Institute of Tropical Medicine
Department of Microbiology
Virology Unit
Nationalestraat 155
Antwerp, 2000
Belgium

Medical Research Council
Fajara, PO box 273
The Gambia

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Development and Application of Assays for Monitoring Drug Resistance in HIV-2 Infected Patients on Antiretroviral Therapy

Ontwikkeling en toepassing van testen voor het aantonen van drug resistentie in HIV-2 geïnfecteerde patiënten op antiretrovirale therapie.

Dissertation for the degree of doctor in Biomedical Sciences at the University of Antwerp to be defended by

Sabelle JALLOW

Promoter: **Prof. Guido Vanham**
Co-Promoters: **Prof. Sarah Rowland-Jones**
Dr. Wouter Janssens

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Sabelle Jallow

Development and Application of Assays for Monitoring Drug Resistance in HIV-2 Infected Patients on Antiretroviral Therapy

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This thesis is dedicated

TO MY FAMILY

I thank God for all I have and all that I am, but especially for my family without whom, I would be completely lost.

TO REGINA SHIKU NYAMU

(1973-2003)

A friend, a sister of my heart and a most beautiful soul.

May you rest in peace.

I allow myself to make the mistakes I need in order to grow and become better. I see that with the bumps and falls that I am refined, the edges softened- the internal beauty revealed.~Anon

List of Abbreviations

3TC	:	Lamivudine
A	:	Adenine
aa	:	Amino acids
Ab	:	Antibody
AIDS	:	Acquired Immunodeficiency Syndrome
AP	:	Alkaline Phosphatase
ABC	:	Abacavir
AMV	:	Avian Myeloblast Virus
APV	:	Amprenavir
ART	:	Anti-RetroTherapy
ARV	:	Anti-RetroVirals
ATV	:	Atazanavir
AZT/ZDV	:	Azidothymidine / Zidovudine
AZT-TP	:	Azidothymidine Triphosphate
b	:	Base
bp	:	Base pair
C	:	Cytosine
CA	:	Capsid
CD4	:	Cluster of Differentiation 4
cDNA	:	Complementary DNA
cpx	:	Complex
CRF	:	Circulating Recombinant Form
D/dig	:	Digoxigenin
d4T	:	Stavudine
ddC	:	Zalcitabine
ddI	:	Didanosine
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide Triphosphates
DLV	:	Delavirdine
DRV	:	Darunavir
ds	:	Double Stranded

env	:	Envelope
EFV	:	Efavirenz
ETR	:	Etravirine
F/flu	:	Fluoresein
FDA	:	Food and drug administration
FI	:	Fusion inhibitor
Fig	:	Figure
FOS-APV	:	FOS-amprenavir
G	:	Guanine
g	:	Grams
gag	:	Group Antigen
GALT	:	Gut-associated lymphoid tissue
gp	:	Glycoprotein
gp120	:	Surface Envelope Glycoprotein
gp160	:	Precursor Envelope Polyglycoprotein
gp41	:	Transmembrane Envelope Glycoprotein
H2O	:	Water
HAART	:	Highly active antiretroviral therapy
HIV	:	Human Immunodeficiency Virus
HIV-1	:	Human Immunodeficiency Virus Type 1
HIV-2	:	Human Immunodeficiency Virus Type 2
HTLV-III	:	Human T-lymphotropic Virus Type 3
HTLV-IV	:	Human T-lymphotropic Virus Type 4
IC50	:	50% inhibitory concentration
IC90	:	90% inhibitory concentration
IDU	:	Intravenous Drug Users
IDV	:	Indinavir
INI	:	Integrase inhibitors
IRF	:	Inter-subtype Recombinant Forms
Kb	:	Kilo base
KD	:	Kilo Dalton

LAV	:	Lymphadenopathy-Associated Virus
LPV	:	Lopinavir
LPV/r	:	Ritonavir-boosted Lopinavir
LTR	:	Long terminal repeat
M group	:	Major group
M	:	Molar
MA	:	Matrix
MHC	:	Major Histocompatibility Complex
mRNA	:	Messenger Ribonucleic acid
MT	:	Mutant
MTCT	:	Mother to child transmission
NC	:	Nucleocapsid
N group	:	Non-M, Non-O group
nef	:	Negative factor
NFV	:	Nelfinavir
NRTIs	:	Nucleoside reverse transcriptase inhibitors
NNRTIs	:	Non-nucleoside reverse transcriptase inhibitors
NSI	:	Non-Syncytia Inducing
NVP	:	Nevirapine
OD	:	Optical density
OH	:	Hydoxyl group
O group	:	Outlier group
OLA	:	Oligonucleotide Ligation Assay
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PIs	:	Protease inhibitors
POD	:	Peroxidase
pol	:	Polymerase
PR	:	Protease

rev	:	Regulatory protein
RNA	:	Ribonucleic acid
RNase A	:	Ribonuclease A
RNase H	:	Ribonuclease H
RRE	:	rev responsive element
RT	:	Reverse transcriptase
RTI	:	Reverse transcriptase inhibitor
RT-PCR	:	Reverse Transcriptase PCR
RTV	:	Ritonavir
SHIV	:	SIV/HIV hybrid
SI	:	Syncytia Inducing
SIV	:	Simian Immunodeficiency Virus
SIVcpz	:	SIV of Chimpanzee
SIVgsn	:	SIV of Greater snot-nosed monkeys
SIVsm	:	SIV of Sooty Mangabey
SIVmac	:	SIV of Sooty Macaque
SIVsm	:	SIV of Sooty Mangabey
SQV	:	Saquinavir
T	:	Thymidine
TAMs	:	Thymidine-analogue resistance mutations
Taq	:	Thermos Aquaticus
tat	:	Transactivator
TDF	:	Tenofovir disoproxil Fumarate
TPV	:	Tipranavir
TM	:	Transmembrane
TMB	:	3,3',5,5'-tetramethylbenzidine
TRIS	:	Tris Hydroxyl Methyl Amino Methane
µM	:	Micro Molar (x 10^{-6})
µl	:	micro litres (x 10^{-6})
UNAIDS	:	The Joint United Nations Programme on HIV/AIDS
UV	:	Ultra Violet
V	:	Volts
V3	:	Third variable domain

vif	:	Viral infectivity factor
VL	:	Viral load
vpr	:	Viral protein R
vpu	:	Viral protein U
vpx	:	Viral protein X
WHO	:	World Health Organization
WT	:	Wild type

AMINO ACID SYMBOLS

A	:	Alanine
C	:	Cytosine
D	:	Aspartic acid
E	:	Glutamic acid
F	:	Phenylalanine
G	:	Glycine
H	:	Histidine
I	:	Isoleucine
K	:	Lysine
L	:	Leucine
M	:	Methionine
P	:	Proline
Q	:	Glutamine
R	:	Arginine
S	:	Serine
T	:	Threonine
V	:	Valine
W	:	Tryptophan
Y	:	Tyrosine

Summary

HIV infection has proven to be one of the most devastating human diseases, resulting in significant increases in morbidity and mortality throughout the world. Despite world-wide efforts, a vaccine is still not available. However, in 1987, the first antiretroviral (ARV) drug was introduced. Since then, several breakthroughs have been accomplished, which resulted in the use of a combination of three or more ARV agents (Highly Active AntiRetroviral Therapy or HAART) to effectively treat HIV infected individuals. The use of HAART has resulted in dramatic decreases in morbidity and mortality, slowing down disease progression and prolonging survival of treated individuals. As such, it has changed the face of this disease from a death sentence into a treatable, chronic infection. However, incomplete viral suppression due to the high mutation rate of HIV and the use of suboptimal therapy, results in drug resistance, which may limit both the magnitude and the duration of the response to treatment.

According to WHO and UNAIDS, HIV has been most devastating in Sub-Saharan Africa, where by the end of 2007, 76% of global AIDS deaths were found. While HAART has reduced mortality in developing countries, high cost of ARVs and insufficient resources in developing countries have limited access to treatment. However, world-wide efforts to increase ARV access to developing nations has resulted in more than 2 million people being treated by the end of June 2006.

HIV-2 was discovered two years after HIV-1 and has been restricted mainly to West Africa, where an estimated 1 million people are infected. High numbers of HIV-2 infection have also been reported in two regions in Southern Africa, Portugal, France and India. In addition, a slow, but continuous spread to all continents has been observed. While HIV-2 is very similar to HIV-1, the majority of HIV-2 infected patients are asymptomatic and have a normal life span with no signs of immunodeficiency. However, 10-15% of HIV-2 patients have a clinical course that is indistinguishable from HIV-1 and therefore need treatment.

Treatment of HIV-1 with optimal regimens and vast knowledge about drug resistance has lead to very efficient management of HIV-1 infection. HIV-1 and HIV-2 share a high sequence similarity especially in their crucial enzymes, the protease (50%), reverse transcriptase (60%) and integrase (60%). These HIV-1 and HIV-2 enzymes are also similar in structure and enzymatic function. Thus, ARVs designed for HIV-1 were expected to be

active against HIV-2. However, HIV-2 is naturally resistant to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and the entry inhibitor T-20; and reduced susceptibility to some protease inhibitors (PIs) has been observed. This is mainly due to the presence of signature HIV-1 drug resistance mutations as natural polymorphisms in HIV-2.

The lower prevalence of HIV-2 relative to HIV-1 and the restriction of the HIV-2 epidemic mainly to West Africa, where treatment has largely been unavailable; has resulted in very limited studies on HIV-2 therapy and the development of resistance. However, a few studies have reported that various HAART regimens fail to suppress HIV-2 viral replication to undetectable levels. With increasing ARV access in Africa, it is important to understand the response of these patients to therapy, to document clinically relevant resistance mutations and to develop and evaluate simple and affordable assays that will be more sustainable for use in resource-limited settings.

Before the advent of HAART in the Gambia, a preliminary dual therapy trial was done with eight HIV-2 infected patients for up to 7 years. We monitored the virological and immunological responses of these patients to Zidovudine (ZDV) and Lamivudine (3TC); and most of the patients failed to suppress the virus to undetectable levels in accordance with previous findings. When HAART became available in the Gambia, patients with CD4 T-cell count below 350cells/ml were treated with ZDV, 3TC and Ritonavir-boosted Lopinavir (LPV/r). We monitored 20 patients, 12 HIV-2 and 8 HIV-1/2 dually infected patients on this therapy and found that with the exception of one patient, viral load was suppressed to undetectable levels in all patients for up to 3 years. The one patient, whose viral load only went down by 2 logs, reported not taking the drugs, because they made him ill. The symptoms he described were associated with LPV/r side effects. This study shows that an efficient and durable suppression of HIV-2 replication can be achieved provided that an optimal regimen is chosen.

Treatment of HIV-2 infected individuals will most likely result in the emergence of drug resistant viruses; as has been observed in HIV-1. Genotyping by sequence analysis will allow us to comprehensively map out all potential HIV-2 drug resistance mutations associated with the ZDV and 3TC use in the reverse transcriptase (RT) region and LPV/r in the protease (PR) region. We successfully developed and optimized a genotypic resistance assay for the amplification and sequencing of the entire PR and RT gene

fragment of HIV-2 with a sensitivity of 91.3%. We used this assay to study drug resistance patterns in our treated patients.

In the dual therapy study, 7/8 patients developed drug-resistance mutations. Four major HIV-1 drug resistance mutations were observed as well as new mutations that might be unique to HIV-2. In the HAART treated patients, only one patient developed drug resistance mutations in both the RT and PR regions. In both studies, some HIV-1 resistance mutations were detected as natural polymorphisms and others were absent, suggesting that HIV-1 and HIV-2 have both similar and different pathways towards resistance.

The Oligonucleotide Ligation Assay (OLA) is a point mutation assay based on the use of differentially–labelled probes that can distinguish between wild-type and mutant sequence. This assay, developed for HIV-1 subtype B, has been shown to be highly sensitive, economical, easier to use and therefore more suitable for resource–poor settings. We contributed to the evaluation of the feasibility of OLA to detect HIV-1 drug resistance mutations at 12 codons of HIV-1 subtype B *pol,* for potential use in resource poor settings, where non-B subtypes are prevalent. Even though the assay successfully genotyped samples from both subtype B and non-B subtypes, we observed a lower sensitivity for non-B subtypes at some codons. Modified probes at codons 151 (RT), 184 (RT) and 90 (PR) increased the sensitivity to 96.1%. We further developed this assay to detect the Q151M and M184V mutations associated with phenotypic resistance to nucleoside reverse transcriptase inhibitors (NRTIs) in HIV-2. We evaluated the assay on 122 samples from different countries and found an overall sensitivity of 98.8%, with a high concordance of 98.4% (Q151M) and 97.5% (M184V) with consensus sequencing.

The high throughput nature of the Oligonucleotide Ligation Assay makes it suitable for use in epidemiological studies and clinical trials. We used the HIV-2 OLA to randomly screen 23 individuals in a cohort of HIV-2 infected individuals about to initiate HAART, for the presence of drug-resistance mutations. The Q151M mutation, associated with phenotypic resistance against most NRTI in HIV-2, was detected in two sequential samples from one individual. The OLA results were confirmed with sequencing. This finding seems to be the result of transmitted drug resistance and represents an important public health issue that would need to be addressed, to provide effective HIV-2 treatment in this population.

While genotyping gives information of genome changes in the presence of drug pressure, a phenotypic resistance assay determines the nature of the mutations and their relevance. Recombinant virus assays are currently the gold standard in terms of determining phenotypic resistance in HIV infection; as they allow characterization of individual mutations without influence or confounding effects of the rest of the genome. We developed a recombinant virus assay to evaluate the genotypic drug resistance mutations found in the HIV-2 RT during this study. We generated mutant viruses that can be used in a phenotypic assay to determine the clinical relevance of the generated mutations as well as the fitness of these mutants relative to the wild type viruses.

While our studies have contributed significantly to knowledge about HIV-2 response to therapy; the importance of selecting an optimal first line regimen; HIV-2 drug resistance and the development of assays to monitor HIV-2 drug resistance; we conclude this study with a call for randomized clinical trials in Africa to determine specific guidelines that are more appropriate for HIV-2 infected patients.

Samenvatting

Infectie met het HIV virus heeft wereldwijd tot één van de ziektes geleid die de grootste tol eist aan menselijke slachtoffers, zowel wat betreft morbiditeit als mortaliteit. Ondanks wereldwijde inspanningen is er nog steeds geen doeltreffend vaccin beschikbaar.

De introductie van een eerste antiretrovirale (ARV) drug in 1987, gevolgd door soortgelijke en alternatieve drugs, heeft geleid tot combinatie therapie (Highly Active AntiRetroviral Therapy of HAART), hetgeen een doorbraak betekende in de behandeling van HIV geïnfecteerde personen. HAART heeft een belangrijke afname teweeggebracht in morbiditeit en mortaliteit, door het afremmen van de progressie naar ziekte en de verlenging van de overlevingskansen van de behandelde personen. Dit betekent dat de diagnose van HIV-infectie niet langer een doodvonnis hoeft te zijn, maar een behandelbare chronische infectie. Een ontoereikende virale onderdrukking, te wijten aan een suboptimale therapie alsook de hoge mutatiesnelheid van HIV, kan echter leiden tot drugresistentie, die de gunstige effecten van behandeling teniet doet.

Volgens gegevens van de WGO en UNAIDS van einde 2007 is Sub-Sahara Afrika het meest getroffen door de HIV epidemie, met 76% van alle wereldwijd voorkomende sterftegevallen. Hoewel HAART het aantal sterftegevallen in de westerse wereld in belangrijke mate heeft teruggedrongen, was er in de ontwikkelingslanden, omwille van de hoge kostprijs van ARV drugs en beperkte middelen, slechts een geringe toegang tot behandeling. Wereldwijde inspanningen om de toegang tot ARV drugs te verhogen voor ontwikkelingslanden, hebben er echter toe geleid dat er tegen juni 2006 twee miljoen mensen behandeld werden.

HIV-2 werd twee jaar na HIV-1 ontdekt en zijn verspreiding is voornamelijk beperkt tot West Afrika, waar er naar schatting één miljoen HIV-2 geïnfecteerden zijn. Een hoge prevalentie aan HIV-2 infecties werd ook beschreven voor 2 regio's in zuidelijk Afrika, Portugal, Frankrijk en Indië. Daarnaast werd een geleidelijke verspeiding waargenomen naar alle continenten. Hoewel HIV-2 veel gelijkenissen vertoont met HIV-1, zijn de meerderheid van HIV-2 geïnfecteerden asymptomatisch, met een normale levensverwachting, en zonder aanwijzingen van immuundeficiëntie. Voor 10-15% van de HIV-2 patiënten is het ziekteverloop echter gelijklopend aan dat van HIV-1 geïnfecteerden, waardoor er ook een nood is aan behandeling van deze patiënten.

Een efficiënte behandeling van HIV-1 geïnfecteerden werd mogelijk door een doorgedreven kennis van drugresistentie en effectieve drug combinaties. HIV-1 en HIV-2 vertonen grote gelijkenissen in hun genetische sequentie, en meer bepaald ter hoogte van cruciale enzymen zoals het protease (PR; 50%), het reverse transcriptase (RT; 60%) en het integrase (60%). Deze HIV-1 en HIV-2 enzymen zijn tevens gelijk in structuur en enzymatische functie. Er werd daarom verwacht dat ARV drugs, die ontwikkeld werden voor HIV-1, ook actief zouden zijn tegen HIV-2. HIV-2 beschikt echter over een natuurlijke resistentie tegen non-nucleoside reverse transcriptase inhibitoren (NNRTIs) en de entry inhibitor T-20; tevens werd een verminderde gevoeligheid voor enkele protease inhibitoren (PIs) waargenomen. Dit is hoofdzakelijk te wijten aan het voorkomen van druggeïnduceerde HIV-1 resistentie mutaties als natuurlijk polymorfisme in HIV-2.

De lagere HIV-2 prevalentie in vergelijking met HIV-1, en de beperking van de HIV-2 epidemie tot voornamelijk West-Afrika waar mogelijkheden tot behandeling zeer beperkt waren, hebben ertoe geleid dat er slechts weinig studies zijn gedaan over HIV-2 behandeling en drugresistentie. Enkele studies hebben echter aangetoond dat verscheidene HAART drug combinaties er niet in slagen om HIV-2 replicatie terug te dringen tot ondetecteerbare waarden. Met de verhoogde toegang tot ARV drugs in Afrika, is het belangrijk om de responsen van deze patiënten op therapie te begrijpen, om de klinisch relevante resistentie mutaties in kaart te brengen, en om eenvoudige en betaalbare testen te ontwikkelen en te evalueren die meer geschikt zijn voor gebruik in centra waar de middelen beperkt zijn.

Voorafgaand aan de introductie van HAART in Gambië, werd een preliminaire bi-therapie trial opgestart met acht HIV-2 geïnfecteerde patiënten gedurende 7 jaar. De virologische en immunologische responsen van deze patiënten op Zidovudine (ZDV) en Lamivudine (3TC) werden in kaart gebracht. De meeste patiënten konden de virale lading niet terugdringen tot ondetecteerbare waarden, wat ook al in andere studies werd aangetoond. Met de beschikbaarheid van HAART in Gambië, werden patiënten met CD4 T-cel aantallen kleiner dan 200cellen/ml behandeld met ZDV, 3TC en Lopinavir/ Ritonavir (LPV/r). Er werden 12 HIV-2 en 8 HIV-1/2 dubbel geïnfecteerde patiënten op deze therapie geplaatst. Met uitzondering van één patiënt werd de virale lading in alle patiënten drie jaar onderdrukt tot ondetecteerbare waarden. De patiënt, wiens virale lading slechts 2 logs gereduceerd werd, gaf aan dat hij de drugs niet nam aangezien hij er ziek van werd. De symptomen van de nevenwerkingen werden gerelateerd tot LPV/r.

Deze studie toont aan dat een efficiënte en langdurige onderdrukking van HIV-2 replicatie kan bekomen worden mits een optimale drug combinatie wordt aangewend.

De behandeling van HIV-2 geïnfecteerde personen zal hoogst waarschijnlijk aanleiding geven tot de ontwikkeling van drugresistente virussen; zoals werd waargenomen voor HIV-1. Genotypering door sekwentie analyse zal ons in staat stellen om alle potentiële HIV-2 drug resistentie mutaties in RT en PR, die respectievelijk gerelateerd zijn tot ZDV en 3TC gebruik, en LPV/r gebruik, in kaart te brengen. We zijn er in geslaagd om een genotypische resistentietest te ontwikkelen door amplificatie en sekwentiebepaling van het volledige RT en PR genfragment. De test werd geoptimaliseerd en bereikte een sensitiviteit van 91.3%. We gebruikten deze test voor de studie van drugresistentie patronen in onze behandelde patiënten.

In de bi-therapie studie ontwikkelden 7 van de 8 patiënten drugresistentie mutaties. Vier belangrijke HIV-1 drugresistentie mutaties werden waargenomen alsook nieuwe mutaties die mogelijk uniek zijn voor HIV-2. In de HAART behandelde patiënten ontwikkelde slechts één patiënt drugresistentie mutaties in zowel RT als PR. In beide studies werden bepaalde HIV-1 resistentie mutaties aangetroffen als natuurlijke polymorfismen, terwijl andere afwezig waren. Dit suggereert dat HIV-1 en HIV-2 over zowel gelijkaardige als verschillende ‘pathways’ beschikken die leiden tot ontwikkeling van resistentie.

De Oligonucleotide Ligatie Assay (OLA) is een punt mutatie test die berust op het gebruik van verschillend gelabelde probes die een onderscheid kunnen maken tussen wild-type en mutante sekwenties. Deze test, die initieel ontwikkeld werd voor HIV-1 subtype B, is zeer sensitief, goedkoper en eenvoudiger in gebruik in vergelijking met sekwentiebepaling, en daarom meer geschikt voor centra met beperkte middelen. We namen deel aan de evaluatie van deze test voor detectie van HIV-1 drugresistentie mutaties ter hoogte van 12 codons van het HIV-1 subtype B *pol*, voor potentieel gebruik in ontwikkelingslanden waar niet-B subtypes prevalent zijn. Hoewel genotypering van zowel subtype B als niet-subtype B stalen mogelijk was met de test, merkten we een lagere sensitiviteit voor sommige codons van niet-subtype B stalen. Geoptimaliseerde probes voor codons 151 (RT), 184 (RT) en 90 (PR) verhoogden de sensitiviteit tot 96.1%. We ontwikkelden ook een OLA voor detectie van de Q151M en M184V mutaties die geassocieerd zijn met fenotypische resistentie tegen nucleoside reverse transcriptase inhibitoren (NRTIs) in HIV-2. Evaluatie van de test op 122 stalen van verschillende

landen gaf een sensitiviteit van 98.8% aan, met een hoge overeenkomst van 98.4% (Q151M) en 97.5% (M184V) met consensus sekwentiebepaling.

De hoge verwerkingscapaciteit van de Oligonucleotide Ligatie Assay maakt deze test geschikt voor gebruik in epidemiologische studies en klinische trials. We gebruikten de HIV-2 OLA voor een random screening van 23 personen in een cohort van HIV-2 geïnfecteerden, naar de aanwezigheid van drugresistentie mutaties voorafgaand aan HAART. De Q151M mutatie, die geassocieerd wordt met fenotypische resistentie tegen de meeste NRTI in HIV-2, werd gedetecteerd in twee opeenvolgende stalen van één persoon. De OLA resultaten werden bevestigd door sekwentiebepaling. Aangezien deze persoon zich aanvankelijk niet bewust was van zijn seropositiviteit en ook geen toegang had tot ARV, wijst dit resultaat op transmissie van drug-resistent virus. Dit is een nieuw gegeven waarmee rekening moet gehouden worden voor de volksgezondheid, om een effectieve behandeling te geven aan deze populatie.

Hoewel genotypering informatie kan verstrekken over veranderingen in het genoom onder druk van drugs, documenteert een fenotypische resistentietest de natuur van mutaties en hun relevantie. Recombinante virus testen worden als gouden standaard beschouwd met betrekking tot het bepalen van fenotypische resistentie in HIV infectie, aangezien zij de karakterisatie toelaten van individuele mutaties zonder rekening te moeten houden met de invloed van de rest van het genoom. We ontwikkelden een recombinante virus test om de genotypische drugresistentie mutaties te evalueren die we documenteerden in het HIV-2 RT tijdens deze studie. We genereerden mutante virussen die kunnen gebruikt worden in een fenotypische test om de klinische relevantie van de gegenereerde mutaties alsook de fitness (replicatie capaciteit) van deze mutanten in vergelijking met de wild type virussen, te bepalen.

Onze studie heeft een significante bijdrage geleverd aan de kennis van responsen van HIV-2 geïnfecteerde patiënten op therapie; het belang van de selectie van een optimale eerstelijns therapie; HIV-2 drugresistentie en de ontwikkeling van testen om HIV-2 drugresistentie in kaart te brengen. We besluiten deze studie met een oproep tot gerandomiseerde klinische trials in Afrika die moeten leiden tot specifieke richtlijnen voor een efficiënte behandeling van HIV-2 geïnfecteerden.

Chapter 1

General introduction

HIV/AIDS: HISTORY AND OVERVIEW

The first awareness of HIV/AIDS occurred around the early 1980's, when clinicians in California and New York noticed a high incidence of a rare form of cancer, Kaposi's Sarcoma, as well as severe opportunistic infections such as Pneumocystis carinii pneumonia, in men who have sex with men (MSM) [1, 2].

The etiological agent of AIDS was discovered in 1983 by a group of scientists and doctors led by Luc Montagnier at the Pasteur Institute in Paris; and named the lymphadenopathy-associated virus (LAV) [3]. A year later, a team led by Robert Gallo in the United States confirmed these findings and renamed the virus human T-lymphotropic virus type III (HTLV-III). [4]. This virus was also independently isolated from both AIDS and asymptomatic patients by a group led by Jay Levy in California and called the AIDS-related Virus [5]. This virus is now known as the Human Immunodeficiency Virus type 1 (HIV-1).

In 1986, another retrovirus was isolated from two AIDS patients from West Africa. This new retrovirus initially called LAV-2 or HTLV-IV, is known called the Human Immunodeficiency Virus type 2 (HIV-2) [6].

ORIGIN AND EPIDEMIOLOGY

The origin of HIV has been a controversial subject for several years. However, advances in molecular biology and epidemiology as well as painstaking research have shown that HIV is due to Zoonotic transmissions from monkeys to humans [7]. In Africa, many non-human primates are infected with a group of retroviruses called Simian Immunodeficiency Virus (SIV), but these viruses do not seem to cause disease in the natural hosts. The Zoonotic nature of HIV was established due to the close phylogenetic clustering of HIV-1 group M and N to SIV from the chimpanzee (*Pan troglodytes troglodytes*), clustering of HIV-1 group O to SIV from the gorilla (*Troglodytes gorilla*) and an even closer clustering of HIV-2 with SIV from the Sooty Mangabey (*Cerocebus torquatus atys*), a West African monkey [7-11].

After the initial outbreak, the Human Immunodeficiency Virus type 1 has reached pandemic proportions. According to UNAIDS/WHO, about 2.5 million people were

newly infected, 2.1 million died during 2007 and 33.2 million people were living with HIV/AIDS by the end of 2007 [12].

HIV is transmitted via direct contact with bodily fluids containing HIV, such as blood, semen or vaginal fluid; with a mucous membrane; or with the bloodstream. Transmission can occur sexually (anal, vaginal or oral sex), via blood transfusion or contaminated needles; and from mother to child either ante-, post- or perinatally.

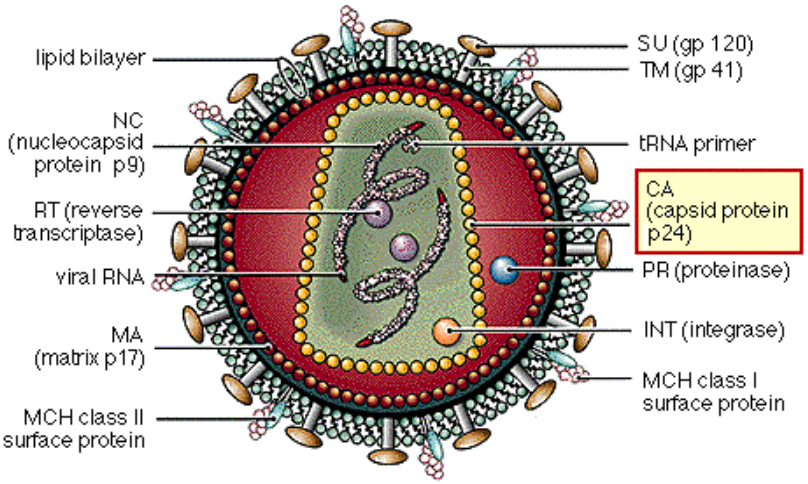
VIROLOGY

The Human Immunodeficiency Virus (HIV) belongs to a group of viruses called Retroviruses, which infect a wide range of animal species and cause a variety of diseases including: tumors, wasting, auto-immune diseases, immunodeficiency syndromes as well as aplastic and haemolytic anaemias [13, 14] . These viruses are so called because they have a unique enzyme, Reverse Transcriptase (RT) that is capable of making a DNA copy from RNA. HIV belongs to the genus Lentivirinae of the Retroviridae family [14].

Morphology

The HIV particle is a spherical enveloped virus of about 100nM in diameter (Fig. 1). Protruding from its lipid envelope are 72 glycoprotein spikes encoded by the viral *env* gene. The inner surface of the envelope is lined with matrix proteins (p17), which surround an icosahedral capsid shell (p24). Within the capsid shell is a cylindrical protein core or nucleocapsid (p7and 9), which contains 2 single genomic RNA and several copies of the enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR) [13-15].

Figure 1.1: The structural components of HIV [a]



Genomic organization

The HIV particle is a diploid RNA virus consisting of two copies of positive sense single-stranded RNA. The genome, which is about 9700 bases long, is flanked on either side by long terminal repeats (LTRs). HIV consists of structural and non-structural genes [15].

Structural genes

HIV has three structural genes namely: *gag, pol* and *env. Gag* encodes a precursor polyprotein that is cleaved post synthetically into the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 protein. *Pol* encodes the viral enzymes protease, reverse transcriptase and integrase. Protease is essential for cleavage of gag-pol polyprotein during maturation; reverse transcriptase is the hallmark of retroviruses and responsible for converting viral RNA to DNA; and Integrase inserts the proviral DNA into the host genome. *Pol* also encodes RNAase H, which degrades viral RNA from the DNA-RNA hybrid [16, 17]. *Env* encodes a 160kDa precursor glycoprotein (gp160), which is post synthetically cleaved into a surface glycoprotein, gp120 and a transmembrane glycoprotein, gp 41. These two glycoproteins, gp120 and gp41, are linked by non-covalent disulphide bridges at the surface of the viral particle [18].

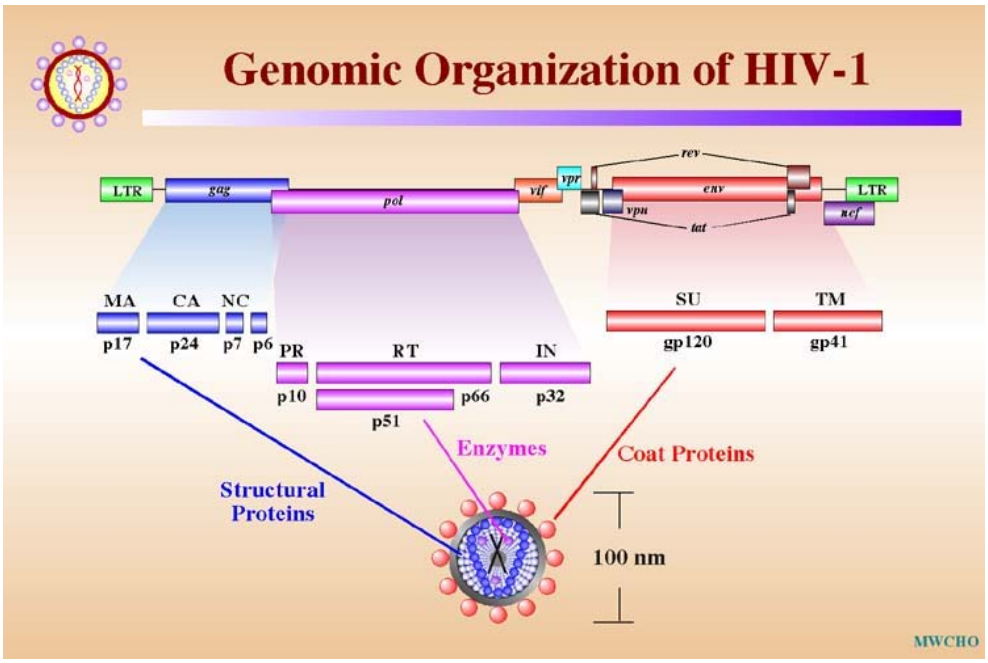


Figure 2: **Organization of HIV genome [b]**

Non-Structural genes

In addition to structural genes, HIV-1 has both regulatory and accessory genes. These genes are encoded by six overlapping open reading frames to produce three regulatory genes: nef, tat and rev; and three accessory genes: vif, vpr and vpu. These enable the virus to manipulate host cell responses and to achieve efficient replication even under host selective pressure [16, 17].

HIV GENETIC VARIATION AND CLASSIFICATION

Genetic variation

HIV is characterized by its extensive genetic variability, which accumulates at a rate of ~ 1 nucleotide substitution per genome per replication cycle [19]. The degree of variation is different for each genomic region; the *gag* and *pol* genes are more conserved and the highest variability is observed in the *env* gene, especially in the envelope glycoprotein gp 120. The highly variable and continuously evolving nature of HIV-1 accounts for the presence of a remarkable pool of genetic variants. Even within an individual, HIV-1 undergoes continuous evolution resulting in swarms of closely related, but distinct variants called quasispecies. Genetic variability gives HIV an enormous flexibility to respond to a large range of *in vivo* selective pressures, such as resistance to drugs and escape from the immune system. Genetic variation may also be reflected in differences in biological phenotype such as mode of transmission, transmissibility, pathogenesis and immunogenesis [20].

Mechanisms of HIV-1 genetic variation

The extraordinary genetic diversity of HIV is mainly due to the high error rate of the reverse transcriptase enzyme coupled with the rapid turnover of the virus and the extensive recombination that occurs during reverse transcription (Fig.3) [19, 20]. In general, RNA viruses have a higher mutation rate than DNA viruses, because their polymerases either lack or do not have adequate proof reading and repair mechanisms [21, 22]. Retroviruses are RNA viruses with an even higher mutation rate due to the high infidelity of the Reverse Transcriptase (RT) enzyme, which has an error rate of ~1 nucleotide substitution/genome/replication cycle [20]. This high mutation rate, in a genome of ~10^4 nucleotides [23], coupled with high turnover number of about 10^9 virions/day [24]; results in about 10^9 new viruses each day. In addition, HIV's high

capacity for recombination was first recognized in 1995 as a major mechanism of HIV variation [25, 26]. HIV is diploid with two copies of single-stranded genomic RNA per virion. If an individual gets infected with two independent viruses, some target cells may contain both viruses. During reverse transcription, the RT may switch back and forth between the two different RNA templates to produce a retroviral sequence that is a recombinant of the two parental strands (Fig.3); such that all subsequent progeny will have this recombinant genotype [24, 26].

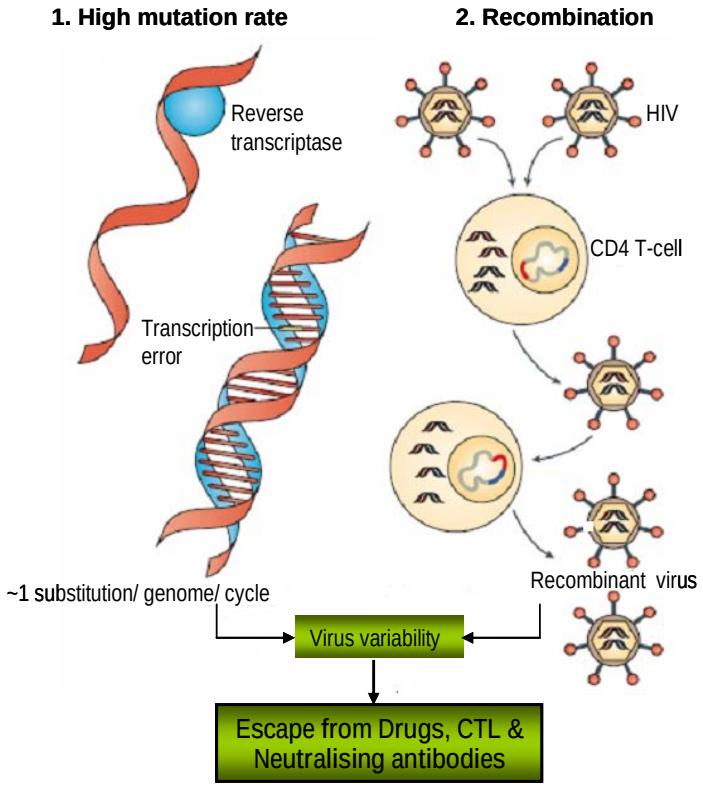


Figure 3: Mechanism of genetic variation [c]

Significance of HIV-1 genetic variation

Genetic diversity can result in differences in biological characteristics. Although HIV-1 and HIV-2 differ by ~50-60% in nucleotide sequence, these differences are manifested in significantly lower transmissibility and reduced pathogenesis in HIV-2 [13, 14, 20, 27, 28]. The genetic diversity of HIV-1 can have many important implications in public health. Diagnostic tests, antiretrovirals, drug resistance assays and HIV vaccines are developed in industrialized countries, where the most common variant is HIV-1 subtype B. These products might not perform optimally, if at all, when used in other regions such as Africa, where non-B subtypes are circulating [29].

Initially, several detection and quantification test kits had a significantly lower sensitivity for HIV-1 non-B subtypes and had to be later replaced, after being modified. In addition, HIV-2 and HIV-1 group O are naturally resistant to the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) [30]. Even within HIV-1 group M, there are variations in drug susceptibility; subtype F is less susceptible to the NNRTI, TIBO R82913 [31]; subtype G is less susceptible to protease inhibitors; and subtype D develops more rapid resistance to the NNRTI, Nevirapine than subtype A [28]. Most candidate vaccines were developed targeting various antigens of HIV-1 subtype B. The extent to which these vaccines will illicit cross protection against other subtypes is unknown [28].

Even within an individual, the presence of viral quasispecies can be both evolutionary and clinically relevant since this genetic variability allows the virus to respond to selective pressure from the host immune system or antiretroviral therapy [32]; resulting in escape mutants (fig 3).

HIV-1 Classification

The HIV-1 pandemic is recognized globally as consisting of many separate epidemics, which are classified into groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs). Phylogenic analysis has shown that HIV-1 can be divided into three groups: group M (main or major), group O (outlier) and group N (new or "Non-M, Non-O"), which cluster distinctively from one another and so each is believed to have arisen from a separate chimpanzee or gorilla to human transmission [28].

Group M is responsible for the global pandemic. Currently, there are 9 subtypes within group M, designated with letters A, B, C, D, F, G, H, J and K, which are roughly equidistant from one another with the exception of clades B and D, which are very close together [33]. The different subtypes show a 25-35% amino acid sequence difference in Env proteins and 20% in Gag proteins [10]. Recombination is now known to be a relatively common occurrence amongst different HIV-1 strains resulting in biologically viable viruses with mosaic genomes [34]. Recombinant viruses with an identical mosaic structure, descended from the same recombination event(s) and contribute to the epidemiology of HIV-1 are called circulating recombinant forms (CRFs). There are 34 CRFs designated, CRF01-CRF34 [35].

