Status of Analyses & Publications of Previous and Active Virology WG Collaborative Studies

- Published:

- Studies completed, manuscripts drafted:
  - NAT and HCV Ag Testing Performance for Reducing the HCV Window Phase (Egypt, France, Germany, Japan, Lithuwania, Poland, USA), led by Syria Laperche
  - Distribution of HIV Viral Loads in NAT Yield Donations and Detection by 4th Generation HIV Ag/Ab assays in Donors from France, Germany, Japan, Poland, South Africa and the United States, led by Leslie Tobler and Mike Busch
  - Rates, Demographics and Virologic Profiles of HIV Elite Controllers Detected Through Donor Screening in the US, France, Germany, South Africa and Australia, led by Mike Busch

- Data compiled, 2 presentations at ISBT Cancun, manuscripts in development:
  - International ID-NAT Study Group (17 countries) Nico Lelie, Steve Kleinman, Brian Custer, Mike Busch
    - HIV Transmission Risk and Efficacy of Screening Strategies
    - HCV Transmission Risk and Efficacy of Screening Strategies
    - Efficacy of HBV Screening Assays in an International Survey
    - Cost Effectiveness of Alternative NAT and Serological Screening Strategies for HIV, HCV & HBV
International survey on NAT testing of blood donations: expanding implementation and yield from 1999 to 2009

Introduction of NAT testing

HCV

- Austria
- Canada
- Ireland
- Japan
- Netherlands
- Spain
- Switzerland
- UK
- USA

- Australia
- Norway
- Singapore
- Slovenia
- France
- New Zealand
- Poland
- Greece
- Portugal
- South Africa
- South Korea
- Slovenia
- Estonia
- Thailand
- Kuwait
- Malaysia
- Latvia
- Taiwan
- Denmark


HIV-1

- Germany
- Austria
- Japan
- USA
- Australia
- Netherlands
- Singapore
- France
- New Zealand
- Spain
- Belgium
- Hong Kong
- Indonesia
- Switzerland
- Poland
- Greece
- Portugal
- UK
- South Africa
- South Korea
- Estonia
- Italy
- Thailand
- Kuwait
- Malaysia
- Slovenia
- Finland
- Israel
- Latvia
- Taiwan
- Denmark


HBV

- Germany
- Austria
- Japan
- Singapore
- France
- Spain
- Poland
- France
- Italy
- Poland
- Greece
- Portugal
- Thailand
- Hong Kong
- Kuwait
- Malaysia
- New Zealand
- Slovenia
- Switzerland
- Finland
- Israel
- Latvia
- Netherlands
- Taiwan (clinical trial)
- Denmark
- Finland
- Iceland
- Israel
- Netherlands
- UK (Scotland)
- Canada
- USA

