Antiviral resistance testing

Erasmus Smit

Purpose of review
Current genotypic resistance tests fail to amplify drug-resistant minority variants when they are present below 20% of the total virus population. Next-generation sequencing (NGS) addresses this issue and is being introduced into diagnostic laboratories. This review gives an overview of the resistance tests currently used and explores the opportunities and challenges that NGS genotypic resistance tests will bring.

Recent findings
The technical challenges of NGS, such as PCR and sequence-related errors, are being addressed and various assays are currently undergoing technical validation for clinical use. Although not conclusive, the data seem to suggest that NGS will be valuable for low genetic barrier drugs and certain types of tests such as the HIV-1 tropism test. Clinical validation of the reporting and interpretation of minority variant results are essential when laboratories start reporting these results.

Summary
The first wave of NGS technology is being rolled out in diagnostic laboratories. Antiviral test benefits include increased sensitivity and eventually cheaper antiviral resistance tests. There is a risk that low percentage minority variants may be over interpreted. This could result in antiviral drugs, which may have been effective, being possibly denied to patients if proper clinical validation studies are not performed.

Keywords
antivirals, minority variants, next-generation sequencing, phenotypic/genotypic resistance tests, quasispecies

INTRODUCTION

Many antiviral treatment guidelines exist that contain advice on when to request antiviral resistance tests. We are, however, on the dawn of more sensitive and cheaper next-generation sequencing (NGS) tests that will change the way we monitor antiviral resistance and influence the clinical management of patients. In spite of a wealth of publications, we still have a lot to learn on how to use the technology in a clinical setting. The aim of this review is to provide an overview of current testing practice and how NGS might change the landscape in the future.

TYPES OF RESISTANCE TESTS

Antiviral susceptibility can be determined by phenotypic and genotypic methods. Phenotypic tests determine resistant phenotype based on the calculation of inhibitory virus growth concentrations for the antiviral drug. The main advantage is an unambiguous interpretation of laboratory findings whilst providing genotype–phenotype correlations of the associated mutational change. It is, however, time-consuming, expensive and not sensitive enough in order to detect minority variants or archived resistance mutations. Phenotypic tests are still largely being used for herpes simplex viruses (HSVs), but disbanding of virus isolation facilities and adoption of NGS technology will make it easier and cheaper to sequence large genes, and therefore the prediction is that genotypic HSV resistance tests will be easier to come by in the future. For HIV-1, their clinical use is restricted to a few specialized laboratories whilst being more popular in certain countries such as the USA. Although not a resistance test per se, phenotypic HIV-1 tropism testing determines the HIV-1 coreceptor usage of patient-derived Env-recombinant viruses to infect reporter cell lines expressing HIV-1 receptors and coreceptors prior to the use of CCR5 antagonists such as maraviroc.

Genotypic resistance testing is faster and is based on PCR amplification and sequencing of viral drug target genes in order to detect drug

Public Health England, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Birmingham, UK

Correspondence to Erasmus Smit, FRCPath, Public Health England, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Bordesley Green East, Birmingham B9 5SS, UK. Tel: +44 121 424 2514; e-mail: Erasmus.Smit@heartofengland.nhs.uk

Curr Opin Infect Dis 2014, 27:566–572
DOI:10.1097/QCO.0000000000000108

KEY POINTS

- Resistance tests should be requested when there has been no clinical improvement or viral load rebound, but it should be kept in mind that for some viral infections there is not always an immediate antiviral response.
- Clinicians are now considering HIV-1 resistance tests as soon as the viral load rebounds above 50 copies/ml. However, there is a greater risk of PCR amplification bias, and the test should be repeated if the viral load rises further.
- Current genotypic resistance tests are insensitive at detecting drug-resistant minority variants.
- NGS increases the ability of genotypic resistance tests to detect minority variants even if they make up as little as 1% of the total virus population.
- Laboratories are switching to NGS technology following technical validation, but clinical validation, reporting and interpretation of minority variants are still lagging behind.

resistance-associated mutations. Various on-line genotypic interpretation systems are available to interpret complex genetic changes using rule-based computational tools. Unfortunately, no database and on-line interpretation tools exist for HSV and varicella zoster virus (VZV) mutations and there can be ambiguity in interpreting numerous amino acid substitutions, whereas only genes encoding the thymidine kinase and DNA polymerase frame shift mutations and stops of translation can be interpreted safely [1–3,4▪].

