Antiretroviral drug resistance testing

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ABSTRACT

While antiretroviral drugs, those approved for clinical use and others under evaluation, attempt in lowering viral load and boost the host immune system, antiretroviral drug resistance acts as a major impediment in the management of human immune deficiency virus type-1 (HIV-1) infection. Antiretroviral drug resistance testing has become an important tool in the therapeutic management protocol of HIV-1 infection. The reliability and clinical utilities of genotypic and phenotypic assays have been demonstrated. Understanding of complexities of interpretation of genotyping assay, along with updating of lists of mutation and algorithms, and determination of clinically relevant cut-offs for phenotypic assays are of paramount importance. The assay results are to be interpreted and applied by experienced HIV practitioners, after taking into consideration the clinical profile of the patient. This review sums up the methods of assay currently available for measuring resistance to antiretroviral drugs and outlines the clinical utility and limitations of these assays.

With the current global pandemic and emerging epidemics in territorially large, highly populated countries like India, China and Russia, human immune deficiency virus (HIV) infection poses a major challenge to the scientific community for successful intervention. The two key factors that are essential for success of antiretroviral (ARV) therapeutic protocols are improvement in host immunity and lowering of the circulating HIV load. With the increasingly greater availability of the current ARV drugs, ARV drug resistance profile has also gradually expanded. In this article, we review the various methods of assaying susceptibility of HIV-1 to antiretroviral drugs, along with their interpretations, indications, benefits and limitations. Various review articles, articles on methodologies, clinical studies and trials, guidelines and recommendations and abstracts of scientific meetings regarding antiretroviral drug resistance testing were reviewed.

Major causes of ARV drug therapy failure

In any HIV-infected individual on ARV drug therapy, the two causes for inability to achieve viral suppression and associated immunological and clinical benefits, are:

a) **Inability of the drug(s) to reach the virus** – factors responsible for this include poor patient adherence due to lack of awareness about dosing schedule requirements,

toxicity and metabolic complications leading to treatment interruptions; and pharmacological factors related to drug absorption, metabolism, activation and interactions.

b) **ARV drug resistance** – leading to failure of the ARV drug to act on the virus.

Evolution of HIV-1 drug resistance

Following primary infection, numerous HIV-1 quasispecies evolve in an individual, each genetically unique, as a result of the ever-mutating nature of HIV-1. Due to lack of proof reading activity in HIV-1 reverse transcriptase (RT) enzyme, about 10^4-10^5 random point mutations occur in HIV-1 daily. This event is coupled with the high replication rate of the virus, occurrence of recombination between variant strains and archiving of various strains within the host cells, leading to generation of multiple HIV-1 quasispecies in the same individual.[1-8]

Upon exposure to ARV drugs, there is positive selection of mutant strains, which are able to escape the selective drug pressure. This leads to emergence of drug resistant HIV-1 mutants gradually replacing the 'wild-type' (wt) virus.

HIV drug resistance–terminologies

a) *Phenotypic resistance:* Ability of HIV to grow in spite of the presence of ARV drug.
b) *Genotypic resistance:* Mutations in HIV genome resulting in diminished susceptibility or response to ARV drugs.
c) *Clinical resistance:* Diminished clinical response in spite of ARV drug therapy.

Assays for HIV drug resistance measurement

The assays available to measure ARV drug resistance are phenotypic assays and genotypic assays.

Phenotypic assays and interpretation

The current available phenotypic assays for testing ARV drug resistance are based on generation of recombinant HIV-1,[9] wherein the protease (PR) and RT region of HIV-1, amplified from patient's plasma sample by reverse transcription - polymerase chain reaction (RT-PCR) is incorporated into recombinant HIV-1 backbone with deleted PR and RT. The fold change in drug concentration required to inhibit the patient virus sample, in comparison to that required for a wt reference strain, is measured. In addition to measurement of diminution in drug sensitivity, there is a direct quantification of the degree of resistance. The result is expressed as the concentration of the drug inhibiting the virus replication by 50% (IC_{50}). The IC_{50} (or IC_{90}) of test sample is compared with a cut-off value, thereby indicating the factor (fold change) by which this IC_{50} can be increased, with reference to IC_{50} of the wt strain and still be classified as sensitive. The different cut-offs that are being used are:

i) **Technical cut-off** - This involves repeated measurement of reference HIV-1 strain sensitivity to any given ARV drug and defines reproducibility and variability of the assay methodology. They represent 95% confidence intervals of the assay for repeated assays.

