Diploma Thesis

Detection of human immunodeficiency virus type 1 (HIV-1) in plasma samples

Submitted by

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Declaration of Originality

I, hereby, declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties.
Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, the 7th of December 2009 *signature*

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Finally, I owe thanks to my parents who made it possible for me to study and supported me the whole time.

Contents

1 Abstract

1.1 English

Background: Detection of human immunodeficiency virus type 1 (HIV-1) through analysis of viral RNA in human blood plasma is one of the major methods in HIV-1 diagnosis.

Objectives: To evaluate the VERSANT HIV-1 RNA Assay 1.0 (kPCR). To compare results with those obtained with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

Methods: For analytical evaluation, the accuracy, linearity, interassay and intra-assay variability of the VERSANT HIV-1 RNA Assay 1.0 (kPCR) were determined. A total of 196 plasma samples of patients with established HIV-1 infection were tested with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

Results: Testing of accuracy revealed results to be within $\pm 0.5 \log_{10}$ unit of the expected results. Determination of linearity gave a quasilinear curve. The coefficients of variation regarding interassay imprecision ranged from 12 to 20%, those of intra-assay imprecision between 8 and 16%. Viral loads, measured with both assays, showed a high correlation (R^2=0.94). The majority of values obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) were largely lower than those obtained with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

Conclusion: The VERSANT HIV-1 RNA Assay 1.0 (kPCR) is suitable for use in the routine diagnostic laboratory.

1.2 German

Hintergrund: Der Nachweis des humanen Immundefizienz-Virus Typ-1 (HIV-1) mittels der im humanen Blutplasma enthaltenen Virus-RNA zählt zu den wichtigsten Verfahren in der HIV-1-Diagnostik.

Ziele: Evaluierung des VERSANT HIV-1 RNA Assay 1.0 (kPCR) und Vergleich der Ergebnisse mit denen des COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

Methoden: Die accuracy, linearity, interassay und intra-assay des VERSANT HIV-1 RNA Assay 1.0 (kPCR) wurden bestimmt. 196 Plasmaproben von PatientInnen mit bekannter HIV-1-Infektion wurden sowohl mit dem VERSANT HIV-1 RNA Assay 1.0 (kPCR), als auch mit dem COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 untersucht.

Ergebnisse: Die Testung der Genauigkeit zeigte, dass die mit dem VERSANT HIV-1 RNA Assay 1.0 (kPCR) erhaltenen Ergebnisse innerhalb von ±0,5 $\log_{10}$ unit der erwarteten Ergebnisse lagen. Die Linearität zeigte eine quasilineare Kurve. Die Interassay lag zwischen 12 und 20%, die Intra-assay zwischen 8 und 16%. Die mit beiden Assays gemessene Viruslast zeigte eine hohe Korrelation (R^2=0,94). Die Mehrheit der mit dem VERSANT HIV-1 RNA Assay 1.0 (kPCR) erhaltenen Werte lag unter denen des COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

Fazit: Der VERSANT HIV-1 RNA Assay 1.0 (kPCR) eignet sich für den Einsatz im diagnostischen Routinelabor.

2 Introduction

Since its first description in 1981, the human immunodeficiency virus (HIV) has spread throughout the world. Every year, millions of people die because of the acquired immunodeficiency syndrome (AIDS), caused by this virus. During the course of HIV infection, opportunistic infections with other viruses and bacteria may affect almost all organ systems.

Although a cure or a save and protective vaccination is not yet available, the introduction of antiretroviral therapy has transformed the disease from fatal to manageable over decades. The therapy elongates the subclinical period after infection, allowing for a relatively non-truculent lifestyle [1]. Currently, access to the expensive medication is not available in all countries, especially in the most affected regions such as the sub-Saharan Africa. The long and ever prolonged subclinical period after the infection is one of the main reasons why the number of infected people develops so rapidly.

Often, people are not aware of symptoms, especially at acute HIV infection, when the viral load is extremely high and thus a high transmission risk exists. Also it is very important to identify infected people during the subclinical period to provide quick and effective treatment.

The virus is usually detected in human blood samples. In transfusion medicine, detection of viral RNA is the commonly used screening test today. This technique is optimal for monitoring treatment response also. Quantitation of plasma HIV RNA is significant because the measured viral load correlates with clinical outcome of patients. Furthermore, either development of resistances against specific drugs of antiretroviral therapy or non-compliance can be detected earlier. To conduct quantitation of HIV RNA in the high-throughput clinical laboratory, commercial molecular assays are available.

In this diploma study, a new commercially available molecular assay for detection and quantitation of HIV-1 RNA, the VERSANT HIV-1 RNA Assay 1.0 (kPCR) was evaluated for the first time. The analytical evaluation included determination of the accuracy, linearity, and interassay and intra-assay variability. For the clinical evaluation, results obtained with the new molecular assay were compared to those

obtained with the routinely used COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. Results of this study were published recently (Katharina T. Troppan, Evelyn Stelzl, Deborah Violan, Michaela Winkler, Harald H. Kessler: Evaluation of the new VERSANT HIV-1 RNA Assay 1.0 (kPCR) for quantitative detection of human immunodeficiency virus type 1 RNA; Journal of Clinical Virology 46, 69-74, 2009).

2.1 The HIV

2.1.1 Definition

The HIV is a retrovirus belonging to the family of *Lentiviruses*. Infections with *Lentiviruses* show a chronic progress and a long latency period with persisting viremia.

There exist two types of HIV, called HIV-1 and HIV-2. Under the electron-microscope, they look extremely similar but they differ from each other in molecular weight and show a genetic homology of only 60%. In comparison with HIV-1, transmission of HIV-2 is less likely, progress of disease is slower, and mortality is lower. HIV-2 accounts for only 1% of worldwide HIV infections, mainly in West-Africa.

HIV-1 is classified into 3 groups (Figure 2-1):
Group M (for major),
Group N (for neo or new), and
Group O (for outliers)

Group M is divided into subtypes A to K. Additionally, 26 circulating recombinant forms (CRFs) have been described up to now. Recombinant forms develop in human cells, when two different subtypes exchange their genetic material.

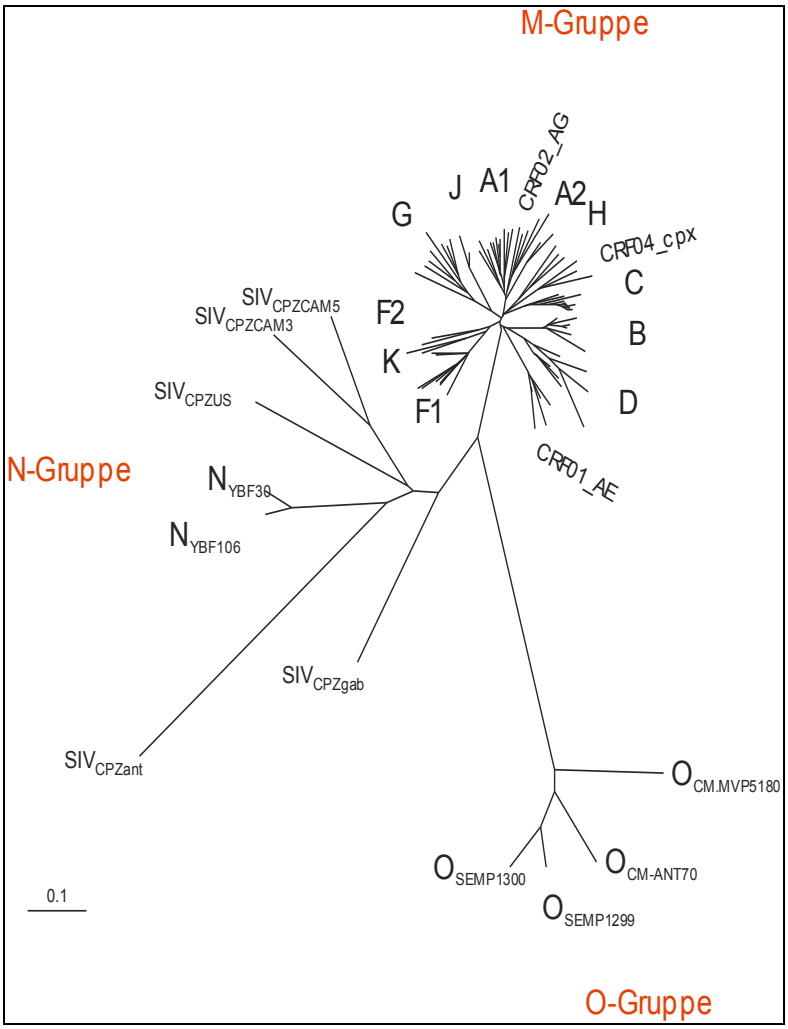


Figure 2-1: Phylogenetic tree of HIV.

In Europe, Australia, and North America, subtype B is mainly found, while in Asia and Southern Africa, subtypes A and C are predominant. Subtype E adds to HIV infections in Southern and South-East Asia. Due to migration, genetic diversity has been increased and non-B subtypes have accelerated spread. In some regions of Austria, up to 30% of non-B subtypes have been detected [2].

For the development of AIDS, the classification of the specific subtype may be important because it might influence speed and severity of the disease. Patients infected with HIV-1 subtype D or recombinant forms have been reported to show faster progression from infection to onset of AIDS [3]. However, differences in clinical outcome of patients under antiretroviral therapy have not been found when patients infected with various subtypes were compared [4].