1997 1999 2004 2005 2006 2007 2008 2009 2010

<table>
<thead>
<tr>
<th>Region/country</th>
<th>Virus</th>
<th>Screened donations since implementation of NAT</th>
<th>NAT-only positives</th>
<th>NAT-only positives/million</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>HCV</td>
<td>2 202 295</td>
<td>81</td>
<td>36.78</td>
</tr>
<tr>
<td></td>
<td>HBV</td>
<td>2 202 295</td>
<td>4</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>HIV-1</td>
<td>71 458 330</td>
<td>44</td>
<td>105.34</td>
</tr>
<tr>
<td>Asia/Pacific</td>
<td>HIV-1</td>
<td>110 860 111</td>
<td>73</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>HCV</td>
<td>139 474 595</td>
<td>206</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>HBV</td>
<td>56 342 555</td>
<td>550</td>
<td>9.76</td>
</tr>
<tr>
<td></td>
<td>HIV-1</td>
<td>87 652 586</td>
<td>45</td>
<td>0.51</td>
</tr>
<tr>
<td>North America</td>
<td>HIV-1</td>
<td>347 374</td>
<td>1</td>
<td>2.88</td>
</tr>
<tr>
<td></td>
<td>HCV</td>
<td>408 167</td>
<td>2</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>HBV</td>
<td>No NAT testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South America</td>
<td>HIV-1</td>
<td>272 520 696</td>
<td>244</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>HCV</td>
<td>303 196 074</td>
<td>680</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>HBV</td>
<td>114 286 214</td>
<td>1884</td>
<td>16.48</td>
</tr>
<tr>
<td>Total (all countries)</td>
<td>HIV-1</td>
<td>272 520 696</td>
<td>244</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>HCV</td>
<td>303 196 074</td>
<td>680</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>HBV</td>
<td>114 286 214</td>
<td>1884</td>
<td>16.48</td>
</tr>
</tbody>
</table>
Global Distribution of HIV-1 Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>205725</td>
<td>60.6%</td>
</tr>
<tr>
<td>C</td>
<td>47853</td>
<td>14.1%</td>
</tr>
<tr>
<td>A</td>
<td>24538</td>
<td>7.2%</td>
</tr>
<tr>
<td>D</td>
<td>10423</td>
<td>3.1%</td>
</tr>
<tr>
<td>F</td>
<td>3218</td>
<td>0.9%</td>
</tr>
<tr>
<td>G</td>
<td>4069</td>
<td>1.2%</td>
</tr>
<tr>
<td>other</td>
<td>18182</td>
<td>5.4%</td>
</tr>
<tr>
<td>total</td>
<td>339246</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
HIV Viral Panels Project Requirements

- Well characterized HIV reference panels encompassing epidemic
- Full length single genome sequencing
- Verified RNA concentration
- Fiebig staging and serological profiles for current assays/platforms including rapid POC assays
- Comparisons of VL from different FDA approved commercially available platforms
- Panels with larger volumes for use on newer diagnostic platforms
- Pilot study completed and published in 2010; Duke awarded 7 year contract for full scale study
Pilot Studies for Development of an HIV Subtype Panel for Surveillance of Global Diversity

Mark Manak,1,2,* Silvana Sina,2 Bharathi Anekella,1 Indira Hewlett,3 Eric Sanders-Buell,2 Viswanath Ragupathy,3 Jerome Kim,4 Marion Vermeulen,5 Susan L. Stramer,6 Ester Sabino,7 Piotr Grabarczyk,8 Nelson Michael,4 Sheila Peel,4 Patricia Garrett,1 Sodsai Tovanabutra,2 Michael P. Busch,9 and Marco Schito10

Table 2. Virus Isolation Rates for Plasma Samples as a Function of Fiebig Stages or Viral Load

<table>
<thead>
<tr>
<th>Fiebig stage</th>
<th>Pos</th>
<th>Total</th>
<th>% Pos</th>
<th>Viral load</th>
<th>Pos</th>
<th>Total</th>
<th>% Pos</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2</td>
<td>27</td>
<td>7.4%</td>
<td>&lt;1,000</td>
<td>0</td>
<td>20</td>
<td>0.0%</td>
</tr>
<tr>
<td>II/III</td>
<td>8</td>
<td>11</td>
<td>72.7%</td>
<td>1,000–10,000</td>
<td>1</td>
<td>14</td>
<td>7.1%</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>7</td>
<td>28.6%</td>
<td>10,000–100,000</td>
<td>10</td>
<td>21</td>
<td>47.6%</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>15</td>
<td>60.0%</td>
<td>100,000–1,000,000</td>
<td>15</td>
<td>20</td>
<td>75.0%</td>
</tr>
<tr>
<td>VI</td>
<td>9</td>
<td>20</td>
<td>45.0%</td>
<td>&gt;1,000,000</td>
<td>4</td>
<td>5</td>
<td>80.0%</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>80</td>
<td>37.5%</td>
<td>Total</td>
<td>30</td>
<td>80</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

Table 3. Correlation of Viral Load Assays

<table>
<thead>
<tr>
<th>Roche TaqMan v2.0 vs.</th>
<th>Roche Amplicor Monitor v1.5</th>
<th>Roche TaqMan v1.0</th>
<th>Abbott m2000</th>
<th>Siemens bDNA v3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtype B</td>
<td>0.997</td>
<td>0.948</td>
<td>0.997</td>
<td>0.952</td>
</tr>
<tr>
<td>Subtype C</td>
<td>0.883</td>
<td>0.872</td>
<td>0.837</td>
<td>0.891</td>
</tr>
</tbody>
</table>

The correlation coefficients for four viral load assays, each vs. the Roche TaqMan v2.0, were calculated separately for U.S. (subtype B; N=20) and South African (subtype C; N=37) samples.