Genotypic HIV-1 tropism testing is not based on specific amino acid changes, but rather a prediction of the coreceptor usage of the circulating viruses based on the positive charge of the V3 loop in the virus' envelope. Most laboratories use a geno-to-pheno coreceptor estimation model after sequencing to determine whether the virus is R5 or X4 tropic. It is generally treated as a 'resistance' test and requested pretreatment to establish whether the virus is R5 tropic. It is used after failure to see whether there has been a switch to X4 virus which has usually been present as minority variants before starting maraviroc, but which was not amplified by the original genotypic test [5,6]. However, only a phenotypic test can establish whether true maraviroc drug resistance has developed in patients who fail therapy without a tropism switch to X4-tropic virus, as no specific mutations have been associated with resistance [7]. Maraviroc resistance is fortunately a rare occurrence.

Not all genotypic tests rely on sequencing, and certain mutations, such as oseltamivir point mutations in the influenza neuraminidase gene, lend themselves well to single nucleotide polymorphisms (SNPs) testing in which case a simple RT-PCR test can quickly and cheaply establish whether resistance is present. Allele-specific RT-PCR assays have also been designed to detect known HIV point mutations, but it is unlikely that their use will move beyond the research setting.

PRINCIPLES REGARDING REQUESTING AND INTERPRETING RESISTANCE TESTS

Antiviral resistance tests are usually requested following suboptimal antiviral response or viral rebound. Both can be measured objectively by quantitative viral load tests for viral infections such as HIV, hepatitis B virus (HBV) or cytomegalovirus (CMV). Lack of clinical improvement can also be an indication of suboptimal antiviral response, for example, influenza or herpetic ulcers.

Routine baseline antiviral resistance tests are also used to identify transmitted drug resistance prior to starting therapy when the prevalence of circulating resistant strains is high enough to warrant screening, for example, HIV and influenza. Certain naturally occurring hepatitis C virus (HCV) polymorphisms such as Q80K, frequently found among genotype 1a in 19–48% of cases, require exclusion before the use of simeprevir [8▪▪]. Clinical guidelines on managing HIV, CMV, HBV and influenza resistance advise on when to test for antiviral resistance, what sample type as well as how to interpret the absence of drug resistance mutations [9–14].

Virus quasispecies with resistance mutations can disappear quickly out of circulation when they are replaced by more 'fit' wild-type viruses when therapy is stopped and therefore minority variants may be missed. This is especially true for many viruses with high virus turnover such as HIV and HCV. Current HIV-1 guidelines recommend that a resistance test be performed whilst on failing therapy or preferably within 4 weeks of stopping [9,15]. It is also known that a HIV-1 CCR5 tropism test result can change over time and a 90-day cut-off has been applied as a practical time period for which the result is valid [10]. This lack of test sensitivity creates uncertainty for resistance tests, affecting all viral infections. NGS technology is able to detect resistant minority variants, also known as low abundance drug resistance variants, even if they only make up 1% of the circulating virus population.

CLINICAL USEFULNESS OF CURRENT TESTS

The clinical added value that resistance tests currently provide is difficult to establish. Most data

exist on the clinical usefulness of HIV-1 drug resistance tests. Poor adherence to antivirals with the subsequent virological failure is not always associated with drug resistance. It is therefore not uncommon to find no resistance mutations in patients with virological failure, especially if the patient is on 'high genetic barrier' drugs such as boosted protease inhibitors and dolutegravir for HIV-1, tenofovir and entecavir for HBV and sofosbuvir for HCV. For 'low genetic barrier' drugs in which single crucial mutations develop easily, drug resistance is assumed upon virological failure, for example, 3TC or FTC in HIV-1 therapy and all the new HCV direct-acting antiviral (DAA) agents apart from sofosbuvir. In these cases, it is best to stop treatment upon virological failure.

Drug resistance is also usually assumed after prolonged therapy and no clinical response in HSV and VZV infections because resistance tests are not readily available with these infections. It is well known that immunocompromised patients are at higher risk of drug resistance, but HSV resistance should also be considered when HSV infections involve immune-privilege sites, for example, herpetic keratitis and encephalitis [4▪]. Therapy changes are also frequently made in clinical practice without a resistance test after a 2-to 3-week period of poor response to CMV treatment in transplant patients. However, CMV resistance takes weeks to months to develop and many patients see an initial increase in viral load on therapy [16,17]. Patients with severe or progressive influenza who do not clinically improve on neuraminidase inhibitors, especially if they are immunocompromised, should have a resistance test [14].

In a complex viral infection like HIV-1, the usefulness of resistance testing is even harder to define. Most prospective studies demonstrated only modest benefit before resistance tests became standard of care in HIV-1 infections [18–21]. There were many confounding factors that influenced the early trials. A meta-analysis of the earlier clinical trials demonstrated a clinical benefit of only 10% increase of undetectable HIV-1 viral load that was seen by 6 months [22,23].