2.1.2 Structure

The HIV-1 virus particle measures about 100nm in diameter. It consists of RNA, a capsid, and an envelope. The envelope contains lipoproteins including the transmembrane glycoprotein *gp41* and the external glycoprotein *gp120* for binding to the human receptor. The nucleocapsid is surrounded by a layer of proteins (*p17*) to ensure the integrity of the virion. The conical inner nucleocapsid, formed by the protein *p24*, contains two copies of positive single-strand RNA and the reverse transcriptase which converts viral RNA into complementary DNA (cDNA) (Figure 2-2).

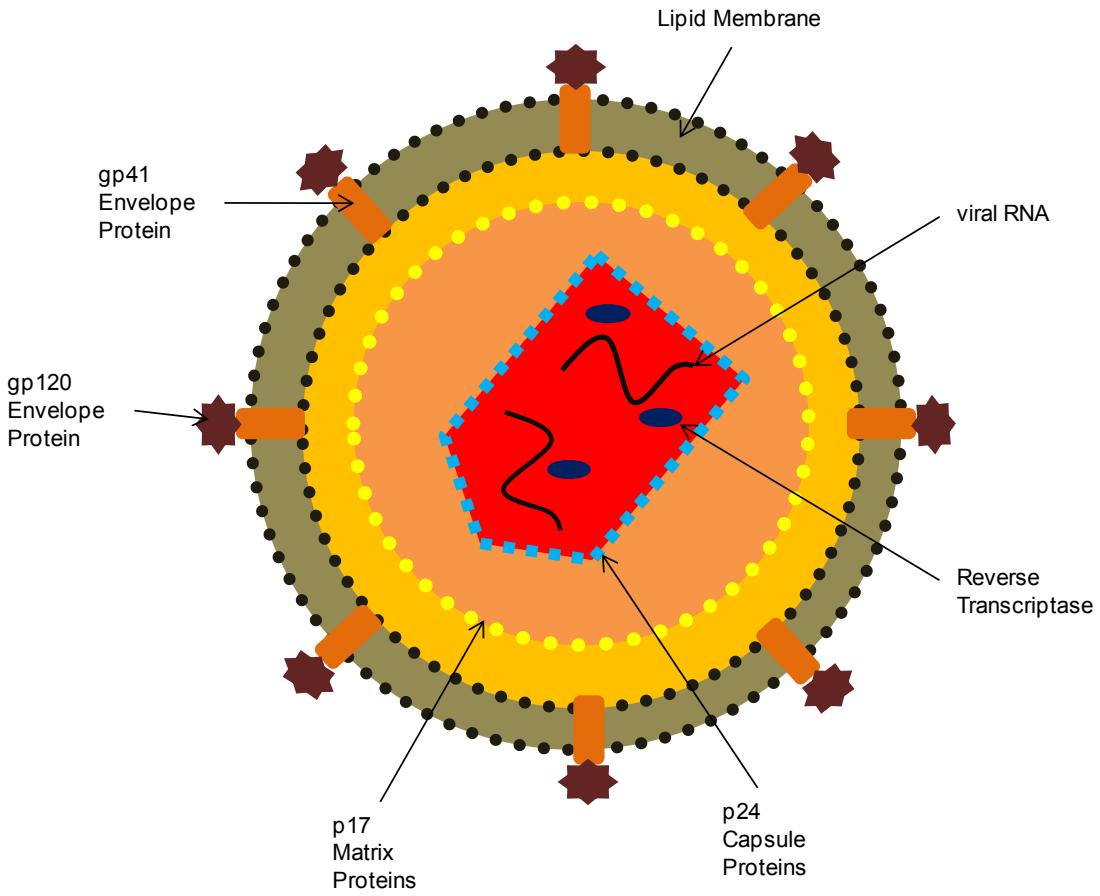


Figure 2-2: Structure of the HIV-1 virus particle.

The viral RNA encodes the nine genes of the virus *gag*, *pol*, *env*, *nef*, *vif*, *vpr*, *vpu*, *tat*, and *rev*.

The *gag* (group antigen) and the *env* (envelope) genes encode the glycoproteins of the viral envelope and the nucleocapsid while the *pol* (polymerase) gene encodes several enzymes including the reverse transcriptase. *Nef*, *vif*, *vpr*, and *vpu* are the so-called accessory genes because they are not necessary for *in vitro* viral replication. *Nef* induces a down regulation of CD4+ cells and of HLA (human leukocyte antigen) class I which allows the virus to escape from cytotoxic T-cells. *Vif* enables the viral replication through binding to a complex molecule, called APOBEC3G, which normally inhibits replication process. *Vpr* and *vpu* ensure that the virus arrives at the right position for all replication steps. *Tat* and *rev* are regulator proteins, responsible for transcription, elongation, transport, and translation. Additionally, two long terminal repeat (LTR) regions comprehend the genes on the 5' and 3' end [5] (Figure 2-3).

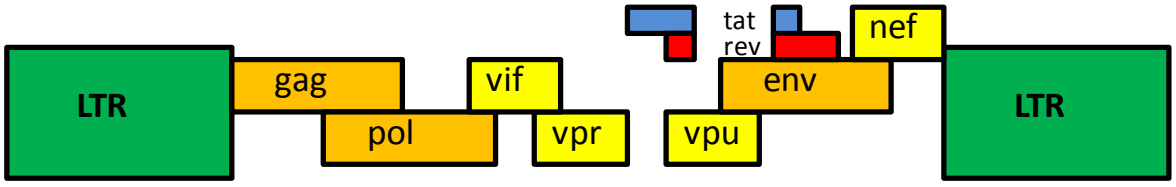


Figure 2-3: Schematic constitution of the HIV-1 virus particle.

2.1.3 Transmission

Three major ways of transmission are known today: sexual contact, vertical transmission, and parenteral transmission.

Sexual intercourse, homo- and heterosexual, with an infected person is the major risk factor for HIV infection. High viral loads can be found in semen and blood, depending on the stage of disease and potential antiretroviral therapy. Shortly after infection, an extremely high viral load can be found in all body fluids. With antiretroviral therapy, the viral load decreases or even may become undetectable. The risk of sexual transmission of HIV-1 may be negligibly low, at least if there are no further sexual transmitted diseases (STDs) present in one of the partners [6].
For vertical transmission, the viral load at delivery is extremely important. With antiretroviral therapy, the probability of vertical transmission can be decreased to

less than 1%. In addition to the scheduled caesarean section, breastfeeding must be avoided.

Today, blood products are checked for HIV-1; the hazard of infection through blood transfusion thus has become extremely low [7]. Needle-sharing among intravenous drug users remains one of the most important transmission ways. Compared to accidental needle stick injuries, the danger of infection by intravenous drug abuse is significantly higher due to aspiration of blood into the syringe. Worldwide, more than 60 cases of HIV infection following accidental blood contact in hospitals have been documented. To prevent nosocomial HIV infection, especially the use of gloves is a mandatory measure of precaution in the health care setting. However, no transmission may occur at bare contact with the skin, through insects, and eating and drinking out of same dishes.

2.1.4 Epidemiology

2.1.4.1 The Situation Worldwide

According to the World Health Statistic Report 2008, the number of people living with HIV worldwide is estimated to be 33.4 million including around 2 million children under the age of 15. The region mostly affected is Sub-Saharan Africa, with a total of 22.4 million people infected.

Every day, about 6800 people are newly infected with HIV (2.5 million per year), most of them in Sub-Saharan Africa. On the other hand, 6000 people die due to AIDS per day. The prevalence of HIV worldwide is approximately 0.8% of the adult population [8] (Figure 2-4).

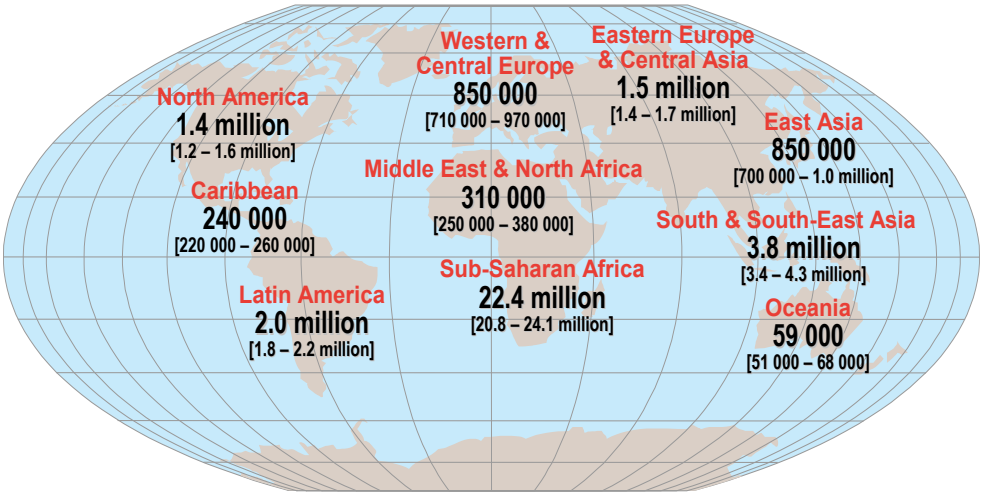


Figure 2-4: Adults and children estimated to be living with HIV, 2008 (UNAIDS Epidemic report 2009).

Today, heterosexual transmission remains the major way of transmission worldwide, followed by drug abuse. Although genetic diversity increases, HIV-1 subtype C remains to dominate and accounts for 55-60% of all HIV-1 infections worldwide. In some regions, especially in South-East Asia, recombinant forms are found in 20% of infected individuals [9].

2.1.4.2 The Situation in Austria

Today, more than 10000 HIV-positives are living in Austria. Two thirds of the infected are men and nearly half of them live in Vienna. More than 50% got infected through sexual contact and about 25% through intravenous drug abuse. Less than 1% has been infected vertically.

