Special article

Updated European recommendations for the clinical use of HIV drug resistance testing

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In most European countries, HIV drug resistance testing has become a routine clinical tool. However, its practical implementation in a clinical context is demanding. The European HIV Drug Resistance Panel was established to make recommendations to clinicians and virologists on this topic and to propose quality control measures. The panel recommends resistance testing for the following indications: i) drug-naive patients with acute or recent infection; ii) therapy failure, including suboptimal treatment response, when treatment change is considered; iii) pregnant HIV-1-infected women and paediatric patients with detectable viral load when treatment initiation or change is considered; and iv) genotype source patient when post-exposure prophylaxis is considered. In addition, for drug-naive patients with chronic infection in whom treatment is to be started, the panel suggests that resistance testing should be strongly considered and recommends testing the earliest sample for drug resistance if suspicion of resistance is high or prevalence of resistance in this population exceeds 10%. The panel does not favour genotyping over phenotype, however it is anticipated that genotyping will be used more often because of its greater accessibility, lower cost and faster turnaround time. For the interpretation of resistance data, clinically validated systems should be used to the greatest extent possible. It is mandatory that laboratories performing HIV resistance tests take regular part in quality assurance programs. Similarly, it is necessary that HIV clinicians and virologists take part in continuous education and meet regularly to discuss problematic clinical cases. Indeed, resistance test results should be used in the context of all other clinically relevant information for predicting therapy response. The panel also encourages the timely collection of epidemiological information to estimate the impact of transmission of resistant HIV and the prevalence of HIV-1 non-B subtypes in the different European countries.

Introduction

In 2001, a European panel presented guidelines for the use of HIV-1 drug resistance testing for treatment management, with special attention to the European setting and the practical use of tests in the laboratory [1,2]. As sufficient new information has now become available, the organizing committee of the *1st European HIV Drug Resistance Workshop: from Basic Science to Clinical Implications*, 6–8 March 2003, Luxembourg, Luxembourg [3] invited that panel to produce updated guidelines, which were presented to the public during the workshop. The current panel consists of the members of the original panel that actively participated in the discussion of the updated guidelines, and new active members were recruited to maintain balanced expertise. The current panel received no funding for its activity.

The current European HIV Drug Resistance Panel consists of experts from mainly European countries, 16 clinicians, 18 academic virologists, 10 representatives from industry involved in the development of assays for resistance testing or of antiviral agents, one statistician/epidemiologist as well as two representatives of international organizations (health policy and patient groups). Discussions were undertaken during five

online and actual discussion meetings from December 2002 to September 2003, during which it became clear that the specificities of the European situation call for recommendations and documentation that are not reflected in other guidelines on the use of drug resistance testing [4–11] as discussed in this article. The recommendations as mentioned in this article were voted on by the panel, the level of consensus in Table 1 reflects the result of this voting. The final document was approved by all panel members.

The recommendations of the panel are intended to help the clinician in designing the best possible long-term therapy strategy for an individual patient (see 'Clinical indications for drug resistance testing' section), to help laboratories in proper reporting to the clinician (see 'Genotyping or phenotyping' and 'Interpretation issues' sections) and to propose measures for quality control to the authorities (see 'Laboratory quality control requirements for sequencing' section). The recommendations summarized in Table 1 are graded to indicate strength of recommendation and level of evidence and in addition contain the level of consensus across the panel. Drug resistance testing will allow identification of drugs with probable reduced activity towards the patient virus, resulting in advice on the exclusion of such drugs in an optimal next therapy. Drug resistance testing is also valuable to provide epidemiological data throughout Europe.

General concepts of anti-HIV drug resistance

Resistance reduces therapeutic options

Current therapeutic choices for the treatment of HIV infections remain limited. While 20 different anti-HIV drugs from four classes are available to the patient, these are used in combinations of three or more drugs [6,12]. Therapy failure with resistance compromising the next-line combination is a common problem, since cross resistance within each class can be extensive [13,14]. With every subsequent therapy failure, multi-class resistance may accumulate, reducing chances of prolonged therapy response.

Virus population dynamics and fitness of resistant virus

Drug resistance is the result of virus population dynamics under drug selective pressure [15]. Resistance is both the cause and the consequence of virus replication in the presence of drug. Virus replication allows the genetic variability of the virus to increase. Residual replication under drug selective pressure has as a consequence that random resistance mutations arising in a minority of the virus population give the variant a selective advantage. Drug selective pressure allows such resistant variants to become predominant due to a shift in the virus population. As a result, the majority virus population becomes fitter, replication increases and further accumulation of resistance mutations is possible. Since resistant virus often shows reduced fitness [16] in absence of drug, more fit wild-type virus can gradually replace the resistant mutant in the absence of selective pressure of the drug, for example during treatment interruption [17,18]. In contrast, although transmitted resistance mutations can revert to wild-type [19,20], they have been reported to persist in plasma for many years after infection [21,22], probably reflecting different population dynamics following infection with a drug-resistant strain in the absence of wild-type virus.

Resistance remains archived in the body

In the case of HIV, which infects long-lived cells, the history of genotypes remains archived [23,24]. Therefore, wild-type or resistant variants acquired during the treatment history of the patient are unlikely to completely disappear from the body with currently available drugs, even though they may have become minor variants. Since current clinically used resistance assays only uncover resistance in the majority virus population in the plasma of the patient at the time of testing, such archived resistant variants may be undetectable on resistance tests. Which resistant strains eventually archived in reservoirs may later reappear and affect future therapeutic attempts is still being investigated. Monitoring minor resistant variants therefore currently remains a research tool.

Superinfection

Superinfections with a new strain carrying different resistance patterns, co-existing with or replacing the original virus have been reported [25–27]. Recent reports of new intersubtype recombinants [28,29] also suggest that superinfection does occur. The low levels of superinfection detected in a large database including resistance genotypic information of successive samples of patients would suggest that superinfection is not a common phenomenon [30]. Considering also the argument that resistance mutations can disappear in absence of therapy, retrospective testing in drug-naive patients is an acceptable strategy. Superinfection risk should, however, always be kept in mind when judging the result of retrospective resistance testing.