The amplification rates of HIV-1 genotypic resistance tests in samples with a low viral load have improved over the years, and resistance testing should definitely be performed upon virological failure above 200 copies/ml. However, guideline guidance varies from above 50 copies/ml to above 1000 copies/ml [10,9]. Unfortunately the success rate of the tests drop substantially the lower the viral load. The stochastic bias of amplifying only some viral quasispecies also increases with low viral load, and therefore the possibility of missing resistant minority variant increases. Reassuringly, studies have shown that it is possible to detect resistance mutations at low viral loads [24,25]. Most clinicians act upon the presence of drug-resistant mutations, but their absence does not totally exclude resistance and the test should be repeated when there is a further rise in viral load. The importance and risk of resistance developing in patients with persistent low-level viraemia and recently reported very low-level viraemia is not yet fully understood. This is mainly due to the inaccuracy and insensitivity of resistance tests at such low viral load levels. It is unlikely that NGS will be more useful for low-level viraemias since all tests that rely on initial PCR amplification step will be influenced by the amplification bias.

NEXT-GENERATION SEQUENCING TESTS

Current diagnostic genotypic resistance tests use an insensitive Sanger sequencing method also known as bulk sequencing, whereby the majority or consensus sequence is displayed with some secondary traces if another quasispecies virus is present at more than 25% of the total. NGS technology, however, uses PCR-amplified single-molecule sequencing, that is, all quasispecies are individually sequenced and displayed which enables it to pick up minority variants even as low as 1% of the total. It is also commonly referred to as ultradeep sequencing or second-generation sequencing. Using NGS to sequence near full-length viral genomes is known as whole-genome sequencing (WGS) and has the advantage of providing additional data to NGS resistance gene sequencing [26▪].

TECHNICAL CHALLENGES

NGS is currently mainly used in research settings, but many laboratories are in the process of validating it for their routine diagnostic work. The unprecedented resolution of the technology can be hampered by reverse transcriptase and PCR errors as well as sequence-related errors which obscure the presence of true low-frequency minority variants [27]. Various filtering and quality-checking software solutions are used and together with the assembling and reporting software are referred to as NGS pipelines. It is well established that all platforms suffer from sequencing errors such as insertions and deletions (indel errors) and low intensities. It is important that quality assessment software minimizes sequencing errors in order not to overcall minority variants. It can, however, be difficult to distinguish assay RT-PCR-derived insertions and deletions from viral genetic changes which confer

drug resistance [28]. The RT-PCR step can even cause in-vitro viral strain recombination as is observed *in vivo* HIV infections [29,30]. Another important quality issue is the increased risk for sample contamination due to the immense sensitivity of the assay.

Apart from the need for automation and other technical issues, the biggest challenge for NGS-based assays is the clinical interpretation of the presence of minority variants. This is complicated by the fact that the unreliability of detecting minority variants at specific percentages, for example 1, 2, 5 and 10%, is exacerbated by low viral load levels (input DNAs) due to sampling bias and unequal amplification during the early cycles of PCR. Any PCR spurious nucleotide misincorporation or unequal amplification of quasispecies templates without mutations could result in over or under calling of minority variants due to the fact that high proportion of sequence reads derive from a few input templates [31,32]. The current NGS platforms tend to sequence short fragments, and it is therefore generally not possible to establish the relationship between genetic changes along the genome sequenced.

The next wave of sequencing technology, the so-called third-generation sequencing, using single-molecule real-time (SMRT) sequencing can do away with a PCR enrichment step, but it then suffers from low signal strength and the need for high viral loads. However, SMRT gives much longer read lengths which will probably give clinicians and researchers the true picture of the breadth and depth of individual circulating viruses by sequencing long enough reads to include the whole viral genome, reliably linking mutations to individual viruses. SMRT instruments are already being used in research settings.

COST

Resistance tests costs have generally been falling as laboratories have introduced more automated sample processing. Testing for SNPs using RT-PCRs is by far the cheapest option, but not all resistance mutations are surrounded by the required conserved sequence, nor is it practical when many different resistance mutations need to be tested for in one virus. It is hoped that the sequence capacity of NGS and low cost per nucleotide sequence will drive down costs further once the labour-intensive and expensive library preparation steps have been automated. It would therefore not cost much extra to sequence all HIV-1 drug-associated genes, that is, Reverse Transcriptase, Protease, Integrase and Envelope when a resistance test is requested [33]. This should ensure further cost savings since the current integrase resistance test is a separate standalone test which is more frequently requested as there is a move to use integrase inhibitors as first-line combination antiretroviral therapy (cART). There is also the possibility of sequencing samples in bulk which can potentially provide resistance testing service to developing countries if samples such as dried blood spots are shipped to a high throughput laboratory [34]. NGS should also bring the cost of sequencing both the thymidine kinase/phosphotransferase and polymerase genes from HSV, VZV and CMV down to a level in which it will become routine to look for resistance earlier in the disease course.