Group O or the "outlier" group was discovered in Cameroon in 1990 [36]. These viruses, which are quite rare and represent a very small percentage of HIV-1 viruses, are mainly confined to Cameroon and a few neighboring countries in West Central Africa [29].

Group N, discovered in 1998, is the most recently identified group. It clusters distinctively from groupes M and O and hence its name "Non-M, Non-O" However this group is even rarer than group O with only a few isolates discovered so far in Cameroon [37].

Geographical distribution of HIV

Distribution of HIV-1 is uneven in different geographical regions. Subtype B is predominant in Europe, North and South America, Japan and Australia; Subtype C is predominant in India and South Africa; CRF02_AG is found mainly in West Africa and the CRF01_AE is prevalent in Thailand and neighbouring countries. In sub-Saharan Africa, all subtypes are found, though subtypes A and C are the most prevalent [28]. Globally, the most predominant is subtype C, representing more than 50% of all infections; followed by subtypes A, B, D and G, which represent 12, 10, 3 and 6% of all infections respectively [38]. About 18% of HIV infections are due to circulating recombinant forms. The predominant CRFs are CRF01_AE and CRF02_AG, which represent 5% each, while the remaining CRFs form 8% of all HIV infections [38, 39].

HIV LIFE CYCLE

HIV-1 replication occurs via eight recognizable steps shown in figure 4; Attachment, penetration or entry, uncoating, replication, gene expression, assembly, release and maturation [39]. Infection starts when the virion attaches specifically to the CD4 receptor of the target cell, via its surface glycoprotein, gp120. Attachment causes a conformational change in gp120, which induces formation of a second binding site on the gp120. The virus uses this second binding site to bind to a chemokine co-receptor, either CCR5 (macrophage tropic viruses) or CXCR4 (T-cell tropic viruses), on the target cell. Binding of the virion to the co-receptor causes further conformational changes in gp120 that lead to the exposure of the fusion domain on gp 41. This domain fuses with the lipid membrane of the target cell, allowing entry into the host cell. Partial uncoating occurs during entry; it is believed that the cellular membrane protein cyclophilin A may be involved in this process [18].

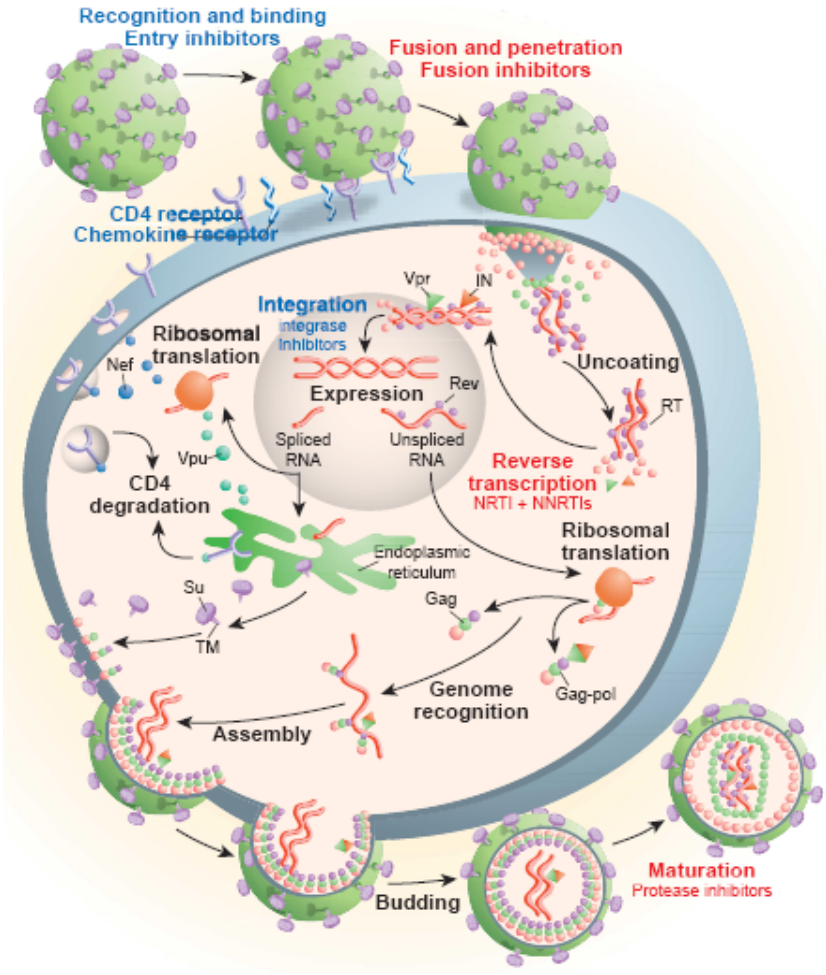


Figure 4: HIV life cycle [d]

The replication step of the life cycle is unique, in that the virus reverse transcribes its RNA genome to make a double stranded DNA copy. This viral DNA is then integrated into the host chromosome by the viral enzyme, Integrase. The integrated viral DNA is referred to as a provirus. Within the host chromosome, the provirus is transcribed into full mRNAs to produce positive-sense, single stranded RNA molecules to serve as the genomes of progeny virions; and also to produce unspliced, singly and multiply spliced mRNAs, which are translated into structural, accessory and regulatory proteins. Assembly occurs in the cytoplasm, close to the plasma membrane. Once the gag (p55) and gag-pol precursors (p160) are translated, they assemble with the genomic RNA and accessory proteins to form the viral core. The envelope proteins are glycosylated in the endoplasmic reticulum, after which they migrate to portions of plasma membrane, whose inner side is lined with matrix proteins [18]. Like most enveloped viruses, release is by budding from the host cell, but unlike most viruses, maturation occurs after release. The

released particles are immature and non-infectious. After release, the viral protease is cleaved from the gag-pol polyprotein. This protease (PR) then cleaves the gag and gag-pol polyprotein at several sites to produce the matrix, capsid, nucleocapsid, p6 proteins, and the three *pol* products, RT, IN and PR itself. Rearrangement of these viral proteins then occurs to produce mature virions [18, 40].

PATHOGENESIS

HIV infects cells in the immune system and the central nervous system resulting in disease that occurs in stages: primary infection, asymptomatic phase, symptomatic phase and AIDS defining illness (Fig. 5). Primary infection or the acute phase is the first stage of HIV disease, when the virus first establishes itself in the body. This phase is characterised by a burst of rapid HIV replication with plasma viral load as high as 10^7 RNA copies/ml as the virus actively reproduces and releases new virus particles into the bloodstream [41]. During acute HIV infection, the virus makes its way to the lymph nodes, a process which is believed to take ~3-5days. In~50% of infected individuals, this stage is accompanied by mononucleosis- or flu-like symptoms, which includes fever, sore throat, skin rash, enlarged lymph nodes, nausea, diarrhoea, thrush, joint aches and general malaise. These clinical syndromes, called seroconversion illnesses, occur ~3-6 weeks after infection. Immunologically, there is a rapid decline of $CD4^+$ T cells, but HIV specific antibodies, which take up to 3 months to develop, cannot be detected at this stage [41-43]. The most severe $CD4^+$ T cell depletion occurs in the gut-associated lymphoid tissue (GALT), where of up to 60% depletion can occur during the first 2-4 weeks of infection. It has been observed that during the first 6 months of HIV therapy, there is persistent viral replication in the GALT, even in the presence of drugs. The GALT is now known to be an important HIV reservoir [44, 45].

Primary infection is followed by the asymptomatic phase or incubation period during which the patient has no clinical signs of illness. Despite this lack of clinical symptoms, called clinical latency, this phase is accompanied by persistent viral replication in lymph nodes with 1-10 billion new virions made daily and $CD4^+$ T lymphocytes being continuously destroyed at a rate that exceeds the body's capacity to produce new $CD4^+$ T-cells. However, the onset of HIV-1 specific cellular immune responses and the subsequent synthesis of HIV-1 specific antibodies occurs at this time, resulting in a drop of HIV particles in the peripheral blood, accompanied by a temporary rise of $CD4^+$ T lymphocytes [41, 42]. This immune control results in a steady state of viremia, when the

rate of new infections equals the death of infected cells [23]. However, the number of $CD4^+$ T lymphocytes decreases gradually, but at a slower pace. This stage can last for years, with ~10% progressing to AIDS within 2-3 years (Rapid progression); 70-80% progressing within 5-10 years (Normal progression); and in ~10% of patients, AIDS is not observed even 10-20 years after infection (Slow progression) [46, 47].

With years of constant activity, the immune system becomes more and more exhausted to a stage when it is no longer capable of keeping the virus in check. Loss of immune control is initially accompanied by mild symptoms, which progressively get worse as the immune system deteriorates. Symptomatic HIV infection is mainly due to the emergence of opportunistic infections and cancers that would normally be prevented by the immune system.

As the immune system worsens, the virus replicates uncontrollably and the $CD4^+$ T lymphocytes drop below 200 cells/µl and lower until these cells are all lost. As a consequence of HIV infection, a generalized, systematic destruction of the immune system leaves the patient so immuno-compromised that the body is ravaged with more severe opportunistic infections and life-threatening, AIDS-defining diseases [42, 48, 49]

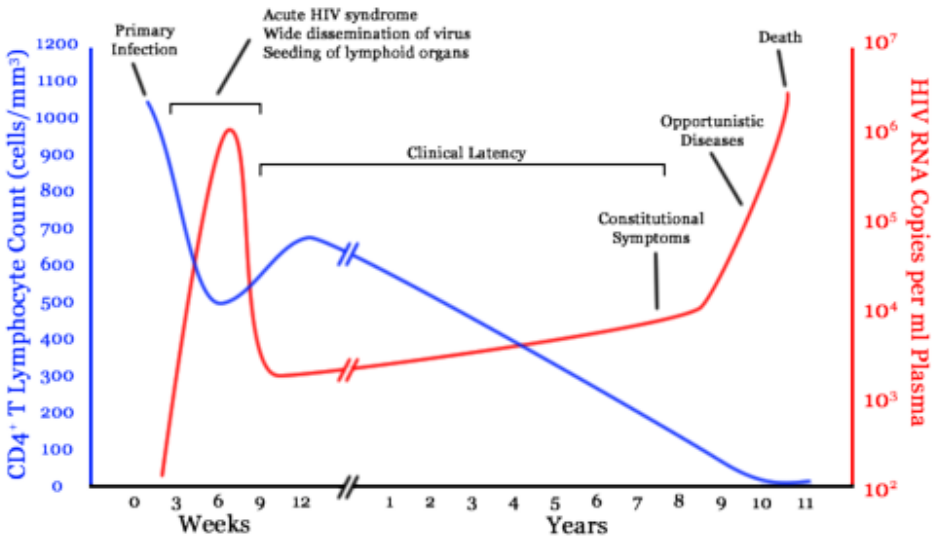


Figure 5: The natural evolution of HIV infection [e]

ANTI-RETROVIRAL THERAPY AND DRUG RESISTANCE

Since the discovery of HIV more than 25 years ago, several breakthroughs have been accomplished, which cumulatively resulted in the use of a combination of antiretroviral drugs to effectively treat HIV infected individuals. Highly Active Antiretroviral therapy (HAART) has changed the face of this disease from a death sentence into a treatable, chronic infectious disease [50]; and has had a dramatic effect on morbidity and mortality, slowing down disease progression and increasing the quality of life of infected individuals with access to treatment. In the absence of treatment, the rate of clinical disease progression varies widely from patient to patient; ranging from 2 weeks (vey fast progressors) up to 20 years (very slow progressors) with a median progression from HIV to AIDS occurring in 9-10 years and a median survival time after developing AIDS of only 9.2 months [51].

In March 1987, the FDA approved the first antiretroviral drug, Azidothymidine (AZT), a drug initially developed in 1964 to treat cancer [52, 53]. Further design and development of anti-HIV drugs stemmed from the discovery that a high viral burden is associated with a faster rate of disease progression [54, 55], suggesting that all steps in the viral life cycle represent potential targets for antiretroviral therapy [41].

Initially, all FDA approved drugs were those targeting the *pol* gene, in particular, the RT and PR enzymes. However, the presence of viral reservoirs; the large number of resistance strains; significant side effects in at least 25% of patients; transmission of resistant strains; difficult drug regiments and high costs of treatment have increased research into new drugs that target other stages of the viral life cycle, such as viral attachment, entry and integration, to produce new drugs with less side effects, lower pill-burden and activity against resistant viruses [18, 56]. Currently, 26 drugs as well as 5 drug combinations have been approved for HIV treatment (Table 1). These drugs target five of the eight replicative steps: reverse transcription, maturation, attachment, entry and integration; giving rise to four classes of drugs; reverse transcriptase inhibitors, protease inhibitors, entry inhibitors and integrase inhibitors, in the order in which they were developed (Table 1).

Reverse transcriptase inhibitors

Reverse Transcriptase is unique to retroviruses, with the exception of the Hepatitis B virus and retrotransposons (mobile genetic elements) [57]. The essential role of RT in the

viral life cycle and its uniqueness to these viruses makes it an ideal target for drug therapy. Drugs targeting RT belong to two classes of compounds: The Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and the Non- Nucleoside Reverse Transcriptase Inhibitors (NNRTIs).

Nucleoside (tide) Reverse Transcriptase Inhibitors (NRTIs) are drugs whose chemical structures resemble that of nucleosides or nucleotides. This class of drugs was the first to be approved and includes 8 nucleoside analogues Zidovudine (AZT, ZDV), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC), Abacavir sulfate (ABC), emtricitabine (FTC) and the only nucleotide analogue, Tenofovir disoproxil fumarate (TDF) [53].

NRTIs are competitive inhibitors whose mode of action is to compete with the natural dNTPs for the active site of the RT enzyme. The characteristic feature of NRTIs is the absence of a free 3'OH group in their sugar ring and its replacement with a less reactive chemical group. The free OH group is required to form a bond with the next nucleotide of the growing nucleotide chain. These substituted nucleosides need to be phosphorylated to their corresponding nucleotide, before they can be recognized by the active site of the RT. Once phosphorylated, these analogues fool the RT into incorporating them into the growing nucleotide chain, where they serve as chain terminators [58].

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) have chemical structures that are completely different from that of regular nucleosides and are therefore not competitive inhibitors, but allosteric inhibitors. NNRTIs bind to a hydrophobic pocket different from, but close to the active site of the RT enzyme. This causes a conformational change in the active site by displacing the catalytic aspartate residues relative to the polymerase binding site, such that it can no longer bind its nucleotide substrate; thus rendering the enzyme inactive [18, 59]. Unlike NRTIs, NNRTIs do not need cellular activation since there is no need for phosphorylation to activate the drug. Hence these drugs are more potent than NRTIs and inhibit the reverse transcription process irreversibly. Four NNRTIs have been approved for HIV treatment, the prototype Nevirapine (NVP), Efavirenz (EFV), Delavirdine (DLV) and Etravirine (ETR) [53, 60].

Protease inhibitors (PIs)

The viral protease (PR) is required for the production of mature infectious particles after release. Due to its crucial function in viral infectivity, this enzyme is one of the most exploited for use in drug design [18]. Currently, there are 10 PIs used for HIV treatment; Saquinavir mesylate (SQV), Ritonavir (RTV), Indinavir (IDV), nelfinavir mesylate (NFV), Amprenavir (APV), Lopinavir + ritonavir (LPV/r), Atazanavir sulfate (ATV), Fosamprenavir Calcium (FOS-APV), Tipranavir (TPV) and Darunavir (DRV). Protease inhibitors can either be non-hydrolysable peptides (mimotopes or peptide mimics) or non-peptide compounds [53, 61].

Protease inhibitors are competitive inhibitors of the protease substrates. They compete with the natural protease substrate for the PR active site; and work by mimicking the proteolytic sites of the protease substrates, the gag and gag-pol polyprotein. Once the PI binds to the active site, it remains bound because it is not cleavable; thereby making the protease enzyme unavailable to act on another substrate [62].

Entry inhibitors

Entry inhibitors have several conceptual advantages over RT and PR inhibitors, mainly because they act extracellularly. Therefore, they neither have the need to cross the cell membrane nor require intracellular activation. A central focus in HIV research is the prevention of HIV transmission; and entry inhibitors provide a way to stop the virus even before it enters the host cell and thus have the potential ability to reduce or even to prevent HIV transmission [18].

HIV entry requires multiple interactions between the Env proteins and the host receptors: interaction between CD4 receptor and gp120, co-receptor binding and membrane fusion; all of which are potential targets for antiretroviral development [18]. Only two entry inhibitors, Maraviroc, an attachment or co-receptor binding inhibitor and T-20, a fusion inhibitor, have been approved for HIV therapy [53].

Attachment inhibitors

Drugs that inhibit co-receptor binding comprise of small inhibitor molecules that target either CCR5 or CXCR4. These drugs are competitive inhibitors and work by binding to either CCR5 or CXCR4; thereby blocking HIV from attaching and entering the host cell. CCR5 and CXCR4 are chemokine receptors; thus some of these drugs are chemokine derivatives. Several regions in CCR5 and CXCR4 involved in binding have negatively

charged amino acids; therefore drugs with an overall positive charge, which can interfere with the negatively charged region of the co-receptor binding site have been developed as well [63].

Maraviroc, a CCR5 inhibitor is the only approved co-receptor binding inhibitor; just recently approved in August 2007. It works by blocking the CCR5 receptor, thereby inhibiting the virus from further conformational changes that will allow fusion with the host membrane. Initially, during the development of CCR5 inhibitors, it was expected that they would sail through the clinical trials, as there are several healthy people with a homozygous CCR5-Δ32 genotype; a 32-bp deletion in CCR5 gene that results in a nonfunctional receptor [64, 65]. However, several unexpected side effects resulted in most of the drugs withdrawn after advanced clinical trials [53].

Fusion inhibitors

Relative to all entry inhibitors, drugs that target fusion are so far the most potent [18]. Gp41 mediates the final stage of viral entry. During co-receptor binding, conformational changes at the N terminal of gp41 result in the formation of a triple-stranded coiled coil. Further conformational changes result in the formation of a six-helix bundle, which is the direct cause of membrane fusion. These fusion inhibitors act to prevent transition from the triple coil to the six-helix bundle and hence prevent fusion [66]. T-20 or Enfuvirtide is the only fusion inhibitor approved by the FDA in 2003 [53, 61].

Integrase inhibitors

The most successful antiretroviral (ARV) drugs developed so far target key enzymes that are essential for viral replication such as the RT and the PR. Much anticipation and speculation has revolved around the third viral enzyme, integrase, responsible for insertion of provirus into the human genome. Like the RT enzyme, integrase is essential for replication and is also unique to HIV without any human analogues. This property has resulted in relatively low toxicities and side effects during clinical trials [67]. The integration process is very complex involving multiple steps (fig 6). First, the integrase binds to viral DNA at the LTR end to form a stable complex and catalyzes the processing of the 3'ends of the DNA. The preintegration complex (HIV DNA-integrase and other HIV proteins) is translocated to the host nucleus and binds to the host DNA. The integrase then catalyzes the strand transfer of the viral DNA into the host DNA and the gaps left after the stand transfer process are repaired by host enzymes. All these steps have been exploited in the development of integrase inhibitors (INI), resulting in

integrase-DNA binding inhibitors, 3'end processing inhibitors, nuclear translocation inhibitors, strand transfer inhibitors and gap repair inhibitors [67] that are in various stages of development.

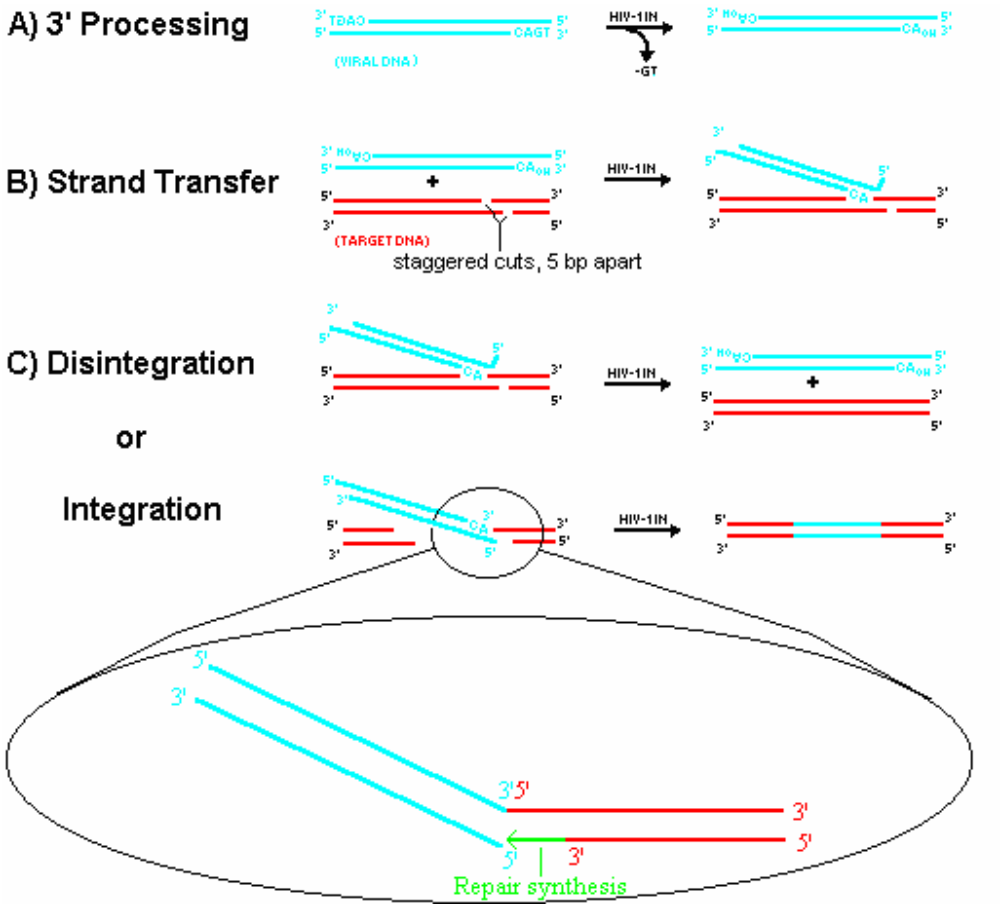


Figure 6: Steps involved in HIV-1 integration [f].

The first integrase inhibitor, Raltegravir was approved by the FDA in October 2007. Raltegravir is a stand transfer inhibitor (STI) and works by specifically blocking the strand transfer step during integration thereby preventing successful integration [53, 56]. During the clinical trials, BENCHMRK I and II, Raltegravir was shown to be a potent drug with a favourable side-effect profile in both treatment naive and experienced patients. These studies showed that Raltegravir is especially useful as salvage therapy in patients with very limited treatment options [68]. This drug is able to achieve virologic suppression to undetectable levels when used as part of a combination therapy [69, 70].

Table 1: 2007 FDA-approved antiretroviral drugs against HIV [53].

Brand Name	Generic Name(s)	Manufacturer Name	Approval Date
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
Retrovir	Zidovudine, Azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87
Videx	Didanosine, Dideoxyinosine, ddI	Bristol Myers-Squibb	09-Oct-91
Hivid	Zalcitabine, Dideoxycytidine, ddC	Hoffmann-La Roche	19-Jun-92
Zerit	Stavudine, d4T	Bristol Myers-Squibb	24-Jun-94
Epivir	Lamivudine, 3TC	GlaxoSmithKline	17-Nov-95
Ziagen	Abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98
Viread	Tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01
Emtriva	Emtricitabine, FTC	Gilead Sciences	02-Jul-03
NRTI combinations			
Combivir	Lamivudine and Zidovudine	GlaxoSmithKline	27-Sep-97
Trizivir	Abacavir, Zidovudine, and Lamivudine	GlaxoSmithKline	14-Nov-00
Epzicom	Abacavir and Lamivudine	GlaxoSmithKline	02-Aug-04
Truvada	TDF and Emtricitabine	Gilead Sciences, Inc.	02-Aug-04
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Viramune	Nevirapine, NVP	Boehringer Ingelheim	21-Jun-96
Rescriptor	Delavirdine, DLV	Pfizer	04-Apr-97
Sustiva	Efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98
Intelence	Etravirine, ETR	Tibotec Therapeutics	18-Jan-08
Multi-class Combination Products			
Atripla	Efavirenz, Emtricitabine and TDF	Bristol-Myers Squibb and	12-Jul-06
Protease Inhibitors (PIs)			
Invirase	Saquinavir mesylate, SQV	Hoffmann-La Roche	06-Dec-95
Norvir	Ritonavir, RTV	Abbott Laboratories	01-Mar-96
Crixivan	Indinavir, IDV,	Merck	13-Mar-96
Viracept	Nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97
Fortovase	Saquinavir (no longer marketed)	Hoffmann-La Roche	07-Nov-97
Agenerase	Amprenavir, APV	GlaxoSmithKline	15-Apr-99
Kaletra	Lopinavir and ritonavir, LPV/r	Abbott Laboratories	15-Sep-00
Reyataz	Atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03
Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03
Aptivus	Tipranavir, TPV	Boehringer Ingelheim	22-Jun-05
Prezista	Darunavir, DRV	Tibotec, Inc.	23-Jun-06
Entry Inhibitors - Fusion Inhibitors			
Fuzeon	Enfuvirtide, T-20	Hoffmann-La Roche &	13-Mar-03
Entry Inhibitors - CCR5 co-receptor antagonist			
Selzentry	Maraviroc	Pfizer	06-Aug-07
HIV integrase strand transfer inhibitors			
Isentress	Raltegravir	Merck & Co., Inc.	12-Oct-07

COMBINATION THERAPY

The initially high efficacy of AZT when it was first approved in 1987 followed by the release of didanosine in 1991 [71], lead to the naïve belief that we could eradicate HIV. However, it soon became obvious that monotherapy had only a brief and limited activity

against HIV-1 [72, 73] and that suppression of viral replication must be continuous and optimal to maximize the effects of treatment. Advanced knowledge of HIV and new drug discoveries has lead to the development of other drugs, such as PIs, as well as other new RTIs. The most significant milestone in the history of HIV therapy occurred in 1996 with the introduction of highly active antiretroviral therapy (HAART); which is the use of three drugs from at least two distinct classes of antiretrovirals (mainly two RTIs + 1 PI). Efficacy of HAART was first presented at the 3rd Conference on Retroviruses and Opportunistic Infections [74, 75]. HAART was found to be sufficiently potent to sustain suppression of plasma viral load below the detection limits of current assays for up to 7 years in adherent patients [76-78]. Sustained suppression of viral replication reduces the rate of disease progression; deter the development of resistance to new agents and allow for immune reconstitution; hence the marked reduction in morbidity and mortality observed with HAART therapy [50]. The long-term virological success of HAART is limited by resistance development, severe side effects and pharmacokinetic interactions; thus new antiretrovirals with improved systemic tolerability profiles that target other critical events of the life cycle, to avoid cross resistance, are required to reduce HIV symptoms, prolong and improve the quality of life of infected individuals [41].

DRUG RESISTANCE

Incomplete suppression of viral replication inevitably leads to the selection of drug resistant strains [60]. The development of genetic mutations in HIV genes targeted for drug therapy, due to the high error rate of reverse transcriptase and the rapid turnover of the HIV-1 population [23, 24], result in the emergence of drug resistant strains. HIV can develop resistance to various anti-retroviral drugs as a result of these mutations, which can be classified as primary or secondary mutations.

For some drugs, a single mutation, called a primary mutation, is sufficient to confer drug resistance. Primary mutations alter the binding of the drug to its target, resulting in an increase in the quantity of the drug required for inhibition. For other drugs, a combination of mutations, called secondary mutations, is required to confer resistance. Secondary mutations do not directly cause resistance, but increase the level of resistance by enhancing the fitness of viruses that already have a primary mutation. In the absence of primary mutations, secondary mutations have little or no effect on the level of resistance [60]. Most mutations are lethal or neutral, and generally result in less efficient replication than in the wild type strain, except in the presence of drug pressure. Mutations are

designated in shorthand with letters and numbers. In the mutation M184V, the first letter represents the amino acid in the wild type (WT), followed by the amino acid or codon number and finally the new amino acid in the mutant that has replaced the one in the WT.

Resistance in NRTIs

Resistance against NRTIs can arise either by mutations that increase the Reverse transcriptase's discrimination between natural nucleotides and NRTIs or by promoting the removal of NRTIs [58]. These mutations occur at the active site of the reverse transcriptase, changing it in such a way that it no longer recognizes the NRTIs as substrate. In AZT resistance, two mechanisms have been identified. The first mechanism is an increased rate of pyrophosphorylysis, the removal of phosphate groups from the terminal nucleotide to form a nucleoside; resulting in the removal of AZT thereby re-activating the RT. The second mechanism is an increased processivity of the RT. Processivity refers to the number of nucleotide additions performed by RT before it disengages from the RNA template and has to reattach; therefore increased processivity is associated with a more efficient the reverse transcription process [74]. Aside from these mechanisms, the RT can excise bound NRTIs and re-continue chain elongation [18, 79]. Thus these inhibitors can only delay viral replication, but do not stop it irreversibly. Both primary and secondary mutations have been associated with NRTI resistance.

Resistance in NNRTIs

NNRTIs have a very low genetic barrier, in that complete resistance occurs rapidly and they do not need the accumulation of several mutations. Resistance is usually due to mutations at the hydrophobic binding pocket adjacent to the active site of the RT. These mutations, mostly primary mutations, cause resistance by altering size, shape or polarity of the binding pocket or by affecting accessibility of NNRTIs to this site [80].

Resistance in protease inhibitors

Unlike the RTIs, high-level resistance to protease inhibitors requires the accumulation of several mutations in the protease gene. These mutations cause resistance to different levels depending on the specific protease inhibitor and as such are referred to as major and minor mutations [81]. In fact, the same mutation can be classified as either major or minor depending on the specific protease inhibitor. Major mutations cause resistance by producing changes in the active site of the protease that result in both a reduced affinity of the protease to these inhibitors as well as increased preferential selection of the natural substrates. Aside from active site mutations, other mutations, called minor mutations

occur away from the active site. These can occur in the *gag* gene to either adapt the *gag* cleavage site or to improve the incorporation of PR into the virion in order to maximize the cleavage activity [82, 83].

Resistance in Entry inhibitors

Resistance to T-20 is due to mutations in the highly conserved 3-amino acid sequence (GIV) at codons 36-38, known to be critical for fusion. In a study of patients receiving T-20 monotherapy, the G36D mutation was the main mutation found and the I37V and V38M mutations were found in only one sample. Other mutations were also found near the GIV region, mostly in conjunction with the G36D mutation. Whilst these other mutations conferred slight increases in drug resistance, the G36D mutation plus either Q32R or Q32H conferred high levels of resistance to the drug [84].

Maraviroc is the only approved attachment inhibitor that blocks the CCR5 co-receptor. HIV can use either, CCR5 (CCR5 trophic), CXCR4 (CXCR4 trophic) or both (dual trophic). Thus viral tropism needs to be determined before treatment with co-receptor blockers. Resistance to co-receptors binding inhibitors occurs via two unusual mechanisms; HIV may bind to CCR5 despite the presence of the inhibitor; and CXCR4 tropism may emerge and dominate [85]. Maraviroc resistance is associated with distinct mutations in the V3 loop of the Env gene, which was not surprising considering the importance of the V3 loop in coreceptor recognition and binding [86]. Mutations in the V3 loop and other regions of gp120 allow the virus to recognize and utilize CCR5 receptors even when they are bound to the inhibitor, either via binding to alternative domains of CCR5, or by binding to the receptor at a higher affinity [85].

Resistance in Integrase inhibitors

Raltegravir is the only available integrase inhibitor, approved in October 2007.Therefore there is not enough experience with his class of drugs to discern their signature resistance pathways. However BENCHMRK-1 and -2 studies of Raltegravir provided some information on drug-resistance mutations. Raltegravir failure was shown to be associated with either the N155H or Q148K/R/H previously described mutational pathways. For each pathway, additional mutations were observed in patients that experienced virologic failure; N155H was found with the V151I, T97A, G163R, and L74M mutations; and Q148K/R/H was found with the G14S/A and E138K mutations. Besides these two pathways, another pathway, Y143R/C + (L74A/I, E92Q, T97A, I203M, and S230R), was observed as another possible pathway involved in Raltegravir resistance [56, 69, 70].

RESISTANCE TESTING

Resistance assays are tools that use different methods to provide complementary information about antiretroviral resistance. After two decades of ART, resistance testing has become an important tool in HIV management, especially when used in conjunction with clinical, immunological and virological parameters. Resistance testing is important in guiding antiretroviral treatment; from the choice of initial regimen to managing treatment failure. In some cases, when 2nd or 3rd line therapy fails, resistance testing is critical to avoid cross resistance with the new regimen. It is also important in developing countries where treatment options are limited as well as in populations with a significant prevalence of transmitted drug resistance. There are two different methods of resistance testing; genotypic and phenotypic testing.

Genotypic Resistance Testing

Genotypic assays give information about the nucleotide sequence of a gene or genotype. Current genotypic resistance tests involve RT-PCR of the gene of interest followed by either sequencing or hybridisation.

For genotypic resistance assays, sequencing is the gold standard. Dideoxynucleotide sequencing is a population-based sequencing method based on the incorporation of fluorescent-labelled dNTPS to give the nucleotide sequence of a PCR-amplified gene and all possible resistance-associated mutations. With the increasing use of genotyping in both clinical and research laboratories, alternate hybridisation methods have been developed. These methods use several molecular biology applications such as Southern blotting, primer-specific PCR, PCR-ligase detection reaction, RNase A mismatch, hybridization against labelled probes, reverse hybridization against immobilized oligonucleotide probes (LiPA) and oligonucleotide ligation assays (OLA)[87]. Though these hybridisation assays are generally cheaper and more sensitive to detect minor variants relative to consensus sequencing, their main disadvantage is that they are single or point mutation assays that can detect only a limited number of mutations. Also these assays are highly sensitive to sequence diversity around the hybridization site [88].

Genotypic drug resistance assays are the most commonly used because they are more readily available, cheaper and rapid to perform relative to phenotypic assays. However, interpretation of genotypic assays requires prior knowledge of resistance markers and thus cannot be used for new compounds whose mutational patterns are unknown [89].

Also, characteristics such as hyper-susceptibility, cross-resistance and re-sensitization are difficult to interpret with genotypic assays [90].

Phenotypic Testing

Phenotype refers to the characteristics or growth properties of a viral isolate, and it is always determined by the genotype. Phenotypic drug resistance assays directly measure the ability of a viral isolate to grow in the presence of specific drugs [60]. Drug susceptibility is defined as the concentration of drug that inhibits viral replication by 50% (IC_{50}) or by 90% (IC_{90}).In terms of drug resistance the phenotype defines the susceptibility of the virus to inhibition by a particular drug [63].

Initial phenotypic resistance assays used Peripheral Blood Mononuclear Cell (PBMC)-based or plaque assays, which were tedious, time consuming and costly [91]. In addition, use of donor PBMCs generated large inter-assay variability. Current phenotypic assays are based on amplification of the gene of interest followed by insertion into a lab clone or backbone lacking the same genes to generate a recombinant virus. These recombinant virus assays are automatable and the use of a common genetic backbone minimizes inter-assay variation. Phenotypic assays provide a direct measure of drug susceptibility and directly accounts for interactions between mutations, in an easy to interpret format [92-94]. These assays are especially important in testing new compounds, since they are the only assays that can determine if a certain mutation confers resistance and the degree to which resistance is conferred. They also give vital information about cross resistance and hypersensitivity [95]. A limitation in these assays is the lack of consensus for the clinical cut-off values. Clinical cut-offs are available for only a limited number of drugs, such that biological cut-offs from *in vitro* susceptibility tests have to be used for clinical isolates from drug naïve patients [96].

VIRAL FITNESS AND DRUG RESISTANCE

Viral fitness is associated with evolutionary adaptations of viruses. The rapid turnover and high mutation rate of HIV allows for a remarkable capacity for rapid evolutionary adaptation [24, 97]. Viral fitness depends on a number of viral factors such as replication capacity, mutation rate and cell tropism and on host factors such as genetic background, immune control and availability of target cells [98]. Generally, viral fitness refers to the ability of a virus to replicate in a given environment, thereby contributing to successive

generations [21, 99]. In the presence of drug pressure, mutant (MT) viruses are fitter than WT viruses, since they have mutations that allow them to circumvent the pressure exerted by the drugs. When virologic failure occurs, due to drug resistance, viral load does not rebound to pre-therapy levels and resistant mutant strains are replaced by wild type viruses when treatment is stopped. It was therefore postulated that acquiring mutations must come at a fitness cost [98, 100, 101]. Resistance mutations usually occur in conserved critical positions, which decrease the intrinsic capacity of the virus to replicate efficiently, thus decreased fitness of resistant viruses should not come as a surprise.

The evolution of viral fitness during ART occurs in two stages. The first is characterized by the selection of viruses with primary resistance mutations that express reduced drug susceptibility, but have an impaired replication potential. The second stage is characterized by the generation and selection of secondary resistance mutations that usually compensate for the fitness loss generated by primary mutations thereby enhancing replication [98]. Although primary drug resistance mutations are usually associated with replication impairment and secondary mutations with an increase in fitness, some primary mutations can be as fit as the wild type (Table 2).

Table 2: Effects of HIV-1 drug resistance mutations on viral fitness/replicative capacity [98, 101]

Drug class	Drug resistance mutation (s)	Viral fitness relative to WT
RTI: NRTIs	K65R	Reduced
	L74V	Reduced
	M184V	Reduced
	T215Y	Reduced
	Q151M	Improved
	M41L	Reduced
	K70R/T215Y	Reduced
	D67N/K70R/T215Y/K219Q (TAM-2)	Improved
RTI: NNRTIs	K103N	Similar/Reduced
	Y181C	Increased
PI	L90M	Similar
	D30N	Reduced
	V82A	Similar/Reduced
FI	I37T	Reduced
	V38M/A	Reduced
	N42T	Reduced

TAM-2: Thymidine analogue mutations type 2

The relationship between viral fitness and drug resistance has been exploited in salvage therapy. The M184V mutation, which causes resistance to the cytosine analogues 3TC and FTC, is associated with a much lower replicative capacity than the wild type. As such patients who have exhausted all therapeutic options can continue to use 3TC or FTC even when they have developed the 184V mutation; because maintaining these drugs in any treatment experienced combination would be beneficial in that it maintains a lower viral load relative to the untreated WT virus [100].

Study of viral fitness in relation to drug resistance has both clinical and epidemiological relevance. Improved knowledge of the evolutionary dynamics of drug resistance will help in modelling the transmission of drug resistant viruses; establishing more optimal and effective therapies; and reducing the rate of disease progression [102].

FIGURE REFERENCES

a. **Figure 1:** The structural components of HIV [103]
b. **Figure 2:** Organization of HIV genome [104]
c. **Figure 3.** Mechanism of genetic variation [105]
d. **Figure 4**: HIV life cycle [79]
e. **Figure 5:** The natural evolution of HIV infection (Adapted from [42])
f. **Figure 6:** Steps of HIV-1 integration [106]

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Chapter 2

Human Immunodeficiency Virus Type 2

HISTORY AND OVERVIEW

Shortly after the discovery of HIV in humans, a similar virus called simian immunodeficiency virus (SIV) was identified when Asian rhesus macaques in US primate centres displayed AIDS-like symptoms [1, 2] and their sera cross-reacted to HIV-1 antigens [3, 4]. Two years after the discovery of HIV-1, researchers found that western blot analysis of sera from Senegalese commercial sex workers cross-reacted preferentially to these SIV antigens compared to HIV-1 [5]. This new human retrovirus, which is more related to SIV than HIV, was later isolated from two West African patients and called Human Immunodeficiency Virus type 2 (HIV-2) [6].

