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Comparison of the Generic HIV Viral Load® assay with the Amplicor™ HIV-1 Monitor v1.5 and Nuclisens HIV-1 EasyQ® v1.2 techniques for plasma HIV-1 RNA quantitation of non-B subtypes: The Kesho Bora preparatory study☆

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A B S T R A C T

The implementation of cost effective HIV-1 RNA quantitation assays in resource-poor settings is of paramount importance for monitoring HV-1 infection. A study comparing the analytical performance of three HIV-1 RNA assays (Generic HIV Viral Load®, Amplicor™ v1.5 and Nuclisens EasyQ® v1.2) was performed on 160 plasma samples from 160 consecutive antiretroviral treatment naive HIV-1-infected pregnant women assessed for eligibility in the Kesho Bora trial aimed at prevention of mother-to-child transmission of HIV-1 in three African countries (Burkina Faso, Kenya and South Africa). Correlation and agreement of results of the three assays were assessed for plasma HIV-1 RNA quantitation in specimens harbouring mainly sub-subtype A1, subtype C, and circulating recombinant form (CRF) 02_AG and CRF06_cpx.

Good degrees of correlation and agreement were observed between these HIV-1 RNA assays. However, nine (9/160, 5.6%) strains detectable with the Generic HIV Viral Load® assay were not detected by either the Amplicor™ ($n=7$) or EasyQ® ($n=2$) test. One strain (0.6%) was missed with the Generic HIV Viral Load® assay. Further, concordantly positive plasma samples harbouring CRF02_AG and CRF06_cpx yielded significantly higher HIV-1 RNA concentrations when tested by Generic HIV Viral Load®, as compared to Amplicor™ v1.5 (mean differences, +0.33 and +0.67 $\log_{10}$ copies/ml; $P=0.0004$ and $P=0.002$, respectively). The Generic HIV Viral Load® assay accurately quantified the majority of the non-B HIV-1 subtypes assessed in this study. Due to its low cost (~10 US $/test), this assay performed with open real-time PCR instruments is now used routinely in the Kesho Bora trial and may be recommended in other African settings.

1. Introduction

Developing nations have achieved significant improvement in programs providing access to antiretroviral (ARV) drugs for the treatment of human immunodeficiency virus type 1 (HIV-1) infection and for the prevention of mother-to-child transmission. There is an urgent need to ensure universal access by 2010 (World Health Organization, 2006).

Extended access to ARVs also necessitates access to regular monitoring of plasma HIV-1 RNA viral load measurements as an indicator of treatment efficacy as well as for early infant HIV-1 diagnosis (and thus, early initiation of treatment) (Calmy et al., 2007; Koenig et al., 2006; Rouet and Rouzioux, 2007). The implementation of cost effective yet sensitive and specific viral quantitation assays is an important priority, particularly in resource-poor settings. The effect of HIV-1 genetic diversity on the accuracy of HIV-1 RNA viral load measurements remains a matter of concern given the ever-increasing pool of diverse recombinant HIV-1 strains generated by the intermixing of viral variants in different parts of the world. The resultant genetic drift, as particularly observed in

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Sub-Saharan Africa, has contributed to a predominance of non-B HIV-1 subtypes, circulating recombinant forms (CRFs), and unique recombinant forms (URFs) in the region (Peeters et al., 2003). However, most of the available tests have been designed mainly for HIV-1 B subtypes. In addition, this complicates the technological and economic challenge of implementing new laboratory techniques in resource-constrained countries (Amendola et al., 2002; Antunes et al., 2003; Damond et al., 2007; Foulongne et al., 2006; Gottesman et al., 2004; Gottesman et al., 2006; Gueudin et al., 2007; Rouet et al., 2007; Wang et al., 2008). Continuous evaluations of available HIV-1 RNA assays using genetically diverse panels are thus of paramount importance.

In light of this requirement and in preparation for a WHO-coordinated trial on prevention of mother-to-child transmission of HIV-1 (the Kesho Bora trial), the analytical performance of three commercial HIV-1 RNA viral load assays on 160 consecutive samples obtained from HIV-1-infected women were compared in three African countries (Burkina Faso, Kenya, and South Africa) where the trial is being conducted.