CLINICAL IMPACT OF NEXT-GENERATION SEQUENCING RESISTANCE TESTS

These tests are undoubtedly more sensitive, but the outcome data are mixed with not all studies showing a clinical benefit [35,36]. NGS is likely to become the new standard of care and there is general agreement that the percentage of minority variant reporting, that is 20, 10, 5, 2 and 1%, requires clinical validation to interpret the risk of virological failure [37]. Ideally, all clinical trials should retrospectively perform NGS analysis and publish their results even if they show no significant difference in order to help with clinically validating low-percentage minority variant reporting.

IMPACT OF NEXT-GENERATION SEQUENCING ON HIV-1 RESISTANCE TESTING

Problems exist with interpreting the published data because of the use of historical cohorts, small studies, over-interpretation, reporting of positive associations and not using the same cut-offs.

Treatment-naïve patients

The greatest impact will be in detecting minority variants in treatment-naïve patients before they commence low genetic barrier NNRTI drugs such as nevirapine and efavirenz. The best data are from a meta-analysis of 10 studies which demonstrated a clinically significant 2.5 to 3 times increase risk of virological failure in patients harbouring these minority variants [38]. There is currently no evidence of a significantly increased risk of virological failure when minority variants are present for high genetic barrier drugs such as boosted protease inhibitors or NRTIs. This is in spite of the fact that minority variants are significantly increased when tested for by NGS [39,40]. It is fair to say that the risk of drug resistance development in modern first-line

cART clinical licensing trials using high genetic barrier drugs is very low, and therefore the argument could be that NGS will not add much value over and above current tests to screen for hidden transmitted drug resistance minority variants prior to starting therapy [41,42]. NGS has the potential to deny treatment to the majority of patients to a drug that they would have responded to because of overcalling of minority variants at low percentages [43].

Treatment-experienced patients

NGS can provide additional information on previous resistance mutations in treatment-experienced patients [44]. The increased sensitivity is useful in cases in which individuals have stopped their medication and in which 'unfit' viruses have become minority quasispecies and in patients with complex and incomplete treatment histories in which resistance tests were either not performed or in which the clinician does not have access to the reports. The added sensitivity will help determine whether drugs such as etravirine will be effective [45,46▪▪].

Tropism detection

Current genotypic tropism tests lack sensitivity to detect minority variant X4 tropic viruses which is more of a risk if the patient has a low CD4 count. Several studies have evaluated the ability of NGS to correctly predict non-R5 HIV-1 variants and the prediction of HIV-1 coreceptor usage has been highly concordant with phenotypic assays (82–87%) [47–49]. Reanalysis of the maraviroc clinical trial data also showed that the ability of NGS in predicting the success of maraviroc-based antiretroviral regimens is as good as the enhanced sensitive phenotypic assay which is the gold standard [47,48].

IMPACT OF NEXT-GENERATION SEQUENCING ON RESISTANCE TESTING FOR VIRUSES OTHER THAN HIV

Very little data is available which compare the clinical outcome based on the presence or absence of minority variants for viruses other than HIV.

Influenza

Antiviral resistance testing involves several laboratory techniques used in research and epidemiology such as specific functional assays, the neuraminidase inhibition assay and molecular techniques (SNP RT-PCR and sequencing) in circulating flu viruses. The specific SNP RT-PCR assays are fast and sensitive and therefore used in routine real-time diagnostic laboratories. However, they only exist for common mutations. A recent publication has once again illustrated how important minority viruses, in this case the R292K mutation in H7N9 viruses from China, can be masked by wildtype viruses when an enzyme-based test was used [50]. Reference laboratories are increasingly using WGS to characterize circulating viruses and to identify common and new resistance point mutations.

Hepatitis B virus

The same concept holds true for hepatitis B in which lamivudine drug resistance can persist at a low level only identifiable by means of NGS following stopping of the drug [51].

Hepatitis C virus

Measuring and recording the resistance mutations upon virological failure is not used in clinical practice, primarily because interpretation and next regimen drug selection are not yet established [8▪▪,52]. Stopping rules following inadequate viral load response or rebound exist to limit the amount of drug resistance developing. As with HIV, drug resistance comes at a fitness cost, and stopping drug therapy results in the least 'fit' variants being quickly replaced by 'fitter' variants. Protease inhibitor mutations have been shown to disappear after a median follow-up period of 30 months for 85% of patients using standard population sequencing [53]. It is unlikely that minority variants will play a significant role in the risk of treatment failure because of the fact that resistance mutations are not archived and that a combination of at least two potent DAA agents is becoming common practice.