ORIGIN OF HIV-2

The origin of HIV-2 has clearly been shown to be due to zoonotic transfer from sooty mangabeys (*Cercocebus atys*) to humans. In HIV-2, the five lines of evidence used to determine zoonotic transfer: similarities in viral genome organization; phylogenetic relatedness; geographic coincidence; prevalence in the natural host; and plausible routes of transmission [7-11]; are all met.

HIV-2 is genetically much more similar to SIV from sooty mangabeys (SIV_{sm}) and SIV macaques (SIV_{mac}) than HIV-1. While HIV-2 shares a 40-50% nucleotide sequence homology with HIV-1, it shares more than 75% nucleotide sequence homology with SIV_{sm} [12-14] as well as the presence of a *vpx* gene, unique to HIV-2 and these SIV, instead of the *vpu* gene found in HIV-1 [10]. Phylogenetically, HIV-2 and SIV_{sm} are so closely related that they cannot be separated into distinct phylogenetic lineages according to species of origin [14-16]. The natural habitat of Sooty mangabeys is Western Africa, which is the epicentre of the HIV-2 epidemic. These monkeys have a high prevalence of SIV_{sm} accompanied with very high viral loads. Despite this high viral

Fig 1: West African girl with pet sooty mangabey [a]

burden, the Sooty mangabeys remain asymptomatic; indicating that they serve as a natural reservoir or host for the virus [16, 17]. In West Africa, there are several interactions between human and primate that can serve as a route of transmission. These monkeys are hunted for food and the bloody hunting and butchering process may expose the hunter to infected blood and bodily fluids. In addition, these primates are commonly kept as household pets (fig 1), which provide further means of exposure and potential transmission [16].

Though HIV-2 was only discovered in 1985, it is now believed that it may have infected humans as early as 1940 [18]. Researchers at the Katholieke Universiteit Leuven in Belgium used timescale analysis and seroprevalence data to track the geographic origin of HIV-2 and to determine when it was first transferred to humans. Their results showed that the putative geographic origin of HIV-2 was Guinea Bissau and that the virus gained epidemic status between 1955 and 1970, a period that coincides with Guinea-Bissau's independence war with Portugal, which took place between 1963 and 1974 [19, 20]. Additional support was obtained when the first cases of HIV-2 in Europe came from Portuguese war veterans, who had served in Guinea Bissau [18, 21].

EPIDEMIOLOGY AND GEOGRAPHICAL DISTRIBUTION

The discovery of HIV-2 was met with the fear of another pandemic. However, unlike HIV-1, HIV-2 has resulted only in an epidemic of about a million infections [22] with limited spread. Whereas HIV-1 is found throughout the world, HIV-2 is restricted mainly to West Africa (fig. 2) with the highest prevalence of 5.9-12.8% found in Guinea Bissau [23, 24]. Other West African countries like The Gambia, Senegal, Cape Verde Islands, Ivory Coast, Ghana and Mali, have moderate to high prevalences of 1-10% [25-30], although prevalence can be as high as 27.5% in high-risk groups such as commercial sex workers in The Gambia [26]. The lowest prevalence of 0.02% was found in Sierra Leone, which remarkably is reported to have the highest HIV-2 diversity [31]. Surveys in other African regions, such as Cameroon, Equatorial Guinea, Gabon and Democratic Republic of Congo found either sporadic cases or no HIV-2 infections [26].

HIV-2 is now being observed with increasing frequency outside West Africa, though only a few regions have significant prevalence of HIV-2. In Europe, Portugal has the highest HIV-2 prevalence of 4% of all reported HIV infections, which can be traced back to the

independence war in Guinea Bissau and the early dissemination of HIV-2 [26, 32]. Angola and Mozambique, former Portuguese colonies in southern Africa that still have ties to West Africa also have high HIV-2 prevalences [26]. Infected individuals have also been identified in other places such as India, Brazil and South Korea [33-37]. HIV-2 has also been reported in countries like the Netherlands, England, France, Sweden, Spain and the USA, mostly in West African immigrants [26, 38-41]. It was concluded that even though HIV-2 infection originated in West Africa, it may come to pose a significant concern in other continents [25].

Figure 2: Distribution of HIV-2 in Africa. Shaded areas represent regions where significant rates of HIV-2 infection have been reported [b].

However, several surveillance studies have revealed a decreasing trend in HIV-2 prevalence in the region of origin. In a study of blood donors in West Africa from 1985 to 1996, nine out of ten nations observed a drop in HIV-2 rates [42]. A 16-year (1988-2003) survey in 23,363 patients aged 15 years or older in the Gambia showed a decline in HIV-2 rates from 7.0% (1988-91) to 4.0% (2001-03) [43]. In Ivory Coast a survey of 19,701 women of reproductive age between 1988 and 1992 showed a decline of HIV-2 prevalence from 2.6% to 1.5% [23, 44]. In a sero-surveillance of men in Guinea Bissau, HIV-2 prevalence decreased from 9.1%(1987) to 4.7%(1996) [24] and a decline from 8.3%(1987) to 3.3%(2001) was observed among pregnant women [43].

CLASSIFICATION

Currently, eight HIV-2 groups, A-H have been reported. Initially, these were called "subtypes", however the phylogenetic distances between these different HIV-2 strains suggest that they arose from eight different cross-species transmissions from sooty mangabeys to humans [15, 31, 45-48],], analogous to the HIV-1 groups. Hence they were renamed as groups instead of subtypes [49]. Unlike HIV-1 with various epidemic subtypes and CRFs, HIV-2 is characterised by an epidemic of only two groups (A and B) and six non-epidemic groups (C–H) [31, 46, 47]. For the non-epidemic groups C to H, only one member of each has been identified, except for group D with two known members [26]. Some of these rare HIV-2 strains are so similar to SIV_{sm} found in the same region that they are believed to represent epidemiological dead end lineages that have failed to establish a successful human to human transmission [49, 50]. Like HIV-1, there is a degree of uneven geographical distribution of the epidemic HIV-2 groups. While group A viruses have been documented in different locations across West Africa and other regions of the world, group B is geographically restricted to Ivory Coast and Ghana; with a few cases documented in Europe and the Middle East [27]. Surprisingly, Sierra Leone with the lowest HIV-2 prevalence, only 0.02% [46], has the highest diversity with four of the eight known HIV-2 groups (A, B, E and F) as well as the A-B recombinant, HIV-2-7312A/B, all present in the population [46, 47].

HIV-2 is also characterised by extensive genetic variation and the capacity to recombine [51], which gives rise to the possibility of intergroup recombination. The first report was an A-B recombinant called HIV-2-7312A/B. This HIV-2 recombinant, isolated from a man in Ivory Coast, consisted of group A in the *env* gene and B in the *gag* and *pol* genes [15, 52]. Recently another A-B recombinant, called CM-03-510-03 was discovered from a woman from Cameroon. Like HIV-2-7312A/B, the *env* gene was from group A and the *gag* and *pol* genes from group B [51]. However, apart from one similar break point, these two isolates have very distinct break points.

TRANSMISSION

Though HIV-2 can be transmitted via the same routes as HIV-1, transmission occurs principally via heterosexual contact. HIV-2 is also transmitted vertically from mother to

child and via blood transfusions, but transmissions via homosexual contact and in intravenous drug users (IVDU) are rare [26]. A glaring difference between HIV-1 and HIV-2 is their frequency of transmission. HIV-2 has a much lower transmission rate than HIV-1 [53]; with sexual transmission reduced by 5-9 fold lower and vertical transmission reduced by 10-20 fold [54]. Lower transmission rate in HIV-2 has been attributed to the reduced viremia observed in HIV-2 patients relative to HIV-1 patients [10, 25, 53].

VIROLOGY

HIV-2 has the same morphology as HIV-1, a spherical enveloped virus of ~ 100nM in size. HIV-1 and HIV-2 also have similar gene expression and produce proteins that are similar in both structure and function. In addition, these viruses share a similar genomic organisation, with one exception (Figure 3). The *vpu* gene of HIV-1 is replaced by another gene called *vpx* that is unique to HIV-2, SIV_{sm} and SIV_{mac}. Despite their high similarity in genomic organisation and gene expression, HIV-1 and HIV-2 share an overall nucleotide sequence homology of only about 30-50%, with HIV-2 having a longer genome by ~500 nucleotides [12]. These differences are manifested in the generation of proteins with slightly different sizes (table 1), antigenicities and immunogenicities.

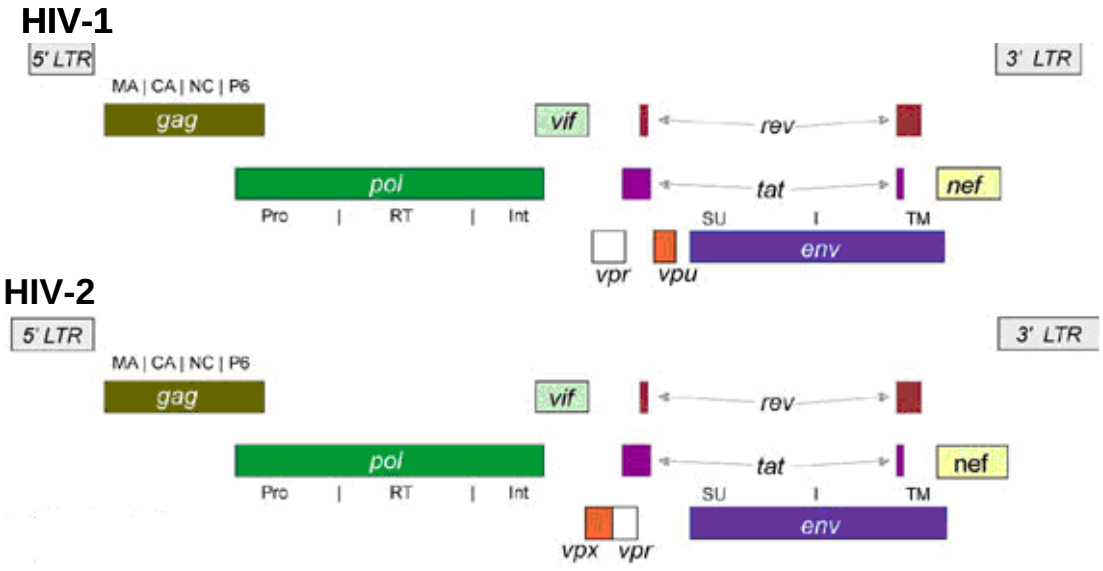

Figure 3: Genomic organisation of HIV-1 and HIV-2 [c].

Differences in HIV-1 and HIV-2 genomic sequence are uneven; the more conserved *gag* and *pol* genes share a homology of ~60% and are antigenically cross-reactive, while the rest of the genes have a similarity of only 30-45% [12] with the biggest difference in nucleotide sequence observed in the *env* genes [54]. HIV-2 encodes three structural genes. *Gag* is translated to a polyprotein, which is cleaved into the matrix (p16), capsid (p26) and nucleocapsid proteins (p8); *pol* encodes protease (p11), reverse transcriptase (p64, p53) and integrase (p34); and *env* encodes the surface glycoprotein gp125 and the transmembrane protein gp36. The HIV-2 structural and regulatory genes are very similar in function to those of HIV-1. HIV-2 encodes six overlapping open reading frames to produce three regulatory genes: *nef, tat* and *rev*; and three accessory genes: *vif, vpr* and *vpx* [10, 25, 55]. These genes are flanked by long terminal repeats (LTR) that do not code for genes, but contain regulatory sites for promoters and enhancers. HIV-2 LTRs are longer than those of HIV-1 [56, 57].

ORF	Protein name	HIV-1	HIV-2
Structural genes			
gag	**Precursor**	**p55**	**p55**
	Matrix	**p17/18**	**p16**
	Capsid	**p24**	**p26**
	Nucleocapsid	**p7**	**p8**
	unknown protein	**p6**	**p6**
pol	**Protease**	**p10**	**p11**
	Reverse transcriptase + RNAse H	**p66**	**p64**
	Reverse transcriptase	**p51**	**p53**
	Integrase	**p32**	**p34**
env	**Precursor**	**gp160**	**gp140**
	Surface glycopreotein	**gp120**	**gp125**
	Transmembrane glycoprotein	**gp41**	**gp36**
Non-structural genes: Regulatory genes			
tat	**TAT**	**p14**	**p20**
rev	**REV**	**p19**	**p19**
nef	**NEF**	**p27-39**	**p31**
Non-structural genes: Accessory genes			
vif	**VIF**	**p23**	
vpr	**VPR**	**p15**	
vpu	**VPU**	**p16**	**not found**
vpx	**VPX**	**not found**	**p16**

Table 1: Comparison of HIV-1 and 2 gene products [e]

Vpx is a small accessory protein of 112-amino-acid that is unique to HIV-2, SIV_{sm} and SIV_{mac}. This protein, believed to be a duplication of the HIV-1 *vpu*, is as abundant as the p26 capsid protein [55] and is necessary for efficient infection of non-dividing cells. In addition, vpx has a nuclear localization signal (NLS) and is therefore very important for nuclear localization of the reverse transcribed viral DNA [58, 59].

LIFE CYCLE

The life cycle of all retroviruses essentially follow the same pattern. Although little work has been done to elucidate the HIV-2 life cycle, the similarities of HIV-1 and HIV-2 in terms of genomic organization, gene expression and transactivtaion, implies that these two viruses have very similar replicative cycles. However, a few differences have been observed.

During entry attachment and co-receptor binding, both HIV-1 and HIV-2 use $CD4^+$ T-cells as their primary receptor for attachment and either CCR5 and/or CXCR4 as their major co-receptor for entry into $CD4^+$ T-cells. However, it was discovered that HIV-2 attachment and entry can also occur independent of $CD4^+$ T-cells binding; instead HIV-2 can use either CCR5 or CXCR4 alone for entry [59-61]. The affinity of HIV-1 to $CD4^+$ T-cell receptor is much higher than that of HIV-2 [62], but HIV-2 binds with a higher affinity to the co-receptors, CCR5 and CXCR4 [10].

In the co-receptor binding step, it was discovered that HIV-2 is more promiscuous in its co-receptor usage. While HIV-1 can, to a lesser extent, use a few other co-receptors such as CCR3; HIV-2 can use a wide variety of other receptors just as efficiently as CCR5 and CXCR4 [54, 63-66]. In addition, HIV-2 isolates can use co-receptors that are not known to be used or are rarely used by HIV-1, such as CCR1, CCR2b, CCR3, CCR4, CCR8, CXCR5, CX3CR1, RDC1, APJ, US28, BOB and BONZO [51, 67]. Also, most primary HIV-2 isolates can infect PBMC homozygous for the Δ32 CCR5 phenotype, unlike HIV-1 [10, 64-66], suggesting that alternate co-receptor usage might also occur *in vivo* in HIV-2.

This broad co-receptor usage is not consistent with the reduced rate of transmission and pathogenesis of HIV-2. Van der Ende *et al*. found that CXCR4 usage is not linked with a

syncytia inducing phenotype as observed in HIV-1 and concluded that CXCR4 use, as well as a wide range of alternate co-receptors, does not result in enhanced pathogenicity of HIV-2 *in vivo* [68].

PATHOGENESIS

Like HIV-1, HIV-2 infects the same cells of the immune system and the central nervous system resulting in disease that occurs in stages: primary infection, asymptomatic phase, symptomatic phase and AIDS defining illness. However, HIV-2 is much less pathogenic than HIV-1.

HIV-2 infected individuals have a much slower disease progression and longer survival [29, 69-71] with some individuals never progressing to AIDS [72, 73]. The asymptomatic phase lasts for much longer periods in the majority of HIV-2-infected individuals, sometimes more than 27 years [74]. The period from infection to clinical AIDS is 3-4 times longer in HIV-2 patients and the period to overt AIDS and death can be 12-13 times longer in HIV-2 relative to HIV-1 [25]. Longitudinal studies in Caio, Guinea Bissau by Schim van der Loeff *et al*. have indicated that 80-85% of HIV-2-infected people do not progress to AIDS [75].

During the last stage of the disease, defined as AIDS, HIV-2 exhibits the same opportunistic infections and diseases as HIV-1. The differences in pathogenesis are much less apparent at this stage of the disease, as both viruses present similar clinical manifestations [76]. However, a few exceptions have been observed. Researchers in Ivory Coast found that extra pulmonary tuberculosis (TB) was less frequent in HIV-2 [77, 78] whereas multi-organ cytomegalovirus (CMV) infections, HIV encephalitis and cholangitis were more frequent in HIV-2 relative to HIV-1[79]. The higher frequency of these diseases associated with severe immunodeficiency in HIV-1, might be due to the fact that HIV-2 infected individuals have a longer clinical AIDS period. In an 11-year study in Senegal, researchers found that oral candidiasis and chronic fever were more frequent in HIV-1 and that bacterial and cryptococcal meningitis was found only in HIV-1 AIDS patients. On the other hand, chronic diarrhoea, especially those caused by bacterial infections were observed more frequently HIV-2 AIDS patients [80]. Studies in the Gambia showed that Kaposi's sarcoma (KS) is less frequent in HIV-2 than in HIV-1 [81]; and that wasting syndrome was more frequent in HIV-2 [82].

WHY IS HIV-2 LESS PATHOGENIC THAN HIV-1?

It has been well established that HIV-2 represents an attenuated version of HIV-1 in terms of pathogenesis and disease progression. Most HIV-2 patients are asymptomatic and have no increased risk of mortality [26]. A similar form of attenuated disease is only observed in SIV infections, where the virus has the advantage of high reproducibility and transmissibility without causing disease in the natural host [83]. While HIV-2 lacks the high viral loads and the high rate of transmission, in a majority of cases it also fails to progress to AIDS. Several studies have been done, looking at the differences between HIV-1 and HIV-2 to elucidate the difference in pathogenesis. Several plausible differences have been observed.

Virological level

In humans, high viral loads during the course of HIV infection have been associated with faster disease progression and higher mortality [22]. Several studies have shown that plasma viral load is significantly much lower or undetectable in HIV-2 even in absence of antiretroviral therapy (ART) [84-86], which may contribute to the reduced pathogenesis of HIV-2 and lower rates of vertical and horizontal transmission. While higher plasma viral loads in HIV-1 infection suggests higher replication rates, studies have observed no difference in proviral DNA of HIV-1 and HIV-2 infected individuals [87, 88]. This indicates that there is little if any difference in the ability to replicate or establish successful infection in human cells between these two viruses [89]. A study that quantified all nucleic acid intermediates found that while the amount of the integrated DNA was similar in HIV-1 and HIV-2; there was a significantly lower quantity of viral messenger RNA (mRNA) in HIV-2 infection [57]. They concluded that although HIV-2 is able to establish a stable, integrated proviral infection *in vivo*, transcription of the provirus into mRNA is attenuated and that reduced viral mRNA levels are consistent with the lower plasma viral loads in HIV-2 [57].

Immunological level

As in HIV-1, $CD4^+$ T-cell count has been shown to be an important predictor of mortality in HIV-2. A high $CD4^+$ T-cell count as well as a slow $CD4^+$ T-cell decline has been associated with slower disease progression and lower mortality [90]. One of the most marked differences between HIV-1 and HIV-2 is the consistently higher $CD4^+$ T-cell count in HIV-2 and that the rate of $CD4^+$ T-cell count decline occurs much slower in HIV-2 than in HIV-1. In fact, it has been shown that at the time of AIDS, HIV-2 patients have higher $CD4^+$ T-cell count than HIV-1 infected patients [82]. The higher $CD4^+$ T-cell

counts in HIV-2 may be related to the lower level of *in vitro* T-cell apoptosis, observed among asymptomatic HIV-2 -infected individuals [91, 92].

Studies of SIV infection in natural monkey hosts, showed that high viral load is not accompanied by a noticeable immune activation [93-95]; unlike HIV infection, where high viral load leads to a considerable immune activation, which leads to $CD4^+$ T-cell depletion and disease [91, 92]. The weaker long-term immune activation observed in HIV-2 is believed to contribute to the slower T-cell depletion and disease evolution [92].

Studies of individuals who have been HIV exposed, but remain uninfected as well as long term non-progressors (LTNPs) has given some evidence of the importance of cell-mediated immune responses in the protection from and control of HIV infection [96, 97]. It was shown that there is an inverse relationship between plasma HIV-1 viral load and the magnitude of the T-helper responses, which indicate that insufficient HIV specific cell-mediated immune responses lead to chronic and progressive HIV infection [98]. HIV-specific memory $CD4^+$ T-cell response has been associated with slower disease progression and better prognosis. Studies from the Gambia have revealed that HIV-2-infected individuals exhibit vigorous virus-specific cytotoxic T-cell responses and that there is a higher frequency of HIV- specific $CD4^+$ T-cell responses during HIV-2 infection [99-103]. Studies on specific $CD4^+$ T-cell responses showed that HIV-2 had a 2.4-fold higher frequency of IFN-γ producing $CD4^+$ T-cells, and a 5.7-fold higher frequency of IL-2-producing $CD4^+$ T-cells compared to HIV-1 [103]. HIV-2-infected individuals have a higher proportion of $CD8^+$ T-cells that retain the ability to simultaneously produce the cytokines IL-2 and IFN-γ [104]. Also, $CD4^+$ T-cells that can simultaneously produce IL-2 and IFN-γ were found exclusively in HIV-2 [103].

HIV-2 infection is associated with an overproduction of β-chemokines by PBMCs *in vitro*, which can prevent infection of R5 tropic viruses [105] and hence might contribute to the lower HIV-2 horizontal and vertical transmission rates. Unlike HIV-1, HIV-2 Env proteins can interact with CD8 on non-permissive T-cells, thereby triggering chemokine production [106, 107]. Increased chemokine production in HIV-2 may account for the better immune control observed in HIV-2.

Studies of HIV, SIV and/or SIV-HIV hybrids (SHIV) have indicated that neutralising antibodies may be important in preventing or modulating HIV infection [93, 108-110]. In HIV-1 infection, it was shown that LTNPs and slow progressors have a broader and higher frequency of neutralising antibodies relative to other HIV-1-infected individuals.

A higher frequency of autologous neutralising antibodies was observed in HIV-2-infected individuals compared to HIV-1-infected individuals, which might contribute to the slower disease progression observed in HIV-2 infection [111].

Molecular Level

Studies done on some SIV strains have shown an association between virulence and mutations in the *env, gag, tat* and *nef* genes as well as in the LTR region[112-115]. These studies lead to the belief that genomic mutations in HIV-2 might be responsible for its reduced pathogenicity [116].

The reduced transcription efficiency of HIV-2 reflected by decreased mRNA production has been shown to be partly responsible for the lower plasma viral load observed in HIV-2 infection [22]. Differences between HIV-1 and HIV-2 mRNA levels may occur after transcriptional initiation. It was found that compared to HIV-1, the HIV-2 LTR is significantly larger; undergoes 5′ LTR splicing; and has a complex secondary structure that may affect transcriptional *trans*-activation [56, 117]. It is postulated that these LTR differences might be translated into a less efficient transcriptional elongation and/or the premature switch from early to late transcripts in HIV-2 infection, resulting in lower mRNA accumulation *in vivo* [22].

Nef is an important factor in the HIV pathogenesis and plays a role in the evasion of host immune responses by down regulating surface expression of MHC class I molecules [118, 119]. Structural studies on the Nef protein have shown that blocking the PxxP domain would probably result in a reduction of viral replication as well as a more efficient recognition of viral particles by the immune system [120]. *In vivo* studies of SIV_{mac} have shown that subtle changes in the *nef gene* can dramatically alter the characteristics of SIV replication [121]. Deletions in the HIV-1 *nef* have been associated with reduced disease progression in a cohort of HIV-1 long-term non-progressors [122]. HIV-2 studies from Ivory Coast, Spain and Portugal have found *nef* deletions in 14% of asymptomatic patients and 4% in AIDS patients [123].

Vpu found exclusively in HIV-1, SIVcpz and SIVgsn [124]; has two primary functions: degradation of the CD4 receptor and enhancement viral particle release [125, 126]. Recent studies have shown that some human cells have proteins that can block the release of retroviruses as well as other enveloped viruses [127, 128]. However, the HIV-1 Vpu counteracts the effects of these inhibitory proteins, thereby enhancing release and

contributing to increased pathogenesis [128]. Simian studies have shown that the presence of Vpu was correlated with a vast increase in plasma viral RNA levels; and that deletions in the vpu gene that were large enough to prevent reversions, resulted in long term non-progressing infections [129, 130]. The absence of vpu in HIV-2 might contribute to the lower viral load and reduced pathogenicity observed during HIV-2 infection. However, other studies have shown that with respect to virus release, the HIV-2 Env protein has a Vpu-like activity that can compensate for the absence vpu gene; but there is no evidence that HIV-2 Env can induce CD4 degradation [131-133].

Not all HIV-2 strains have reduced pathogenicity

Although HIV-2 is generally less pathogenic than HIV-1, some HIV-2 strains are just as pathogenic as most HIV-1 viruses. HIV-2 infection is also clinically diverse with a majority of HIV-2 infected individuals progressing like HIV-1 long term non-progressors [72]; while a small proportion are fast progressors exhibiting very high plasma viral loads and rapid $CD4^+$ T cell decline that leads to disease progression that is indistinguishable from that of HIV-1 infection [22, 134-136].

ANTI-RETROVIRAL THERAPY AND DRUG RESISTANCE

Drug development, susceptibility tests and drug resistance studies has focused almost exclusively on HIV-1, with limited work done on HIV-2. This is mainly due to the lower prevalence of HIV-2 relative to HIV-1 and the restriction of the HIV-2 epidemic mainly to West Africa, where access to treatment has been limited. HIV-1 infection is treated using antiretroviral therapy (ART), which includes the use of drugs that target critical points of the HIV life cycle. Currently approved drugs were designed for HIV-1 subtype B, but due to the highly conserved nature of the HIV-1 and HIV-2 protease and reverse transcriptase, especially around the active sites, the target of most drugs, it was assumed that these drugs would work for both types of HIV infections. The non-nucleoside reverse transcriptase inhibitors (NNRTIs), however, target allosteric sites of the enzyme and it was later discovered that HIV-1 group O and HIV-2 are naturally resistant to these drugs [137-139].

Reverse transcriptase (RT) inhibitors

HIV-1 and HIV-2 RT share a sequence homology of ~60%, with similar structural and catalytic properties [140]. This similarity in sequence and enzymatic activity suggest that

the structures of HIV-1 and HIV-2 RT are most likely very similar. However differences have been observed in their crystal structures [141], which have shown that the functional RT is in the form of an asymmetric heterodimer, with the HIV-2 RT heterodimer being more stable than the HIV-1 RT heterodimer [142, 143].

Nucleoside Reverse Transcriptase Inhibitors (NRTIs) NRTIs are nucleoside analogues that serve as chain terminators. These drugs have a broad spectrum of activity and have activity against both HIV-1 and HIV-2 (Table 2). However, there have been controversial reports of NRTI activity against HIV-2.

A study on HIV-2 susceptibility to the NRTIs, Azidothymidine (AZT), Dideoxycytidine (ddC), and Dideoxyadenosine (ddA), Dideoxythymidine (ddT), Dideoxyguanosine (ddG) and Dideoxyinosine (ddI) using different cell lines showed that HIV-2 was less susceptible to all these drugs than HIV-1. Their results showed that HIV-2 was most susceptibility to ddC with a 3-fold reduction in susceptibility relative to HIV-1; AZT with a 300-fold reduction in HIV-2 susceptibility showed the least activity; and the other NRTIs showed a 10-fold reduction in activity [144]. Another group found that HIV-2 was able to replicate persistently and efficiently at an AZT concentration 3000-fold higher than the EC_{50} of HIV-1 and thus concluded that HIV-2 is naturally resistant to AZT [145]. These studies used both primary isolates and laboratory strains; measured AZT activity using growth kinetics in the presence of AZT; and assessed the evolution of resistance in selection experiments. However, comparison of enzymatic susceptibility of the HIV-1 and HIV-2 RT to AZT-TP showed similar IC_{50} values in both viruses [145]. On the other hand, several other studies have demonstrated that HIV-2 susceptibility to NRTIs is similar to that of HIV-1 both *in vitro* [146-148] and in cell-free assays [149, 150].

A recent publication has compared the methods used in these studies to explain the contradictory results [151]. Previous studies were based on assays that used multiple replication cycles and cytopathic effects to measure viral growth. These experiments may have been confounded by the differences between diverse HIV-2 strains in terms of replication rates, cytopathicities, and multiplicities of infection and therefore may not have accurately reflected intrinsic drug susceptibility [151]. Rapid syncytia formation in HIV-2 destroys cultures faster, saturating the assay and falsely inflating the resultant EC_{50}. This difference in cytopathicity may explain the apparent resistance of HIV-2 to AZT in assays that measure virus-mediated cell killing [151].

To circumvent these problems, this new study used a single replication cycle that is not cofounded by differences in replication capacity and cytopathicity, to compare the activity of AZT and other NRTIs in HIV-1 and HIV-2 infection. They found that HIV-1 and HIV-2 have similar susceptibilities to AZT and other NRTIs [151].

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs are allosteric inhibitors that bind to a hydrophobic pocket close to the active site; where they cause a conformational change by displacing the catalytic aspartate residues and thus rendering the enzyme inactive [152]. These drugs are very specific as they were designed to fit into the HIV-1 subtype B hydrophobic binding pocket. Studies have shown that HIV-2 is naturally resistant to the first generation NNRTIs. There are significant differences in the amino acid residues that make contact with the NNRTI in the binding pocket of HIV-1 and HIV-2. Of special importance are the Tyrosine (aromatic side chain) residues at positions 181 and 188 in HIV-1 that are replaced by Isoleucine and Leucine respectively, in HIV-2 [138]. Despite the significant difference in amino acid side chains, the general structure of the binding pocket is preserved [141]. However, these amino acid polymorphisms are reflected in a significant reduction in the binding capacity of the NNRTI to the HIV-2 binding pocket [141]. HIV-1 mutations at these positions result in the complete resistance to NNRTIs.

Protease inhibitors (PIs)

HIV-1 and HIV-2 proteases have an amino acid sequence similarity of about 50%, less than that observed in their reverse transcriptase enzymes. Although the proteases of these viruses share similarities in structure and enzymatic function, differences have been observed in conformation, substrate specificity and affinity [153, 154]. These sequence differences are reflected in very distinct natural polymorphism in the HIV-1 and HIV-2 proteases. However, most of these polymorphisms occur outside the functionally relevant areas; the region of the active site (25-32), the flap region (48-56), the 2nd loop of the beta sheet (78-87), which were mostly conserved in both viruses [155,156]. Several HIV-2 natural polymorphisms correspond to drug resistance mutations in HIV-1. These include the major drug resistance mutation, M46I to Indinavir and several minor mutations, L10V, V32I, M36I, I47V, A71V and G73A that may decrease the activity of Nelfinavir and Amprenavir [156-159]. Once PI-based ART starts, this background of minor mutations may result in fast acquisition of a multi-PI resistance phenotype [159]. Other studies have shown that while PIs bind to and are effective against HIV-2,

depending on the inhibitor, binding efficiency was 10-100 times less for the HIV-2 protease compared to HIV-1 [154]. *In vitro* studies of individual PIs have shown that while Indinavir, Saquinavir, Lopinavir, Darunavir and Tipranavir were shown to exert full activity against wild-type HIV-2 [160-165],], Nelfinavir had a 6.6-fold reduction and Amprenavir had a 31-fold reduction in susceptibility against HIV-2 [161].

Entry inhibitors

The largest diversity between HIV-1 and HIV-2 is observed in the *env* genes, which share a sequence similarity of less than 35% [54]. Thus it is not surprising that the first entry inhibitor, targeting the HIV-1 subtype B envelop has no activity against HIV-2. Two types of entry inhibitors: fusion inhibitors (FI) and co-receptor binding inhibitors have been approved for HIV therapy. Enfurvitide, a fusion inhibitor, is highly specific and has been found to have no activity against HIV-2 (table 2) [149].

Table 2: Susceptibility of the HIV-2 strains, HIV-2 ROD and EHO to various antiretroviral drugs [f].

Table 1. Susceptibility of two strains of HIV-2 (ROD and EHO) to various anti-HIV-1 compounds in comparison to wild-type HIV-1(III_B)

Compounds	EC_{50}* (µg/ml) (fold ncrease) †		
	HIV-1(III_B)	HIV-2(ROD)	HIV-2(EHO)
Nucleoside reverse transcriptase inhibitors			
Zidovudine	0.0011 ±0.0002	0.00096 ±0.00081 (0.9)	0.00046 ±0.00036 (0.4)
Lamivudine	0.81 ±0.25	0.51 ±0.22 (0.6)	0.16 ±0.05 (0.2)
Stavudine	0.038 ±0.004	0.028 ±0.018 (0.7)	0.0029 ±0.0014 (0.08)
Didanosine	3.0 ±1.3	2.94 ±2.22 (1.0)	0.75 ±0.46 (0.3)
Zalcitabine	0.18 ±0.13	0.054 ±0.039 (0.3)	0.014 ±0.006 (0.08)
Abacavir	1.40 ±0.32	1.85 ±0.21 (1.3)	1.57 ±1.41 (1.1)
Nucleotide reverse transcriptase inhibitor			
Tenofovir	1.15 ±0.52	1.12 ±0.60 (1.0)	1.05 ±0.48 (0.9)
Non-nucleoside reverse transcriptase inhibitors			
Nevirapine	0.019 ±0.005	>40 (>2105)	>40 (>2105)
Delavirdine	0.010 ±0.004	>40 (>4000)	3.55 ±2.26 (355)
Efavirenz	0.00029 ±0.00003	>2 (>6897)	2 (>6897)
Protease inhibitors			
Ritonavir	0.089 ±0.047	0.10 ±0.05 (1.1)	0.056 ±0.007 (0.6)
Indinavir	0.014 ±0.002	0.022 ±0.011 (1.6)	0.009 ±0.001 (0.6)
Saquinavir	0.0075 ±0.0026	0.0035 ±0.0018 (0.5)	0.0019 ±0.0007 (0.3)
Nelfinavir	0.032 ±0.009	0.086 ±0.071 (2.7)	0.040 ±0.022 (1.3)
Amprenavir	0.048 ±0.013	0.42 ±0.07 (8.8)	0.68 ±0.42 (14.2)
Entry inhibitors			
AMD3100	0.0061 ±0.0033	0.021 ±0.006 (3.4)	0.010 ±0.011 (1.6)
Enfuvirtide	0.062 ±0.010	3.95 ±3.77 (64)	5.43 ±2.84 (88)

Maraviroc, a co-receptor binding inhibitor, works by blocking the CCR5 receptor, thereby inhibiting the virus from further conformational changes that will allow fusion with the host membrane. The activity of Maraviroc against HIV-2 has not been tested, though, since Maraviroc binds to the CCR5 receptor, it should work against R5 HIV-2

viruses [166, 167]. Even though CCR5 inhibitors may have activity against HIV-2, the ability to use other co-receptors efficiently by HIV-2 limits their use. Another potential concern is the switch or emergence of X4 viruses, which are associated with faster disease progression. Although R5 to X4 switch is not obvious in HIV-2-infected individuals, a limited number of X4 viruses have been isolated from symptomatic patients [54].

Integrase inhibitors

The integrase inhibitors (INI) work by interfering with the insertion of HIV DNA into host DNA. Being a very important enzyme, the HIV-1 and HIV-2 integrase genes are well conserved, with 60% amino acid sequence homology between the two viruses. The HIV-1 integrase is a 288 amino acids (aa) protein that folds into three distinct functional domains (figure 4): the N-terminal domain (aa 1-50) with the HHCC motifs; the central core domain (aa 51-212) with the catalytic triad DDE (aa 64,16,152); and the C-terminal domain (aa 213-288). The functionally important motifs: the catalytic triad DDE, the HHCC and RKK are 100% conserved in HIV-1 and HIV-2 [168].

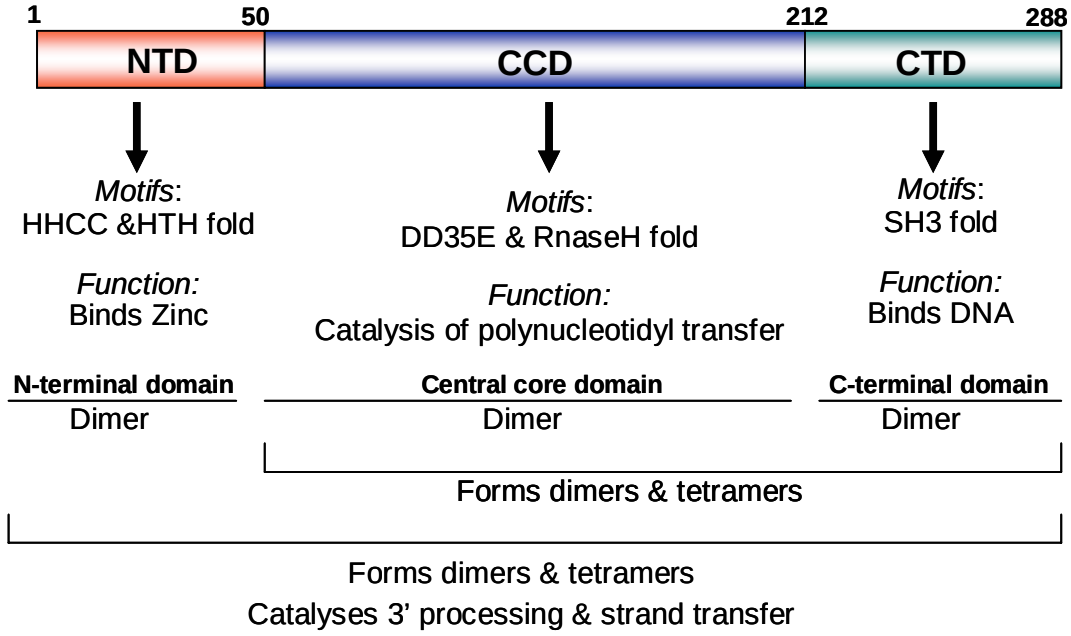

Figure 4: Schematic representation of the HIV-1 integrase gene [d]

Despite this similarity, 9 natural polymorphisms were found in HIV-2 at positions (51, 72, 97, 125, 138, 153, 201, 206 and 280) associated with INI resistance in HIV-1 [168, 169]. Nevertheless, *in vitro* susceptibility of 15 HIV-2 isolates as well as HIV-2 ROD to

Raltegravir has showed similar activity for HIV-1 and HIV-2. *In vivo* studies on two highly treatment-experienced HIV-2 infected individuals showed promising results, with viral loads reduced to undetectable results, when Raltegravir was used in combination therapy [169].

