited infection [76-78]. However, studies on HCV cross-protective immunity are few and results are conflicting [74,79-81]. In Baltimore and Vancouver DU who previously cleared their HCV infection were two to four times less likely to develop new episodes of HCV viremia, compared to DU without previous infection [79,80]. No such protection, however, was observed among a HCV seroconverter cohort of young Australian DU [74]. In addition to HCV reinfection, the recent documentation of recombinant HCV strains [82-84] is de facto evidence that HCV dual infections also occur. When the different HCV strains were contracted simultaneously or within a window period too narrow for the first infection to have resulted in an immunological response, it is defined as HCV coinfection. HCV superinfection refers to a situation in which a subsequent HCV infection is contracted in the presence of a previous HCV strain and the immune response it has generated [68]. The reported prevalence of such HCV superinfection in actively injecting DU varies between 0%-20% [73,85]. The true incidence, however, is hard to establish as current laboratory methodologies make characterisation of superinfection cumbersome [71,75,86]. Figure 3 illustrates the differences between HCV reinfection, coinfection and superinfection.

**Figure 3:** Hepatitis C virus reinfection (A), HCV coinfection followed by viral clearance (B) or chronic infection (C) and HCV superinfection (D). Modified from Blackard et al [71].
Natural history of HCV infection

Clinical course of infection

Less than one third of individuals with an acute HCV infection experiences mostly mild and aspecific symptoms such as fatigue, nausea, loss of appetite, flu-like symptoms, abdominal pain and only occasionally jaundice [3,4,87,88]. Symptoms generally start several weeks after infection, last for 3-12 weeks en disappear spontaneously. Natural viral clearance occurs in a minority (15-50%) of patients [74,89], and has been associated with female sex, younger age at infection, being nonblack, HBV coinfection, negative HIV-status and high CD4 counts in HIV-positives [88,90,91]. Given the asymptomatic onset of infection and slow pathogenesis of chronic disease, HCV diagnosis often occurs late, limiting the possibility to determine natural history of HCV [3,92]. Chronic HCV infection is defined as the persistence of detectable HCV viremia more than 6 months after infection, and may lead to cirrhosis, hepatic failure or hepatocellular carcinoma [4]. Approximately 20 years after onset of chronic infection HCV liver cirrhosis occurs in 6-25% of the patients, of whom per year 1-4% develop hepatocellular carcinoma [3,93-95]. Besides viral factors, different host-related and external factors such as male sex, older age at the time of infection, obesity, alcohol intake and coinfection with HIV or HBV have been associated with accelerated HCV disease progression [3,4,96,97]. New non-invasive methods have been developed that can be used for clinical decision making, including combined serological markers and liver scan techniques to establish the degree of liver fibrosis [98]. However, up till now liver histology is still considered to be the most reliable method to stage and grade the severity of liver disease [93]. Although HCV is a slow progressive disease, and only a minority of all HCV infected individuals will eventually develop end-stage liver disease, chronic HCV infection is now the main indication for liver transplantation in the United States [8]. Worldwide, an estimated 366,000 deaths are attributed to HCV each year, of which 27,000 occur in Western-Europe and the USA [99]. As a result of slow pathogenesis and the fact that a large proportion of individuals were infected before 1990, this number will most likely increase in the years to come [100,101].

HCV treatment

The current standard antiviral treatment of chronic HCV consists of administration of weekly pegylated interferon injection with a daily oral dose of ribavirin. In the absence of HIV-coinfection, HCV treatment duration is usually 12-24 weeks (genotype 2 and 3), or 24-48 weeks (genotype 1 and 4) [5,97,102]. The aim of treatment is to permanently eradicate the viral RNA and to reach a sustained viral response (SVR), which is defined as undetectable HCV RNA 24 weeks after the end of treatment. The most important predictor of SVR, which occurs in approximately 40-80% of HIV-negative cases, is the HCV genotype [103,104].
Chapter 1

Genotypes 1 and 4 are difficult-to-treat genotypes, with approximately 40-60% of treated patients achieving SVR compared to an SVR for up to 90% in patients with HCV genotypes 2 and 3 [5,103,104]. SVR rates over 90% have been reported for all genotypes, when interferon treatment was initiated within 3 to 4 months after acute HCV infection [105,106]. Other predictors of good treatment outcome include a rapid viral response (RVR) during treatment (undetectable HCV RNA levels at week 4) and early viral response (EVR) (a ≥ 2 log decrease in viral load during the first 12 weeks), and low HCV RNA level prior to treatment for genotype 1 [5].