ii) **Biological cut-off** - This highlights biological variations in IC_{50} amongst different wt strains isolated from ARV drug-naïve HIV-infected individuals.

iii) **Clinical cut-off** – This relates to the response to therapy, based on clinical trials. The adjustments in cut-off values for a drug are made after clinical correlation. It is recommended that there be at least two clinical cutoffs: one at which there is some reduction in drug activity and the other at which a drug no longer has specific antiviral activity.[10]

The commercially available phenotypic tests include: Antivirogram® (Tibotec-Virco), Pheno Sense™ (Monogram Biosciences) and Phenoscript™ (Viralliance). Antivirogram® (Tibotec-Virco) assay generates homologous recombinant virus by introducing the PR and RT region of HIV-1, amplified from patient's plasma, along with an HIV-1 vector with deleted PR and RT, in CD 4 + cell culture. Following several replication cycles in different concentrations of ARV drug, cell killing is measured for evaluating the IC_{50}. In the PhenoSense™ (Monogram Biosciences) assay, the recombinant PR and RT region deleted HIV-1 vector has an additional deletion in the envelope gene (*env*) along with firefly *luciferace* gene insertion. The *env* deletion leads to single cycle replication of the recombinant virus, with the *luciferace* acting as a reporter gene, highly sensitive to virus replication. Susceptibility to a drug is measured by comparing luciferase activity produced in the presence and absence of drugs. Phenoscript™ (Viralliance) is an *in vitro* single cycle recombinant virus assay, where three regions – *gag*-PR, RT and *env* are amplified. Thus, this assay measures resistance to fusion inhibitors, in addition to protease inhibitors (PIs) and reverse transcriptase inhibitors (RTIs). The

PCR products are separately co-transfected into cells along with the corresponding plasmids for each drug class (PIs, RTIs, fusion inhibitors). The indicator cells contain the LacZ gene under HIV-1 LTR control, which when infected produce of ß-galactosidase, which is detected colorimetrically.

Genotypic assays

HIV genotypic assays detect known mutations associated with drug resistance. Specific mutations between PR positions 10-93 and RT positions 41-236 are associated with resistance to PIs and RTIs, respectively. Resistance mutations are described as a letter corresponding to 'wt' virus amino acid preceding a number, which shows the position of the relevant codon in the HIV-1 genome PR or RT. The succeeding letter describes the amino acid in the mutant virus. Genotypic assays are commonly used for detection of ARV drug resistance because of lesser cost as compared to phenotypic assays and rapid turnaround time. The sensitivity of most of these assays range between 100-1000 viral RNA copies / ml. These assays involve detection of mutations in the HIV-1 genome, in the regions that are targeted by the different ARV drugs, namely PR, RT and gp 41. Plasma specimen is used for extracting the latest, replicating, positively selected copies of HIV-1, under drug pressure, $T_{1/2}$ of the virus being 6 hours. Following amplification of regions of interest by RT-PCR, the amplicon can be further processed by two different approaches:

a) **Dideoxynucleotide sequencing:** This is the commonest methodology utilized for testing ARV drug resistance worldwide. Along with deoxynucleotide triphosphates (dNTPs), dideoxynucleotide triphosphates (ddNTPs) are introduced for DNA synthesis from the RT-PCR amplicon, in the presence of specific primers. The incorporation of ddNTPs lead to chain termination, finally generating numerous single stranded DNA of varying lengths, differing from each other by one nucleotide length. The DNA strands containing labeled primers/ddNTPs are separated by polyacrylamide gel electrophoresis and detected by fluorometric methods in an automated sequencer. This direct sequencing of amplified RT-PCR is known as viral population-based sequencing. Clonal sequencing is carried out in research setting to study the evolution of HIV-1 drug resistance. The sequences so generated are subjected to computer-based software analysis for generating HIV-1 resistance mutation data. Commercially available genotypic resistance tests include: HIV-1 TrueGene™ (Bayer Healthcare Diagnostics) ViroSeq™ (Celera Diagnostics/Abbott Laboratories), Virco® Type HIV-1 (Virco), GenoSure (Plus)™ (LabCorp) and GeneSeq™ (Monogram Biosciences). Compared to the commercially available genotypic assays, in-house methods are cheaper and are used by majority of the laboratories. However, the in-house methods need certification and validation for routine use.

