**Diploma Thesis**

# DETERMINATION OF HCV AND HIV-1 RNA IN WHOLE SALIVA SPECIMENS


submitted by

**Jasmin Wagner**

Mat. Nr. 0312774


For receiving an academic Medical Degree

**Doktorin der gesamten Heilkunde / doctor of medicine**

**(Dr. med. univ.)**

at the


**Medical University of Graz**

**Institute of Hygiene**

Universitaetsplatz 4, 8010 Graz

**First supervisor: ao. Univ. – Prof. Dr. Harald H. Kessler**


Graz, June 2007

## CONTENTS

# 1. ABSTRACT

## 1.1. German

***Hintergrund*:** Die Hepatitis C Virus (HCV) RNA und die humanes Immundefizienz Virus Typ 1 (HIV-1) RNA könnten im Speichel vorhanden sein und durch Speichel übertragen werden. ***Methoden*:** Es wurden Misch-Speichel- und Blut-Proben von 40 Patienten mit einer chronischen Hepatitis C Infektion ohne Anti-HCV Therapie und von 16 Patienten mit einer HIV-1 Infektion gesammelt. Das Greiner Bio-One Speichel-Sammelsystem wurde für eine standardisierte Sammlung von Misch-Speichelproben verwendet. Die Bestimmung der Viruslast wurde mit kommerziell erhältlichen molekularen Assays durchgeführt. Die daraus erhaltenen Resultate wurden mit den dazugehörigen Resultaten der Blut-Proben verglichen. ***Resultate*:** Die Serum HCV RNA Konzentrationen reichten von $2.7 \times 10^4$ bis $4.4 \times 10^7$ IU/mL. Von den dazugehörenden Misch-Speichelproben wurden 14 positiv getestet, 13 waren unter der Nachweißgrenze und 13 inhibiert. Die 14 HCV RNA positiven Proben zeigten eine Viruslast von weniger als 15 bis $4.9 \times 10^2$ IU/mL. Die Plasma HIV-1 RNA Konzentrationen reichten von $7.4 \times 10^2$ bis $4.1 \times 10^4$ Kopien/mL. Von den dazugehörenden Misch-Speichelproben wurden 2 positiv getestet, 2 waren unter der Nachweißgrenze und 12 inhibiert. Die 2 positiven HIV-1 RNA Proben wiesen eine Viruslast von $1.9 \times 10^2$ und $9.6 \times 10^3$ Kopien/mL auf. ***Fazit*:** Obwohl die infektiöse Dosis unklar bleibt, kann der Kontakt mit kontaminiertem Speichel für die Übertragung von HCV und HIV-1 verantwortlich sein.

## 1.2. English

***Background:*** Both hepatitis C virus (HCV) RNA and human immunodeficiency virus type 1 (HIV-1) RNA may be present in and transmitted through saliva. ***Methods:*** Whole saliva and blood samples were collected from 40 patients with chronic HCV infection without anti-HCV therapy and from 16 patients with HIV-1 infection. The Greiner Bio-One Saliva Collection System was used for standardized collection of whole saliva samples. Viral loads were determined by commercially available molecular assays. Results obtained from blood samples and corresponding whole saliva samples were compared. ***Results:*** Serum HCV RNA concentrations ranged from $2.7 \times 10^4$ to $4.4 \times 10^7$ IU/mL. Of the corresponding whole saliva samples, 14 samples were tested positive, 13 were found to be ULLD, and 13 showed an inhibition. Of the 14 positives, HCV RNA concentrations in saliva ranged from less than 15 to $4.9 \times 10^2$ IU/mL. Plasma HIV-1 RNA concentrations ranged from $7.4 \times 10^2$ to $4.1 \times 10^4$ copies/mL. Of the corresponding whole saliva samples, 2 were tested positive, 2 were found to be ULLD, and 12 showed an inhibition. Of the 2 positives, HIV-1 RNA concentrations in saliva were found to be $1.9 \times 10^2$ and $9.6 \times 10^3$ copies/mL. ***Conclusion:*** Although the infective dose remains unknown, exposure to contaminated saliva may be responsible for transmission of HCV and HIV-1.

# 2. INTRODUCTION

Today, infections with hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) belong to the most frequent chronic infections all over the world. Viral RNA can be detected by reverse transcription polymerase chain reaction (RT-PCR). Transmission of HCV occurs by direct blood contact, intravenous drug abuse, nosocomial, and poor hygienic conditions. The sexual transmission is rare and about 40% of the routes of transmission are unknown. In contrast, HIV is spread by sexual contact, fresh blood and vertical, especially during delivery and weaning. Recent studies have shown that both HCV-RNA and HIV-1-RNA may also be present in the saliva of infected patients indicating the possibility of transmission via contaminated saliva (Gonçalves *et al*, 2005; Lins *et al*, 2005; Hermida *et al*, 2002; Eirea *et al*, 2005; Pastore *et al*, 2006; Arrieta *et al*, 2001; Maticic *et al*, 2001; Rey *et al*, 2001; Tetsuro *et al*, 2005; Baron *et al*, 2000; Vidmar *et al*, 1996).

Human whole saliva may contain a range of infectious agents and, despite several biochemical and antimicrobial mechanisms, transmission of these can occur. Recent studies refer to the importance of HCV particles in human saliva (Ferreiro *et al*, 2005; Mastromatteo *et al*, 2001). However, the role of transmission via saliva remains unclear and there is presently no idea about the viral infective potential (Ferreiro *et al*, 2005).

The use of whole saliva, a mixture of gingival crevicular fluid and saliva secretions, has been shown to be beneficial in the following situations: for epidemiological purposes, especially when collection of blood is difficult (e.g., infants, intravenous drug abusers, hemophiliacs, obese patients); for field collection of samples in developing countries; in non-clinical settings when samples are obtained by persons with minimal training (Cock De *et al*, 2005).

## 2.1. HEPATITIS C VIRUS (HCV)

Hepatitis C is a viral infection of the liver which was called "non A, non B hepatitis" prior to identification of the causative agent in 1989. The discovery and characterization of the HCV led to the understanding of its primary role in post-transfusion hepatitis and its tendency to induce persistent infection.

### 2.1.1. Morphology

The HCV is a small, enveloped, single-stranded, positive-sense, approximately 9500-nucleotide RNA virus classified as a separate genus (Hepacivirus) within the Flaviviridae family (Figure 1, 2). The genomic organization and sequence of HCV resembles that of the pestiviruses and flaviviruses (http://www.who.int/en/; Fauci et al, 1998).

HCV is one of the viruses (A, B, C, D, and E), which together account for the vast majority of cases of viral hepatitis. HCV mainly replicates within hepatocytes in the liver, although there is clear evidence for replication in lymphocytes and/or monocytes. Circulating HCV particles bind to receptors on the surfaces of hepatocytes and subsequently enter the cells (http://www.who.int/en/; Fauci et al, 1998).

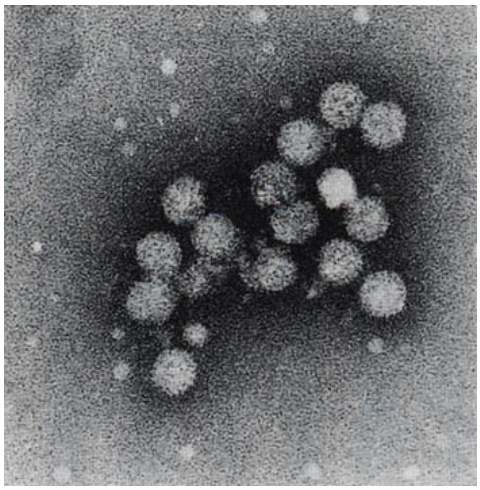

**Figure 1: Electron microscopy picture of the hepatitis C virion**

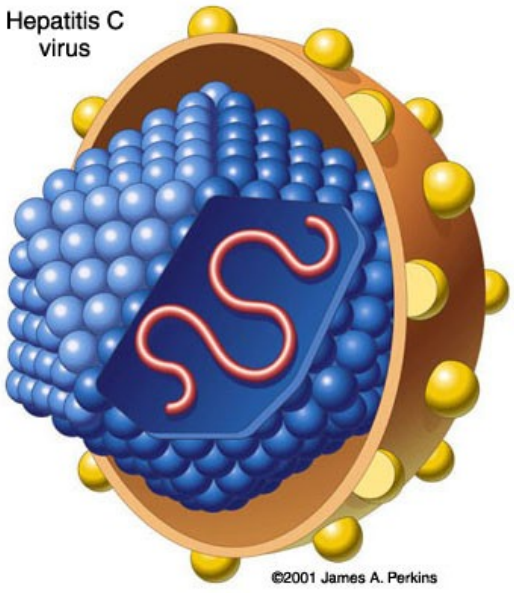


**Fig. 2: Schematic design of HCV**

HCV as a member of the flavivirus family has a high zytopathogenity but the virus specific response of the immune system also leads to massive liver damage. This can be observed by the high expression of MHC-class-I molecules in the infected tissue and the infiltration of the CD8+ cytotoxic T-lymphocytes (http://www.who.int/en/; Fauci *et al*, 1998).

## 2.1.2. Genome and Protein

The HCV genome contains a single large open reading frame (ORF) that codes for a virus polyprotein of approximately 3000 amino acids (Fig. 3). The 5´ end of the genome consists of an untranslated region adjacent to the genes for structural proteins, the nucleocapsid core protein and two envelope glycoproteins, E1 and E2/NS1. The 5´ untranslated region and core gene are highly conserved among genotypes. The envelope glycoprotein's, E1 and E2, are coded for by the hypervariable region, which is responsible for the genetic diversity. It may allow the virus to prevent recognition by antibodies and so it is able to evade host immunologic containment directed at accessible virus-envelop proteins.
The 3´ end of the genome contains the genes for nonstructural (NS) proteins. The NS2 (250 amino acids), NS3 (500 amino acids), and NS4a proteins interact to mediate the processing of the presumed NS region of the polyprotein. NS3 (500 amino acids) is both a proteolytic cleavage enzyme and a helicase, to facilitate unwinding of the viral genome for replication. NS5b is the RNA-dependent RNA polymerase needed for viral replication.

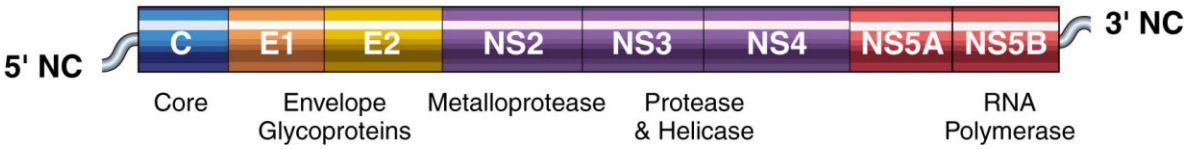


**Fig. 3: Genome of HCV**

At least six distinct genotypes and a number of subtypes have been identified by nucleotide sequencing. Because divergence of HCV isolates within a genotype or subtype and within the same host may vary insufficiently to define a genotype, these intra genotypic differences are referred to as quasispecies. The genotypic

and quasispecies diversity of HCV, resulting from its high mutation rate, interferes with effective humoral immunity. Neutralizing antibodies to HCV have been demonstrated, but they tend to be short-lived and HCV infection has not been shown to induce lasting immunity against reinfection with different virus isolates or even the same virus isolate (Van Soest *et al*, 2006).
HCV is inactivated by exposure to lipid solvents or detergents, heating at 60°C for 10 h or 100°C for 2 min in aqueous solution, formaldehyde (1:2000) at 37°C for 72 h, ß-propriolactone and UV irradiation. HCV RNA is relatively unstable to the storage at room temperature and the repeated freezing and thawing for quantification (http://www.who.int/en/; Kessler *et al*, 2001).