Genotyping and phenotyping

Resistance results from phenotypic changes in target proteins (RT, PRO, ENV) as a result of virus evolution under the selective pressure of the drugs. Such phenotypic changes are always caused by genotypic changes [3]. Thus in theory, genotype predicts phenotype, and phenotype predicts therapy failure. It was therefore

originally anticipated that phenotyping would give a more accurate estimate of resistance and consequent therapy failure than genotyping. Because of technical limitations and differences in genetic barriers to resistance, the relationship between genotype and phenotype towards therapy response is more complex.

Genotypic drug resistance assays aim to detect mutations that are known to confer phenotypic drug resistance or to compromise treatment response. Therefore, genotypic test results always need to be interpreted using prior knowledge of the effect of such mutations. Current clinically used assays all involve sequencing the genes whose gene products are targeted by the drugs: protease, reverse transcriptase and envelope. Phenotypic assays measure the ability of an HIV-1 isolate to grow *in vitro* in the presence of an inhibitor, in comparison with a known susceptible strain. Current clinically used phenotypic assays are all based on recombining the target genes of a patient isolate into the genetic background of a laboratory (subtype B) strain, called a recombinant virus assay. For details about the methodologies, see reviews such as Vandamme *et al.* [31,32], Shafer *et al.* [33] and Schmidt *et al.* [34].

Typically, genotypic assays have a faster turnaround time, 1 to 2 weeks, while for phenotypic assays up to 4 weeks may be needed. Both types of assays start from PCR-amplified target genes. Genotyping then proceeds with sequencing, techniques that, although demanding, can be performed in many dedicated laboratories. Phenotyping requires further processing of the patient-derived amplicon in a biosafety level (BL) 2 or BL3 laboratory and is performed in a few specialized laboratories. In general, the cost of genotyping is 50% or less of the price of a phenotype test. None of the currently clinically used genotypic or phenotypic assays are able to reliably detect minor resistant variants present below 20–30% of the total viral population, although some specialized genotypic assays selectively detect mutations at a much lower detection limit [35,36]. The most significant challenge for resistance assays is, however, the translation of the results obtained into clinically relevant guidance (see 'Interpretation issues' section). Since genotyping relies on prior knowledge of particular mutations and combinations of mutations for their relevance in resistance phenotype or therapy response, this interpretation is complex and needs continuous updating. Phenotypic test results provide quantitative measures of the impact of all mutations, known and unknown, but the levels of phenotypic drug resistance associated with reduced therapeutic response (clinical cut-off, see 'Interpretation issues' section) are difficult to determine for several drugs.

European issues

Transmission of drug resistance

Transmission of resistant virus has been documented in all countries that have surveyed for it (Table 2). However, the data reported are not directly comparable due to differences in sampling strategies, technologies used and criteria used to score the transmission of a resistant virus. In the US, estimates of the proportion of untreated people infected with viral strains displaying resistance towards particular drugs vary between 1% and 27%, while in Europe the range has been 2% to 52% (Table 2). Preliminary results from the Catch study, in which available European sequences from drug-naive patients were retrospectively collected and analysed in a consistent way, suggest a general prevalence of 10.5% in Europe with large inter-country differences [37]. Such geographic differences affect the decision whether or not to test for drug resistance prior to initial therapy. Ideally, in order to calculate the prevalence in treatment-naive individuals, data should be collected prospectively, using representative sampling and a uniform technology and analysis plan, as done in the SPREAD study for Europe [38]. Some mutations reported in drug-naive patients are not the result of transmitted resistance but are natural polymorphisms, especially in non-B subtypes. It is not yet clear how these may affect initial treatment responses. Other mutations, such as reversal mutations (Y215A/C/D/S) that have a wild-type phenotype, are a 'signature' of past drug resistance and are associated with reduced therapy response [39,40]. Therefore, dedicated algorithms that score marker mutations for transmission of resistance may be required [41,42]. The panel encourages the timely collection of epidemiological information on the spread of resistant HIV in the different European countries. Considering the feasibility and value of the results, evaluation of such prevalence is preferentially done using a genotyping sequencing approach. Since current algorithms are not built to assess genotypes in the setting of transmission, estimates on prevalences of transmitted resistance should initially take into account only major resistance mutations [13] but, when available, a dedicated algorithm incorporating marker mutations for the transmission of resistant HIV can be consulted.

Genetic diversity of HIV in Europe

Compared with the US, European countries have a much higher and still-rising prevalence of HIV-1 non-B subtypes and of HIV-2 (Table 3), especially in countries with historical ties to Africa and in some Eastern European countries, where the epidemic is driven by particular non-B subtypes [43,44]. The Catch study [45] reports an overall prevalence of 30% non-B