COMBINATION THERAPY

Treatment of HIV with HAART has drastically changed the course of the disease. HAART involves the use of three or more drugs from at least two different classes to maximally suppress viral replication and deter the emergence of drug resistance mutations. Out of the six types antiretroviral (ARV) drugs currently approved by the FDA: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, co-receptor binding inhibitors and integrase inhibitors, activity against HIV-2 has been shown for only the NRTIs, PIs and INIs. This makes selecting an optimal regimen for HIV-2 very difficult and therefore care should be taken in the choice of drugs so as to preserve future therapy options. Studies on HIV-1 and HIV-2 disease progression have indicated that when AIDS develops in HIV-2 patients, $CD4^+$ T-cell count is higher than in HIV-1 patients [82, 170] and that it is usually more than 200cells/ml. Therefore when HIV-2 patients start therapy at $CD4^+$ T-cell count of 200cells/ml, as in HIV-1, they do so after a longer period of infection. This late initiation of treatment in HIV-2 infection might have contributed to the poorer global treatment response observed in HIV-2 and thus patients might benefit from starting treatment earlier, at higher $CD4^+$ T-cell counts.

DRUG RESISTANCE

The lower prevalence of HIV-2; its restriction to West Africa, where access to treatment was limited; and the lower proportion of patients progressing to the stage where ART is necessary; together have resulted in limited information on treatment of HIV-2 and the emergence of drug resistance. As in HIV-1, resistance emerges as a result of the acquisition of drug resistance mutations within the relevant genes. For each drug class, certain mutations can occur that confer cross resistance to other drugs within the same class. According to the International AIDS Society USA, multi-NRTI resistance occurs

when at least three of the following mutations: M41L, D67N, K70R, L210W, T215Y/F and K219Q/E are present; for the NNRTIs, the presence of K103N or Y188L or at least two of the following mutations: L100I, V106A, Y181C/I, G190S/A and M230L is required. Multi class resistance for the PIs is defined by at least four of the following mutations: L10F/I/R/V, V32I, M46I/L, I54L/M/V, V82A/F/T/S, I84V/A/C and L90M [171]. Due to the limited number of HIV-2 patients treated and the lack of activity of most ARVs against HIV-2, resistance data is only available for the NRTIs and PIs.

HIV-2 NRTI resistance

NRTI resistance in HIV-2 occurs via the same mechanisms as observed in HIV-1. The most common mutations are the M184V and the Q151M. The M184 mutation occurs rapidly and in about 83% of patients receiving a 3TC-containing regimen [172, 173]. This mutation is also associated with phenotypic resistance to 3TC and FTC in HIV-2 infected individuals [174]. The Q151M mutation occurs faster with a much higher frequently in HIV-2 relative to HIV-1. Considering that this mutation causes multi-NRTI resistance in HIV-2, its high frequency in HIV-2 raises real concerns. The Q151M mutation causes phenotypic resistance by decreasing sensitivity to ZDV, ddI, d4T and ddC by 5-10 fold, as in HIV-1 Although HIV-1 and HIV-2 share some classic HIV-2 resistance patterns, preference of alternate resistance pathways have been noticed as well as unique resistance patterns in HIV-2. AZT resistance in HIV-1 occurs by two well documented pathways: the most common and preferred AZT resistance pathway is marked by the accumulation of the six thymidine-analogue resistance mutations (TAMs), M41L, D67N, K70R, L210W, T215Y and K219Q/E [175] and the less common pathway is via the Q151M mutation. These TAM mutations are conspicuously absent in the AZT resistance profiles of HIV-2 patients [158, 172, 176]; instead AZT resistance in HIV-2 involves the Q151M mutation. TAM resistance occurs by nucleotide excision from the nascent nucleotide chain. Structural and binding efficiency studies have indicated that the ATP binding pocket of the HIV-2 RT is shallower and less able to bind ATP appropriately for the excision reaction [177].

HIV-2 PI resistance

PI resistance in HIV-1 occurs by an accumulation of many mutations, though major mutations can cause substantial resistance on their own. However, because most drug resistance mutations in the HIV-1 protease result in decreased fitness, major mutations are accompanied by minor mutations that compensate for the reduced fitness. While HIV-1 and HIV-2 resistance patterns are very similar, the occurrence of 7 HIV-1 drug

resistance mutations as natural polymorphisms has been observed in HIV-2 [158, 159, 176, 178]. Recent studies have shown that these polymorphisms occur at 13 locations in the HIV-2 protease and that while they do not cause major mutations, they may facilitate the time to development of resistance in HIV-2 relative to HIV-1[178]. Several HIV-1 resistance mutations were found to occur at the same positions in HIV-2 as well as new mutations unique to HIV-2 [156, 159, 176, 178]. A study of phenotypic resistance to TPV, NFV, IDV, APV and LPV in HIV-2 has shown that I54M results in 7-15 fold resistance to TPV, NFV and IDV; >22.7 and 120 fold resistance to APV and LPV respectively; I54M-I84V results in 12.7-fold reduction in susceptibility to TPV, and more than 22.5-fold reduction in susceptibility the other PIs; I54M-V71I-L90M resulted in multi-PI resistance of 22-131-fold reduction in susceptibility to all PIs except TPV and the unique mutations V62A+L99F resulted in 124-fold (LPV), 15-fold (NFV) and 9-fold (IDV) reduction in drug susceptibility [178].

FIGURE AND TABLE REFERENCES

a. **Figure 1:** West African girl with pet sooty mangabey: HIV-2 may have been transferred to humans through close contact with sooty mangabeys [179].
b. **Figure 2**: Distribution of HIV-2 in Africa: Shaded areas represent regions where significant rates of HIV-2 infection have been reported (Modified from [25])
c. **Figure 3:** Genomic organisation of HIV-1 and HIV-2 [180]
d. **Figure 4:** Schematic representation of the HIV-1 integrase gene (Modified from [181])
e. **Table 1**: Comparison of HIV-1 and 2 gene products (Modified from [10, 12, 142])
f. **Table 2:** Susceptibility of the HIV-2 strains, HIV-2 ROD and EHO to various antiretroviral drugs [146]

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Chapter 3

Outline and Rationale of Thesis

Outline and Rationale of Thesis

The general aim of this thesis is contribute to the understanding of HIV-2 treatment and the emergence of drug resistance.

Studies on HIV-1 drug resistance have resulted in the generation of optimal ARV regimens; discovery of the benefit of combination therapy; the additive effect of certain drug combinations; the emergence of drug resistance, development of resistance assays and algorithms to interpret resistance in terms of relevance, cross resistance and hypersensitivity issues. The lower prevalence of HIV-2 relative to HIV-1 and the restriction of the HIV-2 epidemic mainly to West Africa, where treatment has largely been unavailable; have resulted in very limited studies on HIV-2 drug resistance. Recently the availability of generic drugs and the scale up of programs to improve ARV access in developing countries have resulted in more than 2 million HIV-infected patients being treated with HAART in sub-Saharan Africa. Therefore, for the proper management of HIV-2 infected patients on ART, there is a need to develop specific assays for the screening and monitoring of HIV-2 infected individuals starting ART. The knowledge of HIV-2 specific characteristics, as compared to HIV-1, is crucial for the choice of initial ARV therapy, as well as for monitoring therapy success.

The Gambia Government has received a grant from the 'Global Fund' to support ARV therapy. The national plan at present indicates 1500 people with HIV should be on treatment by 2009. We thus started this study with several research questions, to promote successful treatment of HIV-2 infected patients in Africa as well as to increase global knowledge of HIV-2 resistance patterns.

Our first aim was to assess the response of HIV-2 infected patients to antiretroviral therapy. Previous studies have indicated that various HAART regimens either fail to suppress HIV-2 replication to undetectable levels or achieve undetectable viral load in only a small percentage (33%) of patients. *In vitro* studies have indicated that HIV-2 is naturally resistant to some ARVs, due to natural polymorphisms that confer resistance. However, *in vivo* studies assessing drug activity are lacking.

In West Africa, co-circulation of HIV-1 and HIV-2 has resulted in patients dually infected with both viruses. Since little is known about HIV-2 response to ART and the development of drug resistance, the presence of HIV-2 in these dually infected

individuals further complicates treatment; therefore having access to ARVs to treat these patients provides a unique opportunity to study the response to treatment and the development of drug resistance in dually infected individuals as well. These studies provide important new information to guide treatment of these patients.

Our second aim was to study and understand the development of drug resistance in patients with virologic failure. Patient samples, both plasma and cells, will be stored before and during treatment. During each clinical visit, the viral load and CD4 T-cell count of each patient will be quantified, and samples corresponding to these measures will be stored. Sequence analysis of pre- and post-therapy samples, in case of viral rebound will allow us to map potential HIV-2 drug resistance mutations and a phenotypic assay will determine the level of resistance, if any, caused by each mutation and in combination with other mutations. This will be the first time HIV-2 specific resistance mutations will be documented. The presence of documented mutations in a database will allow rapid diagnosis in patients and also facilitate quick changes of therapy to prevent excessive immunological damage as a result of virus production.

Treatment of HIV comes with the challenges of monitoring response to therapy and the emergence of drug resistance. In fact, the costs of laboratory tests to monitor HIV had significantly delayed ARV access in developing nations. Current WHO recommendations to countries without the infrastructure for monitoring assays are to use immunological or clinical failure to guide switching to a second line regimen. Therefore, acknowledging the value that these tests bring to the care of patient, assessing alternative methods, and developing resource-appropriate tests to monitor therapy has rapidly become a high priority.

Our third research goal was to develop a cheaper alternative to consensus sequencing to monitor drug resistance in resource poor settings. The oligonucleotide ligation assay (OLA) is a screening tool for the evaluation of the primary mutations associated with drug resistance to the currently available antiretroviral drugs. While sequencing is commonly used to evaluate the presence of drug resistance mutations and provides the opportunity to comprehensively evaluate multiple mutations associated with drug resistance and the identification of new mutations, OLA has several advantages that make it a better alternative in resource poor settings. First, the assay has high throughput that makes it ideal for epidemiologic studies or clinical trials that evaluate a large number of specimens for specific mutations. Second, OLA is highly sensitive in the detection of

small populations of mutant genotypes among wild type viral sequences. Third, the results of the OLA are simple to interpret either visually or by a spectrophotometer. These attributes, together with its low cost, make this assay suitable for genotypic evaluation of HIV-2 drug resistance in laboratories where costly equipment, software, and technical expertise needed for sequence analysis may not be available.

As more people are treated in Africa, cases of transmitted drug resistance will increase in the coming years. Development of both the HIV-1 non-B and the HIV-2 OLA will be useful to screen patients before therapy for the known mutations in the population. Prior knowledge of resistance in patients initiating therapy will allow use of drugs that are active and hence result in effective treatment. These assays can be used in all places where HIV-2 is a problem, in particular the Gambia, Senegal and Guinea Bissau. The national program in the Gambia has expressed an interest in the implementation of this genotyping assay as part of the national ART program.

As a fourth and final aim, we would design a recombinant virus assay for use in a phenotypic resistance assay to evaluate the relevance of mutations found in treated patients. The generation of mutant viruses, will allow definition of individual mutations as primary or secondary mutations, determine the level of resistance and cross resistance to other drugs. Apart from phenotypic resistance, mutant viruses generated from this recombinant virus assay can be used to gain insight as to the fitness of these mutant viruses relative to the wild-type. Knowledge of the fitness in these HIV-2 viruses will allow physicians to identify mutations that could be beneficial to patients who have exhausted their treatment options. This will be especially relevant in Africa, where only a few drugs are available for highly active antiretroviral therapy.

The problems of treating HIV infection in any country, developing or developed are manifold. The opportunity provided by the Global Fund to treat HIV-infected patients in The Gambia with antiretroviral therapy must be exploited to its fullest extent to make the best use of the available therapy. Experience from the treatment of other infections in Africa, such as tuberculosis and malaria, suggests that therapy adherence and the development and spread of drug resistant variants may be a major problem. Any research on the outcomes of using antiretroviral drugs, and especially the development and spread of drug resistance, must necessarily be of value to those providing healthcare in The Gambia. Also, at the global level, documenting HIV-2 resistance markers in an accessible database and generating the tools to identify these mutations will help guide the next

generation of ARV drugs; thereby allowing the development of new drugs that can be used against resistant strains.

RESULTS : PART I

Development, Optimisation and Evaluation of Assays to Detect HIV-2 Drug Resistance Mutations

Chapter 4

A Genotypic Resistance Assay for the Detection of Drug Resistance Mutations in the Protease and Reverse Transcriptase Gene of the Human Immunodeficiency Virus Type 2

A genotypic resistance assay for the detection of drug resistance mutations in the protease and reverse transcriptase gene of the Human Immunodeficiency Virus type 2

Sabelle Jallow[a,b], Steve Kaye[a], Abraham Alabi[a], Akum Aveika[a], Ramu Sarge-Njie[a], Samuel J. McConkey[a], Guido Vanham[b,c], Sarah Rowland-Jones[a] and Wouter Janssens[b]

[a] *Medical Research Council Laboratories, Banjul, The Gambia*
[b] *Department of Microbiology , Institute of Tropical Medicine, Antwerp, Belgium*
[c] *Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*

To be submitted

Abstract

Increasing access to antiretroviral drugs in Africa means that a substantial number of HIV-2 infected individuals will be treated. In HIV-1, resistance to all classes of drugs has been reported, even with the use of Highly Active Antiretroviral Therapy (HAART). Therefore, treatment of HIV-2 infected individuals will most likely result in the emergence of drug resistant viruses; necessitating the study of genotypic resistance patterns of HIV-2 and deciphering the clinical relevance of these genotypic mutations. We successfully developed and optimised a genotypic resistance assay for the amplification and sequencing of the entire protease and reverse transcriptase gene fragment of HIV-2 with a sensitivity of 91.3%. A hemi-nested PCR could be used on negative samples to increase the sensitivity of the assay to 93.8% (RNA) or 98.8% (DNA). This assay could genotype patient samples with viral loads as low as 100 copies/ml, the limit of detection of the viral load assay.

Introduction

The HIV-2 epidemic is restricted mainly to West Africa [1], where antiretrovirals (ARVs) have not been widely available, until recently with the availability of cheaper generic ARVs and scale up programs to increase access in developing nations. Treatment of HIV-2 will result in the emergence of drug resistant variants; as has been observed for HIV-1, necessitating the study of genotypic resistance patterns of HIV-2 and deciphering the clinical relevance of these genotypic mutations.

Monitoring drug resistance is an important aspect in the management and treatment of HIV infection; guiding the initial drug regimen by identifying drugs that are unlikely to suppress viral replication. Also, resistance testing after treatment failure will allow strategic changes in therapy prior to significant deterioration in the patient's clinical or immunologic status [2, 3]. Retrospective and prospective clinical trials have demonstrated the importance of resistance testing [4, 5]; resulting in recommendations of its use in cases of primary infection, chronic infection, treatment failure, pregnancy, post exposure prophylaxis and treatment interruption by several panels of experts, including the International AIDS society, USA and the Euro Guidelines Group [3].

We report the development and optimisation of a genotypic resistance assay to simultaneously elucidate drug resistance mutations in the protease and the reverse transcriptase (RT) gene of the Human immunodeficiency virus type 2.

Materials and Methods

Samples: The PCR method was optimised with CBL23, an HIV-2 reference strain. The amplification procedure was then evaluated on 80 samples from 36 patients, with viral loads of ranging from 100 to 1,000,000 copies/ml (mean = 93,778 and median = 39,162). 70% of samples (56/80) were from treated patients from the Gambia and 30% were from treatment-naïve individuals from Caio, Guinea Bissau (Table 1).

CD4 T-Count and Viral load: CD4 T-cell measurements were done by flow cytometry (Facscan, Becton Dickinson). Plasma HIV-2 RNA copies/ml (viral load) measurements were done using an in-house viral load assay as previously described [6], which had a limit of detection of 100 RNA copies/ml plasma. The assay was modified to include an

internal molecular control for extraction and amplification efficiency as previously described [7].

Table 1: Virological data of the Patients

	Gambia	Guinea Bissau	Total
# of Patients	**13**	**23**	**36**
# of Samples	**56**	**24**	**80**
Mean Viral load (copies/ml)	**95,505**	**89,747**	**93,778**
Median Viral load (copies/ml)	**37,797**	**41,537**	**39,162**
Range of Viral loads (copies/ml)	**100 - 1,000,000**	**7,456 - 582,000**	**100 - 1,000,000**

Primer design: HIV-2 primers were designed using an alignment of HIV-2 reference strains from all available HIV-2 groups. The outer primers SJH21 and SJH22 and the inner primers SJH23 and SJH24 amplify a 1727bp fragment encompassing the entire protease (PR) and reverse transcriptase (RT) gene fragment. In addition, the sequencing primer SHJ26 was designed and used together with two published HIV-2 RT sequencing primers H2Mp6 and JA222 [8, 9] for the sequencing reactions. Compatibility of the PCR primers and the strength of secondary structures were checked with the OLIGO software. The details of these primers are listed in table 2.

Table 2: Primer information

primer name	primer sequence 5'-3'	Length	position on ROD	Reference
SJH21	gaaagaagccccgcaacttccc	22	1861-1882	24
SHJ22	gagtctgttagtgccatcgcaaag	24	3843-3820	24
SJH23	gagaccatacaaagaggtgac	21	1994-2014	24
SJH24	ctattgcaggatccatctgtg	21	3720-3700	24
SHJ26	cagttaggaattccacacccagcagg	26	2651-2678	
H2Mp6	aaaagagatctgtgcaaaaatgg	23	2482-2504	21
JA222	acctccaactaatccttataatacc	25	2530-2554	22

Nucleic acid extraction and PCR amplification: HIV-2 RNA was extracted from 140µl of EDTA plasma from Guinea Bissau samples using QIAamp viral RNA kit (QIAGEN, Venlo, The Netherlands) and 200µl of heparin plasma from the Gambian samples by the method of Boom *et al* [10]. The RNA was eluted into 50µl nuclease-free water.

PCR amplification of the entire protease and RT was optimized using the HIV-2 reference strain CBL23. RNA was extracted from CBL23 culture supernatant with 10^5 RNA copies/ml and a dilution series of 100,000, 2000, 200, 20 and 2 copies/ml was used

with different conditions to obtain a protocol that could amplify up to 200 copies/ml of CBL23 RNA. This protocol includes the use of 3µl of RNA in a single tube reverse-transcription PCR method (Titan one-tube RT-PCR, Roche Applied Science, Lewes UK), using recombinant Avian Myeloblastosis Virus (AMV) reverse transcriptase and Expand High fidelity DNA polymerase. 0.4µM of the outer primers SJH21 and SJH22 were used in a final volume of 25µl. Cycling conditions were 50°C for 45min, reverse transcription; 94°C for 2 min, initial denaturation; 10 cycles of 94°C for 30sec, 60°C for 30sec and 68°C for 120 sec; 25 cycles of 94°C for 30sec, 60°C for 30sec and 68°C 120 sec plus 5 sec/cycle elongation; and a final elongation of 68°C for 10min. Nested PCR was done using 0.5-2µlof first round PCR product ; 0.3µM of each primer, SJH23 and SJH24; 200µM each dNTP and 2.6 units of Expand High Fidelity DNA Polymerase in a final reaction volume of 50µl. Cycling conditions were 95°C for 3 min initial denaturation; 10 cycles of 94°C for 30sec, 54°C for 30sec and 72°C for 105 sec; 25 cycles of 94°C for 30sec, 54°C for 30sec and 72°C for 105 sec plus 5 sec/cycle elongation; and a final elongation of 72°C for 7min to amplify the entire HIV-2 PR and RT genes (1727 bp). PCR products were analysed on 0.8% agarose gels.

Sequence analysis: PCR products were purified using the QIAquick PCR purification system (QIAGEN Benelux B.V., Venlo, The Netherlands) and directly sequenced. Sequencing was done on both strands using the primers SJH23, SJH24, SJH26, JA222, and H2Mp6. Sequencing was done by the VIB Genetic Service Facility (Wilrijk, Belgium) using capillary sequencers (Applied Biosystems 3730 DNA Analyser) combined with ABI PRISM® BigDye™ Terminator cycle sequencing kits. The sequences were assembled using DNAsis software (Hitachi Software Engineering, Molecular Biology Insights, Colorado, U.S.A); aligned with HIV-2 ROD and then edited, translated and analysed with EditSeq and MegAlign (DNASTAR, Lasergene Software, Wisconsin, U.S.A). Viral subtype was determined using an NCBI on-line programme (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and by phylogenetic analysis (DNASTAR). Our samples were aligned with several HIV-2 reference strains (subtypes A, B & G) and the trimmed alignment was used to construct the phylogenetic tree (DNASTAR). SIVmne (Genbank accession #: AF361745) was employed as the outgroup, to root the tree.

Results

All patients were found to be HIV-2 subtype A. Sequences generated in this study have been submitted to Genbank and were assigned the accession numbers AM233873 -

AM233900 and AM408175 - AM408208 [11]. The PCR amplification was optimised with a serial dilution of 100,000, 2000, 200, 20 and 2 RNA copies/ml of CBL23 to obtain a protocol that could amplify down to 200 copies/ml of CBL23 RNA. This protocol is specific and sensitive, resulting in a single positive PCR band with a lower limit of detection of 200 RNA copies/ml. The PCR amplification was evaluated on 80 samples from both treated and untreated patients (Table 1) and was successful for 73 samples (91.3%) with viral loads ranging from 1,000,000 to 100 copies/ml; the limit of detection of the viral load assay. Six out of the seven unsuccessful PCR reactions, with viral loads (VL) 58025, 6016, 3146, 1036, 1354, 100 and 17464 copies/ml, were later successfully amplified using a hemi-nested PCR (use of one of the 1st round primers in the 2nd round PCR) with the primers SJH23 and SJH22 for the first round and SJH23 and SJH24 for the second round. Starting from RNA only two samples with viral loads of 58025 and 6016 copies/ml were amplified with RNA; however DNA amplification resulted in positive amplicons for all the samples except one with a viral load of 17,464copies/ml. The use of this hemi-nested PCR resulted in an in increase in sensitivity to 93.8% (75/80) and 98.8% (79/80) for the RNA and DNA samples respectively. Sequencing was evaluated on 67 of the samples with a positive amplicon. All samples were successfully sequenced.

Drug-resistance mutations: Alignment of the pre- and post-therapy samples from the treated patients with HIV-2 ROD revealed the presence of several mutations that may have arisen as a result of drug pressure. In the HIV-2 RT region, four major HIV-1 NRTI mutations, the K65R, Q151M, M184V and S215Y were observed. V75I, associated with multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance in HIV-1, and K219Q/E, associated with AZT resistance in HIV-1, were found to occur naturally in all the samples.

In addition to the known HIV-1 mutations, several other mutations not previously detected in HIV-1 were observed. These new mutations, K20R, K40R, T53S, A62V, V108G, V111I, I118V, P150Q, I179T, V201A/M, F214L, K223R/E, V263I, K277R, Q333L, I341V, K346R, V356I, V371I and N403S, appeared to emerge as a result of drug pressure in these patients.

Analysis of the HIV-2 PR gene from Protease Inhibitor (PI) naïve patients showed the presence of several mutations, L10V, V32I, M36I, M46I, I47V, A71V and G73A, associated with HIV-1 drug resistance; as natural polymorphisms in a majority of the samples.

Discussion

HIV-2 infected patients are currently treated with drugs that target the protease and reverse transcriptase enzymes encoded by the *pol* gene. Current phenotypic and genotypic assays depend on amplification of the relevant genomic region by RT-PCR from viral RNA in plasma. Therefore, the first step in the development of a phenotypic and a genotypic assay for HIV-2 is the optimization of an RT-PCR to amplify the HIV-2 PR and RT.

Though published HIV-2 primers are available, they either amplify the PR and RT separately or they amplify PR and RT together, but include only part of the RT. This is mainly because most documented RT drug-resistance mutations in HIV-1 were found between codon 41 and 230; resulting in the sequencing of only this region. However, a mutation was later discovered at position 333 [12], which was missed by previous genotyping methods. Despite the 60% sequence homology between HIV-1 and HIV-2 RT [13], some differences have been observed and it is possible that primary mutations could occur at the C-terminal of the HIV-2 RT. Thus we designed primers that will amplify the entire PR and RT.

During this study, the optimised RT-PCR was evaluated on a total of 80 samples from 13 treated and 23 untreated patients from the Gambia and Guinea Bissau to ensure that a fairly representative panel of the HIV-2 epidemic was used. Though our primers were designed to work for all HIV-2 subtypes, all samples available for the evaluation of this assay were HIV-2 subtype A. Our primers successfully amplified the entire protease and RT in 91.3% of the samples evaluated. However, 7 samples from one patient could not be amplified. Since the viral loads of some of these samples were quite high, we assumed that the lack of a positive amplicon was probably due to sequence variability of the samples around the primer annealing region. Therefore, we tried different primer combinations in a hemi-nested PCR and finally produced a combination that gave positive PCR products with a single band. This hemi-nested PCR successfully amplified two of the seven samples with a high viral load starting from RNA and six of the seven samples starting from DNA. One sample could not be amplified; since its viral load was sufficient to produce enough RNA for amplification, lack of a positive amplicon was possibly due to sequence variation around the primer ligation sites.

We report the successful development and optimization of a genotypic resistance assay for the amplification and sequencing of the entire protease and reverse transcriptase gene fragment of HIV-2 with a sensitivity of 91.3%. A hemi-nested PCR could be used on negative samples to increase the sensitivity of the assay to 93.8% (RNA) or 98.8% (DNA). In addition, the assay could be easily adapted to include the integrase gene fragment to accommodate newly approved drugs that have activity against HIV-2.

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Chapter 5

Optimization of the Oligonucleotide Ligation Assay for the Detection of HIV-1 Drug Resistance Mutations in Non-B Subtypes

Optimization of the Oligonucleotide Ligation Assay, a Rapid and Inexpensive Test for Detection of HIV-1 Drug Resistance Mutations, for Non-North American Variants

Ingrid A. Beck[1], MS, Claudia Crowell[1], MD, Robin Kittoe[1], BS, Helba Bredell[2], MS, Molefe Machaba[2], Hons, Carolyn Willamson[2], PhD, Wouter Janssens[3], PhD, **Sabelle Jallow[3]**, PhD, Guido van der Groen[3], PhD, Yiming Shao[4], MD, PhD, Mini Jacob[5], MD, NM Samuel[5], MD, Ivette Lorenzana de Rivera[6], MS, Nicole Ngo-Giang-Huong[7], PhD, Sharon Cassol[8], PhD, George Alemnji[9], PhD, and Lisa M. Frenkel[1], MD.

[1] University of Washington, Seattle, Washington, USA;
[2] University of Cape Town, Cape Town, South Africa;
[3] Institute of Tropical Medicine, Antwerpen, Belgium;
[4] National Center for AIDS/STD Control and Prevention; Beijing, China;
[5] Tamil Nadu Dr. MGR Medical University, Chennai, India;
[6] Universidad Nacional Autonoma, Tegucigalpa, Honduras;
[7] Program for HIV Prevention and Treatment or Faculty of Associated Medical Sciences, Chiang-Mai, Thailand;
[8] MRC Unit for Inflammation & Immunity, Depart. of Immunology, University of Pretoria, Pretoria, South Africa;
[9] University of Yaounde, Yaounde, Cameroon

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ABSTRACT

Objective: We evaluated the feasibility of the oligonucleotide ligation assay (OLA), a specific, sensitive, and economical ligase-based point mutation assay designed to detect HIV-1 drug–resistance mutations at 12 codons of HIV-1 subtype B pol, for potential use in resource-poor settings.

Methods: Specimens from HIV-1–infected individuals collected by 7 international laboratories, including subtypes A, B, C, D, F, G, J, and recombinants AE and AG, were tested by the OLA developed for HIV-1 subtype B. Common polymorphisms that interfered with reactivity of the OLA were identified and modified probes designed and evaluated.

Results: 92.5% (2410) of 2604 codons in specimens from 217 individuals were successfully genotyped by the subtype B OLA. A high rate (range 8.3%–31.2%) of indeterminate results (negative OLA reaction for both mutant and wild type) was observed for 5 codons. Modified probes at reverse transcriptase codons 151 and 184 and protease codon 90 increased the rate of valid OLA to 96.1%.

Conclusions: The OLA designed for HIV-1 subtype B genotyped most pol codons in non-B subtypes from Asia and Africa but was improved by addition of several modified probes. International laboratories experienced in molecular techniques were able to perform the OLA.

INTRODUCTION

Antiretroviral therapy has been instrumental in improving the health of HIV-1 infected individuals and in the reduction of mother-to-child HIV-1 transmission (MTCT). Drug-resistant viruses have been transmitted and selected during antiretroviral therapy (1). Resistant variants have compromised the immunological benefits of ART and interventions designed to reduce MTCT (1). Thus, monitoring drug-resistance in the community and within the individual is recommended to optimize antiretroviral treatment (1).

Consensus sequencing and consensus phenotype are the most commonly used assays for detecting drug-resistant HIV-1 (1). These tests are costly, making them impractical for large-scale studies and inaccessible to most resource poor communities. We have developed a sensitive, specific and inexpensive ligase-based point mutation assay, the Oligonucleotide Ligation Assay (OLA) that detects mutations in HIV-1 *pol* conferring resistance to protease (PR) and reverse transcriptase (RT) inhibitors (2-4). This high-throughput system uses differentially modified oligonucleotides specific for wild-type or mutant sequences and a type-common oligonucleotide. When hybridized to the DNA template [HIV-1 polymerase chain reaction (PCR) amplified from infected individuals' specimens], these oligonucleotides are covalently joined by a thermostable DNA ligase, allowing sensitive detection of both genotypes in a single well of a microtiter plate (5). The current version of the OLA detects primary mutations in 12 codons of HIV-1 subtype B *pol* associated with resistance to most currently FDA-approved antiretroviral drugs. The OLA's main components are available as a kit from the NIH AIDS Reference and Reagent Program (http://www.aidsreagent.org) for no-cost distribution to researchers. Because this test is rapid, inexpensive and simple to perform and interpret, it is suitable for use in resource-limited settings. Limited evaluation of the assay has been performed on HIV-1 non-B subtypes.

The specificity of the OLA for mutant versus wild-type virus is conferred by the ligation reaction, and specifically by the requirement of the DNA ligase that the two bases flanking each side of the oligonucleotide junction be complementary to the target for the ligation to occur (6). Performing the OLA at relatively low temperatures minimizes the effects of polymorphisms within the target sequence, which are common in HIV-1 *pol* (7, 8), while maintaining the specificity at the site of interest. The OLA fails when genetic polymorphisms occur within two bases of the ligation site, or when multiple polymorphisms occur in the subject's HIV-1 within the region complementary to one of

the OLA probes (2, 3). Because genetic variation occurs between HIV-1 subtype B and other subtypes, one aim of this study was to evaluate the OLA probes designed to detect drug-resistance mutations in HIV-1 subtype B on HIV-1 of non-B subtypes, and modify the probes as needed to enable detection of the targeted mutations. Our second aim was to assess the feasibility of using the OLA kits (available from the National Institute of Health) in others' laboratories, especially in Africa and Asia.

MATERIALS AND METHODS

Subject's specimens : Blood specimens were obtained from HIV-1 infected individuals in Belgium (n=44), China (n=30), Honduras (n=39), India (n=45), South Africa (n=43), Thailand (n=20) and Cameroon (n=5) following approval by local committees monitoring research in humans. Specimens consisted of whole blood collected as dry blood spots (DBS) on FTA^{TM} (Whatman, Florham Park, NJ) (N=75, Honduras and India) or 903 specimen collection (Whatman) filter paper (N=15, Thailand and Cameroon), or cDNA derived from the plasma of clinical specimens after RNA extraction and reverse transcription (N=127, Belgium, China, South Africa and Thailand). The specimens from Belgium, China and two regions in South Africa were tested by OLA in their regional laboratories. PCR amplified nucleic acids with invalid or indeterminate OLA results in the regional laboratories were sent to the Seattle Laboratory for repeat testing. Blood specimens collected as DBS in Cameroon, Honduras, India, and Thailand were sent to Seattle and assayed only in Seattle.

Processing of DBS samples : DBS samples were collected, transported and stored at room temperature. Before PCR amplification, 3-mm punches taken from whole blood specimens collected on FTA^{TM} paper (Whatman) were washed following the manufacturer's instructions (9). Specimens collected on 903 paper were extracted using chelex-100 (Bio-Rad, Hercules, CA). Briefly, a circle containing approximately 50ul of whole blood was excised and washed twice with a solution of 0.1% Triton for 15 min. Then, 250ul of 10% chelex-100 were added, incubated for 2h at 56^{o}C, and then boiled for 15 min. The extracted DNA in the supernatant was used directly for PCR.

Polymerase Chain Reaction : Both, DNA bound to the FTA^{TM} paper (1, 3-mm punch) and DNA extracted from the S&S 903 paper (10ul) were amplified by nested PCR using first round primers PRA (CCTAGGAAAAAGGGCTGTTGGAAATGTGG) or IBF1 (AAATGATGACAGCATGTCAGGGAGT) and IBR1 (AACTTCTGTATATCATTGA

CAGTCCA), and second round primers PRB (ACTGAGAGACAGGCTAATTTTTT AGGGA) and IBR2 (CAAA GGAATGGAGGTTCTTTCTGATG). These primers were designed using sequences in the Los Alamos HIV-1 Sequence Database (Los Alamos National Laboratory, http://www.hiv.lanl.gov/content/hiv-db/PRIMALIGN/PRIME.html) to amplify a wide-range of HIV-1 subtypes. Each round of PCR contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 20 pmol of each forward and reverse primer, and 2.5 U of *Taq* DNA polymerase. Cycling conditions consisted of an initial denaturation step of 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 15 sec, 55^{o}C for 20 sec, and 72^{o}C for 2 min in first round or 1 min in second round, and a final extension step of 72^{o}C for 7 min. Plasma HIV-1 RNA was extracted using the QIAmp viral RNA kit (Qiagen, Valencia, CA), reverse-transcribed with Expand Reverse Transcriptase (Roche, Brussels, Belgium) or ThermoScript RT-PCR System (Invitrogen Corp., San Diego, CA.), and amplified with the same primers and conditions described above. The product amplified, an 1168-bp DNA fragment extending from HIV-1 *gag* to codon 228 of the reverse transcriptase gene, was visualized in a 1% agarose gel following electrophoresis and ethidium bromide staining.

Oligonucleotides for ligation detection : Ligation oligonucleotides specific for wild-type or mutant sequences (2-4) were modified at the 5'-end by addition of digoxigenin or fluorescein, respectively. Joining oligonucleotides complementary to common sequences adjacent to both wild-type and mutant codons were biotinylated at the 3' end and phosphorylated at the 5' end. Modified, HPLC-purified oligonucleotides were obtained from MWG-Biotech Inc (High Point, NC).

Oligonucleotide Ligation Assay : The procedure and reaction conditions have been described (2, 3) and a detailed protocol is found at http://depts.washington.edu/idimmweb/faculty/frenkel/OLAmanual1305april04.pdf (OLA Manual, Version 1.4, July 2007). Specimens tested outside the Seattle laboratory were analyzed using Subtype B OLA kits prepared and distributed by the NIH AIDS Reagents and Reference Program that evaluate mutations at codons 30, 50, 82, 84, 88 and 90 of HIV-1 protease, and codons 65, 103, 151, 181, 184, and 215 of HIV-1 reverse transcriptase. These kits contained the same PCR primers, ligation oligonucleotides, and OLA mutant and wild-type controls as used in the Seattle laboratory. Samples with indeterminate results were retested with newly designed OLA probes by the Seattle laboratory only. HIV-1 mutant and wild-type controls for each codon analyzed were described previously (3) and tested in each assay plate. All subjects' samples and controls were analyzed in duplicate. Optical densities at

490 nm (mutant genotype) and 450 nm (wild-type genotype) were measured in a Spectramax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Sequencing: Residual PCR primers and dNTP were removed from the amplified DNA products by treatment with shrimp alkaline phosphatase and exonuclease I (PCR Product Pre-Sequencing Kit, Amersham Laboratories, Arlington Heights, IL). Cleaned PCR products were then directly sequenced using forward primers PRB and RT4 (2), and reverse primers PR2 (3) and IBR2, with fluorescence-labeled dideoxy-chain terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA), and a Prism 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences encoding HIV-1 protease and reverse transcriptase were assembled and edited using Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI).

HIV-1 Subtype Determination: The HIV-1 subtype of the different specimens was determinted by analysis of the specimens' sequences submitted to the Stanford University HIV-1 Database (http://hivdb.stanford.edu), Env and Gag heteroduplex mobility assay (10, 11) or by epidemiologic prevalence.

RESULTS

Blood specimens from 217 HIV-1 infected individuals residing in Belgium (N=44), China (N=30), Honduras (N=36), India (N=39), South Africa (N=43), Thailand (N=20) and Cameroon (N=5) were evaluated for 15 drug-resistance mutations by the OLA. The specimens included HIV-1 subtypes A, B, C, D, F, G, J, and recombinants AE and AG (Table 1). The mutations assayed were the nucleotides that most frequently encode D30N, I50V, V82A/S/T, I84V, N88D and L90M in HIV-1 protease (PR), and K65R, K103N, Q151M, Y181C, M184V, and T215Y/F in HIV-1 reverse transcriptase (RT). The OLA results from the international laboratories were reviewed in Seattle. The OLA was valid for 77.3% of 1069 codons analyzed regionally. Invalid OLA (controls suboptimal on the plate) and indeterminate specimens assayed in international laboratories were repeated in Seattle.

TABLE 1. Evaluation of drug-resistance mutations in 217 blood specimens with diverse HIV-1 subtypes using OLA reagents designed for subtype B, and using reagents modified to improve reactivity of the assay across subtypes

HIV-1 Subtype	Number of specimens evaluated	Number (*) of codons indeterminate by OLA												Total % (^) of indeterminate codons
		Reverse Transcriptase Mutations						Protease Mutations						
		K65R	K103N	Q151M	Y181C	M184V	T215Y/F	D30N	I50V	V82A/S/T	I84V	N88D	L90M	
A	6	0	1	0	0	0	1	0	0	1	0	3	1	9.7
B	70	3	5	7 (0)	2 (1)	1 (0)	2	0	0	1	0	0	0	2.5 (1.4)
C	91	5	1	7 (1)	2	60 (3)	0	0	0	7	1	18	16 (3)	10.7 (3.8)
D	5	1	0	1 (0)	0	0	0	0	0	0	0	1	1	6.7 (5)
F	2	1	0	0	0	0	0	0	0	0	0	1	0	8.3
G	8	0	0	2 (0)	1	2	0	2	0	3	0	1	0	11.4 (9.3)
J	2	0	0	0	0	0	0	0	0	0	0	1	1	8.3
AE	25	1	1	1	3 (2)	1 (0)	0	0	0	6	1	4	2	6.7 (6)
AG	8	0	0	0	3	4 (1)	0	0	0	0	1	1	1	10.4 (7.3)
Total No. for all subtypes	217	11	8	18 (2)	11 (9)	68 (6)	3	2	0	18	3	30	22 (9)	
% indeterminate with Subtype B probes		5.1	3.7	8.3	5.1	31.2	1.4	0.9	0	8.3	1.4	13.8	10.1	7.45
% indeterminate with modified probes **		—	—	0.9	4.1	2.7	—	—	—	—	—	—	4.1	3.9

(*) indicates number of indeterminate samples remaining after testing with modified oligonucleotides

(^) indicates percent of indeterminate samples remaining after testing with modified oligonucleotides

** modified oligonucleotide probes were synthesized and tested only for RT codons 151, 181, 184, and protease codon 90

Specimens collected in Cameroon, Honduras, India, and Thailand were assayed in the Seattle Laboratory whereas specimens collected in Belgium, China, and South Africa were assayed locally in their regional laboratories. The OLA data generated in the international laboratories were reviewed and interpreted in Seattle. The OLA was valid for 77.3% of 1069 codons analyzed regionally. In all, 243 codons had invalid OLA results due to technical factors (controls suboptimal on the plate or replicates with discrepant results). PCR amplicon from the latter and from codons with indeterminate genotypes (negative OLA reaction for both mutant and wild type) was sent to Seattle for repeat testing. All valid OLA results obtained in Seattle and international laboratories were combined in the final
analysis.