Cytomegalovirus

As with HSV, the size of the UL54 (polymerase) gene has always been a barrier to resistance testing, and the smaller UL97 (viral kinase) gene has been used as first-line genotypic testing to exclude ganciclovir resistance since resistance usually develops in this gene first. However, failing treatment for a prolonged period of time on ganciclovir increases the risk of additional UL54 mutations developing. This increases the risk of cidofovir cross-resistance, but not for foscarnet cross-resistance [17]. Resistance during ganciclovir prophylaxis is rare. As with HSV and VZV, NGS technology should eventually provide a cheaper and quicker way of sequencing both relevant genes.

WGS of viruses, such as VZV, HSV and CMV, directly from clinical samples should provide not only information on developing drug resistance, but also better understanding of host–virus interactions [54].

IMPACT OF NEXT-GENERATION SEQUENCING BEYOND PROVIDING RESISTANCE DATA

WGS and third generation sequencing will have an impact beyond resistance testing. Quasispecies relatedness of individual viruses will be able help determine whether a patient has recently been infected, been infected by more than one founder virus or has superinfection. These tests can also help to determine whether different mutations are on the same or different genomes. Further information can also be obtained on the influence of immunological pressures. Antiviral therapy has the potential, by using rule-based computational tools, to be tailored to the individual patient whilst exploiting this additional information to the disadvantage of the virus.

CONCLUSION

Although some resistance tests are as easy as performing real-time PCR, the majority involve PCR amplification, sequencing and at times complex interpretation. NGS has the benefit of improved sensitivity and the feasibility for WGS or at least partial full-length sequencing which will be especially helpful in viral infections in which large genes need to be sequenced, for example, herpes viruses. The increased ability to identify minority variants has been shown to be beneficial, but larger clinical validations are necessary because there is a real danger that patients will be denied certain drugs or given complex four or five drug combinations which will not be necessary. The greatest clinical benefit of NGS so far has been in detecting minority variants in the low genetic barrier HIV cART regimens that contain efavirenz and nevirapine. In light of the available data, switching genotypic HIV-1 tropism testing to the more sensitive NGS method should be considered.

The clinical usefulness of NGS will undoubtedly depend on the clinical scenario, number of available antiviral agents, viral load and percentage minority variant cut-off used. All in all, it is unlikely that minority variants below 20% will increase risk of virological failure beyond that of poor adherence [38]. However, having both poor adherence and the presence of drug resistance, minority variants will increase a patient's risk of virological failure substantially.

Acknowledgements

None.

Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ■ of special interest
- ■■ of outstanding interest

1. Sauerbrei A, Bohn K. Phenotypic and genotypic testing of HSV-1 resistance to antivirals. Methods Mol Biol [internet] 2014; 1144:149–165. http://www.ncbi.nlm.nih.gov/pubmed/24671682. [Accessed 2 August 2014]
2. Andrei G, Georgala A, Topalis D, *et al*. Heterogeneity and evolution of thymidine kinase and DNA polymerase mutants of herpes simplex virus type 1: implications for antiviral therapy. J Infect Dis [internet] 2013; 207:1295–1305. http://www.ncbi.nlm.nih.gov/pubmed/23315323. [Accessed 2 August 2014]
3. Andrei G, Topalis D, Fiten P, *et al*. In vitro-selected drug-resistant varicella-zoster virus mutants in the thymidine kinase and DNA polymerase genes yield novel phenotype-genotype associations and highlight differences between antiherpesvirus drugs. J Virol [internet] 2012; 86:2641–2652. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3302250&tool=pmcentrez&rendertype=Abstract. [Accessed 21 July 2014]
4. ■ Andrei G, Snoeck R. Herpes simplex virus drug-resistance: new mutations and insights. Curr Opin Infect Dis [internet] 2013; 26:551–560. http://www.ncbi.nlm.nih.gov/pubmed/24152761. [Accessed 3 August 2014]

A comprehensive review of the current HSV drug resistance mutations.