Table 1. Recommendations for resistance testing in the European setting

Clinical indication	Communication to clinicians	Motivation	Grading of recommendation, level of evidence and consensus	Comments
Drug-naive patient with acute or recent infection (<6 months)	Test earliest sample for drug resistance, do not delay therapy	Optimal choice of the first regimen is crucial. Resistance testing may not be sufficiently cost-effective in some European countries with low levels of transmission of resistance	AII Panel consensus 82%	If resistance testing is not performed, store the earliest plasma sample for later testing (2 ml of plasma at −80°C)
Drug-naive patient with chronic infection	When treatment start is considered, test earliest sample for drug resistance if suspicion of resistance is high or prevalence of resistance ≥10%, otherwise strongly consider	Optimal choice of the first regimen is crucial. Expected resistance reversal limits the usefulness of testing in this population. Resistance testing may not be sufficiently cost-effective in some European countries with low levels of transmission of resistance	AII (if suspicion of resistance is high or prevalence of resistance ≥10%) BII (otherwise) Panel consensus 72%	Genotyping becomes cost effective when prevalence of resistance is ≥5%, phenotyping when ≥10%. Await resistance test results before starting treatment. Collect epidemiological information on prevalences of transmission of drug resistance
Therapy failure	When treatment change is considered, test sample taken on therapy	Assessing the contribution of resistance in therapy failure improves virological response to next-line therapy. Treatment failure is defined as i) a viral load reduction of less than 1 log within 4 weeks, ii) not reaching undetectable within 6 months and iii) confirmed virological rebound	AI Panel consensus 100%	Resistance testing during treatment interruption is difficult to interpret. Test results need to be interpreted in view of treatment and resistance history
Pregnant women	Test before starting or changing therapy when viral load is detectable	Resistance testing benefits both mother and prevention of transmission to infant Number of cases is limited, cost issues are less important	AIII Panel consensus 100%	
Paediatric patients	Test before starting or changing therapy when viral load is detectable	Choosing optimal therapy is more crucial than in adults, since fewer treatment options are available. Number of cases is limited, cost issues are less important	AIII Panel consensus 100%	
Post exposure prophylaxis (PEP)	Genotype index case when available, do not delay PEP	Resistance test result has to be available within 2 weeks. Number of cases is limited, cost issues are less important	AIII Panel consensus 100%	Genotyping is faster than phenotyping

Table 1. *(Continued)*

Technical issues	Communication to laboratories	Motivation	Level of evidence and consensus	Comments
Which assay to use	No recommendation to choose one assay over the other	Genotyping and phenotyping provide complementary results and have been shown to be associated with therapy response in retrospective studies, in prospective studies genotyping resulted in significantly better therapy outcome. For new drugs and in heavily pretreated patients, phenotyping can provide useful information where genotyping is not easily interpretable. In urgent situations, genotyping is recommended (PEP, acute infection)	I Panel consensus 56%	In practice, genotyping will be used more often, because of its better accessibility, lower cost and faster turnaround time
Interpretation issues	For genotyping, use clinically evaluated interpretation algorithms. For phenotyping, use clinical cut-off when available	Both genotypic and phenotypic results are difficult to interpret. An expert system taking into account clinical relevance is always needed	II Panel consensus 100%	A genotypic report should include a list of mutations and the expert system used (algorithm and version). A phenotype should include fold resistance values, technical, biological and clinical cut-off, if available. Resistance reports should be considered as constraints against the use of drugs with evidence of resistance
Laboratory quality control requirements for sequencing proposed to the authorities	1. Include proper negative and positive controls during extraction/PCR 2. Editing of the sequence should be traceable 3. Resistance-related positions should be evaluated by sequencing in two directions 4. Each laboratory should pass a proficiency panel test at least once a year 5. At least every 2 months or every 50 sequences (whatever comes first), a known genotype should be resequenced 6. Interpretation of the results should be documented	Scoring mutations and thus interpretation of resistance test results is influenced by the laboratory performance	III Panel consensus 97%	Proficiency panel should contain plasma samples, including those with low viral load, different subtypes and samples with mixtures. No major discrepancies and <50% minor discrepancies are required for proficiency panel testing

Table 2. Prevalence of resistance mutations in drug-naive patients

Country or region	% with mutations (excluding secondary PI)*	Selected patient groups
Belgium	30 (LiPA)†	Newly diagnosed 1995–1998 [118]
	8.1 (seq)	Newly diagnosed in 2003 [119]
Denmark	2 (RTI), 0 (PI)	Newly diagnosed in 2000 [120]
France	4	Chronically infected 1998 [121]
	10 (overall), 8 (NRTI), 4 (NNRTI), 6 (PI)	Primary infection 1999–2000 [122]
Germany	9 (RTI), 5 (PI)	Recent seroconverters 1996–1999 [123]
Greece	6	Therapy-naive 2002–2003 [124]
Italy	13 (NRTI),1 (NNRTI), 1 (PI)	Seroconverters 1996–2000 [125]
	8 (RTI), 5 (NNRTI), 1 (PI)	Chronically infected before 2000 [77]
Luxembourg	14, 9 (LiPA)	Newly diagnosed 1996, 1997 [126]
	13	Newly diagnosed 1998–2002 [127]
Poland	52 (LiPA)	Chronically infected in 2000–2001 [128]
Portugal	13	Therapy-naive 2000 [129]
	17	Primary infection 1999 [130]
Serbia-Montenegro	8	Newly diagnosed 2002–2003 [131]
Spain	4	Recently infected 2000–2001 [132]
Sweden	3	Newly diagnosed 1996–1998 [133]
Switzerland	10 (NRTI), 2 (NNRTI), 4 (PI)	Diagnosed 1996–1998 [134]
	9 (NRTI), 1 (NNRTI), 4 (PI)	Seroconverters 1996–1998 [135]
The Netherlands	8	Newly diagnosed 1996–2000 [45]
UK	14	Primary infection 1994–2000 [136]
US military	1 (NRTI), 6 (NNRTI), 1 (PI)	Recently infected 1997–1998 [137]
Seattle/Los Angeles, USA	1 (NRTI), 3 (NNRTI), 0 (PI)	Recently infected 1997–1999 [138]
San Francisco, USA	27	Recent seroconverters 2000–2001 [74]
USA	23	Recent seroconverters 1999–2001 [75]
St Louis, USA	17	Recently infected 1999–2001 [139]
New York, USA	20	Seroconverters 1999–2001 [140]
Boston, USA	18	Chronically infected in 1999 [141]
British Columbia, Canada	2	Primary HIV infection 1996–1998 [142]
	3 (RTI), 4 (PI)	Diagnosed in 1997–1998 [143]
Vancouver, Canada	19 (RTI), 6 (PI)	Primary HIV infection 1997–1999 [144]
Sydney, Australia	9 (RTI), 0 (PI)	Primary HIV infection 1992–2001 [145]

