The primary goal of antiretroviral treatment is to durably suppress the HIV viral replication in order to promote immune reconstitution and reduce AIDS-related morbidity and mortality. Variability in the response to potent antiretroviral regimens is common as a result of multiple factors including adherence, viral resistance, host immunology and drug pharmacology. The increased use of antiretroviral agents in both developed and developing countries has led to an increase in the incidence of drug resistance and subsequently a large pool of resistant virus becoming available to establish new infections. Factors responsible for high rate of HIV-1 drug resistance include absence of a 3’->5’ exonuclease proof-reading mechanism, heterogeneous viral quasispecies and high level of viral production and turnover. Mechanism of mutation involves base substitution, duplication, insertion, and recombination in the RT &/or PR genomes.

**DEFINITION**

The ability of HIV to mutate and reproduce itself in the presence of antiretroviral drugs is called HIV Drug resistance” (WHO). It can also be defined as any change that improves viral replication and survival in the presence of inhibitors. It is an altered phenotype resulting from a change in viral genotype with altered drug susceptibility.

Resistance is of two types;

- **Primary resistance or transmitted resistance**: when an individual is infected by a strain of HIV-1 already resistant to one or more antiretroviral drugs
- **Secondary resistance or acquired resistance**: development of resistance while on antiretroviral therapy.

Development of drug resistance depends on a number of factors: (1) the extent to which virus replication continues during drug therapy, (2) the ease of acquisition of a particular mutation (or set of mutations) i.e. genetic barrier, and (3) the effect of drug-resistance mutations on drug susceptibility and virus fitness.

There are essentially three approaches of detecting antiretroviral resistance in routine clinical practice.

1. Phenotypic Assay
2. Genotypic Assay
3. Virtual Phenotype

**PHENOTYPIC ASSAY**

The most direct method measures the virus phenotypic susceptibility to drugs directly by culturing virus in the presence of increasing concentrations of the drug of interest. The concentration of drug required to inhibit virus replication by either 50% (IC_{50}) or 90% (IC_{90}) relative to a control virus is then taken as a measure of resistance. These assays are similar to antibiotic sensitivity testing, although methods employed to measure antiretroviral drug resistance are more sophisticated. It commonly report results as fold changes in the IC_{50}
Phenotypic assays provide a direct measure of drug susceptibility and offer the important advantage of measuring the cumulative effects of multiple drug mutations. They also detect important interactions between mutations, including hypersusceptibility effects. It provides quantitative assessment of susceptibility & interpretation is straightforward with good reproducibility.

Despite the ongoing technical improvements, a major drawback of phenotypic assays is the long turnaround time, with analyses taking up to 4 weeks to complete and expensive. A more theoretical drawback is the possibility of outgrowth of a major highly resistant strain by a minor non-resistant strain during the initial culture. In most cases, resistance mutations will diminish the replicative capacity or ‘fitness’ of a virus strain. A virus strain without resistance mutations may therefore outgrow a resistant strain in the absence of drug pressure, thereby theoretically underestimating resistance. Clinically determined thresholds not available for all drugs and it detects resistance to single drug, not combinations.

GENOTYPIC ASSAY

Gene sequencing of patient’s virus to detect mutations known to confer drug resistance, and using this information to deduce drug susceptibility (i.e. the likely virus phenotype) to confer drug resistance, and using this information to deduce drug susceptibility (i.e. the likely virus phenotype) to drug resistance. It uses PCR/DNA sequencing technique to make many copies of HIV genetic material and then detect genetic sequence of particular viral enzyme, reverse transcriptase and protease. It has short turnaround time, less expensive, more sensitive to detect emerging resistance.

Genotyping assays present a number of drawbacks. First, the sensitivity to detect minority species is low. A variant that constitutes less than 20 – 25% of the total virus quasispecies population in a patient is generally not detected. Second, genotypic resistance becomes more difficult to predict with an increasing number of resistance mutations after multiple therapy failure because of the interactions between different mutations. Because of this drawback, many experts consider performing both phenotypic and genotypic assays in patients with extensive antiretroviral experience. Third, mutations conferring resistance to antiretroviral drugs at positions not previously characterized as resistance mutations will be missed. Fourth, relatively expensive equipment requiring expert operators is required. Fifth, there is limited data on non Clade B HIV 1 which is very much prevalent in India.