About the HCV-lifecycle, only a few facts are known. It is well established that the envelope glycoprotein (E1 and E2) complex is obviously a candidate ligand for receptors. Several candidates for the receptor have recently been detected. Probably, a receptor complex consisting of CD81- and scavenger receptor class B type I is responsible for binding to HCV. After possible receptor mediated endocytosis, the envelope glycoproteins are thought to mediate fusion. The viral nucleocapsid is thought to be uncoated by unknown mechanism after the release into the cytoplasm. HCV RNA serves as a template and the mRNA is coded for the viral proteins. Polyprotein translation is mediated by binding of the ribosomal 40S subunit to the internal ribosome entry site and of the HCV 5` UTR and the first 30-40 nucleosides to the core-encoding region. The polyprotein precursor is then co- and posttranslational processed by cellular and viral proteases to the mature proteins and later released (Penin *et al*, 2004; Pawlotsky *et al*; 2004).

### 2.1.3. Epidemiology

WHO estimates that about 3% of the world's population has been infected with HCV and that some 170 million are chronic carriers at risk of developing liver cirrhosis and/or hepatocellular carcinoma (Wong *et al*, 2006). These chronic carriers represent a reservoir for HCV to persist. In Austria, up to 80.000 people are infected and about 900 infected patients die of HCV every year (Jonas *et al*, 2004). Some countries have massive HCV problems, especially Egypt. About 15-20% of the population in Egypt is infected. Most patients were infected during

attempts to eradicate endemic bladder schistosomiasis by mass parenteral antischistosomal treatment programs (Frank *et al*, 2000).

## 2.1.4. Transmission

HCV is spread primarily by direct contact with human blood. Transmission through blood transfusions that are not screened for HCV infection, through the reuse of inadequately sterilized needles, syringes or other medical equipment, and through needle-sharing among intravenous drug abusers is well documented (http://www.who.int/en/; Wong *et al*, 2006). Needle stick injuries are also responsible for transmission of pathogens. The estimated risk of transmission through one needle stick injury is 1 - 3% for HCV, 0.3% for HIV and 6-30% for hepatitis B virus (HBV) (http://www.cdc.gov/). Sexual and perinatal transmission may also occur, although less frequently. The vertical transmission risk is estimated at 4 - 7%, and it rises with HIV-1 coinfection and a high maternal HCV viral load (http://www.who.int/en/). Other modes of transmission such as social, cultural and behavioral practices using percutaneous procedures (e.g., piercing, circumcision, tattooing) may occur if inadequately sterilized equipment is used. HCV is usually not spread by sneezing, hugging, coughing, food or water, sharing eating utensils, and casual contact. In both developed and developing countries, high risk groups include intravenous drug abusers, recipients of unscreened blood products, hemophiliacs, dialysis patients, and persons with multiple sex partners who have unprotected sex (http://www.who.int/en/; Fauci *et al*, 1998; Wong *et al*, 2006).

## 2.1.5. Diagnosis

### 2.1.5.1. Clinical Features of Infection

Acute viral hepatitis occurs after an incubation period of 15 - 60 days but the vast majority of acute HCV infections do not show any symptoms (Hof *et al*, 2005). In a few patients, mild flu-like symptoms with little or no jaundice may be observed. For those with symptomatic acute infections, the manifestations are similar to those of hepatitis A virus (HAV) or HBV infection: malaise, vomiting, fatigue, lethargy, anorexia, abdominal pain, myalgia, jaundice, mild hepatosplenomegaly,

maculopapular rash and arthralgia. These symptoms may last for 2-12 weeks. Patients with acute viral hepatitis often report a transient distaste for cigarettes or alcohol or both. Fulminant hepatitis is very rare in the acute infection stage (http://www.who.int/en/; Fauci *et al*, 1998; Wong *et al*, 2006; http://www.cdc.gov/).

A minority of newly infected patients (15 – 50%) is able to clear the infection. In the majority (50 – 85%), the infection gets chronic (Wong *et al*, 2006).

Chronic hepatitis is defined as continuing disease without improvement for at least six months. Chronic infection is often not symptomatic - until evidence of liver failure becomes clinically apparent. Some patients with chronic infection experience malaise, nausea, abdominal pain and pruritus. Fluctuating alanine aminotransferase (ALT) levels are characteristic. In the late stage of chronic HCV infection, the physical examination may reveal signs of liver disease such as spider angiomata, palmar erythema and telangiectasia. Extrahepatic manifestations are uncommon and may include mixed essential cryoglobulinemia, membranous or membranoproliferative glomerulonephritis, non-Hodgkin's lymphoma, Sjögren's syndrome, lichen planus, Wernicke-Korsakoff-Syndrome caused by vitamin B1- deficiency, and porphyria cutanea tarda (http://www.who.int/en/; Fauci *et al*, 1998; Wong *et al*, 2006).

Chronic infection causes chronic inflammation of the liver. Ongoing cycles of inflammation, necrosis, and apoptosis may lead to progressive fibrosis and finally to severe bridging fibrosis with nodular regeneration, cirrhosis. If advanced cirrhosis has developed, jaundice, splenomegaly, ascites, esophageal varices, and hepatic encephalopathy may be observed. The rate of progression to cirrhosis is usually slow, with 20 or more years elapsing between infection and the development of serious complications. Factors that increase the progression rate include sex (men > women), older age at infection acquisition, longer duration of infection, immune suppression (e.g., HIV–HCV co-infection), chronic HBV co-infection, moderate or heavy alcohol use, and obesity (http://www.who.int/en/; Fauci *et al*, 1998; Wong *et al*, 2006). After cirrhosis has developed, the risk of hepatocellular carcinoma is 1 – 4% each year (http://www.who.int/en/).

The differential diagnosis of a symptomatic acute HCV infection includes alcohol or drug abuse, hepatitis A, B, D or E, primary biliary cirrhosis, autoimmune hepatitis, fatty liver, hemochromatosis, Wilson's disease, and α-1 antitrypsin deficiency (http://www.who.int/en/; Fauci *et al*, 1998).

### 2.1.5.2. Laboratory Diagnosis

The biochemical markers are the alanine aminotransferase (ALT), the aspartate aminotransferase (AST), the lactate dehydrogenase (LDH), gamma glutamyle transferase (GGT), alkaline phosphatase (AP), and bilirubin. AST and ALT show a variable increase during the prodromal phase of acute hepatitis and precede a rise of bilirubin. Later a fluctuating ALT level is characteristic for the chronic infection (Fauci *et al*, 1998; http://www.laborlexikon.de/). Another possibility for the staging of fibrosis is the liver biopsy but it is not necessary for the diagnosis of HCV. To establish diagnosis staging is of major importance for assessing the severity of liver disease.

Because of the unspecific nature of the parameters discussed above further tests for detection of the virus are required (http://www.who.int/en/; Fauci *et al*, 1998).

The most practical screening test for the diagnosis of HCV infection is the enzyme linked immunosorbent assay (ELISA) for anti-HCV antibodies, indirect detection of HCV (Fig. 4). Third generation ELISAs detect antibodies to recombinant HCV antigens from the core (C22) and the non-structural regions 3 (C33) and 4 (C-100). ELISAs are easy to perform and the results are highly reproducible. Because of the low specificity of the test, a confirmatory test has to be done following a positive ELISA result (Fig. 4). For this, molecular methods are employed. False positive ELISA results may occur in patients with autoimmune antibodies, paraproteinemia, EBV-infection or other reasons for increased IgG-concentrations (Fauci *et al*, 1998; Wong *et al*, 2006; http://www.laborlexikon.de/; Pawlotsky *et al*, 2002; Lok *et al*, 1997).

The RT-PCR (Fig. 4) is the mostly used direct method to detect HCV RNA in blood (Halfon *et al*, 2006; Cook *et al*, 2006). Serum or EDTA-blood for peripheral blood mononuclear cells (PBMCs) may be used; liver tissue may be an alternative.

Molecular assays are preferred for the confirmation of HCV infection because of their increased sensitivities. With PCR, the diagnostic window can be reduced to 7 - 14 days (Jackson *et al*, 2003). In order to establish duration of anti-HCV therapy, the HCV genotype must be determined prior to start of treatment (Fauci *et al*, 1998; http://www.laborlexikon.de/; Pawlotsky *et al*, 2002).

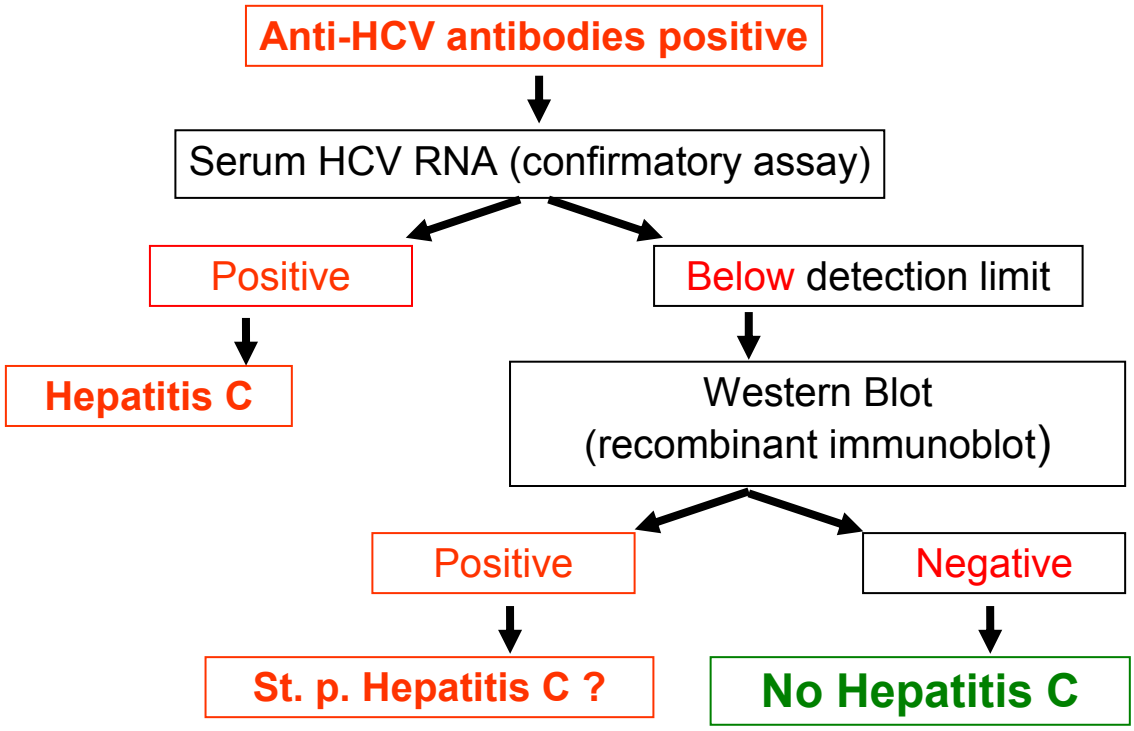


**Fig. 4: Flow chart for diagnosis of HCV**

The recombinant immunoblot assay (RIBA) is a supplemental test that also detects antibodies against HCV antigens (Fig. 4). Multiple HCV antigens are individually displayed on a nitrocellulose strip as bands. The RIBA is considered as positive if at least two reactive bands can be observed (Fauci *et al*, 1998; Pawlotsky *et al*, 2002; Lok *et al*, 1997).

