**ABSTRACT**

Antiretroviral therapy (ART) refers to the use of a combination of three or more Antiretroviral (ARV) drugs to inhibit the replication of Human Immunodeficiency Virus (HIV) and to delay the immune deterioration, in turn to improve the survival rate and quality of life. There has been an increase in the number of individuals receiving antiretroviral therapy (ART) but treatment outcome is hampered by increasing development of drug resistance, the ability of HIV to replicate in the presence of drugs that usually suppress its replication. HIV drug resistance is caused by changes (mutations) in the virus’s genetic structure. The goal of drug-resistance testing is mainly to identify which drugs will be helpful in controlling an HIV infection. In an effort to aid the selection of effective alternative regimens, resistance tests evaluating the susceptibility of HIV to individual antiretroviral drugs have been developed. The 2 types of tests for HIV drug resistance are currently available, (a) phenotypic, in which a viral sample from the patient is subjected to various antiretroviral drugs and growth characteristics are studied and (b) genotypic, in which the HIV genome existing within the patient is identified. Genotypic testing is a baseline screening, because it is more sensitive than phenotypic testing. Hence in this article we have tried to reiterate the genotypic detection techniques of drug resistance which includes dideoxy chain termination sequencing, Microarray, Line probe assay, MS-PCR, Pyrosequencing, Enzyme linked Minisequence assay, Oligonucleotide ligation assay and Southern blotting.

**Keywords:** Antiretroviral, DNA, Drug Resistance, Genotypic techniques, HIV, Sequencing.

**INTRODUCTION**

HIV drug resistance refers to the ability of HIV to replicate in the presence of drugs that usually suppress its replication. HIV drug resistance is caused by changes (mutations) in the virus’s genetic structure. Mutations are very common in HIV because the virus replicates very rapidly and does not contain the proteins needed to correct the mistakes it makes during this process. As such, some degree of HIV drug resistance is anticipated to occur among people receiving treatment even when appropriate regimens are provided and optimal adherence is achieved.

Combinations of antiretroviral drugs are now used for the treatment of HIV infection - so-called highly active antiretroviral therapy (HAART). Current HAART regimens generally comprise three antiretroviral drugs, usually two nucleoside analogues and either a protease inhibitor or a non-nucleoside reverse-transcriptase inhibitor. Food and Drug Administration (FDA)-approved drugs are available for treatment of HIV-infections. These drugs are distributed into six distinct classes based on their molecular mechanism and resistance profiles (Table 1).

Augmenting adherence, carrying out resistance surveillance, and improving treatment monitoring are critical factors for long-term prevention of antiretroviral drug resistance. Assays that detect antiretroviral drug resistance in HIV have recently become available to clinicians. This Resistance assays provide complementary information about antiretroviral resistance by using different technologies. The goal of drug-resistance assay is simply to identify which drugs will be helpful in controlling an HIV infection. The 2 types of tests for HIV drug resistance are termed phenotypic and genotypic.

Genotypic testing is a baseline screening, because it is more sensitive than phenotypic testing for the presence of mixed populations of drug-susceptible and resistant virus and also it is less expensive. The genotypic testing is recommended in cases of acute or recent HIV infection. This resistance testing can improve virological outcome among HIV-infected individuals. HIV genotyping assays basically use a two-step procedure: (1) Polymerase Chain Reaction (PCR) to amplify Ribonucleic acid (RNA) or Dideoxyribonucleic acid (DNA) fragments to sufficient quantities for mutation detection and (2) mutation identification via nucleotide sequencing or hybridization of labeled nucleic acid fragments to oligonucleotide probes. Plasma samples with greater than 1,000 copies of viral RNA per ml are generally required for accurate results. Samples with “undetectable” viral loads (<400 copies/ml) are not suitable for genotyping. Accurate genotyping relies on laboratory quality assurance, skilled technique, and expert clinical interpretation of the mutations identified. The interpretation of resistance-associated mutations is dependent upon an up-to-date, expanding genomic database of sequence variations that correlate with phenotypic drug resistance. (Table 2)
Carbon atom. A dideoxynucleotide stops the growing DNA strand but when it is, (dideoxy thymidine triphosphate ddTTP) can be lack from the critical role played by more than 29 years. The dideoxy method gets its name method since its advent in 1977 and still is in use after colleagues sequencing, was developed termination sequencing method, also known as Sanger dideoxy chain termination technology. The chain determination is most commonly performed using for the study of biological systems today. Sequence DNA sequencing is one Dideoxynucleotide terminator cycle sequencing