b) **Hybridization methods:** The entire sequence of the amplified product or specific drug- resistance mutations can be detected by this method. The gene chip (Affymetrix) containing numerous probes, detects each nucleotide of the test isolate amplicon. This kit is no longer available commercially. The major drawbacks of this

method were lesser reliability as compared to dideoxynucleotide sequencing and diminished performance in sequencing non-B subtypes and detecting insertions and deletions.[11-14]

The INNO-LiPA HIV-1 line probe assay (Murex Innogenetics) detects specific drug resistance point mutations. Hybridization of biotin labeled test isolate amplicon with probes for specific codons (wild-type and mutants), attached to a nitrocellulose strip, lead to the production of colour in the presence of avidin-enzyme complex and substrate. The results of direct sequencing and LiPAs have been shown to be highly concordant.[15] Though cheap, this suffers from drawbacks like poor hybridization and associated non-interpretation of 10% of results and ability to detect known mutations only.[15,16]

Genotypic assay interpretation

Interpretation of the genotype can be based either on rule-based algorithms or on "virtual" phenotype (Virco, Belgium). The computerized rule-based algorithms classify the virus as "susceptible", "probably resistant" and "resistant". These are designed by expert panels, such as the French ANRS (Agence Nationale de Recherches sur le SIDA) AC11 research group (http://www.hivfrenchresistance.org), the International AIDS Society-USA (IAS-USA) panel[17] and the European HIV drug resistance guidelines panel.[18] These algorithms need to be updated frequently and ideally should be based on studies correlating the virus genotypic profile at baseline with the virological response to treatment.[17] While the commercially available kits provide for sequence analysis and interpretation of mutations, there are on-line databases available, free of cost, for test isolate sequence interpretation, for example: Stanford HIV RT protease sequence database (http://hivdb.stanford.edu/hiv), Los Alamos HIV sequence database (http://hiv-web.lanl.gov), HIVresistanceWEB (http://www.hivresistanceweb.com), HIV genotypic drug resistance interpretation – ANRS AC11 (http://www.hivfrenchresistance.org). In these databases, the sequences submitted by the user are compared with reference subtype B sequence and the differences from the reference sequence are used for checking the database. In these databases, compilations of published protease and reverse transcriptase gene sequence data exist. Based on rule-based algorithms, the query sequences are interpreted for drug resistance.

In "virtual" phenotyping, the genotypic mutation profile of a given test isolate is compared with the available paired genotypes and phenotypes in the database. Limitations of this include representation of virus strains in database from Europe only and inclusion of pre-selected codons considered relevant for a particular drug and not the entire sequence of virus, for matching the submitted genotype.

Role of inhibitory quotient: Genotypic and phenotypic

The inhibitory quotient (IQ) is the plasma concentration of an antimicrobial drug divided by the susceptibility of the microorganism to that drug. The IQ is being used as a way to integrate ARV drug exposure and viral susceptibility. The phenotypic inhibitory quotient (PIQ), which is based on phenotype testing, is the trough plasma drug level (C_{trough}) divided by the IC_{50}. Genotypic inhibitory quotient (GIQ) is

the ratio of C_{trough} to the number of mutations. Since the effects of PI-associated mutations are considered to be gradual, GIQ has been used for PIs. The virtual inhibitory quotient (VIQ) is the ratio of C_{trough} to the fold change by virtual phenotyping (VFC) or the VIC_{50} and the normalized inhibitory quotient (NIQ) is defined as $IQ_{reference}/IQ_{population}$ or $VIQ_{reference}/VIQ_{population}$. The prognostic value of PIQ, GIQ and NIQ, as predictor of virologic response to ART, has been demonstrated. However, the difference in the equations used by various studies is a limiting factor.[19]

