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# **Study on Hepatitis C virus (HCV) subtypes in Sweden before and after the universal screening of blood donors**

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## Abstract

Since the discovery in 1989 of hepatitis C virus (HCV) as the infectious agent responsible for the vast majority of post-transfusion non-A non-B hepatitis, blood transfusions are no longer a source for HCV transmission in Sweden. Anti-HCV testing was implemented for all blood donations in 1992. Since then intravenous drug use (IDU) has become the major route of transmission in the western world. Six genotypes and more than 80 subtypes of HCV have now been identified world-wide. These genotypes and subtypes are determined by genetic divergences between the HCV strains. Subtypes 1a, 1b, 2b, 2c, and 3a have global spread, while the other subtypes have a more limited geographical distribution.

Little was known on the prevalence of HCV among blood donors and on which genotypes and subtypes of HCV were circulating in Sweden before the testing of all blood donations was implemented. The prevalence of anti-HCV was therefore investigated in sera sent to the Swedish Institute for Infectious Disease Control (SMI) from 412 patients; 241 were sampled between 1970 and 1991 before the universal screening in 1992, while 171 were sampled between 1992 and 2002. The samples derived from 193 (47%) blood donors, (104 sampled before, and 89 after 1992), and from seven other groups of patients. Two groups had suspected known routes of infection, intravenous drug use (IDU) 33 patients and hemodialysis, 16 patients, while it was unknown for the other patients.

Anti-HCV was detected in 120 (29%) samples. The highest frequency was found among IDUs, (91%). Before general screening was implemented, 2.8% of the blood donors were positive for hepatitis C, whereas 28% of those sampled after 1992 were anti-HCV positive. Those latter samples were sent to SMI due to anti-HCV reactivity in a primary test at the blood centre. HCV RNA could be detected by PCR in 56 (47%) of the anti-HCV positive samples, the subtype could be determined by sequencing in 45 (80%) of those. The subtypes found were 1a in 31 %, 1b in 18%, 2b in 22%, and 3a in 27%. One sample was of subtype 2c. There was a tendency of increase of genotype 2 and a decrease in subtype 1a with time. 1a was found in 38% of the samples collected before 1992, while it was only found in 19% of the samples from 1992 or later. On the other hand genotype 2 was found in 17% sera sampled before 1992 and in 37% of the samples collected 1992 or later. It is not known if this genotype has recently been introduced into Sweden. Further analysis on larger series of samples is needed to confirm these preliminary results.

**Key words:** HCV (Hepatitis C Virus), Blood donors; Sweden, NS5B region, sequencing.

**Abbreviations:** HCV= Hepatitis C Virus, IDU = Intravenous Drug User, MSM = Men having Sex with Men, tx = kidney transplanted patients.

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## Introduction

Hepatitis C infection is a global health problem. Estimations indicate that at least 175 million persons are infected worldwide, which is equivalent to a prevalence of 3% <sup>4,7</sup>. The prevalence varies between countries. In Sweden it is estimated that round 0,5% of the population is infected <sup>1</sup>. Blood transfusion was one leading cause for the spread of hepatitis C virus (HCV) in most European countries since World War II. Implementation of an all-volunteer blood donor system (1980), effective virus-inactivation procedure for blood derivatives (1987) and the introduction of first (1990) and second-generation anti-HCV tests for blood donors (1992) drastically reduced transfusion-associated transmission in developed countries <sup>27</sup>.

## Route of transmission

The route of transmission is mainly by parental route as blood/blood products and/or other body fluids. Articles that might be contaminated with blood, e.g. syringes, toothbrushes or razors could transmit the disease <sup>2</sup>. There are high rates of hepatitis C in intravenous drug users (IDU) <sup>3</sup>. The possibility of sexual transmission cannot be eliminated, but the risk seems to be low 2-12% <sup>8, 28</sup>. Vertical transmission from mother to child occurs with a rate of 5-10% <sup>8</sup>. This risk can be lowered by caesarean section <sup>8</sup>.

## Symptoms

Hepatitis C has an incubation period of 2-26 weeks <sup>11</sup>. Almost all patients develop a vigorous antibody and cell-mediated immune response, which fails to eliminate the infection but may contribute to liver damage <sup>8</sup>. In most cases, the infection develops as an asymptomatic clinical picture with severe consequences, since about 75-80% of the cases develop chronic hepatitis <sup>6</sup>. If symptoms occur it include loss of appetite, vomiting, stomach pain, extreme fatigue and a yellowing of the skin and eyes (jaundice) <sup>29</sup>. However, as mentioned, most acute infections go unnoticed with no symptoms. This infection is one of the most ordinary causes of liver cirrhosis and liver cancer (hepatocellular carcinoma, HCC) world-wide. In Sweden it is estimated that 45 000 individuals are chronically infected by HCV. Of those 25-30% are at risk of premature death as a consequence of their liver disease <sup>1</sup>.

## **Therapy**

The HCV infection can be treated more or less successfully depending on infecting genotype. Alpha-interferon and ribavirin in combination are presently the only approved therapy. Alpha interferon is normally induced by the host at virus infections and has a general antiviral effect. Ribavirin affects the nucleotide pools of cells and may work as a mutagen by inducing mutations, and thereby defective viral RNA molecules. This therapy is expensive and has a large number of side effects, but 40-80% of patients, depending on infecting genotype, can be healed. Successful treatment is defined as no detection of HCV-RNA 6 months after termination of the treatment (sustained virological response, SVR). Genotypes 1 and 4 are more difficult to treat than the other genotypes, probably since these genotypes may have lower sensibility for interferon. The therapy with interferon and ribavirin is effective for 40-50% of patients with genotype 1 or 4 at 48 weeks therapy, and for 80% with genotype 2 or 3 with 24 weeks therapy <sup>15</sup>.

## **HCV- life cycle**

The hepatitis C virus must attach to and enter into a cell (hepatocyte) in order to infect liver cell and thereby carrying out its life cycle and reproduction. HCV must complete a few key steps to carry out its life cycle, (figure 1). 1) Hepatitis C uses its envelope proteins on its lipid coat to attach to a receptor site on the surface of the liver cell. 2) The virus penetrates, probably through endocytosis of the host cell. It releases the genomic RNA from the virus particle into the cytoplasm. 3) The RNA translates the polyprotein and replicase complex associated with intracellular membranes are formed. 4) The viral plus-strand RNA is template for synthesis of minus-strand RNA. 5) Production of new plus-strand RNA molecules which in turn can be used for synthesis of new minus strands or as new mRNAs for polyprotein expression as viral genomic RNAs or packaged into progeny virions. 6) Envelope proteins are associated with a membranous well formed by cellular lipid membranes. The viral core particle encompassing genomic viral RNA associated with the envelope proteins on the membranous well, thereby forming a mature virus particle which is secreted using cellular pathways through endoplasmic reticulum (ER). 7) The virus particles are released into the blood stream and may infect new hepatocytes. <sup>16</sup>.