*Secondary PI mutations (listed as minor mutations in D'Aquila *et al.* [13] were not taken into account. †LiPA, line probe assay. LiPA is a resistance test based on reverse hybridization of amplified HIV gene fragments to short oligonucleotide probes for wild-type (WT) or mutant (MT) sequences at key resistance codons, immobilized on a nitrocellulose strip [146]. Seq, nucleotide sequencing; RTI, reverse transcriptase inhibitor; PI, protease inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor, NNRTI, non-nucleoside reverse transcriptase inhibitor. Sequencing results are given unless otherwise indicated.

strains but with only few data from Eastern Europe. It may be relevant to be aware of the subtype when performing resistance testing, both for technological and interpretation reasons. Not all resistance assays perform equally well on B and non-B subtype strains [46–48] although continuous efforts are made to improve assays in this respect. Although in general, similar drug resistance mutations are found in subtype B and non-B strains, some differences in resistance pathways according to subtype have been reported. For example, in patients failing a nelfinavir-containing therapy, subtype B strains most commonly develop the D30N marker mutation, whereas those infected with subtypes A, C or G more frequently develop the L90M mutation [49–52]. Similarly, V106M, upon failure with a non-nucleoside reverse transcriptase inhibitor (NNRTI), is more often observed in subtype C [53,54] while L210W is less often observed upon failure with nucleoside reverse transcriptase inhibitors (NRTIs) in subtype F [55]. Non-B subtype strains also have a higher prevalence of secondary – or minor – protease inhibitor (PI) resistance mutations [56,57] and new variants at NNRTI resistance-related positions [51]. Recently, a new mutation 89L/I was identified, associated with PI therapy failure and phenotypic drug resistance in subtypes G and F but not in subtype B [58,59]. Finally, genotypic drug resistance interpretation systems are more frequently discordant for some non-B subtypes [60]. Since treatment response is usually measured using virological criteria, an additional complexity is added since not all viral load assays perform equally well across subtypes [61–63].

Table 3. Prevalence of non-B subtypes in Europe and the US

Country	% non-B	Selected patient population
Belgium	32	Newly diagnosed 1985–1994 [147]
	49	Newly diagnosed in 1999 [148]
Denmark	38	Newly diagnosed 2000 [120]
Finland	26	Surveillance 1988–1994 [149]
France	16	Recently diagnosed 1996–1998 [150]
	19	Primary infection 1999–2000 [122]
Germany	33	Recent seroconverters [151]
Greece	33	Patients genotyped for drug resistance in 2002 [152]
Italy	7	Seroconverters 1996–2000 [125]
Luxembourg	20	Newly diagnosed 1983–2000 [153]
Poland	12	Cross-section of population 2001–2002 [154]
Portugal	18, 13	Cross-section of population 1998–2000: Lisbon, Porto [155]
	50	Lisbon hospital 1998–2000 [156]
Scotland	43	Heterosexually infected 1995–1997 [157]
Spain	22	Newly diagnosed 2000–2004 [158]
Sweden	30	Newly diagnosed 1993 [159]
Switzerland	30	Newly diagnosed 1996–1998 [134]
	31	Recent seroconverters 1996–2001 [160]
The Netherlands	40	Heterosexually infected 1988–1996 [161]
UK	25	Cross-section of population 1997 [162]
USA	7	Military seroconverters 1997–2000 [163]
USA	1	Cross-section of Bronx, New York [164]
USA	10	Chronically infected, Boston 1999 [141]
British Columbia, Canada	4	Therapy-naive, diagnosed in 1997–1998 [165]

Therefore the panel recommends collecting more data on therapy response in non-B subtypes, differentiating between the different non-B subtypes and planning clinical studies with respect to the subtype differences in Europe as well. Epidemiological data on the prevalence of non-B subtypes should be collected by subtyping sequences obtained from HIV drug-resistance testing, for example.

The highest prevalence of HIV-2 outside Africa is found in Portugal (up to 4%, [64]). Therapeutic decisions are not easy to make when treating HIV-2 patients. Fewer drugs are available, since the virus has a natural resistance to NNRTIs, while resistance pathways for PIs and NRTIs are not yet fully characterized [65–69,70]. Follow-up is also hampered by the limited availability of technologies such as viral load assays and resistance assays. More data are needed before any recommendations can be made for HIV-2 resistance testing.

Implementation throughout Europe

The implementation of resistance testing is not uniform throughout Europe, since reimbursement policies are country-, and in some cases, region-dependent. Yet the same standards should be valid in all European countries. The panel therefore encourages the authorities to take action in order to facilitate the implementation of these standards. The World Health Organization has made suggestions on how resistance testing can be implemented when confronted with limited financial resources [71]. The present guidelines should help in the optimal use of limited financial resources. Resistance test results should also be made available to surveillance programs to allow timely dissemination of epidemiological information.

Continuous education is essential to remain aware of the powers and limitations of HIV drug-resistance testing. HIV resistance expertise contributes greatly to therapy response [72,73]. Education of clinicians and virologists is difficult to organize at a pan-European level, however the panel recommends HIV clinicians and virologists take part in continuous education programs and to consult with each other on a regular basis.

Clinical indications for drug resistance testing

Drug-naive patients: acute or recent infection

The rationale for treating patients with acute or recent HIV infection is to preserve and improve the immune response and to reduce virus spread and viral heterogeneity [49]. Thus, when a clinician decides to treat, treatment initiation cannot await resistance test results. Optimal choice of the first regimen is crucial. Although

only retrospective data are currently available [74–77], it is anticipated that resistance in drug- naive patients may reduce the efficacy of the initial and/or subsequent regimens. Because of reduced fitness in the absence of drugs, transmitted resistance may revert to wild-type [58,59], as argued above. Therefore, early or retrospective testing of the earliest sample may be more representative of potential transmitted resistance. In view of this scientific evidence, the panel considers resistance testing in acute or recent infection more useful than resistance testing in chronic infection. In those patients, the tested virus population is likely to be more representative of the transmitted virus compared with drug-naive chronically infected patients. Identifying such transmitted resistance is valuable information for the clinician when starting treatment of acute infection. In addition, the information can be easily stored to help guide the first treatment choice in case treatment is not to be initiated.