In 2008, 505 newly acquired infections were documented (more than one newly acquired infection per day). Homosexual contact is still the major way of transmission; however, the number of heterosexual transmissions increases significantly.

Today, 1224 people suffer from AIDS in Austria. From 1983 onwards, 1495 people died of the disease. Every year about one million HIV antibody checks are performed, the major part financed by donation [10].

2.1.5 History

HIV-1 is thought to have developed from a simian immunodeficiency virus. It originated in Western Africa and jumped species from non-human primates to humans.

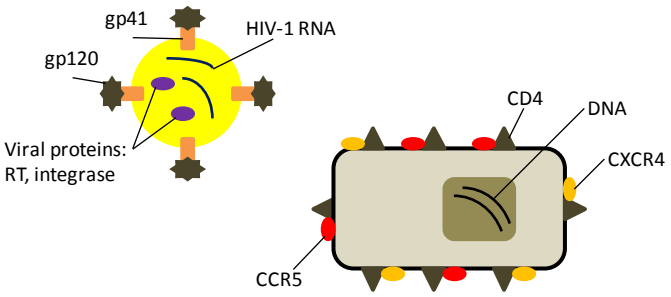
In 1981, a report about five cases of *Pneumocystis carinii* pneumonia in homosexual men was published [11]. Articles on cancer, especially Kaposi's sarcoma and other life-threatening opportunistic infections followed. Not only homosexual men but also hemophiliacs and intravenous drug users were affected. In August 1982, the Centers for Disease Control and Prevention (CDC) named it "acquired immunodeficiency syndrome". In 1983, Luc Montagnier and his team at the Pasteur Institute reported the isolation of a new retrovirus from lymphoid tissue. They named it lymphadenopathy-associated virus (LAV). About one year later, Robert Gallo discovered the virus, too and called it human T-cell lymphotropic virus type III (HTLV-III). Later it was found, that both viruses were identical and they were thus united under the new name "human immunodeficiency virus".

Soon it was clear that the virus was not only transmitted by sexual contact but also by blood products. Work was intensified to develop safe blood products and blood-screening was introduced. In 1996, the virus had reached the whole world and the pandemic is still going on [12]. In 2008, the Nobel Prize for Physiology or Medicine was awarded half to Luc Montagnier for discovery of HIV.

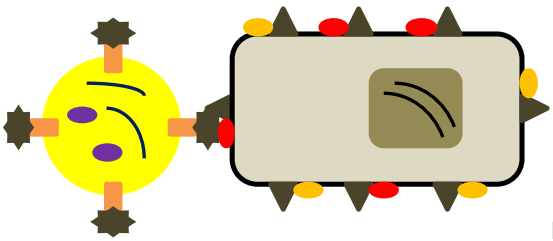
2.1.6 Etiopathology

The target cells of HIV are T-lymphocytes, monocytes, macrophages, eosinophil granulozytes, dendritic cells, and microglia cells. All of them expose a CD4+ receptor on their surface, the basic contact point for the V3 loop of the external glycoprotein (gp120) of HIV. Additionally, a co-receptor is required to start the entry process. The most important co-receptors are the chemokine receptors CCR5, responsible for most new infections, and CXCR4, mainly used in late stages of infection. A deletion mutation, the Δ32 mutation, which alternates expression of CCR5 on the cell surface, confers resistance against HIV-1 [13].

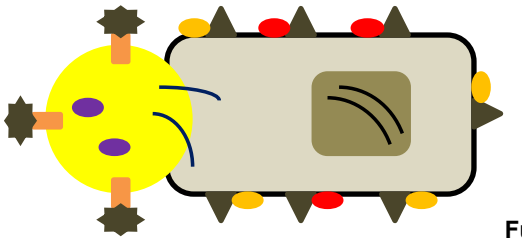
After binding of receptors, fusion takes place. The viral core is then released into the cell cytoplasma, the so-called uncoating. The virus' own reverse transcriptase converts viral RNA into cDNA. With the viral integrase, viral cDNA is inserted into the host's DNA. The cell now produces viral proteins which move towards the cell surface. The new viruses are assembled by means of the protease which cuts the proteins into appropriate parts. Finally, the new virus particles are released from the cell through budding. Antiretroviral therapy uses almost every single step to intervene in the viral replication cycle [9]. Figure 2-5 illustrates a schematic design of the HIV-1 life cycle.



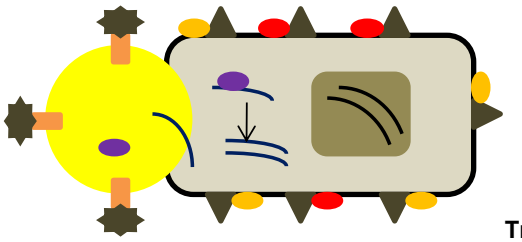
HIV-1 particle and CD4+ target cell



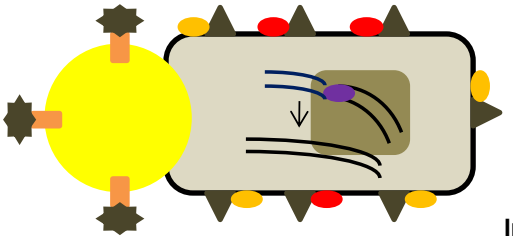
Binding of receptors



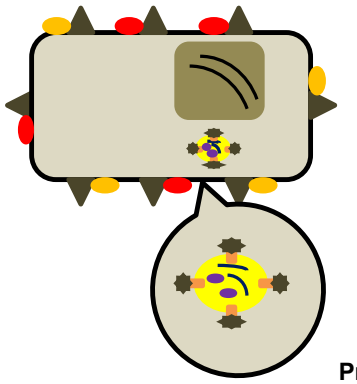
Fusion and releasing of viral core into cytoplasma



Transcription of virus RNA into DNA



Integration of virus DNA into cell DNA



Production of new virus particles and budding

Figure 2-5: HIV-1 life cycle.

2.1.7 Clinical Course of Infection

The clinical manifestations of HIV infection are divided into different stages of disease (Table 2-1).

About one to six weeks after the infection, the patient develops clinical presentation similar to mononucleosis including fever, lymphadenopathy, and sometimes splenomegaly, exanthema, and myalgia. While the antibody ELISA is usually negative in this stage, the viral load, measured by molecular assays, is extremely high. At this stage, patients are often not aware of the disease and the risk of transmission of infection to other people is thus increased significantly.

In the first clinical stage, also called latency phase, patients are asymptomatic but potentially virulent. This stage takes up to ten years, sometimes even longer if no immunodeficiency or malnutrition exists. An estimated 40% of patients develop a so-called lymphadenopathy-syndrome (LAS), characterized by persisting (longer than 3 month) lymphadenopathy, but the lack of general symptoms. One to three month after infection, HIV antibodies are detectable. The viral load usually remains relatively high, while CD4+ cell count begins to decrease slowly.

Clinical stage two is characterized by beginning opportunistic infections, mostly caused by viruses. Opportunistic infections are favored by a defect of the immune system. These infections often show atypical and complicated progress as well as difficulties in treatment.

In the third clinical stage, the patients start losing weight; additionally, chronic fever and diarrhea appear and opportunistic infections become more and more frequent. The CD4+ cell count decreases continuously while the viral load begins to increase.

Once less than 200 CD4+ cells/µl are counted, the patient is in the fourth stage, the full picture of AIDS. Kaposi's sarcoma, dementia, encephalopathy, and *Pneumocystis jiroveci* pneumonia are only three of multiple disease patterns occurring in various combinations in this final stage.

For vertical HIV-1 infections of children, these stages are not applicable. Their disease evolution is characterized by prematurity, dystrophia, and severe damages of the central nervous system (CNS) such as ataxia [14].

Primary HIV-1 infection	• Asymptomatic • Acute retroviral syndrome
Clinical stage 1	• Asymptomatic • Persistent generalized lymphadenopathy
Clinical stage 2	• Moderate unexplained weight loss • Recurrent respiratory infections • Herpes zoster • Minor mucocutaneous manifestations
Clinical stage 3	• Severe weight loss • Unexplained chronic diarrhea for more than 1 month • Unexplained persistent fever for more than 1 month • Oral candidiasis • Oral hairy leukoplakia • Pulmonary tuberculosis within the last 2 years • Severe presumed bacterial infections • Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis • Unexplained anemia (hemoglobin under 8g/dl) • Neutropenia (neutrophils under 500 cells/µl) • Thrombocytopenia (platelets under 50 000 cells/µl)
Clinical stage 4	• HIV wasting syndrome • *Pneumocystis jiroveci* pneumonia • Recurrent severe or radiologic bacterial pneumonia • Chronic herpes simplex infection for more than 1 month • Esophageal candidiasis • Extra pulmonary tuberculosis • Kaposi's sarcoma • CNS toxoplasmosis • HIV encephalopathy • Extra pulmonary cryptococcosis • Disseminated non-tuberculosis mycobacteria infection • Lymphoma • Invasive cervical carcinoma

Table 2-1: WHO clinical staging of HIV/AIDS for adults and adolescents [15].

2.1.8 Diagnosis

2.1.8.1 Tests for screening for HIV infection

Table 2-2 provides an overview of tests used for first diagnosis of HIV-1 infection and monitoring of HIV-1 infection.

Indirect	***Direct***	***Combination***
Antibody ELISA	(Culture)	Antigen/antibody
Immunoblot	Antigen ELISA (p24)	
(Indirect immunofluorescence test)	Molecular assays (HIV-1 RNA)	

Table 2-2: Methods available for first diagnosis and monitoring of HIV infection.

