World Health Organization Generic Protocol for surveillance of initial drug-resistant HIV-1 among children < 18 months of age newly diagnosed with HIV
### Acronyms and abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>3TC</td>
<td>lamivudine</td>
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<tr>
<td>ABC</td>
<td>abacavir</td>
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<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<td>ANC</td>
<td>antenatal clinic</td>
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<td>ART</td>
<td>antiretroviral therapy</td>
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<td>ARV</td>
<td>antiretroviral drug</td>
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<tr>
<td>AZT</td>
<td>zidovudine (also ZDV)</td>
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<td>BAN</td>
<td>Breastfeeding, Antiretrovirals, and Nutrition study</td>
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<tr>
<td>CBV</td>
<td>combivir (zidovudine + lamivudine)</td>
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<tr>
<td>CDC</td>
<td>U.S. Centers for Disease Control and Prevention</td>
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<tr>
<td>d4T</td>
<td>stavudine</td>
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<tr>
<td>DBS</td>
<td>dried blood spot</td>
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<tr>
<td>ddl</td>
<td>didanosine</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EFV</td>
<td>efavirenz</td>
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<td>EIA</td>
<td>enzyme immunoassay</td>
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<td>ETV</td>
<td>etravirine</td>
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<tr>
<td>FDC</td>
<td>fixed-dose combination</td>
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<td>FTC</td>
<td>emtricitabine</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HIVNET</td>
<td>HIV Network for Prevention Trials</td>
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<td>HIVDR</td>
<td>HIV drug resistance</td>
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<tr>
<td>IMPAACT</td>
<td>International Maternal Pediatric Adolescent AIDS Clinical Trial</td>
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<td>KiBS</td>
<td>Kisumu Breastfeeding Study</td>
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<tr>
<td>LPV/r</td>
<td>lopinavir/ritonavir</td>
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<tr>
<td>MCH</td>
<td>Maternal and Child Health</td>
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<tr>
<td>mL</td>
<td>milliliter</td>
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<tr>
<td>MTCT</td>
<td>mother-to-child transmission (of HIV)</td>
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<td>NFV</td>
<td>nelfinavir</td>
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<tr>
<td>NNRTI</td>
<td>non-nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>NRTI</td>
<td>nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>NVP</td>
<td>nevirapine</td>
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<tr>
<td>PACTG</td>
<td>Pediatric Clinical Trials Group</td>
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<td>PEPFAR</td>
<td>President’s Emergency Plan for AIDS Relief</td>
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<td>PEPI</td>
<td>Malawi Post-Exposure Prophylaxis of Infants study</td>
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<td>PETRA</td>
<td>Perinatal Transmission Study</td>
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<tr>
<td>PI</td>
<td>protease inhibitor</td>
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<tr>
<td>PITC</td>
<td>Provider-initiated (HIV) testing and counseling</td>
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<tr>
<td>PLHIV</td>
<td>people living with HIV</td>
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<tr>
<td>PMTCT</td>
<td>prevention of mother-to-child transmission (of HIV)</td>
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<tr>
<td>SAINT</td>
<td>South African Intrapartum Nevirapine Trial</td>
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<tr>
<td>sd-NVP</td>
<td>single-dose nevirapine</td>
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<td>SIMBA</td>
<td>Stopping Transmission from Mother-to-child via Breastfeeding in Africa</td>
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<td>SWEN</td>
<td>Six Weeks Extended-dose Nevirapine study</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>TDF</td>
<td>tenofovir disoproxil fumarate</td>
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<td>TOPS</td>
<td>Treatment Options Preservation Study</td>
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<tr>
<td>VCT</td>
<td>Voluntary (HIV) Testing and Counseling</td>
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<td>VL</td>
<td>viral load</td>
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<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>ZDV</td>
<td>Zidovudine (also AZT)</td>
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PROTOCOL OVERVIEW

This generic protocol describes survey method to assess HIVDR using remnant dried blood spot specimens from a representative sample of children <18 months being tested for early infant diagnosis in resource-limited countries.

Countries should adapt this protocol to reflect the national situation and submit the national protocol for clearance to the appropriate institutions.

1. INTRODUCTION

A. Literature Review

Background

As of December 2010, the number of children less than 15 years living with HIV was estimated to be 3.4 million [3 000 000-3 800 000] [1]. In 2010, an estimated 390 000 [340 000–450 000] children under 15 years were newly infected with HIV, down from 500 000 [320 000–670 000] in 2001; the decrease is attributed to expansion of PMTCT. An estimated 250 000 [220 000–290 000] children died from AIDS-related illnesses.

In 2010, 48 % [44 %–54 %] of pregnant women living with HIV in low- and middle-income countries received the most effective regimens to prevent mother to child transmission (excluding single dose nevirapine).

Before antiretroviral drugs (ARVs) were used for mother to child transmission prevention, (PMTCT), mother to child transmission (MTCT) rates in untreated non-breastfeeding populations in high-income countries ranged from 14% to 32% vs. 25% to 48% among breastfeeding populations in resource-poor settings [2]. In the absence of antiretroviral prophylaxis, the risk of postnatal infection appears highest in the 4–6 weeks of the infant’s life, ranging from 0.7%-1% per week [3-5].
However, the risk continues for the duration of breastfeeding; in two large studies, the risk of late postnatal transmission after age 4-6 weeks was 8.9 infections per 100-child-years of breastfeeding (~0.17% per week) and was constant throughout the breastfeeding period [6, 7]. The proportion of transmission occurring during different time periods for children with prolonged breastfeeding is 10-25% in utero, 35-50% intrapartum, 20-25% during first 2 months of breastfeeding, and 20-25% after the first two months of breastfeeding.

In well-resourced health care systems, universal HIV testing for pregnant women, provision of ARVs either for prophylaxis or for the mother’s health, elective caesarian delivery, and avoidance of breastfeeding has reduced MTCT of HIV infection to 1%-2% [8-10]. In the absence of any ARVs, studies have demonstrated that the probability of MTCT during breastfeeding is between 14%-37% depending on duration of breastfeeding [5, 11, 12]. With breastfeeding, studies show that the prophylactic use of ARVs in pregnant women and their children reduces postnatal MTCT to an estimated 1-7%, depending on the timing, type, and duration of the ARV regimen used, adherence to the regimen, duration of breastfeeding, and maternal underlying disease (e.g., CD4 count, HIV RNA level, WHO disease stage) [13-21]. Breastfeeding is associated with significantly lower mortality in children in resource-limited countries, especially when the child is HIV-infected [7, 22-25].

WHO recommends [26] that mothers known to be HIV-infected who breastfeed their infants should exclusively breastfeed for the first 6 months of life, introducing appropriate complementary foods thereafter, and continue breastfeeding for the first 12 months of life and that ARV prophylaxis should be provided (as detailed below). Breastfeeding should only stop once a nutritionally adequate and safe diet without breast milk can be provided. HIV infected mothers of infected infants are advised to continue breastfeeding for at least two years [26].
Current WHO recommendations for PMTCT and pediatric ART

WHO PMTCT Guidelines

As access to PMTCT services has increased, the total number of children being born with HIV has decreased. In 2010, an estimated 390 000 [340 000–450 000] children under 15 years were newly infected with HIV (a drop of 24% from five years earlier) [27]. Today, the vast majority of children who acquire HIV infection have not received PMTCT interventions. WHO published new recommendations for PMTCT in 2010 [28]. The recommendations are:

1. Initiation of ART for their own health is recommended for all HIV-infected pregnant women with a CD4 cell count < 350 cells/mm³, irrespective of WHO clinical staging; and for all HIV-infected pregnant women in WHO clinical stage 3 or 4, irrespective of CD4 cell count.

2. Initiation of ART for eligible pregnant women regardless of gestational age and continuation of ART throughout pregnancy, breastfeeding, and thereafter.

3. The preferred first-line regimen for pregnant women is ZDV +3TC + [NVP or EFV]. An alternate regimen of TDF + [FTC or 3TC] + [NVP or EFV] is also acceptable.

4. Infants born to women receiving ART for their own health should receive daily ZDV or NVP from birth to four - six weeks regardless whether they are breastfeeding or not.

5. All HIV-infected pregnant women not receiving ART for their own health should start ARV prophylaxis to prevent MTCT as early as 14 weeks gestation, or as soon as possible when women present late in pregnancy, in labor, or at delivery.

There are two options for PMTCT ARVs for women not receiving ART for their own health.

<table>
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<tr>
<th>Option A</th>
<th>Option B</th>
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<tr>
<td><strong>Maternal PMTCT regimen</strong>: Antepartum daily ZDV; plus sd-NVP + ZDV +3TC during labour and delivery; plus daily ZDV +3TC continued for 7</td>
<td><strong>Maternal PMTCT regimens</strong>: Antepartum daily triple ARV prophylaxis until delivery or, if breastfeeding, until one week after all exposure</td>
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</table>
| Days postpartum. | to breast milk has ended. Recommended maternal regimens include:  
| | ZDV +3TC +LPV/r  
| | ZDV +3TC +ABC  
| | ZDV +3TC +EFV  
| | TDF+3TC (or FTC) +EFV |

| **In breastfeeding children**, daily NVP should be given to the child from birth until one week after all exposure to breast milk has ended or a minimum six weeks, if breastfeeding stops earlier. | **In breastfeeding infants**, maternal triple ARV prophylaxis should be coupled with daily NVP or twice daily ZDV to the infant from birth until four to six weeks of age. |

| **In infants receiving only replacement feeding**, Sd-NVP at birth plus daily NVP or twice daily ZDV should be given to the infant from birth until four to six weeks of age. | **In infants receiving only replacement feeding**, maternal triple ARV prophylaxis should be coupled with daily NVP or twice daily ZDV to the infant from birth until four to six weeks of age. |

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**WHO recommendations on HIV diagnosis and antiretroviral treatment for children**

WHO also revised ART and HIV diagnostic recommendations for infants and children in 2010.[29] WHO recommends that children known to be exposed to HIV receive a diagnostic HIV PCR (nucleic acid) test at four to six weeks of age, or at the earliest opportunity thereafter, and, if negative at that time, repeated at approximately six weeks after the complete cessation of
breastfeeding (when the child is < 18 months old) or earlier if symptoms suggestive of HIV infection occur [29].