Performance of the PCR primers

Of 127 plasma specimens collected in Belgium, China, South Africa and Thailand, 100% amplified with the primer sets PRA/IBR1 and PRB/IBR2 (N=64/64) or IBF1/IBR1 and PRB/IBR2 (N=63/63). However, only 77 (77.8%) of 99 specimens received as DBS from Honduras, India, Cameroon and Thailand, amplified when initially tested with primers PRA/IBR1 and PRB/IBR2. Nineteen of those that failed were tested again with the first round forward primer PRA replaced by IBF1, which amplified 13, suggesting that the primer IBF1 performed better on non-B specimens. The 9 DBS specimens that failed amplification were not evaluated for PCR inhibitors, degradation of the nucleic acid, or the HIV-1 DNA content.

Performance of the ligation oligonucleotides

A total of 2604 codons were genotyped by the subtype B OLA reagents of which 92.5% (2410) were successfully evaluated (Table 1). The remaining 194 codons had indeterminate results, defined as a negative OLA reaction for both mutant and wild type resulting from failure of the probes to either hybridize or ligate. High rates of indeterminate OLA were observed at RT codons 184 (31.2%) and 151 (8.3%) and PR codons 88 (13.8%), 90 (10.1%), and 82 (8.3%). The indeterminate results at codons 184, 88, and 90 were primarily in subtype C viruses whereas those testing indeterminate at codons 151 and 82 were distributed among multiple subtypes. Lower rates of indeterminate results were observed in RT codons 65 (5.1%), 181 (5.1%), and 103 (3.7%). And the fewest indeterminate results were at PR codons 30 (0.9%), 50 (0%), and 84 (1.4%) and RT codon 215 (1.4%). Genetic polymorphisms were the likely cause of

assay failure; therefore, specimens with indeterminate OLA results were sequenced, and when common polymorphisms were identified, new probes were designed and tested.

Optimization of the ligation oligonucleotides for non-B HIV-1 subtypes.

A review of HIV-1 *pol* sequences from a panel of non-B HIV-1 clones (12), from subjects with non-B subtypes residing in the Seattle area (N=17, data not shown), and from the Los Alamos HIV-1 Sequence Database, allowed us to identify single base polymorphisms of moderate prevalence within two bases of the ligation site of the oligonucleotide probes. Probes complementary to polymorphisms in RT codons 151, 181 and 184 (Table 2) were synthesized and evaluated in specimens that tested indeterminate with the subtype-B probes. The modification of the probes for M184V at codon 183 (TAC →TAT) decreased the rate of indeterminate results from 31.2% to 2.7% across subtypes, and from 65.9% (60/91) to 3.3% (3/91) for subtype C (Table 1). The modified oligonucleotide probe for codon 151, complementary to a polymorphism CAG to CAA at this codon, reduced the rate of indeterminate tests from 8.3% to 0.9%. A third probe with the first nucleotide of the common probe for RT codon 181 modified from T to C, only modestly reduced the rate of indeterminate tests from 5.1% to 4.1%.

TABLE 2. Sequence of oligonucleotide probes for HIV-1 subtype B OLA and modifications made for detection of drug-resistance mutations in non-B subtypes

HIV-1 *pol* region	Mutation detected	Oligonucleotide probes	Sequence: genotype-specific*common[a]
PR	L90M	WT[b]	CAACATAATTGGAAGAAATCTGT*TGACTCAGATTGGTTGCACTTT
		Mutant	----------A*----------
		Modified[c]	----------**A**---*--------**C**----**A**-----**A**--
RT	Q151M	WT	CAGTACAATGTGCTTCCACA*GGGATGGAAAGGATCAC
		Mutant	----------AT*----------
		Modified	----------*__A__----------
	Y181C	WT	ACAAAATCCAGACATAGTTATCTA*TCAATACATGGATGATTTGTATGTA
		Mutant	----------G*----------
		Modified	----------*__C__----------
	M184V	WT	AGACATAGTTATCTATCAATACA*TGGATGATTTGTATGTAGGATC
		Mutant	----------G*----------
		Modified	----------**T**-*----------

[a] Bases comprising the codons of interest are shaded
[b] WT, wild-type
[c] Nucleotide modifications are in boldface type
* Indicates the ligation site

Specimens that had indeterminate test results with the subtype B probes, or remained indeterminate after evaluation with the modified OLA probes, were subsequently sequenced to identify additional interfering polymorphisms. A polymorphism four bases from the ligation site on the genotype-specific probe, and several others in the region of the common probe were frequently detected in sequences of the specimens with indeterminate results at codon 90 of protease, especially those with HIV-1 subtype C. Modified probes for L90M that included these base changes (Table 2) were evaluated, and decreased the indeterminate rate from 10.1% to 4.1% (Table 1).

The specimens that tested indeterminate with the subtype B and the modified oligonucleotides for codons 151, 181, 184 and 90 had sequences that were not complementary to the probes (Table 3). Of note, the polymorphism incorporated into the modified 181 common probe was not prevalent among the specimens we tested, and when observed this mutation occurred with several additional polymorphisms in the region of both the genotype-specific and the common probes that precluded their ligation. Also of interest, a polymorphism two bases from the ligation site of RT codon 103 (AAA→AGA) encoded a K103R mutation that has not been associated with resistance (13) (Table 3), and was observed only in subtype B specimens from Honduras. Indeterminate reactions at PR codons 82 and 88 included polymorphisms within 2 nucleotides of the ligation site in a subset of specimens, and multiple other polymorphisms in the remaining samples (Table 3). Modified oligonucleotide probes were not tested for RT codon 103 or PR codons 82 and 88, due to the relatively low prevalence of particular interfering polymorphisms, and heterogeneity in the sequences in regions corresponding to the OLA probes.

Comparison of genotypes detected by OLA and consensus sequencing

Consensus sequences generated in the Seattle laboratory for specimens with indeterminate OLA results (N=48 specimens for protease, N= 37 specimens for RT) were compared to the genotypes obtained by OLA (excluding the one codon per subject that tested indeterminate). Genotypes were concordant for 408 (96%) of the 425 codons analyzed. The discordant genotypes (4%) corresponded to mixtures of wild-type and mutant virus detected by the OLA when consensus sequencing detected only wild-type variants. No drug-resistant genotypes detected by sequencing were missed by the OLA.

TABLE 3. Nucleotide sequences of OLA probes and the specimens with indeterminate genotype by OLA, showing the region complementary to the probes on either side of the ligation site

HIV-1 Subtype	RT codon/ Specimen	Results of direct sequencing (5'→3')
Con B	Codon 65 wt	CTCCAGTATTTGCCATAAAGAA*RAAAGACRGTACTAAATGGAGAA
	Codon 65 mut	----------G*----------
B	Dzy8	----------G---*A--GA--A------G--------
B	Dzy17	-----A----------C--*A--GA-TA------G--------
B	Dzy19	-----A----------*G--GA--A--GA--G--------
C	Efc32	-----A-------T-----A--*G--G---A-------G-------
C	Efc38	-----A----------A--*G--GA--A------GG-------
C	Efc50	-----A-------A-----A--*G--G---A-------G-------
C	Ena9	-----A----------GA--*G--G---A-------G-------
D	Dzy1	-----A----------*A--GA--A------GG-------
F	Dzy7	----------T---------*A-----AA--T-C-GT-CT-AGT
AE	Dxy39	-----A-------T----G---*A--G---A-C--C--------G-
Con B	Codon 103 wt	ACATCCCGCAGGGTTAAAAAAGAAA*AAATCAGTAACAGTACTGGATGTGGGT
	Codon 103 mut	----------C*----------
A	Dzy30	G-----A--G--T----------G*----------A--------G
B	Dub35	---------G---C--C------G-*----------
B	Dtr80	------T----------G*----------
B	Dtx67	----------G-*----------R---
B	Duf36	----------G--G-*----------A---------
B	Duf37	----------G-*----------A
AE	Dzy31	G-----A--G--T--G---C-----*----------A--------G
Con B	Codon 151 wt	CAGTACAATGTGCTTCCACA*GGGATGGAAAGGATCAC
	Codon 151 mut	----------AT*----------
	Codon 151 mod	----------*A----------
AE	Dxy35	----------G--G--*----------
C	Ena31	--A--T--------G-----*---G----------
Con B	Codon 181 wt	ACAAAATCCAGACATAGTTATCTA*TCAATACATGGATGATTTGTATGTA
	Codon 181 mut	----------G*----------
	Codon 181 mod	----------*C----------
B	Dtx74	----------A----G-AW--T--*----------
C	Ena28	-AG---------AT----C--T--*------T-------RC---------
C	Ena45	----------A-----C--T-G*------TG-------C---------
G	Dzy40	-A----------A--G--G-----*C--G---G----------A------
AE	Esp1	-A----------A--G-----T--*----------C--A------
AE	Esp8	-A----------A--G-----T--*----------C----------
AG	Dzy3	-A----C-----G--G--G-----*C-----TG----------A------
AG	Dzy45	-A----------AC-G--G-----*-------G----------A------
AG	Eib35	-A--T-------G-----G--T--*C-----T----------A------
Con B	Codon 184 wt	AGACATAGTTATCTATCAATACA*TGGATGATTTGTATGTAGGATC
	Codon 184 mut	----------G*----------
	Codon 184 mod	----------T-*----------
C	Ena45	---A-----C--T-G------TG*-------C----------C---
C	Efc38	---A-----C----------TG*-A-----C----------C---
C	Efc54	---A-----C--------G--T-*-A-----C----------C--
G	Dzy14	---AG-------T-G--------*----------A-----G-C---
G	Dzy40	---A--G--G-----C--G---G*----------A----------
AG	Eib35	---G-----G--T--C-----T-*----------A----------
Con B	Codon 215 wt	CAACATCTGTTGAGGTGGGGATTTAC*CACACCAGACAAAAAACATCAGAA
	Codon 215 mut F	----------TT*----------
	Codon 215 mut Y	----------TA*----------
A	Dzy30	GCT-----A--A--A-----GC----*T----------G--------
B	Dzy17	--G-------G--A------G--CTT*---------AC----G
B	Epw1	----------G-------G-----*----------

TABLE 3. (continued) Nucleotide sequences of OLA probes and the specimens with indeterminate genotype by OLA, showing the region complementary to the probes on either side of the ligation site

HIV-1 Subtype	PR codon/ Specimen	Results of direct sequencing (5'→3')
Con B	Codon 30 wt	TATTAGATACAGGAGCAGATG*ATACAGTATTAGAAGAAATGAAT
	Codon 30 mut	--------------------A*----------------------
G	Dzy14	----------------------*--------C--AC---C--AG--
G	Dzy15	----------------------*--------C--AC---C--AG--
Con B	Codon 82 wt	TATTAGTAGGACCTACACCTGT*CAACATAATTGGAAGAAATCTGT
	Codon 82 mutA	---------------------C*----------------------
	Codon 82 mutS	--------------------AG*----------------------
	Codon 82 mutT	--------------------AC*----------------------
A	Dzy2	----G---------------TC*---------------G---A---
B	Dtx34	-----A----R--C-----CA-*T----------------------
C	Ena3	-----A----G-----------*---------------G---A---
C	Ena26	----------G--------C--*----------------CA---
C	Ena42	-R---A----G--------C--*---------------G---A---
C	Eek36	-----------GAC--------*---------------G---A---
C	Eew44	-------G-----C--------*------------C------A---
C	Edw35	-------RR----C--------*T-------------G---A---
C	Esp7	-------G-----C--------*-------------------A---
G	Dzy23	--------------------A-*------------G------A---
G	Dzy44	--C-----------------A-*-------------------A---
G	Dzy40	--C-----------------A-*------------G------A---
AE	Dzy31	----G---------T-C--C--*------------C-----CA---
AE	Dyf31	-------------------A--*-------------C-----A---
AE	Dye8	-G-----------------A-*-------------C-----A---
AE	Esp1	-------------C--G-----*-------------C-----A---
AE	Esp8	-------------C--G-----*-------------C-----R---
AE	Esp10	-------------C--G-----*-------------C-----A---
Con B	Codon 84 wt	AGGACCTACACCTGTCAACA*TAATTGGAAGAAATCTGTTGACT
	Codon 84 mut	-------------------G*----------------------
C	Ena43	G--R---------A-----T-*--------------A--------
AG	Dzy38	--------------C-----*--------C-G--CG-RA----C
Con B	Codon 88 wt	CCTGTCAACATAATTGGAAGAA*ATCTGTTGACTCAGATTGGTTG
	Codon 88 mut	---------------------G*----------------------
A	Dzy2	---TC---------------G-*--A-------------------
A	Dzy12	--------------------G-*--A-------------------
A	Dzy30	---A-------------------*--G-------C-----G--A--
C	Dzy24	----------------------*--A----------AC----A--
C	Dzy25	----Y----R----------G-*--------------Y----A--
C	Dzy29	----C-------G----------*--T--A--------T-A--A--
C	Ena2	--------------------G-*--A----------C----A--
C	Ena21	-----------------G----*--A----------C----A--
C	Ena26	--C--------------------*-CA----------C----A--
C	Ena28	--------------------G-*-CA----------C----A--
C	Ena34	--------------------G-*--A----------C----A--
C	Ena39	-----------------G----*--A----------AC----A--
C	Ena42	--C-----------------G-*--A----------C----A--
C	Ena8	-----Y----------------*-CA----A------C----A--
C	Ena29	------R----------------*-CA----------C----G--
C	Edw35	-----T--------------G-*--A----A------C--R-A--
C	Eek36	--------------------G-*--A----------C----G--
C	Eew56	--------------------G-*--T----------C----A--
C	Esp7	----------------------*--A-------C---C----A--
D/F	Dzy11	----C----R----------*--T----A--C--A-----C--
F	Dzy34	----C-------G----------*--T--A--------T-A--A--
G	Dzy13	---------G-------G----*--A--A-------A--------
J	Eig31	-----T----------------*-CA----A-----A-----A--
AE	Dzy31	--C--------------C----*-CA----------------C--
AE	Esp1	------------------C---*--A----------C-------
AE	Esp8	------------------C---*--R----------C-------
AE	Esp10	------------------C---*--A----------C-------
AG	Dzy38	----C-------------C-G-*-CG-RA----C----------

Con B	Codon 90 wt	CAACATAATTGGAAGAAATCTGT*TGACTCAGATTGGTTGCACTTT
	Codon 90 mut	----------A*
	Codon 90 mod	----------A---*--------C----A-----A--
A	Dzy27	---------W---------A-AC*----------T-----
C	Edw35	T----------G---A---*-A------C--R-A-----A--
D/F	Dzy11	----R----------T---*-A--C--A-----C--------
J	Eig31	T----------CA---*-A-----A-----A--T-----
AE	Dye1	----------C-----A---*--R--M----------T-----
AE	Dxy34	----------C-----A-A-*----------T-----
AG	Dzy38	----------C-G--CG-RA*----C----------T-----

* indicates ligation site; Con B = consensus B; wt = wild type; mut = mutant; PR = protease

DISCUSSION

The utility of the OLA, a sensitive, simple, and economical assay, designed for the detection of HIV-1 drug–resistance mutations in subtype B US variants (2,3,14), was demonstrated for a diverse array of HIV-1 subtypes collected outside North America. In addition (3), international laboratories experienced with molecular techniques were able to implement the OLA following the kit instructions.

In this study we demonstrated that reagents developed to detect primary drug-resistance mutations in HIV-1 subtype B prevalent in the United States can effectively assay most (89-93%) viruses of non-B subtypes and nearly all (97%) B subtypes from Central America, Asia and Europe. In addition, modifications of several of these reagents increased the reactivity of the OLA to 97% of RT and 95.2% of PR codons in specimens contributed by 7 sites in Africa, Asia, Central America and Europe. While these assay performance rates may not be representative of an extensive sampling of global HIV-1 variants and subtypes, our study demonstrates several attributes of the OLA: First, the available reagents genotype the majority of codons associated with high-level HIV-1 resistance to most NRTI, NNRTI and PI currently in use (1). Second, the subtype B reagents have a high rate of reactivity across most non-B HIV-1 subtypes. Third, publicly available databases and consensus sequences of regional specimens can be used to modify the reagents to increase the OLA reactivity for most codons. Similarly, sequence data can be used to develop reagents for prevalent regional genotypes, such as K103R in RT that was prevalent among Honduran specimens, or additional codons, as has been done for RT codons 106 and 190 (unpublished). Fourth, the OLA detects minority mutant genotypes that are not apparent when the same PCR amplicon is evaluated by consensus sequencing (14).

Among the specimens tested, the OLA failed most frequently at PR codons 82, 88 and 90, and at RT codons 151 and 184, with indeterminate rates ranging from 8.3 to 31.2%.

Other codons had indeterminate rates of 5% or less, similar or only slightly higher than the 2-3.5% observed in specimens from ARV naïve (Ellis, submitted) and highly treated North Americans infected with HIV-1 subtype B (2, 3). Interfering genetic polymorphisms, especially those located within 2 bases of the ligation site, are the most common cause of assay failure (2, 3). Single nucleotide modifications within 2 bases of the ligation site of the probes for RT codons 151 and 184, and changing several nucleotides in the probes for PR codon 90 restored the reactivity of the OLA probes for most specimens we evaluated with initial indeterminate results. However, heterogeneity in the target sequences at other codons precluded simple modifications to improve the performance of several probes. It is possible that the use of mixed bases or promiscuous non-standard nucleotides, such as deoxyuridil or deoxyinosine, may improve the OLA performance at the highly polymorphic regions probed for PR codons 82 and 88, and RT codons 65 and 181. We plan on exploring these modifications, and to incorporate the optimized probes to the next version of the OLA kits available from the NIH AIDS Reagent and Reference Program.

Other groups have used the OLA for drug-resistance genotyping. Rates of indeterminate results were similar to our present study when testing subtype B and non-B viruses from Spain (15). Testing of RT codons 65, 103, 151, 181, 184 and 215 in HIV-1 subtype C South African specimens (16) had an overall low reactivity rate due to high genetic variation in the region of the OLA probes. In our cohort, the highest rates of indeterminate OLA results were observed among subtype C specimens at RT codon 184 (66%), and PR codons 88 (19.8%) and 90 (17.6%). The modified codons 184 and 90 probes reduced the indeterminate results for subtype C specimens to 3.3% at both codons, demonstrating that optimization of OLA probes can enable genotyping of specific resistance mutations in subtype C viruses. In addition, a recent report on the development of an OLA for detection of Q151M and M184V in HIV-2 infections (17) shows that virus-specific primers and OLA probes will be needed to evaluate certain drug resistance mutations.

Multiple assays designed to detect point mutations in HIV-1 have been developed for the evaluation of minor populations of drug-resistant HIV-1 (18–23). These assays vary in the sensitivity and specificity of detecting mutations and in the required laboratory equipment and technical expertise. Few have reported performance across HIV-1 subtypes, although modifications in each assay's reagents should allow the detection of targeted mutations. The line probe assay (18) and mutagenically separated PCR assay

(19) have reported variable reactivity for non-B specimens (19, 24) .Both the LigAmp assays, which utilize DNA ligase for specificity, similar to the OLA, and real-time PCR (21) and a selective mutation-specific PCR real-time assay (22), have been shown to be very sensitive for the evaluation of nevirapine-resistance mutations K103N and Y181C in women infected with HIV-1 subtypes A, C, and D (22,25,26). These real-time PCR-based assays seem to require specific primers/probes for each subtype, in addition to more expensive equipment and reagents compared with the OLA, that might limit use for the screening of multiple mutations in resource-limited communities. An allele-specific real-time PCR developed for multiple drug-resistance codons (23) has not yet been modified for non-B subtypes. Parallel allele-specific sequencing (27) is a new primer extension modality that detects minor genotypes, including linked mutations, that likewise, has not been modified for non-B subtypes.

Screening for all resistance-associated mutations by a point mutation assay such as the OLA is impractical. Therefore the OLA kits were designed to evaluate only mutations associated with high-level resistance to FDA-approved antiretrovirals. As the guidelines for surveillance of drug-associated resistance mutations in HIV-1 infected individuals have been modified since the development of the Subtype B OLA (28-30), we have developed probes for detection of the G190A mutation in RT associated with resistance to NNRTI, and V106M (unpublished), a mutation frequently detected in NNRTI treated individuals infected with subtype C (31, 32). V82I, a protease mutation not currently detected by the OLA assay, has not been associated with drug resistance, however it has been described as a frequent mutation in subtype G viruses of treatment naïve persons (33). As only one nucleotide change is required to mutate to 82T or 82M, it may be useful to design probes for the detection of V82I when analyzing specimens of subtype G variants.

Similar to previous comparisons, OLA and consensus sequence genotypes derived from the same amplicon demonstrated concordance. Except, consistent with past evaluations, the OLA appeared more sensitive compared to consensus sequencing for the detection of minor mutant variants in mixed viral populations (2, 3, 14).

In addition to being sensitive and highly specific, the OLA is simple, has a high throughput and a low cost. Utilizing only a thermocycler and a spectrophotometer, one codon can be genotyped in forty-three specimens on a single 96-well plate in less than 5 hours, with OLA reagents costing less than US$1.50/codon/specimen. As part of this

project, several international laboratories implemented the OLA and tested their specimens regionally. The majority of OLA performed well.

Nucleic acids amplified from plasma HIV-1 RNA as well as proviral DNA, including specimens collected on filter paper have been genotyped by OLA. Of note, the sensitivity of the OLA, and especially for detection of minority mutant genotypes, may be less when evaluating specimens collected on filter paper, as suggested by a 9.1% (9/99) PCR failure on dry blood spots collected for this study. The decreased sensitivity is likely due to the small volume of blood collected, and consequently low HIV-1 DNA copy number (34), the presence of PCR inhibitors or DNA degradation.

Other important limitations of the OLA are that novel mutations are not identified, and only a selected number of resistance mutations are evaluated. Sequencing, on the other hand, gives comprehensive information on all the possible mutations within the region probed. Sequencing may be preferred for genotyping of specimens from highly drug-experienced individuals, with the caveat that mutations at low-levels may not be detected. In contrast, the OLA, with a higher sensitivity for detection of low-level mutations, high throughput, low cost and simplicity, may be of great utility for the surveillance of drug-resistance in settings where the antiretrovirals mutational pattern is predictable, such as ARV roll-out programs adopted by developing countries recently offering antiretroviral treatment (35), and the various programs implemented for the prevention of mother to child transmission of HIV-1 (36).

In summary, the OLA is a sensitive, rapid, economical and simple test for the detection of primary drug-resistance mutations in HIV-1 subtype B and non-B subtypes prevalent in Asia and Africa. The OLA is easily implemented in laboratories experienced in molecular techniques, and can be adapted to evaluate resistance mutations in HIV-1 variants circulating in a particular geographical region. Therefore, as more antiretroviral agents become available in resource limited areas, the OLA could be a valuable tool to monitor HIV-1 drug-resistance.

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Chapter 6

Development and Evaluation of an Oligonucleotide Ligation Assay for the Detection of Drug Resistance Associated Mutations in the HIV-2 *pol* Gene

Development and evaluation of an Oligonucleotide Ligation Assay for the detection of drug resistance associated mutations in the HIV-2 pol gene

Sabelle Jallow *[1,2], Steve Kaye[1,6], Martin Schutten[3] , Eleonor Brandin[4,5], Jan Albert[4,5], Samuel J. McConkey[1,7], Tumani Corrah[1], Hilton Whittle[1], Guido Vanham[2], Sarah Rowland-Jones[1] and Wouter Janssens[2]

[1] *Medical Research Council Laboratories, Banjul, The Gambia*
[2] *Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*
[3] *Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands*
[4] *Department of Virology, Immunology and Vaccinology, Swedish Institute for Infectious Disease Control, Solna, Sweden.*
[5] *Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden.*
[6] *Present address: Department of Retrovirology, Imperial College London, United Kingdom.*
[7] *Present address: Department of International Health and Tropical Medicine, Royal College of Surgeons in Ireland, Dublin.*

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ABSTRACT

HIV-2 is naturally resistant to several antiretroviral drugs, including all the non-nucleoside reverse transcriptase inhibitors (nNRTI) and the entry inhibitor, T-20; and may have reduced susceptibility to some protease inhibitors. These resistance properties make treatment of HIV-2 patients difficult with very limited treatment options. Therefore, early detection of resistance mutations is important both for understanding treatment failures and as a guide to subsequent therapy decisions. With the Global Fund Initiative, a substantial number of HIV-2 patients in West Africa will receive antiretroviral therapy. Therefore developing cheaper and more sustainable resistance assays, such as the Oligonucleotide Ligation Assay (OLA), is a priority. In this study, we designed oligonucleotide probes to detect the Q151M mutation associated with phenotypic resistance to Zidovudine, Didanosine, Zalcitabine and Stavudine; and the M184V mutation associated with phenotypic resistance to Lamivudine and emtricitabine in HIV-2. The assay was successfully developed and evaluated on 122 samples from The Gambia, Guinea Bissau, The Netherlands and Sweden. The overall sensitivity of the assay was 98.8%, with 99.2% for Q151M and 98.4% for M184V. OLA results were compared with sequencing to give a high concordance of 98.4% (Q151M) and 97.5% (M184V). OLA demonstrated a higher sensitivity to detect minor variants as a mixture of wild-type and mutant viruses in cases when sequencing detected only the major population. In conclusion, we have developed a simple, ease-to-use and economical assay to genotype drug resistance in HIV-2 that is more sustainable for use in resource-poor settings than consensus sequencing.

Introduction

HIV-2, the other causative agent of AIDS (10), has a relatively lower prevalence than HIV-1. HIV-2 is restricted mainly to West African countries such as Senegal, Guinea Bissau and The Gambia (29), where its prevalence varies from 1-10% of the adult population (11, 17, 21, 26, 31, 35), although prevalence can be as high as 28% in high-risk groups such as commercial sex workers in The Gambia (29). Cases of HIV-2 infection occur in countries outside of West Africa, such as Portugal and in other countries with former socio-economical links to Portugal, including Southwest India, Angola, Mozambique and Brazil (2, 27, 29).

HIV-2 is naturally resistant to some currently available antiretroviral drugs, such as the non-nucleoside reverse transcriptase inhibitors (nNRTIs) and the entry inhibitor, T-20, due to the presence of natural polymorphisms that confer resistance (24). In addition, HIV-2 has several natural polymorphisms associated with major and minor resistance to some protease inhibitors, notably amprenavir and nelfinavir (28). These polymorphisms make the nucleoside reverse transcriptase inhibitors (NRTIs) central to HAART regimens used in the treatment of HIV-2 infection. Thus early detection of resistance mutations to NRTIs is very important to explain treatment failures and guide subsequent treatment decisions.

HIV is characterized by its extensive genetic variability resulting into different strains classified into groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs). While the HIV-1 pandemic is recognized globally as consisting of many separate epidemics (25) with nine subtypes and 34 CRFs (http://www.hiv.lanl.gov/content/index), HIV-2 is characterised by an epidemic of only two subtypes (A and B) and five non-epidemic subtypes (C–G)(23). Only one member each of subtypes C, E, F and G, and two members of subtype D, have been identified (29). Whilst HIV-2 subtype A viruses have been documented in different locations across West Africa and other regions of the world, subtype B is geographical restricted mainly to Ivory Coast and Ghana; with a few cases documented in Europe and the Middle East (18).

The Oligonucleotide ligation assay (OLA) is a point mutations assay based on the covalent joining of two adjacent differentially labelled oligonucleotide probes by a DNA ligase, when they are hybridized to a complementary DNA template (15, 20). OLA has been used for the evaluation of the primary mutations associated with HIV-1 drug

resistance to currently available antiretroviral drugs (4, 13, 14, 34). Each primary drug resistance point mutation of interest is analysed with a set of three labelled oligonucleotide probes: a 5'-digoxigenin-labelled probe to detect the wild-type (WT) sequence; a 5'-fluorescein labelled probe to detect the mutant (MT) sequence and a 3'-biotinylated oligonucleotide probe that hybridizes on the 3' side of the mutation site for both WT and MT targets (34). OLA is a simple assay in which the probes are annealed to a PCR fragment derived from the patient sample; the WT or MT probe ligated to the common probe and the ligated product detected in an enzyme linked immunosorbent assay (ELISA) (4, 32) (Figure 1). Routinely, genotypic resistance testing involves sequencing of the protease and RT genes. However, OLA has been shown to be simpler, rapid, more sensitive and economical without need for expensive equipment and technical expertise. Excluding PCR costs necessary for both methods, OLA costs ~$1 for the two mutations per sample whereas sequencing costs at least $10. Like all ligation assays, the potential disadvantage of OLA is that the presence of other mutations around the ligation site may result in failure of the assay (indeterminate result). The occurrence of indeterminate results with OLA is related to the high level of genetic variability in HIV such that the HIV-1 OLA has been modified for some non-B subtypes (33). For HIV-2, only two subtypes A and B are of epidemiological importance.

In HIV-2 reverse transcriptase enzyme, as is the case for HIV-1, the Q151M mutation is a multi-NRTI resistance mutation associated with phenotypic resistance to zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), abacavir (ABC) and stavudine (d4T); whilst the M184V mutation is associated with phenotypic resistance to lamivudine (3TC) and emtricitabine (FTC) (1) in HIV-2. We report the development and evaluation of an oligonucleotide ligation assay to detect the mutations Q151M and M184V associated with high-level resistance to NRTIs in HIV-2.

Materials and Methods

Patients: A total of 122 samples were obtained from 64 patients from the Gambia (n = 42), Guinea Bissau (n = 24), The Netherlands (n = 26) and Sweden (n = 30). The samples from the Gambia included 32 samples from eight patients treated with Combivir (AZT/3TC) as previously described (19) and ten samples from five patients on triple therapy (AZT + 3TC + Lop/r). The 24 samples from Guinea Bissau were from 23 subjects included in the Caio HIV-2 cohort (29). The 26 samples from the Netherlands were from 16 treatment-experienced HIV-2 infected patients, mostly of West African

origin who were genotyped at the Erasmus Medical Centre in Rotterdam. The 30 Swedish samples were from 12 patients; six untreated, one treated successfully and five failing therapy, as previously described (8). These samples were selected from 20 of 23 known cases of HIV-2 in Sweden. Most of these patients were immigrants from different West African countries (8).

Nucleic acid extraction and PCR amplification: HIV-2 RNA was extracted from 140µl of EDTA plasma from Caio samples using the QIAamp viral RNA kit (QIAGEN, Venlo, The Netherlands) or 200µl of heparin plasma from Gambian samples by the method of Boom et al (5). The RNA was eluted into 50µl nuclease-free water and 3µl of the eluate was reverse transcribed and amplified using Titan one-tube RT-PCR (Roche Applied Science, Lewes, UK). DNA was extracted from Caio samples using the QIAamp DNA kit (QIAGEN). The inner primers SJH21 (forward-5'GAAAGAAGCCCCGCAACTTC CC, position 1861 to 1882) and SJH22 (reverse-5'-GAGTCTGTTAGTGCCATCGCA AAG, position 3843 to 3820) were used. All primer positions refer to HIV-2 ROD [Genbank accession #: M15390]. This PCR reaction was performed in a final volume of 25µl. Cycling conditions were 50°C for 45 min, reverse transcription, 94°C for 2 min, initial denaturation, 10 cycles of 94°C for 30 sec, 60°C for 30 sec and 68°C for 120 sec, 25 cycles of 94°C for 30 sec, 60°C for 30 sec and 68°C 120 sec plus 5 sec/cycle elongation and a final elongation of 68°C for 10min.

Nested PCR was performed using primers SJH23 (forward-5'- GAGAGACCATACAAA GARGTG, position 1992 to 2012) and SJH24 (reverse-5'CTATTGCAGGATCCATC TGTG, position 3719 to 3699). The reaction mix contained 2.6 units of Expand High Fidelity Polymerase (Roche), 0.3µM of each primer and 200µM of each dNTP in Expand reaction buffer. Cycling conditions were 95°C for 3 min initial denaturation, 10 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 105 sec, 25 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 105 sec plus 5 sec/cycle elongation, and a final elongation of 72°C for 7min to amplify the entire HIV-2 protease and RT encoding region (1727 bp). PCR products were analysed on 0.8% agarose gels. Samples from The Netherlands and Sweden were supplied as PCR amplicons amplified from the protease and RT coding region of pol.

Figure 1: OLA schema

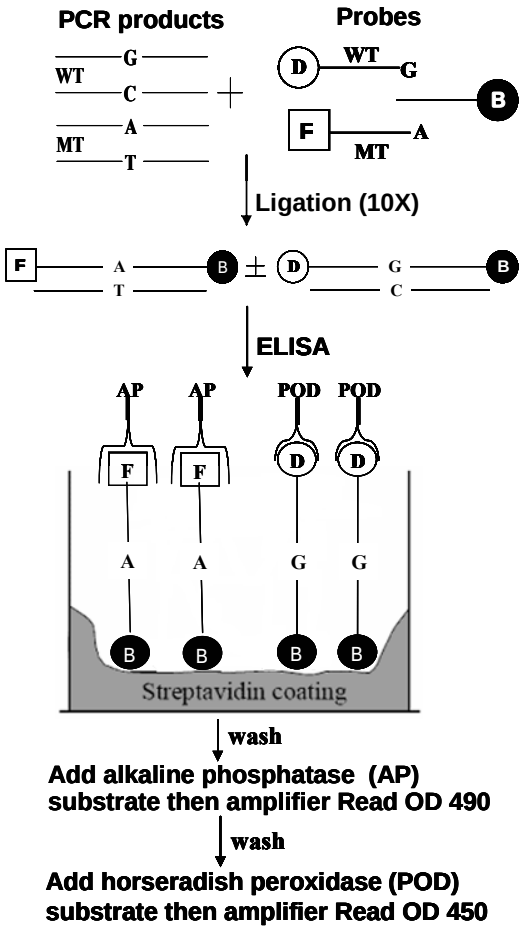


Figure 1: OLA schema. OLA involves generation of PCR fragments; the use of three labeled probes, digoxigenin (D) for wild-type (WT), fluorescein (F) for mutant (MT) and Biotin (B) for the common probe. These probes anneal to the PCR product and the wild-type or mutant probe is ligated to the common probe. Ligated products are captured on streptavidin-coated microtiter wells and detected by ELISA with horseradish peroxidise (POD) labeled anti-D antibodies and alkaline phosphatase (AP) labeled anti-F antibodies.

Figure 2: OLA genotyping (visual results).

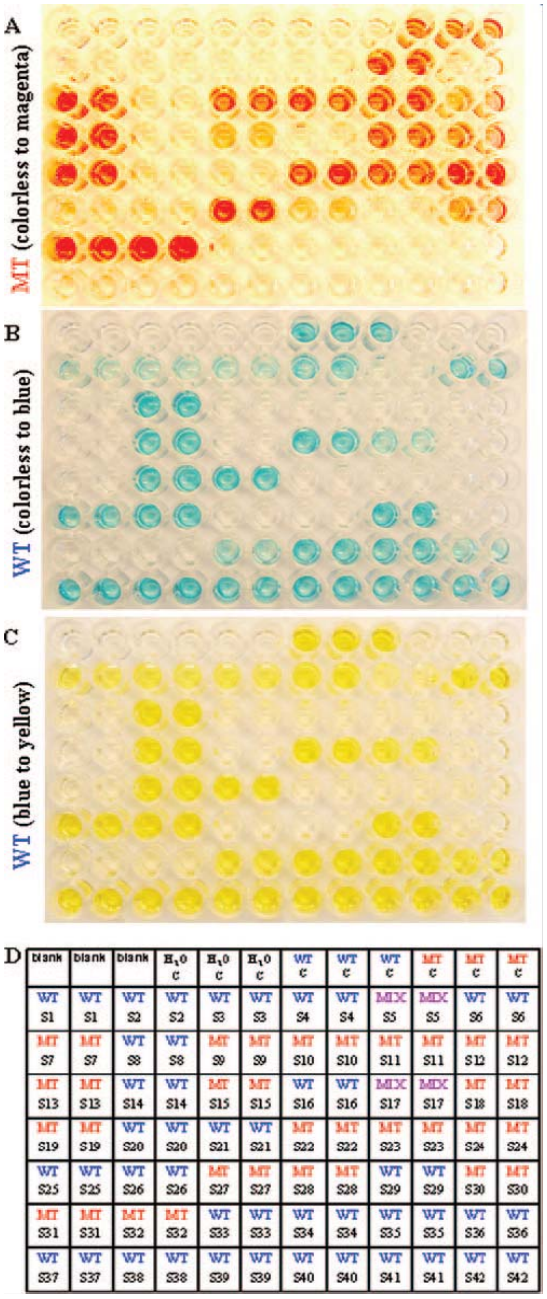


Figure 2: OLA genotyping (visual results). In (A) samples with mutant (MT) genotype produce a deep magenta color upon addition of alkaline phosphatase substrate and amplifier, while negative samples remain colorless/clear. In (B), after washing the plate, addition of horseradish peroxidase substrate turns wild-type (WT) samples blue and addition of the amplifier in (C) turns them yellow; while the negative samples remain clear. 2D shows the final interpretation of the results. The first row has the controls (C), blank, H2O, WT & MT; in triplicate and subsequent rows have the samples (S) in duplicate. Samples 5 & 17 have a mixture (MIX) of WT and MT viruses (positive for magenta, blue and yellow).

Sequencing: PCR products were purified using the QIAquick PCR purification system (QIAGEN) and directly sequenced. Sequencing was done on both strands using the primers SJH23, SJH24, JA220 (reverse -5'-GTCTTTATYCCTGGGTAGATTTGTG-3', position 3183 to 3207) (8), JA222 (forward-5'-ACCTCCAACTAATCCTTATAATACC, position 2530 to 2555) (8), H2Mp4 (reverse -5'-CCCAAATGACTAGTGCTTCTT-3', position 3527 to 3707) (11) and H2Mp6 (forward-5'-AAAAGAGATCTGTGCAAAAA TGG-3', position 2482 to 2504) (11). Sequencing was done by VIB Genetic Service Facility (Wilrijk, Belgium) using capillary sequencers (Applied Biosystems 3730 DNA Analyser) combined with ABI PRISM® BigDye™ Terminator cycle sequencing kits. The sequences were assembled using DNAsis software (Hitachi Software Engineering, Molecular Biology Insights, Colorado, U.S.A); aligned with HIV-2 ROD and then edited, translated and analysed with EditSeq and MegAlign (Lasergene Software, DNASTAR Wisconsin, U.S.A). Viral subtype was determined using an NCBI on-line programme (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and by phylogenetic analysis (DNASTAR). Multiple sequence alignments our samples with several HIV-2 reference strains (subtypes A, B & G) were performed with CLUSTAL version W of Megalign (DNASTAR). Consequently, the trimmed alignment was used to construct the phylogenetic tree (DNASTAR). SIVmne (Genbank accession #: AF361745) was employed as the outgroup to root the tree.