### 2.6.1 Treatment

The current standard therapy uses a combination of weekly subcutaneous pegylated interferon-α (peg-IFN) with daily oral ribavirin. This combination is more effective than monotherapy with peg-IFN and superior to the combination of

standard IFN and ribavirin. This combination clears the virus in about 45 – 80% of patients, depending on viral genotype (Wong *et al*, 2006).

The duration of therapy and the response to the therapy depend on the HCV genotype. Genotype 1 and 4 patients require 48 weeks of therapy for approximately 50% sustained virological response (SVR), while genotype 2 and 3 requires 24 weeks of therapy for 80 - 90% viral elimination. SVR is the primary marker of therapeutic outcome. This is achieved if serum HCV RNA levels are under the lower limit of detection (ULLD) six months after completion of therapy. Rapid virological response (HCV RNA level ULLD) may be achieved within 4 weeks after the initiation of therapy. These patients are also referred to as "super-responders" and may require a shorter duration of therapy.

Peg-IFN is the most frequently used cytokine in treatment with an immunoregulatory and anti-inflammatory function. IFN inhibits the production of viral proteins by destroying the viral RNA and inhibiting the translation. The progression of hepatic disease can be thus inhibited and IFN is currently the only drug that can eradicate HCV. The therapeutic responses to IFN can be classified as SVR, relapse, or nonresponse. SVR means complete elimination of HCV, which is defined as loss of detectable HCV RNA during therapy and its continued absence for at least 6 months after the termination of therapy. Relapse is defined as being ULLD at the end of IFN treatment but HCV-positive within 6 months after the termination of therapy. Nonresponse is defined as the absence of an ULLD condition even with IFN administration (Wong *et al*, 2006).

Revolutionary progress in the development of IFN agents was recorded with the development of peg-IFN and its introduction to clinical use. Pegylation is defined as modification of a drug by the addition of an artificial polymer, polyethylene glycol (peg), for the purpose of delaying drug elimination, lowering its antigenicity, and modifying the drug's effect. The most beneficial effect of peg-IFN is that it delays drug elimination, making it possible to maintain a stable blood concentration with once weekly administration (Wong *et al*, 2006; Hayashi *et al*, 2006).

Studies revealed that the antiviral effect is determined mainly by viral load and the viral genotype. Genotypes 1a and 1b are those most difficult to treat, especially in

patients with a high viral load. The adverse effects include flu-like symptoms, lose of weight, alopecia, thrombopenia and depressions (Fauci *et al*, 1998; Wong *et al*, 2006).

Ribavirin, developed in 1972, is a synthetic nucleic acid analogue with a purine skeleton. It has antiviral activity in vitro to a wide variety of RNA and DNA viruses, and it is orally administered. The adverse effects of ribavirin include hemolytic anemia and potential teratogenicity. Hematopoietic growth factors such as erythropoetin and granulocyte-colony stimulating factor help to reduce therapy-mediated anemia and granulocytopenia while improving patient compliance and thereby leading to better viral clearance. Contraception is absolutely required during administration of ribavirin. Ribavirin is contraindicated for patients with renal failure, because it is excreted by the kidney and cannot be eliminated by dialysis (Fauci *et al*, 1998; Wong *et al*, 2006; Dixit *et al*, 2006).
Important for the treatment of HCV is also vaccination against HAV and HBV to avoid co-infection, which may lead to more progressive liver damage (Fauci *et al*, 1998).

### 2.1.7. Perspectives

Promising agents for future anti-HCV therapies are classified as HCV-specific inhibitors targeting its protease and polymerase activities, IFN inducers, or less toxic ribavirin-like agents. A number of drugs are in preclinical or clinical trials (Hayashi *et al*, 2006).

A new and promising therapy, currently in clinical development, is VX-950 and peg-interferon. VX-950 is a NS3/NS4a protease inhibitor. This combination has shown better results in reducing HCV RNA viral load and HCV RNA “negativity” than the standard combination therapy peg-interferon and ribavirin. VX-950 has also shown to improve the sensitivity for interferon. Mutations on the binding site have been observed but viruses show a low replication-rate and disappear as soon as the therapy is stopped. In June 2006, the phase II study was started in the USA and Europe to investigate new combination therapies and their duration.

Parallel, the phase II b study was started with non-responders (Davis *et al*, 2006).

New targets for the HCV-therapy are the toll-like-receptors (TLRs). Especially TLR9 seems to be the best target and agonists have been produced until now, e.g. Actilon. Actilon is a synthetic oligonucleotide and a selective TLR9-agonist which enhances the ability of dendritic cells to activate killer T-cells against the virus (Fig. 5). It is a stimulator of the innate and adaptive immune system. Actually the drug is in phase II (http://www.coleypharma.com/) .

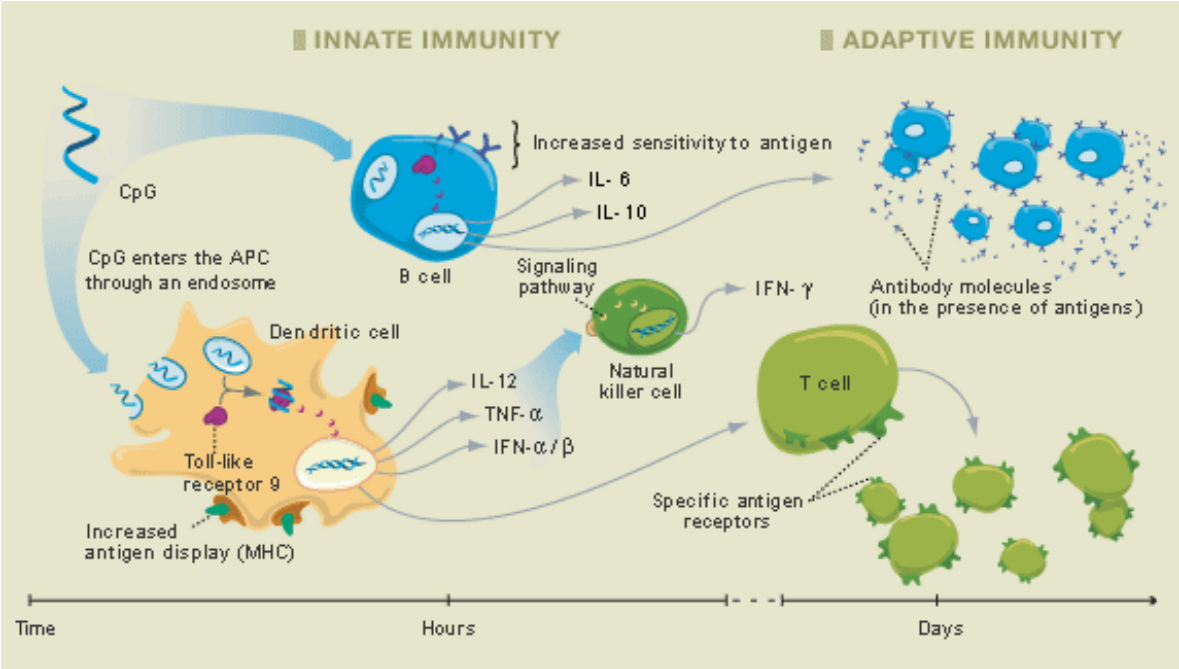


**Fig. 5: Mechanism of Actilon**

Recently, a new T-cell HCV genetic vaccine capable of protecting chimpanzees from acute hepatitis induced by challenge with heterologous virus was described. Suppression of acute viremia in vaccinated chimpanzees occurred as a result of massive expansion of peripheral and intrahepatic HCV-specific CD8 T-lymphocytes that cross-reacted with vaccine and virus epitopes. These findings show that it is possible to elicit effective immunity against heterologous HCV strains by stimulating only the cellular arm of the immune system and suggest a path for new immunotherapy against highly variable human pathogens like HCV, HIV or malaria, which can evade humoral responses. But until now no vaccines are available (Folgori *et al*, 2006).

## 2.2. Human Immunodeficiency Virus (HIV)

The first cases of AIDS (Acquired Immunodeficiency Syndrome) were described in 1981, when the Center of Disease Control and Prevention reported a cluster of *Pneumocystis carinii* pneumonia in five homosexual men in Los Angeles. The disease was originally named GRID, Gay-Related Immune Deficiency. Three years later, 1984, Luc Montagnier and Robert Gallo isolated the responsible retrovirus referred to as HIV.

In the last 25 years, AIDS killed more than 25 million people making it to one of the most destructive epidemics in history. Despite recent improvements in antiretroviral treatment and care in many regions of the world, the AIDS epidemic claimed 3.1 million lives in 2005 and a curable treatment is not yet available.

### 2.2.1. Morphology

HIV type 1 (HIV-1) and HIV type 2 (HIV-2) are members of the retrovirus family and the subfamily lentivirus. It is the cause of the symptom-complex AIDS.

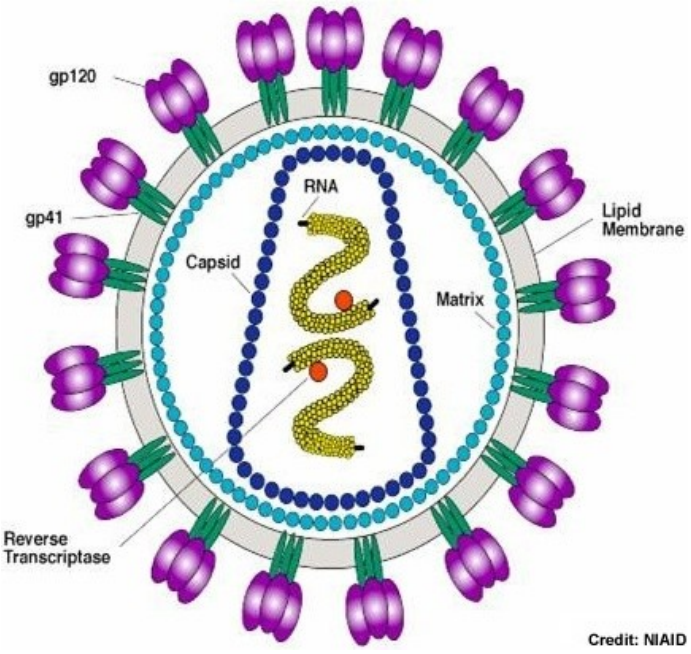


**Fig. 6: Schematic design of HIV-1**

The HIV virion is an icosahedra structure consisting of a glycoprotein gp120 outer membrane, gp41 trans-membrane components of the envelope, p18 inner membrane, p24 core protein, the RNA and the enzyme reverse transcriptase, integrase and protease (Fig. 6). The RNA is single-stranded, positive sense and with a genome size less than 10 kb (Fauci *et al*, 1998).