The first diagnosis of HIV-1 infection is based on two different test systems. The enzyme-linked immunosorbent assay (ELISA) is the most commonly used screening method. This assay detects antibodies directed against HIV-1 and HIV-2. The sensitivity of ELISA is very high. In contrast, the ELISA lacks specificity. To exclude false-positives, a confirmatory assay must be added. Another problem of the antibody ELISA is the serodiagnostic window. Seroconversion, that means appearance of antibodies, does not occur earlier than six to twelve weeks after infection. It may also show false-negative results in the late stage of disease because of exhaustion of antibody production. Furthermore, the ELISA is not suitable for testing newborns of HIV-1 positive mothers because of diaplacentar transfer of maternal antibodies.

Any positive result obtained with the ELISA must be confirmed, mostly done by an immunoblot. Today, the traditional Western Blot assay has been largely replaced by the recombinant reverse line-probe assay.

The indirect immunofluorescence test and viral culture are not used in the routine diagnostic laboratory.

The antigen ELISA detects the presence of the HIV-1 capsid protein p24 in the patient's blood. This assay is used within the time period after infection but prior to the appearance of antibodies.

With molecular assays, the diagnostic window can be shortened best. These assays have the capacity to detect HIV-1 RNA in human plasma samples ten to

fourteen days after infection; they are thus used for screening of blood products. Quantitative molecular assays are employed for monitoring treatment response in the management of HIV-1 infected patients. Figure 2-6 shows appearance of diagnostic markers.

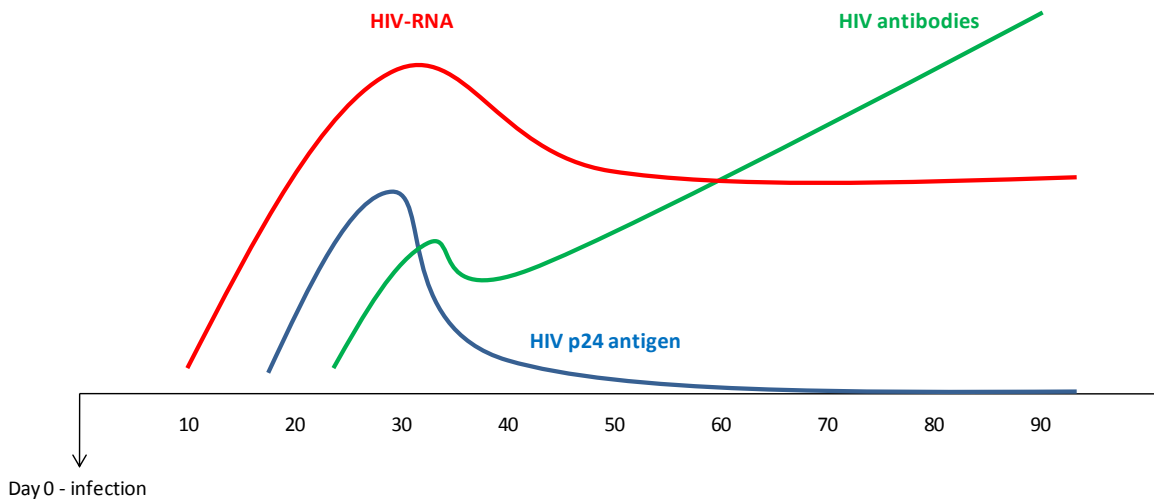


Figure 2-6: Appearance of diagnostic markers in early HIV infection.

Today, combination assays, testing for antigen and antibodies simultaneously, thus allowing for earlier detection of HIV infection, have largely replaced single parameter tests.

The CD4+ cell count is employed as a marker of progression of HIV-1 infection. Low CD4+ cell counts are associated with increased appearance of opportunistic infections. The value of 200 CD4+ cells per µl (normal range, 500 to 1500 cells per µl) is a criterion of AIDS [16].

Developed countries can afford all of those assays. However, in developing countries, laboratory instruments required are hardly ever available. Efforts to design inexpensive and better manageable techniques for these countries include oral fluid testing [17].

2.1.8.2 Supplementary tests

For identification of resistance to antiretroviral medication, genotypic testing based on sequence analysis of the target genes such as the protease and the reverse

transcriptase genes is performed. Use of an HIV-1 sequence database allows for detection of mismatches [18]. The TruGene HIV-1 genotyping assay is a largely automated assay and allows for detection of drug resistance mutations by analyzing sequences of the protease and the polymerase genes. Additionally, it provides an easy determination of HIV-1 subtypes.
To determine the co-receptor status of the individual virus, the sequence of the V3 loop of the gene encoding gp120, is analyzed [19].

2.1.9 Therapy

Today, five different classes of antiretroviral therapy (ART) agents are available. They include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, and integrase inhibitors [5].

2.1.9.1 NRTIs

The NRTIs were the first drugs, introduced in 1987, targeting the reverse transcriptase. They compete against physiologic nucleosides for integration in the newly produced DNA. Once integrated in the cDNA strand, they interrupt further production of cDNA immediately.
Today, several drugs including *Tenofovir*, *Lamivudin*, *Abacavir*, *Emtricitabin*, *Zidovudin*, *Didanosin*, *Stavudin*, and *Retrovir* are available. Adverse effects may include nausea, diarrhoea, cephalea and/or fatigue which are treated symptomatically. Long-term adverse effects include myelotoxicity, lactic acidosis, neuropathy, and pancreatitis which are caused by mitochondrial toxicity [20].

2.1.9.2 NNRTIs

The NNRTIs bind to the reverse transcriptase, directly stopping the replication process. The major problem with NNRTIs (*Delaviridin*, *Efavirenz*, *Etravirin*, and *Nevirapin*) is the rapid evolvement of cross-resistance. Nevertheless, they have shown good results in combination with NRTIs [20].

2.1.9.3 PIs

The PIs inhibit the virus protease. Today, several PIs are available including *Amprenavir*, *Indinavir*, *Tipranavir*, *Saquinavir*, *Lopinavir*, *Darunavir*, *Atazanavir*, *Nelfinavir*, *Fosamprenavir*, and *Ritonavir*.

Gastrointestinal problems may represent a major problem for patients. Furthermore, long-term consequences, such as kidney stones, hyperbilirubinemia, and hyperlipidemia have been reported [21].

2.1.9.4 Entry Inhibitors

Entry inhibitors interfere at the three key stages when HIV-1 enters the CD4+ cell. Attachment inhibitors, for instance *TNX355*, inhibit binding of gp120 to CD4+ receptors. Presently, entry inhibitors are only used in clinical trials.

Recently, the co-receptor antagonist *Maraviroc* has been introduced. Through binding to the co-receptor CCR5 and modifying the molecule, the virus is impaired to bind to the receptor [22]. Prior to the use of this specific medication, testing for the co-receptor status is obligatory.

The fusion inhibitor *Enfuvirtide* interferes at the last step of the entry process. Through attaching to gp41, the fusion of the virus particle and the target cell is inhibited [23].

Due to the novelty of these substances, there is still little information about adverse effects or possible resistances.

2.1.9.5 Integrase Inhibitors

Raltegravir, the presently exclusive representative of this class, inhibits the viral integrase. Through inhibition of the binding of integrase to cell DNA, the integration of viral cDNA into cell DNA is stopped. Only marginal adverse effects have been reported up to date [24]. However, further studies are needed.

2.1.9.6 Treatment Recommendations

The starting point of antiretroviral therapy has not been clearly defined. Increasing disease-free survival time and preservation of immunological function must be weighed against risks due to drug toxicity and emergence of viral resistance.

Attention should be paid to co-morbidities and patients compliance as well. Recommendations for initiating antiretroviral therapy are given in Table 2-3.

	Recommendation
Symptomatic HIV disease	Antiretroviral therapy
Asymptomatic HIV disease	
<350/µl CD4 cells	Antiretroviral therapy
>350/µl CD4 cells	Individualized start of antiretroviral therapy

Table 2-3: Recommendations for initiating antiretroviral therapy.

Antiretroviral therapy consists of a combination of at least two different classes of drugs. Several criteria, such as drug interactions, primary drug resistance, co-morbidities, and desire for pregnancy have to be taken into account.
Initial therapy is usually started with two NRTIs plus either one NNRTI or one PI. Besides baseline testing for resistance, the treatment response must be monitored by frequent determination of viral load in plasma. Reduction of the HIV-1 RNA plasma viral load under the detection limit of 50 HIV-1 copies/ml within the first 12 weeks of therapy is essential.
Virological failure must be overcome by replacing single agents, for instance an NNRTI through a PI or vice versa. Another possibility is the addition of new class medications such as *Maraviroc* or *Raltegravir*.
Special treatment recommendations exist for pregnant women, co-infected patients, and patients with specific opportunistic infections [1].

2.1.10 Prevention

Prevention strategies are classified into four major categories: prevention for HIV-1-negative people, especially high risk groups, for people with maximum probability of exposure but before exposure, for those shortly after exposure, and for already infected people (secondary prevention) [25].
Broadly targeted behavioral interventions should be the major measure with education as the most important issue. Further issues include the use of condoms,

fewer sexual partners, and avoidance of needle-sharing among intravenous drug users. In addition to educational efforts, various studies have shown that male circumcision can reduce the risk of HIV-1 transmission by around 60% [26]. Otherwise, through application of a diaphragm, no protective benefit was observed [27].

Another measure to prevent transmission of HIV-1 may be the topical microbicide technology. However, the first trials evaluating surfactants and membrane disruptors revealed disappointing results. Vaginal milieu protectors, guaranteeing a certain pH-value have shown better results [28].

After exposure, post exposure prophylaxis may avoid an infection. Besides flushing and disinfection, a combination of antiretroviral drugs, taken within 48 hours after the event, may be able to decrease the risk of infection.