Genotype-phenotype discordances

The various causes for discordances between genotypic- and phenotypic assay results are as under:

1. ***Genotypic mixtures*** – In test specimen harboring mixtures of wild type and drug resistant mutant strains, genotypic assays may interpret the results as resistant, while the phenotyping may be unable to detect the same, especially when the proportions of mutant strain are low.[20]

2. ***Transitional mutations*** – Some mutations may not lead to drug resistance, but may indicate evolving resistance. These will be detected by genotypic assays, but interpreted as susceptible by phenotyping.[21]

3. ***Thymidine analogue mutations (TAMs)*** – Nucleoside analogue-associated mutations (NAMs) are associated with resistance to multiple nucleoside RTIs and TAMs are a subset of NAMs. Though zidovudine and stavudine are the only FDA approved thymidine analogues, TAMs, namely M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, have been associated with resistance to other NRTIs as well except lamivudine and emtricitabine.[22] While the genotypic assay detects individual TAMs, phenotypic assay cut-offs (biological and clinical), especially for stavudine, didanosine and tenofovir, may overlap, and hence, may be difficult to interpret.

4. ***Antagonistic mutations*** – Presence of one mutation may reverse the effect of a second mutation. For example, M184V partially reverses the resistance conferred by TAMs to zidovudine, stavudine and tenofovir. As a result, phenotyping of a test isolate with such a combination of mutations will be interpreted as susceptible, while genotyping will identify both the mutations as resistant.[23]

5. ***Atypical mutations*** – Atypical resistance mutations that are not mentioned in the algorithms utilized for interpretation of genotypic assays will be missed out. Phenotypic assays will be able to detect resistance in such circumstances.[24] Identification of new changes in the amino acid sequence associated with susceptibility leads to upgradation of mutation lists of HIV-1. For example, following completion of the study by the GenPheRex and PhenGen study groups of MASTER cohort, the IAS mutation list was updated and substitutions 54AST and 73CT in protease region were considered as resistance related mutations.[25]

6. ***Complex patterns of mutations*** - While several mutations associated with drug resistance are documented, complex interactions between them may lead to cross resistance and hypersusceptibility. Demonstration of such associations necessitates revision of genotypic rule-based

algorithms for improving their concordance with phenotyping. Use of a new lopinavir genotypic algorithm, taking into account cross-resistance between protease inhibitors lopinavir and amprenavir, led to improvement in genotypic-phenotypic concordance from 80 to 91%.[26] RT mutations (M41L, M184V, L210W and T215Y) associated with hypersusceptibility to NNRTIs have been associated with better virological and immunological responses to efavirenz-based ARV therapy.[27] The evolution of incremental resistance to lopinavir (emergence of new mutation[s] and/or at least a twofold increase in phenotypic resistance compared to baseline isolates) has been reported to be highly dependent on the baseline phenotype and genotype. Mutations at positions 82, 54 and 46 in PR have been suggested to be important for conferring high-level resistance. In baseline isolates with eight or more mutations, associated with lopinavir resistance and/or displaying > 60-fold-reduced susceptibility to lopinavir, emergence of incremental resistance was uncommon. This provides insight into suitable upper genotypic and phenotypic breakpoints for lopinavir-ritonavir.[28] Following the evaluation of clinical significance of such data, algorithms will require updating.

The advantages and disadvantages of genotypic and phenotypic tests are tabulated in Table 1.

Clinical trials of resistance testing

A number of clinical trials have been conducted to look into the utility of ARV drug resistance testing. Most of the studies have shown greater benefits, immunologic and virologic, with the use of genotypic assay data in clinical management. Findings of clinical trials of resistance testing are summarized in Table 2.