### 2.2.2. Genome and Replication

Like other retroviruses, HIV-1 has genes that encode the proteins of the virus (Fig. 7): *gag* encodes the proteins that form the core of the virion (including p24), *pol* encodes the proteins responsible for reverse transcription and integration, and *env* encodes the envelope glycoproteins. HIV-1 contains at least six other genes (*tat, rev, nef, vif, vpr, vpu*), which code for proteins involved in the regulation of gene expression. Long terminal repeats (LTR) flank those genes containing regulatory elements involved in gene expression, such as the TATA promotor sequence (Fauci *et al*, 1998; Simon *et al*, 2006).

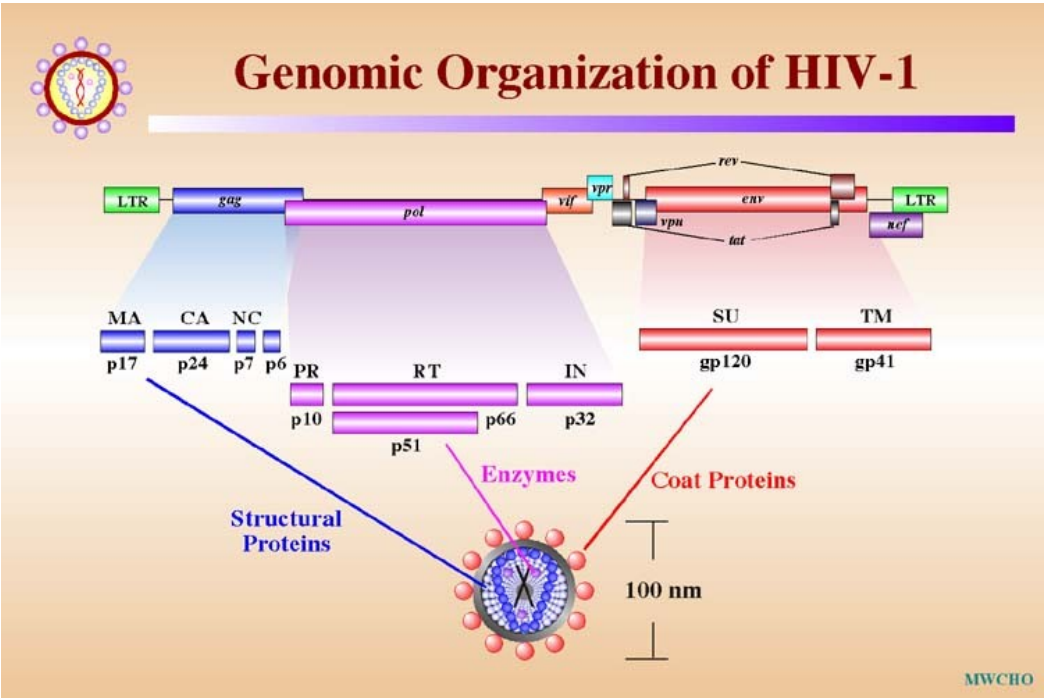


**Fig. 7: Genomic organisation of HIV-1**

Based on their genome, HIV-1 viruses are divided into three groups; the M, N, and O groups. These groups probably result from distinct cross-species transmission

events. Pandemic HIV-1 has diversified into at least nine subtypes and many circulating recombinant types within the M group. Subtype C viruses continue to dominate and account for 55 - 60% of all HIV-1 infections worldwide (Simon *et al*, 2006). Infections with two or more genetically distinct viruses may lead to new recombinant viruses. This diversity is also an immense challenge for the development of any preventive or therapeutic intervention. Emerging evidence suggests that clinical progression to AIDS might be more rapid in individuals with dual infection (Simon *et al*, 2006).

The HIV-1 life cycle (Fig. 8) is complex and its duration depends on target cell type and cell activation. In the first step, the virus enters the cell without causing any lethal damage but this process may stimulate intracellular signal cascades, which might facilitate viral replication. The two molecules gp120 and gp41 are forming the spikes on the vision's surface. During the entry process, gp120 takes contact with the cell membrane, binding to the CD4+ receptor on the surface of T helper-cells and macrophages. The actual fusion event takes place within minutes by pore formation to release the viral core into the cell cytoplasm. The viral genome is reversed transcribed into DNA by the virus own reverse transcriptase enzyme. During this step, mutations can easily appear.

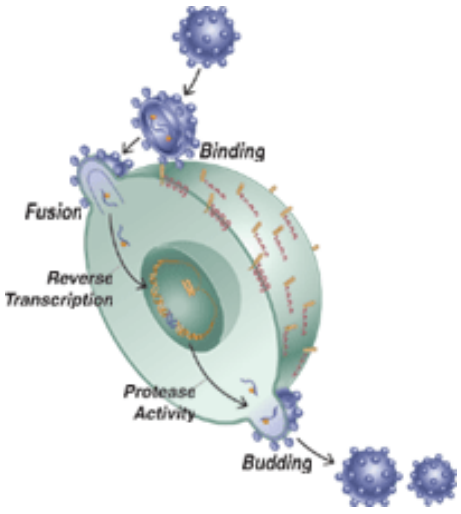


**Fig. 8: Lifecycle of HIV-1**

At the midpoint of infection, the viral protein integrase in conjunction with the host DNA repair enzymes inserts the viral double stranded genome into gene-rich, transcriptional active domains of the hosts DNA, transforming the cell into a potential virus producer. This is called the latent stage of HIV infection. To actively produce virus, different transcription factors have to be present. The most important one is the nuclear factor NF-B, which is up-regulated when the T cells become activated. The produced viral particles leave the cell via vesicular (Fauci *et al*, 1998; Simon *et al*, 2006).

The cytoplasmatic molecules of the producer cell and components from its cell surface lipid bilayer are incorporated into the new viral particle; virions bear characteristics of the producer cell. After the production of the virus, the CD4+-cells are attacked from the host immune system. Other non-infected cells are also killed, which leads to CD4+ T cell deficiency. The change of the cytokine concentration leads to a reduction of T helper cells and cytotoxic T cells. Now the host is exposed to opportunistic infections and special tumors, especially the Kaposi-sarcoma and lymphomas (Fauci *et al*, 1998; Simon *et al*, 2006).

### 2.2.3. Epidemiology

In 2005, about 40.3 million were estimated to be infected with HIV all over the world, Joint United Nations Programme. Every year about 4.9 million people get newly infected even though organizations all over the world have running prevention programs. In 2005, a total number of 3.1 million AIDS deaths occurred.

### 2.2.4. Transmission

The majority of HIV infections are acquired through unprotected sexual contact, homo- or heterosexual. Sexual transmission occurs when there is contact between sexual secretions of one partner with the rectal, genital or oral mucous membrane of the other partner. Infectious fluids include semen, vaginal fluid, and whole saliva.

The second important way of transmission is the direct contact with blood and blood products. This transmission route is especially important for intravenous drug abusers, hemophiliacs and recipients of blood and blood products. It is also important for patients and health care workers in countries with poor hygienic standard, especially those with reuse of injection equipment. People, who receive tattoos, piercing or scarification procedures, are also in danger.
Another important way is the mother to child transmission, the vertical route. Transmission may occur *in utero* and at childbirth. The risk of transmission can be reduced with adequate treatment to decrease the viral load and Caesarean section. The virus has also been found in breast milk.
HIV has also been detected at low concentrations in whole saliva, tears and urine of infected individuals but concentrations are negligible (Fauci *et al*, 1998).

## 2.2.5. Diagnosis

### 2.2.5.1. Clinical Manifestations

The clinical manifestations of the HIV infection are divided in different stages (Fig. 9).

The acute infection, 2 – 6 weeks after infection, is often asymptomatic or related to unspecific mononucleosis-similar symptoms. This stage is also called seroconversion-illness. Clinical manifestations that have been observed include fever, pharyngitis, lymphadenopathy, headache, arthralgia, myalgia, malaise, lethargy, nausea, vomiting, neurologic, and dermatologic symptoms. Usually they persist one to several weeks and gradually subside as an immune response to HIV develops and the levels of plasma viremia decrease (Fauci *et al*, 1998; Hof *et al*, 2005).

The second stage is the clinical latency, the sub-clinical persistence. The median time of this stage is approximately 10 years. Even if there are no symptoms, viral replication continues. The rate of disease progression is directly correlated with the HIV RNA level (Fauci *et al*, 1998; Hof *et al*, 2005).

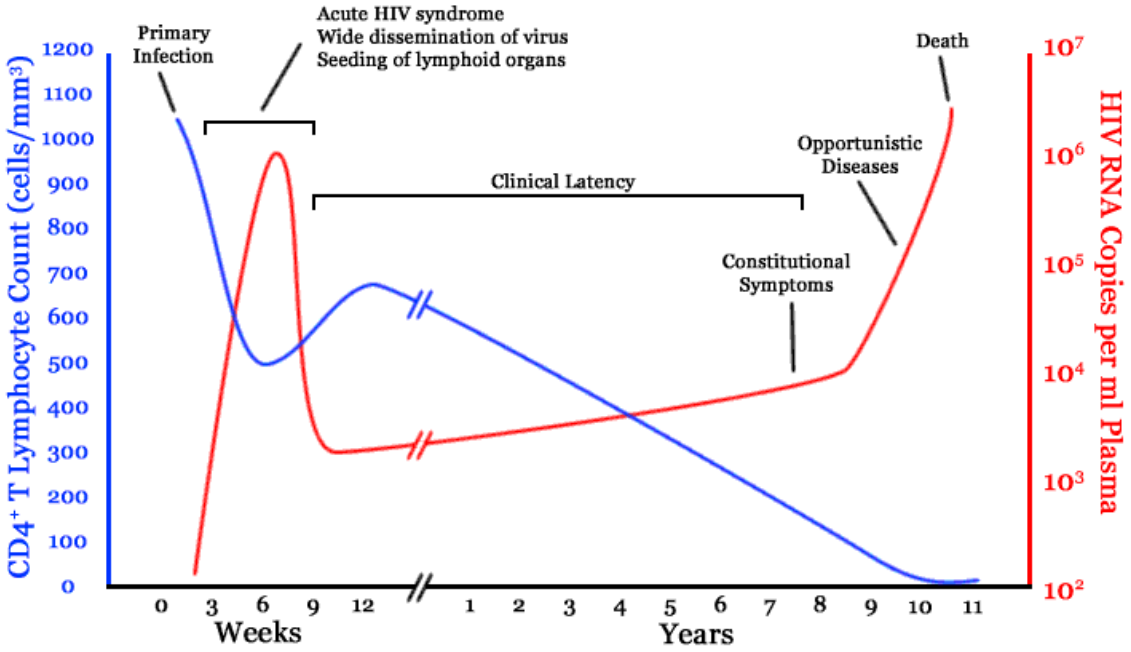


**Fig. 9: Progression of HIV-1 infection**

The third stage is called persistent generalized lymphadenopathy and is characterized by swelling of one or more lymph nodes for several months (Fauci *et al*, 1998; Hof *et al*, 2005).