People who are already infected with HIV-1 should invariably undergo antiretroviral therapy. Only antiretroviral treatment may lead to long lasting viral suppression and reduction of morbidity and mortality. This may also be essential for minimizing the risk of further infections.

In developed countries, prevention of mother-to-child transmission has been very successful. Antiretroviral therapy during pregnancy and birth has been proven to be effective in conjunction with replacing breastfeeding through infant formula feeding. In developing countries, where possibilities for antiretroviral therapy are not provided comprehensively, up to 30% vertical transmissions of HIV-1 have been reported. Strategies to ensure worldwide availability of ART are urgently needed [25].

2.2 Polymerase Chain Reaction (PCR)

2.2.1 History and Principles

Since its invention in 1984 by Kary Mullis (who therefore received the Nobel Prize in Chemistry in 1993) the polymerase chain reaction (PCR) has revolutionized molecular detection of pathogens. With PCR, it is possible to amplify a single piece of DNA and generate millions of copies of a certain DNA fragment within a few hours. The essential player is an enzyme, called polymerase, which builds a new DNA strand, complementary to one of the original strands. In the first years, PCR applications were very expensive and elaborate with a lot of hands-on time necessary. After each replication cycle, a new polymerase had to be added because it did not withstand the high temperatures necessary for denaturation of the double-strand DNA. To overcome this, the Taq polymerase, an enzyme of the heat-resistant bacterium *Thermus aquaticus* was introduced. This allowed automation of the process and triggered a dramatic increase in publications mentioning PCR. In 1995, only seven publications using the key word PCR were found in a search, while in 2003, the same entry gave 2291 results. In 2008, there were more than 350.000 entries concerning PCR [29].

Today, qualitative and quantitative PCR assays have been established which can be used for a wide range of applications including detection and monitoring of infectious diseases, detection of genetic diseases, oncology, forensic DNA-analytics, and pharmacogenomics. While qualitative PCR allows bare confirmation of a pathogen, quantitative PCR determines the amount of DNA or cDNA [16]. With real-time PCR, which allows for amplification and detection of amplification products simultaneously, another step into full automation has been made. During each cycle of PCR, the amount of amplified DNA can be measured, using a fluorescent reporter signal. The high analytical sensitivity (down to five or fewer copies per ml) of real-time PCR together with the decrease of cross-contamination has contributed to the widespread use of real-time PCR today.

2.2.2 The PCR Process

The whole PCR process takes place in a thermal cycler, an instrument which automatically performs the different steps. Reagents needed for PCR include target DNA, primers specific for beginning and end of the amplification segment, DNA-polymerase incorporating nucleotides complementary to the strand of origin, the nucleotides Adenine, Guanine, Cytosine, and Uracil as building bricks for the new DNA strand, and a buffer solution to obtain the optimal condition. In case of target RNA, a reverse transcriptase has to be added.

The PCR technology works after the role model of natural DNA replication. In a human cell, the two strands of the DNA double helix are unwinded and newly synthesized. Many proteins accomplish the build of a daughter strand, wherein one parent strand acts as a template.

The PCR process comprises the following steps achieving an exponential increase of amplification products, according to the formula $N=N_0\text{-}2^n$, where n is the number of amplified products [30]. In theory, twenty cycles yield a million copies and thirty cycles yield a billion copies. In practice, there is a nearly exponential kinetic first part, then follows a log linear phase and when reaction components are exhausted, the reaction slows down to the plateau phase.

In the sample preparation step, the nucleic acids have to be extracted from either blood samples or other DNA-sources. The core PCR technique consists of three reaction steps, called denaturation, annealing, and elongation (Figure 2-7). First, the double-strand DNA is heated to about 95°C, which separates the complementary strands from each other by destroying the hydrogen bonds between them. The stronger covalent chemical bonds within the linear DNA remain intact. During the annealing, the primers bind to the complementary area on the separated DNA strands of origin or onto already amplified fragments, at temperatures of about 55°C. These primers, whose specifity for the exact sequence is very high, indicate the start and end point of the new strand. To assure specifity of the PCR, the selection of the correct primer is critical. For the next step the elongation, a temperature of about 72°C is needed, for the DNA polymerase to build new complementary DNA strands. It binds to the specific sequence, marked by the primers and synthesizes the complementary strand, using the free nucleotides. At the end of each cycle, the new amplification

products also act as targets for the next cycle. These steps can be repeated several times, leading to an exponential growth of DNA fragments. As a control for the correct performance of the PCR and to determine inhibition, an internal control (IC) or quantitation standard (QS) can be added.

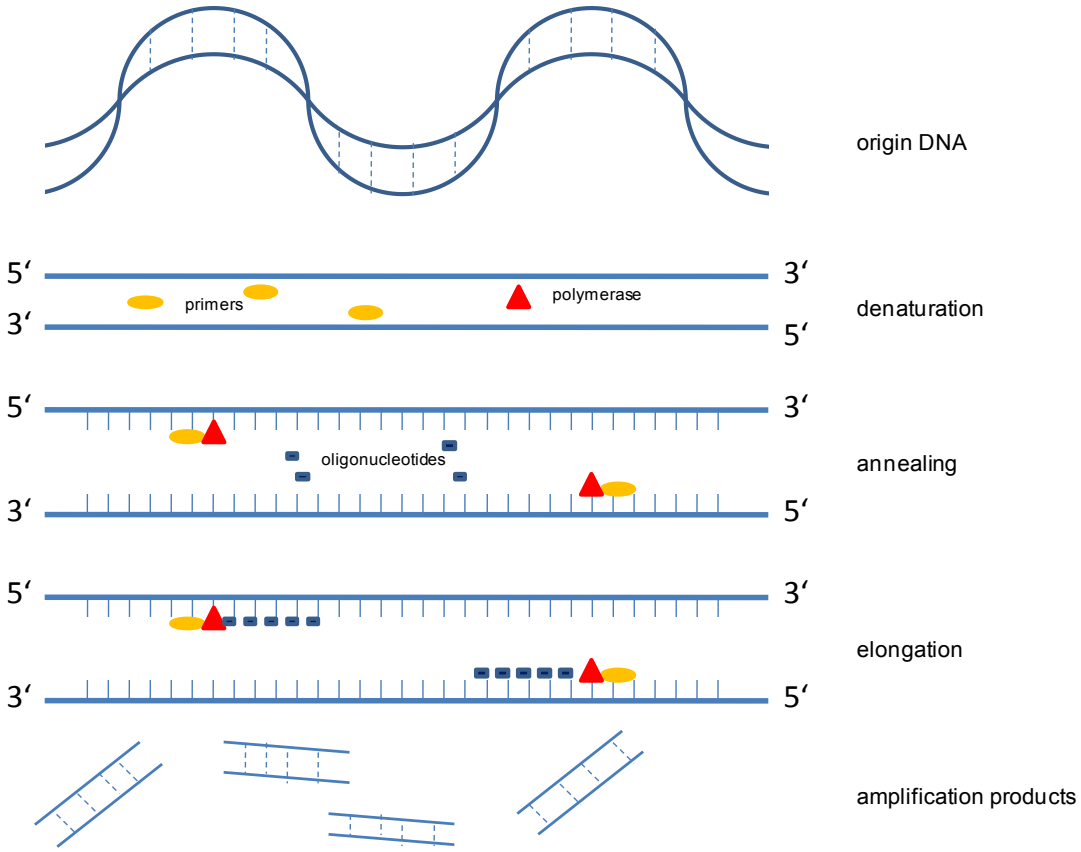


Figure 2-7: Principle of the PCR.

DNA can be amplified directly by PCR. In contrast, RNA must be transcribed into DNA, for instance when diagnosing HIV, because RNA is no efficient substrate for the Taq DNA polymerase [16]. Therefore the reverse transcriptase PCR is used. In this part process, the enzyme reverse transcriptase converts RNA into cDNA, and then amplification as described earlier occurs.

When performed with the original PCR technique, the above mentioned steps were a time-consuming process. The invention of real-time PCR in 1991 allows for simultaneous amplification and quantification of the targeted DNA molecule. Collection of data during the phase of PCR amplification reduces cross-contamination and saves a lot of time, compared to end-point PCR detection.

2.2.3 Advantages of PCR

Values of sensitivity and specificity for PCR are reported to be very high, i.e. both positive and negative samples are identified correctly as such. However, a small percentage of false-positive or false-negative results remain. This occurs when introducing contaminated DNA (false-positive) or presence of inhibitors (false-negative) in blood samples. To avoid these errors, PCR reagents contain Uracil N glycosylase to control contamination. Purification of DNA is the method of choice to elude inhibition.

While other molecular-assays often need days to obtain results, PCR is able to give same-day results, important in diagnosis and for therapy. Nevertheless, this promptness increases monetary cost, compared to conventional diagnostic laboratory methods. In addition to initial acquisition and production costs, the reagent charges for each sample and also the requirement for separate rooms for the different PCR steps to avoid contamination are not inexpensive [31].

The above described advantages and disadvantages of PCR, compared to other molecular-based methods, are listed in Table 2-4.

Advantages	***Disadvantages***
High sensitivity	False-positive results possible
High specificity	False-negative results possible
Promptness: same-day results possible	Expensive

Table 2-4: Advantages and disadvantages of PCR.

2.2.4 Detection Methods

For the detection of amplification products, different detection formats exist. Due to almost exclusive use of real-time PCR today, only these detection methods will be described in this text. Combining of thermal cycling, fluorescence detection and software analysis in one instrument enables cycle-by-cycle detection.