Table 1: Advantages and disadvantages of genotypic and phenotypic tests

	Genotypic assay	Phenotypic assay
Advantages	Less expensive	Direct and quantitative measure
	Short turn around time (< 1 week)	Non-B subtypes can be evaluated
	Detection of mutation may precede phenotypic resistance	Any new ARV agents can be assessed
	Sensitivity to detect mixtures (wild + mutants) higher	Net effect of mutations (including cross
	Detects TAMs associated with didanosine, stavudine and tenofovir resistance	resistance mutations) measured
Disadvantages	Interpretation complex, when many mutations present	Expensive
	Discordance with phenotypic assay	Turn around time: 2-4 weeks
	Algorithms based on subtype B	Cut-offs – not defined for all drugs
	Indirect measure	Inter-assay standardization not defined
	Minor quasispecies (20-30%) – not detected	Minor quasispecies (20-30%) not detected
		TAMs associated with didanosine, stavudine and tenofovir resistance – detection levels below technical reproducibility

Table 2: Clinical trials of antiretroviral resistance testing

Name of study	Study design	Result
Viradap[29]	Genotype versus standard of care	Genotyping beneficial
GART (CPCRA046)[30]	Genotype + expert advice versus standard of care	Genotyping beneficial
ARGENTA[31]	Genotype versus standard of care	Outcome related to adherence
Havana[32]	Genotype versus expert advice versus genotype + expert advice versus standard of care	Genotyping beneficial
VIRA3001[33]	Phenotype versus standard of care	Phenotyping beneficial
CCTG575[34]	Phenotype versus standard of care	No benefit
NAVRAL[35]	Genotype versus phenotype versus standard of care	Difference not significant
CERT[36]	Genotype versus phenotype versus standard of care	Phenotyping beneficial; genotyping-no benefit
ERA[37]	Genotype + phenotype versus genotype alone	Difference not significant
GenPhenRex[38]	Virtual phenotype versus phenotype	Difference not significant
Realvirfen[39]	Virtual phenotype versus phenotype	Difference not significant
RADAR[40]	Rule-based genotype versus virtual phenotype (with and without concentration-controlled intervention)	Difference not significant

Table 3: Guidelines for antiretroviral drug resistance testing

Presentation	IAS-USA[10]	DHHS[41]	EuroGuidelines[18]
Failure of subsequent drug regimens	Recommended	Recommended	Recommended
Primary/acute HIV infection	Consider testing	Consider testing	Consider testing
Established (untreated) HIV infection	Consider testing	Not recommended	Consider testing
Pregnancy	Recommended	As for non-pregnancy	Recommended
Postexposure prophylaxis	—	—	Recommended (for index case)
Pediatrics	—	—	Recommended (if mother is viraemic)

IAS- International AIDS society; DHHS- US Department of health and human services.

Guidelines for ARV drug resistance testing

Based on evidence, various expert panels have issued guidelines recommending ARV drug resistance testing in different clinical settings [Table 3].

As per World Health Organization draft guidelines for surveillance of HIV drug resistance, surveillance and monitoring of drug resistance is likely to become an important part of expanded access to HIV treatment, by contributing to the evaluation of the efficacy of regimens and programmes and providing important public health information.[42]

Limitations of HIV-1 drug resistance testing

The ultimate goal of drug resistance testing should be prediction of response to ARV therapy.[43] However, the utility of drug resistance testing is limited by a number of factors.

i) *Inability to detect minor quasispecies of HIV-1 population* – unless the resistant mutant strain is more than 20-30% of the circulating population, the same may go undetected by current available assay methods. It has been demonstrated that such minor quasispecies, not detected by current commercial assays, can influence virologic response to therapy.[44,45] This inadequacy has been dealt with the use of newer sensitive assays like single genome sequencing,[46] quantitative real-time polymerase chain reaction using allele-discriminating oligonucleotides[47] and LigAmp assay.[48,49]