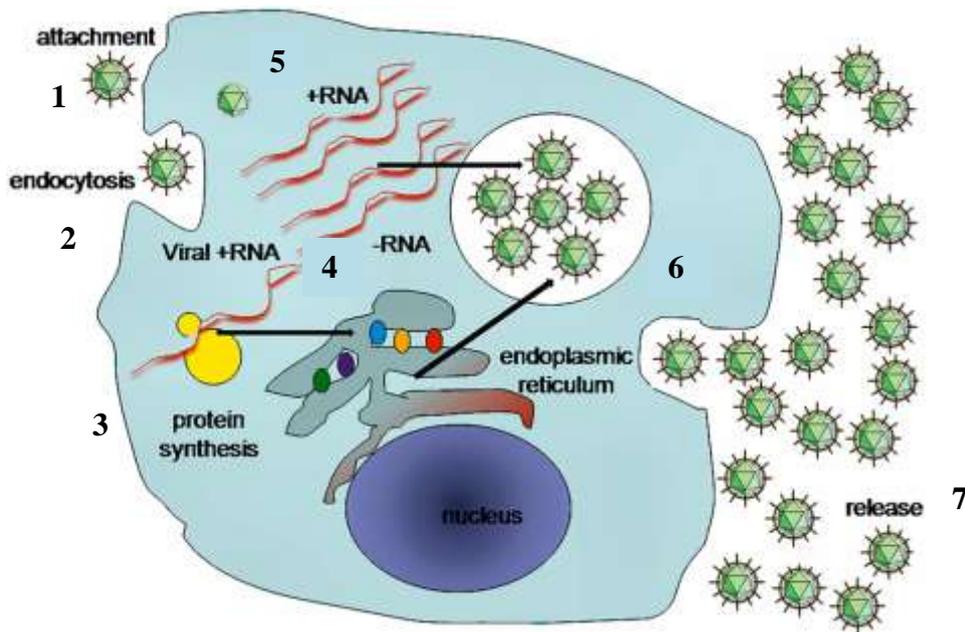


Figure 1. Important key steps of the hepatitis C virus life cycle <sup>11</sup>.

Each surviving virus, those which are not destroyed by the immune system or other environmental factors, can produce a hundred or thousands of offspring when infecting a cell. Over time, this endless cycle of reproduction results in significant damage to the liver, as millions upon millions of cells are destroyed by viral reproduction or by the immune system attacking infected cells <sup>16</sup>.

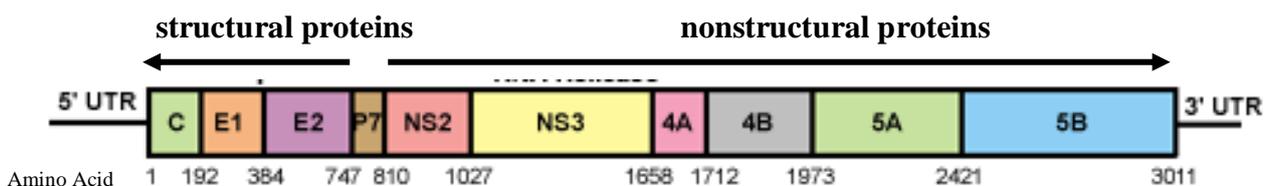


Figure 2. HCV genome consists of a single positive-sense RNA molecule approximately 9.6 kb in length <sup>8</sup>.

### HCV- genome

The HCV genome is a positive stranded RNA consisting of 9 600 nucleotides. It encodes for a single long open reading frame of about 3000 amino acids. The polyprotein is further cleaved into one capsid protein, two envelop glycoproteins and seven non-structural proteins NS2-NS5B, figure 2. The non-structural protein NS5B is a RNA-dependant RNA polymerase (RdRp) essential for viral replication <sup>9</sup>.

Since the RdRp of HCV has a high error rate and no proof reading capacity several mutants are produced during the replication cycles. Thus, an infected individual carries not one unique virus but a whole population of related viruses, termed a quasispecies. The majority of the quasispecies are not viable, but some with no or non-essential amino acid changes may have a higher probability of survival either through higher viability or through escape from the host immune response <sup>5,9</sup>.

### **HCV genotypes & subtypes**

Based on genetic differences of complete genomes, HCV can be subdivided into 6 genotypes. Each genotype being further divided in to subtypes designated with small letters (a, b, c, etc) <sup>1,25</sup>. Some genotypes have a more geographically restricted distribution than other. Subtypes 1a, 1b, 2b, 2c and 3a are distributed worldwide. In West Africa most subtypes of genotype 2 are predominant, and most subtypes of genotypes 3 are found in India. Genotype 4 is mainly found in the Middle East and Northern Africa, Genotype 5 is most common in South Africa and France, while type 6 is restricted to South East Asia (Thailand, Vietnam and Indonesia). In Sweden subtypes 1a and 3a are most common, but 1b, 2b and 2c have also been reported. It is not yet clear whether immunity to one type prevent subsequent infection with another <sup>11,17</sup>.

One larger study on the prevalence of the different HCV subtypes in Sweden has been performed on newly notified cases in the county of Södermanland during 2002 to 2006. In this study there was a decline in the number of reported cases during the years from 130 to 73 new reported cases. There was no significant variation in the subtype distribution. Most strains belonged to subtype 1a, (39%) and 3a, (38%) while 17% were subtype 2b and 6% were 1b <sup>10</sup>.

Little data is available on which prevalent HCV genotypes were circulating in Sweden before 1992 when anti-HCV testing was implemented. We therefore investigated the anti-HCV prevalence in serum samples from blood donors sampled before 1992, and in donors with anti-HCV reactivity in the first generation anti-HCV ELISA at the blood centres. We have also investigated the prevalence of anti-HCV in seven other groups of patients sampled 1970-2002. The HCV strains in anti-HCV positive sera were typed by sequencing the NS5B region to identify if there were differences in subtype distribution during a 30-year period.

**Specific aims were:**

To investigate the prevalence of HCV genotype/subtype in blood donors before 1992 when anti-HCV testing was implemented, by sequencing the NS5B region and compare it with HCV strains from newly identified hepatitis C cases in Sweden.

## Materials & Methods

### Materials

This study was conducted on 412 samples collected in 1970-2002, table I. The serum samples from blood donors sampled after 1992 had shown reactivity against HCV in the first generation anti-HCV ELISA. The other samples were from patients with non-A-non-B hepatitis.

Table. I. Selection of the materials investigated in this study.

Patient group	No. samples before 1992	No. samples after 1992	Total
Blood donors	104	89	193
IDU*	32	1	33
Dialysis patients	16	0	16
Patients at health control	13	16	29
Other	44	1	45
Unknown	29	64	93
MSM**	2	0	2
Kidney transplanted patients	1	0	1
<b>Total</b>	<b>241</b>	<b>171</b>	<b>412</b>

\*Intravenous Drug User \*\* Man having Sex with Man

Each serum sample was at the first thawing aliquotated into two tubes, one was used for serological testing, for HCV RNA detection, typing and the other tube was saved. All the samples were stored at -70°C.

### Methods

#### 1. Serological test:

The 412 samples were analyzed for anti-HCV with an indirect Enzyme-Linked Immunosorbent Assay, ELISA (Monolisa anti-HCV PLUS Version 2, Bio-Rad, Belgium). The assay was performed according to the manufacturer's instructions. It is based on one solid phase and one liquid phase. The solid phase is coated with purified antigen from the non structural region and the structural region of hepatitis C virus. The liquid phase (conjugate) consisting of peroxidase labelled goat-antihuman IgG antibodies.

