Original Article

Set up of Genotyping Test for Detection of HIV-1 Drug Resistance

Baesi K[1], Saberfar E[1*], Haji-Abdolbaghi M[2], Goodarzi Z[1]

1. Research Center of Virus and Vaccine, Baqiatallah University of Medical Sciences, Tehran, Iran.
2. Iranian Research Center for HIV/AIDS (IRCHA), Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Background and Aims: Genotyping assay has been accepted as a guidance in the therapeutic management of Human Immunodeficiency virus 1 (HIV-1). But, it is not commonly used in our country due to its high running cost. The aim of this study is evaluate an in-house genotyping resistance test (GRT) for HIV-1.

Methods: HIV-1 RNA of 20 samples were extracted from plasma and RT Nested- PCR was performed and the final products were sequenced. Stanford HIV Sequence Database was used for genotyping and interpretation of resistance data.

Results: Subtype A was the dominant viral subtype in these patients. Also the results of drug resistance interpretation showed that drug-naïve patients are susceptible to drugs and for patients taking the drugs; 66.6% susceptible for AZT, 50% high- level resistance for 3TC, 66.6% low- level resistance for ABC, 66.6% susceptible for TDF, 50% high- level resistance for NVP and 50% high- level resistance for EFV.

Conclusion: Our method is able to amplify and sequence HIV-RNA from plasma samples from a random selection of patients encompassing different subtypes. The results of this study may have important consequences for survey and clinical management of patients with AIDS in Iran.

Keywords: Human Immunodeficiency Virus Type 1; Subtypes; Genotyping; Drug Resistance

Introduction

The use of potent antiretroviral therapy has reduced morbidity and mortality of patients with human immunodeficiency virus type 1 (HIV-1) infection (1), and often results in substantial recovery of impaired immunologic function (2).

Recently, the highly active antiretroviral therapy (HAART), based mainly on combinations of reverse transcriptase (RT) and protease (PR) inhibitors are widely used to treat human immunodeficiency virus infections (3). The rapid replication rate of HIV and its inherent genetic variation have led to the identification of many HIV variants and also is one of the primary reason for HAART failure over the time (4)

Drug-resistant variants that evolve during treatment can be transmitted from one person to another and such spread of drug-resistant strains through the population compromises the future use of any antiretroviral drug (5).

Currently HIV drug resistance methods are phenotyping assays that measure drug inhibition of HIV-1 in vitro and Genotyping assays, which are based on direct detection of

*Corresponding author: Esmail Saberfar, PhD. Research Center of Virus and Vaccine, Baqiatallah University of Medical Sciences, Tehran, Iran. Tel: (+98) 21 66 94 96 44; Fax: (+98) 21 66 94 96 43 Email: saberfar@yahoo.com

drug resistance mutations (6). The later assays are less expensive and faster than phenotyping assays and are the most commonly used method for HIV-1 drug resistance. In addition, some idea of potential resistance to a new drug in an established class can be gained from knowing the resistance mutations for other members of the class. Genotypic assay also permits determination of viral subtype and ruling out of contamination or sample mix-up by phylogenetic analysis (7).

The aim of this study was to design and evaluate a RT Nested-PCR genotyping system for the sensitive amplification and sequencing of the reverse transcriptase (RT) gene to detect drug resistance mutations among HIV-1 strains.

Methods

Sample collection
EDTA Blood sample were collected from twenty HIV-infected patients that referred to Infectious Disease Division of the Imam Khomeini Hospital. Patients were divided into two groups; 8 cases were naïve patients for evaluating the baseline resistance therapy and the remaining 12 samples were included patients that have taken antiviral drugs. The plasma was separated and stored at −80°C until RNA extraction.