2. Materials and methods

2.1. Studied population and samples

The Kesho Bora ("A better future" in Swahili) study is a randomized controlled clinical trial assessing the efficacy of maternal ARVs given during pregnancy, labour and postpartum breastfeeding to reduce HIV-1 transmission in three African countries (Burkina Faso, Kenya and South Africa) (Kesho Bora Study Group, 2009). During the first 3 months of the study, an additional blood sample was obtained at enrollment from untreated HIV-1 infected women who provided written informed consent to participate. The study protocol was reviewed and approved by the WHO ethics committee and by the Institutional Review Boards in all three countries.

All plasma specimens were prepared from EDTA-anticoagulated blood. They were then processed, aliquoted and frozen at −80 °C within 6 h. For samples from Burkina Faso and Kenya, one frozen (−80 °C) aliquot was sent to France for HIV-1 RNA quantitation and HIV-1 genotyping. A second frozen (−80 °C) aliquot from the same sample was shipped to Belgium for HIV-1 RNA quantitation. South African samples were quantified and genotyped *in situ* in Durban.

2.2. Plasma HIV-1 RNA quantitation assays

HIV-1 RNA was quantified in all plasma samples using the Generic HIV Viral Load® assay (Biocentric, Bandol, France) (Rouet et al., 2007). This assay is a quantitative real-time reverse transcription (RT)-PCR technique targeting a conserved region within the LTR gene. Viral RNA extraction was done with the manual Qiagen spin column (QIAamp Viral RNA Mini Kit, Qiagen, Courtaboeuf, France). The standard curve was established by using a commercial standard (Optiquant® quantification panel HIV RNA N°6, Acrometrix Inc., CA, USA), included in the kit, and calibrated against the WHO international HIV RNA standard. A commercial low positive control (target value: 6200 copies/ml [3.8 $\log_{10}$/ml]; accepted ranges, 3.5–4.1 $\log_{10}$/ml), also included in the kit, was used to assess inter-assay reproducibility and inter-laboratory comparability (Rouet et al., 2008). The lower detection limit (LDL) of the assay was 300 copies/ml (2.48 $\log_{10}$ copies/ml), with an input volume of 200 μl of plasma. HIV-1 RNA was quantified in two distinct real-time PCR platforms: the ABI PRISM 7000 (Applied BioSystems, Foster City, CA, USA) in the EA 4205 laboratory in Montpellier (France) for samples from Burkina Faso and Kenya; and the MiniOpticon (BioRad, Marne-La-Coquette, France) in the Africa Center Virology Laboratory in Durban for specimens from South Africa.

All plasma specimens from Burkina Faso and Kenya were also tested for HIV-1 RNA using the Cobas Ampliprep/Cobas Amplicor™ HIV-1 Monitor test v1.5 (Roche Diagnostics, Branchburg, NJ, USA) (Germer et al., 2007) at the AIDS Reference Laboratory of the University Hospital, Ghent, Belgium. The Amplicor™ v1.5 assay, an end-point RT-PCR assay targeting a consensus region of the *gag* gene, has a LDL of 400 copies/ml (2.60 $\log_{10}$ copies/ml) using 200 μl of plasma.

The samples from Durban also were assessed for HIV-1 RNA with the real-time Nuclisens EasyQ® assay v1.2 (BioMérieux, Boxtel, The Netherlands) (Stevens et al., 2007) at the Africa Center Virology Laboratory, following HIV-1 RNA extraction by the semi-automated Nuclisens miniMag extraction procedure. This real-time nucleic acid sequence-based amplification (NASBA) assay, targeting a consensus region in the *gag* gene, has a dynamic range of 50–3,000,000 International Units (IU)/ml using 1.0 ml of plasma. According to the manufacturer, 1 IU/ml is equivalent to 1 copie/ml.

2.3. HIV-1 genotyping assays

All specimens from Burkina Faso and Kenya were genotyped in Montpellier using the protocol of the ANRS Resistance Study Group, as described elsewhere (Pasquier et al., 2001).

A 621 bp region of the HIV-1 *env* (C2-V5) gene was sequenced in order to genotype the first 22 isolates from Durban, according to a previously published protocol (Gordon et al., 2003).

2.4. Statistical analysis

Statistical analyses were performed using SPSS 15.0 (SPSS, Illinois, USA). The sensitivity of the Biocentric test was calculated in comparison with positive HIV-1 RNA results obtained with the Roche or BioMérieux assay. The correlations between HIV-1 RNA results obtained with the three assays were assessed using the two-tailed Spearman rank test. A Bland–Altman difference plot was generated for bias and agreement (Bland and Altman, 1995). A percent similarity model was applied to determine the accuracy and precision of the assays (Stevens et al., 2005). Comparisons between HIV-1 RNA values were made using the paired Student's *t*-test or Mann–Whitney–Wilcoxon matched-pairs tests.