<table>
<thead>
<tr>
<th>Class of drugs</th>
<th>Name of the drugs</th>
<th>Reference</th>
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<tr>
<td>Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (Nnrtis).</td>
<td>Abacavir (ABC, Ziagen), Didanosine (Ddi, Videx), Emtricitabine (FTC,Emtriva), Lamivudine (3TC, Epivir), Stavudine(D4t, Zerit), Zalcitabine (Ddc, Hivid), Zidovudine (AZT, Retrovir), And Tenofovir Disopropl Fumarate (TDF, Viread)</td>
<td>Young FE. et al.,1988(^3)</td>
</tr>
<tr>
<td>Non–Nucleoside Reverse Transcriptase Inhibitors (Nnrtis).</td>
<td>Etravirine, Delavirdine, Efavirenz, and Nevirapine</td>
<td>Kohlstaedt LA et al., 1992(^4)</td>
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<tr>
<td>Integrase Inhibitors.</td>
<td>Raltegravir (RAL), MK-0518</td>
<td>Espeseth AS et al., 2000 (^5)</td>
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<tr>
<td>Protease Inhibitors (Pis).</td>
<td>Amprenavir (Apv, Agenerase), Atazanavir (Atz, Reyataz), Darunavir (Tmc114, Prezista), Fosamprenavir (Lexiva), Indinavir (Idv,Crixivan), Lopinavir (Lpv), Nelfinavir (Nfv, Viracept), Ritonavir (Rtv, Norvir), Saquinavir (Sqv, Fortovase/ Invirase), And Tipranavir (Tpv, Aptivus)</td>
<td>Park J, et al.,1993(^6)</td>
</tr>
<tr>
<td>Fusion Inhibitors.</td>
<td>Fuzeon</td>
<td>Wilen CB et al., 2011(^7)</td>
</tr>
<tr>
<td>Co Receptor Antagonists.</td>
<td>Selzentry</td>
<td>Dragic T et al., 2000(^8)</td>
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Table 1: FDA approved drugs for treatment of HIV infections

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<th>Mutation Identified</th>
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<td>Microarray (GENECHIP)</td>
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<td>Mellors, J. W. 1998(^12)</td>
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<td>Pyrosequencing</td>
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<td>Protease inhibitors (PI)</td>
<td>-</td>
<td>O’Meara et al., 2001(^13)</td>
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<td>RT</td>
<td>AZT, del, ddc, and 3TC</td>
<td>69,70,74 And 215.</td>
<td>Stuyver, L et al., 1997(^14)</td>
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<td>Enzyme Linked Minisequence Assay</td>
<td>RT and POL</td>
<td>(1)Nucleoside analogue resistance &amp; (2) protease inhibitor resistance</td>
<td>(1)Codon 41, 67, 70, 215, 184 &amp; (2)30.46,48,82,84, 90</td>
<td>Wataru Sugiura et al, 2003(^15)</td>
</tr>
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<td>Point Mutation Assay(Or) Ms-Pcr</td>
<td>RT</td>
<td>Zidovudine</td>
<td>(1)41 and 70 (2)41, 67, 70, 215, 210,219</td>
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<td>-</td>
<td>P. Scott Eastman et al., 1995(^20)</td>
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Table 2: List of drug-resistance mutations identified within the HIV-1 Protease and RT Genes using genotypic detection techniques