The fourth stage is called early symptomatic disease or AIDS related complex. The viral load is continuously increasing and at a certain point, usually after the CD4+ T cell count has fallen under 500/µL, patients begin to develop symptoms including generalized lymphadenopathy, oral lesions (Candida infection, oral hairy leukoplakia due to Epstein-Barr virus), reactivation of herpes zoster, thrombocytopenia, peripheral neuropathy, and/or tuberculosis (Fauci *et al*, 1998; Hof *et al*, 2005).

The last stage is called AIDS and characterized by opportunistic infections and malignancies. Opportunistic infections may be caused by viral, bacterial and fungal pathogens as for example *pneumocystis carinii* pneumonia, cytomegalovirus infection (retinitis, hepatitis, pneumonia, encephalitis), herpes virus infection, toxoplasmosis encephalitis, and esophageal and pulmonal candida infection. Encephalopathies are leading to dementia. AIDS-associated malignancies include

the Kaposi sarcoma, which is due to human herpes virus 8, and lymphomas (Fauci *et al*, 1998; Hof *et al*, 2005).

#### 2.2.5.2. Laboratory Diagnosis

HIV tests are able to detect antibodies, antigens or HIV-1 RNA in serum, plasma, whole saliva, dried blood spots, and urine. They are important for the primary diagnosis and monitoring of treatment.
The diagnostic window for antibody tests is 22 days, for antigens 16 days and for molecular assays 7-14 days (Fauci *et al*, 1998; Jackson *et al*, 2003).

The standard screening test for HIV is the ELISA, with a sensitivity of over 99.5%. Results are reported as positive (highly reactive), negative (non-reactive), and indeterminate. While the ELISA is extremely sensitive, it is not ideal with regard to specificity. False-positive ELISA tests may be associated with antibodies to class II antigens, autoantibodies (e. g. systemic lupus erythematodes), antibodies directed to other infectious agents, and the rheumatoid factor. A confirmatory test is thus needed by a positive or indeterminate ELISA test result. Moreover, the serologic window period, must be taken into consideration with regard to anti-HIV antibody testing. During this window period the infected person may transmit to others (Fauci *et al*, 1998).

The most commonly used confirmatory test is the western blot. Today, recombinant western blot assays are usually employed. The western blot is considered to be positive if at least two bands (to p24, gp41, and gp120/160) are visible (Fauci *et al*, 1998).

The p24 antigen test detects the presence of the p24 protein of HIV, a major core protein. Monoclonal antibodies specific to p24 are mixed with the persons blood. Presence of p24 is detected with an ELISA (Fauci *et al*, 1998).

With RT-PCR, a target sequence of a highly conserved region of the HIV-1 genome is detected. The viral genome is extracted from the patient's plasma. Today, conventional PCR has been replaced by real time PCR in the routine

diagnostic laboratory (Drosten *et al*, 2006; Perrin *et al*, 2006; Swanson *et al*, 2006). The lower limit of detection is 40 copies/mL. The amount of HIV-1 RNA in plasma is correlated to viral replication. Today, it is employed as standard assay for monitoring of antiviral therapy (Fauci *et al*, 1998).

The close relationship between clinical manifestations of HIV and CD4+ T cell count has made the determination of the CD4+ T cell level to a routine method for the evaluation of HIV infected individuals and gives information about the prognosis (Fauci *et al*, 1998). Patients with an initial diagnosis of HIV infection should have CD4+ T cell measurement performed every 6 months. With the CD4+ T cell count below 500/µL, an antiviral therapy is indicated and patients with a declining CD4+ T cell count should receive antiretroviral drugs. Patients with CD4+ T cell counts below 200/µL are at high risk of infection with pneumocystis carinii pneumonia, while patients with CD4+ T cell counts below 100/µL are at high risk of infection with the cytomegalovirus and the mycobacterium avium-intracellulare (Fauci *et al*, 1998).

### 2.2.6. Treatment

The cornerstone of medical management of HIV infection is antiretroviral therapy. Suppression of the HIV replication is an important component in prolonging life as well as improving the quality of life of HIV infected patients. Also important is the treatment of the opportunistic infections. The agents have to comply with certain requirements: good activity against the virus, minimal toxicity (because they have to be applied lifelong), oral intake, good penetration into the central nervous system, and reduction of the viral load. Presently, three main drug groups are available: nucleoside reverse transcriptase inhibitors (NRTIs), non nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) (Fauci *et al*, 1998).

Agents of the NRTIs include zidovudine, stavudine, didanosine, abacavir, tenofovir, lamivudine and emtricitabine. NRTIs are nucleoside analogues that act as DNA chain terminators because of their inability to form a 3`-5` phosphodiester

linkage with another nucleoside. The substances are applied orally. Major side effects include leucopenia, periphery neuropathy and pancreatitis (Fauci *et al*, 1998).

The group of the NNRTIs consists of nevirapine, efavirenz, and delavirdine. They are active inhibitors of reverse transcriptase by binding to regions outside the active side. The effect of NNRTIs is very selective to HIV-1 and a monotherapy leads to a rapid emergence of drug-resistant-mutants. The main side effects are a maculopapular rash and an elevation of the hepatic enzymes (Fauci *et al*, 1998).

The PIs, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, fosamprenavir, lopinavir, ritonavir, tipranavir and atazanavir, are selective to the HIV-1 protease. They inhibit the split of the inactive precursor-proteins and consequently the maturation of the virus. The side effects are an elevation of the hepatic enzymes, redistribution of the body fat and metabolic disturbances. The PI-monotherapy leads to a rapid development of drug-resistant isolates (Fauci *et al*, 1998).

Additionally, a fusion-inhibitor has recently been introduced. The drug is called Enfuvirtide and binds to the viral fusion-proteins to inhibit the necessary conformation-change (Fauci *et al*, 1998).

The standard HIV-therapy is called highly active antiretroviral therapy (HAART). The basic combination consists of two NRTIs and one PI. Different combinations can be given and therapy has to be arranged individually with regard on side effects and compliance. HAART has proven to be the best combination to reduce the viral load, increase CD4+ T cell count, and prolong life (Simon *et al*, 2006). If an HIV infection becomes resistant to HAART, this may be overcome by increasing the dosage or by switching to another antiviral drug.
Even though HAART is a good therapy-combination, it is not possible to cure the disease and HIV may also be transmitted during therapy. This is another reason why it is very important to give the patients detailed education about their illness and how to cope with it (Simon *et al*, 2006).

Alternative drugs including integrase inhibitors and more selective fusion inhibitors are under investigation.

A vaccine to overcome HIV is the biggest hope but the development remains an enormous challenge. There are currently several vaccine candidates in phase I trials and four vaccines in phase I/II, including the promising Merck adenovirus vector vaccine now in phase IIb. This vaccine may stimulate anti-HIV cell mediated immunity. Unfortunately currently only one vaccine has proceeded into a phase III clinical trial (Simon *et al*, 2006).

## 2.3. SALIVA

For diagnostic purposes, whole saliva offers several advantages over serum. It can be collected non-invasively, no collection-training is necessary, and it may be more cost-effective. It may be a good option for large screening programs (Kaufman *et al*, 2002; Crouch *et al*, 2005; Streckfuss *et al*, 2002).

### 2.3.1. Production and Contents

What we commonly call “saliva” is indeed a mixture of several fluids and is therefore correctly called “whole saliva” or “mixed saliva”. Components of whole saliva include the saliva, gingival crevicular fluid (GCF), expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses, fungi, desquamated epithelial cells, other cellular components, and food residues (Kaufman *et al*, 2002).
The saliva is produced in the major salivary glands including parotid, submandibular and sublingual glands and in the minor salivary glands. The gland-specific saliva can be separately collected for the diagnosis of gland-specific diseases as infections and obstructions. The saliva contents water, several anorganic ions ($Na^+$, $K^+$, $Cl^-$, $HCO_3^-$, and $Ca^{2+}$), and proteins (mucins, the enzymes α-amylase and lipase, growth factors, and immunoglobulins). It is alkaline and hypotonic. The saliva is produced in two steps. First, the primary saliva is produced in the acini by secretion. It is isotonic and has a similar electrolyte-constitution as plasma. $Na^+$ and $Cl^-$ are reabsorbed; $K^+$ and bicarbonate are added to create hypotonic saliva in the ductal region of the glands. The average saliva production is 0.5 – 1.5 l per day. Functions of the saliva include the cleaning of the oral cavity by moistening the mucus membranes of the upper GI-tract and supplying enzymes that are needed for digestion (Fauci *et al*, 1998).

Whole saliva contains immunoglobulins (Ig) that originally come from the salivary glands and serum. The dominant immunoglobulin is the secretory IgA, produced in the plasma cells of the salivary glands and it is the most important immune defense mechanism in the whole saliva. In contrast, IgM and IgG are primarily

derived from the serum via GCF and they are present in lower concentrations in the whole saliva. Antibodies against viruses can also be detected in the whole saliva and may be helpful for the diagnosis of infections (Kaufman *et al*, 2002).

Research groups have discovered endogenous inhibitors of HIV-1 in the whole saliva (Shugars *et al*, 1999). That's the explanation for the low risk of transmission via whole saliva. Proposed endogenous inhibitors are the amylase, complement system, cystatins, defensins, histatins, lactoferrins, lysozymes, proline rich peptides, salivary mucins, secretory leucocyte protease inhibitor (SLPI), statherin, thrombospondin, and virus-specific antibodies (Shugars *et al*, 1999). SLPI is a 12 kDa mucosal protein that blocks the HIV infection in several cell-culture systems. Similar concentrations of this inhibitor have been observed in infected and uninfected individuals. The mechanism of inhibition has not been identified but its role is in the early stage of infection where SLPI may be present during viral exposure. In the stage after viral inoculation, no further inhibitory effect has been observed (Shugars *et al*, 1999).

### 2.3.2. Collection Devices

Whole saliva can be collected with or without stimulation. Stimulated whole saliva is collected by masticatory action or by gustatory, mechanical or olfactory stimulation (Kaufman *et al*, 2002). The flow of whole saliva also depends on hydration, exposure to light, body position, and seasonal and diurnal factors (Kaufman *et al*, 2002).

For the collection of whole saliva, different collection devices are available. Prior to collection, a 30-minutes period without smoking, drinking, eating or brushing teeth has to be met. The easiest way to collect whole saliva is by unstimulated spitting into a sterile tube (Lins *et al*, 2005). For fluid-stimulation, a simple sterile rubber can be used. The donor has to chew the rubber for a specified time, then spitting it into a tube. After centrifugation, the whole saliva is ready for use in the diagnostic laboratory (Gonçalves *et al*, 2005).

The saliva flow can also be stimulated by applying a few drops of 0.1 mol/l citric acid on the anterior dorsal surface of the tongue every minute for three minutes and finally spitting into a tube (Hermida *et al*, 2002).

There are several collection devices commercially available: Oracol (Malvern Medical Development; Cock De *et al*, 2005), OraSure (Epitope; Judd *et al*, 2003), Omni-Sal (Saliva Diagnostic System; Chohan *et al*, 2001; Van Doornum *et al*; 2001), and Salivette (Sarstedt; Van Doornum *et al*, 2001; Judd *et al*, 2003).

## 2.4. Aim of the study

The aim of the study was to determine HCV RNA levels or HIV-1 RNA levels in whole saliva samples collected from patients with chronic hepatitis C without anti-HCV therapy or patients with HIV-1 infection. Results obtained from whole saliva samples were compared with those obtained from blood samples.

