Pyrosequencing of HIV-1 reverse transcriptase to reveal minority populations of resistant virus before start of an NNRTI-based regimen.

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Population Genotyping
 Minority drug resistant HIV variants: the variants below threshold of population sequencing (15 to 20%)

- Common approaches
  - Point mutation assays (< 0.1%)
  - Clonal sequencing
  - Ultra deep sequencing

Do Minority drug resistant HIV variants have a major clinical impact
Do Minority drug resistant HIV variants have a major clinical impact

- **Halvas et al., 1 March 2010, JID**
  - Compared NNRTI minority DRV in NNRTI treatment experienced versus NNRTI treatment naive patient samples (P=0.22)

- **Paredes et al., 1 March 2010, JID** (allele specific PCR, minority DRM < 1%, drug naive patients)
  - Compared the presence K103N and Y181C in plasma samples of 51 randomly selected individuals + 127 norandomly selected subjects who experienced VF and 144 successfully suppressed subjects (Y181C was associated with 3.5 fold increased risk of VF)

- **Balduin et al., J Ci Vir 2009** (allele specific PCR, minority DRM < 1%, drug naive patients)
  - There is some evidence for a higher risk of NNRT- treatment failure in patients with K103N minorities (24 versus 15%; p=0.3)

- **Geretti et al., JAIDS 2009** (allele specific PCR, minority DRM < 1%, drug naive patients)
  - Evaluated 93 patients, 18 experienced VF (75 patients successfully treated, in 7 of the 18 plasma samples of the patients who experienced DRV were detected (with population genotyping N=3, with allele specific PCR N=4)

**Conclusion of these studies:**
- Low- frequency mutants (K103N, Y181C) are associated with VF
Research question

Does ultradepsequencing reveal the presence of minority DRM that are subsequently associated with virological failure upon initiation of an NNRTI based regime

Definition of VF*:

- Primary failure: not achieving undetectable VL at 6 months
- Secondary failure: achieving undetectable VL at 6 months with subsequent rebound viremia

*(Geretti et al., j AIDS 2009)
70 plasma samples of patients starting an NNRTI based regimen between 1999 and 2008 were randomly selected for deep sequencing on the GS-FLX (Roche)

VL ranges between: $4 \times 10^3 > 10 \times 10^6$

The design of the study and the experiment aimed a sensitivity of 1% (i.e. 5000 reads per sample)

Sensitivity dependant on

- Input (VL)
- Protocol: from extraction $\rightarrow$ amplification $\rightarrow$ sequencing
- Sequence coverage
Assay design
amplicon preparation

1. cDNA synthesis
   • random hexamers
   • AccuScript RT (error rate 6.3x10^{-5})*

2. ‘Outer PCR’
   • Primers# with partial overlap
   • Seven replicates
   • Amplicon length: 447 bases
   • Phusion Taq polymerase (error rate 4.4x10^{-7})*

3. ‘Inner PCR’ aa 59-190
   • Re-use of two primers
   • With addition of:
     • sequencing adaptors A&B
     • ‘key’ sequence
     • multiplex identifiers (MIDs)
   • Phusion Taq polymerase

# Primer design by evaluation on 266,781 Virco sequences
*Regular Taq polymerase: error rate 1,11x10^{-4}
Clinical outcome

70 plasma samples of patients starting an NNRTI based regimen

- Good response after 24 till 48 weeks N=48
- Primary failure N=3
- Secondary failure N=2
- LTFU N=3
- Intolerance N=8
- Q-ART N=6
RESULTS

Only samples present and with Stanford score above > 5


= detection of DRM populations
# RESULTS

= detection of DRM populations present >1%

= detection of DRM populations present <1%

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 |
RESULTS

- detection of DRM populations present >15%
- detection of DRM populations present <15% and > 1%
- detection of DRM populations present <1%

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RESULTS

In patients on triple ART having baseline plasma harbouring minority DRM >1 and <15%,

- L100I (N=2) (1.07-1.49%)
- V108I (N=2) (1.12-2.25%)
- A98G (N=1) (3.32%)

Was not associated with virological failure (all 5 patients achieved undetectable VL)
## RESULTS

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- = primary failure + adherent
- = secondary failure

Positive control

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Presented at the 8th European HIV Drug Resistance Workshop, March 17-19 2010, Sorrento, Italy
Prim failure: One patient was non adherent

One patient sample harboured DRM: V90I (0.04%) and V106I (0.49%), suboptimal nevirapine level

One patient sample harboured Q151M (0.26%); V90I (0.03%); V108I (0.08%)

Sec failure: One patient sample harboured V106A (99.21)

One patient sample harboured K101E (0.26%)

WT by population sequencing at failure

M184I and 188L by population sequencing at failure

population sequencing at failure: No results (203 copies)

V106A present in population sequencing at failure

HIV RNA load returned to <50 copies/ml
Fisher’s exact test could not demonstrate association between virological failure or inadequate response and the presence of minority variants present between 1 and 15%.

In patients on triple ART having baseline plasma harbouring minority DRM >1 and <15%, L100I (N=2), V108I (N=2) and A98G (N=1) were not associated with FV (all 6 patients achieved undetectable VL).

A swarm of minority DRM <1% are found in the A98G, L100I, K101G, V108I and in E138A, but these are below the predefined error rate of 1%.

K103N and Y181C were not present as minority in our analysis.
Ultrasensitive sequencing technology, applied to randomly selected patients initiating a NNRTI-based first-line regimen in a local HIV clinic failed to show a relationship between virologic failure and the presence of minority variants present above 1.

Our data indicate that expensive methods like pyrosequencing to determine minority species will have to be evaluated in regions with more prevalent baseline resistance in order to establish the eventual threshold of cost-effectiveness before the introduction in routine diagnostic settings.
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