**Genotyping Detection Technique**

**Dideoxynucleotide terminator cycle sequencing**

DNA sequencing is one of the most important platforms for the study of biological systems today. Sequence determination is most commonly performed using dideoxy chain termination technology. The chain termination sequencing method, also known as Sanger sequencing, was developed by Frederick Sanger and colleagues\(^{21}\), has been the most widely used sequencing method since its advent in 1977 and still is in use after more than 29 years. The dideoxy method gets its name from the critical role played by synthetic nucleotides that lack the -OH at the 3’ carbon atom. A dideoxynucleotide (dideoxy thymidine triphosphate ddTTP) can be added to the growing DNA strand but when it is, chain elongation stops because there is no 3’-OH for the next nucleotide to be attached. For this reason, the dideoxy method is also called the chain termination method.\(^{22}\) Most commercially available genotypic tests rely on automated sequencing technology. Viral RNA is extracted from plasma sample and reverse transcribed into complementary DNA (cDNA). The Protease (PR or POL) and Reverse transcriptase (RT) coding regions of the cDNA are then amplified by polymerase chain reaction (PCR), and the nucleotide sequence of the PCR product (or amplicons) is determined on an automated DNA sequence. (Figure 1) The sequencing assays may lead to a useful clinical niche with further development which could be cheaper and faster to detect minority subpopulations (to 10% or less).
been associated with zidovudine resistance mutations. The following fields are mainly involved for microarray application: cancer, toxicology, cardiology, nutrition & aging, bacteriology, and mycology have all benefited from this technology.

**Line probe assay**

To begin with, HIV RNA preparation, cDNA synthesis, and PCR with biotinylated primers are performed. Biotinylated DNA is hybridized with specific oligonucleotide probes immobilized in parallel lines on membrane-based strips. After hybridization, streptavidin labeled with alkaline phosphatase is added and binds to biotinylated hybrids. Incubation with a chromogen results in a purple-brown precipitate visible to the naked eye. The locations of these colored bands on the strip determine the presence of specific wild-type codons, mixtures of codons, and mutant codons.

Line probe assay (LiPA) for the rapid and simultaneous characterization of the following variations in the RT gene: M41 or L41; T69, N69, A69, or D69; K70 or R70; L74 or V74; V75 or T75; M184, I184, or V184; T215, Y215, or F215; and K219, Q219, or E219. Detection of the wild-type RT gene and selected mutations associated with genotypic resistance to Zidovudine (AZT), Didanosine (ddI), Zalcitabine (ddC), and Lamivudine (3TC). LiPA is suitable for detecting mixed populations and easy to implement in clinical laboratories and might be useful for epidemiological surveys of primary HIV-1 resistance.

**Point mutation assays (or) Mutagenic ally separated PCR (MS-PCR)**

The principle of mutagenic ally separated PCR (MS-PCR), first developed by Rust et al., can be applied to the HIV-1 nonnucleoside analogue reverse transcriptase (RT) and protease inhibitor-resistant mutation detection. The MS-PCR assay is a rapid, simple, and inexpensive assay that is highly sensitive in detecting mutant targets, including minor populations. Thus, it could be used as a powerful tool for epidemiological surveillance of drug-resistant mutations in developing countries.

Viral RNA is extracted from patient plasma and one-step RT-PCR with mutagenically separated primer pair and PCR for targeted sequence is performed. Two competitive detection primers are required for each detection point. One is for wild-type detection and the other is for mutant detection and there are three basic rules for the primer design: the 3’ end of each detection primer is designed to correspond to the wild-type or the mutant nucleotide pattern; to distinguish wild-type and mutant amplicons on electrophoresis, wild-type detection primers must be 20 bp longer than the mutant primers.

MS-PCR is sensitive and specific for the detection of mutations in HIV-1, and can be adapted easily to test for resistance at any codon of interest. A rapid zidovudine (ZDV) resistance genotypic assay was developed based on the mutagenically separated PCR (MS-PCR) technique to
detect two ZDV-resistant mutations, M41L and K70R in CRF01_AE (subtype E) and also M41L, D67R, K70R, T215F/Y, L210W, and K219Q mutations are known to be associated with ZDV resistance in subtype B isolates.  