#### Procedure according to the manufactures protocol:

Serum samples and all kit reagents were room temperatured before performance of the assay. 80 µL of sample diluents (<0.1% sodium azide and 0.01% thimerosol) were added to each microtiterplate well coated with antigens, figure 3a. 20 µL of negative control (human serum

negative for anti-HCV) were added to two wells and 20  $\mu\text{L}$  of positive control (human serum positive for HCV antibodies) to three wells. One well used as blank was left empty. 20  $\mu\text{L}$  of the sample were added to the remaining wells. The plate was incubated for 1 hour  $\pm$  5 minutes at  $37^\circ\text{C} + 1^\circ\text{C}$ . If anti-HCV antibodies were present, they would bind to the antigen in the solid phase, figure 3b. The liquid phase was removed and the wells were rinsed with washing solutions (Tris NaCl buffer, pH 7,4), to remove unbounded proteins from the serum samples. The washing step was repeated twice. The microtiterplate was blotted on absorbent tissue to remove excess liquid from the wells. 100  $\mu\text{L}$  conjugate solution were added to each well. The plate was covered with adhesive seal and incubated for  $30 \pm 5$  minutes at  $37^\circ\text{C} + 1^\circ\text{C}$ . If HCV antibody was present in the sample, it is bound to the antigen on the solid phase, and the enzyme-conjugated antihuman antibody IgG will bind to this complex during the incubation, figure 3c. The washing step was repeated as described earlier. 100  $\mu\text{L}$  of newly prepared peroxidase substrate (citric acid and sodium azide, pH 4.0 with  $\text{H}_2\text{O}_2$  0.015% and DMSO 14%), were added to each well and incubated for  $30 \pm 5$  minutes at room temperature in a dark place, figure 3d. If antibodies are present, they have bound the conjugate labelled with enzyme peroxidase. This enzyme converts hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) into water and oxygen. This leads to a change of the pH and thereby a change in the color since the substrate contains a pH indicator. The reaction was stopped by adding 100  $\mu\text{L}$  stopping solution (sulphuric acid, 1M). The absorbance was read within 4-30 minutes at wavelength 450/620-700 nm using a spectrophotometer. A colour change indicates that the sample contains antibodies against the HCV antigen on the wells. The intensity of the absorbance is proportional to the concentration of the anti-HCV antibodies in the serum sample.



Figure. 3. A schematic picture representing the procedure of ELISA. a. serum is added to the well containing antigens in the solid phase. b. If antibodies are present in the sample it will bind to the antigen. c. Antibody enzyme conjugate detect the antibodies attached to the antigens. d. A colour change based on the peroxidase reaction indicates if the sample contains antibodies.

### 1:1 Interpretation of the results:

To validate the obtained results from the ELISA test, the positive and negative control samples should fulfil the following criteria.

### 1:2 Negative control:

The absorbance of each negative control should be lower than 0,150.

### 1:3 Positive control:

The mean absorbance value of the positive controls should be higher than or equal to 1,000 and lower or equal to 2,400. If the absorbance value of one of the three positive controls differs more than 30% from the mean, the calculation of the mean absorbance should be done with the two remaining control values. If the negative control, and /or more than one positive control, falls outside the mentioned value interval the test should be repeated.

The mean absorbance of the positive controls is determined by dividing the sum of the optical density (OD) for all control values with the number of controls. The threshold value is thereafter calculated as the mean OD of positive controls divided by 5, table II. The presence or absence of anti-HCV antibodies is determined for each sample by comparing the absorbance value of the sample with the threshold value.

Table II. An example of calculation of the threshold value. Samples with an absorbance lower than 0,354 are considered negative in this test.

Sample	Optical Density (OD)	Criteria for acceptance
Negative control	0,130	OD < 0,150
Positive control 1 (D1)	1,862	OD ≥ 1,000
Positive control 2 (E1)	1,587	
Positive control 3 (F1)	1,865	
<b>Total</b>	<b>5,314</b>	
<b>Mean value OD =</b>	<b>Total OD / 3 = 5,314 / 3 = 1,771</b>	
<b>Threshold value Vs =</b>	<b>Mean value / 5 = 1,771 / 5 = 0,354</b>	

The validity criteria were as follows; samples with OD lower than the threshold value are considered negative. A sample with OD higher than or equal to the threshold value is considered positive.

## **2. RNA extraction**

Viral RNA was extracted from 118 samples. 500  $\mu\text{L}$  lysis-buffer (0,5% SDS, 10 M pH 8,0 EDTA and RNase free water) were added to 100  $\mu\text{L}$  serum in 1,5 eppendorf tubes. 100  $\mu\text{L}$  of freshly prepared mix of 500  $\mu\text{L}$  water saturated phenol, 50  $\mu\text{L}$  of 3,0 M NaOAc pH 5.0 and 5  $\mu\text{L}$  beta-mercaptoethanol were added to the tubes. The eppendorf tubes were vortexed for 30 seconds and then centrifuged at 14000 rpm for 10 minutes at 4°C. Two liquid phases are established after the centrifugation step.

The upper aqueous phase was transferred into new a eppendorf tube containing 500  $\mu\text{L}$  isopropanol and 2  $\mu\text{L}$  glycogen. The tubes were mixed carefully and centrifuged for 30 minutes at 4°C to precipitate RNA. The supernatant was discarded and the pellet was washed with 80% EtOH by careful mixing. The tubes were thereafter centrifuged for 1 minute at 4°C. The upper phase was discarded and the pellet was dried briefly at 89-90°C. The RNA pellet was dissolved in 25  $\mu\text{L}$  RNase free water and stored directly at -70°C.

## **3. cDNA synthesis**

The RNA was converted into cDNA with the use of reverse transcriptase. cDNA was synthesized from RNA by addition of a reaction mixture consisting of 3  $\mu\text{L}$  Milli-Q water, 5  $\mu\text{L}$  5' first strand buffer, 2,5  $\mu\text{L}$  DTT 100 mmol/L, 2,5  $\mu\text{L}$  dNTPs 10 mmol/L (dATP, dTTP, dGTP and dCTP), 1  $\mu\text{L}$  random primer, 0,5  $\mu\text{L}$  RNaseOUT, 0,5  $\mu\text{L}$  Superscript II (or III). 15  $\mu\text{L}$  of reaction mixture and 10  $\mu\text{L}$  RNA were added to a final reaction volume of 25  $\mu\text{L}$  in each eppendorf tube. The reaction mixture together with RNA was first allowed to react at room temperature for 15 minutes and incubated at 42°C for 120 minutes. After incubation the samples were stored at -20°C.

### **PCR procedure:**

The DNA template is in this study a cDNA molecule consisting of a region of the fragment that will be amplified. Primers are short DNA fragments containing sequences complementary or identical to the target region on the cDNA. During the PCR the primers bind to the DNA template and are thereby initiating for the DNA polymerase, which synthesizes the new DNA strand. The DNA polymerase uses the dNTPs which stands for deoxynucleotide-triphosphates (dNTPs) to assemble the new strand. There are four types of dNTPs (dATP, dCTP, dGTP, dTTP), one for each nucleotide occurring in the DNA strand.