All children < 24 months of age should start ART as soon as they are diagnosed as HIV-infected, regardless of clinical or immunologic status [29]. Children previously exposed to any NNRTI, either through their mothers during pregnancy, delivery, or breastfeeding, or having received an NNRTI directly, should start on triple ARV therapy including a boosted PI. All children not known to have been previously exposed to an NNRTI should start on standard NVP-containing ART. WHO-recommended ART regimens are ZDV +3TC + NVP for children without prior NNRTI exposure and ZDV+3TC+ LPV/r for children with prior NNRTI exposure [29].

The implementation of early pediatric LPV/r-based ART in low resource settings represents a global challenge: the public health approach to the global ART scale-up has been based on two NRTIs and one NNRTI, which are widespread and available, are relatively cheap, exist in generic formulations and pediatric co-formulated tablets.

The pediatric PI-based ART regimen, commonly reserved for second-line therapy but recommended as the first-line in children previously exposed to NNRTIs, has limited availability and relatively high cost and present a number of challenges. These include:

1. LPV/r is currently the only protease inhibitor option, as the pediatric dosing and/or formulations are not available and/or approved for other agents.
2. Only liquid formulations are available for children (which are poorly palatable and require supply through a cold chain) [30].
3. Interactions between LPV/r and rifampin complicate the use of LPV/r in children with TB [31].
4. LPV/r is associated with long term metabolic complications [32-34].
5. There are no ARV drug classes other than NRTIs, NNRTIs, and PIs available in resource-limited countries, so there are no clear options for second-line therapy for children whose first line regimen is PI-based, unless the NNRTI class is re-introduced.

**Resistance among children who become infected despite prophylaxis for MTCT**

*Resistance in children infected after prophylaxis with an NNRTI for MTCT*

In children, NNRTI resistant HIV can be selected after exposure to maternal or pediatric NNRTIs in the antenatal, intrapartum, and postpartum periods (including during breastfeeding); or transmitted *in utero*, at birth, or during breastfeeding. Although no studies have evaluated resistance in children infected despite EFV ART regimens taken by the mother for her own health or for prophylaxis during breastfeeding, NNRTI-resistant HIV developing from exposure to EFV is generally cross-resistant to all NNRTIs, and EFV has been demonstrated to appear at high levels in breast milk [35].

Although combination regimens for use in PMTCT have been recommended for resource-limited countries since 2006 [36], many countries continue to offer single-dose nevirapine (sd-NVP), associated with development of high rates of NNRTI resistance among both HIV-infected mothers and infants who become infected. Sd-NVP continues to be used because of cost and feasibility issues. Recent recommendations to prolong pediatric NVP during the postpartum period and breastfeeding [28] will prevent many pediatric HIV infections, but a large proportion of children infected despite NVP postnatal prophylaxis may have NNRTI HIVDR [37, 38].

Using a random effects model, Arrivé’s meta-analysis[39] of seven studies of infants who become infected despite sd-NVP prophylaxis (without additional ARV drugs) showed 52.6% (n=201; CI 37.7-67.0) NNRTI resistance detected at four to eight weeks using standard assays.
The NEVEREST study assessed pre-ART NNRTI resistance in 257 HIV-1 infected children < 2 years of age with prior exposure to sd-NVP [41]. A clear age-trend was evident in population-based sequencing of pre-treatment specimens with 46% of children 0-6 months having NNRTI resistance, 24% at 6-12 months, 20% at 12-18 months and 0% at 18-24 months (p<0.001). An additional 9-10% in each age group had NNRTI resistance mutations detected by a more sensitive method, AS-PCR.

Moorthy and colleagues[40] compared 49 Indian infants who received sd-NVP at birth with 25 Indian infants who received six-week extended-dose NVP (“SWEN”) during breastfeeding. For infants diagnosed with HIV in the first six weeks of life, those who received SWEN were significantly more likely to have NNRTI resistance mutations detected by standard population sequencing than those who received sd-NVP (92% of 12 vs. 38% of 29; p=0.002). For infants whose infections were detected late in breastfeeding, both groups had a similar prevalence of NNRTI resistance (15% of 13 infants who had received SWEN vs. 15% of 20 infants who had received sd-NVP).

Church and colleagues[37] genotyped samples from 24 infants who were infected despite sd-NVP at birth and 25 who were infected despite sd-NVP at birth and six weeks postpartum NVP prophylaxis. In both study arms, infants who were HIV infected at birth frequently had NVP resistance detected. When infants were six weeks old, population-based sequencing detected NVP resistance in a higher proportion of infants in the extended NVP arm than in the sd-NVP arm (21 of 25 [84%] vs. 12 of 24 [50%]; P < .01). Infants in the extended NVP arm who were infected postpartum were more likely to have resistance detected at six weeks, compared with infants in the sd-NVP arm. The use of extended NVP prophylaxis was also associated with detection of NVP resistance by ViroSeq at six months: seven of seven [100%] infants in the extended NVP arm had resistance detected, compared with one of six [16.7%] infants in the SD NVP arm (P < .005).

NNRTI resistance is often found at higher rates in infants in observational studies as opposed to clinical trials like those reviewed by Arrivé.[39] Explanations include insufficiently strict
methodology for accurate evaluations, varying periods of exposure, or “real-world” conditions such as lack of adherence to ARVs. The South African Intrapartum Nevirapine Trial (SAINT) examined 40 HIV-infected infants born to mothers who had received two doses of NVP and found NNRTI mutations among 21 (53%)[44]. In relatively small studies, HIV-infected infants exposed to NVP alone antepartum/intrapartum and postpartum were reported to have NNRTI resistance at rates between 38% and 92%[45-49].

**Resistance in infants exposed to combination NNRTI/NRTI prophylaxis for MTCT**

Arrivé’s metanalysis of seven studies showed that NNRTI resistance prevalence was reduced to 16.5% (n=138; CI 8.9–28.3) in the group of eight study arms combining NVP with ZDV or ZDV+3TC,[39] compared to the above-reported 52.6% (n=201; CI 37.7–67.0) in infants who had been exposed to NVP-only prophylaxis.

The Malawi Post-Exposure Prophylaxis of Infants (PEPI) study compared 43 infants receiving extended NVP after birth to 45 infants receiving extended NVP and ZDV.[41] At 14 weeks of age, the proportion of infants with NVP resistance was lower in the extended NVP and ZDV arm than in the extended NVP arm (28/45, 62.2% vs. 37/43, 86.0%; P< 0.015). None of the infants had ZDV resistance. Addition of extended ZDV to extended NVP was associated with reduced risk of NVP resistance at 14 weeks if HIV was diagnosed and prophylaxis was stopped by six weeks (54.5 vs. 85.7%, P<0.007) but not if prophylaxis was continued beyond six weeks (83.3 vs. 87.5%, P< 1.00).

Eshleman and colleagues[42] analyzed the development of NVP resistance in HIV-1-infected Malawian infants who received regimens containing sd-NVP for prophylaxis of MTCT. All infants received sd-NVP, and some received ZDV as well. Mothers did or did not receive sd-NVP on the basis of when they arrived at the hospital for delivery. In infants six to eight weeks of age, NVP resistance was less frequent when infants had received sd-NVP plus ZDV and mothers had not received sd-NVP than when infants had received sd-NVP alone and mothers had received sd-NVP
(4/15 [27%] vs. 20/23 [87%]; P < .001). The risk of MTCT of HIV-1 was comparable with these regimens. The authors suggest on the basis of these results that maternal sd-NVP may not be necessary if infants receive sd-NVP plus ZDV.

Pillay and colleagues[43] genotyped samples from 53 HIV-infected Thai infants at six weeks, 23 of whom had received NVP during the six weeks and 30 of whom had both received NVP and been exposed to NVP through their mothers. Three of the 23 (13%) who had received NVP directly had NNRTI resistance mutations vs. eight of the 24 (33%) who had ingested NVP and also been exposed to maternal NVP.

Church and colleagues[44] pooled data from four NNRTI resistance studies in 82 Ugandan infants infected with HIV despite sd-NVP prophylaxis and tested at six to eight weeks: HIVNET 012 (1997-1999, sd-NVP arm only), the Repeat Pregnancy study (2004-2006, prospective portion only), the Pathophysiology of Breast Milk study (2003-2004), and the Ugandan component of the SWEN study (2004-2006, sd-NVP arm only). Testing was done by ViroSeq and a more sensitive method, LigAmp, was also used to detect three NNRTI mutations. The investigators found a higher proportion of NNRTI resistance in infants infected in utero compared to those infected later (OR=3.5 [95% CI: 1.3-9.4], p=0.01 for ViroSeq; OR=3.9 [95% CI: 1.3-11.4], p=0.01 for LigAmp). Of the 36 who had NNRTI resistance at 6-8 weeks, 12/27 with available specimens still had detectable NNRTI mutations at 6-12 months.