**Pyrosequencing**  
Pyrosequencing is a novel DNA sequencing technology, developed at the Royal Institute of Technology, and it is the first alternative to the conventional Sanger method for de novo DNA sequencing. This method relies on the luminescent detection of pyrophosphate that is released during primer-directed DNA polymerase catalyzed nucleotide incorporation. This technique is a widely applicable, alternative approach for the detailed characterization of nucleic acids. Pyrosequencing has potential advantages of accuracy, flexibility, parallel processing, and can be easily automated.  

Furthermore, the technique avoids the need for labeled primers, labeled nucleotides, and gel electrophoresis. Pyrosequencing has been successful for both confirmatory sequencing and denovo sequencing. It employs a series of four enzymes to accurately detect nucleic acid sequences during the synthesis. In Pyrosequencing the sequencing primer is hybridized to a single-stranded DNA biotin-labeled template and mixed with the enzymes; DNA polymerase, ATP sulfurylase, Luciferase and Apyrase, and the substrates adenosine 5′-phosphosulfate (APS) and luciferin. (Figure 3)

**Figure 3: Diagrammatic representation of Pyrosequencing**  
In Pyrosequencing nucleotide removal is performed in two different ways: (i) the solid phase pyrosequencing, which utilizes a three coupled enzymatic procedure with washing steps and (ii) the liquid-phase pyrosequencing technique, which employs a cascade of four enzymes with no washing steps.

**Solid phase Pyrosequencing**  
The biotin labeled DNA template with annealed primer is immobilized to streptavidin coated magnetic beads. The immobilized, primed single-stranded DNA is incubated with three enzymes; DNA polymerase, ATP sulfurylase and luciferase. After each nucleotide addition to the reaction mixture, the DNA template is immobilized by a magnet system and the unincorporated nucleotides are removed by a washing step.

**Liquid phase Pyrosequencing**  
Pyrosequencing by the liquid-phase approach came about by the introduction of a nucleotide-degrading enzyme, called apyrase. The implementation of this enzyme in the Pyrosequencing system excluded the use of solid-phase separation, and consequently, eliminated extra steps such as washes and repetitive enzyme additions. Apyrase shows high catalytic activity and low amounts of this enzyme in the reaction system efficiently degrade the unincorporated nucleoside triphosphate to nucleoside diphosphate and subsequently to nucleoside monophosphate. In addition, apyrase stabilizes the baseline with no fluctuations in the sequencing procedure.

**Enzyme-Linked Minisequence Assay**  
Enzyme linked mini-sequence assay (ELMA) mainly used for monitoring drug resistance in the developing countries. In addition ELMA detects minor resistant populations (10%) by the assay. The high sensitivity and specificity of the assay mainly recommend it as a first screening assay for drug resistance surveillance. In ELMA, two modifications to the standard hybridization method were introduced. First, a relatively low annealing temperature was selected for the hybridization reaction. For this low temperature minimized the effect of unexpected mutations within the target sequence and decreased the risk of false-negative results. Second, a 1-base extension reaction of the probe with tagged deoxynucleotide was added after the hybridization step.  

The assay consists of four major steps: (i) extraction and amplification, (ii) hybridization, (iii) extension, and (iv) visualization. In the first step, extraction and amplification, target DNA fragments with one or more detection points are amplified from patient plasma viral RNA. In the second step, hybridization, the denatured amplified target DNA fragments are captured by the corresponding oligonucleotide probe applied to an enzyme-linked immunosorbent assay (ELISA) plate. The third step is an extension step. A biotinylated dNTP is incorporated on the 3′ end of each oligonucleotide probe. The final step is visualization, in which the incorporated biotinylated dNTP is visualized by using horseradish peroxidase (HRP)-conjugated avidin and HRP as substrates. The basic technology of ELMA is a combination of DNA hybridization and point mutation detection by 1-base elongation with a biotinylated deoxynucleotide, i.e., a minisequence. This assay detects five mutations conferring nucleoside analogue resistance (M41L, D67N, K70R, T215Y, and M184V) and six mutations conferring protease inhibitor resistance (D30N, M46I, G48V, V82A, I84V, and L90M).
Oligonucleotide ligation assay