Most efficient virus isolation was observed for plasma samples at Fiebig stages II and V, although virus could be isolated at each stage. Efficiency of virus isolation went up as viral load increased. Virus isolation was not achieved for samples with viral loads below 1000 copies/ml.
Overview of EQAPOL Viral Diversity Program

• EQAPOL (External Quality Assurance Oversight Laboratory)
  • Seven year NIAID contract
  • Encompasses multiple EQA programs (ELISpot, Flow Cytometry Luminex) and the Viral Diversity Program

• Viral Diversity Program Goals
  • Create HIV panels of plasma and viral isolates representative of worldwide viral diversity
  • Grow 50 high-titer/high-volume cultures per year
  • Characterize viruses
  • Conduct work in a GCLP environment
  • Make viruses available for order through a web-based system
Viral Diversity Program Workflow

**Collect viral specimens**
- Sources include National Blood Collection organization (BSRI), FDA, Instituto de Salud Carlos III and First Affiliated Hospital of China Medical University

**Perform Initial Characterization**
- Fiebig staging, VL, p24, pre-culture sequencing, sterility testing

**Culture to High-titer, High-volume**
- Two step culture process
- Results in culture supernatant and HIV-spiked plasma

**Characterize Virus**
- TCID$_{50}$, Final VL testing (multiple platforms), sequencing, coreceptor usage, sterility testing

**Distribute HIV to Research Laboratories**
- Inventory available through EQAPOL web-based system
Two-step culture process

**Step 1: Small-Scale Culture**

- **Source Viral Specimen**
  - Plasma, PBMCs, previously-cultured supernatant

- **Feeder Cells**
  - Pooled cryopreserved PBMCs

- **Culture**
  - **Master Lot**
    - $\approx 40$ mL
    - Average titer $>4.90 \times 10^9$ cp/mL
    - Average culture time is 8.2 days

**Step 2: Large-Scale Culture**

- **Aliquot of master lot**

- **Feeder Cells**
  - Pooled cryopreserved PBMCs

- **Culture**
  - **High Titer Culture Supernatant**
    - $\approx 250$ $1$ mL aliquots
    - Average titer $>4.48 \times 10^9$ cp/mL
    - Culture time generally 4-7 days

- **HIV Spiked-plasma**
  - $\approx 100$ $1$ mL aliquots at $1 \times 10^7$ cp/mL
  - $\approx 20$ $1$ mL aliquots at $5 \times 10^7$ cp/mL
Characterization Performed on All Final Viral Products

- **Sterility Testing**
  - Endotoxin
  - Mycoplamsa
  - Bacterial Inoculation

- **Viral Load**
  - TCID\textsubscript{50}
    - TZM-bl cell-based assay
  - Roche 2.0
  - Abbott (FDA)
  - Future: bDNA

- **Sequencing**
  - Data uploaded to GenBank

- **Coreceptor Usage**
  - Phenotype
  - NP-2 Cell Assay

- **Pre-culture Testing**
  - Fiebig staging
  - VL and p24
  - Sterility
  - Pre-culture sequencing

**Viral Products**
(Culture supernatant and spiked plasma)
Sample Certificate of Analysis (COA)

- All viral products generated for EQAPOL will have a COA
- COA will be signed by EQAPOL Central Quality Assurance Unit
- COA will be available for download on EQAPOL web-based system

CERTIFICATE OF ANALYSIS

**Product Information**

Virus Name: DEMB94ZA001.01

Product Type (HIV-spiked Serum or Cell Culture Supernatant): Cell Culture Supernatant