# 3. MATERIALS AND METHODS

Whole saliva and blood samples were collected from patients with chronic hepatitis C (*n*=40) or HIV-1 infection (*n*=16). The study was approved by the local ethic committee, Medical University of Graz. All participants gave written informed consent.

## 3.1. Collection of whole saliva samples

Whole saliva samples were collected using the new Greiner Bio-One Saliva Collection System (SCS; Greiner Bio-One, Kremsmünster, Austria). This new whole saliva collection system includes one tube containing 6 mL of rinsing solution, one tube containing 4 mL of saliva extraction solution (SES), one collection beaker, and two 3.5 mL scaled vacuum sample transfer tubes containing sodium azide in crystalline form as preservative (Fig. 10).


**Fig. 10: Greiner Bio-One Saliva Collection System (SCS).**

Intake of food or liquids and smoking was not permitted within 30 min prior to the whole saliva collection. Immediately before whole saliva collection, the oral cavity was cleaned with the rinsing solution. After emptying the oral cavity, it was rinsed with SES for 2 min and the entire content was spitted into the beaker. After this,

the beaker content referred to as ‘whole saliva sample’ (SES plus whole saliva) was transferred into the evacuated sample transfer tubes. Sample transfer tubes containing the whole saliva sample were centrifuged at 2200 x g for 10 min. Samples were stored at room temperature when analyzed within 6 hours, otherwise frozen at -20°C.

## 3.2. Quantitation of saliva in whole saliva samples

The quantity of saliva in whole saliva samples is given in percent by volume (vol.-%). For determination of HCV RNA and HIV-1 RNA concentrations in saliva, the quantity of saliva in whole saliva samples needs to be calculated by means of the Saliva Quantification Kit (SQK; Greiner Bio-One). The SQK includes 5 calibrators containing saliva quantities of 10, 25, 45, 65, and 80 vol.-%, 2 controls containing 30 and 70 vol.-%, 1 vial containing preserved SES (20 mL), and 1 vial containing preserved artificial saliva (20 mL) (Fig.2).


**Fig. 11: Greiner Bio-One Saliva Quantification Kit (SQK).**

The determination of saliva quantity in whole saliva samples is based on the food dye tartrazine included in the SES. With the 5 calibrators included in the kit, a calibration curve was generated using a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Two hundred

µL of sample were taken (system water as cuvette blank value) and the optical density of the internal standard (tartrazine) was determined photometrically at 450 nm.

If the saliva quantity of a whole saliva sample was found to be in-range (between the lowest and the highest calibrator), the value measured was considered as valid. If the saliva quantity of a whole saliva sample was found to be either below the lowest calibrator or above the highest calibrator, the whole saliva sample was either concentrated with preserved artificial saliva or diluted with preserved SES included in the kit and re-analyzed afterwards. The two controls containing saliva quantities of 30 and 70 vol.-% were included in each run.

## 3.3. Collection of blood samples

Blood samples were obtained from HCV-infected patients with 9-mL serum tubes (Vacuette; Greiner Bio-One). Within 2 h of drawing blood, tubes were centrifuged at 1500 x *g* for 20 min at room temperature. After centrifugation, serum aliquots were prepared and immediately frozen at -20°C until tested.

EDTA whole blood samples were obtained from patients with HIV-1 infection using 3.0-mL Vacuette EDTA tubes (Greiner Bio-One). Within 2 h of drawing, tubes were centrifuged at 1500 x *g* for 20 min at room temperature. After centrifugation, plasma aliquots were prepared and immediately frozen at -20°C until tested.

## 3.4. Quantitation of HCV RNA

For quantitation of HCV RNA in whole saliva and serum samples, the commercially available IVD-labeled COBAS AmpliPrep/COBAS TaqMan HCV Test (Roche Molecular Systems, Branchburg, NJ, USA) was used according to the instructions of the manufacturer. This assay is based on automated HCV RNA extraction (1.1 mL input volume) on the COBAS AmpliPrep (Roche Molecular Systems) instrument followed by real-time PCR on the COBAS TaqMan analyzer (Roche Molecular Systems).

The COBAS AmpliPrep instrument lyses virus particles by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer and separates with

magnetic glass particles from other substances like salts, proteins and other cellular impurities. The processed specimen, containing the magnetic glass particles as well as released RNA, is added to the amplification mixture and transferred to the COBAS TaqMan analyzer. Both the target RNA and the Quantitation Standard are reverse transcribed, amplified, and simultaneously detected by evaluating the intensity of emission of the dual labeled fluorescent reporter.

The lower limit of detection of the COBAS AmpliPrep/COBAS TaqMan HCV Test is 15 IU/mL. If the assay gave a positive result below 15 IU/mL, it was referred to as weak-positive.

## 3.5. Quantitation of HIV-1 RNA

For quantitation of HIV-1 RNA in whole saliva and plasma samples, the commercially available IVD-labeled COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche Molecular Systems) was used according to the instructions of the manufacturer. This assay is based on automated HIV-1 RNA extraction (1.1 mL input volume) on the COBAS AmpliPrep (Roche Molecular Systems) instrument followed by real-time PCR on the COBAS TaqMan analyzer (Roche Molecular Systems).

The lower limit of detection of the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test is 40 copies/mL. If the assay gave a positive result below 40 copies/mL, it was referred to as weak-positive.

# 4. RESULTS

## 4.1. HCV RNA in serum and saliva

Serum and whole saliva samples were obtained from 40 patients with chronic HCV infection and without anti-HCV therapy. Samples were tested for HCV RNA with the commercially available molecular assay (lower limit of detection 15 IU/mL). The serum HCV RNA concentration ranged from 2.7 x $10^4$ to 4.4 x $10^7$ IU/mL. Of the corresponding whole saliva samples, 14 samples were found to be positive for HCV RNA, 13 samples were found to be ULLD, and 13 samples showed an inhibition (Table 1a-c). Of the 14 HCV RNA positive saliva samples, the viral load in saliva ranged from weak positive (< 15 IU/mL) to 4.9 x $10^2$ IU/mL (Fig. 12).

| HCV RNA in serum (IU/mL) | HCV RNA in saliva (IU/mL) |
|---|---|
| 32.100.000 | 79 |
| 29.500.000 | 75 |
| 9.850.000 | 75 |
| 9.400.000 | 161 |
| 8.980.000 | 234 |
| 5.090.000 | 137 |
| 4.820.000 | <15 |
| 4.640.000 | 75 |
| 3.710.000 | 27 |
| 3.490.000 | 489 |
| 1.630.000 | 184 |
| 829.000 | <15 |
| 456.000 | 158 |
| 346.000 | 160 |

**Table 1a: Results obtained from 14 patients with HCV RNA in saliva.**

| HCV RNA in serum (IU/mL) | HCV RNA in saliva |
|---|---|
| 21.900.000 | ULLD |
| 11.000.000 | ULLD |
| 8.490.000 | ULLD |
| 7.720.000 | ULLD |
| 7.680.000 | ULLD |
| 5.990.000 | ULLD |
| 3.370.000 | ULLD |
| 1.680.000 | ULLD |
| 1.430.000 | ULLD |
| 829.000 | ULLD |
| 773.000 | ULLD |
| 633.000 | ULLD |
| 582.000 | ULLD |

**Tab. 1b: Results obtained from 13 patients without measurable HCV RNA in saliva; ULLD, under lower limit of detection.**

| HCV RNA in serum (IU/mL) | HCV RNA in whole saliva |
|---|---|
| 44.100.000 | INHIBITED |
| 43.600.000 | INHIBITED |
| 15.000.000 | INHIBITED |
| 8.570.000 | INHIBITED |
| 6.230.000 | INHIBITED |
| 5.770.000 | INHIBITED |
| 4.730.000 | INHIBITED |
| 3.290.000 | INHIBITED |
| 2.840.000 | INHIBITED |
| 709.000 | INHIBITED |
| 322.000 | INHIBITED |
| 252.000 | INHIBITED |
| 26.700 | INHIBITED |

**Tab. 1c: Results obtained from 13 samples of patients with inhibition in saliva.**

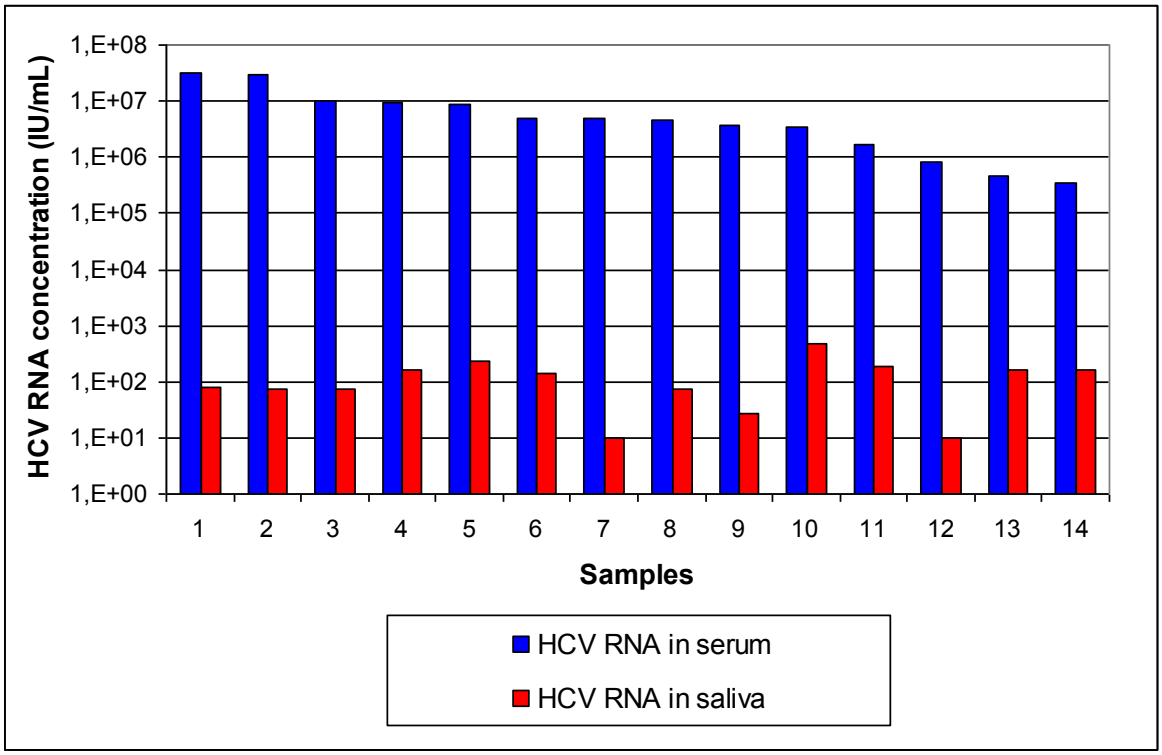


**Fig. 12: Comparison of HCV RNA concentrations in serum and in saliva.**

## 4.2. HIV-1 RNA in EDTA plasma and saliva

A total of 16 EDTA whole blood and whole saliva samples obtained from patients with HIV-1 infection were tested with the commercially available molecular assay (lower limit of detection 40 copies/mL). The plasma HIV-1 RNA concentration ranged from 7.4 x $10^2$ to 4.1 x $10^4$ copies/mL. Of the corresponding whole saliva samples, 2 were found to be positive, 2 ULLD, and 12 showed an inhibition (Tab. 2). Of the two HIV-1 RNA positive samples, the viral load in saliva was found to be 1.9 x $10^2$ and 9.6 x $10^3$ copies/mL, respectively (Fig. 14).