Acute or recent infection is defined as documented seroconversion within the previous 6 months [12]. Resistance testing of the earliest sample available is recommended, irrespective of whether treatment is to be initiated (Table 1). However, treatment initiation should not be delayed because of resistance testing, rather the initial therapy can be revised if necessary once test results are available.

Considering the limited resources for resistance testing in some European countries, the issue of cost effectiveness weighs heavily on the discussion about performing resistance testing for drug-naive patients. Therefore, in areas where current rates of transmission of resistance are very low in patients at risk and when treatment is not to be initiated, an acceptable alternative can be to store the earliest plasma sample available. Storing samples in general is a useful strategy, allowing retrospective testing with updated techniques and knowledge.

Drug-naive patients: chronic infection

A recent study suggested that for chronically infected patients, carrying a resistant virus before starting therapy is associated with a worse virological outcome of the first regimen [78]. The rationale of testing for drug resistance in chronic infection is therefore the same as for acute or recent infection: optimal choice of the first regimen is crucial. However, because of the reduced efficiency of detecting possible transmitted resistance mutations after a prolonged period of absence of drug selective pressure, as argued above, and because of the far larger number of patients potentially involved, cost issues become more important for this patient population. Therefore, when treatment is to be initiated, the panel recommends testing for drug resistance if suspicion of transmission of resistance is high (for example, partner has resistance) (Table 1). The panel recommends testing the earliest sample available, taking care, however, with the interpretation of the results when superinfection is suspected, as argued above. Since starting therapy is not an urgent issue in this population, the resistance test result can be awaited before starting therapy.

The panel also recommends considering the local prevalence of resistance mutations in the drug-naive population and to test for drug resistance when this prevalence exceeds 10%. Even if the prevalence is below 10%, we still suggest that resistance testing be strongly considered (Table 1). The data obtained by the Catch study (prevalence of drug resistance in European drug-naive patients is 10.5%; [45]) and the information provided in Table 2 could be considered indicative if more recent data on the drug-naive population in the geographic area are not at hand. In most European countries the 10% cut-off is exceeded and hence resistance testing is recommended. The 10% cut-off is based on cost-effectiveness arguments as outlined in Weinstein *et al.* [79] and in Holtzer & Youle [80], whose calculations were based on the virological benefit of drug resistance testing in prospective trials with drug-experienced patients. Taking into account the observed degree of resistance in the reported prospective studies and the reduction in virological failure in the arm on resistance testing, it can be argued that the probability of failure decreases by 25% for patients with resistance that receive resistance testing. However, it is likely that the virological benefit of drug resistance testing in a chronically infected drug-naive population will be more modest, depending on the efficiency of detecting transmitted drug resistance, the set of mutations transmitted and consideration of the power of the first regimen. If one assumes that resistance testing will result in a 5% reduction in probability of failure to the first regimen [80], then genotyping would be cost-effective when the prevalence of drug resistance is more than 5% in the relevant drug-naive population, while phenotyping is cost-effective at a prevalence of more than 10%. Such models require validation. Therefore, the prevalence cut-off for resistance testing mentioned here should be considered with great caution, and should be adapted as soon as more data become available. In countries with limited resources, it may be useful to prioritize resistance testing based on prevalences in the relevant risk group populations, if such data are available. Should resistance testing not be performed, it is always useful to store a sample for later testing or for epidemiological purposes.

Drug-exposed patients: treatment failure

Today, additional prospective data confirm that drug resistance testing at therapy failure improves virological

response to the next-line therapy (Table 4). In several studies using an intention-to-treat and primary endpoint analysis, virological benefit of resistance testing versus no resistance testing to guide the next therapy is significant but limited (Viradapt, Gart, Havana and Argenta), while some studies could not show an improved virological response (Kaiser, VIRA3001, Narval, CCTG575 and CERT). More detailed analysis of the VIRA3001 results favoured phenotyping over no resistance testing, while additional analysis of the Narval data indicate a virological benefit of genotypic drug resistance testing. The limited effect of drug resistance testing is most probably due in part to lack of therapeutic options in the face of the extensive pre-existing resistance found in these heavily pretreated study populations. Suboptimal interpretation of resistance data are also in part responsible for the only modest benefits seen. In addition, treatment failure can be caused by other factors such as weak drug potency, lack of drug adherence and pharmacokinetic issues. Resistance testing should allow proper evaluation of resistance as one of the causes of treatment failure, allowing exclusion of drugs for which resistance is found from the next-line therapy, provided sufficient treatment options remain available.

The panel maintains its recommendation to test for drug resistance at treatment failure when treatment change is being considered (Table 1). Moreover, current concepts of treatment failure are extended to also include suboptimal virological response, since suboptimal virological response can be due to the presence of (a limited number of or minor variants of) resistance mutations. The panel therefore defines treatment failure as i) a viral load reduction of less than 1 log within 4 weeks, ii) not reaching undetectable viral load within approximately 6 months (current detection limits of viral load assays are 50 RNA copies/ml), iii) confirmed virological rebound; in accordance with the treatment guidelines [6,12,81].