3. Results

Overall, 160 HIV-1-infected pregnant women from Bobo-Dioulasso, Burkina Faso ($n = 40$), Nairobi, Kenya ($n = 34$), Mombasa, Kenya ($n = 41$), and Durban, South Africa ($n = 45$) agreed to participate. Out of the 160 samples tested for HIV-1 RNA, 150 were concordantly positive with two techniques. Precisely, 107 samples (out of a total of 115 West and East African samples) were concordantly positive with the Biocentric and Roche assays whereas 43 (out of a total of 45 South African specimens) were concordantly positive with the Biocentric and BioMérieux assays.

Discrepant results, observed in 10 samples, were distributed as follows: nine samples were HIV-1 RNA detectable with the Biocentric test (range, 2.71–4.27 $\log_{10}$ copies/ml) but undetectable with either the Roche ($n = 7$) or the BioMérieux ($n = 2$) techniques. There was sufficient sample available for genotyping for eight of these specimens which included one subtype C, one CRF06_cpx strain, and one unclassified strain. Genotyping failed in the remaining five samples (most probably due to the low amount of viral RNA present in the specimens). One specimen (a CRF02_AG strain) was negative with the Biocentric test but positive (5.12 $\log_{10}$ copies/ml) with the Roche technique. Thus, among the 151 samples found positive with either the Roche or BioMérieux assays, 150 were also positive with the Biocentric kit, leading to an overall sensitivity of 99.3% (95% CI,

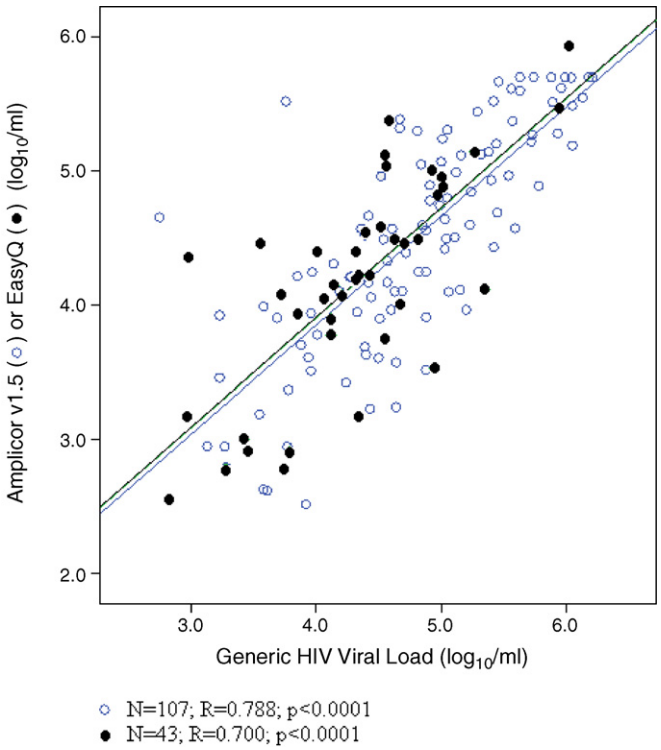

Fig. 1. Direct assay correlation between Generic HIV Viral Load® and Amplicor™ v1.5 (empty circles) or Nuclisens EasyQ® (black circles).

96.4–100) for the Biocentric assay. When performing the Biocentric assay, results obtained for the low positive control were strictly within the accepted ranges (see Section 2), regardless the use of two distinct real-time PCR machines (ABI PRISM 7000 in France vs. MiniOpticon in South Africa).

As shown in Fig. 1, HIV-1 RNA values of the 150 concordant positive samples were well correlated ($R = 0.788$ between Generic HIV Viral Load® and Amplicor™ v1.5; and $R = 0.700$ between Generic HIV Viral Load® and Nuclisens EasyQ® v1.2).