Clade: B

**Final Harvest Date:** 08/30/2011

**Viral Load of Product:** $1.5 \times 10^{10}$ copies/mL

**Co-receptor Usage (determined using cell culture supernatant):** CCR5

**TCID$_{50}$ of Product (cell culture supernatants only):** $2.5 \times 10^4$ TCID$_{50}$/mL

**Sterility Information**

*Mycoplasma* Testing: **PASS**

*Endotoxin Testing:* Concentration: 0.05 EU/mL **PASS**

*Bacterial Testing:*

- Soybean Casein Digest Medium: **PASS**
- Fluid Thioglycolate Medium: **PASS**

**Source Specimen Information**

**Fiebig Stage of Source Plasma (if available):** II

**Country of Origin for Source Specimen:** South Africa

**Year of Donation for Source Specimen:** 1994

**Additional Information about Source Specimen:**

________________________________________  _______________________

Quality Assurance Signature                  Date
Current Diversity of Panel

- Algeria
- Angola
- Bolivia
- Brazil
- Cameroon
- China
- France
- Germany
- Greece
- Nigeria
- Poland
- South Africa
- Spain
- Tanzania
- Uruguay
- US
- Venezuela
EQAPOL Web-based Application

• Web-based application developed for EQAPOL Viral Diversity:
  • Data regarding culture process
  • Viral characterization results
  • Inventory of viral products
  • Allows external users to order viral products
  • Tracks shipping and receipt of viral products
  • Track sites participating in the program

• To use the system, users must request access via email: EQAPOL@duke.edu
Is the virulence of HIV changing? A meta-analysis of trends in prognostic markers of HIV disease progression and transmission

Joshua T. Herbeck\textsuperscript{a}, Viktor Müller\textsuperscript{c}, Brandon S. Maust\textsuperscript{a}, Bruno Ledergerber\textsuperscript{d}, Carlo Torti\textsuperscript{e}, Simona Di Giambenedetto\textsuperscript{f}, Luuk Gras\textsuperscript{g}, Huldrych F. Günthard\textsuperscript{d}, Lisa P. Jacobson\textsuperscript{h}, James I. Mullins\textsuperscript{a,b} and Geoffrey S. Gottlieb\textsuperscript{b}

\textbf{Results:} Baseline CD4\textsuperscript{+} T-cell counts showed a summary trend of decreasing cell counts [effect $= -4.93$ cells/\textmu{l} per year, 95\% confidence interval (CI) $-6.53$ to $-3.3$]. Set point viral loads showed a summary trend of increasing plasma viral RNA loads (effect $= 0.013 \log_{10}$ copies/ml per year, 95\% CI $-0.001$ to $0.03$). The trend rates decelerated in recent years for both prognostic markers.

\textbf{Conclusion:} Our results are consistent with increased virulence of HIV-1 over the course of the epidemic. Extrapolating over the 30 years since the first description of AIDS, this represents a CD4\textsuperscript{+} T cells loss of approximately 148 cells/\textmu{l} and a gain of $0.39 \log_{10}$ copies/ml of viral RNA measured during early infection. These effect sizes would predict increasing rates of disease progression, and need for ART as well as increasing transmission risk. © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins

\textit{AIDS} 2012, \textbf{26}:193–205
<table>
<thead>
<tr>
<th>Author, year</th>
<th>N</th>
<th>Effect size, [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galai, 1996</td>
<td>683</td>
<td>4.20 [-8.34, 16.74]</td>
</tr>
<tr>
<td>Sinicco, 1997</td>
<td>285</td>
<td>-11.25 [-37.40, 14.90]</td>
</tr>
<tr>
<td>Easterbrook, 2000</td>
<td>1093</td>
<td>3.20 [-4.82, 11.22]</td>
</tr>
<tr>
<td>Dorrucci, 2005</td>
<td>1251</td>
<td>-7.30 [-11.42, -3.18]</td>
</tr>
<tr>
<td>Müller, 2006</td>
<td>4089</td>
<td>-1.85 [-3.73, -0.03]</td>
</tr>
<tr>
<td>Dorrucci, 2007</td>
<td>3687</td>
<td>-6.61 [-8.71, -4.51]</td>
</tr>
<tr>
<td>Herbeck, 2008</td>
<td>375</td>
<td>-7.90 [-16.43, -0.63]</td>
</tr>
<tr>
<td>Crum-Cianflone, 2009</td>
<td>2174</td>
<td>-7.63 [-13.67, -1.59]</td>
</tr>
<tr>
<td>Gras, 2009</td>
<td>578</td>
<td>-4.69 [-7.28, -2.10]</td>
</tr>
<tr>
<td>Müller, 2009</td>
<td>4563</td>
<td>-5.91 [-7.60, -4.22]</td>
</tr>
<tr>
<td>Potard, 2009</td>
<td>1441</td>
<td>-5.76 [-11.17, -0.35]</td>
</tr>
<tr>
<td>Troude, 2009</td>
<td>833</td>
<td>-5.08 [-12.04, 1.88]</td>
</tr>
</tbody>
</table>

Summary estimate: $Q = 22.89$ $P = 0.018$

**CD4⁺ T cells/µl per year**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>N</th>
<th>Effect size, [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Müller, 2006</td>
<td>2264</td>
<td>0.012 [0.000, 0.024]</td>
</tr>
<tr>
<td>Dorrucci, 2007</td>
<td>1584</td>
<td>0.035 [0.021, 0.049]</td>
</tr>
<tr>
<td>Herbeck, 2008</td>
<td>357</td>
<td>-0.005 [-0.029, 0.019]</td>
</tr>
<tr>
<td>Crum-Cianflone, 2009</td>
<td>1267</td>
<td>0.000 [-0.014, 0.014]</td>
</tr>
<tr>
<td>Gras, 2009</td>
<td>612</td>
<td>0.016 [0.002, 0.030]</td>
</tr>
<tr>
<td>Müller, 2009</td>
<td>2396</td>
<td>-0.013 [-0.023, -0.003]</td>
</tr>
<tr>
<td>Potard, 2009</td>
<td>1402</td>
<td>0.056 [0.029, 0.083]</td>
</tr>
<tr>
<td>Troude, 2009</td>
<td>903</td>
<td>0.013 [0.001, 0.025]</td>
</tr>
</tbody>
</table>

Summary estimate: $Q = 49.12$ $P = 2.2e-08$

**Log₁₀ HIV RNA copies/ml per year**

**Fig. 1.** Forest plots of trends in prognostic markers of HIV disease progression. Forest plot for (a) trends in baseline CD4⁺ T-cell count; and (b) trends in set point plasma HIV-1 RNA loads (log₁₀-transformed). The 95% confidence intervals for each study are represented by horizontal lines, and the effect sizes (regression slopes) are represented by squares, with the squares area equal to the weight of the study (with the weight calculated as the inverse of the variance). Confidence interval for summary effect size is represented by the width of the diamond shape.
Phylogenetic Approach Reveals That Virus Genotype Largely Determines HIV Set-Point Viral Load

Samuel Alizon\textsuperscript{1*}, Viktor von Wyl\textsuperscript{2}, Tanja Stadler\textsuperscript{1\#}, Roger D. Kouyos\textsuperscript{1\#}, Sabine Yerly\textsuperscript{3}, Bernard Hirschel\textsuperscript{4}, Jürg Böni\textsuperscript{5}, Cyril Shah\textsuperscript{5}, Thomas Klimkait\textsuperscript{6}, Hansjakob F. Vernazza\textsuperscript{8}, Enos Bernasconi\textsuperscript{9}, Manuel Battegay\textsuperscript{10}, Philippe Bürgisser\textsuperscript{11}, Amalio Telenti\textsuperscript{12}, Huldrych F. Günthard\textsuperscript{2\#}, Sebastian Bonhoeffer\textsuperscript{1\#}, the Swiss HIV Cohort Study