The panel recommends testing a sample taken when on therapy. Treatment interruption to induce resistance reversal is a controversial strategy [82–84] and resistance testing during treatment interruption can give misleading results [85]. Therefore, the panel cannot make any recommendation with regard to resistance testing during treatment interruption until further studies clarify the utility of this therapeutic strategy and under which conditions it can be applied. However, storing a sample can be considered in order to keep all options open. In situations when restarting treatment after an interruption, with no information on resistance history or no on-therapy sample from previous failure stored, clinicians may chose to consider results of a resistance test on an off-treatment sample to help guide the next therapy. However, interpretation may be very difficult since, due to the usually

Table 4. Prospective studies for resistance testing

Study	Design	Primary endpoint	Difference in virological response between arms*
Viradapt [166]	G vs SOC[†]	ΔVL W12: −1.04 vs −0.46 log	0.58 log; *P*=0.01
		ΔVL W24: −1.15 vs −0.67 log	0.48 log; *P*=0.05
Gart [167]	G+EA[‡] vs SOC	ΔVL W8: −1.19 vs −0.61 log	0.58 log; *P*<0.001
Kaiser [168]	P vs SOC	ΔVL W12: −0.2 vs −0.4 log	0.2 log; NS
VIRA3001 [169]	P vs SOC	%<400[§] W16: 46% vs 34%	12%; *P*=0.079
Narval [170]	P vs G vs SOC	%<200 W12: 35% vs 44% vs 36%	−1% (P), 8% (G); NS
Havana [72]	G vs −G	%<400 W24: 49% vs 36%	13%; *P*<0.05
	EA vs −EA	%<400 W24: 47% vs 37%	10%; NS
Argenta [107]	G+EA vs SOC+EA	%<500 W12: 27% vs 12%	15%; *P*=0.01
		%<500 W24: 21% vs 17%	4%; NS
CCTG575 [171]	P vs SOC	%<400: W24 48% vs 48%	0%; NS
		ΔVL: −0.71 vs −0.69 log	0.02 log; NS
CERT [92]	P vs G vs SOC	Days to failure: 736 vs 799 vs 585	151 (P), 214 (G); NS
RealVirFen [172]	P vs VP	ΔVL W24: −0.92 vs −1.3	0.4 log; *P*=0.01
GenPherex [173]	P vs VP	%<400 W48: 20% vs 24%	4%; NS
Vihres [174]	P+EA vs G+EA	%<200 W48: 38% vs 28%	NS
ERA [91]	G+P vs G	ΔVL W48: −1.38 vs −1.37	NS

*Difference between arms expressed in difference in viral load drop (log_{10} copies/ml) or difference in % reaching undetectable (%), or difference in time to failure, significance according to an intention-to-treat analysis; [†]SOC, standard of care, at that time without resistance testing. Four of the recent studies (RealVirFen, GenPherex, Vihres and ERA) did not include an arm without resistance testing. [‡]EA, expert advice consisting of an expert panel; [§]%<: % of patients reaching undetectable viral load according to the indicated detection limit in copies/ml. For CERT, primary endpoint was time to persistent failure despite treatment change in days (persistent failure defined as VL >log 3 AND <ΔVL of 1 log at W4, or VL >200 copies/ml at W6 or viral rebound). G, genotyping; P, phenotyping; VP, *Virtual*Phenotype (see Table 5); ΔVL, viral load response expressed in log_{10} copies/ml; W, week after change of therapy; NS, not significant.

better fitness of wild-type virus in absence of drug selective pressure, some resistant variants may not be detected any more in the majority population, although they have been archived in the body.

Special populations: pregnant women, paediatric patients and post-exposure prophylaxis

The panel maintains its recommendations to test pregnant women and paediatric patients with detectable viral load before starting or changing (prophylaxis) therapy (Table 1). Optimal therapy in pregnant women not only serves the mother but also contributes to prevention of transmission to the infant. The risk of development of resistance in mothers receiving short-term treatment (to prevent transmission) with single drugs of low genetic barrier (such as lamivudine, NNRTIs) is substantial [86,87] and resistance can continue to develop even after stopping treatment, especially for drugs with low genetic barrier and a long half-life [88,89]. Therefore, special care should be taken when following up pregnant women and mothers. In paediatric patients, choosing the best treatment is even more crucial than in adults, since fewer treatment options are available. In both special populations, the cost issues are less of a concern, since these populations are still small in Europe.

The recommendation to test for drug resistance in cases of post-exposure prophylaxis (PEP) also still holds (Table 1). Treatment should, however, be initiated without delay. In such cases, genotypic results on a sample from the index case, when available, can be taken into account when modifying the treatment, if necessary. The purpose of PEP is to prevent or abrogate infection in the recipient by treating with a powerful combination for a period of 4 weeks. Any resistance result should therefore return to the clinician within a period useful to change the prophylaxis itself. Phenotypic test results may not be available within this time frame (see also 'Technical considerations' section), hence the recommendation is to genotype as soon as possible but within 2 weeks at the latest.

Technical considerations

Genotyping or phenotyping?

The panel discussed at length the issue of which test to use and reached the recommendation not to favour genotyping over phenotyping with the lowest level of consensus compared with the other recommendations in this document (Table 1). The panel felt that genotyping and phenotyping provide complementary information, however we recommend genotyping in specific urgent situations, such as for PEP, or for treatment of acute infection, because of the rapid turnaround time. In practice, genotyping is more often performed than phenotyping because of its better accessibility, lower cost and faster turnaround time.

Relatively few clinical studies have made direct comparisons between the two methodologies. Both types of assays have shown to be correlated with therapy response in retrospective studies [90]. A prospective direct comparison between genotyping and phenotyping did not show a significantly better therapy response in either the genotyping or phenotyping arm compared with no resistance testing (Narval, CERT, see Table 4). In an intention-to-treat analysis using primary endpoints, none of the prospective studies comparing phenotyping to no resistance testing (termed, at that time, standard of care) could convincingly show a benefit of resistance testing (Kaiser, VIRA3001, CCTG575, see Table 4), although for VIRA3001, secondary endpoint analysis favoured phenotyping over no resistance testing, while the Kaiser study was underpowered to detect a difference because of under-enrolment. All four prospective studies using genotyping resulted in a better therapy outcome (Viradapt, Gart, Havana, Argenta, see Table 4). Among direct comparisons of genotyping to phenotyping, one of the five studies (RealVirFen) showed a significant benefit of genotyping over phenotyping (Narval, CERT, RealVirFen, GenPherex, Vihres, see Table 4, considering *Virtual*Phenotype as a genotypic analysis). A more detailed analysis of the Narval results shows that randomization to the genotyping arm was significantly associated with good therapy response, indicating a virological benefit of genotypic drug resistance testing. In addition, phenotypic tests did not provide benefit over and above genotypic testing in highly drug-experienced patients enrolled in the ERA trial [91]. However, Wegner *et al.* [92] provide indications that for heavily pretreated patients for which interpretation of the genotype is very complicated, phenotyping may improve therapy outcome.