VIRTUAL PHENOTYPIC ASSAY

It is a mixture of the first two methods, whereby the virus under investigation is sequenced and identified changes are matched to a large dataset of genotype–phenotype pairs of previously sequenced viruses for which the phenotype is known.

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTI) MUTATION

The NRTI resistance mutations include M184V, thymidine analog mutations (TAM), mutations selected by non-thymidine analogs, multi-nucleoside resistance mutations. There are two biochemical mechanisms of NRTI resistance. The first mechanism is mediated by mutations that allow the RT enzyme to discriminate against NRTIs during polymerization, thereby preventing their addition to the growing DNA chain relative to the natural dNTP substrates. The second mechanism is mediated by mutations that promote the hydrolytic removal of the chain-terminating NRTI and thus enable continued DNA synthesis. This mechanism of resistance has also been referred to as pyrophosphorolysis, nucleotide excision, and primer unblocking. The hydrolytic removal requires a pyrophosphate donor, which in most cells is usually ATP.

M184V

M184V is the most commonly occurring NRTI resistance mutation. In vitro, it causes high level resistance to lamivudine (3TC) and emtricitabine (FTC), low-level resistance to didanosine (ddI) and abacavir, (ABC) and increased susceptibility to zidovudine (ZDV), stavudine (d4T), and tenofovir (TDF). The phenotypic and clinical significance of M184V is influenced by the presence or absence of other NRTI resistance mutations. For example, the presence of K65R or L74V in combination with M184V is sufficient for high-level resistance to both ABC and ddI. In contrast, three or more TAM plus M184V are required for high-level ABC and ddI resistance.

Thymidine analog mutations are selected by the thymidine analogs ZDV and d4T. Thymidine analog mutations decrease susceptibility to these NRTI and to a lesser extent to ABC, ddI, and TDF. Thymidine analog mutations are also common in viruses from persons who began therapy in the pre-HAART era with incompletely suppressive dual NRTI regimens, but are becoming less common in areas in which the fixed-dose combinations of TDF/FTC and ABC/3TC have become the most common NRTI backbones. However, even in these areas, TAM and in particular the partial T215 revertants remain the most common type of transmitted NRTI resistance mutation. Thymidine analog mutations accumulate in two distinct but overlapping patterns. The Type I pattern includes the mutations M41L, L210W, and T215Y. The Type II pattern includes D67N, K70R, T215F, and K219Q/E. Mutation D67N also occurs commonly with type I TAM. However, K70R and L210W rarely occur together. Type I TAM causes higher levels of phenotypic and clinical resistance to the thymidine analogs and cross-resistance to ABC, ddI, and TDF than do the type II TAM. Indeed, the presence of all three Type I TAMs markedly reduces the clinical response to ABC, ddI, and TDF. The clinical significance of the type II TAM is not as well characterized.
K65R causes intermediate resistance to TDF, ABC, ddI, 3TC, and FTC, low-level resistance to d4T, and increased susceptibility to ZDV. L74V causes intermediate resistance to ddI and ABC, and a slight increase in susceptibility to ZDV and TDF. L74I has similar phenotypic properties to L74V, but is found primarily in viruses with multiple TAM, possibly because it increases ZDV and TDF susceptibility less than L74V.

**MULTI NUCLEOSIDE RESISTANCE MUTATIONS**

Amino acid insertions at codon 69 generally occur in the presence of multiple TAM, and in this setting are associated with intermediate resistance to 3TC and FTC and high-level resistance to each of the remaining NRTI. **Q151M** is a 2-bp mutation (CAG→ATG) that is usually accompanied by two or more of the following mutations: A62V, V75I, F77L, and F116Y. The Q151M complex causes high-level resistance to ZDV, d4T, ddI, and ABC, and intermediate resistance to TDF, 3TC, and FTC. This complex developed in 5% of patients who received ddI in combination with ZDV or d4T, but is rarely selected by 3TC or FTC containing regimens. Q151M may be uncommon because the two intermediate amino acids Q151L (CAG→CTG) and Q151K (CAG→AAG) are poorly replicating and rarely observed. Q151M is a common genetic mechanism of NRTI resistance in HIV-2-infected persons. The optimal NRTI combination to use in patients with codon 69 insertions or Q151M is not known.