HIV RNA extraction
Viral RNA was extracted from 200 micro liter of each plasma sample using the (PureLink viral RNA/DNA kits, invitrogene, Germany) according to the manufacturer's protocol. Extracted RNA was either immediately reverses transcribed or stored at −80°C until use.

cDNA synthesis and Nested PCR amplification
Denaturation of viral RNA was performed at 70 °C for 10 min, and then cDNA synthesis was performed at 42 °C for 60 min, using 10 U of M-MuLV reverse transciptase (fermentas) and 1 nM of antisense outer primers (Table 1). plus 1.0 mM dNTP, 10 U RNAse inhibitor (fermentas).

First-round PCR was conducted using 5 micro liter cDNA, 1X PCR Buffer, 0.2 ml of 10 nM dNTPs, 0.1U of 5U polymerase, 1.5 ml 50 nM MgCl2, 0.3 nM of 12.5 nM primer solution containing outer primers (Table 1) and 15.5 ml of Double Distillated Water. Briefly the amplification was performed denaturation at 94°C for 5 min, followed by 35 cycles of with denaturation at 94°C for 50s, annealing at 54°C for 45s, extension at 72°C for 75 s, followed by a final extension phase at 72°C for 5 min.

An aliquot (about 3 nl) of the primary PCR products was used for 35 cycles of nested PCR as follows; initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 40 s, and polymerization at 72°C for 60 s, with a final elongation at 72°C for 5 min.

The PCR products were isolated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by an ultra-violate (UV) transluminator.

DNA purification and sequencing
The PCR products were purified and then subjected to bi-directional sequencing with primers F2 and R2 and automated sequencer ABI version 3700 (Applied Biosystem, Foster city, CA, USA).

Drug resistance interpretation
The RT sequences were then multiple aligned by BioEdit software (version 5.0.6) and then analyzed through the Stanford University HIV Drug Resistance Database HIVdb program (version 4.2.6 [http://hivdb.stanford.edu/]) for genotypic resistance interpretation.

Subtyping of the strains
The determination of subtypes was accomplished by submitting the obtained RT sequence to the recently developed REGA HIV-1 subtyping tool http://www.bioafrica net/subtypetool/, available also through http://hivdb.stanford.edu), which combines phylogenetic analysis with bootscanning methods for the genetic subtyping of subgenomic fragments and full-length of HIV-1 (8).

Results

Among the 20 diagnosed HIV-1 infected patients enrolled in the study, 15 patients were males and 5 patients were females. 14 patients

Table 1. The primers first round (F1, R1) and second round (F2, R2) used for amplification.

Primer		sequence		product
F1	5'	**ACVCCTGTCAACATAATTGG**	3'	Round 1: 1039 bp
R1	5'	**CTAYTAANTCTTTTGHTGGGTC**	3'	Round 2: 744 bp
F2	5'	**ATTAAAGCCAGGAATGGATGG**	3'	
R2	5	**TTYTGTATRTCATTGACAGTCCAG**	3'	

reported intravenous drug users, 4 patients reported hemophiliacs, and 2 patients had sexual contacts. Nested PCR amplification of the 20 cDNA samples gave suitable product for all except two samples that failed to amplification.

Sensitivity and specificity of in-house genotyping system

The in-house primer sets were identified to have 100% specificity to the HIV-1 *pol* gene among the sequences submitted to the NCBI blast algorithm.

HIV drug resistance

The mutations indicative for resistance found in this study are E44A, K65R, M184V/L and K103N.

Zidovudine (ZDV), Lamivudine (3TC), Abacavir (ABC), Stavudine (d4T), Tenofovir (TDF), Nevirapine (NVP) and Efavirenz (EFV) drugs prescribed in the Iranian HIV infected patients. The drug resistance interpretation in this study showed: 80% susceptible, 20% low- level resistance for

AZT, 50% susceptible, 20% potential low-level resistance and 30% high- level resistance for 3TC, and 50% susceptible, 40% low- level resistance, 10% intermediate resistance for ABC, 40% susceptible, 20% potential low level resistance, 40% low-level resistance for D4T, 80% susceptible, 10% potential low-level resistance, 10% low- level resistance for TDF, 60% susceptible, 10% potential low-level resistance and 30% high- level resistance for NVP and 70% susceptible, and 30% high-level resistance for EFV. The results summarized in figure 1. All drug-naïve patients were susceptible to nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) drugs.