The Treatment Options Preservation Study (TOPS)[45] included three arms: sd-NVP alone, administered to the mother at the onset of labor and to the infant, was compared to maternal sd-NVP with AZT plus 3TC, given as Combivir (CBV) for 4 (NVP/CBV4) or 7 (NVP/CBV7) days, initiated simultaneously with sd-NVP in labor; newborns received the same regimens as their mothers. An unplanned interim analysis resulted in early stopping of the sd-NVP arm. In the infants with intra-uterine HIV-1 infections and follow-up after exposure to ARVs, NNRTI-resistant mutations were
seen in seven of the eight HIV-infected infants treated with sd-NVP only, four of 25 of those who received NVP/CBV4, and none of the ten who received NVP/CBV7. The difference between NVP alone and the combined NVP/CBV arms is statistically significant (Fisher’s exact, two-sided, p<0.001).

In the BAN (Breast feeding, Antiretrovirals, and Nutrition) study in Malawi, Farr and colleagues[46] evaluated 14 HIV-infected infants at six weeks. Among them, one of six (17%) whose mothers had received NVP/ZDV + 3TC and three of eight (38%) whose mothers received sd-NVP had NNRTI resistance mutations.

**Resistance in infants exposed to NRTI prophylaxis for MTCT**

In the Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 study, no NRTI resistance was detected among six infants infected despite maternal prophylaxis with ZDV during pregnancy, labor, and during the six weeks after birth.[47] In the SAINT study, no infants born to 37 women who received seven days of ZDV/3TC had NRTI resistance mutations.[39] In PACTG 185, a higher prevalence of ZDV resistance (30%) was found among infants infected despite maternal ZDV prophylaxis. The assessment was performed at the age of six weeks.[48]

In the Stopping Infection from Mother-to-child via Breastfeeding in Africa (SIMBA) study,[38, 49] infants whose mothers had received ZDV and ddI from 36 weeks to one week postpartum were randomized to receive either NVP or 3TC until one month after stopping breastfeeding or until HIV diagnosis was confirmed. Samples were available from 26 of 30 infected infants. Of those who received NVP prophylaxis, 12/13 (92%) had NNRTI resistance mutations, all of which were still present in the nine samples collected six months after discontinuation of prophylaxis. Among the infants who had received 3TC, 9/13 (69%) had the M184V/I mutation conferring 3TC resistance; it was no longer present among the seven samples collected five months following discontinuation of prophylaxis. No infants had ZDV or ddI resistance mutations.
Resistance in infants exposed to maternal HAART

In the Kisumu Breastfeeding Study (KiBS) pregnant women received a HAART regimen, ZDV +3TC +NVP or ZDV +3TC +NLF, from 34 weeks through six months of breastfeeding. 29 infants were HIV-infected, of which 24 were diagnosed by PCR < 24 weeks and whose specimens were genotyped. ARVs taken by the mothers of these 24 infants included the NVP-based regimen for 14 (58%) and the NFV-based regimen for 10 (42%). Genotypic resistance was detected among 16 infants including 6 (43%) of 14 infants of mothers taking NVP and 10 (100%) of 10 infants of mothers taking NFV (p = 0.006). Genotypic resistance mutations to NRTI were M184V (n = 13), K65R (n = 4), D67N (n = 2), and T215Y (n = 2), and to NNRTI were Y181C (n = 3), K103N (n = 2), G190A (n = 2), and K101E (n = 1). Among infants exposed to maternal NVP and NRTIs, four (67%) of six infants with resistance had an NRTI mutation and all six had an NNRTI mutation. Among infants exposed to maternal NFV and NRTIs, 10 (100%) of 10 infants had a NRTI mutation, but none had a major protease inhibitor mutation[58].

Lidstrom and colleagues[59] found that among seven infants who had received sd-NVP and extended NVP prophylaxis, and whose mothers had started NNRTI-based HAART by six months, six had both NNRTI and NRTI resistance mutations at six weeks, the seventh had NNRTI mutations alone.

Lidstrom with another set of colleagues[60] genotyped 14-weeks samples from four HIV-infected infants who had had sd-NVP, two of whom had also had extended NVP prophylaxis and two of whom had had extended NVP/ZDV prophylaxis. All four had mothers who had started on an NRTI/NNRTI HAART by 14 weeks. At fourteen weeks all four infants had NNRTI mutations and three infants (75%) had NNRTI/NRTI mutations. Among 84 infants infected in utero whose mothers did not start HAART by 14 weeks, many had NNRTI mutations, but none had NRTI mutations detectable by bulk sequencing.
Fogel and colleagues found that initiation of maternal ART within 14 weeks of delivery was associated with detection of multi-class HIVDR in breastfeeding infants.[50]

**ARV drug concentrations and drug-resistant HIV in breast milk**

Mirochnick and colleagues[51] assessed ARV levels in breast milk of 67 mothers receiving AZT, 3TC, and NVP from 34 weeks gestation to six months postpartum. Plasma ARV levels were measured in their 67 infants at two, six, and fourteen weeks. The median concentrations in breast milk of ZDV, 3TC, and NVP during the study period were 14 ng/mL, 1214 ng/mL, and 4546 ng/mL, respectively. ZDV was not detectable in any infant plasma samples obtained after the day of delivery, while the median concentrations in infant plasma samples from postpartum week biologically significant, at all measurement points for 3TC and NVP. During weeks 2, 6, and 14, the median infant NVP concentration was 896.9 ng/mL, well above the median HIV IC50 of 17 ng/mL for NVP but below the suggested target trough NVP concentration of 3,000 ng/mL when NVP is used for treatment (1; Nevirapine package insert, revised April 2007 [Boehringer Ingelheim]).

The maternal breast milk and infant plasma concentrations and the breast milk/plasma ratio observed for NVP in this study are similar to those reported in a study of EFV transfer from breast milk.[35] In that study, the median breast milk EFV concentration was 3450 ng/mL, the mean EFV breast milk/plasma ratio was 0.54, and the median infant EFV concentration was 870 ng/mL, just below the suggested target trough EFV concentration of 1,000 ng/mL when EFV is used for treatment.

Kunz and colleagues[52] measured NVP levels in breast milk of 44 Ugandan women for up to 48 days after intrapartum sd-NVP was received. Between days six and nine all breast milk samples exhibited NVP concentrations higher than the IC50 for NVP, the median being 114 ng/mL (IQR 63 ng/mL- 206 ng/mL). Between days 13 and 16, 42% of the women still had measurable NVP concentrations above the IC50. NVP was not measurable in any sample from day 16 after NVP.
intake onwards. These ARV levels could prevent infection in the infant, but might equally lead to the acquisition of resistance in infants previously infected with wild-type HIV.[51, 53]

Lee and colleagues[54] evaluated NNRTI-resistant HIV in the breast milk of 20 Zimbabwean women who had received intrapartum sd-NVP. Of these, 13/20 (65%) had at least one NNRTI mutation in the RT sequence amplified from breast milk. Four infants were HIV-infected; of these, two were thought to have been infected *in utero*, and the mother of one had wild-type HIV in her breast milk.

Farr and colleagues[46] compared the breast milk of Malawian women who received sd-NVP to others who received sd-NVP + a seven-day ZDV/3TC “tail” at two to six weeks. Seven women had sufficient viral loads in breast milk to test for viral resistance to nevirapine. In the sd-NVP group, three of three women had at least one NVP resistance mutation. In the NVP/ZDV + 3TC group, none of four women had NVP resistance mutations in breast milk.

**Effects of PMTCT-associated resistance on pediatric ART outcomes**

The P1060 (Cohort I) study [55] was a randomized Phase II treatment trial conducted in six African countries comparing ART with ZDV +3TC and LPV/r or NVP in HIV-infected children 6 to 36 months old and previously exposed to sd-NVP prophylaxis. Primary study endpoints were virologic failure (HIV RNA level < 1 log10 copies below entry at 12-24 weeks or confirmed HIV RNA > 400 copies/mL at 24 weeks) or treatment discontinuation for any reason including death and toxicity. The study showed that LPV/r was superior to the NVP arm for time to primary endpoint. DSMB terminated enrollment early because a higher proportion of children receiving NVP-based ART (39.6%) vs. LPV/r-based ART (21.7%) experienced a primary endpoint by week 24, with a 18.6% difference between arms; 95% confidence interval 3.7-33.6%, nominal p-value =0.015. Baseline NVP resistance was detected in 18/148 (12%) children and was associated with treatment failure.[55]
In the Botswana Mashi (“milk”) trial, Lockman and colleagues[56] compared the outcome of NNRTI-based ART for 30 HIV-infected infants who had been previously randomized either to receive sd-NVP (n=15) or placebo (n=15); their mothers were randomized to the same prophylactic regimens to prevent MTCT. All mothers received antenatal ZDV from week 34. Three infants who received placebo and two who received sd-NVP died before the six-month visit and could not be evaluated. Using a Kaplan-Meier analysis, the authors reported that virologic failure by the six-month visit occurred in significantly more infants who had received a sd-NVP than in infants who had received placebo (10/12 [76.9%] vs. 1/13 [9.1%]; P<0.001). Findings were substantially unchanged at 12 months and 24 months; one more infant in the placebo group had virologic failure by 24 months after ART initiation, but the difference in failure rates between the two groups was still highly significant. Subsequently the study was revised so that all infants received sd-NVP but mothers continued to be randomized to placebo vs. sd-NVP. The authors updated the report on this study in 2009.[57] In the original study period, the NNRTI-based HAART of 11 of 15 infants in the infant sd-NVP/maternal sd-NVP group (73%) failed vs. four of 15 in the infant placebo/maternal placebo group (27%) (p=0.008). In the revised study period, six of 14 of the infant sd-NVP/maternal sd-NVP group failed (43%) and four of 12 of the infant sd-NVP/maternal placebo group failed (33%); these outcomes were not significantly different (p=0.96), indicating either that the sd-NVP infant exposure was not associated with failure or that the sample size was too small to detect differences in the failure rates of the two groups.