5. Tsibris AMN, Korber B, Arnaout R *et al*. Quantitative deep sequencing reveals dynamic HIV-1 escape and large population shifts during CCR5 antagonist therapy in vivo. Nixon DF, editor. PLoS One [internet] 2009; 4:e5683. http://dx.plos.org/10.1371/journal.pone.0005683. [Accessed 7 August 2014]
6. Archer J, Rambaut A, Taillon BE, *et al*. The evolutionary analysis of emerging low frequency HIV-1 CXCR4 using variants through time: an ultra-deep approach. Kosakovsky Pond SL, editor. PLoS Comput Biol [internet] 2010; 6:e1001022. http://dx.plos.org/10.1371/journal.pcbi.1001022. [Accessed 7 August 2014]
7. Delobel P, Cazabat M, Saliou A, *et al*. Primary resistance of CCR5-tropic HIV-1 to maraviroc cannot be predicted by the V3 sequence. J Antimicrob Chemother [internet] 2013; 68:2506–2514. http://www.ncbi.nlm.nih.gov/pubmed/23794603. [Accessed 3 August 2014]
8. ■■ Poveda E, Wyles DL, Mena A, *et al*. Update on hepatitis C virus resistance to direct-acting antiviral agents. Antiviral Res [internet] 2014; 108:181–191. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24911972. [Accessed 9 July 2014]

A good review which provides interpretation to the latest resistance data for HCV DAAs.

9. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf. [Accessed 02 August 2014]
10. Asboe D, Aitken C, Boffito M, *et al*. British HIV Association guidelines for the routine investigation and monitoring of adult HIV-1-infected individuals 2011. HIV Med 2012; 13:1–44.
11. Vandamme A-M, Camacho RJ, Ceccherini-Silberstein F, *et al*. European recommendations for the clinical use of HIV drug resistance testing: 2011 update. AIDS Rev 2011; 13:77–108.
12. Vandekerckhove LPR, Wensing AMJ, Kaiser R, *et al*. European guidelines on the clinical management of HIV-1 tropism testing. Lancet Infect Dis [internet] 2011; 11:394–407. http://www.ncbi.nlm.nih.gov/pubmed/21429803. [Accessed 29 July 2011]
13. Kotton CN, Kumar D, Caliendo AM, *et al*. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation [internet] 2013; 96:333–360. http://www.ncbi.nlm.nih.gov/pubmed/23896556. [Accessed 27 May 2014]
14. Pozo F, Lina B, Andrade HR De, *et al*. Guidance for clinical and public health laboratories testing for influenza virus antiviral drug susceptibility in Europe. J Clin Virol [internet] 2013; 57:5–12. http://www.ncbi.nlm.nih.gov/pubmed/23375738. [Accessed 12 June 2014]
15. British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2012 (Updated November 2013. All changed text is cast in yellow highlight.). HIV Med [internet]. 2014; 15:1–85. http://www.bhiva.org/documents/Guidelines/Treatment/2012/hiv1029_2.pdf.
16. British Transplantation Society guidelines for the prevention and management of CMV disease after solid organ transplantation. 3rd ed. 2011.
17. Drew WL. Cytomegalovirus resistance testing: pitfalls and problems for the clinician. Clin Infect Dis [internet] 2010; 50:733–736. http://www.ncbi.nlm.nih.gov/pubmed/20100090. [Accessed 22 June 2014]
18. Clevenbergh P, Durant J, Halfon P, *et al*. Persisting long-term benefit of genotype-guided treatment for HIV-infected patients failing HAART. The Viradapt Study: week 48 follow-up. Antivir Ther [internet] 2000; 5:65–70. http://www.ncbi.nlm.nih.gov/pubmed/10846595. [Accessed 3 August 2014]
19. Baxter JD, Mayers DL, Wentworth DN, *et al*. A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. CPCRA 046 Study Team for the Terry Beirn Community Programs for Clinical Research on AIDS. AIDS [internet] 2000; 14:F83–F93. http://www.ncbi.nlm.nih.gov/pubmed/10894268. [Accessed 3 August 2014]