Construction of reference plasmids: Reference plasmids with the wild type and the mutant sequence, for both the Q151 and M184 mutations, were generated to serve as controls. Previously documented Gambian pol sequence samples (19), DTS 3-12 (WT 151 & WT 184) and DTS 3-18 (MT 151 & MT 184), were amplified, purified and inserted into TA plasmid vectors (TOPO TA cloning kit and TOP10 cells; Invitrogen, Merelbeke, Belgium). The resulting plasmids, named p12 and p18 for the WT and MT plasmids respectively, were transformed into competent E. coli and amplified. The plasmid DNA was extracted and purified with the QIAgen Plasmid mini purification kit (QIAgen), sequenced, and analysed by OLA.

Oligonucleotide probes: An alignment of the reverse transcriptase region of HIV-2 reference strain sequences, mostly subtypes A and B, retrieved from GenBank, was used to design the Q151M and M184V probes. The probes were designed to detect all HIV-2 subtypes, especially relevant subtypes A and B. The probes were differentially labelled to simultaneously detect both mutant and wild type sequences (Table 1) in a single assay.

Table 1: Differentially labelled oligonucleotide probes to detect mutations in HIV-2 associated with drug-resistance to NRTIs

Mutation	Probe	Label	Sequence 5' to 3'
Q151M	Wild-type	Digoxigenin	*dig*-TACATATATAAAGTCTTGCCACA
	Mutant	Fluorescein	*flu*-TACATATATAAAGTCTTGCCAAT
	Common	Biotin	RGGRTGGAAGGGATCACCAGCA-*bio*
M184V	Wild-type	Digoxigenin	*dig*-GATGTCATTATCATTCARTAYA
	Mutant	Fluorescein	*flu*-GATGTCATTATCATTCARTAYG
	Common	Biotin	TGGATGATATCTTAATAGCTAGT-*bio*

Oligonucleotide Ligation Assay (OLA): The OLA protocol and reaction conditions used were as previously described (4, 13), with the following modifications: 2µl of pol amplicon; 0.167U of Ampligase DNA Ligase (Epicenter Technologies, Cambridge, UK.); 0.333pmol for all Q151M probes and 0.5pmol for all M184V probes (Table 1). All patient samples were done in duplicate and the controls in triplicate. The controls consisted of blanked wells, water only, 100% WT plasmid control (p12) and 100% MT plasmid control (p18). Cut-off values for the optical readings were established as:

- For WT probes: Mean OD (negative control) + 6 standard deviation OD (negative control)
- For mutant probes: Mean OD (negative control) + 9 standard deviation OD (negative control)

Fluorescein, used to label the mutant probe, is rapidly degraded by sunlight and sensitive to high temperatures (smith and pretorious). Therefore the absorbance readings for the mutant probe were subjected to a more stringent cut-off value.

In addition to the OD readings, visual results from the ELISA were recorded and used together with the OD readings to ensure accurate cut-off values and efficiency of the assay when used in field conditions (no spectrophotometer). Samples are defined as mutant only (magenta colour &/ positive OD490), wild type only (yellow colour &/ positive OD450), mixture of mutant and wild type (magenta + yellow colour &/ positive OD490 and positive OD450) and indeterminate (no colour change &/ negative OD490 and negative OD450).

The HIV-2 Nucleotide sequences generated in this study have been submitted to EMBL, Genbank and DDBJ sequence databases and were assigned the accession numbers AM233873 to AM233900 and AM408175 to AM408208.

Results

The OLA was successfully developed for both the Q151M and M184V mutations. Analysis of the 122 RT sequences showed that 121/122 patients were HIV-2 subtype A and 1/122 was subtype B (data not shown). The probes were optimised to give a sensitivity of detection of 99.2% for the Q151M mutation (Table 2) and 98.3% for the M184V mutation (Table 3). Sensitivity (# positive samples X 100 / the total number of samples) is defined as the ability of the probes to detect positive results (WT and/or MT).

Table 2: Comparison of OLA with consensus sequencing for the Q151M mutation

Interpretation	[a]Genotype Q151M		# samples	%
	Sequencing	OLA		
Concordance	WT (CAR)	WT (CAR)	101	96.72
	MT (ATG)	MT (ATG)	17	
	Mix (ATG and CAR)	Mix (ATG and CAR)	0	
Detection of minor variants by OLA	WT (CAR)	MT (ATG)	0	1.64
	MT (ATG)	Mix (ATG and CAR)	1	
	WT (CAR)	Mix (ATG and CAR)	1	
Alternate mutation	Q151L (CTG)	Mix (ATG and CAR)	1	0.82
False negative	MT (ATG)	WT (CAR)	0	0
Indeterminate result	WT (CAR)	indet	1	0.82
	MT (ATG)	indet	0	
Total			122	100

[a]WT/wild-type: Q = CAR = CAA & CAG; MT/mutant: M = ATG; MIX = mixture of WT & MT viruses; Other = other mutation apart from Q151M; Indet = Indeterminate (neither WT nor MT).

Table 3: Comparison of OLA with consensus sequencing for the M184V mutation

Interpretation	[a]Genotype Q151M		# samples	%
	Sequencing	OLA		
Concordance	WT (ATG)	WT (ATG)	70	95.90
	MT (GTG)	MT (GTG)	45	
	Mix (GTG and ATG)	Mix (GTG and ATG)	2	
Detection of minor variants by OLA	WT (ATG)	MT (GTG)	0	1.64
	MT (GTG)	Mix (GTG and ATG)	1	
	WT (ATG)	Mix (GTG and ATG)	1	
Alternate mutation	M184I (ATA)	WT (ATG)	1	0.82
False negative	MT (GTG)	WT (ATG)	0	0
Indeterminate result	WT (ATG)	indet	2	1.64
	MT (GTG)	indet	0	
Total			122	122

[a]WT/wild-type: M = ATG; MT/mutant: V = GTG; MIX = mixture of WT & MT viruses; Other = other mutation apart from M184V; Indet = Indeterminate (neither WT nor MT)

For the Q151M mutation, sequence analysis gave 103 WT viruses, 18 MT viruses and 1 sample with an alternate mutation (Q151L); OLA identified 101 WT viruses, 17 MT viruses, 3 samples with a mixture of WT and MT viruses and 1 indeterminate result (Table 4). For the M184V mutation, the sequenced results indicated 72 WT viruses, 47 MT viruses, 2 samples with a mixture of WT and MT viruses (M184V and M184I) and 1 sample with an alternate mutation (M184I); OLA showed 70 WT viruses, 46 MT viruses, 4 samples with a mixture of WT and MT viruses and 2 indeterminate results (Table 5).

Table 4: Sequencing and OLA results for the Q151M mutation

Country	No. of patients	No. of samples	No. of samples with indicated genotype[a]							
			*Sequence				OLA			
			WT	MT	Mix	Other	WT	MT	Mix	Indet.
The Gambia	13	42	38	4	0	0	38	4	0	0
Guinea Bissau	23	24	22	2	0	0	22	2	0	0
The Netherlands	16	26	17	9	0	0	16	9	0	1
Sweden	12	30	26	3	0	1	25	2	3	0
Total	64	122	103	18	0	1	101	17	3	1

[a] MIX, mixture of WT and MT viruses; Other , other mutation apart from Q151M; Indet , Indeterminate (neither WT nor MT)

Table 5: Sequencing and OLA results for the M184V mutation

Country	No. of patients	No. of samples	No. of samples with indicated genotype[a] *Sequence WT	MT	Mix	Other	OLA WT	MT	Mix	Indet.
Gambia	13	42	20	19	2	1	21	18	3	0
Guinea Bissau	23	24	24	0	0	0	23	0	0	1
The Netherlands	16	26	11	15	0	0	11	15	0	0
Sweden	12	30	17	13	0	0	15	13	1	1
Total	64	122	72	47	2	1	70	46	4	2

[a] MIX, mixture of WT and MT viruses; Other , other mutation apart from M184V; Indet , Indeterminate (neither WT nor MT)

Comparison of OLA with consensus sequencing gave a high overall concordance of 96.3%, with 96.7% for the Q151M mutation and 95.9% for the M184V mutation. Concordance was defined as the same result by both OLA and sequencing (Table 2 & 3).

In 6.6% of the samples, OLA detected variants which were not detected by sequencing. OLA genotyped seven of eight samples as a mixture of WT and MT viruses, that were identified as MT (five of seven) and WT (two of seven) by sequencing and one of eight as a WT virus that sequencing identified as an alternate mutation, M184I (Table 6 & 7).

Table 6: Discordant results between OLA with consensus sequencing for the Q151M mutation

Sample	Result by[a] Sequencing	OLA	Interpretation
B25 (p18 Apr-00)	ATG (M)	ATG & CAR (Q & M (mix)	OLA detects WT minor population
B26 (p18 Jun-00)	CTG(L)	ATG & CAR (Q & M (mix)	OLA detects WT & MT minor populations, sequencing detects alternate MT (Q151L)
B28 (p18-01)	CAG(Q)	ATG & CAR Q & M (mix)	OLA detects MT minor population
S26 (02-7282)	MWR (K, I, M, Q and/or L)	— (indeterminate)	OLA fails to detect both WT & MT

[a]MWR are degenerate bases: M, A and C; W, A and T; R, A and G. MWR translates to a mixture of any of the following codons encoding for: AAA and AAG (K); ATA (I); ATG (M); CAA and CAG (Q); CTA and CTG (L).

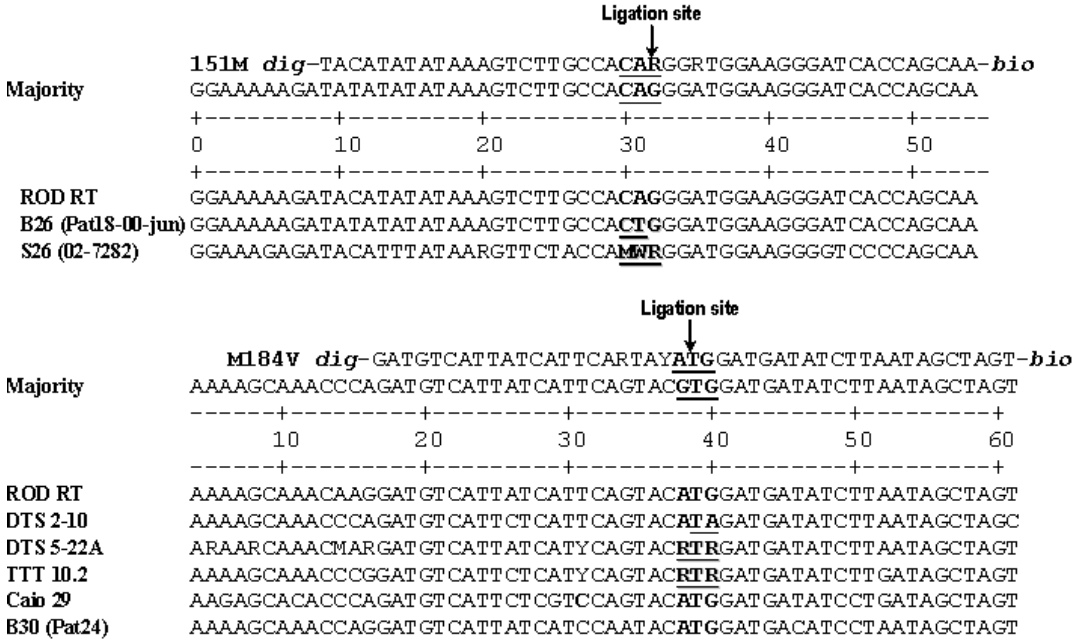
In some cases, alternate mutations were observed at the target codon. These alternate mutations were identified by sequencing, which reports the major population, but not by OLA whose probes were not designed to detect these mutations. However, OLA was able to report the presence of the minor WT and MT populations that were present in addition to the major population with an alternate mutation (Tables 6 & 7).

Table 7: Discordant results between OLA with consensus sequencing for the M184V mutation

Sample	Result by:		Interpretation
	Sequencing	OLA	
B1 (Pat1)	ATG (M)	ATG & GTG (M & V [mix])	OLA detects MT minor population
DTS 2-9	GTG (V)	ATG & GTG (M & V [mix])	OLA detects WT minor population
TTT 10.2	ATA (I)	ATG & GTG (M & V [mix])	OLA detects WT & MT minor populations, sequencing detects alternate MT (M184I)
DTS 5-22A	ATA (I)	ATG & GTG (M & V [mix])	OLA detects WT & MT minor populations, sequencing detects alternate MT (M184I)
DTS 2-10	ATA (I)	ATG (M [WT])	OLA detects WT minor population, but sequencing detects alternate MT (M184I)
B30 (Pat24)	ATG (M)	— (indeterminate)	OLA fails to detect both WT & MT
Caio 29	ATG (M)	— (indeterminate)	OLA fails to detect both WT & MT

Ligation assays are sensitive to sequence variation, such that the presence of other mutations, within two bases of the ligation site, results in failure of the assay (i.e., an indeterminate result) (4). Of the 244 codons analyzed, we observed a total of three indeterminate results, one of three for Q151M and two of three for M184V. For the Q151M mutation, alignment of the samples shows the presence of other bases at the ligation site for sample S26 (02-7282), which had the sequence of degenerate bases MWR (M, A + C; W, A + T; R, A + G) at codon 151 (Fig. 3). MWR translates to a mixture of any of the following codons coding for lysine (AAA and AAG), isoleucine (ATA), methionine (ATG), glutamine (CAA and CAG), and leucine (CTA and CTG). However, the indeterminate result of OLA for sample S26 (02-7282) suggests that the base combinations resulting in lysine, isoleucine, and/or leucine are most likely present (Tables 6 and 7). For the M184V mutation, analysis of the sequence alignment for samples B30 (Pat24) and Caio 29 did not show additional changes around the ligation site that would account for the indeterminate results observed (Fig. 3).

Figure 3. Alignment of samples with alternate mutations and indeterminate results with HIV-2 ROD. Underlined codons mark positions 151 (upper alignment) and 184 (lower alignment). The degenerate codons MWR and RTR correspond to the following codon and amino acid combinations. MWR codon: AAA and AAG (K), AUA (I), AUG (M), CAA and CAG (Q), and CUA and CUG (L). RTR codon: ATA (I), AUG (M), and GTA and GTG (V).



Discussion

The HIV-2 epidemic is restricted mainly to West Africa (29), where antiretroviral drugs have not been widely available. However, this situation is changing rapidly with the recent initiation of the Global Fund that provides funding for the treatment of HIV-infected individuals. Therefore, in the next few years, it is likely that drug resistant strains of HIV-2 will emerge in developing countries, as has been observed for HIV-1. Hence the development of economical resistance assays is crucial for the effective management of HIV-2 patients on ART, especially in resource-poor settings.

We have developed, optimised and evaluated an OLA to detect the mutations Q151M and M184V associated with phenotypic drug resistance in HIV-2 (1, 24). This assay is rapid, economical, highly sensitive and specific for the detection of resistance mutations in HIV-2.

In this study, a total of 244 codons were evaluated in samples from 64 patients from different geographical regions to ensure that a representative panel of the HIV-2 epidemic was used. Though our probes were designed to work for all HIV-2 subtypes, most of the samples available for the evaluation of this assay were HIV-2 subtype A; with only one subtype B sample.

OLA has been reported to be a highly sensitive assay, which allows the detection of variants representing as little as 5% of the total viral population within a sample (4, 13). This high sensitivity reduces the potential for false negative detection of resistance, when mutant viruses are present at low levels (<20%), such as when resistant viruses are first emerging or when selective pressure by a drug has been removed and mutants are supplanted by fitter wild-types (4). The overall sensitivity of detection of the assay was 98.8% for the two mutations tested; with 99.2% sensitivity for the Q151M mutation and 98.4% for the M184V mutation. The documented higher sensitivity of OLA to detect minor variants was observed in this study (4, 13, 15, 33). OLA detected resistance mutations, which were not detected by sequencing, in 6.6% of the samples (Table 4). The presence of these mutant variants as a mixture of MT and WT signal indicate that they represent only a proportion of the quasispecies, i.e. that they are minor variants. It is unlikely that they represent false-positive OLA results, because subsequent samples from these patients showed the presence of the mutant sequences by both OLA and sequencing (Table 4). Thus, OLA appears to have a lower limit of detection of minor variants.

Another attribute of OLA is its high specificity, which depends on the requirements of the DNA ligase and not the DNA annealing conditions (15, 20). The specificity of ligation between the MT/WT probe to the common probe depends on three factors; specificity of hybridization of the probes to their complementary sequences on the template, hybridization of the probes in the 5’ to 3’ orientation and perfect base pairing at the target junctions (ligation site) of the probes. These conditions, when fulfilled, allow non-stringent annealing conditions without compromising specificity (31). However, this highly specific nature of OLA is also the cause of assay failure. The presence of mutations located within two bases on either side of the ligation site may result in failure of the DNA ligase to join the adjacent wild type or mutant probe to the common probe and resulting in an indeterminate result (4). Overall, 3 indeterminate results (1.2%) were observed in this study. In one of three samples, lack of a result was due to the present of alternate mutations at the ligation site, but for the other two, there were no changes around the target site that could explain the indeterminate results observed.

Another cause of indeterminate results is the presence of alternate mutations for which the probes were not designed. When alternate mutations are encountered, their relevance should be balanced with the cost of additional new probes. In HIV-1, the Q151L is a rarely observed transitional mutation that appears to precede the emergence of Q151M (http://hivdb6.stanford. edu/). Q151L is a potential intermediate of Q151M. Also, the much lower replicative fitness of viruses bearing the Q151L variation than those with Q151M further supports the role of Q151L as a transient intermediate of the Q151M mutation. (16). The M184I mutation is associated with 1000-fold phenotypic resistance to lamivudine similar to the M184V mutation (6, 12); and it was reported that the M184I appears earlier (12) and is then outgrown by the 184V mutant (3, 22), which has superior RT polymerase function (4, 7, 9). It seems that both the Q151L and the M184I mutations are transitional mutations with a much lower fitness. Their presence indicates the near emergence of the Q151M and the M184V mutations respectively. Though important, these mutations are only transient and therefore do not warrant the development of additional probes to detect them.

The Oligonucleotide ligation assay, in addition to being simple, highly specific and sensitive also has high through-put, allowing at least 43 samples as well as 3 different controls to be genotyped on a single 96-well Elisa plate. It is also highly adaptable such that additional probes can be easily incorporated as new mutations are discovered. Though data on HIV-2 drug resistance is very limited, some genotypic HIV-2 mutations have been identified (15, 24). However, phenotypic resistance assays are necessary to determine their clinical relevance. As more phenotypic resistant HIV-2 mutations are identified, additional probes can be incorporated into the assay to detect them.

In conclusion, we have successfully developed the oligonucleotide ligation assay for the detection of the mutations Q151M and M184V associated with drug resistance to the nucleoside reverse transcriptase inhibitors. The economical nature of this assay, its higher sensitivity, ease of use and high concordance with sequencing makes it a more practical alternative to consensus sequencing and sustainable for use in resource-poor settings.

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Chapter 7

Development of a Recombinant Virus Assay for Evaluation of Drug Resistance Mutations in the Protease and Reverse Transcriptase Gene of the Human Immunodeficiency Virus Type 2

Development of a recombinant virus assay for evaluation of drug resistance mutations in the protease and reverse transcriptase gene of the Human Immunodeficiency Virus type 2

Sabelle Jallow[a,b], Stefan Herrman [b], Betty Willems [b], Katrien Fransen [b], Martin Schutten [c], Guido Vanham[b,d], Sarah Rowland-Jones[a,e] and Wouter Janssens[b]

[a] *Medical Research Council Laboratories, Banjul, The Gambia*
[b] *Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*
[c] *Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands*
[d] *Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*
[e] *Weatherall Institute of Molecular Medicine, Medical Research Council, Human Immunology Unit, John Radcliffe Hospital, Oxford, United Kingdom.Department of Microbiology , Institute of Tropical Medicine, Antwerp, Belgium*

Abstract

A recombinant virus assay for the generation of mutant HIV-2 viruses for the evaluation of drug susceptibilities is described. An infectious molecular clone of HIV-2ROD (pPK59ROD) was used to generate a deleted molecular clone lacking the entire protease (PR) and reverse transcriptase (RT) of HIV-2. A PCR fragment covering the entire PR and RT with an overlap of at least 100 base pairs on either side of the PR-RT deleted clone was inserted into another vector. This vector was used to introduce mutations into the RT region by site directed mutagenesis. The linearized PR-RT deleted clone and the mutated PCR fragment were transfected into CEM-SS cells by electroporation. Homologous recombination within these cells resulted in the generation of recombinant mutant HIV-2 viruses.

Introduction

Phenotypic resistance assays measure the susceptibility of the virus to inhibition by a particular drug [1, 2]. Although phenotypic resistance assays are generally more expensive, labour-intensive and take several weeks to perform; interpretation of mutations in new compounds cannot be done without prior correlation with phenotypic resistance. In addition, determination of the clinical relevance of new and documented mutations in a different virus such as HIV-2 whose mutational patterns are unknown can only be done using phenotypic resistance assays [1, 3].

Several methods have been used to determine phenotypic resistance of HIV-1, including culture assays in PBMCs [4], non-culture based biochemical methods that measure enzymatic activity [5] and recombinant virus assays [2]. Initial phenotypic resistance assays in PBMCs from HIV sero-negative donors required the preparation of high-titre stocks of primary virus isolates [4], and so were not only laborious and time-consuming, but PBMC from different donors resulted in significant inter-assay variability [6].However, most of these problems were overcome by the revolutionary and innovative method of generating recombinant viruses for drug susceptibility testing [2]. Recombinant virus assays are generated by amplifying the gene of interest from patient plasma and inserting it into a molecular clone of HIV-1, such that the resultant recombinant viruses have a common genetic backbone, which minimizes inter-assay variation [2, 6-9].

We have developed recombinant virus to allow evaluation of genotypic resistance mutations identified in the reverse transcriptase (RT) gene of the Human immunodeficiency virus type 2.

Materials and Methods

Molecular clones, cells lines and bacteria cells : An infectious molecular clone of HIV-2 containing the HIV-2 ROD backbone in the pPK59 plasmid (pKP59ROD) was a kind gift from Dr. Martin Schutten. This molecular clone, which is the same as pROD10, was fully sequenced.

The following cell lines used in this study were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS from Dr. Peter L. Nara [10, 11] and MT-4 from Dr. Douglas Richman. These cells were grown in RPMI 1640 (Bio Whitaker, Verviers, Belgium) supplemented with 100 U of penicillin/ml, 100 µg of streptomycin/ml (Boehringer Ingelheim, Brussels, Belgium), 10% Fecal Calf serum and L-glutamine (Gibco BRL, Invitrogen, Merelbeke, belgium). Cells were maintained at 37^{o}C with 5% CO_2 and used in the logarithmic phase of growth. Cells were discarded after a maximum of 20 passages.

Bacterial cells used for transformation are JM110 and TOP10 cells (Strategene, Huissen, The Netherlands). JM110 are both Dam^{-} and Dcm^{-} methylation, such that they can be used to prepare methylation-free pPK59ROD for restriction with SexAI, a methylation-sensitive restriction enzyme. For mutagenesis experiments methylated plasmids are required; therefore pPK59ROD was also transformed in TOP10 cells.

Transformation : Transformation of the full molecular clone into JM110 or TOP10 cells was done with 10ng of plasmid DNA according to the user manual, except that all incubation steps were done at 30°C instead of 37°C; to ensure that parts of the plasmids are not lost during bacterial expansion.

Nucleic acid extraction: Plasmid DNA was extracted with either the QIAfilter Plasmid mini or midi or maxi kits (QIAGEN, Venlo, Belgium) according to the instruction manual. Extracted DNA were dissolved into 20, 50 and 200µl of nuclease free water respectively and stored at -20°C.

Mutagenesis : We used the QuikChange II Site-Directed Mutagenesis Kit (Stratagene,), which uses a high fidelity enzyme (*Pfu* Ultra HF DNA polymerase) to introduce RT mutations in the HIV-2 PRRT plasmid; according to the instruction manual with slight modifications. We used the protocol in a final volume of 25ul with 2.5ng of plasmid DNA and 62.5ng of each primer. We designed 15 oligonucleotide primers to generate 14 single mutations and 5 multiple mutations in the RT gene (Table 1). For the multiple mutations, we used previously generated plasmids with a RT-mutation to add a new mutation. The generated plasmids were sequenced to confirm the presence of the mutation and the absence of erroneous mutations.

Amplification and sequence analysis of HIV-1 PR and RT sequences : The PR and RT genes were amplified in a single round PCR from the PRRT plasmids with introduced RT mutations. Briefly, 5ng of plasmid DNA was used to amplify the PRRT fragment using 0.3μM of each primer SHJ21 and SJH22 [12]with the Expand High Fidelity Kit (Roche Applied Science, Vilvoorde, Belgium). PCR products were purified using the QIAquick PCR purification system (Qiagen) and directly sequenced. Sequencing was done using any of the following primers SJH23, SJH24, SJH26 [12], JA222 [13], and H2Mp6 [14] as previously described [12].

Electroporation : Electroporation protocols for HIV-1 recombination [2, 15] were modified for HIV-2 transfection into MT-4 cells. In these protocols, C8166 cells were electroporated at 300V and 500μF [2] and MT-4 cells at 300V and 300μF [15]. We modified these protocols for HIV-2 transfection into MT-4 cells. Briefly, cells that were split 1:2 24 hours before electroporation were resuspended in ice cold PBS at 3.125 X 10^6 cells/ml. 800μl of these cells are mixed with 10μg of the undeleted full molecular clone or 10μg of the linearized pKP59RODBglII with 2μg of PCR product. Electroporation was done at 300V with either 300 or 500μF. The electroporated product was chilled briefly on ice and incubated at 20°C for 30 minutes to increase DNA uptake. 5ml of medium was added and the electroporated cells were subsequently maintained in a humidified incubator with 5% CO_2 for 48 hours at 37°C. An additional 5ml of medium is added after 48 hrs and further incubated for 5days. Virus was harvested and detection was done using the INNOTEST HIV Antigen mAb (Innogenetics, Gent, Belgium).

Results

Generation of the deletion vector pKP59RodΔPRRT

Sequence analysis of the complete genome of the infectious molecular clone pKP59ROD, allowed identification of restriction sites that will remove the protease (PR) and reverse transcriptase (RT) genes as well as a list of enzymes that do not cut this clone. The restriction enzymes, *XhoI* (Roche Applied Science) and *SexA1* (New England Biolabs, Westburg, Leusden, Belgium), removed a 1629 base pair (bp) fragment from pKP59ROD. The *XhoI – SexAI* digested plasmid was run on a 0.8% agarose gel and purified QIAquick Gel Extraction Kit (Qiagen). We designed an adaptor for ligation of the deleted clone as well as the simultaneous introduction of a unique restriction site with *BglII* to produce a new vector called pKP59RODBglII (figure 1).

Generation of a vector with the HIV-2 PRRT : PCR amplification of the PR and RT from the pKP59ROD plasmid DNA was done with the primers SJH21 and SJH22 primers. An additional round of PCR with Taq polymerase was done to generate 3'-A overhangs for cloning into a TOPO vector. The PCR fragment was then cloned into the PCR2.1-TOPO vector (Invitrogen, Merelbeke, Belgium) to produce a plasmid called pTopoPRRT, which was then transformed in Top 10 cells.

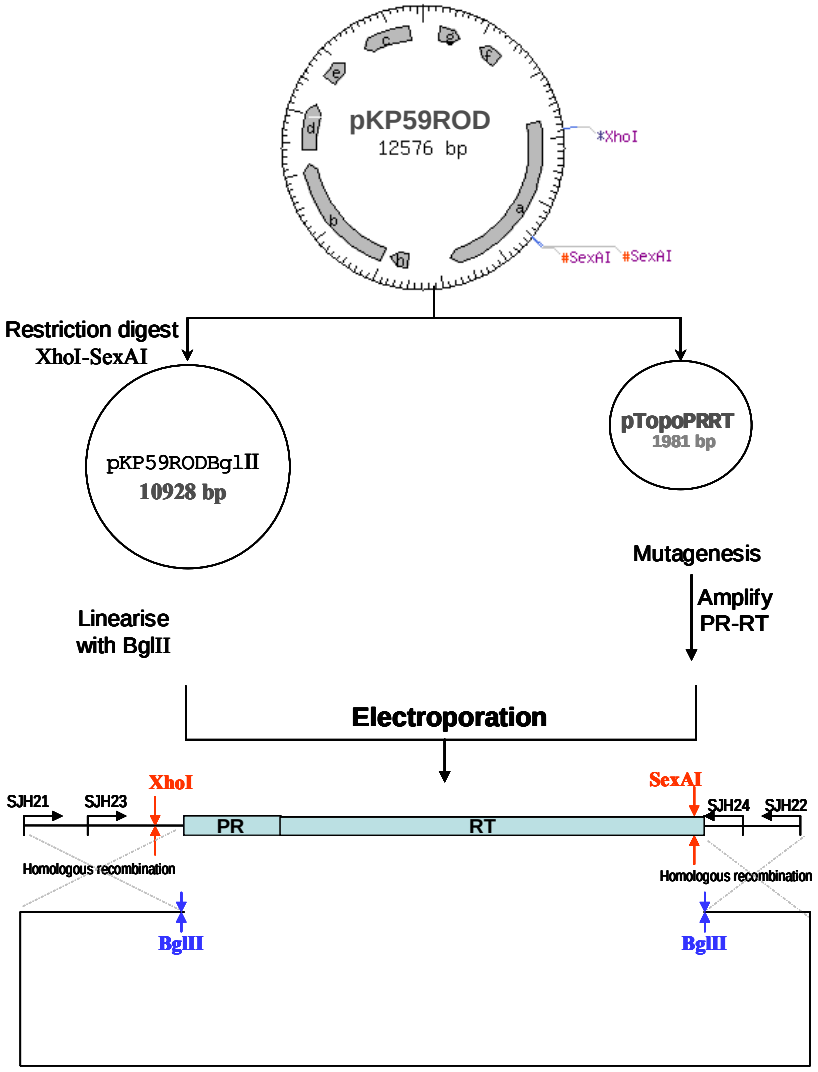

Figure 1: Generation of recombinant HIV-2 viruses.

Generating plasmids with potential drug resistance mutations

We have previously identified new and documented potential drug resistance mutations in Combivir treated HIV-2 patients [16]. These mutations were introduced artificially into

our pTopoPRRT plasmid by site-directed mutagenesis. We successfully generated 14 plasmids each harbouring a single RT mutation and 5 plasmids with at least 3 RT mutations. These mutations are listed in table 1.

Table 1. Introduced mutations and HIV-2 mutagenesis primers:

Mutation	Probe	Sequence
K40R	H2-K40R-S	gaa atc tgt gaa aga atg gaa aaa gaa ggc cag cta gag gaa gc
	H2-K40R-AS	gct tcc tct agc tgg cct tct ttt tcc att ctt tca cag att tc
A62V	H2-A62V-S	ccc cca cat ttg taa tca aga aaa agg aca aaa aca aat gga gg
	H2-A62V-AS	cct cca ttt gtt ttt gtc ctt ttt ctt gat tac aaa tgt ggg gg
K65R	H2-K65R-S	cat ttg caa tca aga gaa agg aca aaa aca aat gga gga tgc
	H2-K65R-AS	gca tcc tcc att tgt ttt tgt cct ttc tct tga ttg caa atg
N69S	H2-N69S-S	gca atc aag aaa aag gac aaa agc aaa tgg agg atg c
	H2-N69S-AS	gca tcc tcc att tgc ttt tgt cct ttt tct tga ttg c
K70S	H2-K70S-S	gca atc aag aaa aag gac aaa aac agc tgg agg atg c
	H2-K70S-AS	gca tcc tcc agc tgt ttt tgt cct ttt tct tga ttg c
V111I	H2-V111I-S	gag aag aat tac tgt act aga tat agg gga tgc tta ctt ttc c
	H2-V111I-AS	gga aaa gta agc atc ccc tat atc tag tac agt aat tct tct c
I118V	H2-I118V-S	gct tac ttt tcc gta cca cta cat gag gac ttt aga cc
	H2-I118V-AS	ggt cta aag tcc tca tgt agt ggt acg gaa aag taa gc
Q151M	H2-Q151M-S	gat aca tat ata aag tct tgc caa tgg gat gga agg gat cac c
	H2-Q151M-AS	ggt gat ccc ttc cat ccc att ggc aag act tta tat atg tat c
M184V	H2-M184V-S	cat tca gta cgt gga tga tat ctt aat agc tag tga cag g
	H2-M184V-AS	cct gtc act agc tat taa gat atc atc cac gta ctg aat g
V201A	H2-V201A-S	gac aga ttt aga aca tga cag agc agt cct gca gct aaa gg
	H2--V201A-AS	cct tta gct gca gga ctg ctc tgt cat gtt cta aat ctg tc
F214L	H2-F214L-S	cta aat ggc cta gga ctt tct acc cca gat gag aag ttc c
	H2-F214L-AS	gga act tct cat ctg ggg tag aaa gtc cta ggc cat tta g
S215Y	H2-S215Y-S	cta aat ggc cta gga ttt tat acc cca gat gag aag ttc c
	H2-S215Y-AS	gga act tct cat ctg ggg tat aaa atc cta ggc cat tta g
K223R	H2-K223R-S	cca ccc cag atg aga agt tcc aaa gag acc ctc cat acc
	H2-K223R-AS	ggt atg gag ggt ctc ttt gga act tct cat ctg ggg tgg
Q333L	H2-Q333L-S	gct aga agc aac agt cca aaa gga tct aga gaa tca gtg g
	H2-Q333L-AS	cca ctg att ctc tag atc ctt ttg gac tgt tgc ttc tag c
A62V+K65R +N69S	H2-A62V+K65R-S +N69S-S	ccc cca cat ttg taa tca aga gaa agg aca aaa gca aat gga gg
	H2-A62V+ K65R-S +N69S-AS	cct cca ttt gct ttt gtc ctt tct ctt gat tac aaa tgt ggg gg

Mutations	
A62V+ K65R + N69S +V111I	Mutagenesis of mutated plasmid
A62V+ K65R +N69S +Q151M	Mutagenesis of mutated plasmid
A62V+ K65R +N69S +V111I+Q151M	Mutagenesis of mutated plasmid
V111I + Q151M	Mutagenesis of mutated plasmid

Generation of recombinant viruses : Electroporation of the full molecular clone, pKP59ROD into MT-4 cells with the protocols described for HIV-1 did not result in virus production. To determine these cells can support HIV-2 growth, we infected MT-4 cells with HIV-2 ROD virus supernatant. This virus grew very poorly in these cells and did not produce a cytopathic effect. We optimised the protocol for use in CEM-SS cells, which are known to support production of HIV-2. The HIV-1 plasmid, pNL4-3lucR$^-$ was used to optimise the electroporation protocol in CEM-SS cells. We electroporated 10µg of pNL4-3lucR$^-$ into 2.5 x 10^6 CEM-SS cells, using three voltage conditions of 200, 250 and 300V with three sets of capacitances of 950, 500, 300µF. 48 hours post-infection, we quantified the number of viable cells and measured infection (successful electroporation) of CEM-SS cells by quantifying luciferase activity. Higher voltages of 250 and 300V resulted in the death of the CEM-SS cells, such that there was neither luciferase activity nor viable cells were measured. The optimal voltage for these cells was found to be 200V. The number of viable cells was highest for 300µF > 500µF > 950µF; while luciferase activity was highest for 500µF > 300µF > 950µF. Thus we concluded that electroporation of CEM-SS cells at 200V and 500µF was the best for virus production. These conditions when used with 10µg of linearised pKP59RODΔBglII together with 2µg of PCR product from the mutated pTopoPRRT plasmids. This resulted in the production of recombinant virus with the mutation of interest.

Discussion

We have successfully developed and optimised a recombinant virus assay; akin to the one that has been developed for HIV-1 [15]. However, electroporation into MT4 cells as used for HIV-1 did not produce viruses with our molecular clone and growth of HIV-2ROD virus stock into these cells yielded both poor viral growth and no cytopathic effect (CPE). Another study on phenotypic resistance of HIV-2 to Lopinavir/r also reported poor growth of HIV-2 isolates in MT-4 cells as well as the lack of CPE [17, 18]. However, optimisation of the protocol for use with CEM-SS cells resulted in virus production.

We developed mutant recombinant viruses that have exactly the same backbone as the wild-type of HIV-2ROD, except for the introduced mutation of interest. These viruses can be further used in drug susceptibility assays to determine phenotypic resistance as a result of individual as well as multiple mutations by simply comparing the 50% inhibitory concentration (IC_{50}) of each mutant relative to the wild-type.

Previous studies in HIV-1 have revealed that the development of drug resistance is usually associated with a loss in fitness [19, 20]. In fact, this reduction in viral fitness due

to the presence of certain mutations has been exploited in salvage therapy. The M184V mutation to Lamivudine has a much lower replicative capacity than the wild type and can therefore be maintained in patients who have exhausted all therapeutic options; to keep the viral load lower compared to that of the untreated wild-type [19, 21]. The mutant viruses we have generated can also be used in growth competition assays with the wild-type to determine the relative fitness of the mutant viruses compared to the wild-type. This might result in identification of viruses with significantly reduced fitness for use in salvage HIV-2 therapy.

Development of an HIV-2 recombinant virus and the generation of viruses with new and previously documented mutations will play an important role in deciphering the clinical relevance of these potential drug resistance mutations and the natural polymorphism in a phenotypic resistance assay and therefore allow efficient management and treatment of HIV-2 infected patients.

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RESULTS : PART II

Response of HIV-2 and HIV-1/2 Dually Infected Patients to Anti-Retroviral Therapy

Chapter 8

Virological and Immunological Response to Combivir and the Emergence of Drug Resistance Mutations in a Cohort of HIV-2 Patients in The Gambia

Virological and immunological response to Combivir and the emergence of drug resistance mutations in a cohort of HIV-2 patients in The Gambia

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Sabelle Jallow[a,b], Steve Kaye[a], Abraham Alabi[a], Akum Aveika[a], Ramu Sarge-Njie[a], Saihou Sabally[a], Tumani Corrah[a], Hilton Whittle[a], Guido Vanham[b,], Sarah Rowland-Jones[a] , Wouter Janssens[b] and Samuel J. McConkey[a]

[a] *Medical Research Council Laboratories, Banjul, The Gambia*
[b] *HIV and Retrovirology Research Unit, Department of Microbiology , Institute of Tropical Medicine, Antwerp, Belgium*

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Abstract

We monitored the virological and immunological responses of eight HIV-2 infected patients to Zidovudine and Lamivudine and the emergence of drug-resistance mutations. Most patients failed to suppress the virus to undetectable levels. 7/8 patients developed drug-resistance mutations; some HIV-1 resistance mutations were detected as natural polymorphisms and others were absent, suggesting that HIV-1 and 2 have both similar and different resistance pathways. For effective HIV-2 therapy, it is important to identify critical drug resistance mutations.

Introduction

The use of ART has resulted in dramatic decreases in mortality in the developed world. Due to the conserved nature of the protease and reverse transcriptase (RT) enzymes, antiretroviral drugs developed for HIV-1 subtype B, were expected to have similar efficacy for all HIV viruses. However, HIV-1 group O and HIV-2 were later discovered to be naturally resistant to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) [1].