Coovadia and colleagues[58] assessed treatment outcomes in HIV-infected children under the age of two years who had been exposed to sd-NVP prophylaxis and who met immunologic and clinical criteria for ART (n=322). These children were started on LPV/r, D4T, and 3TC in Johannesburg, South Africa. Children who achieved and maintained HIV RNA < 400 copies/mL for ≥3 months were eligible for randomization (n=195) either to remain on the same regimen (control
n=99) or to substitute NVP for LPV/r (switch n=96). The children were followed for 52 weeks.

Plasma viremia greater than 50 copies/mL occurred less frequently in the switch group (Kaplan-Meier probability, 0.438; 95% CI, 0.334-0.537) than in the control group (0.576; 95% CI, 0.470-0.668) \( (P=.02) \). Confirmed viremia greater than 1000 copies/mL occurred more frequently in the switch group (0.201; 95% CI, 0.125-0.289) than in the control group (0.022; 95% CI, 0.004-0.069) \( (P=_-.001) \). CD4 cell response was better in the switch group (median CD4 percentage at 52 weeks, 34.7) vs. the control group (CD4 percentage, 31.3) \( (P=.004) \). Older age (relative hazard [RH], 1.71; 95% CI, 1.08-2.72) was associated with viremia greater than 50 copies/mL in the control group.

Inadequate adherence (RH, 4.14; 95% CI, 1.18-14.57) and drug resistance (RH, 4.04; 95% CI, 1.40-11.65) before treatment were associated with confirmed viremia greater than 1000 copies/mL in the switch group. Thus, while viral suppression to <50 copies/mL was more frequent in the children switching to NVP then those on LPV/r, the children on NVP whose VL was not suppressed were more likely to have VL increase to >1,000 copies/mL than those who stayed on LPV/r.

The authors state that their data indicate that children who switch to NVP-based therapy once they have achieved viral suppression after an average of nine months of therapy based on LPV/r are more likely to achieve viremia less than 50 copies/mL than children who kept their original regimen. However, a sizable minority (20%) experienced breakthrough viremia greater than 1000 copies/mL that required consideration for therapy change. This outcome was strongly related to pretreatment NNRTI mutations and was rare (2%) among children who maintained their original regimen. The authors claim that the study provides proof of concept that re-use of NVP following successful suppression on LPV/r-based therapy is possible under some circumstances for HIV-infected children exposed to sd-NVP prophylaxis, but also say that the “seemingly inconsistent results highlight the promise and pitfalls of switching NVP-exposed children back to an NNRTI-based regimen.” [58]
Moorthy and colleagues[59] also studied a subset of South African children between 2 and 24 months of age from the NEVEREST trial who had failed sd-NVP PMTCT and who were treated with LPV/r, 3TC, and d4T before being randomized to continue the LPV/r regimen or being switched to an NVP, 3TC, and d4T regimen. The infants started ART at median nine months of age and received LPV/r for a median of nine months. 63 children in the NVP arm and 61 children in the LPV/r arm had complete RT sequences at endpoint. Children with viral loads of ≥1000 copies/mL at two or more visits within 52 weeks were defined as having virologic failure. Ultradeep pyrosequencing was successful in 96 children. Of those, 59 (62%) had archived NNRTI mutations. Confirmed viremia of ≥1000 copies/mL by 52 weeks was more common in children who switched to NVP-based ART than those who continued to receive LPV/r-based ART (14/63 [22%] vs. 2/61 [3%]; P=.002).

In summary, more than 1000 new pediatric HIV infections occur each day primarily through MTCT, and the vast majority of children become infected with HIV due to slow scale-up of appropriate PMTCT programs. A relatively small proportion of children become infected despite PMTCT prophylaxis and the use of sd-NVP; extended NVP prophylaxis either alone or in combination with NRTIs, or maternal use of NNRTI-based HAART, places children at risk for acquiring NNRTI resistance mutations if they become HIV-infected. NRTI prophylaxis to prevent MTCT can also lead to infection with NRTI-resistant HIV. Scale-up of PMTCT with the use of more efficacious regimens should result in a dramatic decrease in the incidence of pediatric HIV infection, but the widespread use of an NNRTI for infant or maternal PMTCT prophylaxis or as a drug in a maternal HAART regimen during pregnancy and breastfeeding will result in an increase in the proportion of children infected despite prophylaxis or maternal HAART who are infected with NNRTI-resistant HIV.
Response to NNRTI-based first-line ART in HIV-infected children may be compromised by prior exposure to NNRTIs for PMTCT or for maternal health. NRTI mutations resulting from prior NRTI exposure may also reduce response to ART. Infants initiating treatment during the first months of life may be at highest risk for virologic failure, but the high risk of mortality precludes delaying ART initiation. LPV/r-based ART is the recommended regimen for HIV-infected children with prior NNRTI exposure for PMTCT prophylaxis. However, it is costly and not yet widely available in low and middle-income countries. The South African study described above demonstrates that in some circumstances NVP-containing regimens can be used after suppression is maintained on a boosted PI regimen in children previously exposed to sd-NVP. But there are limited options currently available for children who cannot tolerate LPV/r or whose first-line LPV/r-based ART fails [68]. Alternative approaches and new ARVs are urgently needed to ensure safe and successful lifelong ART for infants and children with HIV infection.

B. The WHO HIVDR Prevention and Assessment Strategy

WHO recommends that countries adopt the HIVDR Prevention and Assessment strategy [60], including this protocol and two additional pediatric assessments:

1. **HIV Drug Resistance Early Warning Indicators (EWI) at sites providing ART to pediatric populations.** The HIVDR EWI monitoring do not involve genotyping, but assess factors associated with the emergence of HIVDR in a representative sample of children receiving ART at representative ART sites in each country. Factors monitored include: ART prescribing practices, losses to follow-up at 12 months, the proportion of children still on appropriate first-line ART at 12 months, the proportions of children whose ARV drugs are picked up on time and ARV drug stock-outs.
2. Surveys to Monitor HIV Drug Resistance Prevention and Associated Factors in Sentinel Pediatric Antiretroviral Treatment Sites. WHO has developed a protocol to evaluate the effect of maternal and pediatric ARV history, baseline drug resistance, and other factors on ART outcomes in children. The protocol specifies obtaining a baseline genotype in a cohort of approximately 130 children before ART is started and following them for 12 months to evaluate the factors associated with viral suppression and the development of drug resistance, including the child’s and the mother’s ARV history intrapartum, at delivery, and postpartum. A viral load and genotype are obtained after twelve months of treatment.

The survey has been piloted in a cohort of older children in one country; data are being analyzed.

This generic protocol aims to describe rational and methods to conduct survey of drug-resistant HIV-1 among children < 18 months of age newly diagnosed with HIV.

2. JUSTIFICATION FOR THE SURVEY

Studies like those discussed in the literature review have estimated the prevalence of DR-HIV among children associated with various PMTCT regimens administered to mothers and children. However, no widespread surveillance systems have been implemented to evaluate the association of PMTCT and DR-HIV among children in the field, because of expense, difficulties with specimen collection, and the difficulty of comparing children in different programs and receiving different regimens. With new virological diagnostic methods for children < 18 months of age involving dried blood spots (DBS) being implemented in a large number of resource-limited countries [61], remnant DBS are likely to be available for drug resistance surveys. This represents an important opportunity to evaluate resistance routinely among children < 18 months of age.
newly diagnosed with HIV, providing critical information to support optimal regimen selection. HIV has been successfully amplified and genotyped for drug resistance testing from DBS in a large number of studies.[62-70]

Plasma is considered the optimal specimen type for genotyping, but collection, transport, and storage of plasma specimens is not feasible in many resource-limited settings, particularly in remote sites. A number of different methodologies for performing HIVDR genotyping using dried blood (or serum) spots, including some comparisons of various storage conditions, have been developed and reported in the literature [62, 70, 71]. Despite the potential advantages of DBS as a method of specimen collection, there are several disadvantages, the foremost being reduced sensitivity of viral RNA amplification. In specimens with low viral loads, pro-viral DNA from peripheral blood mononuclear cells (PBMC) may contribute a significant proportion of information to genotyping results. Thus in some patients with low viral loads, genotyping results from DBS specimens may not reflect the current status of replicating viruses circulating in the patient’s plasma as accurately. However, HIV-infected children < 18 months of age generally present with a high viral load because their immature immune system is unable to control viral replication. In the population targeted in this protocol, low viral loads are likely only to be seen in children being tested while on postnatal ARV prophylaxis (NVP or AZT).