As shown in Fig. 2A, the overall mean difference (δ) in the HIV-1 RNA values obtained with the Biocentric and Roche assays was +0.30 $\log_{10}$/ml (mean, 4.78 $\log_{10}$ copies/ml vs. 4.48 $\log_{10}$ copies/ml, respectively) (*t*-test, $P < 0.0001$). Four (3.7%) samples showed HIV-1 RNA levels more than ±2 standard deviations (SDs) from the mean δ, with RNA values for two samples (one CRF02_AG and one strain which could not be amplified for genotyping) being higher using the Biocentric assay and two samples (one sub-subtype A1 and one CRF01_AE) with higher levels using the Roche test. The percentage similarity model (Fig. 3A) showed overall good accuracy (percentage similarity = 103.9%) and precision (SD, 7.0%) between the two methods. When analyzing the effect of HIV-1 genotypes on plasma HIV-1 RNA values, the mean δ between Generic HIV Viral Load® and Amplicor™ v1.5 was not statistically significant for the A1 sub-subtypes (+0.05 $\log_{10}$/ml; Wilcoxon test, $P = 0.21$), which represented 52% of HIV-1 genotypes circulating in Kenya. In samples harbouring CRF02_AG or CRF06_cpx (representing 83% of strains from Burkina Faso), significantly higher HIV-1 RNA concentrations were obtained with the Generic HIV Viral Load® assay, as compared with the Amplicor™ v1.5 test (+0.33 and +0.67 log10/ml, respectively; Wilcoxon test, $P < 0.001$ and $P = 0.002$, respectively).

As shown in Fig. 2B, the mean δ between the Biocentric and BioMérieux assays was +0.15 $\log_{10}$ copies/ml (mean, 4.32 $\log_{10}$ copies/ml vs. 4.17 $\log_{10}$ copies/ml, respectively) (*t*-test, $P = 0.071$). For three (6.9%) samples (harbouring subtypes C), the difference between the assays exceeded two SDs. These included two specimens that displayed higher viral loads with the Biocentric test and one that was higher with the BioMérieux assay. The percentage similarity model (Fig. 3B) comparing the two techniques revealed overall good accuracy (percentage similarity = 102.6%) and precision (SD, 7.3%).

4. Discussion

Due to the high and continuous genetic diversity of HIV-1 on its entire genome, designing a HIV-1 RNA viral load assay that is able to detect the full spectrum of HIV-1 group M genotypes/CRFs with equal efficiency remains problematic. Significant discrepancies in plasma HIV-1 RNA quantitation of distinct non-B strains have been recently reported in Spain (Holguin et al., 2008).

In the present study, plasma HIV-1 RNA concentration values obtained with three different commercial HIV-1 RNA assays were correlated among samples harbouring non-B subtypes/CRFs from sub-Saharan Africa. The studied sampling consisted mainly of CR02_AG and CR06_cpx, sub-subtype A1 and subtype C, predominantly circulating in West, East, and Southern Africa, respectively. However, some significant discrepancies in HIV-1 RNA concentrations were observed among these three assays. Nine samples were missed by either the HIV-1 *gag*-based Amplicor™ ($n = 7$) or EasyQ® ($n = 2$) assay. Also, one sample was missed by the Generic HIV Viral Load® technique, emphasizing that there is no perfect HIV-1 RNA quantitation technique. These 10 discrepant samples were not further investigated. They are likely due to mismatches between the target regions within the *gag* (for the Roche and BioMérieux techniques) or LTR (for the Biocentric assay) gene and the corresponding probe and/or primers. In addition, when comparing HIV-1 RNA