A PCR is normally performed in three steps, forming a cycle that is repeated between 20 and 40 times. The steps in each cycle are showing in figure 4:

1. Denaturation: This step is usually performed at 95°C about 30 seconds. Due to the high temperature double-stranded DNA melts apart into two single strands.
2. Annealing: The mixture is cooled down to 45-60°C for 20 seconds. This allows the added primers to bind to the complementary regions of the template DNA. The annealing temperature is chosen depending on the sequence of the primers.
3. Extension: The last step is carried out at 72°C for seconds to minutes depending on DNA fragment length given that the Taq polymerase has been used. During this step the DNA polymerase binds to the primer bound to the template DNA, and synthesizes a complementary strand <sup>14</sup>.

#### **4. 1<sup>st</sup> Round PCR (Regular PCR)**

1<sup>st</sup> round PCR was synthesized from cDNA by addition of a reaction mixture consisting of a mix of 5 µL Taq buffer general (or Reaction buffer IV), 1 µL 5 mmol/l dNTP, 0,063 µL of respective internal primers for NS5B (101M and 120) figure 5, 0,2 µL Taq polymerase 5U/µL and 5 µL MgCl<sub>2</sub>. The reaction volume was adjusted with sterile distilled water to 45 µL per sample and 5 µL of cDNA were added to each eppendorf tube and

The first PCR amplifications were performed by an initial cycle of 94°C for 3 min followed by 40 cycles at 94°C for 20 s, 55°C for 20 s and 72°C for 50 s, and a final extension at 72°C for seconds to minutes depending on DNA fragment length.

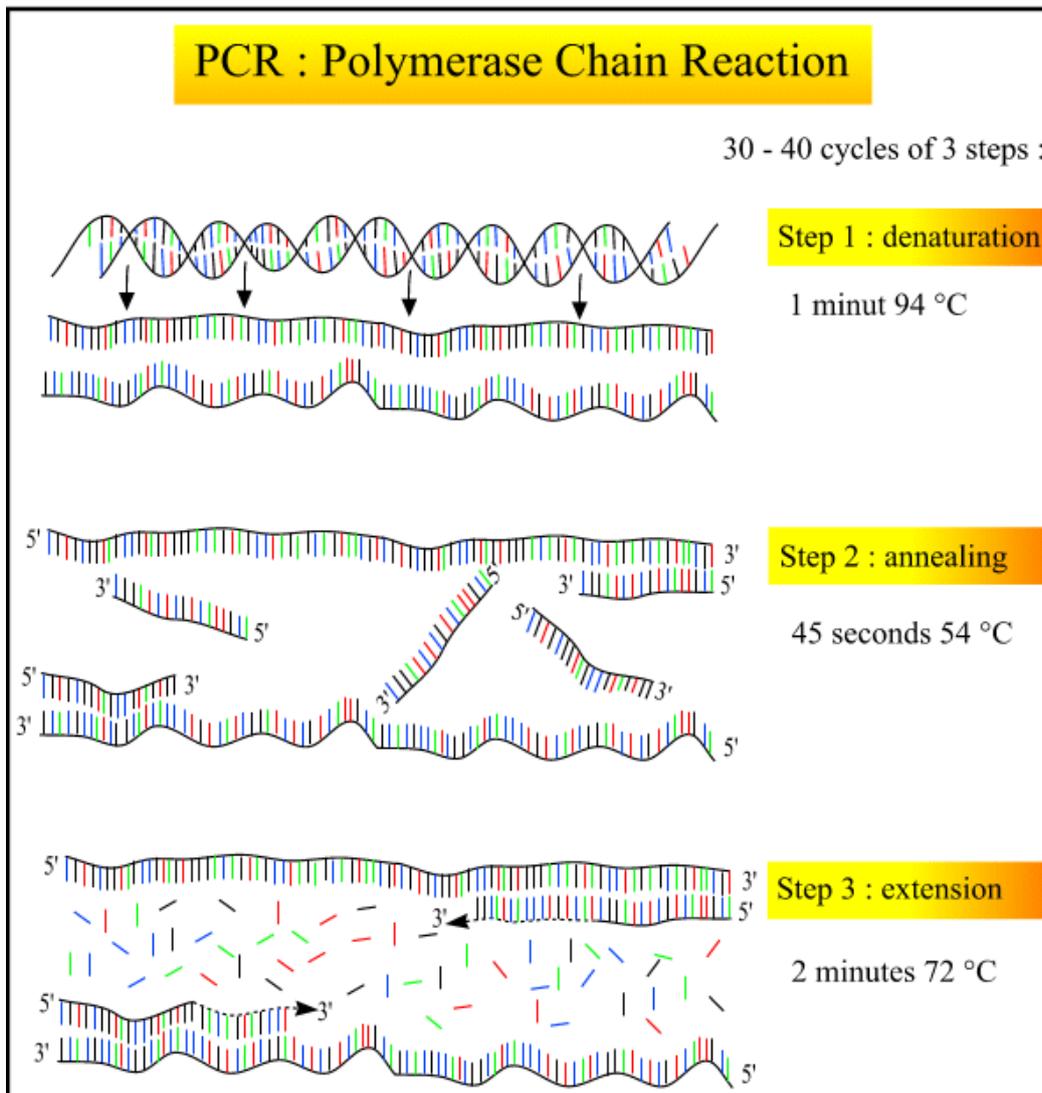


Figure. 4. The different steps in PCR <sup>14</sup>.

## 5. 2<sup>nd</sup> Round PCR (Nested-PCR)

Nested PCR increases the specificity and sensitivity of DNA amplification. The second amplification was performed by addition of a reaction mixture consisting of a mix of 5  $\mu$ L Taq buffer general (or reaction Buffer IV), 1  $\mu$ L 5 mmol/l dNTP, 0,2  $\mu$ L Taq polymerase 5U/ $\mu$ L, 6  $\mu$ L MgCl<sub>2</sub>, 0,063  $\mu$ L of each inner primers (101M and 105) figure 5. The reaction volume was adjusted with sterile distilled water to 47  $\mu$ L per sample and 3  $\mu$ L of the first amplification product were added to each eppendorf tube.

The second PCR amplifications was performed by an initial cycle of 94°C for 3 min followed by 40 cycles at 94°C for 20 s, 55°C for 20 s and 72°C for 50 s, and a final extension at 72°C for 4 min.

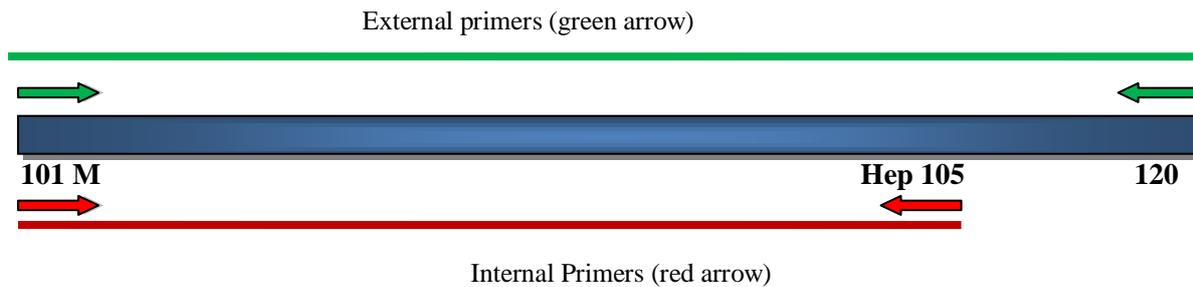


Figure. 5. Displaying the internal and external primers used. Primer 101M was used both as internal and external.