ii) *Clinical utility* – The utility of assay interpretations has been established for improving short-term response to ARV therapy.[29,31-33,36] In a meta-analysis of nine published randomized trials that were specifically designed to assess the clinical utility of drug resistance testing, resistance testing increased the proportion of patients who achieved undetectable viral load by an average of 7%. This was suggested to be an over-estimate of the impact of resistance testing in clinical practice because of the idealized design and analytical approaches used in most of the studies.[50] In the treatment of naïve patients, transmitted mutations may be detected in recently infected patients and be undetectable in chronically infected, as a result of overgrowth of the wild type strains in the absence of drug pressure.[51] However, the effect of resistance mutations on ARV therapy in the treatment of naïve population has been questioned wherein individuals with transmitted multidrug-resistant HIV-1 have partially responded to standard initial ARV therapy.[52, 53]

In patients who have undertaken multiple treatments, the presence of multiple resistance mutations may make the interpretation of genotypic assay difficult. The use of phenotypic testing in such patients is beneficial (in combination with genotyping), since it provides a quantitative assessment of the likely activity of each ARV agent. The genotypic algorithms and phenotypic cut-off values, while highlighting the drugs that will not be effective, fail to guarantee the success of the subsequent therapeutic regimen.

Since different approaches to monitoring resistance reflect different interpretation of the results, the prediction of drug-resistance from a given HIV sequence might be contradictory and requires accurate standardization and unique interpretative rules.

iii) *Cross-resistance* - The K65R mutation in RT leads to cross-resistance to multiple NRTIs like tenofovir, abacavir, didanosine, zalcitabine, lamivudine and possibly stavudine.[54] Similarly, TAMs are associated with cross-resistance to all other nucleoside analogues, with the exception of lamivudine and emtricitabine. Of these, mutations at positions 65 and 215 have been demonstrated to play a central role in reducing phenotypic susceptibility to tenofovir. Furthermore, the resensitizing effect of M184V mutation has been suggested to be minor.[55] A 20 to 30-fold increase in resistance to all available NNRTIs has been shown in the presence of K103N mutation in RT.[56] There is a high degree of cross-resistance between saquinavir, nelfinavir, indinavir and ritonavir. V82A (/T/F/S) in PR occurs mainly with indinavir, lopinavir and ritonavir and in combination with other mutations, leads to cross-resistance to other PIs.[57] The presence of such cross-resistant mutations makes application of genotypic interpretation difficult for choosing ARV drugs that may be clinically suitable for achieving viral suppression.

iv) *Reservoirs and archives* – The standard assay procedures use plasma samples for detection of resistance to mutation. As a result, drug resistant strains archived in proviral DNA and harbored in different body compartments remain undetected. These may emerge rapidly after exposure to ARV therapy under selective pressure.

Conclusion

ARV drug-resistance testing has emerged as a recommended tool for clinical management of HIV-1 infected patients. It has been established as the standard of care to guide treatment after ARV drug failure.[18,41,58] While the phenotypic and genotypic assay results are complimentary to each other, both being equally important, it is essential to understand the importance of expertise and capability of laboratories generating these drug-resistance reports and data. Stringent quality assurance protocols should be adhered to and appropriate technical expertise should be made available. The needs of laboratory certification, periodic proficiency testing and staff training are mandatory. The interpretations of drug-resistance assays can be challenging and need to be updated constantly. The drug-resistance report should be interpreted in conjunction with detailed clinical history, including current and past ARV regimens and possible reasons for failure of therapy.

The ever-growing research activities investigating varied fields like utility of ultrasensitive assay techniques for detection of drug resistance,[47,59] highlighting of clinical relevance of transmission of drug-resistant viruses[60] and emphasizing the need for sequencing HIV-1 *gag* gene for extensive analysis of *gag* cleavage sites and their role in conferring resistance to PIs,[61] are likely to expand the application of drug resistance assays. Other key research areas like the issue of coreceptor switch leading to drug resistance associated with use of CCR5 antagonists[62] and evaluation of the significance of

ribonuclease (RNase) H domain of HIV RT mutations associated with TAMs,[63] etc. are being investigated. The applications of drug resistance assays are bound to expand with incorporation of newer methods, extended regions of HIV-1 to be investigated, and up gradations of associated rules and recommendations.

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