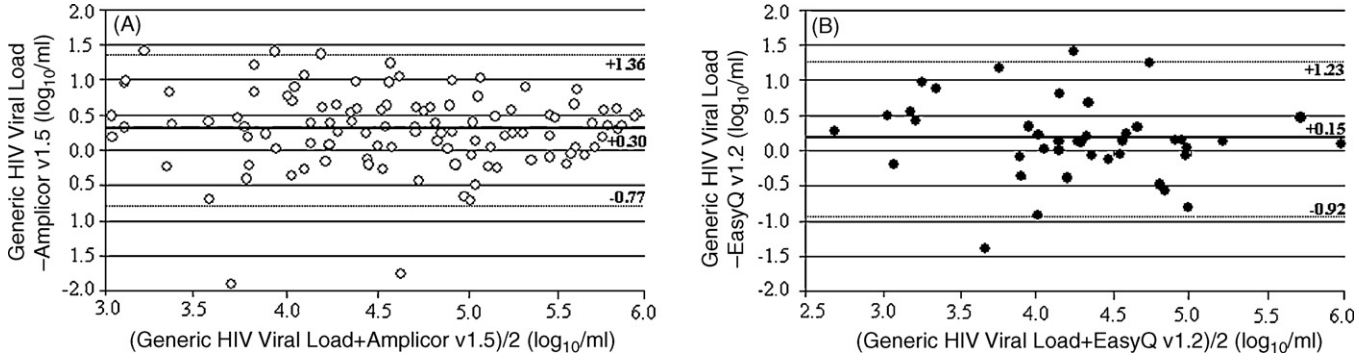

Fig. 2. Bland–Altman difference plots. (A) Difference between Generic HIV Viral Load® and Amplicor™ v1.5 (*Y*-axis) against the mean values obtained for each sample with the two tests (*X*-axis). The bias on the difference was +0.30 $\log_{10}$ copies/ml (SD, 0.54) with limits of agreement ranging from +1.36 to −0.77. (B) Difference between Generic HIV Viral Load® and Nuclisens EasyQ® (*Y*-axis) against the mean values obtained for each sample with the two tests (*X*-axis). The bias on the difference was +0.15 $\log_{10}$ copies/ml (SD, 0.55) with limits of agreement ranging from +1.23 to −0.92.

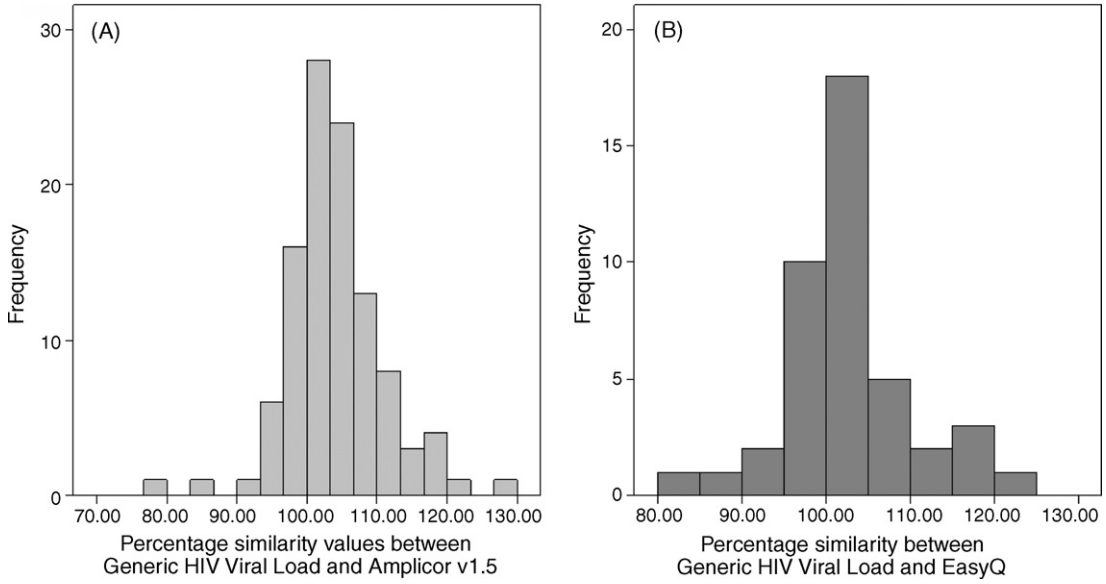

Fig. 3. Percent similarity plots. (A) Agreement between Generic HIV Viral Load® and Amplicor™ v1.5 assays. (B) Agreement between Generic HIV Viral Load® and Nuclisens EasyQ®.

results obtained with the Generic HIV Viral Load® assay and the Amplicor™ v1.5, approximately 38% (41/107) of values differed by >0.5 $\log_{10}$/ml as follows: 36 (~33%) specimens yielded higher values by the Biocentric assay, whereas five (~5%) samples yielded higher values with the Roche assay. Very similar percentages in terms of differences in HIV-1 RNA results have been previously described between these two assays (Rouet et al., 2007). When comparing HIV-1 RNA values obtained with the Generic HIV Viral Load® assay and the EasyQ® technique, about 28% (12/43) of results differed by >0.5 $\log_{10}$/ml (19% were lower by the EasyQ® test, whereas 9% were lower by the Generic HIV Viral Load® assay).