Oligonucleotide ligation assay (OLA) is a rapid, sensitive, and specific method for the detection of known single nucleotide polymorphisms (SNPs). Ligation assays have a number of advantages in genotyping mutations within the HIV pol gene compared to other phenotypic or genotypic approaches. First, because of their highly specific nature, ligation assays yield clear positive or negative outcomes that are easy to interpret visually or that can be interpreted by a spectrophotometer and computer program with samples on a micro titer plate. RNA was obtained by silica extraction of plasma or cerebrospinal fluid with the QIAmp Viral RNA kit (QIAGEN) and cDNA was synthesized by using SuperScript II RNase H reverse transcriptase. The cDNA was amplified by nested PCR and followed by ligation. This method is based on the joining of two adjacent oligonucleotide probes (Capture and Reporter Oligos) using a DNA ligase while they are annealed to a complementary DNA target (e.g. PCR product).

These capture and reporter oligo probes differ only in sequence at the last base at the 3' end. The reporter probe is a common probe that is complementary to the target DNA sequence immediately downstream (3') of the SNP site. This probe is modified with a phosphate at its 5' end, and has no fluorescent modification. Allele discrimination occurs by the ability of DNA ligase to join perfectly matched probes; a 3' mismatch in the capture probe will prevent ligation.

Robert W. Shafer et al., 1996 studied the sequence-specific PCR used in six laboratories and a ligase detection reaction used in one laboratory to detect the Zidovudine-resistance mutation at codon 215 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase DNA.

Reva E. Edelstein in 1997 studied the detection of mutations in the pol gene of human immunodeficiency virus type 1 associated with resistance to Zidovudine, didanosine, and by genotyping by an oligonucleotide ligation assay specific codons in the pol gene amplified by PCR and also is ideally suited for typing point mutations in the human immunodeficiency virus type 1 (HIV-1) genome, including their specificity, sensitivity, and compatibility with DNA amplification by PCR.

Southern blotting

Southern hybridization assay was developed by Southern (1975) involving restriction enzyme digestion and agarose gel electrophoresis of the target DNA prior to hybridization. The different bands on the agarose gel are transferred by capillary action onto a nitrocellulose or nylon membrane in a blotting apparatus. During the transfer, each of the DNA bands is transferred onto the membrane in the same relative position that it had in the gel. After the transfer, the target DNA is probed and detected by autoradiography. (Figure 4) The main drawback with this membrane is its fragile nature.

The transfer of DNA to a solid membrane support and detection of virus-specific sequences using radiolabeled viral nucleic acids was described by P. Scott Eastman in 1995. In this hybridization assay, he determined the HIV genotypic Zidovudine resistance. Biotinylated PCR product was hybridized with enzyme-labeled probes for wild-type or resistant mutant sequences and detected calorimetrically or chemiluminescently in a micro plate format. Changes in mutant-to-wild-type ratios allow the monitoring of longitudinal patient samples.

Figure 4: Diagrammatic representation of Southern Blotting

CONCLUSION

In the past few years the selection of a combination regimen continues to be challenging because of increasing development of drug resistance. Nevertheless, the number of available antiretroviral agents has markedly increased. Genotyping assays that detect antiretroviral drug resistance in HIV have recently become available to clinicians, which is based on determination of the nucleotide sequence of regions that confer phenotypic resistance and it determines the presence of mutations that are known to confer decreased drug susceptibility. Hence we suggest that genotyping resistance testing may be useful in the assessment of the success of salvage antiretroviral therapy and may improve short-term virological response when compared to other detection testing. We also state that the genotyping resistance testing is recommended to help guide the choice of new drugs for pregnant women and also for the patients non-responsive to treatment.

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