Abstract

HIV virulence, i.e. the time of progression to AIDS, varies greatly among patients. As for other rapidly evolving pathogens of humans, it is difficult to know if this variance is controlled by the genotype of the host or that of the virus because the transmission chain is usually unknown. We apply the phylogenetic comparative approach (PCA) to estimate the heritability of a trait from one infection to the next, which indicates the control of the virus genotype over this trait. The idea is to use viral RNA sequences obtained from patients infected by HIV-1 subtype B to build a phylogeny, which approximately reflects the transmission chain. Heritability is measured statistically as the propensity for patients close in the phylogeny to exhibit similar infection trait values. The approach reveals that up to half of the variance in set-point viral load, a trait associated with virulence, can be heritable. Our estimate is significant and robust to noise in the phylogeny. We also check for the consistency of our approach by showing that a trait related to drug resistance is almost entirely heritable. Finally, we show the importance of taking into account the transmission chain when estimating correlations between infection traits. The fact that HIV virulence is, at least partially, heritable from one infection to the next has clinical and epidemiological implications. The difference between earlier studies and ours comes from the quality of our dataset and from the power of the PCA, which can be applied to large datasets and accounts for within-host evolution. The PCA opens new perspectives for approaches linking clinical data and evolutionary biology because it can be extended to study other traits or other infectious diseases.
Adaptation of HIV-1 envelope gp120 to humoral immunity at a population level

Evelien M Bunnik¹, Zelda Euler¹, Matthijs R A Welkers¹, ¹,³, Brigitte D M Boeser-Nunnink¹, Marius I Grijzen², Jan M Prins² & Hanneke Schuitemaker¹, ³

By comparing HIV-1 variants from people who became infected at the beginning of the epidemic and from people who have recently contracted the virus, we observed an enhanced resistance of the virus to antibody neutralization over time, accompanied by an increase in the length of the variable loops and in the number of potential N-linked glycosylation sites on the HIV-1 envelope gp120 subunit. The enhanced neutralization resistance of HIV-1 in contemporary seroconverters coincided with the poorer elicitation of neutralizing antibody responses, which may have implications for vaccine design.

Figure 2: Increased Env length and increased number of PNGs in the viral envelope in contemporary subtype B HIV-1 as compared to historical isolates. (a–f) The lengths of gp120 (a), the V1 loop of gp120 (b) and the V4 loop of gp120 (c) and the number of PNGs in gp120 (d), the variable (V) regions of gp120 (e) and the V1 loop of gp120 (f) are shown for viruses isolated during primary infection from participants of the Amsterdam Cohort Studies who seroconverted between 1985 and 1989 (n = 14) or between 2003 and 2006 (n = 20). Each data point represents the average value for all viruses from one seroconverter. (g–j) Similar comparisons are shown for subtype B envelope sequences from the Los Alamos database, with a documented year of virus isolation between 1985 and 1988 (n = 27) or between 2003 and 2005 (n = 72) for the length of gp120 (g), the length of the V1V2 loop of gp120 (h) and for the number of PNGs in gp120 (i) and in the V1 loop of gp120 (j). In all panels, horizontal bars represent the median. Differences between groups were evaluated with a Mann-Whitney U test, aa, amino acid residues. Amsterdam Cohort Studies have been conducted in accordance with the ethical principles set out in the declaration of Helsinki, and written informed consent was obtained from each cohort participant prior to data and

...
Objectives of REDS-III HIV Diversity Project

- Comparison, training and adoption of improved sequencing methods for pol gene and/or full genome to enable high yield of sequence data and better drug resistance classification and subtype assignment than in REDS-II

- Perform detailed env diversity analysis targeting NAT yield and recent SC incident cases to characterize T/F viruses, and to provide data for validation of deep sequencing study (using 454 deep sequencing platform)

- Demonstrate evolution of T/F viruses in blood donations with incident HIV infections by performing deep sequencing study on 300 acutely infected blood donor samples, 100 from the US, 100 from Brazil and 100 from SA, with 50 from 15-20 years ago and 50 recent NAT yields or very recent SCs per country