Overall, CRF02_AG and CRF06_cpx yielded higher HIV-1 RNA values when measured by the Biocentric assay than by the Roche assay, whereas no significant difference was observed for sub-subtypes A1. The 45 specimens from South Africa assessed for HIV-1 RNA by the Generic HIV Viral Load® and EasyQ® assays were assumed to harbour almost exclusively subtype C virus because this is the predominant (>90%) HIV-1 clade among heterosexual populations in South Africa (Pillay et al., 2002). Further, all 22 samples from this cohort that were genotyped were classified as subtype C. No statistically significant difference in viral load results was observed among the 43 specimens found positive with the two assays.

In conclusion, this study from four independent African laboratories demonstrated that the Generic HIV Viral Load® assay is a robust test able to accurately quantify most of non-B HIV-1 subtypes circulating in these settings. This together with its low cost (~10 US $/test) (Steegen et al., 2007) prompted the decision to use this commercial kit in the Kesho Bora trial. Therefore, using well-validated real-time PCR assays and open real-time PCR machines (Rouet et al., 2008) can be recommended for routine use in resource-constrained settings facing the highest HIV-1 genetic diversity and having to achieve high quality performance in HIV care. Feasibility pilot studies using this open-ended assay are in progress in these different African countries, including the usefulness of dried blood spots as alternatives to plasma specimens (Gampini et al., 2009).

Conflicts of interest

None declared.

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Appendix A. The Kesho Bora Study Group

A.1. Study sites

(1) Bobo-Dioulasso, Burkina Faso (Centre Muraz): Nicolas Meda (Principal Investigator), Paulin Fao, Odette Ky-Zerbo, Clarisse Gouem (Study coordinators), Paulin Sombda, Hervé Hien, Elysée Ouedraogo, Dramane Kania (Investigators), Diane Valéa (Laboratory Coordinator), Sayouba Ouedraogo (Data Manager), François Rouet (Inter-Sites Laboratory Coordination).
(2) Durban, South-Africa (University of KwaZulu-Natal): Nigel Rollins (Principal Investigator), Lynne McFetridge, Kevi Naidu (Study Coordinators), Johannes Viljoen (Laboratory Coordinator).
(3) Mombasa, Kenya (International Centre for Reproductive Health): Stanley Luchters and Mark Hawken (Principal Investigators), Eunice Irungu (Study Coordinator), Christine Katingima and Gina Ouattara (Investigators), Kishor Mandaliya (Laboratory Coordinator).
(4) Mtubatuba, South Africa (Africa Centre for Health and Population Studies): Marie-Louise Newell (Principal Investigator), Stephen Mepham (Study Coordinator), Johannes Viljoen (Laboratory Coordinator), Ruth Bland (Investigator).
(5) Nairobi, Kenya (NARESA): Ruth Nduati (Principal Investigator), Judy Kose (Study Coordinator), Ephantus Njagi (Laboratory Coordinator), Peter Mwaura (Data Manager).

A.2. Supporting institutions

(1) Université Montpellier 1, EA 4205 "Transmission, Pathogenèse et Prévention de l'infection par le VIH"; and CHU Montpellier, Laboratoire de Bactériologie-Virologie, Montpellier, France:

Philippe Van de Perre, Pierre Becquart (until December 2006), Vincent Foulongne, Michel Segondy (Laboratory Coordination).
(2) Institut de Recherche pour le Développement (IRD), Montpellier, France: Kirsten Simondon and Cécile Cames (Nutrition Coordination).
(3) Université Aix-Marseille, France: Alice Desclaux and Saskia Walentovitz (Anthropology).
(4) Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, USA: Jennifer Read (Sponsor Representative and Co-Investigator).
(5) Agence Nationale de Recherches sur les SIDA et les hépatites virales, France: Brigitte Bazin and Claire Rekacewicz (Sponsor Representatives).
(6) Centers for Disease Control and Prevention, USA: Allan Taylor, Nicole Flowers, Michael Thigpen, Mary Glenn Fowler, Denise Jamieson (Sponsor Representatives and Co-Investigators).
(7) International Centre for Reproductive Health (ICRH), Ghent, Belgium: Patricia Claeys, Marleen Temmerman (Sponsor Representatives).

A.3. Study coordination

World Health Organization, Geneva, Switzerland: Isabelle de Vincenzi (Study Coordinator), Philippe Gaillard (Site Coordinator), Tim Farley (Project Manager), Eduardo Bergel (Study Statistician), Sihem Landoulsi (Data Manager).

A.4. Financial support

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