In most settings, three to five DBS are collected from each child for PCR testing and other purposes.

World Health Organization pediatric ART guidelines state that children who have received NNRTI-based PMTCT previously should be started on a protease inhibitor (PI)-based regimen.[29] Despite these guidelines, in many countries all children are still started on NNRTI-based regimens, because of cost and feasibility. For this reason, it is important to know what proportion of children < 18 months of age who will be given an NNRTI-based regimen have mutations that may be
associated with regimen failure. Even in countries where pediatric PI-based regimens are available, PMTCT ARV exposure may not be routinely recorded for children starting ART, or in many cases, previous ARV experience may be reported as “none” or “unknown”, so children who have been exposed to ARV drugs antepartum/intrapartum or postpartum may be started on an NNRTI-based regimen. It is important to evaluate the proportion of children whose PMTCT history is reported as “none” or “unknown” have mutations associated with resistance that may affect their treatment outcome.

The World Health Organization recommends a small-sample method that classifies transmitted HIV drug resistance for adults as <5%, \( \geq 5\% - <15\% \), or \( \geq 15\% \) [82]. This methodology is not suitable for estimating initial resistance among children < 18 months of age. As noted in the literature review, studies have demonstrated that where PMTCT is used, the prevalence of HIVDR among children < 18 months infected with HIV despite PMTCT is likely to be > 15%; a more precise method of estimating prevalence is required. Secondly, adults who have been recently infected will not require ART for many years, whereas children < 18 months require ART as soon as they are diagnosed with HIV, so more precise estimates of resistance prevalence to specific drugs are required to support ART decisions for this group in countries where a population-based approach to ART is used. This protocol describes a project to use remnant diagnostic DBS to survey initial resistance among a representative sample of children < 18 months diagnosed with HIV in resource-limited countries.

**Intended/potential use of survey findings**

The target audience for the survey findings will be clinicians, policy-makers tasked with decision-making on ART regimens, community members, and caregivers providing care to children living with HIV. The expansion of PMTCT options is likely to lead to changing patterns of DRMs in children
infected despite PMTCT exposure. The estimation of prevalence of DR-HIV to specific ARVs will support decision-making about pediatric ART regimens on a population basis in specific countries. The survey can potentially provide information about the risk of resistance related to different pre-treatment ARV exposures, when information regarding exposure is available and provided that groups exposed to different regimens are sufficiently large. If current regimens or current record-keeping are found to be non-optimal, results may provide opportunities for corrective action. If information regarding previous ARV experience is reported as “unknown” for large proportions of children and high levels of DR-HIV are detected in this group, recommendations to improve record keeping system and to strengthen patient information flow will be needed. In addition, targeted early virologic monitoring (when possible) may need to be explored to assess the efficacy of first line regimens. This project may also provide useful information for the periodic review of the WHO PMTCT ARV guidelines.

3. SURVEY OBJECTIVES AND HYPOTHESIS TO BE TESTED

Purpose

Overall Purpose: to assess initial drug-resistant HIV among children < 18 months of age and newly diagnosed with HIV in resource-limited countries in order to inform selection of first-line ART regimens for this population in each participating country and support global decision-making on regimens for this population.

Objectives

- To describe the prevalence of initial NNRTI and NRTI resistance in children newly diagnosed with HIV who are < 18 months of age
To describe the prevalence of initial NNRTI and NRTI resistance in children newly diagnosed with HIV who are < 18 months of age and whose previous ARV exposure is recorded as “none” or “unknown.”

Hypothesis

As ARV use for PMTCT increases, there will be a relatively small proportion of children who become infected with HIV despite PMTCT prophylaxis. However, among those infected, an increasing proportion will harbor drug-resistant strains of HIV.

4. PROCEDURES/METHODS

A. General Approach

The default survey methodology is retrospective: the evaluation on genotyping will be based on stored remnant DBS collected for pediatric PCR diagnosis and abstraction of routinely collected information available at the diagnostic laboratory. Remnant DBS specimens will be stored and handled appropriately to optimize amplification for genotyping, and transported to the selected laboratory. The relevant portions of the RT region of the pol gene of HIV will be sequenced using standard sequencing methods. Amplification and sequencing will be performed at laboratories accredited for DBS genotyping by the WHO HIVResNet Laboratory Network or, alternatively, by one of the two laboratories recommended by WHO: the CDC Global AIDS Program Laboratory and the Institut de Recherche pour le Développement Laboratory in Montpelier. Demographic information and clinical data, including PMTCT regimen exposure will be abstracted from laboratory requisition forms into a national or area-based HIV drug resistance database, with no identifying information recorded. The analysis will consist of a description of the prevalence of drug resistance...
mutations, and the prevalence of high, intermediate, and low levels of resistance for each relevant ARV as determined by Stanford drug resistance score.[72] Prevalence will be estimated with 95% confidence intervals based on ARV-exposure (Yes/None or Unknown). If sample sizes are sufficient, separate analyses based on age at diagnosis and specific ARV-exposure histories may also be performed. If for these analyses, sample sizes for a specific survey are insufficient, multi-year or multi-country survey results could be combined to achieve sufficient power, if survey procedures are identical.

**Survey investigators**

WHO and CDC recommend that the survey be coordinated by a subgroup of the national HIV drug resistance working group (HIVDR-WG), usually convened by the Ministry of Health to develop an HIVDR prevention and assessment strategy. The HIVDR-WG generally includes all national and international partners involved in national ARV care, including clinicians, HIV surveillance and ARV monitoring and evaluation experts, PMTCT planners and evaluators, laboratory experts and HIVDR researchers. WHO and CDC staff in-country, and at the central level, will provide technical support for implementation and analysis, but CDC staff will not interact with participants nor be involved in collection of identifiable data. Countries will be requested to share data which will not include elements that could identify individuals through the WHO HIVDR database, but not required to do so. Laboratories must share sequences with a WHO HIVResNet virologist at WHO Headquarters in Geneva for quality assurance at the same time as they are sent to the country.

**Survey Design**
This is a retrospective cross-sectional survey of DR-HIV prevalence among children < 18 months of age diagnosed with HIV by PCR methodology using remnant DBS specimens. Specimens will be provided by all laboratories in a country where PCR is performed for HIV diagnosis of children < 18 months of age or from a representative sample of these laboratories where remnant DBS are routinely available and appropriately stored.

Patient data will be abstracted from laboratory requisition forms that accompany DBS samples, which may be supplemented by records or registers maintained at sites where blood is drawn. A survey identification number (SID) will be assigned to the data abstraction form and the DBS. A logbook will be maintained in a locked cabinet linking the SID to other identifying information for purposes of comparison of re-abstraction of data to evaluate reliability of data abstraction (see page 42) and for de-duplication of specimens, but will be destroyed before data analysis is performed. Age or date of birth, sex, and ANC site will be used for de-duplication. No identifying information will be retained; the analysis will be unlinked and anonymous.

Ideally, especially during the transition to more complex PMTCT regimens taking place in many countries, the survey should be completed annually, and integrated into routine programme monitoring as PMTCT is scaled up in resource-limited countries.

Case Definition

Drug-resistant HIV: The survey will use the Stanford algorithm for the interpretation of drug resistance.[72] Any mutation or combination of mutations that produces low, intermediate, or high level resistance to a relevant NRTI/NNRTI ARV drug or NRTI/NNRTI drug class is defined as DR-HIV to that drug or drug class. A Stanford classification of "potential" drug resistance [72] is not classified as drug resistance for the purpose of this survey.
Study Population

DBS specimens collected for resistance genotyping will be selected from all HIV positive DBS from children < 18 months of age diagnosed with HIV in participating laboratories. DBS are likely to originate from three main sources:

1. **Routine follow-up of HIV-exposed children through immunization clinics, PMTCT programmes Maternal and Child Health (MCH) or Antenatal (ANC) clinics.**
2. **HIV testing of symptomatic children < 18 months of age presenting to MCH clinics, hospitals or other medical facilities**
3. **Children < 18 months of age who are tested in Provider Initiated Testing and Counseling (PITC) sites or Voluntary Counseling and Testing (VCT) sites.**

The population to be represented is either all children < 18 months of age diagnosed as HIV-positive by PCR nationally, or all children < 18 months of age diagnosed by PCR in one or more specific areas of the country. Ideally, each Early Infant Diagnostic (EID) laboratory in the country will participate in the survey and will contribute to the overall sampling; this is particularly feasible in countries with a limited number of EID diagnostic laboratories. In countries with a larger number of EID laboratories, the survey might include only specimens from defined geographic areas (districts, provinces or regions) and results will only represent the area(s) from which participants are enrolled.

Participant inclusion criteria

A specimen will be considered eligible under the following conditions:

1. **DBS tested HIV-positive by PCR from a child < 18 months of age.**
2. If DBS for PCR is collected from a child at different time points, to increase the chance of HIVDR detection, the most recent DBS specimen is selected for genotyping. DBS these should be clearly labeled with a unique ID so that the child is not counted twice or more times.

3. At least one viable remnant DBS is available that is not required for clinical testing or quality assurance (two-four DBS would be optimal.)

4. From time of blood draw, DBS specimen has been stored no longer than 14 days at room temperature, then stored at -20°C or -70°C with no thawing before genotyping will be performed.