## 6. Analysis of PCR and nested products:

### 6:1 Agarose gel electrophoresis:

The first and nested PCR products were separated on 2% agarose gel. This was done by mixing 48 mL of 2% agarose. 2g agarose per 100 MI 1xTBE was first mixed. The agarose was solubilised by heating in a microwave oven. It was then cooled before pouring the gel in a casting tray, ensuring that no air bubbles were trapped in the gel. After the gel had solidified at room temperature, the gel was carefully moved to the electrophoresis machine containing sufficient amount of 1xTBE buffer. 10 µL one kb ladder was used as a marker of the size of the products and loaded in the first well, figure 9. 11,5 µL of the PCR or nested product were mixed with 2,5 µL 6x loading buffer and loaded on the gel in the respective wells. The voltage was set at 100V for 40 minutes. The movement of the dye in the gel indicated the migration of the DNA from cathode to anode. After 40 minutes the gel was soaked in a GelRed bath for at least of 15 minutes. The DNA fragments were visualized under ultraviolet light and photographed, figure 6.

### 6:2 Quality control

Positive controls were used in cDNA, PCR and nested PCR analysis for ensuring that all reagents and mixtures were correct. A dilution serie of a known HCV-RNA positive sample was used as control of the sensitivity of the PCR. This sample, VHE no 3453/91, was diluted in 1:10, 1:100, 1:1000 and 1:10 000 in negative serum.

Negative control: Water was used as negative control to identity any contaminations. In this reaction mixture no serum was added.

To ensure the accuracy of the cDNA synthesizes, a previously extracted HCV RNA was used as a template. When the PCR was run, a previously cDNA positive sample was used to



liquid were discarded and 500  $\mu\text{L}$  wash buffer were added into the HiBind columns. The tubes were centrifuged for 1 minute at 10 000 rpm. The liquid were discarded and the HiBind DNA columns were centrifuged for 2 minutes at max speed (14000 rpm) to dry the column matrix. The HiBind DNA columns were placed into clean eppendorf tubes and 40  $\mu\text{L}$  elution buffer (10mM Tris, pH 8,5) were added. The eppendorf tubes together with the HiBind DNA columns were centrifuged for 1 minute at max speed 14 000 rpm to elute DNA. The quality of the purified DNA at 1:10 dilution in water was determined by measuring the absorbance, using a spectrophotometer at a wavelength 260-280 nm. Water was used as blank, the absorbance was proportional to the concentration of the purified product. The concentration values of the purified products were between 8-25 ng/ $\mu\text{L}$ .

### **7.2 Sequencing with Big Dye Terminator v3.1 Cycle sequencing kit:**

Cycle sequencing was performed using the Big Dye Terminator v3.1 Cycle sequencing kit. Sanger sequencing or chain termination sequencing with fluorescent nucleotides according to the manufacturers procedure.

**Sanger sequencing** is a useful technique to determine the order of the nucleotides. An oligonucleotide primer is extended by DNA polymerase in the presence of each of the four nucleoside triphosphates and a low concentration of fluorescent labeled dideoxynucleoside triphosphate bases. The DNA polymerase produces a new chain complementary to one strand of the DNA by elongating a primer bound to the template DNA. When the polymerase uses a dideoxynucleotide there is a termination of the chain elongation. The dideoxynucleotide is a chain terminator since it does not have a 3'OH, which is required for subsequent extension of the chain, (figure 7). The ABI machine separates the different elongated and terminated chains. It detects the labeled terminating dideoxynucleotide with laser. Since the four bases are labeled with different fluoresceints which gives different energy, each dideoxynucleotide is detected as different colors <sup>18, 19</sup>. Thereby the nucleotides are detected and represented as 'peaks' of different colors, which can then be interpreted to determine the base sequence, (figure 8).

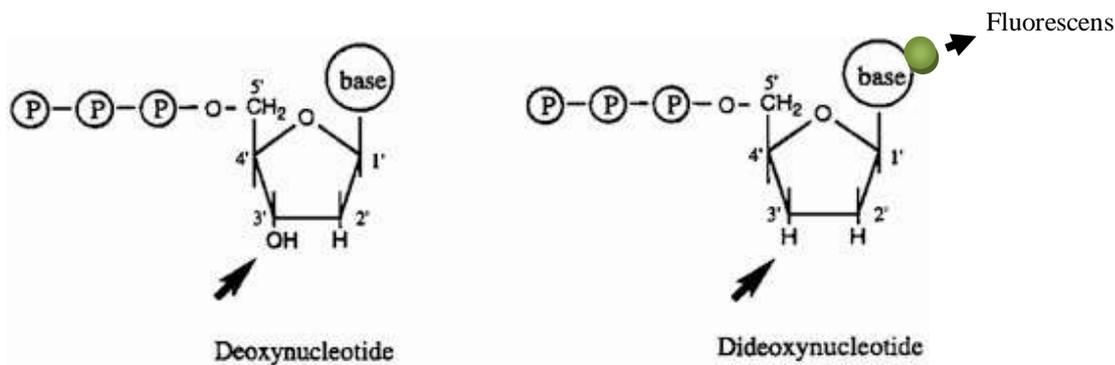


Figure. 7. Structure of a deoxynucleotide and a dideoxynucleotide. The arrows indicate the single difference in the structures of these two molecules <sup>19</sup>.

### 7.3 HCV sequencing with Big Dye:

The sequencing procedure was carried out according to the manufacturer. The primer hep 105 (figure 5) was used as sequencing primer and was diluted 1:20. 10 ng of the amplified PCR product were used in the sequencing reaction. A mixture consisting of 4  $\mu\text{L}$  5x buffer, 1,3  $\mu\text{L}$  terminators mix (consisting of dioxynucleotides and polymerase) and 1  $\mu\text{L}$  of primer were mixed and 6,3  $\mu\text{L}$  of the purified template were added to each PCR tube. The reaction volume was adjusted with sterile distilled water to 20  $\mu\text{L}$  per sample. The PCR programme for sequencing was run for 35 cycles; 95°C, 10''; 50°C, 10''; 60°C, 4'; followed by 4°C hold.

### 7.4 Precipitation of the sequenced product:

The precipitation procedure was carried out according to the manufacturer. 80  $\mu\text{L}$  EtOH were added to each eppendorf tube. 20  $\mu\text{L}$  of the sequenced PCR products were added and mixed carefully by pipette. The eppendorf tubes were left in a dark place at room temperature for at least 15 minutes. The orientations of the tubes were marked to keep track of the invisible precipitate. The tubes were centrifuged for 20 minutes at max speed 14 000 rpm. The supernatant was carefully aspirated. 250  $\mu\text{L}$  70% ethanol were added to the tubes and mixed carefully. The orientation of the tubes were marked and centrifuged for 10 minutes at max speed 14 000 rpm. The supernatant was removed and the pellet was dried at 80°C for a few minutes. 12  $\mu\text{L}$  HiDi were mixed with each sample and the mixture added into an optical 96-well reaction plate (MicroAmp), according to the protocol, (Excel sample sheet, V3-800 base pair).