In the TaqMan technology (Figure 2-8), a reporter fluorophore and a fluorescent quencher dye, bound to the 5’ and 3’ ends of the probe, are used. In the native state both dyes are in close interaction and the fluorescence of the reporter dye is mostly absorbed by the quencher and hardly an emitted fluorescence is detected.

After annealing, reporter and quencher dye are released (separated), so the reporter is de-suppressed and fluoresces under a certain wavelength, whose intensity is proportional to the amount of the present transcript. This means, the higher the DNA products of a specimen, the higher is the fluorescence level of the probe.

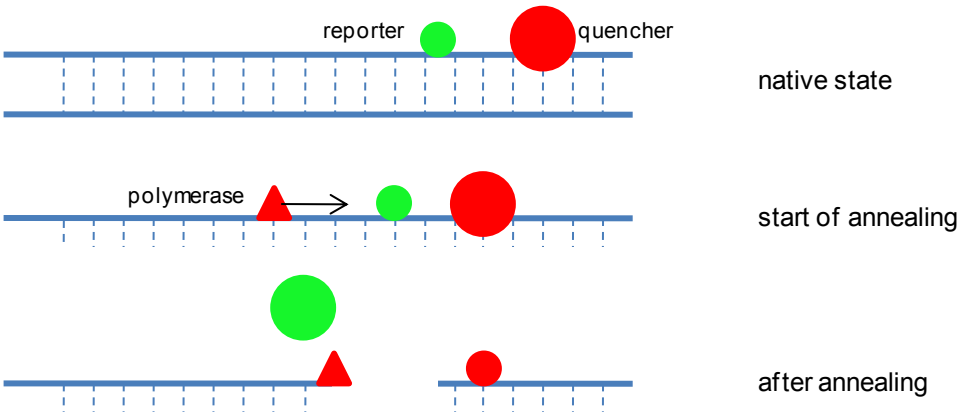


Figure 2-8: Principle of the TaqMan method.

The TaqMan method is one of the most widely used detection technologies, other methods will be explained shortly in the following paragraph:

Of similar accuracy and performance as the TaqMan system is the LightCycler or hybridization technology, which uses two fluorescent oligonucleotides in the probe, one for the donor at the 3' and the other for the acceptor at the 5' end. Hybridized to the target in a head-to-tail position, the fluorophores are brought into each other's close proximity, giving an increasing amount of measured fluorescence, proportional to the increasing amount of DNA.

The molecular beacons method is a non-fluorescent detection, forming stem-loop hairpin structures, which are complementary to the target on DNA. A quencher again blocks the reporter until hybridization of the target occurs.

Another technique, not used for multiplexed analysis, is the SYBR Green technology. After replicating the target sequence, multiple molecules of SYBR Green bind to the amplification product dsDNA (double-strand DNA) and emit a fluorescent signal [32].

3 Materials and Methods

3.1 Specimens used in this study

In this study, a total of 196 EDTA plasma samples were tested. All specimens were obtained from patients with established HIV-1 infection, with or without antiretroviral therapy and had already been tested with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0. For the determination of the HIV-1 subtype, the TRUGENE HIV-1 Genotyping Kit (Siemens) had been used according to the manufacturer`s package insert. The subtype of the nucleotide sequence generated was determined by homology analysis using an HIV-1 sequence database (www.geno2pheno.org). Subtypes obtained from clinical samples are shown in Table 3-1.

Number of samples	*Subtype*
14	A
49	B
19	C
27	D
23	F
11	G
53	CRFs

Table 3-1: Distribution of subtypes obtained from samples investigated in this study.

3.2 Molecular Assays

Basic features of the molecular assays used in this study are shown in Table 3-2.

	Siemens Health Care Diagnostics	***Roche Molecular Diagnostics***
Assay Name	VERSANT HIV-1 RNA Assay 1.0 (kPCR)	COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0
Target sequence	pol gene	gag gene + LTR
Sample volume required	650 µl or more	1000 µl
Detection method	TaqMan	TaqMan
Range of linearity	3.7×10^1 to 1.1×10^7 copies/ml	2.0×10^1 to 1.0×10^7 copies/ml
Time to results (24 samples)	5h 40min	5h 40min

Table 3-2: Characteristics of the two molecular assays.

3.2.1 The VERSANT HIV-1 RNA Assay 1.0 (kPCR)

The Versant HIV-1 RNA Assay 1.0 (kPCR) uses a reverse transcription, real-time PCR and detection procedure for quantifying HIV-1 RNA in human EDTA plasma samples. This molecular assay targets a highly conserved region within the HIV-1 integrase section of the *pol* gene. The HIV-1 RNA of HIV-1 group M and HIV-1 group O can be quantified over the range of 3.7×10^1 (lower limit of detection) to 1.1×10^7 copies/ml. Positive results below the lower limit of detection are referred to as “weak positive”. The Versant HIV-1 RNA-Assay 1.0 (kPCR) is performed on

two automated instruments, the sample preparation (SP) module and the amplification and detection (AD) module.

The HIV-1 RNA is extracted on the VERSANT SP Module (Figure 3-1). In the first step, the reagents (the enzyme mix, the primer mix, the internal control (IC), two calibrators, and three external controls) are placed into the control carrier. After this, the sample tubes containing approximately 650µl (depending on the sample vial) of EDTA plasma are loaded onto the sample carrier. A maximum of 96 samples can be tested simultaneously. For the lysis step, 20µl of proteinase K, 500µl of the plasma sample, 825µl of lysis buffer, 25µl of magnetic silica beads, and 15µl of the IC are mixed automatically by the SP module. The HIV-1 RNA is released from the viral capsid and binds, together with the IC, to the magnetic beads. Three wash steps remove remaining plasma components (e.g. proteins). Then, 70µl of elution buffer are added and the whole dilution is incubated for 16 minutes. Finally, 55µl of the eluate is transferred into a well of the PCR plate which contains the primer/probe mix (buffer, dNTPs, and synthetic oligonucleotides and probes specific for HIV-1) and the enzyme mix (reverse transcriptase, DNA polymerase, and uracil N glycosylase to control contamination).

Figure 3-1: The VERSANT SP Module.

The PCR plate is manually placed into the AD module which performs real-time PCR and detection (Figure 3-2). In a first step, the PCR plate is heated by the thermo cycler of the AD module. Then, reverse transcription and amplification by

real-time PCR is done simultaneously. In a final step, detection and quantitation of the amplification products are provided by use of the TaqMan technology [33].

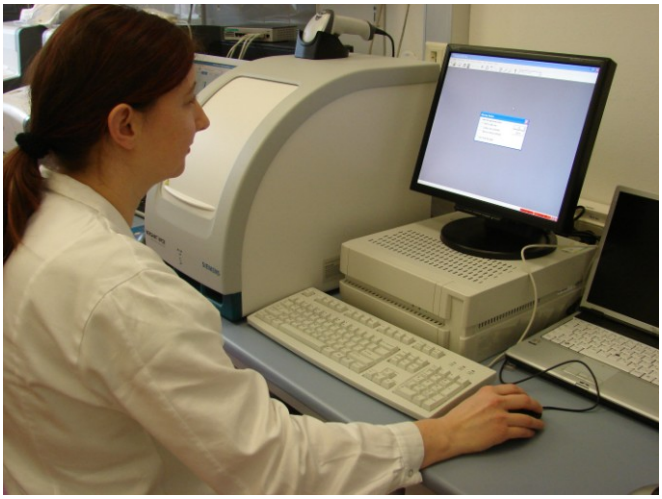


Figure 3-2: The VERSANT AD Module.

3.2.2 The COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0

The COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 is a reverse transcription and nucleic acid amplification test, using real-time PCR for quantitation of HIV-1 RNA in human EDTA plasma samples. This molecular assay utilizes highly conserved regions of both the HIV-1 *gag* gene and the HIV-1 *LTR* region. It allows quantitation of HIV-1 RNA of HIV-1 group M and HIV-1 group O over a range of 2.0 x 10^1 (lower limit of detection) to 1.0 x10^7 copies/ml. Positive results below the lower limit of detection are referred to as “weak positive”. The COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 is performed on two instruments, the COBAS AmpliPrep Instrument for automated specimen processing and the COBAS TaqMan Analyzer for automated amplification and detection.

The HIV-1 RNA is extracted on the COBAS AmpliPrep Instrument (Figure 3-3). In the first step, 850 µl of EDTA plasma, the lysis reagent, the protease, a known number of HIV-1 Quantitation Standard (QS; armored RNA molecules), and magnetic glass particles are mixed and incubated. The HIV-1 RNA and the HIV-1 QS bind to the surface of the magnetic glass particles. By several washing steps,

remaining plasma components (e.g. proteins) are removed. Finally, the processed specimens are added to the specially designed amplification tubes containing the amplification mix.

Figure 3-3: The COBAS AmpliPrep.

The carrier containing the specially designed amplification tubes is transferred manually to the COBAS TaqMan Analyzer (Figure 3-4). There, the mixture is heated and reverse transcription and amplification occur simultaneously. For detection of amplification products, the TaqMan technology is used [34].

Figure 3-4: The COBAS TaqMan.

3.3 Goals of this Study

- To evaluate the performance of the VERSANT HIV-1 RNA Assay 1.0 (kPCR).
- To compare results obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) with those obtained with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0.

The performance of the VERSANT HIV-1 RNA Assay 1.0 (kPCR) was evaluated using verification steps as recommended recently [35]. Verification steps included evaluation of accuracy, linearity, and interassay and intra-assay imprecision.