Possible causes for the apparently greater usefulness of genotyping may be that genotyping allows the detection of mutations such as reversal mutations (215A/C/D/S) as 'signatures' of past drug resistance [39,40]. These may not contribute to a significantly reduced phenotypic susceptibility by themselves, but may contribute to therapy failure. Due to virus population dynamics, as discussed above, (renewed) selective pressure of drugs to which resistance has been archived may result in a quick shift of the virus population in favour of the archived resistance variant. Some secondary PI mutations have been reported to be associated with therapy failure, although they only contribute to phenotypic resistance in the presence of primary mutations [93,94]. Finally, genotyping also allows the detection of mutations associated with failure of drugs for which clinically relevant phenotypic

cut-offs are within the reproducibility range of the assay, as shown for stavudine [95,96], or are not sufficiently documented.

Both phenotyping and genotyping are essential when new drugs become available for which reliable genotypic interpretation systems are still being developed, such as for the entry inhibitor T-20.

To guarantee optimal results from resistance testing, the sample should be plasma obtained before starting, stopping or changing therapy, with a viral load above the detection limit of the resistance test, typically around 1000 RNA copies/ml. Storage conditions should be such that the integrity of the virus RNA is maintained. Storing a 2 ml plasma sample at –80°C will serve this purpose. Transportation should be performed by specialized carrier services.

Interpretation issues

Interpretation of resistance is crucial. Since therapy response is dependant on many more factors than resistance only, resistance test results should be interpreted in the context of all factors important for therapy response. A first challenge is to interpret genotypic and phenotypic resistance test results into different levels of constraint against the use of particular drugs. Our knowledge of mutations conferring cross-resistance or antagonism, of reversal mutations and of clinically relevant phenotypic cut-offs is still insufficient.

Resistance is not a discrete variable. However, most genotypic interpretation systems consider discrete categories (for example, susceptible, reduced susceptibility and resistant), a necessity resulting from the need to give simple and straightforward advice, but also because such systems are more easily designed [97]. Therefore, residual drug activity may remain, even when drugs are scored as resistant. The most frequently used clinically available systems are listed in Table 5. These systems are based on two different concepts. Some systems try primarily to predict phenotype under the assumption that phenotype predicts therapy response, such as Geno2Pheno and *Virtual*Phenotype, both of which use databases of genotypic and correlated phenotypic data. Other systems try primarily to predict therapy response, such as all the other systems

Table 5. Clinically available genotypic drug interpretation systems

Interpretation system*	Source	Clinical evaluation	Levels	Access
Stanford, β-test version	Experts rules-based	Yes, retrospectively [100]	S/PL/LL/IR/HR	http://hivdb.stanford.edu/
*Virtual*Phenotype	Database (>29 000 G/P)	Yes, retrospectively [90,102,169] and prospectively [172,173]	Quantitative. Resistance 'likely' or 'unlikely', for rare patterns	http://www.vircolab.com
Geno2pheno, v2.1	Database (>600 G/P)	Yes, retrospectively [175]	S/R Quantitative	http://www.genafor.org
RetroGram™, v1.6	Experts rules-based	Yes, retrospectively (V1.4) [100,102] and prospectively (V1.0) [72]	A/B/C/D/U	http://www.retrogram.com
Rega v6.3, Belgium	Experts rules-based	Yes, retrospectively (V5.5) [99,100], (V6.0) [101]	S/I/R	http://www.kuleuven.ac.be/rega/cev/links/ http://www.ablnetworks.org http://hivdb.stanford.edu/pages/asi/
CHL v3.2, Luxembourg	Experts rules-based	Yes, retrospectively [100]	S/I/R	http://www.ablnetworks.org
ANRS v2003, France	Experts rules-based	Yes, retrospectively [170]	S/I/R	http://www.hivfrenchresistance.org/index.html http://hivdb.stanford.edu/pages/asi/
TruGene™, VGI/Bayer	Experts rules-based	Yes, retrospectively [102,107]	S/I/R	http://www.trugene.com
ViroSeq™, ABI/Abbott	Experts rules-based	No	S/PM/P/HM/H	Sven.Thamm@abbott.com
GeneSeqHIV (v 3.0)	Experts and Database (>38 000 G/P) rules-based	No	S/R	http://www.virologichiv.com

*These algorithms are regularly updated, please visit the indicated websites. A, B, C and D are a ranking system used for Retrogram, where A indicates no evidence for resistance and D indicates evidence for high level resistance, while U is used to indicate uncertainties. S, susceptible; PL, possible low-level resistance; LL, low-level resistance; IR or I, intermediate resistance; HR, high level resistance; R, resistance; PM, possible multi-NRTI resistance; P, possible resistance; HM, high-level multi-NRTI resistance; H, high-level resistance; G/P, genotype/phenotype.

listed in Table 5. The latter systems currently rely on 'rules' devised by experts using information extracted from databases of genotypic and correlated phenotypic or treatment response data (for example, [98]). The aim of interpretation systems is to predict therapy response, thus they are validated for their clinical relevance by evaluating their predictive power for therapy response and failure in retrospective and, when possible, prospective studies (for example, [47,99–101]). In a retrospective analysis of the Genpherex results, Retrogram and Trugene, two rules-based systems trying to predict therapy response, performed better than *Virtual*Phenotype (Virco), which tries to predict phenotype, or better than real phenotyping [102]. Even among systems that have been shown to be correlated with therapy outcome, significant discrepancies in interpretation exist, particularly for non-B strains [99,100,103–105].