The sequenced products were separated in an ABI machine, which detected the order of the nucleotides through laser reading of the fluorescent dyes.

### 7.5 Blast search and DNA assembly:

Sequences were analyzed using SeqMan program (DNASTAR) and EditSeq programme. Each sequence was analyzed with visual correction. The height of each signal for each nucleotide was checked, figure 7. Double or triple peaks were occasionally found. The nucleotides at these positions were designated as ambiguities. They ambiguities were designated according to the Nucleotide single letter codes table, table III.

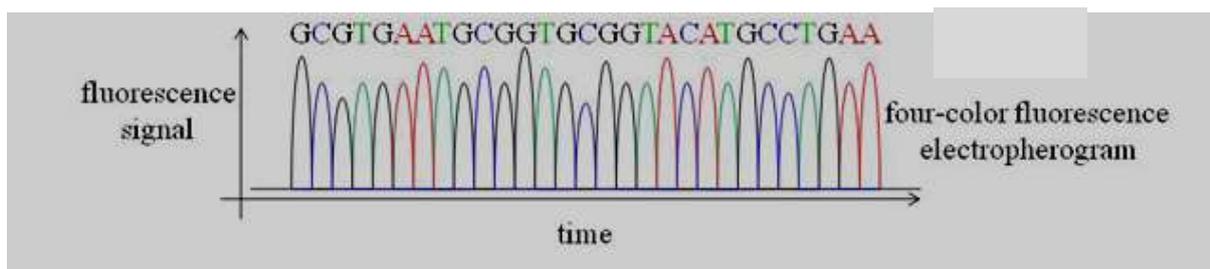


Figure 8. Displaying the peaks of different colours representing different bases A, T, C and G <sup>18</sup>.

Table III. Nucleotide single letter codes.

SLC (Single Letter Code)	Meaning	Origin of Designation
<b>G</b>	G	Guanine
<b>A</b>	A	Adenine
<b>T</b>	T	Thymine
<b>C</b>	C	Cytosine
<b>R</b>	G or A	po <b>R</b> ine
<b>Y</b>	T or C	p <b>Y</b> rimidine
<b>M</b>	A or C	a <b>M</b> ino
<b>K</b>	G or T	<b>K</b> eto
<b>S</b>	G or C	<b>S</b> trong interactions (3 H-bonds)
<b>W</b>	A or T	<b>W</b> weak interactions (2 H-bonds)
<b>H</b>	A or C or T	Not-G, <b>H</b> follows G in the alphabet
<b>B</b>	G or T or C	Not A, <b>B</b> follows A
<b>V</b>	G or C or A	Not-T, (not-U), <b>V</b> follows U
<b>D</b>	G or A or T	Not-C, <b>D</b> follows C
<b>N</b>	G or A or T or C	a <b>N</b> y

The sequence obtained was translated into amino acids in the EditSeq programme. If the sequence reading frame was in anti-sense direction, the sequence was converted into sense direction; thereafter translated into amino acids by translating the nucleotides in triplets, figure 10. There are two amino acid signatures verifying the correct reading frame, TERLY, placed after 40-50 base pair and GDD, placed at the end of the sequence, figure 9.

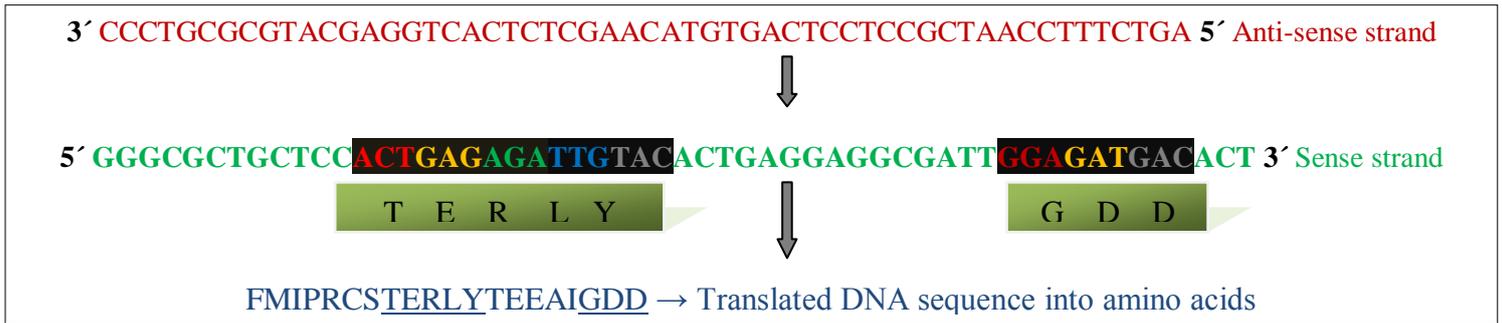


Figure 9. Part of the NS5B region displaying different proofreading's and the translations step from nucleotides to amino acids.

Table IV: Amino acid abbreviations 3 and 1-letter <sup>12</sup>.

Amino acids	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

		Second Letter					
		T	C	A	G		
First Letter	T	TTT } Phe TTC } TTA } Leu TTG }	TCT } TCC } Ser TCA } TCG }	TAT } Tyr TAC } TAA } Stop TAG } Stop	TGT } Cys TGC } TGA } Stop TGG } Trp	T	C
	C	CTT } CTC } Leu CTA } CTG }	CCT } CCC } Pro CCA } CCG }	CAT } His CAC } CAA } Gln CAG }	CGT } CGC } Arg CGA } CGG }	T	C
	A	ATT } ATC } Ile ATA } ATG } Met	ACT } ACC } Thr ACA } ACG }	AAT } Asn AAC } AAA } Lys AAG }	AGT } Ser AGC } AGA } Arg AGG }	T	C
	G	GTT } GTC } Val GTA } GTG }	GCT } GCC } Ala GCA } GCG }	GAT } Asp GAC } GAA } Glu GAG }	GGT } GGC } Gly GGA } GGG }	T	C
						A	G

Figure. 10. The Standard Genetic Code <sup>13</sup>.

Viral genotype and subtypes were identified after analysis of the NS5B sequences obtained (GenBank accession number) using software BLAST for performing sequence alignment. BLAST is a genetic sequence comparison program developed at the National Center for Biotechnology Information (NCBI). It finds regions of similarity between sequences.

## **Results**

The 412 analysed samples were collected during the period 1970-2002. The samples were subdivided into eight different groups, based on patient category and presumed risk factor for HCV infection (Table V). The largest group consisted of blood donors with 193 samples; 104 were sampled before 1992 and 89 after 1992. Thirty-three were IDUs; 32 of them sampled before 1992 and 1 after 1992. All 16 dialysis patients were sampled before 1992. For 29 patients the reason for sampling was elevated transaminases, detected during health control, 13 were sampled before 1992 and 16 after 1992. Two samples derived from men having sex with men (MSM) and one sample was from a kidney transplant patient. There were several reasons for testing another 45 patients; none of which known as a risk factor for hepatitis C, 44 were sampled before 1992 and 1 after 1992. For 93 patients the reason for testing for hepatitis was not given in the patient forms, 29 were sampled before 1992 and 64 after 1992.