The accuracy is the degree of conformity of the measured quantity to its real value. Derived from comparison of the results with those obtained with a reference method, an error value is calculated and quoted as $\log_{10}$ unit difference. To determine accuracy of values obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR), the Quality Control for Molecular Diagnostics (QCMD) 2009 HIV-1 RNA proficiency panel (www.qcmd.org) was used as the reference material. This panel consists of 10 plasma samples containing various concentrations of HIV-1 RNA, calculated from all data returned by participants of QCMD. The accuracy of values obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) was expressed by the log difference to those stated for the members of the QCMD panel.

The linearity, defined as the linear range of quantification, was determined by a dilution series. A high-titer routine clinical sample was diluted with a plasma sample negative for HIV-1 RNA in 0.5log steps and used for detection with the VERSANT HIV-1 RNA Assay 1.0 (kPCR). Each dilution was tested three times. Linearity was further characterized employing the equation:

$y = a \pm b*x$

a … if different from 1, the deviation is a measure for debility in linearity

b … if different from 1, it gives the multiplying factor of the linear quantity offset

With regression analysis, an error term was calculated, representing the variation of experimental data around the ideal straight. The closer this regression coefficient is to 1, the better is the linearity.

The interassay imprecision is the level of deviation of results from one run to another. This was ascertained by testing eight samples with different concentrations of HIV-1 RNA five times on five days. The intra-assay imprecision, i.e. the level of deviation of results within a single run, was tested by analyzing four samples with various concentrations of HIV-1 RNA five times each in a single run. Both the interassay and the intra-assay imprecision are expressed numerically as the standard deviation or the coefficient of variation of the results.

4 Results

4.1 Evaluation of the VERSANT HIV-1 RNA Assay 1.0 (kPCR)

When 10 members of the QCMD 2009 HIV-1 RNA proficiency panel were tested with the VERSANT HIV-1 RNA Assay 1.0 (kPCR), both of the negative members were found to be negative. Of 8 positive members, 6 gave values within ±0.5$\log_{10}$ unit of the expected panel results. Another two positive members of the QCMD panel tested weak positive with the VERSANT HIV-1 RNA Assay 1.0 (kPCR).

Testing of linearity showed a quasilinear curve from 3.4 x 10^1 to 3.4 x 10^5 HIV-1 RNA copies/ml with a regression coefficient of 0.982 (Figure 4-1).

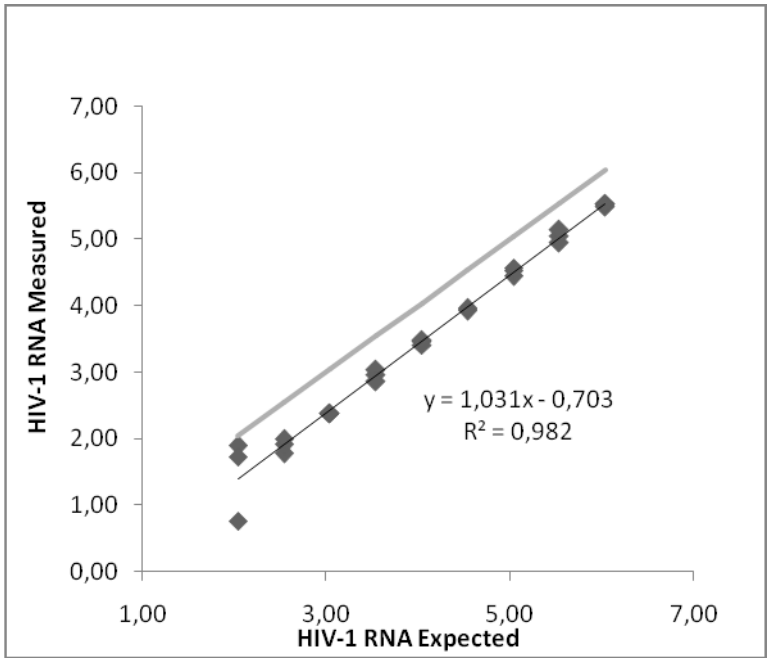


Figure 4-1: Linearity testing.

The coefficients of variations of the interassay imprecision varied from 12 to 20% while those of the intra-assay variation were found to be between 8 and 16% Table 4-1 and Table 4-2).

Sample no.	Copies of HIV-1 RNA/ml detected		Coefficient of variation (%)
	Mean	*Standard Deviation*	
1	3.2×10^2	6.4×10^1	20
2	4.4×10^2	6.7×10^1	15
3	6.2×10^2	9.6×10^1	15
4	3.2×10^3	4.7×10^2	15
5	9.5×10^3	1.1×10^3	12
6	3.1×10^4	4.6×10^3	15
7	1.0×10^5	1.9×10^4	19
8	2.3×10^5	3.1×10^4	13

Table 4-1: Results of interassay testing.

Sample no.	Copies of HIV-1 RNA/ml detected		Coefficient of variation (%)
	Mean	*Standard Deviation*	
1	4.2 x 101	6.6 x 100	16
2	1.1 x 102	1.0 x 101	9
3	4.0 x 102	3.1 x 101	8
4	6.4 x 103	9.8 x 102	15

Table 4-2: Results of intra-assay testing.

4.2 Comparison of molecular assays

A total of 196 human plasma samples including various HIV-1 subtypes were tested with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. The VERSANT HIV-1 RNA Assay 1.0 (kPCR) gave an invalid result for three samples, which were excluded from further investigation. Of the remaining 193 samples, there were consistent results in 164 samples: 136 samples were found to be positive, 4 samples weak positive, and in 24 samples the target was not detected. The remaining 29 samples showed discrepant results. Two samples were not detected with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) but gave positive results with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. Seven samples showed weak positive results with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 but the target was not detected with the VERSANT HIV-1 RNA Assay 1.0 (kPCR). Another 14 samples gave positive results with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 but showed weak positive results with the VERSANT HIV-1 RNA Assay 1.0 (kPCR). Four samples gave weak positive results with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) while they were not detected with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. The VERSANT HIV-1 RNA Assay 1.0 (kPCR) reported 2 samples as positive which were not detected with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. There were no samples with positive results obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) and weak positive results with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. The majority of discrepancies were found with samples containing HIV-1 subtype G, followed by CRFs. The majority of results obtained from samples containing HIV-1 subtypes A or B were concordant (Table 4-3).

		VERSANT HIV-1 RNA Assay 1.0 (kPCR)		
		Positive	*Weak positive*	*Target not detected*
COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0	*Positive*	136	14	2
	Weak positive	0	4	7
	Target not detected	2	4	24

Table 4-3: Results obtained with the two molecular assays.

Except for a single sample, all results obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR) were found to be lower than those obtained with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. This sample contained a circulating recombinant form of HIV-1. The median difference of results obtained with both of the molecular assays was -0.58log_{10} unit. Results obtained from 7 samples showed deviations of more than ±1.0log_{10} unit; results obtained from 78 samples were found to be within ±0.5 and ±1.0log_{10} unit and results obtained from 50 samples (including the sample with a higher result obtained with the VERSANT HIV-1 RNA Assay 1.0 (kPCR)) were found to be within ±0.5log_{10} unit. When results obtained from samples with different HIV-1 subtypes were compared, the median difference ranged from -0.36log_{10} unit in samples with HIV-1 subtype G and -0.67log_{10} unit in samples with CRFs (Table 4-4).

HIV-1 subtype	***Median log_{10} unit difference***
A	-0.56
B	-0.63
C	-0.64
D	-0.58
F	-0.42
G	-0.36
CRF	-0.67

Table 4-4: Median difference between results.

When viral loads of 136 plasma samples were compared, a high correlation (R^2 = 0.94) was found (Figure 4-2). The regression coefficient ranged from 0.8895 in samples with HIV-1 subtype B (Figure 4-3) to 0.9825 in samples with HIV-1 subtype F (Figure 4-4).

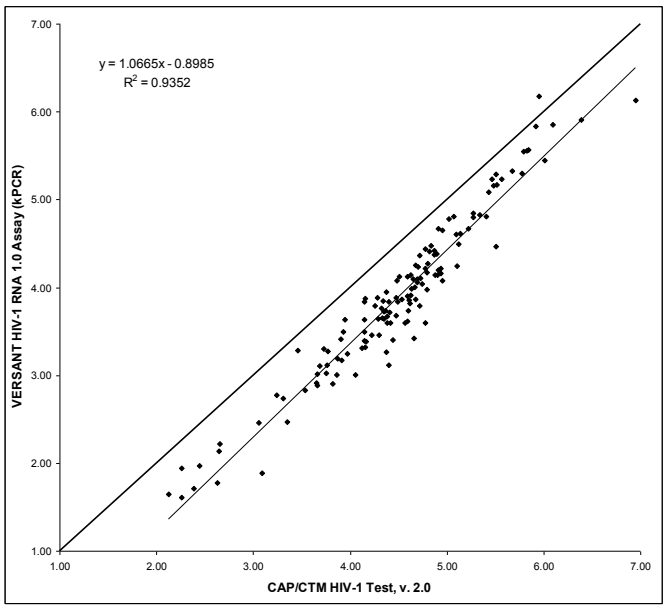


Figure 4-2: Correlation between the results for 136 plasma samples.

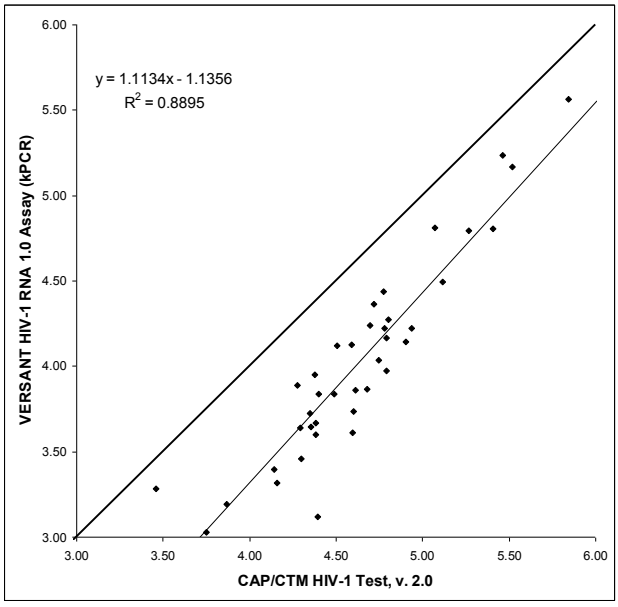


Figure 4-3: Correlation between the results for 41 plasma samples (HIV-1 subtype B).