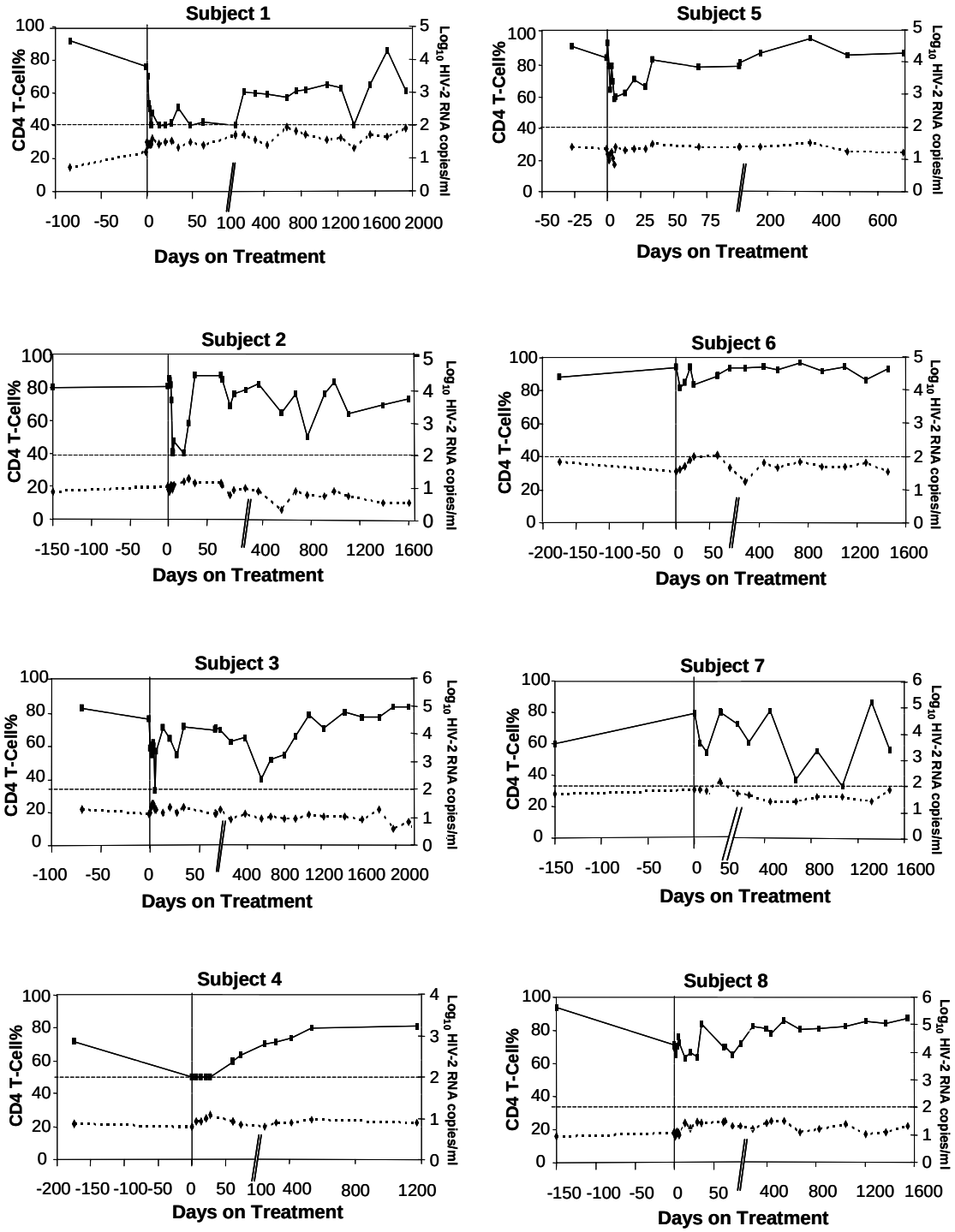
Research on HIV-2 drug resistance is limited [2], due to the lower worldwide prevalence of HIV-2 and its restriction mainly to West Africa, where access to treatment was limited. However, with the Global Fund, a substantial number of HIV-2 patients will be treated and hence it is important to understand HIV-2 response to ART and the emergence of drug-resistance mutations.

We have monitored the emergence of drug-resistance mutations in HIV-2 infected patients treated with Zidovudine (AZT) and Lamivudine (3TC). Although such treatment would now be considered sub-optimal, at the time of the study no other ART was available in The Gambia.

Materials and Methods

Between 1998 and 2001, before the advent of ART in the Gambia, eight treatment-naïve HIV-2 infected patients were treated with Combivir (AZT+3TC). The Joint Gambian government–Medical Research Council Ethics Committee approved this study.
CD4 measurements were done using Flow cytometry (Becton-Dickinson). Plasma HIV-2 RNA copies/ml were measured with an in-house viral load assay (100copies/ml limit of detection), as previously described [3, 4]. HIV-2 RNA was extracted from plasma [5], reverse transcribed and amplified to produce the entire HIV-2 protease and RT genes. Purified PCR products were directly sequenced on both strands and analysed with DNAstar software (Lasergene). Phylogenetic analysis showed HIV-2 viruses from all patients to be subtype A. Sequences generated were assigned the accession numbers AM233873 to AM233900.

Figure 1: *Virological and immunological responses of HIV-2 infected patients treated with AZT and 3TC. Time lines depicting changes in log of plasma HIV-2 RNA copies/ml (continuous line with squares) and changes in % of CD4+ T-Cells (dotted line with diamonds) over time. The horizontal dashed line at log 2 plasma HIV-2 RNA copies/ml represents the limit of detection of the viral load assay (i.e.100copies/ml).*



Results

Response to therapy for each patient was computed with respect to reduction in viral load. In patient 1, 2 and 4, viral load dropped to undetectable levels. Patient 3, 5 and 7 had moderate responses of 1-2 logs drop in viral load whilst patients 6 and 8, had insignificant virologic responses (<0.5log drop in viral load). At the time of genotyping, all subjects had experienced viral load rebound (Figure 1).

Analysis of the RT sequences for the presence of drug-resistance mutations showed four major HIV-1 NRTI mutations: K65R, Q151M, M184V and T215Y/F. The most common mutation, M184V, was found in 7/8 patients, whilst the others were each found in one patient. Minor mutations, N69S and A62V were each found in one patient whilst V75I and K219Q/E occurred naturally in all patients.

Some commonly detected HIV-1 mutations were not observed in our study, K70R/S previously reported in HIV-2 patients [2, 6, 7] was absent. Except for S215Y and the K219Q/E polymorphism, the HIV-1 thymidine-analogue mutations (TAMs), M41L, D67N, K70R, L210W, T215Y and K219Q/E, were also absent. However, several new potential HIV-2 drug-resistance mutations including K20R, K40R, A62V, I118V, F214L and Q333L were detected.

Analysis of the protease sequences revealed several natural HIV-2 polymorphisms previously detected as drug-induced mutations in HIV-1**.** Several minor mutations, L10V, V32I, M36I, I47V, A71V and G73A were found in all the patients and the major mutation, M46I, was found in 7/8 patients.

Discussion

Several studies have shown that ART in HIV-1 infected individuals usually results in significant reduction in viral load [8, 9], but we observed a range of responses, from good to insignificant, in our patients. Previous studies also show that HAART reduces HIV-1 viral load to undetectable levels, but in HIV-2 various HAART regimens failed to achieve this: instead a drop of only 0.4–2 logs was observed [9, 10].

The Lamivudine resistance mutation, M184V, commonly observed in HIV-1 infected patients taking this drug, was the mutation most frequently observed (7/8) in this study. This mutation has been associated with phenotypic resistance to 3TC in HIV-2 [9].

In contrast, the absence of the classic AZT resistance mutations suggests that HIV-2 may have a different AZT-resistance pathway from that observed in HIV-1, consistent with other findings [11]. Another possibility is the documented natural resistance of HIV-2 to AZT [12]. This study suggested that the lack of selective pressure by AZT on these viruses might explain the failure of AZT-related mutations to develop in the HIV-2 RT.

The multi-NRTI mutation, Q151M (1/8), although rare in HIV-1, has been reported to be more frequent in HIV-2 [1, 9]. K65R together with A62V, N69S and M184V were observed in patient 6. This accumulation of A62V, K65R, N69S, Q151M and M184V, previously reported in HIV-2 [2] might represent a multi-NRTI mutation complex.

Apart from the M184V resistance pathway, HIV-2 may have another mechanism towards Lamivudine resistance; which may explain the absence of M184V in patient 1 even after five years on Combivir. Even though the known HIV-1 mutations were absent, K20R, R22K, I181V, D195G, V201A and Q333L that could represent an alternative resistance pathway, were present. In HIV-1, the G333D/E, present naturally in 50% of infected patients [13] facilitates dual resistance to both AZT & 3TC [14]. Thus Q333L, also found naturally in HIV-2 subtype B and G might be the HIV-1 G333D/E equivalent and cause reduced susceptibility to AZT and / 3TC in HIV-2.

The presence of PI mutations as natural polymorphisms may cause reduced sensitivity to some PIs in HIV-2 [15], especially M46I, which is associated with high-level resistance to Indinavir. Thus PIs should be selected with care in HIV-2 treatment.

Conclusions

We report for the first time the emergence of HIV-2 resistance mutations attributed to a single drug regimen, Combivir (AZT+3TC). We have shown that whilst HIV-2 has some equivalent HIV-1 mutations, others were either absent or pre-existed as natural polymorphisms. A phenotypic resistance assay is necessary to determine the clinical relevance of these new and previously documented mutations. It is clear from this and other studies that some antiretroviral drugs may not provide sufficient HIV-2 inhibition.

Thus for effective and optimal HIV-2 treatment, the differences between HIV-1 and 2 should be taken into account in the development of new drugs.

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Chapter 9

Virological Response to Highly Active Antiretroviral Therapy (HAART) and The Emergence of Drug-Resistant Mutations in HIV-2 and HIV-1/HIV-2 Dually Infected Patients in The Gambia

Virological response to Highly Active Antiretroviral Therapy (HAART) and the Emergence of Drug-Resistant Mutations in HIV-2 and HIV-1/HIV-2 Dually Infected Patients in The Gambia

Sabelle Jallow[1,2], Abraham Alabi[1], Ramu Sarge-Njie[1], Kevin Petterson[1], Christopher Akolo[1], Akum Aveika[1], Hilton Whittle[1], Guido Vanham[2,3], Sarah Rowland-Jones[1,4] and Wouter Janssens[2].

[1] *Medical Research Council Laboratories (MRC), Banjul, The Gambia*
[2] *Unit of Viroloy, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*
[3] *Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*
[4] *Weatherall Institute of Molecular Medicine, Medical Research Council, Human Immunology Unit, John Radcliffe Hospital, Oxford, United Kingdom.*

Submitted: J. of Clin. Microbiology

Abstract

Background: Drug design, antiretroviral therapy (ART) and the study of drug resistance has been focused almost exclusively on HIV-1, resulting in limited information on the response to ART and the emergence of drug resistance in HIV-2 and HIV-1/2 dually-infected patients. However, a few studies have reported that various HAART regimens fail to suppress HIV-2 viral replication to undetectable levels. With the increasing access to antiretrovirals in Africa, it is important to understand the response of these patients to therapy and document clinically relevant resistance mutations.

Methods: Twenty patients, 12 HIV-2 and 8 HIV-1/HIV-2 dually-infected patients were put on a fixed regimen of Zidovudine (ZDV), Lamivudine (3TC) and Ritonavir-boosted Lopinavir (LPV/r) and followed up longitudinally for up to three years. Clinical, immunological and virological data were collected for the patients before and during therapy. In events of virological failure, the entire HIV-2 protease and RT genes were amplified from RNA and genotyped for drug resistance-associated mutations

Results: In 19 out of 20 patients, viral load was reduced to undetectable levels. One patient had a viral suppression of two logs followed by viral rebound to pre-therapy levels. This patient reported adverse effects associated with LPV/r that caused him to stop taking all the drugs. Viral rebound in most patients was due to blips or problems of non-adherence as the viral loads went back to undetectable levels. However, in two patients, viral rebound was clearly due to the emergence of documented and new drug resistance mutations, in both the protease and RT genes.

Conclusions: The combination of ZDV, 3TC and LPV/r is able to provide efficient and durable suppression of HIV-1 and HIV-2 for up to three years in HIV-2 and dually infected patients. However, the emergence of new and documented drug resistance mutations, observed during our study, can compromise the efficacy of this treatment.

Introduction

Development of drug-resistance has posed a major obstacle in the effective treatment of the human immunodeficiency virus (HIV), limiting both the magnitude and the duration of the response to treatment; as well as reducing the number of active antiretroviral (ARV) drugs available for HAART [1].

Drug development, susceptibility tests and drug resistance studies have focused almost exclusively on HIV-1, with limited work done on HIV-2. This is mainly due to the lower prevalence of HIV-2 relative to HIV-1 and the restriction of the HIV-2 epidemic mainly to West Africa, where access to treatment has been limited. Currently approved drugs were designed for HIV-1 subtype B, but due to the highly conserved nature of the critical HIV-1 and HIV-2 enzymes, protease and reverse transcriptase, especially around the active sites, it was assumed that these drugs will be active for both types of HIV infections. The non-nucleoside reverse transcriptase inhibitors (NNRTIs), however, target allosteric sites of the enzyme and it was later discovered that HIV-1 group O strains and HIV-2 are naturally resistant to these drugs [2-5]. In addition, HIV-2 has been found to be naturally resistant to the entry inhibitor, T-20, and may have reduced susceptibility to some protease inhibitors [6-8]. Therefore, drugs used for the treatment of HIV-2 should be carefully selected to allow optimal and durable viral suppression. The presence of HIV-2 in HIV-1/HIV-2 dually infected individuals complicates treatment of these patients. Therefore, the optimal regimen for the treatment of dually infected patients should include drugs with simultaneous activity against both HIV-1 and HIV-2.

In the developed world as well as a few countries in Africa, HIV-2 infected individuals have been treated with ARV [9-16], using drugs and protocols developed for HIV-1. However, increasing accessibility to these drugs in Africa means that a substantial number of HIV-2 individuals will be treated; making the study of HIV-2 response of treated patients to HAART and development of resistance to ARV a priority.

We have monitored the response to treatment and the emergence of potential drug resistance associated mutations in HIV-2 and HIV-1/ HIV-2 dually infected patients treated with a combination of ZDV, 3TC and LPV/r for up to three years. The presence of drug resistance mutations was analysed in relation to the viral load and CD4 counts of these patients.

Materials and Methods

Study subjects

The Genito-urinary Medicine clinic at the Medical Research Council Laboratories in The Gambia follows a cohort of HIV-2 patients, who are routinely monitored for CD4 T-cell count, plasma viral load and clinical signs and symptoms. Antiretrovirals became available in the Gambia in 2004 and patients reaching the criteria of CD4 T-cell count ≤350 cells/ml are now treated according to the national guidelines. HIV-2- and HIV1/2 dually- infected patients are put on a fixed regimen of ZDV, 3TC and LPV/r as the first line regimen. Study subject details are shown in Table 1. The Joint Gambian government – Medical Research Council Ethics Committee approved this study.

Table 1: Epidemiological, immunological and virological data of the Patients

				HIV	Approved	Start	CD4 and VL at start of ART			
	reference #	sex	age	Status	ARV	ARV	CD4%	abs CD4	VL HIV-1	VL HIV-2
Patient 1	20041287	M	44	2	03/05/2005	20/05/2005	10	300	-	315,118
Patient 2	20020797	M	54	2	31/05/2005	13/06/2005	5	90	-	108,650
Patient 3	19921115	M	42	2	19/04/2005	05/05/2005	16	180	-	
Patient 4	20047429	F	30	2	28/06/2005	22/07/2005	4	130	-	50,950
Patient 5	20053368	M	48	2	16/08/2005	17/08/2005	10	130	-	
Patient 6	2003A182	F	43	2	16/08/2005	01/09/2005	5	70	-	38,645
Patient 7	20051154	F	31	2	25/10/2005	17/11/2005	12	180	-	54,324
Patient 8	20052184	M	31	2	25/10/2005	17/11/2005	7	50	-	120,695
Patient 9	20054262	F	46	2	15/11/2005	28/11/2005	8	230	-	311,055
Patient 10	2001A731	F	50	2	15/11/2005	28/11/2005	16	130	-	3,162
Patient 11	20009737	F	31	2	30/11/2004	10/03/2005	4	10	-	484,047
Patient 12	19934174	F	37	2	06/09/2005	19/12/2005	19	210	-	5,656
Patient 13	19813502	F	46	1 &2	25/01/2005	14/03/2005	11	220	111867	144691
Patient 14	19927800	F	40	1 &2	07/12/2004	25/01/2005	7	40	380,315	167
Patient 15	19972375	F	28	1 &2	19/04/2005	05/05/2005	5	160	78,487	5,735
Patient 16	20018919	M	49	1 &2	01/03/2005	04/04/2005	3	40	10,570	41,506
Patient 17	20034661	F	38	1 &2	28/06/2005	22/07/2005	4	210	1,000,000	7,941
Patient 18	19965521	F	36	1 &2	15/11/2005	28/11/2005	12	300	42,462	100
Patient 19	20056249	M	30	1 &2	07/02/2006	02/03/2006	7	60	951,770	100
Patient 20	19936825	F	51	1 &2	16/08/2005	02/03/2006	14	180	1,000,000	37,334

CD4 T-cell Count and Viral load

CD4 T-cell measurements were done using flow cytometry (Facscan, Becton Dickinson). Plasma HIV-2 RNA copies/ml (viral load) was quantified using an in-house viral load assay [17], which had a limit of detection of 100 RNA copies/ml plasma. This assay was modified to include an internal molecular control for extraction and amplification efficiency [18].

Nucleic acid extraction, PCR amplification and Sequence analysis

HIV-2 RNA was extracted from 140µl of EDTA plasma using the QIAamp viral RNA kit (QIAGEN, Venlo, The Netherlands) and eluted into 50µl nuclease-free water. The entire HIV-2 protease and reverse transcriptase (RT) were amplified using a single tube reverse-transcription PCR method (Titan one-tube RT-PCR, Roche Applied Science, Lewes UK) starting from 3µl of RNA followed by a nested PCR with Expand High Fidelity DNA polymerase and 0.5-1µl of first round product as previously described [19]. Purified PCR products were directly sequenced on both strands and analysed with DNAstar software (Lasergene, DNASTAR Wisconsin, U.S.A) [19]. For the dually infected patient, genotyping was done for only HIV-1, where viral rebound was observed. The inner primers SJH11A (forward-5'-AAAAGGGCTGTTGGAAAT GTGG-'3, position 2018 to 2105) and SJH12A (reverse -5'-CCTAATGCATACTGTGAGT CTG -'3, position 3936 to 3912); and the outer primers SJH13a (forward-5'-GAGAGACAGGC TAATTT TTTAGGG, position 2071 to 2094) and SJH14 (reverse-5'- CCTATTAGCTGCCCC ATCTACATA-'3, position 3893 to 3870) were used to amplify the entire HIV-1 protease and RT. These primer positions refer to HIV-1 HXB2 [Genbank accession #: K03455]. The PCR conditions used for HIV-2 were also used for HIV-1 except for the annealing temperatures; 56°C was used for the first round and 57°C was used for the nested PCR. Three sequencing primers were used to generate the HIV-1 protease and RT: SHJ13A, SJH14 and SJH15 (forward-5'- GATGTGGGGGAYGCATATTTTCAG-'3, position 2877 to 2901). Viral subtype was determined using an NCBI on-line programme (http://www.ncbi.nih.gov/projects/ genotyping/). All HIV-2 strains were subtype A and the HIV-1 strain in the dually infected patient was CRF_02_AG. Sequences generated were assigned the accession numbers (to be submitted).

Results

Response to therapy

For the HIV-2 infected patients, viral load was initially reduced to undetectable levels (i.e. < 100 copies/ml, the limit of detection) in all, but one patient (Patient 1), accompanied by a general increase in CD4 T-cell count from pre-therapy levels. Viral load in Patient 1 was reduced by two logs during the first two weeks of therapy, but rapidly rebounded to pre-treatment levels. In 7 patients (patients 3, 4, 5, 6, 8, 10 and 12), viral load is still undetectable after a range of 3 to 28 months on therapy. However, in Patient 12 viral load dropped slowly and only became undetectable after 5 months. In the

rest of the patients, (patients 2, 7, 9 and 11), viral load was undetectable for a range of 3 to 15 months, but viral rebound was observed afterwards (Figure 1). In patients 2 and 11, the viral load rebounded and returned back to undetectable levels several times, indicating there might have been problems with adherence.

A very good virological response to therapy was observed for both HIV-1 and HIV-2 in the dually infected patients as shown by the decline of their viral loads to undetectable levels. In seven out of eight patients, viral loads are still undetectable after a range of 18 to 36 months on therapy. In patient 19, the HIV-1 viral load increased by 2 logs while HIV-2 viral load remained undetectable. Reduction in viral load was accompanied by a general increase in CD4 T-cells in all dually-infected patients.

Figure 1: Virological and immunological responses of HIV-2 infected patients treated with AZT 3TC and LPV/r. Time lines depicting changes in log of plasma HIV-2 RNA copies/ml (blue line) and changes in % of CD4+ T-Cells (pink line) over time. The limit of detection of the viral load assay is log 2 or 100copies/ml).

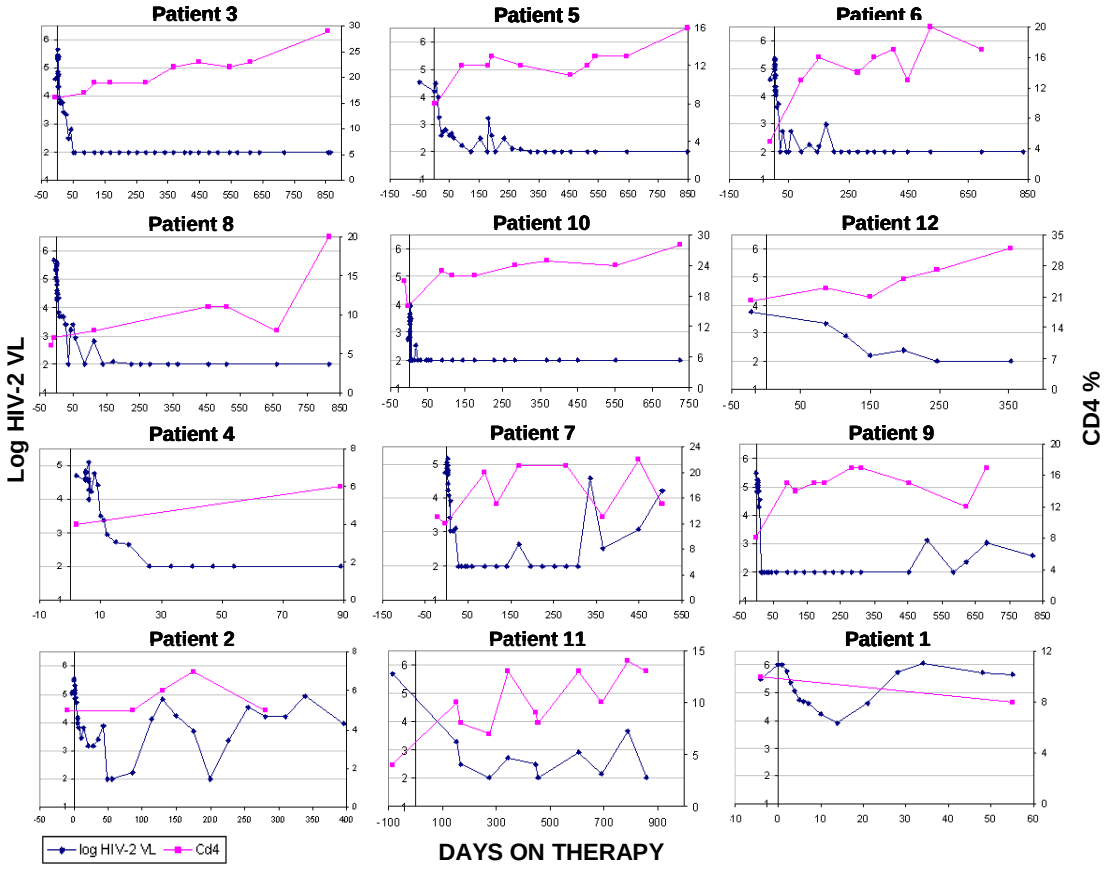
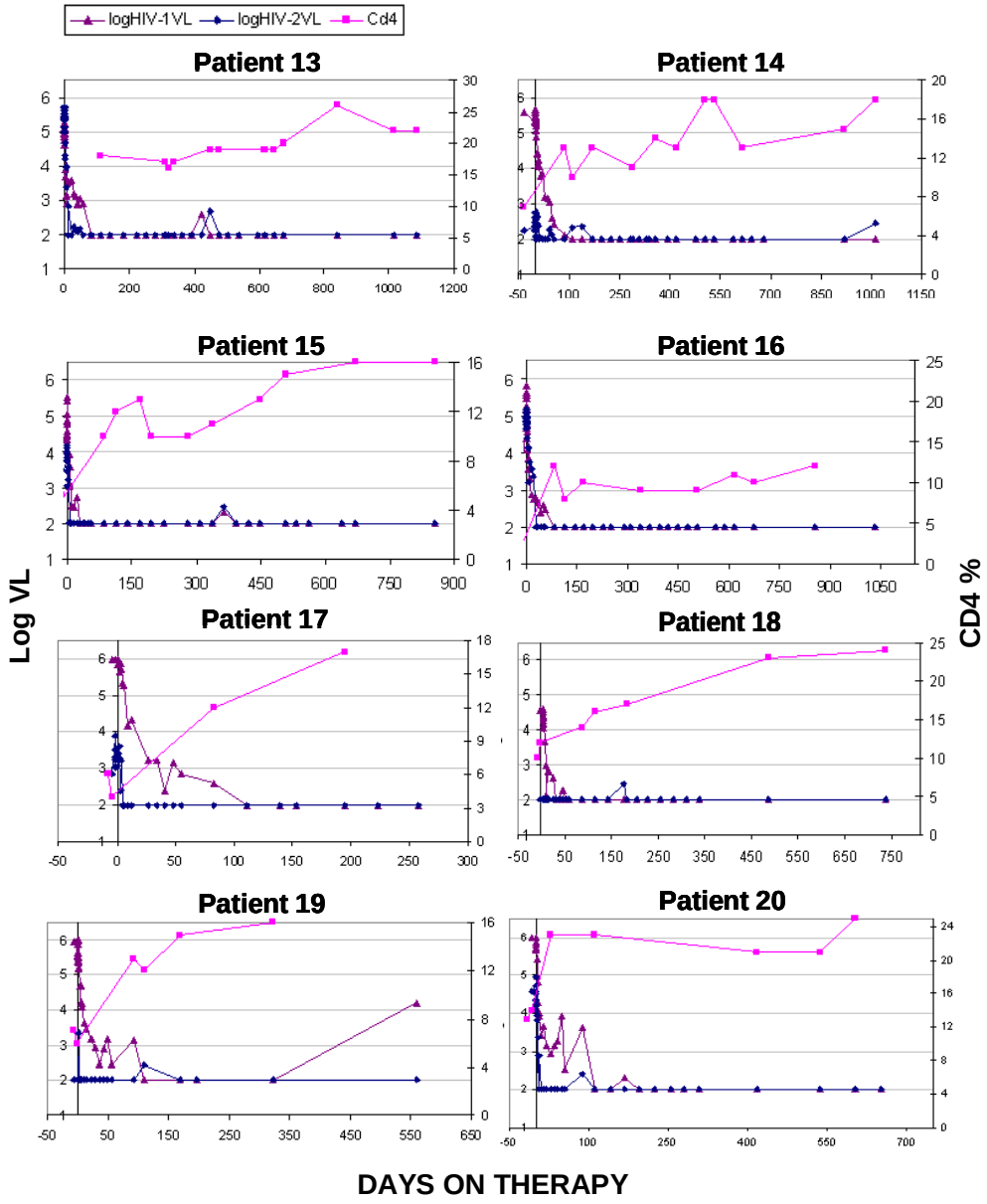


Figure 2: Virological and immunological responses of HIV-1 and HIV-2 dually infected patients treated with AZT, 3TC and LPV/r. Time lines depicting changes in log of plasma HIV-2 RNA copies/ml (blue line), log of plasma HIV-1 RNA copies/ml (purple line), and changes in % of CD4+ T-Cells (pink line) over time. The limit of detection of the viral load assay is log 2 or 100copies/ml).



Drug-resistance mutations

Sequence analysis was performed for ten patients (nine HIV-2- and one dually-infected) with detectable viral load despite therapy. The HIV-2 protease and reverse transcriptase (RT) region of the sequences were aligned and analysed for drug-resistance mutations.

The alignment of the patient samples for different time points, before and after therapy, shows mutations that may have arisen as a result of AZT, 3TC and LPV/r pressure. A major HIV-2 protease inhibitor (PI) mutation, V47A [20] and a primary HIV-1 nucleoside reverse transcriptase inhibitor (NRTI) mutation, M184V/I were observed in Patient 2 and Patient 11. Patient 2 had the M184I mutation after 4 months on ART, and the V47A and M184V mutation after 8.5 months on AZT, 3TC and LPV/r (Table 2 & 3). In addition, the mutation F53V, whose position is known to harbour a PI resistance in HIV-1 (F53Y/L), emerged due to drug pressure in Patient 2 [21]. Patient 11 had two PI mutations, V331 and V47A, and the 3TC mutation M184V at the time of genotyping.

Drug-resistance mutations: Potential HIV-2 specific mutations

In addition to the known HIV-1 mutations, several other mutations not previously detected in HIV-1 were observed. In the protease sequences, the K45N (Patient 1), H14R and M95I (Patient 11) mutations seemed to emerge as a result of LPV/r pressure; and in the RT sequences the mutations, L260P (Patient 1), and V371I (Patient 2), seemed to emerge presumably as a result of ZDV and 3TC pressure.

Polymorphisms in the protease and reverse transcriptase gene

Analysis of the HIV-2 protease gene from pre-therapy samples showed the presence of several mutations that are associated with drug resistance when found in HIV-1. Eight minor HIV-1 mutations, L10V, K20R, L33V, M36I, I62V, A71V, G73A and I93L and three major mutations, V32I, M46I, I47V, were present in all the patients (Table 2). Also, polymorphisms were found at positions K76V (M in HIV-2) and V82A/T/F (I in HIV-2), known to harbour major HIV-1 PI mutations and a minor mutation E34Q (A in HIV-2). [12, 13, 15].

On the other hand, analysis of the HIV-2 RT gene from pre-therapy samples revealed only a few mutations associated with HIV-1 drug resistance. Two RT mutations, V75I associated with multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance in HIV-1, and K219Q/E, associated with AZT resistance in HIV-1, were found to occur naturally in all the samples (Table 3). Polymorphisms were also found at positions T69D (N in HIV-2), L210W (N in HIV-2), T215Y/F (S in HIV-2) and G333D/E (Q in HIV-2), known to harbour HIV-1 RT mutations (Table 3).

Table 2: Drug resistance mutations emerging as a result of PI exposure. *Mutations in red represent known drug resistance HIV-1 mutations and those in blue represent HIV-2 polymorphism that could affect drug susceptibility.*

	Common Mutations in HIV-1 patients exposed to PIs																											
	L10F/I/C/V	G16E	K20M/R/V	L24I	D30N	V32I	L33F/I/V	E34Q	M36I/L/V	M46I/L	I47A/V	G48V	I50V	F53Y/L	I54V/A/M	D60E	I62V	L63P	I64L/M/V	A71V/T	G73A/S	T74P	L76V	V82A/T/F	I84V	N88S	L90M	I93L/M
HIV-1 WT	**L**	**G**	**K**	**L**	**D**	**V**	**L**	**E**	**M**	**M**	**I**	**G**	**I**	**F**	**I**	**D**	**I**	**L**	**I**	**A**	**G**	**T**	**L**	**V**	**I**	**N**	**L**	**I**
Pat 19 t = 0	I	E	R	L	D	V	L	E	I	M	I	G	I	F	I	D	I	P	I	A	G	T	L	V	I	N	L	I
Pat 12 t = 559	I	E	I	L	D	V	L	E	I	M	I	G	I	F	I	D	I	P	I	A	G	T	L	V	I	N	L	I
HIV-2 WT	**V**	**E**	**V**	**L**	**D**	**I**	**V**	**A**	**I**	**I**	**V**	**G**	**I**	**F**	**I**	**K**	V	E	I	**V**	**A**	**T**	**M**	**I**	**I**	**N**	**L**	**L**
Pat 1 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 1 t = 55	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t =0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t = 130	V	E	V	L	D	I	V	A	I	I	V	G	I	V	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t = 255	V	E	V	L	D	I	V	A	I	I	A	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t = 310	V	E	V	L	D	I	V	A	I	I	A	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t = 395	V	E	V	L	D	I	V	A	I	I	A	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 2 t = 511	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 5 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	I	A	T	M	I	I	N	L	L
Pat 6 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 6 t = 174	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 7 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 7 t = 336	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 7 t = 504	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 8 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 9 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	R	V	E	I	I	A	T	M	I	I	N	L	L
Pat 9 t = 816	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 11 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 11 t = 775	V	E	V	L	D	I	I	A	I	I	A	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 11 t = 945	V	E	V	L	D	I	I	A	I	I	A	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 12 t = 0	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L
Pat 12 t = 107	V	E	V	L	D	I	V	A	I	I	V	G	I	F	I	K	V	E	I	V	A	T	M	I	I	N	L	L

Table 3: Drug resistance mutations emerging as a result of NRTI exposure. *Mutations in red represent known drug resistance HIV-1 mutations and those in blue represent HIV-2 polymorphism that could affect drug susceptibility*

	Common Mutations in HIV-1 patients exposed to NRTIs															
	M41L	K65R	D67N	T69D	K70R	L74V	V75I	F77L	Y115F	F116Y	Q151M/L	M184V	L210W	T215Y/F	K219Q/E	G333D/E
HIV-1 WT	**M**	**K**	**D**	**T**	**K**	**L**	**V**	**F**	**Y**	**F**	**Q**	**M**	**L**	**T**	**K**	**G**
Pat 19 t = 0	M	K	D	T	K	L	V	F	Y	F	Q	M	L	T	K	G
Pat 12 t = 559	M	K	D	T	K	L	V	F	Y	F	Q	M	L	T	K	G
HIV-2 WT	**M**	**K**	**D**	**N**	**K**	**L**	**I**	**F**	**Y**	**F**	**Q**	**M**	**N**	**S**	**E**	**Q**
Pat 1 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 1 t = 55	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 2 t =0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 2 t = 130	M	K	D	N	K	L	I	F	Y	F	Q	I	N	S	E	Q
Pat 2 t = 255	M	K	D	N	K	L	I	F	Y	F	Q	V	N	S	E	Q
Pat 2 t = 310	M	K	D	N	K	L	I	F	Y	F	Q	V	N	S	E	Q
Pat 2 t = 395	M	K	D	N	K	L	I	F	Y	F	Q	V	N	S	E	Q
Pat 2 t = 511	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 5 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	C
Pat 6 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 6 t = 174	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 7 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 7 t = 336	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 7 t = 504	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 8 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 9 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 9 t = 816	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 11 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 11 t = 775	M	K	D	N	K	L	I	F	Y	F	Q	V	N	S	E	Q
Pat 11 t = 945	M	K	D	N	K	L	I	F	Y	F	Q	V	N	S	E	Q
Pat 12 t = 0	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q
Pat 12 t = 107	M	K	D	N	K	L	I	F	Y	F	Q	M	N	S	E	Q

Discussion

Currently approved ARV drugs were designed and optimised for treatment of HIV-1 subtype B infections. These drugs were expected to have a similar efficacy in HIV-2 patients as in HIV-1, due to the high structural and enzymatic function similarities between the protease and reverse transcriptase genes and proteins of these two viruses [3]. However, it was discovered that HIV-2 is naturally resistant to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and the entry inhibitor, T-20; and may have reduced susceptibility to some protease inhibitors [3, 12]. These resistance properties restrict treatment of HIV-2 and dually infected patients with HAART by limiting the drugs available for second and subsequent regimens. The use of a potent and effective first line regimen to which the virus is sufficiently sensitive in HIV-1 can result in full viral suppression for more than seven [22]. Our study reports durable and efficient suppression of both HIV-1 and HIV-2 in most of twenty treated patients. Even though previous studies have reported that various HAART regimens suppress HIV-2 viral replication by only 0.4 - 2 logs and failed to achieve undetectable viral load levels [10, 23]; viral suppression to undetectable levels was achieved in all, but one HIV-2 patient and was maintained for up to 3 years (range 3-36 months). Reduction in viral load was accompanied by a general increase in CD4 T cells. Viral load in Patient 1 dropped by 2 logs and then proceeded to increase to pre-therapy levels. It was later discovered that Patient 1 had stopped taking the drugs due to adverse effects associated with the PI (LPV/r). These findings indicate that the combination of Zidovudine, Lamivudine and Lopinavir/r is effective in achieving an efficient and durable viral suppression in HIV-2 and dually infected patients.

The presence of several HIV-1 PI mutations as natural polymorphisms in HIV-2 has resulted in reduced susceptibility to some protease inhibitors [3, 12]. A recent study on the activity of currently approved PIs against the HIV-2 protease has shown that Lopinavir, Saquinavir, Tipranavir, and Darunavir exhibit the highest potency in this order and that Atazanavir, Nelfinavir and Amprenavir show the lowest potency in this order [24]. Sequence analysis of the protease region during this study has revealed the natural presence of eight minor HIV-1 PI mutations, L10V, K20R, L33V, M36I, I62V, A71V, G73A and I93L and three major mutations, V32I, M46I, I47V , which may explain the reduced susceptibility of HIV-2 wild type viruses to several protease inhibitors [25].

There are relatively fewer on the selection of drug resistance mutations in patients taking a Ritonavir-boosted PI relative to un-boosted PIs. However, it has been reported that while the same mutations usually emerge in boosted and un-boosted PIs the relative frequency of mutations may differ [25], and that an accumulation of several mutations is often necessary to cause significant resistance to boosted PIs[25]. According to the International AIDS Society of USA (IAS-USA), an accumulation of 6 or more of the PI mutations listed in table 2 is associated with resistance to LPV/r in HIV-1 PI-experienced patients [26, 27], however resistance is rare in patients whose first PI is LPV/r, with some exceptions [25]. In HIV-1, LPV/r resistance is associated with the accumulation of specific resistance mutations in the HIV-1 protease gene (L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M) [27]. Even in patients whose first PI is LPV/r, a few specific mutations, most notably I47A and V32I, are associated with high-level resistance to LPV/r [28-30]. A recent study on the phenotypic susceptibility of HIV-2 to LPV/r has shown that the presence of the V47A resulted in substantial reduction in susceptibility to Lopinavir [20]. In our study, the protease sequences, after viral rebound, showed the V47A (Patient 2 and Patient 11), V331 (Patient 11) and F53V (Patient 2) mutations emerging as a result of LPV/r pressure in a background with the L10V, K20R, **V32I,** L33V, M36I, M46I, I62V, A71V, G73A and I93L mutations. Therefore, the observed virological failure in these patients could be partly attributed to high level drug resistance to LPV/r.

Analysis of the RT sequences in patients with viral rebound revealed first the M184I (Patient 2) and later the M184V (Patient 2 and Patient 11) mutations emerging due to drug pressure. These mutations are associated with 1000-fold phenotypic resistance to Lamivudine [19, 21]. In addition, the presence of M184V in a background with the V75I and K219Q/E mutations, as well as polymorphisms at positions known to harbour HIV-1 RT mutations, may result in high level resistance to both ZDV and 3TC. As previously observed, the accumulation of the six thymidine-analogue resistance mutations (TAMs), M41L, D67N, K70R, L210W, T215Y and K219Q/E [31] marking classic AZT resistance in HIV-1 were absent in the HIV-2 sequences [9-12, 14].