HCV treatment causes a wide range of side effects in the majority of patients [107,108]. Serious side effects cause 10-15% of all patients to eventually discontinue treatment. Therefore, patients with mild disease activity can be given the option of deferring therapy until new drugs become available. New drugs are being developed which directly target HCV RNA and viral enzymes or influence host-virus interactions [109-116]. Despite toxicity issues and rapid selection of resistance which did restrain some of the initial enthusiasm, several of these new compounds are very promising and are expected to be registered within the next three years. Two protease inhibitors, telaprevir (VX-950) and boceprevir (SCH503034) have recently entered phase III clinical trials. Treatment regimens that include one of these new-generation anti-HCV drugs, referred to as STAT-C (specifically targeted antiviral therapy for HCV) have achieved SVRs up to 65-75% and 50% in treatment-naïve patients and treatment-experienced patients who were nonresponsive to interferon/ribavirin, respectively [113-116]. Future treatment of chronic HCV infection will probably be more effective and shorter, and consist of a combination of peginterferon and ribavirin together with one or more new drugs.

Injecting DU still have limited access to HCV antiviral treatment, as effectiveness of HCV treatment in this population is often questioned [117,118]. Besides the negative impact that ongoing drug use might have on treatment adherence and hence response to HCV therapy, drug users successfully treated for HCV remain at risk for HCV reinfection when they continue to inject drugs [71,74]. Recent studies, however, suggest that when HCV care is integrated with methadone provision in a multidisciplinary setting, drug users can be successfully treated [119,120]. Especially in countries such as the Netherlands, where HCV incidence has drastically declined as a result of reduced injecting risk behaviour and successful harm reduction strategies, the risk of HCV reinfection will be limited [121,122].

**HIV-HCV coinfection**

Due to shared routes of transmission, an estimated 15-30% of HIV infected persons in the Unites States and Europe are coinfected with HCV [123]. Following the introduction of highly active antiretroviral therapy (HAART) for HIV and the consequent reduction in HIV-related morbidity and mortality, HCV has emerged as an increasingly significant problem in this group. HIV/HCV coinfection profoundly influences the natural history of HCV, resulting in
increased hepatic-related morbidity and mortality. HIV/HCV coinfection has been associated with lower rates of spontaneous HCV clearance, higher levels of HCV viremia, and accelerated HCV disease progression [124-126]. Moreover, HCV might induce hepatotoxicity due to certain HIV-inhibitors, causing a forced switch or stop of HAART, or in serious cases hospital admission or death [127]. Persistent viral HCV eradication succeeds in only a minority (30% - 40%) of HIV-infected patients, and less then 20% of patients with high viremia of HCV genotype 1 or 4 [5,6,128]. Also in HIV-positive patients, results of interferon treatment in the acute phase of HCV infection are promising, but the SVR is not as high as observed among HIV-negatives [129,130].

Molecular Epidemiology

HCV genotyping

Molecular epidemiology is a two-sided approach in which the detection and characterisation of the genetic variability of a microorganism is combined with demographic and behavioural characteristics of its host. Due to its extensive genetic variation, HCV is an excellent candidate to be studied using molecular epidemiological techniques [55,64]. As HCV genotype distribution depends on geographic area, mode of transmission and its genetic variation increases over time, it provides information about the historical origin and spread of the virus [65,131]. The degree of genetic diversity (or similarity) among different HCV viral variants provides information on the time that has passed since two viruses separated from a common ancestor, and hence the likelihood that two strains were acquired in the context of the same transmission network. Molecular epidemiology can therefore be used as a tool to identify the (common) source of infection [132-134], to elucidate (new) transmission networks [38,44], to evaluate the impact of prevention measures [135] and when necessary develop new strategies to mitigate further spread of the virus [136]. For example, the degree of diversity among HCV and HIV isolates obtained from children attending a hospital in Bhengazi, Lybia, proved that the date of origin of four separate HIV and HCV outbreaks in this hospital varied between 1985 and 1997 [137]. As the foreign staff of this hospital that were found guilty of deliberately infecting 426 children with HIV only arrived in March 1998, these outbreaks were clearly due to a longstanding infection control problem instead of the arrival of the foreign staff.