M184V is the most commonly occurring NRTI resistance mutation. *In vitro*, it causes high-level resistance to emtricitabine (FTC) and lamivudine (3TC), low-level resistance to abacavir (ABC) (8), (9). K103N is currently the most clinically important NNRTI resistance mutation because it causes 20 to 50- fold resistance to each of the available NNRTIs (10).

HIV subtyping

According to the REGA HIV-1 subtyping tool, 11 out of 18 (61%)patients included in the study were infected with the subtype A and 7(39%) with the subtype B of HIV-1 strains.

Discussion

It has been demonstrated that monitoring of patients with AIDS who are under treatment has substantial benefits in terms of survival and quality of life for these patients (11). However, a better access to antiretroviral drugs
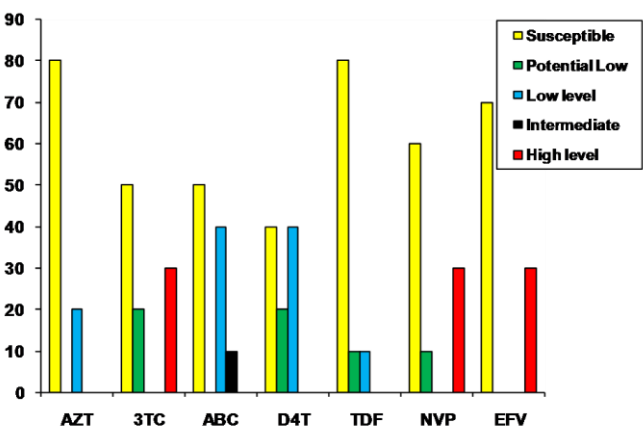
Fig. 1. Percentage of drug resistance among HIV infected patients.

without adequate control and monitoring might result in widespread transmission of drug resistant virus.

Previous studies have shown that patients whose physicians have access to drug resistance data, particularly genotypic resistance data, respond better to therapy than the control patients whose physicians do not have access to these assays (6, 12).

Albeit, in our study, drug-naïve patients were susceptible to NRTIs and NNRTIs drugs, Surveys in North America and Europe, where the history of antiretroviral therapy (ART) is extensive, have shown that transmitted or primary HIV-1 drug resistance is present in 8%–20% of ART-naıve persons (13-14). And they have demonstrated that transmitted of these drug resistance variants could reduce the efficacy of first-line regimens. As antiretroviral drug use expands to cope with the pandemic, adequately assessing the prevalence and transmission of drug resistance variant will be increasingly important for optimizing treatment efficacy (15).

Genotypic testing is the most commonly used method of detecting resistant HIV-1 isolates and is one of the earliest applications of gene sequencing for clinical purposes (16). The main objective of this study was to present an RT-PCR genotyping system for the monitoring of HIV-infected patients under treatment. The method described is able to amplify and sequence HIV-RNA from plasma samples from a random selection of patients encompassing different subtypes. The method is therefore suitable for detection of drug resistance and can be performed at a significant lower price than the current commercial genotyping assays. Also in this study we have shown that 11 out of 18 (61%) patients were infected with the subtype A and 7(39%) with the subtype B of HIV-1 strains. Although small in terms of patient numbers, this was comparable with pervious study that reported from Iran (17). All these data together indicate that subtype A is likely to be the dominant viral subtype circulating in the Iranian patients. In conclusion, the results of this study may have important consequences for the prevention and clinical management of HIV-1 infection in our country. Also this method could be the ideal combination for a fast and low-priced monitoring of patients under treatment.

Acknowledgments

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