The presence of these high level protease and RT inhibitor resistance mutations in Patient 2 (after five months on HAART) and Patient 11, explains the observed virological failure. Although it has been reported that drug resistance is rare in patients taking LPV/r as part of the first line therapy [28-30], the presence of background mutations may facilitate the time to development of resistance in HIV-2 relative to HIV-1 [32], as observed in this

study. In HIV-1, the emergence of the LPV/r mutation I47A is a two-step process, going from I → V→ A, while in HIV-2 [20], it occurs in a single step from V→ A; making emergence of this mutation easier and faster in HIV-2. However, HIV-2 V47A mutants were found to retain susceptibility to other PIs and seemed to be hyper-susceptible to Atazanavir and Saquinavir [20]. This finding makes these drugs useful as second line PIs for our HIV-2 patients, especially Saquinavir which has been reported to have a potent activity against HIV-2 [24].

Sequence analysis from other patients with viral rebound did not indicate the presence of known drug resistance mutations. However, the viral load in subsequent samples returned to undetectable levels, indicating that the rebound was in fact only a viral blip or a consequence of poor adherence.

We report the response of twenty HIV-2 and dually infected patients to a HAART regimen and the finding that the combination of Zidovudine, Lamivudine and Lopinavir/r is effective in achieving an efficient and durable viral suppression in both HIV-2 and dually infected patients. After more than three years, only three patients need second line therapy, one due to adverse reactions to LPV/r and the rest due to the emergence of drug resistance mutations. New potential drug resistance mutations, not previously observed in HIV-1, were observed in this study. These mutations could be primary determinants of drug resistance in HIV-2 and so there is a need to develop phenotypic resistance assays for HIV-2 to determine their clinical relevance. In conclusion, HIV-2 response to HAART can be similar to HIV-1 response providing efficient and durable viral suppression for up to 3 years, if an optimal drug regimen is chosen; further highlighting the importance of choosing protease and RT inhibitors that are active against HIV-2.

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Chapter 10

Presence of a Multi-drug Resistance Mutation in a Treatment-Naïve HIV-2 Individual in Caio, Guinea Bissau

Presence of a multi-drug resistance mutation in a treatment-naïve HIV-2 individual in Caio, Guinea Bissau

Sabelle Jallow[1,2], Aleksandra Leligdowicz[1,3], Thushan De Silva [1], Abraham Alabi[1], Ramu Sarge-Njie[1], Tim Vincent[1,4], Peter Aaby[4], Tumani Corrah[1], Hilton Whittle[1], Matthew Cotton[1], Guido Vanham[2,5], Sarah Rowland-Jones[1,3] and Wouter Janssens[2]

[1] *Medical Research Council Laboratories, Fajara, The Gambia*
[2] *Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*
[3]*Weatherall Institute of Molecular Medicine, Medical Research Council Human Immunology Unit, John Radcliffe Hospital, Oxford, United Kingdom*
[4]*Projecto Saude Bandim, Bissau, Republic of Guinea-Bissau.*
[5]*Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*

To be submitted

Abstract

Background: The use of HAART in the developed world has resulted in dramatic decreases in morbidity and mortality, slowing down disease progression and prolonging survival; however, emergence of drug resistant strains, may limit both the magnitude and the duration of the response to treatment. High occurrences of transmitted drug resistant virus in primary HIV-1 infections strongly argue for the use of resistance testing in treatment-naïve individuals before the initiation of therapy**.**

Design: We screened 23 individuals for the presence of drug-resistance mutations, selected randomly from a cohort of HIV-2 infected individuals about to initiate HAART.

Methods: Twenty-three HIV-2 infected individuals from a cohort in Caio, Guinea Bissau were randomly selected for genotypic resistance studies. The entire HIV-2 protease and reverse transcriptase genes were amplified from plasma RNA, sequenced and analysed for resistance-associated mutations.

Results: The Q151M mutation, associated with phenotypic resistance to zidovudine, didanosine, zalcitabine, abacavir and stavudine in HIV-2, was detected in three sequential samples from one individual.

Conclusions: We report the possible transmission of a drug resistance in HIV-2. Although unauthorised use of ARVs cannot be completely ruled out, this finding, in a drug-naïve population, presents an important public health issue that would need to be addressed, to provide effective HIV-2 treatment in this population.

Introduction

Drug resistance is the main problem in achieving an efficient and durable suppression of viral replication. Emergence of drug-resistance is associated with: suboptimal therapy due to non-adherence to toxic and complex regimens; initiation of therapy late in the course of infection; pre-existence of drug-resistant variants within the quasispecies and the transmission of resistant variants at the time of the infection [1]. Transmission of drug resistance has been reported in several developed countries with prevalences ranging from 10-30% of primary HIV-1 infections [1-4]. High occurrences of transmitted drug resistant virus in the United States (US) have led to new guidelines that propose the use of resistance testing in treatment-naïve individuals before the initiation of therapy [5].

Increasing access of antiretrovirals (ARVs) in Africa has made the study of drug resistance in HIV-2 a priority, especially in West Africa. ARVs have not been available officially in Guinea Bissau until 2008. In preparation of the initiation of therapy, 23 patients were randomly screened for the presence of drug resistance mutations, so as to maximise the efficiency of the first line regime. We report the presence of a multidrug resistance mutation in a treatment-naïve HIV-2 infected individual, in Caio, Guinea Bissau.

Materials and Methods

Patients: Caio Cohort

The Caio Cohort was initiated in 1988 to study the epidemiology of HIV-2. Caio is an isolated rural community in the northwestern part of Guinea Bissau. This area is very poor, without running water and electricity. A sero-survey of the adult population was done between 1989 and 1992 to determine HIV status. A second survey was done between 1996 and 1998 of the same adults in this region. Each year, the births, deaths, immigration into and emigration out off Caio were recorded. From the questionnaires, it was established that many of the male villagers mostly live and work in larger towns in the sub-region or in Portugal or France and a substantial number of the women work or have worked as commercial sex workers in neighbouring countries. Two more re-surveys of this population were done in 2003 and 2006. Between each survey, HIV-infected individuals were followed up more regularly and had free access to the clinic. All study participants in the cohort were antiretroviral therapy (ART) naïve and gave informed

consent. HIV results were made available upon request; although the majority of subjects did not seek to know their results.

The Joint Gambian government – Medical Research Council Ethics Committee, and the National AIDS Control Programme Committee of Guinea Bissau approved this study.

CD4 T-cell count and viral load

CD4 T-cell measurements were done using Flow cytometry (FACScan by Becton Dickinson). Plasma HIV-2 RNA copies/ml (viral load) was quantified using an in-house viral load assay [6], which had a limit of detection of 100 RNA copies/ml plasma. The assay was modified to include an internal molecular control for extraction and amplification efficiency [7].

Genotyping

HIV-2 RNA was extracted from plasma from 140□l of EDTA plasma using the QIAamp viral RNA kit (QIAGEN, Venlo, The Netherlands) eluted into 50□l nuclease-free water. 3□l of RNA was reverse transcribed and amplified to produce the entire HIV-2 protease and RT genes. Purified PCR products were directly sequenced on both strands and analysed with DNAstar software (Lasergene, Wisconsin, U.S.A). Sequences generated were assigned the accession numbers AM408175 to AM408208. Patients were analysed by two genotypic methods, sequencing and the Oligonucleotide Ligation Assay (OLA). OLA is a hybridization-based point mutations assay that can distinguish wild-type from mutant sequence using differentially-labelled oligonucleotide probes [8, 9]. HIV-2 OLA for the Q151M and M184V mutation has been previously described [8].

Results

Phylogenetic analysis showed HIV-2 viruses from all patients to be subtype A. Of the 23 patients genotyped, 22 had wild-type HIV-2. As previously observed with other HIV-2 wild type viruses, some HIV-1 drug resistance mutations in the protease and reverse transcriptase (RT) were found as natural polymorphisms in these HIV-2 viruses [10-14]. In one patient, a multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance mutation, Q151M was discovered. Two subsequent samples from this patient were also genotyped, and the Q151M mutation was still present. The sequencing and OLA results were concordant.

Case Report

The index patient is a 68 year old woman from Caio, Guinea Bissau. During the first sero-survey in 1989, the patient tested negative for both HIV-1 and HIV-2. During the second survey in 1997, the patient tested positive for HIV-2. In the 2003 survey, the index patient was still infected with only HIV-2, had a viral load of 37,427copies/ml and a CD4T-cell count of 123cells/ml (11%). The last survey, in 2006, showed that the patient was still singly infected with HIV-2 with a viral load of 25,836copies/ml and a CD4T-cell count of 130cells/ml (9%). Clinical examination in 2006 showed that the patient had evidence of HIV disease, presenting with lymphadenopathy (axillary, mandibular, inguinal), but she had never had TB, Herpes Zoster or oesophageal candidiasis. During two subsequent follow ups in 2006-2007 and 2007, the index patient refused to be bled.

During each survey, the cohort members go through 2 questionnaires, one with a field worker to determine clinical history and one with a physician during a clinical examination. At both stages, past or present use of ARV was inquired about. Also, queries with respect to general drug usage were made: cohort members were asked if they were on any long-term treatment, what the drugs were and where they came from, if they were from relatives in Europe or other big cities. If a patient did not know the purpose of the drugs they were using, a field worker would be sent to their home to record what the drugs were. During these surveys, no ARV usage was recorded and the replies did not suggest ARV therapy among any of the HIV-positive patients.

The index patient has been followed up since 1989, but has never requested for her HIV results. During the 2007 clinical examination, the physician and field worker noted that the patient was unaware of her HIV status.

All three samples (1997, 2003, 2006) available for this patient have been genotyped. Both samples revealed the presence of the Q151M mutation, associated with phenotypic resistance to the nucleoside reverse transcription inhibitors (NRTIs), Zidovudine (ZDV), Didanosine (ddI), Zalcitabine (ddC), Abacavir (ABC) and Stavudine (d4T) [19].

The questionnaires and the fact that the patient does not seem to know her HIV status indicates that the patient has never been treated and thereby suggests that the index patient might have been infected with a drug resistant HIV-2 strain.

Discussion

In HIV-2, limited treatment options due to natural resistance, has made choosing a sufficiently potent and durable first line regimen even more important. Widespread use of ARVs for the treatment of HIV-1 has led to an increase in the transmission of drug resistant viruses. Consequently, it is important to screen treatment-naïve patients starting therapy for the first time.

Although time of seroconversion cannot be determined in this patient, several factors indicate that the presence of the Q151M mutation is most likely due to transmission of drug resistance and not due to prior drug exposure.

Drug resistance mutations can occur either by natural evolution or be selected in the presence of drug pressure [15]. However, primary mutations, which cause high-level resistance, are not expected to occur naturally in treatment-naïve individuals, because acquisition of primary mutations is associated with a significant loss in fitness in the absence of drug pressure [16, 17] Therefore, the presence of primary mutations in untreated patients is believed to indicate transmission of drug-resistant HIV, while the presence of secondary mutations in treatment-naïve patients is associated with natural polymorphisms [18]. The Q151M has not been observed as a natural polymorphism in any of the known HIV viruses; its presence has been associated solely with NRTI treatment.

The Q151M mutation is a primary drug resistance mutation associated with multi-NRTI resistance. It is very rare in HIV-1, but has been reported to be more frequent in HIV-2, where it causes low-level phenotypic resistance to ZDV, ddI, and ddC, ABC and d4T [10, 15]. Unlike most mutants, viruses with the Q151M mutation are more fit than the wild-type virus in the absence of drugs [17]. The improved fitness of Q151M mutants may explain the apparent persistence and stability of this mutation in our patient. Also, it has been demonstrated that unlike the setting of treatment interruption, transmitted drug-resistant viruses can persist for more than 9 months. During transmission of drug resistance, these resistant variants represent the majority of the quasispecies and in most cases wild-type virus will not have been transmitted. In this case, there will be no latent or archived wild-type viruses to later out-compete the transmitted resistant viruses [16, 19].

The fact that antiretrovirals have not been widely used in West Africa, and were not available in Guinea Bissau until 2007, as well as the fact that the patient was unaware of her HIV status, seemed to indicate that the patient has not been on ARVs. In addition, initial demographic studies in Caio suggest that an elderly woman is not likely to travel out of Caio; and questionnaires to gather information about ARV usage suggested that the patient did not have prior ARV experience. Furthermore, the remoteness and lack of most basic necessities in Caio argue against possible ARV usage. However, prior ARV use cannot be completely ruled out, since individual and unauthorised ARV use does occur frequently in some African countries. In either case, the presence of a multi-NRTI resistance mutation in a patient initiating therapy, whether from prior drug exposure or from transmission of drug resistance, is of major public health concern. This finding in West African, where there is a large population of HIV-2 infected individuals with fewer treatment options, provides strong support for the routine use of genotypic resistance tests in new patients starting therapy to optimise the first line regime and allow for a more effective treatment.

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Chapter 11

OPINION PIECE: A Call for Randomized Controlled Trials of Antiretroviral Therapy for HIV-2 Infection in West Africa

OPINION PIECE: A Call for Randomized Controlled Trials of Antiretroviral Therapy for HIV-2 Infection in West Africa.

Geoffrey S. Gottlieb[1], Serge-Paul Eholié[2], John N. Nkengasong[3], **Sabelle Jallow[4,6],** Sarah Rowland-Jones[4], Hilton C. Whittle[4] and Papa Salif Sow[5]

[1]Department of Medicine-Allergy & Infectious Diseases, University of Washington, Seattle, Washington, USA;
[2]Service des Maladies Infectieuses et Tropicales, Centre Hospitalier Universitaire de Treichville, Abidjan, Côte d'Ivoire,
[3]Centers for Disease Control and Prevention, Atlanta, Georgia, USA,
[4]Medical Research Council Laboratories, Fajara, The Gambia,
[5]Clinique des Maladies Infectieuses Ibrahima Diop Mar - CHU de Fann, Universite Cheikh Anta Diop de Dakar, Senegal.
[6]Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium.

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HIV-2 is endemic in West Africa, but unlike HIV-1 has had limited spread to other other locales [1]. HIV-1 has emerged in West Africa more recently and because HIV-1 and HIV-2 co-circulate in this region, some individuals become dually infected with both viruses [2, 3]. Compared to HIV-1 infection, HIV-2 is characterized by a much longer asymptomatic stage, lower plasma viral loads, slower decline in CD4 count, decreased mortality rate due to AIDS, lower rates of mother to child transmission, genital tract shedding, and sexual transmission [1, 4-12]. Nonetheless, a significant proportion of HIV-2 infected individuals eventually progress to AIDS [6, 13-15]. The clinical consequences of dual infection with both HIV-1 and HIV-2 need to be more fully understood, but current data suggest the majority of such patients also eventually progress to AIDS [2, 16-20].

Antiretroviral therapy (ART) is becoming increasingly available in West Africa where HIV-2 infects up to 1-2 million people. As ART "scale-up" programs proliferate in West Africa, significant numbers of HIV-2 and dually infected individuals will have access to and will be treated with antiretrovirals (ARV) developed against HIV-1 [22-25]. However, HIV-2 is intrinsically resistant to the non-nucleoside reverse transcriptase inhibitors (NNRTI) and T-20 (enfurvirtide) and reports suggest that HIV-2 may be partially resistant to some protease inhibitors (PI) (e.g. Amprenavir, atazanavir and nelfinavir) and have a low genetic barrier to nucleoside reverse transcriptase inhibitors (NRTI) resistance [26-38, 62]. In addition, at least one recent report from Burkina Faso suggested the NRTI mutations M184V and Q151M may rarely be found in ARV-naïve individuals . The presence of HIV-2 in dually infected patients complicates treatment; requiring drugs that are active against both HIV-1 and HIV-2. Clinical trials in dually infected patients will allow identification of drugs that have the best activity in these patients, so as to provide efficient and durable viral suppression.

Several observational cohort studies in developed countries have shown variable but generally poor outcomes of ART for HIV-2 infection [15, 40-48] with similar results reported from the few small cohort studies from resource limited settings in Senegal, The Gambia and Cote d'Ivoire, West Africa [24, 25, 49].

Recently, there has been an effort in Europe to develop an international consortium (ACHIeV$_2$E (A Collaboration on HIV-2 Infection)) of HIV-2 cohorts, to better study HIV-2 infection and standardize its management, including HIV-2 viral load testing, and

treatment . These efforts should ultimately lead to better care of HIV-2 infected individuals in the developed world and West Africa.

To date there has not been a single randomized clinical trial of ART for HIV-2 infection. This is despite greater than 20 years since the discovery of HIV-2 and advent of AZT, greater than 10 years since the landmark studies showed the benefit of HAART for HIV-1 [52-54], and currently 6 different classes of ARV available for HIV-1 (NRTI, NNRTI, PI, Fusion Inhibitors, CCR5-coreceptor blockers, and integrase inhibitors) of which 4 show *in vitro* and/or *in vivo* activity against HIV-2 [37, 38, 55-57]. The WHO currently recommends for first line therapy of HIV-2 infected patients, 2 NRTI and a protease inhibitor boosted by ritonavir ; they make no explicit recommendations for dually infected patients or for second line HIV-2 (or dual) infection regimens and little such data are available. In resource limited West Africa biological monitoring (CD4 counts) is limited and HIV-2 viral load and genotypic or phenotypic resistance testing are not commercially available, standardized nor routinely obtainable; thus assessing regimen failure is often made on clinical criteria and data suggest that CD4 count recovery with ART in HIV-2 infection is often poor [47, 48] . Consequently, an assessment of the relative efficacy of potential first line and salvage regimens including the newer generation of protease inhibitors, integrase inhibitors and CCR5 co-receptor blockers among HIV-2 (and dually) infected patients is needed. A key issue in West Africa where HIV and TB are common is to find the ART regimens for HIV-2 that can be given with concurrent treatment for TB, given interactions between protease inhibitors and rifampicin . Finally, albeit rare, proven regimens to prevent mother to child transmission of HIV-2 (or dual infection) are needed .

We believe the 1-2 million HIV-2 infected people and communities in West Africa most affected by HIV-2 (and dual infection) deserve the same evidence-based medicine and efforts that those with HIV-1 have justly demanded . Rigorous, well designed randomized controlled trials, that demonstrate which ARV regimens are effective in treating HIV-2 infected people, are urgently needed to guide patients and the clinicians who care for them. The outcome of such trials will also be beneficial in treating patients dually infected with HIV-1 and HIV-2, although ultimately trials of ART in these patients are also needed. However, the apparent decline of HIV-2 in some West African locales [3, 60, 61] highlights the importance of involving multiple West African countries to establish large multi-center collaborative studies and trials. We believe the best place to carry out such trials is in West Africa, the locale where HIV-2 is endemic and effective

treatment is most likely to serve the local population. We, therefore call on the wider AIDS community to support efforts for evidenced based treatment of HIV-2 infection.

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Chapter 12

General Discussion and Future Perspectives

DISCUSSION AND FUTURE PERSPECTIVES

HIV-2 is endemic in West Africa with an estimated one million infections . In this region co-circulation with HIV-1 has resulted in individuals infected with both HIV-1 and HIV-2 [1]. While several studies have shown that ART in HIV-1 infected individuals usually results in significant reduction in viral load [2, 3], there have been very limited studies done in HIV-2 [3] and to my knowledge no studies have been done in dually infected individuals. We have studied the response of HIV-2 and dually infected individuals to ART. In chapter 7, study of eight HIV-2 infected patients treated with ZDV and 3TC (dual therapy) revealed an overall poor response relative to that observed in HIV-1 patients treated with ZDV and 3TC [2]. Undetectable viral load was observed in only 3 patients; while a drop of only 0.5–2 logs was observed in the rest of the patients. While these results agreed with previous findings that various HAART regimens usually fail to reduce viral load to undetectable levels in HIV-2 infected patients [2, 4] or that only minority of patients (1/3) achieve undetectable viral loads [5], our finding in chapter 8 on patients treated with HAART revealed viral suppression to undetectable levels in all patients, except one patient who had stopped taking the drugs due to adverse effects. In 19 out of these 20 HIV-2 and dually infected patients treated with Zidovudine (ZDV), Lamivudine (3TC) and Ritonavir-boosted Lopinavir (LPV/r), an efficient and durable suppression of viral replication was achieved for up to 3 years; accompanied by a general increase in CD4 T-cell count. This indicates that choosing an optimal regimen is crucial in achieving treatment success.

Recently the availability of cheaper generic ARVs, as well as efforts from several international organizations like the Global Fund and WHO/UNAIDS “3 by 5” initiatives to scale up ARV access in Africa have resulted in about 2 million HIV-infected individuals in sub-Saharan Africa being treated with HAART by the end of June 2006 [6, 7]. However, treatment of HIV comes with the challenges of monitoring treatment and the emergence of drug resistance; especially in developing where limited resources, inadequate infrastructure and technical expertise make the use of advanced monitoring techniques very difficult. Current treatment strategies used in sub-Saharan Africa are based on WHO recommendations to switch to a second-line regimen when clinical or immunological failure occurs [8]. However, since ARVs suppress viral replication, virological tests such as viral load and resistance testing are the best indicators of treatment success and not clinical response. When treatment failure occurs, viral load will increase, followed by reduced or loss of immune control and finally clinical failure

occurs. The time between treatment and clinical failure would have allowed the accumulation of additional mutations that could cause cross resistance to other drugs within the same class and thereby limit the number of drugs that can be used in a second line regimen. The development of alternative monitoring assays that are cheap and easy to use, without the need for expensive equipment and technical expertise have become a priority in resource-poor settings.

Drug resistance is monitored by genotypic and phenotypic resistance assays. The first step in the development of a genotypic and phenotypic assay for HIV-2 is the development of an RT-PCR to amplify the HIV-2 protease (PR) and reverse transcriptase (RT) regions, which are targeted by a majority of current ARVs. In chapter 3, we developed a genotypic resistance assay for the amplification and sequencing of the PR and RT gene fragment of HIV-2 with a sensitivity of 91.3%. This assay is cost effective as it allows amplification of both the PR and RT in a single PCR reaction instead of two separate reactions for each region. This assay was subsequently used to decipher genotypic resistance patterns in treated HIV-2 and dually infected patients in the Gambia. In patients treated with only ZDV and 3TC, analysis of the RT region revealed four major HIV-1 NRTI mutations, the K65R, Q151M, M184 and S215Y and the presence of two other mutations V75I, associated with multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance in HIV-1, and K219Q/E, associated with ZDV resistance in HIV-1 as natural polymorphisms. In addition, several new mutations including K20R, K40R, T53S, A62V, V108G, V111I, I118V, P150Q, I179T, V201A/M, F214L, K223R/E, V263I, K277R, Q333L, I341V, K346R, V356I, V371I and N403S, appeared to emerge as a result of drug pressure. The RT gene of patients treated with HAART revealed only the M184I/V mutation emerging as a result of 3TC pressure. During our study, we noticed an absence of the thymidine-analogue resistance mutations (TAMs) observed during ZDV resistance in HIV-1 [9]; indicating that HIV-2 might have a different pathway towards ZDV resistance. A study has shown that while ZDV resistance in HIV-1 occurs mainly via the TAM pathway, HIV-2 utilises the Q151M mutational pathway [10]. TAM resistance occurs by nucleotide excision from the nascent nucleotide chain. However, the ATP binding pocket of the HIV-2 RT is shallower and is therefore less able to bind ATP appropriately for the excision reaction [9]; thus making the Q151M pathway more efficient for ZDV resistance in HIV-2 .

Analysis of the PR sequence revealed 13 major and minor protease inhibitor (PI) mutations, L10V, K20R, V32I**,** L33V, M36I, M46I, I47V, I62V, A71V, G73A and I93L,

as natural polymorphisms in our patients. HIV-1 drug resistance mutations occurring in HIV-2 as natural polymorphisms have been associated with natural drug resistance to NNRTIs and the entry inhibitor T-20 [11-13] as well as facilitating PI resistance in HIV-2 by accelerating the time to resistance [14]. In a patient on HAART, sequence analysis when the virus rebounded, revealed the PI mutation, V47A associated with high level resistance to LPV/r in HIV-2. In HIV-1, the I47A mutation occurs in a two-step process, going from I → V→ A, while in HIV-2, it occurs in a single step from V→ A; making the emergence of this mutation easier and faster in HIV-2 [15]. A phenotypic susceptibility study in HIV-2 has shown that V47A mutants retained susceptibility to other PIs and seemed to be hyper-susceptible to Atazanavir and Saquinavir [15]. This finding makes these drugs useful as second line PIs for our HIV-2 patients, especially Saquinavir which has been reported to have a potent activity against HIV-2 [16].

Although consensus sequencing is currently the gold standard for genotyping, increasing use of genotypic assays in both clinical and research settings has resulted in the development of cheaper alternatives, such the oligonucleotide ligation assay (OLA) [14]. OLA has several advantages that make it a better alternative for use in resource–poor settings; it is relatively cheaper and rapid to perform; has high throughput, which makes it ideal for epidemiological studies and clinical trials; and it is so sensitive that it can detect minor variants representing as little as 5% of the total viral population [17-19]. The main disadvantage of this assay is that the presence of sequence diversity around the ligation site results in assay failure, because the probes will not anneal to the target sequence [17-19]. This assay has already been developed for HIV-1 subtype B; but when we evaluated it for HIV-1 non-B subtypes, sensitivity was not optimal for the PR probe at position 90 and the RT probes at positions 151 and 184. Therefore this assay was further optimised, resulting in a sensitivity of 96.1% (chapter 5). In addition we developed and optimized the OLA to detect the Q151M and M184V mutations in HIV-2 infected individuals (chapter 4); and achieved an overall sensitivity of 98.8% with a high concordance of more than 97.5% with consensus sequencing. To date only these two mutations, Q151M and M184V, have been shown to result in phenotypic resistance against NRTIs in HIV-2. However, the versatility of this assay means that it can be easily modified to include new HIV-2 mutations with phenotypic resistance against NRTIs or PIs. Availability of both the HIV-1 non-B and the HIV-2 OLA will allow detection of drug resistance mutations in all our patients, including the dually infected patients.

After successful development of the HIV-2 OLA, we used it to screen treatment-naïve patients before the initiation of therapy, in chapter 9, and found a multidrug resistance mutation, Q151M, in one patient from rural Guinea Bissau. Most indications suggested that the patient might have been infected with a drug resistant strain of HIV-2. This finding argues for the screening for transmitted drug resistance in treatment-naïve HIV-2 infected patients so as to maximise the efficiency of the first line regimen.

Phenotypic resistance assays are crucial in determining the *in vitro* growth characteristics of mutant viruses; the level of drug resistance; and cross resistance to other drugs within the same class. There have been only a couple of studies on phenotypic resistance in HIV-2 to NRTIs, both of which have utilized PBMC-based assays [3, 20]. The use of recombinant viruses to determine phenotypic drug resistance has resulted in vast knowledge of resistance patterns and the relevance of individual mutations in HIV-1 [21-23]. In chapter 10, we designed a recombinant virus assay to evaluate phenotypic drug resistance in HIV-2. We generated mutant viruses from the list of genotypic mutations observed in the patients treated in chapters 7 and 8. These recombinant viruses will allow determination of phenotypic resistance to these mutations and the relative fitness of these mutant viruses relative to the wild-type. These data will contribute towards the study of HIV-2 drug resistance, salvage therapy and therefore lead to a better management of HIV-2 infection.

Although the ART scale up has resulted in a substantial number of treated patients in Africa, only a few drugs are available, mainly the NRTIs (ZDV, 3TC, d4T, ddI & TDF), the NNRTIs (NVP & EFV) and mostly only one of the PIs, Lopinavir/r. Even though the WHO recommends change of all components of the regimen in case of failure [8], a second line consisting of new drugs is not always possible; especially for HIV-2 whose first line regimen includes a PI. We therefore end this study with a call for randomized clinical trials in HIV-2 infected individuals to determine the best first line regimen that will allow maximum suppression for very long periods, as has been achieved with HIV-1 [24]. In additional, these trials will allow identification of second and subsequent regimens that can suppress the mutated virus after first line therapy. Studies comparing HIV-1 and HIV-2 disease progression have shown that when AIDS develops, the CD4 T-cell count in HIV-2 infected patients is usually more than 200 cells/ml and higher than that observed in HIV-1 infected patients at the same stage of disease [24, 25]. Therefore HIV-2 patients with CD4 T-cell of 200cells/ml will have been infected for longer periods than their corresponding HIV-1 counterparts. Initiation of HIV-2 therapy at a CD4 T-cell

200 cells/ml might thus be too late and might have contributed to the poorer overall treatment response observed in HIV-2. Instead of relying on HIV-1 guidelines, randomized clinical trials for HIV-2 patients will generate results that would lead to new guidelines that are more appropriate for HIV-2; thereby resulting in a better management and treatment of HIV-2 infected individuals.

CONCLUSION

Our findings have indicated that treatment of HIV-2 infected individuals with can result in efficient and durable suppression of viral replication to undetectable levels; providing that a maximally suppressive therapy is used. During treatment failure, mutations emerge in the HIV-2 protease and reverse transcriptase genome, at positions both similar and dissimilar to that observed in HIV-1. In addition to these potential drug resistance mutations, several HIV-1 drug resistance mutations occur as natural polymorphisms in HIV-2 and therefore contribute to the reduced susceptibility of HIV-2 to some antiretrovirals, especially the protease inhibitors. These studies have also resulted in the identification of drugs that will allow suppression of mutant viruses in a second line regimen. Therefore, our studies have contributed to the knowledge of HIV-2 response to therapy, the understanding of resistance patterns and the value of suppressive therapy. We conclude this study with a call for randomized clinical trials in HIV-2 to generate a solid basis to guide treatment of HIV-2 infected individuals.

FUTURE PERSPECTIVES

Scale up programs to increase access to antiretrovirals to all those who need them in Africa, will require a huge effort. Treatment of HIV-2 is often difficult with limited treatment options, due to natural resistance or reduced susceptibility to currently available drugs. Therefore, more studies should be done on both HIV-2 and dually infected individuals, to determine optimal treatment combinations, discern resistance patterns, cross resistance and hypersensitivity issues to allow better management of treated patients. We will continue following up treated patients in the Gambia by studying virological, immunological and clinical parameters of these patients. Results from these patients can guide treatment of subsequent patients in the surrounding countries, most hit by HIV-2, as well as globally.

Several genotypic resistance mutations have been identified in the HIV-2 reverse transcriptase. We have generated 20 mutant viruses, which we will use in growth competition assays with wild-type HIV-2 to determine if these mutations cause resistance, to classify the drug resistance mutations as primary or accessory mutations and to determine the level of resistance. In addition, we can generate mutations in the wild-type HIV-2 to produce the sequence found in HIV-1 at drug resistance sites to determine the clinical relevance of the natural polymorphisms found in HIV-2.

Once we have defined drug resistance mutations by a phenotypic assay, the information can be used to update current algorithms to allow correct interpretation of HIV-2 drug resistance based on genotypic resistance data. In addition, the discovered primary RT mutations will be incorporated into the HIV-2 oligonucleotide ligation assay. This assay will also be extended to include major PI mutations; to provide an assay for all important HIV-2 mutations in the PR and RT regions. As more patients are treated in Africa, the incidence of transmitted drug resistance will increase as has been observed in developed countries. Therefore, the HIV-1 non-B and the HIV-2 oligonucleotide ligation assays will be useful in screening patients before therapy to ensure that the drugs used in the first line regimen are all active as well as for monitoring treated patients for the emergence of known drug resistance mutations.

We will participate in future randomised clinical trials in West Africa to determine optimal regimens, identify drugs that are active in vivo and identify second and salvage regimens for HIV-2 infected individuals; as well as the best time to initiate therapy.

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Curriculum Vitae

Sabelle Jallow

Personal Information

Age: 34 **Nationality:** Gambian **Marital Status:** Single

Objective

To establish a career in medical research, more specifically in the research of human infectious diseases, their treatment and prevention.

Education

May 2004 – present **Joint PhD project** Belgium/ The Gambia

Universiteit Antwerpen (UA)
Institute of Tropical Medicine (Antwerp, Belgium)
Medical Research Council (The Gambia)

PhD (September 2009)

- **Development and application of assays for monitoring drug resistance in HIV-2 infected patients on antiretroviral therapy**

Sept 2001– 2003 Interuniversity program Molecular Biology Belgium

Vrije Universiteit Brussels (VUB)
Katholic Universiteit Leuven (KULeuven);
Universiteit Antwerpen (UA)

Master of Science in Molecular Biology (Great Distinction)

- Thesis: "**Contributions to the development of Human Immunodeficiency Virus Type 1 drug resistance monitoring assays; taking into account genetic variability."** Institute of Tropical Medicine, Antwerp, Belgium
- President Interuniversity Program Molecular Biology (IPMB) class of 2001-3

Sept '93 – Oct '96 **Mount St. Vincent's University** Halifax, NS, Canada

Bachelor of Science in Chemistry (Distinction)

- Major in Chemistry, Minor in Biology and Mathematics.
- Dean's List (ave >80% with no subject below 70%).
- Executive Member: Science Club and International Student Club

Professional Experience

Feb 2004 – Present ***MRC Laboratories*** Fajara, The Gambia

Higher Scientific Officer

- Principle investigator of the HIV drug resistance study at MRC
- Development, assessment and implementation of new assays that measure viral resistance to anti-retroviral drugs
- Research into innate resistance as a result of natural polymorphisms
- Analysis and Data Management of all genotyping Results.
- Development of a HIV-2 sequence and drug-resistance mutation database

Sept '99 – Sept 2001 ***MRC Laboratories*** Fajara, The Gambia

Scientific Officer

- Research into MHC associations with diseases of interest (HIV, Malaria, TB , Measles..)
- Management of HLA Typing Laboratory
- Analysis and Data Management of all DNA Haplotyping Results.

Feb '99 – Sept '99 **Depart. of State for Health** Banjul, The Gambia

Laboratory Technician

- Making medications: eye drops, chloroquin syrup...
- Identifying illegal drugs by chemical analysis for the National Drug Squad.

Publications

OPINION PIECE: A Call for Randomized Controlled Trials of Antiretroviral Therapy for HIV-2 Infection in West Africa. Geoffrey S. Gottlieb, Serge-Paul Eholié, John N. Nkengasong**,** *Sabelle Jallow***,** Sarah Rowland-Jones, Hilton C. Whittle and Papa Salif Sow. *Accepted: AIDS.* June 2008

Optimization of the Oligonucleotide Ligation Assay (OLA), a Rapid and Inexpensive Test for Detection of HIV-1 Drug-Resistance Mutations, for Non-B Subtypes. Ingrid A. Beck, Claudia Crowell, Robin Kittoe, Helba Bredell, Molefe Machaba, Carolyn Willamson, Wouter Janssens, *Sabelle Jallow,* Guido van der Groen, Yiming Shao Mini Jacob, NM Samuel, Ivette Lorenzana de Rivera, Nicole Ngo-Giang-Huong, Sharon Cassol, George Alemnji, and Lisa M. Frenkel. *Journal of AIDS*; Aug 2008; 48(4):418-427

Development and evaluation of an Oligonucleotide Ligation Assay for the detection of drug resistance associated mutations in the HIV-2 *pol* gene. **Jallow S**; Kaye S; Schutten M; Brandin E; Albert J; McConkey S.J; Corrah T; Whittle H; Vanham G; Rowland-Jones S; Janssens W. *J. Clin. Microbiol*; May 2007. 45 (5): 1565-1571.

Virological and immunological response to Combivir and emergence of drug resistance mutations in a cohort of HIV-2 patients in The Gambia. **Jallow S**; Kaye S; Alabi A; Aveika A; Sarge-Njie R; Sabally S; Corrah T; Whittle H; Vanham G; Rowland-Jones S; Janssens W; McConkey S.J. *AIDS;* June 26, 2006. 20(10):1455-1458.

Vigorous but Short-Term Gamma Interferon T-Cell Responses against a Dominant HLA-A*2 –Restricted Measles Virus Epitope in Patients with Measles. Jaye A, Herbert CA, **Jallow S**, Atabani S, Klein MR, Hoogerhout P, Kidd M, Van Els CACM, Whittle HC. *J of Virology* 2003; 77:5014-5016.

Conferences

Oral presentation - **HIV-2 Therapy and Resistance: The Gambian experience**. S Jallow, Alabi A; Petterson K; Togun T; Aveika A; Whittle H; Vanham G; Rowland-Jones S and Janssens W. *UK-Ghana Royal Society HIV workshop;* April 28-30, 2008; London, Great Britain.

Poster presentation - **Development of the Oligonucleotide Ligation Assay for the Detection of the Q151M Mutation Associated with Multi-Drug Resistance to the Nucleoside Reverse Transcriptase Inhibitors in HIV-2.** S Jallow, Kaye S, M Schutten, Brandin E, Albert J, Vanham G, Whittle H, Rowland-Jones S, Janssens W. *5th European HIV Drug Resistance Workshop,* March 28 – 30, 2007; Cascais, Portugal.

Poster 40 - **Virological Response to Highly Active Antiretroviral Therapy (HAART) and the Emergence of Drug-Resistant Mutations in HIV-2 and HIV-1/HIV-2 Dually Infected Patients in The Gambia.** Jallow S; Alabi A; Sarge-Njie R; Petterson K; Akolo C; Aveika A; Whittle H; Vanham G; Rowland-Jones S and Janssens W. *7th Annual Symposium on Antiviral Drug Resistance: Targets and Mechanism*. November 12-15, 2006; Chantilly, Virginia, USA.

Poster Presentation 2.7 - **Development of the Oligonucleotide Ligation Assay for the Detection of the M184V Mutation Associated with Resistance to Lamivudine in HIV-2.** S Jallow, S Kaye, M Schutten, S Rowland-Jones, W Janssens*. International Symposium on HIV & Emerging Infectious Diseases*. Jun 21-23, 2006; Toulon France.

Oral presentation - **Anti-Retroviral Therapy (ART) Monitoring and the Development of the Oligonucleotide Ligation Assay for Detecting Critical Drug-resistant Mutations in HIV-2 Patients in Preparation of the Global Fund Initiative in The Gambia**. S Jallow, S Kaye, A Alabi, A Aveika, S McConkey, S Rowland-Jones, W Janssens. *Retrovirology* 2005, 2 (Suppl 1):S134 (8 Dec. 2005) [2005 *International Meeting of The Institute of Human Virology;* 29 August - 2 September 2005; Baltimore, USA.]

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- Travel grant: *The Institute of Human Virology meeting (2005 & 2006)*
- NCI-NIH Travel grant: *7th Annual Symposium on Antiviral Drug Resistance: Targets and Mechanism* (2006)
- ITM-DGOS PhD studentship (Belgium, 2004-2008)
- VLIR Research Back Pack grant for PhD students (Belgium, 2005)
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- Sr. Patricia Mullins Award for Excellence in Chemistry (Canada, 1994)
- Dr. E. Margaret Fulton Scholarship for Academic Achievement (Canada, 1994 & 1995)

Skills

Laboratory

- HLA genotyping using sequence specific primers
- Polymerase Chain Reaction (PCR)
- DNA & RNA extraction; Cell culture and drug assays
- Cell analysis by FASCAN (Flow Cytometry)
- Enzyme-Linked ImmunoSorbent Assay (ELISA)
- Oligonucleotide Ligation Assay (OLA)
- Cloning, generation of recombinant viruses

Computer:

- Operating Systems: MS DOS, Windows, UNIX
- Primer and probe design
- Editing, generating and analysis of Sequences: DNAstar, DNAsis, Oligo

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