Phylogenetic analysis

Dissimilarity between viral variants is often expressed as the genetic distance. The genetic distance is deduced from the percentage nucleotide differences between two sequences, taking into account both the likelihood that such a mutation occurs and the possibility that
more than one event has taken place at the same site in the sequence (multiple hits). The genetic distance among genes of different HCV viral strains can be represented in a phylogenetic tree (Figure 4), comparable to a pedigree showing which genes are most closely related [138]. The genetic distance between any two sequences existing taxa in the tree equals the sum of the lengths of all horizontal tree branches that connect the two existing taxa. The joint origin of two branches are called internal nodes, which are hypothetical taxa representing the common progenitor of the existing taxa that it connects [138]. As the genetic variability of HCV is not uniformly distributed over its genome, the genomic region selected to study transmission networks is crucial. Regions with low genetic variability (5'NTR, core) are very suitable for diagnostic testing, but as genetic diversity is limited they provide little to no information about specific epidemiological links between different viral strains [70]. Highly variable regions, e.g. the hypervariable region of the E2 region, loose their epidemiological information quickly as a result of rapid and continuous genetic modifications [139]. Rapid evolution of the virus, even within one patient, creates a lot of phylogenetic noise that even within short time periods severely disturbs the interpretation of epidemiological signals. The NS5B region which codes for the RNA polymerase, has intermediate variability and appears to have the highest phylogenetic signal among the different genes and can be employed reliably for phylogenetic analysis in the absence of full-genome sequences [139].

**Figure 4:** Schematic example of a rooted phylogenetic tree. Branches connect the external taxa at internal nodes. that represents the most common recent ancestor. Genetic distance between two external taxa can be calculated by summing the horizontal branch lengths that connect both taxa. In this figure the genetic distance between HCV subtype 4a and 4d equals AA’ + BB’ + CC’, the node represents the most recent progenitor of HCV subtypes 4a and 4d. Shaded boxes reflect HCV subtype variation; shaded circles reflect HCV quasispecies variation [138].
Outline of this thesis

The studies in this thesis were performed to improve our understanding of the spread of HCV in the Netherlands, using a molecular epidemiological approach. Based on the genetic variability of the HCV NS5B region as well as demographic and behavioural data of the infected patients, we studied the spread of HCV among Dutch donors, injecting and non-injecting drug users and men who have sex with men.

In the Netherlands little is known about the seroprevalence and spread of HCV in the general population. Chapter 2 describes the prevalence and incidence of HCV among voluntary Dutch blood donors in the period 1997-2002, as well as the residual risk of transmitting HCV through contaminated blood products in the Netherlands. Molecular epidemiological techniques were used to elucidate HCV risk profiles of subjects unaware of their positive HCV status, thereby improving future transfusion policy and blood safety.

Chapter 3 includes three studies on the molecular epidemiology of HCV among drug users in Amsterdam, a population that has been heavily affected by the HCV epidemic. The first study describes trends in HCV prevalence and risk behaviour among young drug users in Amsterdam over time. The second study investigates the spread of HCV and its underlying risk behaviour among drug users that deny injection drug use. The third study looks at the frequency of which HCV reinfection and superinfection occur in actively injecting drug users, in order to evaluate the existence of (partial) protective HCV immunity. All studies were conducted using data from the Amsterdam Cohort Studies among drug users.

Chapter 4 includes two studies that describe the emergence of HCV as a sexually transmitted infection among HIV-positive men who have sex with men (MSM). The first study describes a rise in HCV incidence among HIV-positive but not HIV-negative MSM in Amsterdam, using data from (i) the Amsterdam Cohort Studies among homosexual men and (ii) homosexual men diagnosed with acute HCV in 4 major Amsterdam hospitals, including the STI-clinic. The second study aims to determine whether sexual transmission of HCV among HIV-positive MSM is restricted to small isolated outbreaks, or whether these outbreaks form part of a larger interconnected international transmission network. Through an international collaboration, we collected data from HIV positive MSM diagnosed with acute HCV attending urban HIV treatment centres in England, the Netherlands, France, Germany and Australia.

In the general discussion, chapter 5, main findings concerning the spread of HCV among the different risk groups in the Netherlands are discussed and related to recent literature. Their implications for prevention are discussed and recommendations for future research are presented.
1.5 References


Introduction


Introduction


Chapter 1


[113] www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html. HCV advocate


[115] www.hivandhepatitis.com/hep_c.html. HIV and Hepatitis.com


Introduction