### **Detection of anti-HCV**

The prevalence of HCV for all groups independent of year of sampling was 29% (120/412), table V. There was no difference in prevalence between IDUs sampled before or after 1992. In this study there was only one IDU sampled after 1992, but the prevalence of anti-HCV among IDUs is higher than 80% in other Swedish studies from 2002 and onwards. There were however differences in the other groups, although the figures are small. More than half of those sampled due to elevated liver enzymes at health controls before 1992 were positive for anti-HCV compared to only one of 16 (6.2%) among those sampled later. There were opposite results for those sampled for unknown reasons, with 47% anti-HCV positive patients sampled after 1992 compared to 17% sampled before.

Table V. Distribution of numbers of Anti-HCV positive for different groups. N = number of patients.

Group	N	No. Anti-HCV positive before		No. Anti-HCV positive after		TOTAL	
		1992	N	1992	N	Anti-HCV positive	Total tested
Blood donors	104	3 (2.8%)	89	25 (28%)		28 (14%)	193
IDU	32	29 (90%)	1	1		30 (91%)	33
Dialysis	16	7 (44%)	0	0		7 (44%)	16
Health control	13	7 (54%)	16	1 (6,2%)		8 (28%)	29
Other	44	10 (23%)	1	1		11 (24%)	45
Unknown	29	5 (17%)	64	30 (47%)		35 (38%)	93
MSM	2	1 (50%)	0	0		1	2
Transplantation	1	0	0	0		0	1
<b>Total</b>	<b>241</b>	<b>62 (26%)</b>	<b>171</b>	<b>58 (34%)</b>		<b>120 (29%)</b>	<b>412</b>

### Detection of HCV RNA

HCV RNA could be detected by nested PCR of the NS5B region in 56 (47%) of the 120 anti-HCV positive samples. All three anti-HCV positive blood donors sampled before 1992 were HCV RNA positive, while it was detected in 25 (28%) of those sampled after 1992. HCV RNA could also be detected in the majority (80%) of the samples from IDUs, regardless of year of sampling. Half of those detected as HCV infected due to health controls, and 57% of those on dialysis had detectable HCV RNA. The frequency of HCV RNA was lower, 17% and 27% respectively, in the anti-HCV positive patients with unknown or other reasons for sampling.

Table VI. The genotype distributions of different groups and subtypes studied during the time 1970-1991 for samples tested both with anti-HCV and PCR. N= number of patients.

Route of infection	N	No. Elisa positive	No. PCR positive	N typed	Subtypes				
					1a	1b	2b	2c	3a
Blood donors	104	3	3	2	2				
IDU	32	29	24	18	7	1	5		5
Dialysis	16	7	4	2		2			
Health Control	13	7	4	4	1	1			2
Other	44	10	3	0					
Unknown	29	5	1	2	1	1			
MSM	2	1	1	1					1
Transplantation	1			0					
<b>Total</b>	<b>241</b>	<b>62</b>	<b>40*</b>	<b>29</b>	<b>11</b>	<b>5</b>	<b>5</b>	<b>0</b>	<b>8</b>

\* 11 PCR positive samples could not be genotyped

Table VII. Genotype distribution in relation to sampled between groups 1995 and 2002. N= number of patients.

Patient group	N	Anti-HCV positive	HCV RNA positive	N	Subtype				
					1a	1b	2b	2c	3a
<b>Blood donor</b>	89	25	11	11	3	1	2	1	4
<b>IDU</b>	1	1	0	0					
<b>Control</b>	16	1	0	0					
<b>Other</b>	1	1	0	0					
<b>Unknown</b>	64	30	5	5		2	3		
<b>Total</b>	<b>171</b>	<b>58</b>	<b>16</b>	<b>16</b>	<b>3</b>	<b>3</b>	<b>5</b>	<b>1</b>	<b>4</b>

Table VIII. Route of infection and distribution of genotypes of HCV strains isolated from 412 patients, during 1970-2002.

Route of infection	N	%	No. Elisa positive	No. N. PCR positive	Subtype 1a	Subtype				Total genotyped
						1b	2b	2c	3a	
<b>Blood donors</b>	193	46%	28 (14%)	14 (50%)	<b>5</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>4</b>	<b>13</b>
<b>IDU</b>	33	8%	30 (91%)	24 (80%)	<b>7</b>	<b>1</b>	<b>5</b>	<b>0</b>	<b>5</b>	<b>18</b>
<b>Dialysis</b>	17	4%	7 (41%)	4 (57%)	0	<b>2</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>2</b>
<b>Health Control</b>	29	7%	8 (28%)	4 (50%)	<b>1</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>2</b>	<b>4</b>
<b>Other</b>	45	10%	11 (24%)	3 (27%)	0	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>Unknown</b>	93	25%	35 (38%)	6 (17%)	<b>1</b>	<b>3</b>	<b>3</b>	<b>0</b>	<b>0</b>	<b>7</b>
<b>MSM</b>	2	0.4%	1	1	0	<b>0</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>1</b>
<b>Trans-plantation</b>	1	0	0	0						<b>0</b>
<b>Total</b>	<b>412</b>	<b>100%</b>	<b>120 (29%)</b>	<b>56* (47%)</b>	<b>14</b>	<b>8</b>	<b>10</b>	<b>1</b>	<b>12</b>	<b>45</b>

\* 11 PCR positive samples could not be genotyped.

### Genotyping by sequencing the NS5B region

Out of 56 PCR positive products, 45 was be genotyped, while for 11 samples the amount of PCR product was too low, or the sequences were unreadable due to double and/or triple nucleotide signals.

In the whole material genotype 1 was the most common genotype found in 22 samples (49%) followed by genotypes 2 and 3, in 11 and 12 samples respectively. There were 16 (55%) genotype 1 strains in sera sampled before 1992, which was a slightly higher frequency compared to those sampled after 1992 with 49% genotype 1 positive samples. On the other hand there was a tendency of increase of genotype 2 strains, with 17% genotype 2 strains sampled before 1992 compared to 38% in those sampled after 1992.

The most common subtype found among the 14 strains from blood donors was 1a followed by 3a, in 5 and 4 strains, respectively. There were only two sequenced strains sampled from blood donors before 1992. They were both of subtype 1a. Subtypes 1a and 3a, 54% and 50% respectively were dominating among IDU's followed by 2b and 1b, table VIII.

Both genotyped samples from dialysis patients were of subtype 1b. Among those detected as hepatitis C infection through health control two were infected by 3a, one with 1a and one with 1b. Only one strain from a MSM could be genotyped and was of subtype 3a, table VIII.