20. Tural C, Ruiz L, Holtzer C, *et al.* Clinical utility of HIV-1 genotyping and expert advice: the Havana trial. AIDS [internet] 2002; 16:209–218. http://www.ncbi.nlm.nih.gov/pubmed/11807305. [Accessed 3 August 2014]
21. Hirsch MS, Brun-Vézinet F, Clotet B, *et al.* Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA Panel. Clin Infect Dis 2003; 37:113–128.
22. Torre D, Tambini R. Antiretroviral drug resistance testing in patients with HIV-1 infection: a meta-analysis study. HIV Clin Trials [internet] 2002; 3:1–8. http://www.ncbi.nlm.nih.gov/pubmed/11819179. [Accessed 4 August 2014]
23. Panidou ET, Trikalinos TA, Ioannidis JPA. Limited benefit of antiretroviral resistance testing in treatment-experienced patients: a meta-analysis. AIDS [internet] 2004; 18:2153–2161. http://www.ncbi.nlm.nih.gov/pubmed/15577648. [Accessed 3 August 2014]
24. Prosperi MCF, Mackie N, Di Giambenedetto S, *et al.* Detection of drug resistance mutations at low plasma HIV-1 RNA load in a European multicentre cohort study. J Antimicrob Chemother [internet] 2011; 66:1886–1896. http://www.ncbi.nlm.nih.gov/pubmed/21624929. [Accessed 8 August 2014]
25. Santoro MM, Fabeni L, Armenia D, *et al.* Reliability and clinical relevance of the HIV-1 drug resistance test in patients with low viremia levels. Clin Infect Dis [internet] 2014; 58:1156–1164. http://www.ncbi.nlm.nih.gov/pubmed/24429430. [Accessed 28 May 2014]
26. ■ Capobianchi MR, Giombini E, Rozera G. Next-generation sequencing technology in clinical virology. Clin Microbiol Infect 2013; 19:15–22.

A good review for anyone who is interested in additional NGS applications in virology.

27. Wang C, Mitsuya Y, Gharizadeh B, *et al.* Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. Genome Res [internet] 2007; 17:1195–1201. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1933516&tool=pmcentrez&rendertype=Abstract. [Accessed 12 June 2011]
28. Winters MA, Merigan TC. Insertions in the human immunodeficiency virus type 1 protease and reverse transcriptase genes: clinical impact and molecular mechanisms. Antimicrob Agents Chemother [internet] 2005; 49:2575–2582. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1168704&tool=pmcentrez&rendertype=Abstract. [Accessed 8 August 2014]
29. Shao W, Boltz VF, Spindler JE, *et al.* Analysis of 454 sequencing error rate, error sources, and artifact recombination for detection of Low-frequency drug resistance mutations in HIV-1 DNA. Retrovirology [internet] 2013; 10:18. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3599717&tool=pmcentrez&rendertype=Abstract. [Accessed 9 June 2011]
30. Burke DS. Recombination in HIV: an important viral evolutionary strategy. Emerg Infect Dis 1997; 3:253–259.
31. Kozarewa I, Ning Z, Quail MA, *et al.* Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nat Methods [internet] 2009; 6:291–295. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2664327&tool=pmcentrez&rendertype=Abstract. [Accessed 30 May 2014]
32. Casbon JA, Osborne RJ, Brenner S, Lichtenstein CP. A method for counting PCR template molecules with application to next-generation sequencing. Nucleic Acids Res [internet] 2011; 39:e81. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3130290&tool=pmcentrez&rendertype=Abstract. [Accessed 23 May 2014]
33. Gibson RM, Meyer AM, Winner D, *et al.* Sensitive deep-sequencing-based HIV-1 genotyping assay to simultaneously determine susceptibility to protease, reverse transcriptase, integrase, and maturation inhibitors, as well as HIV-1 coreceptor tropism. Antimicrob Agents Chemother [internet] 2014; 58:2167–2185. http://www.ncbi.nlm.nih.gov/pubmed/24468782. [Accessed 9 June 2014]
34. Dudley D, Chin E, Bimber B. Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. PLoS One [internet]. 2012; 7 (5). http://dx.plos.org/10.1371/journal.pone.0036494.g003. [Accessed 22 September 2014]
35. Gianella S, Delport W, Pacold ME, *et al.* Detection of minority resistance during early HIV-1 infection: natural variation and spurious detection rather than transmission and evolution of multiple viral variants? Society 2011; 85:8359–8367.
36. Stekler JD, Ellis GM, Carlsson J, *et al.* Prevalence and impact of minority variant drug resistance mutations in primary HIV-1 infection. PLoS One [internet] 2011; 6:e28952. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3241703&tool=pmcentrez&rendertype=Abstract. [Accessed 8 August 2014]
37. Gianella S, Richman D. Minority variants of drug-resistant HIV. J Infect Dis [internet]. 2010; 202. http://jid.oxfordjournals.org/content/202/5/657.short. [accessed 22 September 2014]
38. Li JZ, Paredes R, Ribaudo HJ, *et al.* Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. JAMA [internet] 2011; 305:1327–1335. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3325645&tool=pmcentrez&rendertype=Abstract. [Accessed 5 August 2014]
39. Simen BB, Simons JF, Hullsiek KH, *et al.* Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis [internet] 2009; 199:693–701. http://www.ncbi.nlm.nih.gov/pubmed/19210162. [Accessed 18 October 2010]
40. Lataillade M, Chiarella J, Yang R, *et al.* Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naïve subjects in the CASTLE study. Popul (English Ed) 2010; 5:1–7.
41. Sax PE, DeJesus E, Mills A, *et al.* Co-formulated elvitegravir, cobicistat, emtricitabine, and tenofovir versus co-formulated efavirenz, emtricitabine, and tenofovir for initial treatment of HIV-1 infection: a randomised, double-blind, phase 3 trial, analysis of results after 48 weeks. Lancet [internet] 2012; 379:2439–2448. http://www.ncbi.nlm.nih.gov/pubmed/22748591. [Accessed 6 August 2014]
42. Walmsley SL, Antela A, Clumeck N, *et al.* Dolutegravir plus abacavir-lamivudine for the treatment of HIV-1 infection. N Engl J Med [internet] 2013; 369:1807–1818. http://www.ncbi.nlm.nih.gov/pubmed/24195548. [Accessed 6 August 2014]
43. Deeks SG. Transmitted minority drug-resistant HIV variants: a new epidemic? PLoS Med 2008; 5:1014–1016.
44. Le T, Chiarella J, Simen BB, *et al.* Low-abundance HIV drug-resistant viral variants in treatment-experienced persons correlate with historical antiretroviral use. PLoS One [internet] 2009; 4:e6079. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698118&tool=pmcentrez&rendertype=Abstract. [Accessed 9 July 2014]
45. Codoñer FM, Pou C, Thielen A, *et al.* Added value of deep sequencing relative to population sequencing in heavily pre-treated HIV-1-infected subjects. PLoS One [internet] 2011; 6:e19461. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3094345&tool=pmcentrez&rendertype=Abstract. [Accessed 27 June 2014]
46. ■■ Pou C, Noguera-Julian M, Pérez-Álvarez S, *et al.* Improved prediction of salvage antiretroviral therapy outcomes using ultrasensitive HIV-1 drug Resistance Testing. Clin Infect Dis [internet] 2014; 1–11. http://www.ncbi.nlm.nih.gov/pubmed/24879788. [Accessed 9 June 2014]