For the interpretation of phenotypic results, appreciation of several parameters is important. Assay reproducibility, often captured as a technical cut-off, indicates what differences in levels of resistance can be reliably measured. Some phenotype assays provide biological cut-offs indicating the variation in phenotype of isolates from untreated individuals, below which a sample cannot be confirmed to have acquired resistance as a result of drug selective pressure. A currently developing area is the definition of clinically relevant cut-offs, providing guidance as to levels of reduced drug susceptibility that compromise therapeutic response to each drug. To date, clinical cut-offs have only been defined for a few drugs, often only in specific patient populations or drug combinations. In a retrospective multivariate analysis, it was shown that some clinically relevant cut-offs are very close to the biological cut-offs [106].

For genotypic interpretation systems, the panel recommends use of clinically validated algorithms. A genotypic report should include a list of observed drug-related resistance mutations and an interpretation of the results with indications of which expert system was used (identifying the algorithm and version). For phenotypic interpretation, consideration of the clinical cut-off of the assay is recommended, if available. A phenotypic report should include a list containing the observed fold-reduced susceptibility towards clinically used drugs and an interpretation of the results with indications of technical, biological and preferentially clinical cut-offs when available. Resistance test results should be interpreted as different levels of constraint against a particular drug, since therapy response is dependant on many more factors than resistance only. Interpretation system developers should update their system regularly and compare its performance with other available systems.

The panel recognizes that there is still much room for improvement of interpretation systems. This can be done by analysing large clinical datasets with improved analysis methods (for example, [98], The Forum For Collaborative HIV Research, www.hivforum.org), by comparing interpretation systems and, preferentially, the individual rules in retrospective analysis [100,105], by performing basic research to better understand the relationship between mutations and resistance and by integrating pharmacological information.

Proper interpretation can only be done in view of treatment history and previous resistance test results. The clinician is therefore encouraged to provide the laboratory with a complete treatment history. Interpretation should also be done in the context of adherence. Lack of adherence can result in suboptimal therapy response in absence of resistance, but lack of adherence is also correlated with resistance development [107,108]. For drugs with a low genetic barrier, such as 3TC and the current NNRTIs, absence of resistance in the face of virological failure should alert the physician to poor adherence. Another important issue is the measurement of drug levels. Drug levels and the inhibitory quotient (IQ) are correlated with therapy response ($IQ=C_{trough}/IC_{50}$, C_{trough} is a measure of the drug level, IC_{50} is a measure of resistance to the drug) [84,109–115]. Insufficient drug levels may put the patients at higher risk of treatment failure and development of resistance mutations, and intermediate resistance may be overcome by high drug levels, as in the case of boosted PI. Measures of drug levels can potentially be used to decrease dosing in case of toxicity, to prompt increase in dosing to prevent or overcome therapy failure, or to stress adherence if required. Integrating drug level monitoring with resistance test results has not been sufficiently evaluated prospectively [84,111,115] therefore no recommendations can be made on this issue. Although the role of pharmacology in the interpretation of resistance testing is still undergoing definition [116], if drug levels are available, discussion with a HIV pharmacology expert may benefit the interpretation of resistance testing results and the selection of an optimal therapy.

The panel recommends clinicians and virologists to discuss resistance test results of complex cases in the context of all other factors that can influence therapy response and to remain aware of the difficulties associated with interpretation of HIV drug resistance.

Laboratory quality control requirements for sequencing

Laboratories engaging in drug resistance genotyping should comply to a set of minimal quality control requirements. Quality controls have revealed large

differences between laboratories, in particular in samples containing mixtures [117].

The panel estimates that the following minimal quality control requirements should be mandatory: i) inclusion of proper negative and positive controls during extraction/PCR; ii) editing of the sequence should be traceable; iii) resistance-related positions should be evaluated by sequencing in two directions; iv) participation, at least once a year, in a proficiency panel test; v) at least every 2 months or every 50 sequences (whatever comes first), a known genotype should be resequenced; and vi) interpretation of the results should be documented. In addition, it is advisable to compare consecutive sequences of the same patient and samples tested simultaneously (for example, phylogenetically or using a BLAST approach), which may allow identification of contamination or superinfection. The proficiency panel should contain plasma samples, including those with low viral load, different subtypes and samples with mixtures. Mutants present as mixtures identified in a standard sequencing assay should be scored.

Conclusions

As outlined above, several arguments have stimulated the panel to draft specifically European recommendations. First of all, it is important to keep the guidelines updated, even though for some indications the recommendations have not changed but are supported by more scientific evidence, such as for testing, in case of treatment failure. A major motivation was to document the high prevalence of non-B subtypes in Europe, along with its consequences for performance of resistance assays and interpretation of results. Another important European issue is the prevalence of drug resistance in drug-naive populations, which vary according to geographical area, as documented here. Cost issues weigh heavily on the implementation of resistance testing and many considerations are appreciated differently in the different countries. Since there is no uniform implementation strategy in Europe, the current document is essential in national efforts to help achievement of this implementation.

The clinical implications of resistance data depend on many factors. Clinicians receiving resistance test results should be aware of the difficulties associated with interpretation of the data and should not only rely on resistance test results when suggesting a new therapy. Resistance information should be integrated into the clinical judgement, which includes taking into account factors such as the power of the combination chosen, therapy history, resistance history, regimen convenience and tolerability, drug adherence and drug levels, drug toxicities and interactions, availability of potentially active drugs, and so on. Virologists and clinicians should discuss difficult cases and clinicians should seek expert advice in order to optimize the use of resistance data.

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