Participant Exclusion Criterion

1. Child is ≥ 18 months of age.

2. Child is receiving three or more ARV drugs for the purpose of treatment of HIV (rather than prophylaxis to prevent HIV infection) at time of blood draw.

B. Sampling

Laboratory and Sample Selection

When possible, all laboratories performing HIV EID will participate in the surveillance round, thus creating a census of all laboratories. If only a subset of laboratories are able to participate, a sample of those will be selected, based on probability proportional to size (PPS) cluster sampling. Once a laboratory is selected, the number of specimens to genotype will be calculated using simple random sampling without replacement. This means that once a specimen is selected, it is removed from the group of specimens available for selection.
Sample size

All laboratories in a country performing DNA-PCR for HIV testing in infants and children, or a representative sample of these laboratories, should participate in this survey.

Our sample size calculations are based on an assumed "true DR-HIV prevalence" of 50%; 95% confidence intervals (CI) of +/- 7%; and power (1-β)=0.80. The assumed prevalence of 50% is the most conservative assumption which will give the largest sample size and the most precise confidence intervals (CI). Precise CI are especially important when prevalence is low. A non-amplification rate of 20% is assumed to allow for storage and transport problems affecting the amplification rate for DBS. The formulae for the calculations can be found in Appendix 1.

Using the normal approximation to a binomial distribution in PASS 2008 software[73] (http://www.ncss.com) a range of 95% CIs of 14%(+/-7%) for a prevalence of 50% requires a sample size of 196. However, because the amplification rate from DBS is expected to be 80%, the effective sample size is 196/.8 = \textbf{245}.

The sample sizes below are suggested for one of three possible sampling strategies:

1) **There is only one laboratory from which DBS will be selected.**

In countries where only one laboratory participates, \textbf{the sample size will be 245}. All specimens from the laboratory will be listed, in any order, to create the sampling frame. From the sampling frame a simple random sample may be drawn using a random number table or it can be done in most spreadsheet software (i.e., Excel). The instructions for using Excel to draw a random sample are found in Appendix 2. These instructions can also be used for selecting simple random samples within the laboratories for the other sampling strategies. If sample size doesn't reach the 245 sample as recommended, the survey should still be conducted but loss in precision will have to be considered.
2) There are multiple laboratories from which DBS will be selected and ALL laboratories will participate.

A design effect represents the ratio of within stratum variance to total variance and the use of a design effect of 2 is recommended for this survey. The final effective sample size if more than one laboratory participates will therefore be $245 \times 2 = 490$. The sample size for each laboratory will be subsequently calculated by applying a proportional sampling approach (see page 32).

3) There are multiple laboratories from which DBS will be selected and a sample of laboratories will participate.

If there are many laboratories in the country, it may not be feasible for all laboratories to participate. A sample of laboratories will be selected and then a sample of specimens will be selected within each laboratory. As a guide, it is recommended that at least one-third of the national laboratories should be included in the sample, to allow for potential differences between laboratories. As an example, we will sample five laboratories in countries with 15 eligible laboratories.

In countries where a sample from multiple laboratories participate in the survey, there will be an impact of the clustering of data on the sample size in the form of intracluster correlation. The design effect is a multiplier that is linearly related to the intracluster correlation. Again, a design effect of 2 is recommended for this survey, leading to an effective sample size of 490 samples. In this case, the country should use Probability Proportionate to Size (PPS) cluster sampling to randomly sample laboratories. The basic steps for PPS and potential scenarios are described in details in Appendix 1; moreover, a tutorial accessible to everyone can be found on http://www.cdc.gov/globalhealth/FETP/modules/MiniModules/PPS/page01.htm.

Once the participating laboratories have been selected, laboratory sample sizes should be calculated. When multiple laboratories will participate, proportional allocation will be used to
determine the sample sizes for each laboratory based on the number of children < 18 months of age diagnosed as HIV positive in the target year. This method was chosen because these laboratories are representative of geographic regions and will be treated as strata. The method is as follows:

A. From each participating lab, list the total number of eligible specimens (Nh) for the target year

B. Sum the total numbers of specimens across all participating labs (N) for the target year

C. For each lab, calculate the proportion of specimens that make up the total of eligible specimens (Nh/N)

D. Multiply by the total sample size to get the sample size for the lab, nh = (Nh /N ) * 490.

Example 1: Assuming that in country Y there are two laboratories, one (Lab A) where 2500 children < 18 months are diagnosed as HIV-infected by PCR in the target year, the other (Lab B) where 500 children < 18 months are diagnosed as HIV-infected by PCR in the target year. Country Y will estimate the proportion of HIVDR with 95% CI width 14% for a sample size of 490. The total number of children diagnosed as HIV-infected is 3000 (2500+500). Lab A has 2500/3000=83.3333% of the total. .83333 X 490 = 408.3317. Rounding down gives 408 specimens to be sampled from Lab A, leaving 490 – 408 = 82 to be sampled from Lab B.

Example 2: In country Z there are three laboratories, one (Lab A) where 2000 children < 18 months are diagnosed as HIV-infected by PCR in the target year, the second (Lab B) where 200 children < 18 months are diagnosed as HIV-infected by PCR in the target year, and the third (Lab C) where 150 children < 18 months are diagnosed as HIV-infected by PCR in the target year. Country Z will estimate the proportion of HIVDR with 95% CI width 14% for a sample size of 490. The total
number of children diagnosed as HIV-infected is 2000+200+150=2350. Lab A has 
2000/2350=85.1063% of the total. .851063 X 490= 417.02087. Rounding down gives 417 
specimens to be sampled from Lab A. Lab B has 200/2350=8.5106% of the total. .085106 X 
490=41.70194. Rounding up gives 42 specimens to be sampled from Lab B. Lab C has 
150/2350=6.3829% of the total. .063829 X 490 = 31.27621. Rounding down gives 31 specimens to 
be sampled from Lab C. 417 + 42 + 31 = 490. 

Once the sample size to be contributed by each participating laboratory has been 
calculated, simple random sampling of specimens without replacement for the purpose of 
genotyping is performed within each laboratory, using the following method:

Step One: Sampling Frame

A sampling frame should be comprehensively compiled, containing every member of the 
population of interest, listed in any order.

For example, it should be a list of all eligible specimens in the target year in each selected 
laboratory. At this stage, no members should be excluded for any reason so this list will be the 
true, master sampling frame.

Step Two: Draw a Random Sample

A. Number your eligible specimens from 1 to X, where X represents the number of specimens 
B. Accounting for the sample size being defined previously, samples from the sampling frame 
should be selected randomly. Countries may find it feasible to use a random number generator 
such as the one found at http://www.random.org/integers/. Be sure to include only one HIV-
positive specimen per child. Simply specify the numbers between which you want to generate
random numbers (for instance, 1-300 if X= 300 of eligible samples), and the number of random numbers you want to generate (sample size) and let the random number generator do the work for you. You may get repetitions (198 is repeated twice below), in which case you should rerun the program and take the next numbers generated up to the number you need to replace the repetitions.

Here are your random numbers:

130 116 198 180 79
14 267 260 124 124
249 198 245 24 171
263 208 137 248 282
220 78 97 286 202
62 48 224 173 119

Appendix 3 contains a description of how to use Excel to select a simple random sample of specimens for each laboratory.

If countries have insufficient numbers of eligible specimens to reach the required effective sample size, the country should genotype all eligible specimens but reduction in precision should be considered by calculating CI around the estimated prevalences of NRTI and NNRTI resistance.

C. Laboratory Methods

The survey will use remnant DBS available after all diagnostic, clinical, and quality assurance tests have been performed. National or site-based guidance should be followed for collecting DBS for HIV diagnosis by PCR.

To be suitable for genotyping, DBS should ideally be handled, transported and stored as outlined in the WHO recommendations for HIVDR testing on DBS[74] (also see below).
DBS handling and storage

To ensure ability to amplify specimens for genotyping, DBS should be handled as described below:

- As humidity and UV light can damage DBS, always keep them in **zip-lock bags with desiccant bags, in the dark.** Change desiccant bags when humidity indicators change color.
- DBS should not be kept and/or transported at ambient temperature for more than 14 days from collection.
- If DBS can be transported to the genotyping lab **within 14 days from the date of collection,** they can be kept and/or transported at ambient temperature.
- If genotyping cannot be performed **within 14 days from the date of collection,** DBS should be transported to a central laboratory with a constant electricity supply and should be frozen in a -70°C freezer or, if not available, in a non-frost-free -20°C (or lower) freezer (for up to two years). DBS should be transferred to -20°C or lower to the genotyping laboratory.

For more detailed information on processing, handling, transport within the country, storage, and shipment outside the country, refer to the WHO manual for HIVDR testing using DBS specimens

(http://www.who.int/hiv/pub/drugresistance/dried_blood_spots/en/index.html)[74]

Country-specific quality assurance and appropriate supervision for specimen handling, transport, and storage should be included in the Standard Operating Procedures (SOPs) developed for this protocol.