Although a smallish retrospective study, this is an example of the type of study which needs to be performed in order to generate more clinical validation data for NGS.

47. Swenson LC, Mo T, Dong WWY, *et al.* Deep sequencing to infer HIV-1 co-receptor usage: application to three clinical trials of maraviroc in treatment-experienced patients. J Infect Dis [internet] 2011; 203:237–245. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3071057&tool=pmcentrez&rendertype=Abstract. [Accessed 7 August 2014]
48. Swenson LC, Mo T, Dong WWY, Zhong X, Woods CK, Thielen A, *et al.* Deep V3 sequencing for HIV type 1 tropism in treatment-naive patients: a reanalysis of the MERIT trial of maraviroc. Clin Infect Dis [internet]. 2011; 53. http://cid.oxfordjournals.org/content/53/7/732.short. [Accessed 22 September 2014]
49. Saliou A, Delobel P, Dubois M, *et al.* Concordance between two phenotypic assays and ultradeep pyrosequencing for determining HIV-1 tropism. Antimicrob Agents Chemother [internet]; 55:2831–1836. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3101380&tool=pmcentrez&rendertype=Abstract. [Accessed 7 August 2014]
50. Yen H, McKimm-Breschkin J, Choy K. Resistance to neuraminidase inhibitors conferred by an R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. MBio [internet]. 2013; http://mbio.asm.org/content/4/4/e00396-13.short. [Accessed 22 September 2014]
51. Margeridon-Thermet S, Svarovskaia ES, Babrzadeh F, *et al.* Low-level persistence of drug resistance mutations in hepatitis B virus-infected subjects with a past history of Lamivudine treatment. Antimicrob Agents Chemother [internet] 2013; 57:343–349. http://aac.asm.org/content/57/1/343.long. [Accessed 7 August 2014]
52. Schneider MD, Sarrazin C. Antiviral therapy of hepatitis C in 2014: do we need resistance testing? Antiviral Res [internet]. 2014; 105:64–71. http://www.sciencedirect.com/science/article/pii/S0166354214000540. [Accessed 7 August 2014]
53. Sherman KE, Sulkowski MS, Zoulim F, *et al.* Follow-up of SVR durability and viral resistance in patients with chronic hepatitis C treated with telaprevir-based regimens: interim analysis of the extend study. Hepatology 2011; 54:485A–486A.
54. Depledge DP, Palser AL, Watson SJ, *et al.* Specific capture and whole-genome sequencing of viruses from clinical samples. Jhaveri R, editor. PLoS One [internet] 2011; 6:e27805. http://dx.plos.org/10.1371/journal.pone.0027805. [Accessed 4 July 2014]