| HIV-1 RNA in plasma (copies/mL) | HIV-1 RNA in saliva (copies/mL) |
|---|---|
| 40.700 | 9620 |
| 33.000 | 190 |

**Tab. 2: Results obtained from 2 patients with HIV-1 RNA in saliva.**

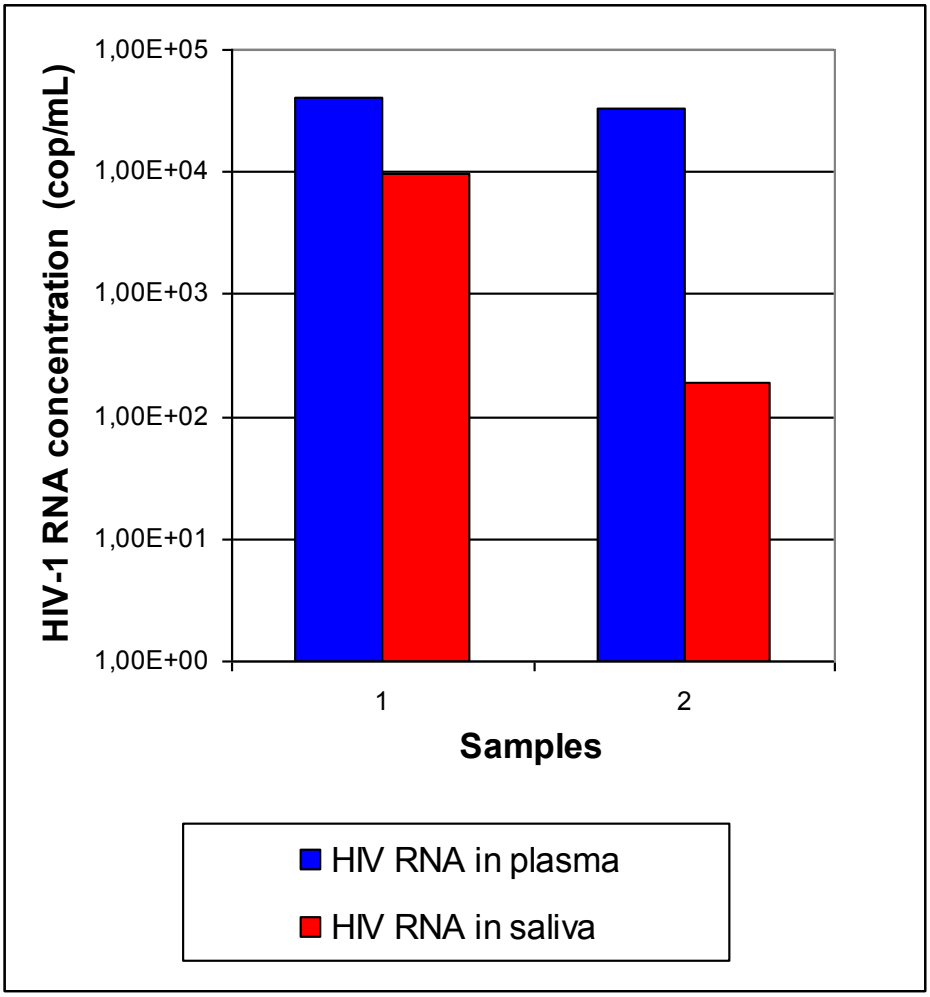


**Fig. 13: Comparison of HIV-1 RNA concentrations in plasma and in saliva.**

# 5. DISCUSSION

Infections with HCV and HIV-1 are among the most frequent chronic infections worldwide. HCV has infected about 3% of the world´s population. It is spread primarily by direct contact with human blood. Transmission occurs through not screened blood transfusions, through the reuse of inadequately sterilized medical equipment, and through needle-sharing among intravenous drug abusers. The vertical route is also a well documented but rare way of transmission (http://www.who.int/en/; Wong *et al*, 2006). However, in about 40% of all patients, the route of transmission is still unknown.
The Joint United Nations Programme-UNAIDS estimated about 40.3 million people infected with HIV all over the world in 2005. Every year about 4.9 million people get newly infected. The majority of HIV infections are acquired through unprotected sexual contact, homo- or heterosexual. The second way of transmission is the direct contact with blood and blood products, especially affecting intravenous drug abusers, hemophiliacs and recipients of blood and blood products. Although medication is available, HIV infection requires lifelong treatment that provides a life prolonging effect but no cure.

The aim of the study was to determine HCV RNA levels or HIV-1 RNA levels in whole saliva samples collected from patients with chronic HCV infection without anti-HCV therapy or patients with HIV-1 infection. Results obtained from whole saliva samples were compared with those obtained from blood samples. In this study, HCV RNA was detected in 14 of 40 (35%) whole saliva samples obtained from patients with chronic hepatitis C without anti-HCV therapy. In contrast, HIV-1 RNA was detected in only 2 of 16 (12.5%) whole saliva samples obtained from patients with HIV-1 infection. Conflicting results have been reported on the presence of HCV RNA in whole saliva (Hermida *et al*, 2001; Gonçalves *et al*, 2005); similarly, the presence of HIV-1 RNA in whole saliva has been found to vary strongly (Melvin *et al*, 1997; Robin *et al*, 2000). Differences in patient selection, in PCR techniques or with handling the whole saliva collected may have contributed to large variations. In this study, whole saliva samples were collected with the Greiner Bio-One SCS, a new standardized and liquid based collection

system. For detection and quantitation of HCV RNA and HIV-1 RNA, commercially available IVD/CE-labeled molecular assays were employed.

Whole saliva from patients with HCV infection may be potentially infectious. It has been postulated that only 10 to 100 viral particles might be required for infection (http://www.rki.de). Nevertheless, the exact infective dose has not been determined yet. Kissing and biting as possible ways of transmission of HCV thus cannot be excluded. Indeed, transmission of HCV by biting has been reported (Dusheiko *et al*, 1990). However, in patients with chronic hepatitis C, HCV-specific IgG and in lesser extent IgA, directed to E1/E2 surface glycoproteins, have been found in saliva and cervicovaginal fluid suggesting minor infectivity of saliva and cervicovaginal secretions (Belec *et al*, 2003).
In this study, HCV RNA concentrations in saliva were found to be significantly lower when compared to those in serum. However, in concordance with other studies, no correlation between viral loads in serum and those in saliva were found (Maticic *et al*, 2001; Roy *et al*, 1998).

Similar to HCV, whole saliva from patients with HIV infection may be infectious. It has been postulated that only 100 viral particles might be required for infection (http://www.rki.de). Nevertheless, the exact infective dose has not been determined yet. Kissing and biting as possible ways of transmission of HIV thus cannot be excluded. Indeed, transmission of HIV-1 by biting has been reported (Vidmar *et al*, 1996).
In this study, HIV-1 RNA concentrations in saliva were found to be significantly lower when compared to those in plasma. These results are in concordance with those reported previously (Luizzi *et al*, 1996; Shugars *et al*, 1998).

The major problem observed in this study was the high number of inhibitions occurring in whole saliva samples. Due to inhibition the determination of the RNA concentration failed in 13 of 40 samples obtained from patients with chronic HCV infection and in 12 of 16 samples obtained from patients with HIV-1 infection. In molecular assays, amplification may fail because of interference from PCR inhibitors. Besides heparin, serum drug levels may act as inhibitors. Additionally, endogenous inhibitors may be present in oral fluid (Shugars *et al*, 1999). Proposed

endogenous inhibitors include amylase, complement system, cystatins, defensins, histatins, lactoferrins, lysozymes, proline rich peptides, salivary mucins, secretory leucocyte protease inhibitor (SLPI), statherin, thrombospondin, and virus-specific antibodies. Inhibition can easily be detected by inclusion of an internal control. However, the mechanism of inhibition remains unclear.

In conclusion, both HCV RNA and HIV-1 RNA can be detected in whole saliva samples of patients with chronic HCV infection and those with HIV-1 infection. Nucleic acid concentrations were found to be low. Although the infective doses have not been defined, a potential risk of transmission through saliva must be considered.

# 6. REFERENCES


Arrieta JJ, Rodríguez-Iñigo E, Ortiz-Movilla N, Bartolomé J, Pardo M, Manzarbeitia F, Oliva H, Mariscal Macías D, Carreño V. In Situ Detection of Hepatitis C Virus RNA in Salivary Glands. AJP. 2001;158:259-264

Baron S, Poast J, Richardson CJ, Nguyen D, Cloyd M. Oral Transmission of Human Immunodeficiency Virus by Infected Seminal Fluid and Milk: A Novel Mechanism. J Infect Dis. 2000;181:498-504

Bèlec L, Legoff J, Si-Mohamed A, Bloch F, Mbopi Keou FX, Becquart P, Matta M, Prazuck T, Petite JP, Gutmann L, Payan C. Mucosal humoral immune response to hepatitis C virus E1/E2 surface glycoproteins and HCV shedding in saliva and cervicovaginal fluids from chronically HCV-infected patients. J Hepatol. 2003;38:833–842

Centers for Disease Control and Prevention [homepage on the Internet] Atlanta: Centers for Disease Control and Prevention [daily updated; downloaded August 26, 2006].Available from: http://www.cdc.gov/

Chohan BH, Lavreys L, Mandaiya KN, Kreiss JK, Bwayo JJ, Ndinya-Achola JO, Martin Jr. HL. Validation of a Modified Commercial Enzyme-Linked Immunoassay for Detection of Human Immunodeficiency Virus Type1 Immunoglobulin G Antibodies in Saliva. CDLI. 2001;8:346-348

Cock De L, Hutse V, Vranckx R. Correlation between detection of antibodies against hepatitis C virus whole saliva and hepatitis C virus RNA in serum. Eur J Clin Microbiol Infect Dis. 2005;24:566-568

Coley Pharmaceutical Group, Inc.[homepage on the Internet] Massachusetts. built@zoomedia [updated 2006 August 9; downloaded August 28, 2006] Available from: http://www.coleypharma.com/

Cook L, Sullivan K, Krantz EM, Bagabag A, Jerome KR. Multiplex real-time reverse transcription-PCR assay for determination of HCV genotypes. J Clin Microbiol. 2006;44:4149-4156

Crouch DJ. Whole saliva collection: The neglected variable in whole saliva testing. Forensic Sci Int. 2005;150:165-173

Davis GL. New Drug Targets and Advances in Therapeutics for the Treatment of Viral Hepatitis. In: Davis GL, Gish RG. Hep DART 2005: Frontiers in Drug Development for Viral Hepatitis; 2005 Dec. 11-15; downloaded March 20, 2006. Available from: http://www.clinicaloptions.com/

Dixit NM, Perelson AS. The metabolism, pharmakokinetics and mechanisms of antiviral activity of ribavirin against hepatitis C virus. Cell Mol Life Sci. 2006;63:832-842