**NNRTI RESISTANCE**

Each of the primary NNRTI resistance mutations – K103N/S, V106A/M, Y181C/I/V, Y188L/C/H, and G190A/S/E – cause high-level resistance to nevirapine and variable resistance to efavirenz, ranging from about twofold for V106A and Y181C, six-fold for G190A, 20-fold for K103N, and more than 50-fold for Y188L and G190S. In contrast, patients with any single one of the primary NNRTI resistance mutations may benefit from etravirine salvage therapy, although the mutations at position 181 and to a lesser extent 190 compromise etravirine response and may provide the foundation for the development of high-level etravirine resistance. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are generally the preferred agents in currently recommended combinations for the treatment of HIV-1 infection. Although there are 5 NNRTIs approved by the US Food and Drug Administration (FDA); efavirenz (EFV), delavirdine (DLV) and nevirapine (NVP), Etravirine only EFV and NVP are available in India.

HIV-2 is intrinsically resistant to most NNRTIs. Although NNRTIs are generally well tolerated with adverse events occurring within the first 6 weeks and being predictable and manageable, a major limitation for all currently available NNRTIs is the low genetic barrier to resistance. A single mutation in the NNRTI-binding pocket may confer high-level resistance to one or more NNRTIs. Several studies have shown that a single dose of NVP used to prevent mother-to-child transmission of HIV-1 can select for NNRTI-resistant mutants. In a recent study, using standard resistance testing, resistance was demonstrated in as many as 40% of women after a single-dose of NVP. Such mutations appear to be transient with detectable resistance no longer found in plasma 12–24 months post partum. There is evidence, however, to suggest that single-dose NVP does impact on the future response to NNRTI-containing regimens. Furthermore, resistance mutations have been observed following exposure to a single dose of NVP even when added to other therapy.

Multiple studies of EFV administered to treatment-naive patients in combination with tenofovir, stavudine (d4T) or zidovudine (ZDV) and in combination with lamivudine or emtricitabine have shown that the NNRTI resistance mutations – primarily K103N and Y181C –are the most common mutations associated with virological failure, usually occurring in 50–70% of cases. Treatment failure on NVP-containing regimens appears to produce similar results, although data are more limited.

**MUTATIONS AT CODON 103**

K103N occurs in ~30% of patients with virological failure while receiving NVP and reduces susceptibility to NVP and DLV by 50-fold. The mutation also occurs in ~50% of patients failing EFV and reduces susceptibility to EFV by 25-fold. K103N alone is sufficient to prevent a virological response to EFV.

**MUTATIONS AT CODON 181**

NVP usually selects for Y181C when administered without a concomitant thymidine analogue. This mutation confers very high-level resistance (50- to 100-fold) to NVP and DLV. However, when ZDV or d4T are included in the regimen, the K103N mutation is preferentially selected. Y181C occurs in ~<5% of patients developing virological failure while receiving EFV and reduces EFV susceptibility by ~2-fold. Despite this, in patients failing NVP-containing regimens with Y181C, there is only a transient response to EFV-containing salvage regimens.

**PROTEASE INHIBITORS**

More mutations are selected by the PI than by any other ARV class. The effect of PI resistance mutations on individual PI may be difficult to quantify when many mutations are present in the same virus isolate or when mutations occur in unusual patterns. The effect of PI resistance mutations on drug susceptibility can also be modulated by gag cleavage site mutations and possibly other parts of gag that influence Gag-Pol processing. Although multiple protease mutations are often required for HIV-1 to develop clinically significant resistance to a Ritonavir-boosted PI, some mutations indicate
that a particular PI, even when boosted, may not be effective.

- Major mutations – 30, 46, 50, 83, 84, 90
- Minor mutations -10, 20, 24, 32, 35, 54, 71, 73, 77 etc.
- Signature mutation – D30N (Nelfinavir), 150L (Atazanavir)

- Boosted PI – high genetic barrier (LPV/r ≥ 6 & DRV/r ≥ 10)

**INDICATIONS OF DRUG RESISTANCE TESTING**

The Euro Guidelines Group for HIV-1 Resistance, the International AIDS Society (IAS)-USA Consensus Panel on Resistance Testing and the Panel on Clinical Practices for Treatment of HIV-1 Infection of the US Department of Health and Human Services (DHHS) have published recommendations on the use of resistance testing:

- Before initiation of therapy, Primary (acute and early) infection
- First evaluation of chronic HIV-1 infection
- Treatment initiation for chronic HIV-1 infection
- In antiretroviral-treated patients during treatment failure
- In specific settings like Pregnancy
- Others – post exposure prophylaxis

**PREREQUISITE FOR DRUG RESISTANCE TESTING**

- Plasma samples to be tested for drug resistance should contain at least 500-1000 RNA copies/ml to ensure successful PCR amplification required for all sequencing approaches;
- It is preferable that the blood sample for resistance testing be obtained while the patient is on the failing regimen of not less than 4 weeks, if possible.
- Resistance testing should be performed by laboratories that have appropriate operator training, certification, and periodic proficiency assurance.