Genotyping should be performed in a WHO HIVResNet-accredited laboratory using a method that has been accredited for DBS genotyping by WHO HIVResNet.
5. VARIABLES AND DATA HANDLING

Required Variables

1. Date of birth; if not available, age of child in months at time of blood draw
2. Gender
3. Site name where DBS was collected
4. Site type where DBS was collected (e.g., ANC/MCH site, VCT site, pediatric clinic, pediatric hospital)
5. Date of DBS collection
6. Child receiving ARVs for its own treatment (not PMTCT) at time of specimen collection (yes/no)
7. Date of first freezing DBS at -20 C or -70 C
8. Date of PCR assay
9. Date of genotyping

Optional variables

1. Is child breastfeeding on date of sample collection? (yes/no)
2. Were ARVs received by mother for PMTCT or maternal health: (yes/no). If yes, circle those that apply: antepartum; intrapartum; postpartum; during breastfeeding.
3. Which ARVs were received by mother for maternal health or prophylaxis: sd-NVP; sd-NVP + ZDV; ZDV + 3TC; or other [specify]; a three-drug ARV regimen [specify regimen]
4. Were ARVs received by child for PMTCT? (Yes/No)
5. Which ARVs were received by child for PMTCT: sd-NVP; extended NVP (that is, NVP daily for more than one day); extended ZDV (that is, ZDV daily for more than one day); extended NVP + ZDV (that is, NVP + ZDV daily for more than one day); other [specify]

Data abstraction

Information should be abstracted from the laboratory forms accompanying the DBS. If all the required variables listed are not available on the DBS laboratory form, extra fields may be added to the laboratory form for the duration of the study.

The WHO HIV Drug Resistance Database, version 2, supports data entry of these variables and importation and interpretation of sequences according to the latest Stanford HIV drug resistance database algorithms. The Database is available from the WHO HIV Drug Resistance team or from the CDC Global AIDS Program ESI Branch.

Training for survey personnel

Data abstractors will receive training in data abstraction based on the specific records routinely used in the country.

Quality assurance for abstraction and data entry

A quality assurance process should take place for abstraction and data entry into the electronic database, if used. Country-specific data quality assurance procedures, including review of procedures to ensure that data are being abstracted from the laboratory forms and entered appropriately on survey forms should be included in the SOPs for this protocol.
Data Security

A data security plan should be developed and included in each country’s protocol. All electronic files, including data files and analysis files, should be password-protected and the passwords shared only among data entry staff and data supervisors. Files should never be taken off-site except to implement a secure backup. Plans for secure backup of analysis files should be developed and included in the protocol. A data confidentiality guide for staff entering or accessing data should be developed and summarized in the protocol. The protocol should include a clear statement of who owns the data and who will have access to it, including, if appropriate, access to a public dataset. A data retention plan consistent with local policies should also be included in the protocol; that is, it must be stated how many years the data will be retained and when they will be finally destroyed. It is recommended that countries state in the protocol the number of years from the final analysis that the data will be destroyed.

6. ETHICAL CONSIDERATIONS

Consent

The default survey methodology is to base the evaluation on remnant specimens and information collected in a previous year.

All DBS samples used for this survey will be remnant specimens originally collected for diagnostic purposes. Caregivers will have given verbal consent for collection and HIV testing of the DBS at the time of collection. The remnant specimens will be tested anonymously; therefore, a “non-research” determination will be requested from the IRB/ERC on the basis that this survey is routine surveillance not requiring consent. No personal identifiers will be abstracted. Individual results will not be returned to the caregiver, clinician or clinic. It is presumed that children with
resistance mutations will have started HIV treatment before results will be available; if WHO recommendations are followed, children exposed to NNRTIs during PMTCT will have been started on a PI-based regimen. Survey results will be made available to policy makers, pediatricians and other clinicians, participating laboratories, researchers interested in HIVDR, and community groups by the national HIVDR WG, and including in the annual HIVDR report that should be circulated nationally.

Confidentiality

Procedures to protect confidentiality of children and mothers are already in place at laboratories where HIV PCR testing is taking place. No identifying data will be abstracted. Data abstraction forms and the HIVDR database will be stored under lock and key or under password protection, accessible only to survey staff and national HIV drug resistance working group members actively involved in the survey.

Risks to survey participants

There are no additional physical risks to survey participants. No additional blood will be collected. Because the data will not include identifying information, a breach of confidentiality is unlikely to result in disclosure of HIV status.

Benefits to survey participants

No results will be returned. Participants will receive no immediate or direct benefit from the survey results. Survey findings will be used to inform regimen selection for pediatric HIV care in resource-limited settings, and may lead to improved ARV regimens offered to children or specific
groups of children. Better information to inform regimen selection may benefit survey participants in the future.

7. DATA ANALYSIS and DISSEMINATION OF RESULTS

Data analysis plan Prevalence, along with 95% confidence intervals, of relevant mutations and combinations of mutations leading to classifications of high, intermediate, or low resistance to relevant drug classes and drugs [defined according to the Stanford HIVDR Database website[72] (http://sierra2.stanford.edu/sierra/servlet/JSierra)] will be calculated for children < 18 months of age for each laboratory. Results from all laboratories will be further summarized in following ways. First, the mean prevalence of HIVDR to relevant drug classes and drugs from each laboratory along with the 95% confidence intervals (CI) will be reported. Secondly, the median prevalence of HIVDR across laboratories, along with the 25th and 75th percentiles for laboratory prevalences, will be presented as a robust estimate of overall prevalence and dispersion.

If the sample size is sufficient, a separate analysis of HIVDR prevalence with 95% confidence intervals will be performed to evaluate the association the “none” and “unknown” exposure classification with resistance, by laboratory. To evaluate this association across laboratories a mixed effects model will be employed, with laboratory HIVDR prevalence as the dependent variable, laboratory as the random variable, and exposure classification as the independent predictor variable. Often, data have a clustered structure. Classical statistics assumes that observations are independent and identically distributed. Applied to clustered data, this assumption may lead to false results, with specific concern towards the estimation of standard errors. In contrast, the mixed effects model treats clustered data adequately and assumes two sources of variation, within cluster and between clusters. If the sample size is sufficient, separate
analyses will be performed evaluating the association of ARV exposure antepartum, intrapartum and postpartum and the association of various combinations of ARVs on development of HIVDR.

For analyzing trends in HIVDR prevalence over at least three successive surveillance rounds we will employ the following strategy. First, laboratory-specific HIVDR prevalences will be plotted as line graphs for visual analysis, for laboratories that are consistently evaluated over the successive years. This will allow countries to see the HIVDR prevalence in all labs over time and detect obvious trends. Second, if new laboratories are added in successive years a secondary analysis including all laboratories may be employed to evaluate the impact of surveillance expansion on the overall trend. Third, the use of box-and-whisker plots may be used to visualize changes in median and mean HIVDR prevalence. A box-and-whisker plot is a way of graphically summarizing data through five-number summaries: the minimum, 25\textsuperscript{th} percentile, median (or 50\textsuperscript{th} percentile), 75\textsuperscript{th} percentile, and the maximum. An example of a line graph and boxplot is shown below.
Lastly, we may employ the use of mixed-effects logistic regression models for a statistical analysis of trends, with HIVDR prevalence from each laboratory as the dependent variable, laboratory as the random variable, and year as the independent predictor. This analysis will give us the result of a statistical test, including p-value, of the trend in HIV-DR over surveillance rounds.

A data validation plan to compare the data recorded at each laboratory with data recorded at all or selected sites should be developed.

Potential biases

The survey will not necessarily represent all children < 18 months of age diagnosed with HIV-1 in the country. Limitations include:

1. Some facilities may use rapid serologic tests for HIV diagnosis of children < 18 months of age not followed by DBS collection for diagnostic PCR. Those clinics, likely to be rural sites and smaller sites, may not be well represented in the survey.
2. Larger, more urban or semi-urban sites that collect DBS for diagnostic PCR are more likely to be represented in the survey. These sites may be more likely to make available PMTCT regimens consisting of ≥ 2 ARV drugs rather than sd-NVP or other simpler PMTCT regimens. Children who themselves received sd-NVP or who were exposed to sd-NVP through their mothers may be under-represented.

3. If PMTCT sites contribute most diagnostic specimens, children with "no" or "unknown" ARV exposure may not be well-represented in the survey.

4. If non-PMTCT sites contribute a higher number of diagnostic specimens, children with recorded ARV exposure may not be well-represented in the survey.

5. If EID coverage is not high, the sample will not be representative of the children <18 month of age infected with HIV.

**Dissemination of results**

HIVDR-WG should analyze and disseminate the results to participating sites and laboratories, policy makers, participants, community groups, and others within a year of completion of the survey. WHO and CDC recommend that the results are also submitted in the form of an article to a peer-reviewed journal. On request, WHO and CDC staff will provide technical assistance in the data analysis, interpretation and reporting.

**8. RESOURCES REQUIRED FOR THE SURVEY**

The sequencing of over 200 DBS, which will be required for most surveys, is expensive (US $150-$300/test). In countries where required variables are not routinely included on the laboratory forms additional abstraction may require substantial person-hours. Data validation may
also require additional hours. WHO and CDC will seek funding centrally to support surveys in selected countries. HIVDR-WGs are encouraged to incorporate the survey into their national strategic plan, in Global Fund applications, and into their PEPFAR country operation plans, and to seek partnerships with academic institutions or NGOs to support the surveys.
Reference List


(10) Cibulka NJ. Mother-to-child transmission of HIV in the United States. Many HIV-infected women are now planning to have children. What are the risks to mother and infant? Am J Nurs 2006; 106:56-63.


(37) Church JD, Omer SB, Guay LA, et al. Analysis of nevirapine (NVP) resistance in Ugandan infants who were HIV-infected despite receiving single-dose (SD) NVP versus SD NVP plus
daily NVP up to 6 weeks of age to prevent HIV vertical transmission. J Infect Dis 2008; 198:1075-82.