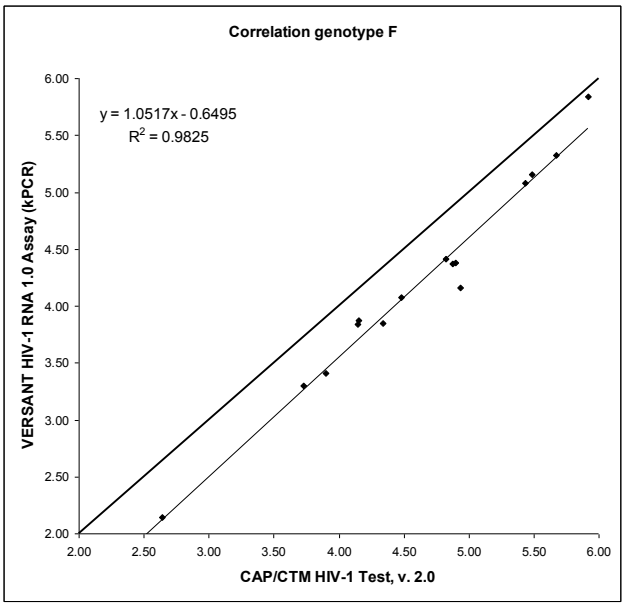


Figure 4-4: Correlation between the results for 16 plasma samples (HIV-1 subtype F).

5 Discussion

HIV is among the five most hazardous infectious diseases worldwide, besides infectious diarrhea, pneumonia, malaria, and tuberculosis [14]. Once infected, there is no escape from developing AIDS.

As long as the rate of new HIV infections and the number of people living with HIV increases rapidly, the detection of the virus remains to be targeted. Besides the early detection of infected people, providing an established and clinically validated tool for controlling the progress of already infected patients is necessary. The quantitation of HIV-1 RNA in human plasma represents a reliable method, for both timing of initial therapy and monitoring treatment response. Based on measuring the increase of the plasma HIV-1 load, clinicians can decide if their patients need antiretroviral therapy or if a switch in medication is required [36].

Today, several techniques for quantifying the viral load are commercially available. Molecular assays are based on target (real-time PCR or nucleic acid sequence based amplification) or signal amplification (branched DNA) techniques. The use of the identical assay for therapeutic monitoring is required to ensure comparability of the results. Low detection limit and rapidity have made real-time PCR playing the leading role on the market [37].

For the use in high-throughput laboratories, a maximum of automation and standardization is required. Therefore, automated sample preparation systems have been introduced. In comparison to quantitative assays using manual sample preparation, they show less hands-on time, quicker results, and low labor-intensity [38].

The first fully automated quantification system on the market was the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0. It combines automated extraction of nucleic acids, performed on the COBAS AmpliPrep instrument, with a real-time PCR, accomplished by the COBAS TaqMan analyzer. A sequence derived from a highly conserved region within the HIV-1 *gag* gene is used as target. The quantification standard included in this assay enables avoiding contamination and inhibition. It has been shown that the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0 provides high sensitivity, specificity, and accuracy [39]. Additionally, results were found to be strongly

correlated with other PCR-based tests. The broad measuring range and the reliable quantification of HIV-1 RNA also contribute to the advantages when using this assay.

A study of Guedin et al. compared the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0 with the Abbott RealTime HIV-1 (m1000sp + m2000rt) [40]. The Abbott system targets the *pol* region of HIV-1 RNA. Plasma samples belonging to HIV-1 group M, HIV-1 group O, and HIV-2 were tested. The mean HIV-1 load obtained from HIV-1 group M supernatants was found to be 0.51 $\log_{10}$ unit lower with the COBAS system. One supernatant (subtype G) was not detected by the COBAS system, even when diluted 10^{-3} and therefore excluded from statistical analysis. The Abbott RealTime HIV-1 detected 7 of 8 HIV-1 group O supernatants, while the COBAS system did not detect any. However, it must be noted that the COBAS system has been designed for HIV-1 group M exclusively. Both systems gave negative results when testing HIV-2 which is within expectations considering their genetic diversity. The COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0 underestimated nearly half of the samples studied. The median log difference was especially high when recombinant forms were tested. These results underline the importance of specific primer and probe selection. Another study even questioned the reliability of the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 1.0 for viral load quantification in HIV-1 infected patients with non-B subtypes [41].

To overcome these concerns, the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 was developed and brought on the market. It uses the highly conserved regions of the HIV-1 *gag* gene and, additionally, the HIV-1 *LTR* region to guarantee improved sensitivity and more quantifiable results. According to Scott et al., the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 was able to quantify 38.6% more samples than the previous version, extending the dynamic range from $4 \times 10^1 - 1 \times 10^7$ copies/ml to $2 \times 10^1 - 1 \times 10^7$ copies/ml [42]. Furthermore, detection of HIV-1 group O should be possible with this assay.

Recently, Siemens introduced the VERSANT HIV-1 RNA Assay 1.0 (kPCR), utilizing a highly conserved region within the HIV-1 integrase section of the *pol* gene. In this study, the VERSANT HIV-1 RNA Assay 1.0 (kPCR) was evaluated and results were compared with those obtained with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0. The new assay provided

reliable results regarding linearity, accuracy, and interassay and intra-assay variability. Compared to the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0, results of the VERSANT HIV-1 RNA Assay 1.0 (kPCR) were found to be lower, with a median log difference between both assays of −0.58 $\log_{10}$ unit but without any consequence on detection of samples close to the lower limit of detection. Both of the assays may thus be used for monitoring of antiretroviral therapy. However, it must be noted again that one and the same patient must be followed-up by the identical assay. If a switch to another assay is inevitable, re-baselining is required.

Due to the increasing spread of genetic diversity, reliable detection of rare HIV-1 subtypes and recombinant forms is required. In this study, the two assays compared showed a high correlation of results for all HIV-1 subtypes tested. This is in contrast to another study reporting severe discrepancies in detection of HIV-1 subtype G and CRFs [43].

In conclusion, the new VERSANT HIV-1 RNA Assay 1.0 (kPCR) was found to be suitable for monitoring viral load of patients with antiretroviral therapy. Because of its high level of automation it is useful for high-throughput laboratories.

6 Future Aspects

Molecular diagnosis will remain the gold standard to monitor the plasma HIV load in patients with antiretroviral therapy in the near future. Major efforts of the diagnostic industry aim at the further development of established products. Providing maximum standardized and automated assays with an extended dynamic range are among the major goals of development.

Other attempts in the fight against HIV focus on the improvement of clinical guidelines. In the United States, the screening for HIV of all adolescents and adults with increased risk for HIV infection is strongly recommended by the U.S. Preventive Services Task Force [44]. This includes men who have sex with men, persons having unprotected sex with multiple partners, past or present intravenous drug users, people being treated for STDs, and persons with a history of blood transfusion between 1978 and 1985. These services should improve health outcomes and the benefits thereof outweigh its harms. Complete screening of the population is recommended especially in regions with at least 1% HIV prevalence.

Across European countries, differences in testing strategies and practices can be noticed. Most European countries offer routine HIV testing in clinics, largely anonymous and free of charge. In contrast, testing of pregnant women is not ensured in the majority of countries. The discussion about making HIV testing part of routine clinical practice is still in progress but no coherent or comprehensive HIV testing strategy has been established yet [45].

In developing countries with limited resources, HIV testing is restricted by cost and lack of infrastructure. Therefore, techniques employing a simple and inexpensive format to detect infections early are sought-after. Newly introduced rapid HIV antibody tests are inexpensive, can be interpreted visually, and do not require further instruments [46]. They can be done by laymen and show high sensitivity and specificity. These tests give widespread access to testing and may play an important role in reducing new infections in future.

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8 List of Figures and Tables

8.1 Figures

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9 Abbreviations

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
CCR	chemokine receptor
CD	cluster of differentiation
CDC	Centers for disease control and Prevention
CNS	central nervous system
CRF	circulating recombinant form
CXCR	chemokine receptor
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dsDNA	double strand DNA
dNTP	deoxynucleoside triphosphate
ELISA	enzyme-linked immunosorbent assay
env	envelope
gag	group antigen
gp	glycoprotein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV	human T lymphotropic virus
IC	internal control
LAS	lymphadenopathy syndrome
LAV	lymphadenopathy-associated virus
LTR	long terminal repeats
NASBA	nucleic acid sequence based amplification
nef	negative factor
NNRTI	nucleoside/nucleotide reverse transcriptase inhibitors
NRTI	non-nucleoside reverse transcriptase inhibitors
PCR	polymerase chain reaction
kPCR	kinetic polymerase chain reaction
rtPCR	real-time polymerase chain reaction
PI	protease inhibitor
pol	polymerase
PR	protease

QCMD	quality control for molecular diagnostics
QS	Quantitation Standard
rev	regulator of virion
RNA	ribonucleic acid
RT	reverse transcriptase
STD	sexual transmitted disease
tat	transactivator
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U

10 Curriculum Vitae

Personal Information	
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