Dusheiko GM, Smith M, Sheuer PJ. Hepatitis C virus transmitted by human bite. Lancet 1990;336:503-504

Drosten C, Panning M, Drexler JF, Hansel F, Pedroso C, Yeats J, de Souza Luna LK, Samuel M, Liedigk B, Lippert U, Sturmer M, Doerr HW, Brites C, Preiser W. Ultrasensitive monitoring of HIV-1 viral load by a low-cost real-time reverse transcription-PCR assay with internal control for the 5`long terminal repeat domain. Clin Chem. 2006;52:1258-1266

Eirea M, Diz Dios P, Hermid AM, Rodriguez I, Castro A, Ocampo A. Detection of HCV-RNA in Saliva of HIV-HCV Coinfected Patients. AIDS Res Hum Retroviruses. 2005;21:1011–1015

Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL. Harrison´s Principles of Internal Medicine. 14th ed. Singapore; McGraw-Hill 1998

Ferreiro MC, Dios PD, Scully C. Transmission of hepatitis C virus by saliva?. Oral Dis. 2005;11:230-235

Folgori A, Capone S, Ruggeri L, Meola A, Sporeno E, Bruni Ercole B, Pezzanera M, et al. A T-cell HCV vaccine slicing affective immunity against heterologous virus challenge in chimpanzees. Nat Med. 2006;12:190-197

Frank C, Mohamed MK, Strickland TG, Lavanchy D, Arthur RR, Magder LS, El Khoby T, Abdel-Wahab Y, Aly Ohn EL, Anwar W, Sallam I. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 2000;355:887–891

Gonçalves PL, Cunha CB, Busek SCU, Oliveira GC, Ribeiro-Rodrigues R, EL Pereira F. Detection of Hepatitis C Virus RNA in Saliva Samples from Patients with Seric Anti-HCV Antibodies. Braz J Infect Dis. 2005;9:28-34

Halfon P, Bourliere M, Penaranda G, Khiri H, Ouzan D. Real-time PCR assays for HCV RNA quantitation are adequate for clinical management of patients with chronic HCV infection. J Clin Microbiol. 2006;44:2507-2511

Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. J Gastroenterol. 2006;41:17-27

Hermida M, Ferreiro MC, Barral S, Laredo R, Castro A, Diz Dios P. Detection of HCV RNA in saliva of patients with hepatitis C virus infection by using a highly sensitive test. J Virol Methods. 2001;101:29–35

Hof H, Dörries R. Medizinische Mikrobiologie.3rd ed. Stuttgart: Georg Thieme Verlag. 2005

Jackson BR, Busch MP, Stramer SL, AuBuchon JP. The cost-effectiveness of NAT for HIV, HCV, and HBV in whole-blood donations. Transfusion. 2003;43:721-729

Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO). AIDS epidemic update December 2005 Special Report on HIV Prevention. Switzerland

Jonas S, Jessner W, Rafetseder O, Wild C, Chronische Hepatitis C Implikationen für Therapie und ökonomischen Ressourceneinsatz in Österreich. June 30, 2004, Wien.

Judd A, Parry J, Hickman M, McDonald T, Jordan L, Lewis K, Contreras M, Dusheiko G, Foster G, Gill N, Kemp K, Main J, Murray-Lyon I, Nelson M. Evaluation of a modified commercial assay in detecting antibody to hepatitis C virus in whole salivas and dried blood spots. J Med Virol. 2003;71:49-55

Kaufman E, Lamster IB. The Diagnostic Applications of Saliva- a Review. Critical Reviews in Oral Biol Med. 2002;13:197-212

Kessler HH, Stelzl E, Raggam RB, Haas J, Kirchmeir F, Hegenbarth K, Daghofer E, Santner BL, Marth E, Stauber RF. Effects of Storage and Type of Blood Collection Tubes on Hepatitis C Virus Level in Whole Blood Samples. J Clin Microbiol. 2001;39:1788-1790

Laborlexikon [homepage]. Dorsten: Hagemann O [updated July 12, 2006; downloaded August 20, 2006]. Available from: http://www.laborlexikon.de/

Lins L, Almeida H, Vitvisk L, Carmo T, Parana R, Mitermayer G Reis. Detection of Hepatitis C Virus RNA in Saliva Is Not Related to Oral Health Status or Viral Load. J Med Virol. 2005;77:216–220

Lok ASF, Gunaratnam NT. Diagnosis of Hepatitis C. Hepatology 1997;26:48S-56S

Luizzi G, Chirianni A, Clementi M, Bagnarelli P, Valenza A, Cataldo PT, Piazza M. Analysis of HIV-1 load in blood, semen and saliva: evidence for different viral compartements on a cross-sectional and longitudinal study. AIDS 1996;10:F51-F56

Mastromatteo AM, Rapaccini GL, Pompili M. Hepatitis C virus infection: other biological fluids than blood may be responsible for intrafamiliar spread. Hepatology 2001;48:193-196

Maticic M, Poljak M, Kramar B, Seme K, Brinovec V, Meglic-Volkar J, Zakotnik B, Skaleric U. Detection of Hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. J Periodontol. 2001;72:11-16

Melvin AJ, Tamura GS, House JK, Hobson AC, Cone RW, Frenkel LM, Burchett S. Lack of detection of human immunodeficiency virus type 1 in the saliva of infected children and adolescent. APAMed. 1997;151:228-32

Pastore L, Fiore JR., Tateo M, De Benedittis M, Petruzzi M, Casalino C, Genchi C, LoMuzio L, Angarano G, Serpico R. Detection of hepatitis C virus-RNA in saliva from chronically HCV-infected patients. Internat J Immunopathol Pharmacology 2006;19:217-24

Pawlotsky JM. Use and Interpretation of Virological Tests for Hepatitis C. Hepatology. 2002;36:565-573

Pawlotsky JM. Pathophysiology of hepatitis C virus infection and related liver disease. Trends in Microbiol. 2004;12:96-102

Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural Biology of Hepatitis C Virus. Hepatology. 2004;39:5-19

Perrin L, Pawlotsky JM, Bouvier-Alias M, Sarrazin C, Zeuzem S, Colucci G. Multicenter Performance Evaluation of a New TaqMan PCR Assay for Monitoring Human Immunodeficiency Virus RNA load. J Clin Microbiol. 2006;44:4371-4375

Rey D, Fritsch S, Schmitt C, Meyer P, Lang JM, Stoll-Keller F. Quantitation of Hepatitis C Virus RNA in Saliva and Serum of Patients Coinfected with HCV and Human Immunodeficiency Virus. J Med Virol. 2001;63:117-119

Robert Koch Institut [homepage]; Robert Koch Institut; [updated 2007 March 26; download March 27, 2007]; Available from: http://www.rki.de

Shepard RN, Schock J, Robertson K, Shugars DC, Dyer J, Vernazza P, Hall C, Cohen M, Fiscus SA. Quantitation of Human Immunodeficiency Virus Type 1 RNA in Different Biological Departements. J Clin Microbiol. 2000;38:1414-8

Roy KM, Bagg J, McCarron B, Goad T, Cameron S, Pithie A. Predominance of HCV type 2a in saliva from intravenous drug users. J Med Virol. 1998;54:271-275

Shugars DC, Wahl SM. The role of oral environment in HIV-1 transmission. J Am Dent Assoc. 1998;129:851-8

Shugars DC, Alexander AL, Fu K, Freel SA. Endogenous salivary inhibitors of Human Immunodeficiency Virus. Archives of Oral Biol. 1999;44:445-453

Simon V, Ho DD, Abdool Karim Q. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet 2006;368:489-504

Streckfuss CF, Bigler LR. Saliva as a diagnostic fluid. Oral Dis. 2002;8:69-76

Swanson P, Holzmayer V, Huang S, Hay P, Adebiyi A, Rice P, Abravaya K, Thamm S, Devare SG, Hackett J Jr. Performance of the automated Abbott RealTime HIV-1 assay on a genetically diverse panel of specimens from London: comparison to VERSANT HIV-1 RNA 3.0, AMPLICOR HIV-1 MONITOR v1.5, and LCx HIV RNA Quantitative assays. J Virol Methods. 2006;137:184-192

Tetsuro S, Kazuhiko O, Tazuko S, Takahiro M, Chiaki A, Munehiro M, Tomonori M, Tatsuo M. Quantitative Detection of Hepatitis C Virus (HCV) RNA in Saliva and Gingival Crevicular Fluid of HCV-Infected Patients. J Clin Microbiol. 2005;43:4413-4417

Van Doornum GJJ, Lodder A, Buimer M, Van Ameijden EJC, Bruisten S. Evaluation of hepatitis C antibody testing in saliva specimens collected by two

different systems in comparison with HCV antibody and HCV RNA in serum. J Med Virol. 2001;64:13-20

Van Soest H, Boland GJ, Van Erpecum KJ. Hepatitis C: changing genotype distribution with important implications for patient management. Netherland J Med. 2006;64:96-99

Vidmar L, Poljak M, Tomazic J, Serne K, Klavs I.Transmission of HIV-1 by human bite. Lancet. 1996;347:1762-1763

Wong T., Lee SS. Hepatitis C: a review for primary care physicians. C Med Assoc J. 2006;17:649-59

World Health Organization (WHO) [homepage], Geneva: World Health Organization; [daily updated; downloaded July 18, 2006]. Available from: http://www.who.int/en/

# 7. LIST OF FIGURES AND TABLES

# 8. ABBREVIATIONS

**ALT** = alanine aminotransferase

**AP** = alkaline phosphatase

**AST** = aspartate aminotransferase

**GCF** = gingival crevicular fluid

**GGT** = gamma glutamyle transferase

**HAART** = highly active antiretroviral therapy

**HAV** = hepatitis A virus

**HBV** = hepatitis B virus

**HCV** = hepatitis C virus

**HIV** = human immunodeficiency virus

**HIV-1** = human immunodeficiency virus type 1

**IFN** = interferon

**IG** = immunoglobulin

**LDH** = lactate dehydrogenase

**NNRTI** = non nucleoside reverse transcriptase inhibitors

**NRTI** = nucleoside reverse transcriptase inhibitors

**NS** = nonstructural

**PBMC** = peripheral blood mononuclear cells

**PCR** = polymerase chain reaction

**PEG-IFN** = pegylated interferon

**PI** = protease inhibitors

**RIBA** = recombinant immunoblot assay

**RT-PCR** = reverse transcription polymerase chain reaction

**SCS** = saliva collection system

**SES** = saliva extraction solution

**SLPI** = secretory leucocyte protease inhibitor

**SQK** = saliva quantification kit

**SVR** = sustained virological response

**TLR** = toll like receptor

**ULLD** = under the lower limit of detection

# 9. ACKOWLEDGEMENT

First of all, I would like to thank Harald H. Kessler for supporting me to finish this difficult work whenever I needed help. Also thanks for the chance to learn so much about these interesting topics.

I also want to thank Reinhard B. Raggam who always had an ear for my questions and for spending so much time on stimulating and inspiring discussions.

Special thanks also to Evelyn Stelzl. Your great ideas always helped me to improve my diploma thesis and often lead me to other important points of view.

Also thanks to the whole crew of the molecular diagnostic laboratory. It really was a pleasure for me to gather my first scientific experiences at this great institute.

Thanks also to my parents who always encouraged me to go my way and made everything possible to me.