Appendix 1

Sample size calculations

A sample size calculation should be performed for each participating laboratory and for the country overall.

The first stage of the calculation is for the overall sample size required for the country to pool data: We assume:

- a 50% prevalence
- a 95% confidence level, and
- a ± 6% confidence interval
- Power = .80

\[
n = \frac{Z^2 p(1-p)}{e^2}
\]

where,

- 1.96 is the Z value for 95% confidence level
- e is 0.07, which represents the acceptable confidence interval of ± 7%
- \( p=0.50 \), that is, the estimated prevalence is 50% (which gives the most conservative estimate for the sample size).

An adjustment for “non-response” – that is, an adjustment for an inability to amplify and sequence the genetic material from some percentage of the DBS should also be considered. This adjustment is calculated as follows:

\[
n' = \frac{n}{A}
\]

\( A \) = the proportion that we expect to be amplifiable for sequencing. A conservative non-amplification rate is 20%, which corresponds to an “A” of .80. The text shows examples of the use of these calculations.

\[
n' = \frac{n}{1+n} \cdot \frac{1}{N}
\]

\( N \) = the number of children newly diagnosed with HIV by DBS-PCR in the year of the survey.
If there is only one laboratory participating in the country, the effective sample size is $196/0.8 = 245$. In countries where all laboratories participate in the survey, proportional allocation will be used to determine the sample sizes for each laboratory based on the number of children < 18 months of age diagnosed as HIV positive in the target year. This method was chosen because these laboratories are representative of geographic regions and will be treated as strata. The design effect represents the ratio of within stratum variance to total variance. We will account for a design effect of 2 in this survey. The final effective sample size if all laboratories participate is $245 \times 2 = 490$.

We will account for a design effect of 2 in this survey. The final effective sample size if all laboratories participate is $245 \times 2 = 490$. Proportionate sample sizes for each lab based on the number of children < 18 months of age diagnosed as HIV positive should be calculated as follows: Calculate the proportion of the total number of children < 18 months of age diagnosed in each laboratory in the target year, and multiply that proportion by 490.

E. From each participating lab, list the total number of eligible specimens (Nh) for the target year

F. Sum the total numbers of eligible specimens across all participating labs (N) for the target year

G. For each lab, calculate the proportion of specimens that make up the population (Nh/N)

H. Multiply by the total sample size to get the sample size for the lab, $n_h = \left(\frac{Nh}{N}\right) \times 490$.

If multiple laboratories participate, but their number is too large and a sample of laboratories is required, a sample of laboratories will be selected and then a sample of specimens will be selected within each laboratory. Select the number of laboratories based on feasibility if your country has a large number of laboratories. **At least one-third of the laboratories should be included in the sample.** As an example, we will sample seven laboratories in countries with $\geq 15$ eligible laboratories.

In countries where a sample from multiple laboratories participate in the survey there will be an impact of the clustering of data on the sample size in the form of intracluster correlation. The design effect is a multiplier that is linearly related to the intracluster correlation. Again, we will
account for a design effect of 2, making the effective sample size 490. In this case, the country should use Probability Proportionate to Size (PPS) cluster sampling to randomly sample laboratories. (A tutorial accessible to everyone can be found on http://www.cdc.gov/globalhealth/FETP/modules/MiniModules/PPS/page01.htm)

The basic steps for PPS sampling are as follows a) List laboratories in alphabetical order along with their numbers of eligible specimens, b) Calculate the cumulative numbers of eligible specimens for each laboratory listed, c) Determine the sampling interval, d) Select a random starting point, f) Select laboratories based on the random starting point, sampling interval, and cumulative totals of eligible specimens.

a) List the laboratories in alphabetical order (not in order of numbers of eligible specimens) and their numbers of eligible specimens.

b) Calculate the cumulative total of eligible specimens for each laboratory.

For example:

<table>
<thead>
<tr>
<th>Lab Name</th>
<th>Number of eligible specimens</th>
<th>Cumulative totals of eligible specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab A</td>
<td>111</td>
<td>111</td>
</tr>
<tr>
<td>Lab B</td>
<td>53</td>
<td>164</td>
</tr>
<tr>
<td>Lab C</td>
<td>20</td>
<td>184</td>
</tr>
<tr>
<td>Lab D</td>
<td>300</td>
<td>484</td>
</tr>
<tr>
<td>Lab E</td>
<td>15</td>
<td>499</td>
</tr>
<tr>
<td>Lab F</td>
<td>125</td>
<td>624</td>
</tr>
<tr>
<td>Lab G</td>
<td>357</td>
<td>981</td>
</tr>
<tr>
<td>Lab H</td>
<td>45</td>
<td>1026</td>
</tr>
<tr>
<td>Lab I</td>
<td>353</td>
<td>1379</td>
</tr>
<tr>
<td>Lab J</td>
<td>105</td>
<td>1484</td>
</tr>
<tr>
<td>Lab K</td>
<td>115</td>
<td>1599</td>
</tr>
<tr>
<td>Lab L</td>
<td>25</td>
<td>1624</td>
</tr>
<tr>
<td>Lab M</td>
<td>600</td>
<td>2224</td>
</tr>
<tr>
<td>Lab N</td>
<td>400</td>
<td>2624</td>
</tr>
<tr>
<td>Lab O</td>
<td>201</td>
<td>2825</td>
</tr>
</tbody>
</table>

c) Determine the sampling interval
The sampling interval is determined by dividing the cumulative population size over all eligible laboratories by the number of laboratories to be sampled. In the case of our example, the cumulative population size is 2825. Therefore the sampling interval is 2825/7 = 404.

d) Pick a random starting point between 1 and the sampling interval 404, using a random number table or a random number generator. A random number generator can be found on http://www.cdc.gov/globalhealth/FETP/modules/MiniModules/PPS/page06.htm. To select the first lab, obtain a random number between 1 and the sampling interval 404. In our example, our random number and starting point is 320.

e) Select laboratories based on the random starting point, sampling interval, and cumulative total of eligible specimens. Select a laboratory, which has the number you are using within its cumulative total of eligible specimens.

1. Select the first laboratory in which your random start falls within the cumulative total of specimens. In our example, Lab C has a cumulative population size of 184 and Lab D has a cumulative population size of 484. Our random number, 320, is greater than the cumulative DBS total of Lab C but less than that of Lab D, so Lab D will be selected.

2. Add the sampling interval to the initial random number and then select the first laboratory listed in which the cumulative total of specimens includes the total of the sampling interval plus the random number. 320+404=724, so we would select Lab G. 724+404=1128; select Lab I. Continue adding the sampling interval to the result obtained until all seven laboratories have been selected. Below is the selection process for our example.

<table>
<thead>
<tr>
<th>Lab Name</th>
<th>Number of eligible specimens</th>
<th>Cumulative totals of eligible specimens</th>
<th>Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab A</td>
<td>111</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Lab B</td>
<td>53</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Lab C</td>
<td>20</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>Lab D</td>
<td>300</td>
<td>484</td>
<td>320</td>
</tr>
<tr>
<td>Lab E</td>
<td>15</td>
<td>499</td>
<td></td>
</tr>
<tr>
<td>Lab F</td>
<td>125</td>
<td>624</td>
<td></td>
</tr>
<tr>
<td>Lab G</td>
<td>357</td>
<td>981</td>
<td>320 + 404 = 724</td>
</tr>
</tbody>
</table>
A laboratory may be selected more than once if it is a laboratory with an eligible population size larger than the sampling interval plus the total. A tool to calculate the sample of labs can be found in Appendix 2.

If countries have insufficient numbers of eligible specimens to reach the required effective sample size of 490 the country should genotype all eligible specimens and calculate CI around the estimated prevalences of NRTI and NNRTI resistance.

Available calculators/software:

- PS – Power and Sample Size Calculation
- STATCALC module - Epi Info™
- Russ Lenth’s Java Applets for Power and Sample Size

The sample sizes in the examples in the text were calculated using PASS 2008 and checked using the Vanderbilt PS – Power and Sample Size Calculation software.
Appendix 2
Appendix 3

Using Excel to draw a simple random sample

1. Open Excel
2. You may require Administrative rights on your computer to install the “Analysis ToolPak” or you may have to have someone with Administrative rights to install it for you.
3. Go to Office Button at top left corner -> Excel Options -> Add-Ins and select “Analysis ToolPak”. This will enable you to use the Sampling Tool in Excel.
4. List the entire population of eligible specimens for each laboratory in Column A, in any order. Note, do not label the columns in the first row at this time. All data in the columns must be numbers only for the Sampling Tool to work.
5. Number the specimens from 1 to Nh, where Nh is the total number of eligible specimens in the laboratory in Column B. This can be done my putting the number “1” in the first row, selecting the column by clicking on the column header, then go to Home -> Fill -> Series and entering Nh in the “Stop Value” box. This will automatically number all the rows from 1 to Nh.
6. Go to Data -> Analysis and select the Sampling Tool.
7. Fill out the following:
   a. For Input Range select all of the Lab ID Numbers from Column B by clicking the column heading
   b. For Sampling Method make sure “Random” is selected
   c. In the Number of Samples box enter in the sample size for the laboratory
   d. For Output Range select Column C by clicking the column heading.
8. Column C will now contain a simple random sample of Sample ID Numbers. If you prefer, at this time you may insert a row at the very top and label your columns.