## Discussion

### Blood donors before 1992:

In this study the prevalence of hepatitis C antibodies was 2.9% in the blood donors collected before 1992, although it was a small number analyzed. Only two of these could be sequenced. They were both of subtype 1a. A prevalence of 2,9% is higher than other studies on the prevalence of hepatitis C in the normal population in Sweden at that time. In Sweden, the prevalence of HCV infection was estimated that 0,2%-0,5% of Swedish blood donors had antibodies in the beginning of the 1990's, when the screening of blood donors was introduced [1]. The prevalence of HCV in southern Europe ranges between 2,5% and 3.5%, but in northern Europe it is now lower and is below 1% [24]. Previous studies have shown a predominance of genotype 1 among Swedish blood donors, which was also confirmed in this study [23]. To confirm our results larger series of samples collected before 1992 is needed

### Blood donors after 1992:

The most common subtype found in the 11 strains from blood donors sampled after 1992 was 3a followed by 1a, in 4 and 3 strains, respectively. These samples were sent to SMI due to anti-HCV reactivity in a primary test at the blood centre. The prevalence of anti-HCV among blood donors was therefore higher (28%) than in samples collected before 1992. The infecting subtypes in these blood donors were in agreement with the distribution of HCV subtypes in Europe from other studies [22].

In 1992, more than 4,000 individuals with a history of former blood transfusion, IDU, elevated liver enzymes, liver disease of unknown cause or a diagnosis of NANB hepatitis were diagnosed with an HCV infection in Sweden and reported to SMI. Most of them had been infected for a long time without knowing it. The annual reporting has since then declined to less than 2,000 notifications per year. However, the number of notifications of younger people, 15-24 years old, has remained the same over the years, indicating that the epidemic has been ongoing mainly among IDUs with the same intensity during the last decade's [15,23].

### **Intravenous Drug Users**

It was noted that IDUs independent of year of sampling had no differences in prevalence of HCV infection. This should be confirmed by studies on larger series of samples. The IDUs had the highest frequency of anti-HCV (91%) among all the groups, before the screening was implemented 1992. This confirms Swedish studies showing that the prevalence of anti-HCV among IDUs is still high, more than 80%. In 1990s; over 90% of Swedish IDU's were anti-HCV which supports the findings in our study <sup>24</sup>. This means that the frequency of anti-HCV among IDUs has not changed over the past 30 years. The HCV subtypes among IDUs independent of year of sampling were 1a 29% followed by 3a 28% and 2b with 21% and a few 1b 4,2%. Our findings support other European studies showing 1a and 3a as the prevalent types among European IDUs <sup>22</sup>. Other studies have also found genotype 2b and 1b to be common among IDUs in Europe and Asia <sup>22, 26</sup>. Drug addiction is an important route of infection in most countries and also in Sweden <sup>23, 24</sup>. Studies have shown that hepatitis C became more prevalent in the 1970s as a result of the increase of IDUs in Sweden.

### **HCV infection detected through health control**

Before 1992, 7 out of 13 (54%) were anti-HCV among those diagnosed through health control. After 1992 only 1 out of 16 (6%) were anti-HCV positive. Four strains of HCV infection from patients detected through health control could be genotyped. Two were infected with subtype 3a, one with 1a and one with 1b. Some of these patients could also have been infected through IDU's since the genotypes among these correspond with the genotypes among IDU's.

### **Unknown route of infection**

The number of patients with unknown source of infection was relatively high, especially after 1992. 30 (47%) out of 64 were positive by anti-HCV, 5 (17%) of those were also HCV RNA positive by PCR. Before 1992 the number of anti-HCV positive patients in this group was 5 (17%) out of 29, one was HCV-RNA positive. Persons in this group could also have been using or only testing intravenous drugs once in their younger years. Since 80% of IDU have hepatitis C, there is a high risk that a person can get infected through IDU even if only tried once.

### **Distributions of HCV genotypes:**

The results obtained in present study shows a tendency of an increase of genotype 2 and a decrease in a subtype 1a with time. This may indicate that genotype 2 has recently been introduced into Sweden. Other European countries as Lithuania, Estonia and St Petersburg, have had a shift in genotypes from 1 to 3a <sup>11</sup>. One larger study on the prevalence of the different HCV subtypes in Sweden has been performed newly notified cases in the county of Södermanland during 2002 to 2006 <sup>10</sup>. In that study there was a decline in the number of reported cases during the years from 130 to 73 new reported cases. There was no significant variation in the subtype distribution. Most strains belonged to subtype 1a, (39%) and 3a, (38%) while 17% were subtype 2b and 6% were 1b <sup>11</sup>. This is in accordance to the genotypes found in the present study with predominance of subtypes 1a and 3a followed by genotype 2.

The prevalence of HCV infection in Sweden is estimated to be round 0, 5%. Of 31 European countries (East and West) reporting HCV infection, the total prevalence appears to be 1.2%. In Western Europe, France has prevalence larger than 1%, the prevalence in Romania is the highest in Eastern Europe at 4.9%. Sweden may have a lower prevalence of HCV than European countries <sup>20</sup>.

### **Anti-HCV in relation to HCV RNA:**

The level of optical density in the anti-HCV test was related to the positivity for HCV RNA by PCR. Most of the PCR positive samples had a strong reactivity by anti-HCV, in contrast to a few samples with weak reactivity by anti-HCV that presented detectable HCV-RNA. The reason to the antibody positive and HCV-RNA negative cases might be that these are low amounts of HCV RNA in the blood in serum plasma or false reactivity in the ELISA test. The virus could also be present in peripheral blood mononuclear cells (PBMC) and not in the serum/plasma. One more explanation could also be that the patients who completed their therapy regime or cleared the infection were still anti-HCV positive even when they were no more PCR positive.

There could be samples from individuals in this study lacking anti-HCV but still HCV-RNA positive. Such results could be due to that the antibody tests are unable to identify subjects in the early stage of infection, in what is known as the diagnostic window period, during which specific antibodies have not yet been produced, but the virus is present in the plasma. This

period prior to seroconversion may last up to 2 months in immunocompetent subjects and as long as 6 to 12 months in immunodeficient patients.

According to some studies, a new test has recently been developed to detect the HCV core protein (HCV antigen [Ag]) in serum, which may be more sensitive than the detection of HCV RNA by PCR. This protein may be an ideal target for the development of methods to detect an HCV Ag and more importantly to identify samples from individuals in the early stage of infection <sup>21</sup>.

### **Study improvements:**

Viral RNA in serum samples is fairly unstable and susceptible to degradation; thus, improper specimen handling can cause false-negative results regarding HCV RNA detection. HCV is relatively unstable to storage at room temperature and repeated freezing and thawing. This means that sera must be tested immediately or frozen as quick as possible and stored at -70°C. Hepatitis C is a notifiable disease by law since 1990.

Inexperience in performing in laborative tasks could influence the test results. Experience in laborating techniques is important since RNA is easily degraded. Practice and carefulness should always be taken on account to give more reliable results.

Although serological and molecular aspects of HCV infection in this study is obtained through testing a small population, this study confirmed which genotypes of HCV were circulating in Sweden after 1992 when anti-HCV testing was implemented. But more samples are needed to be tested to investigate if a shift in genotypes has occurred. It is important to expand this study, which also can be used for contact tracing, to follow changes in subtype distribution, and to identify recombinants or even new subtypes or genotypes.

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