**LIMITATIONS OF RESISTANCE TESTING**

- Lack of uniform quality control across different laboratories
- High cost in comparison to other test routinely done in HIV care
- Can’t be reliably performed with HIV RNA < 500-1000 copies/ml
- Can’t detect minority populations (<20%), especially common after drug discontinuation.
- Limited data and reliable interpretation for non-B subtypes specially for PIs

- The genotypic data for the strains from different regions of India including south, showed the predominant subtype to be Clade C. The Stanford HIV genotypic drug resistance database algorithm database generates mutations based on a consensus on Clade B sequence.

**DRUG RESISTANCE TESTING IN CLINICAL PRACTICE**

Genotypic assay is preferred in treatment naïve subjects – acute or chronic infection, early virologic failure and when patient is no longer on therapy.

Phenotypic or virtual phenotype assay is preferred or combined phenotypic or genotypic assay preferred in cases with high level resistance to NRTI or PIs on genotype and subjects with multiple regimen failure with limited treatment options.

**INDIAN SCENARIO**

In India few studies are available, these are important because these information provide resistance pattern of Clade C virus whereas most studies in western population is of Clade B. In resource-limited settings like India, WHO recommends a standardized, public-health approach to facilitate the rapid expansion of access to ART in contrast to the individualized patient-management strategies in developed countries, based on routinely available diagnostic monitoring. Standardized first-line ART regimens consist of a non-nucleoside reverse transcriptase inhibitor (NNRTI) and a dual nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) backbone, available as generic fixed-dose combinations. Recommended second-line regimens combine a ritonavir-boosted protease inhibitor (PI) with two previously unused and/or recycled NRTIs.

Routine HIV viral-load monitoring is not generally available in the National ART programme of India and treatment failure is frequently suspected by immunological and/or clinical failure. However, all suspected treatment failure cases are confirmed by viral load assay to determine eligibility of second-line ART. Typically, virological failure precedes immunological failure and lastly clinical failure occurs. So, virological breakthrough may be detected late while the failing regimen is continued, thus facilitating the acquisition and accumulation of drug resistance-associated mutations. Drug-resistant HIV variants may compromise the effectiveness of subsequent lines of treatment and their transmission to newly infected individuals has severe public health consequences.
in 60% of patients. Twenty-five percent of patients had ≥3 NNRTI mutations.  

One study from CMC, Vellore\textsuperscript{11} demonstrated that among the 20 strains, 19 showed resistance to both nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), one strain to NNRTIs and five strains showed protease inhibitors (PI) resistance and 3-class resistance. The most common mutation conferring NRTI resistance was M184V (90%) while K103N (45%) was the most common mutation conferring NNRTI resistance. The M46I mutation was seen in 20% of the PI sequences. 

One study from Mumbai\textsuperscript{12} revealed different drug-resistant mutations and all RT (Reverse Transcriptase) sequences clustered with subtype C. Here drugs used as 1st line were AZT or d4T + 3TC+ NVP or EFV. Among the major mutations, NTRIs were (in decreasing frequencies) M184V, T215Y/F, D67N, M41L, K70R, K219Q/E, L210W, V75M, Q151M, L74I, K65R and T69 del while those to NNRIs were K103N, Y181C,G190A, Y188L, V106M and K101E. V90I, which was associated with resistance to Etravirine in the ARNS algorithms, was observed in the samples. Most of the patients had TAMs with a predominance of the TAM 1 pathway. So, when failure is diagnosed late on WHO clinical and/or immunological criteria, there is an accumulation of highly significant and crucial drug resistance mutations including K65R substitution which can impair second line therapy including tenofovir. 

However, in resource restricted settings, availability of drug resistance assay is virtually non-existent and physicians are to rely on obtaining detailed ARV history, reviewing adherence data, ruling out drug interactions, drug toxicities and virologic failure before deciding on switching on to second line ART in absence of drug resistance studies. 

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