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Full Length Research Paper

Evaluation of different deoxyribonucleic acid (DNA) extraction methods using dried blood spot for early infant diagnosis of HIV1 in Sub-Saharan Africa

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Mother-to-child transmission leads to death of 50% of infected children in the first 2 years of life. Early diagnosis is essential for timely treatment. Dried blood spots (DBS) collected on filter paper provide a simple approach for sample collection especially in resource limited settings, because storage and shipment are very easy. The aim of the study was to standardize reproducible, affordable and not expensive deoxyribonucleic acid (DNA) extraction procedures. 146 samples were collected in the context of DREAM Program activity (Drug Resources Enhancement against AIDS and Malnutrition), a large public health programme in Africa to treat AIDS, and nine experimental methods were compared with the HIV-DNA extraction from whole blood using Amplicor HIV-1 DNA Roche Molecular Systems. The results obtained demonstrates that it is possible to extract DNA form DBS using a Chelex resin based method with comparable efficiency to that of a commercial standardized kit, as Qiagen, decreasing test costs by about 50% and with an excellent correlation with whole blood extraction.

Keys words: Early infant diagnosis, human immunodeficiency virus type 1 (HIV-1), dried blood spots (DBS), extraction.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) transmission during pregnancy and breastfeeding leads to a high rate of infant mortality, especially during the first 2 years of life of the child. Nevertheless, over the past few years, early diagnosis of infant HIV-infection combined with antiretroviral treatment and prophylaxis of opportunistic infections have significantly reduced the morbidity of the virus and improved the quality of life of many HIV-1 infected infants. Early HIV diagnosis and early antiretroviral therapy reduced infant mortality by 76% and HIV progression by 75% (Violari et al., 2008). Early detection of HIV infection status in exposed children has been identified as the first step towards improving paediatric HIV/AIDS care in developing countries. Serological tests are not useful in the early identification of infected infants since maternal antibodies are transplacentally transferred and remain detectable in children's bloodstream up to the age of 18 months. Thus, to prevent adverse outcomes, it is necessary to implement molecular early infant diagnosis of HIV-1 infection for the detection of HIV-DNA in order to determine the real status of infection in the child and eventually start the antiretroviral therapy (Read et al., 2007). The

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amplification of the integrated viral genome by Polymerase Chain Reaction (PCR) is the preferred method for early diagnosis of HIV infection in children (Fischer et al., 2004). Thus, early diagnosis remains a challenge, particularly in sub-Saharan Africa, where in 2008, 390,000 children were estimated infected (UNAIDS-WHO, 2009).

In place of PCR, it is possible using rapid/serological testing algorithms (Menzies et al., 2009), or tests as Total Nucleic Acid (TNA) (Ou et al., 2007) and HIV-1 p24 antigen (Schüpbach, 2002) and, recently, Abbott RealTime HIV-1 Qualitative (Huang et al., 2011). However, PCR is the gold standard, and the Roche Amplicor DNA PCR version 1.5 assay has been validated in many Countries. The assay is standardized, available in kit format, has the most widespread use, and is therefore recommended for EID programs. Overall, results to date have shown good performance, but a slightly reduced sensitivity was seen when dry blood spot (DBS) specimens were used (Stevens et al., 2008). However, as with any molecular test, there are issues related to staff expertise and training, instrument and space requirements, and cost. For these reasons, continued effort is required in the development and validation of additional point-of-care (POC) tests to support HIV patient care, and in quality assurance in manufacturing and in test performance in the field to ensure appropriate use of existing and new POC tests (Anderson et al., 2011).

DBS have greatly facilitated genetic and metabolic screening of newborns in different countries by simplifying all aspects of sample collection, storage, and transport (Schulze et al., 2003; Neto et al., 2004; Al-Dirbashi et al., 2006; De Wilde et al., 2008) and was applied also to identify other viruses as an alternative to viral culture (Barbi et al., 2000). DBS can overcome the blood samples and logistical obstacles that limit access to infant diagnosis in low resource settings. DBS are easy to collect and store, and can be a convenient alternative to plasma (Hamers et al., 2009). DBS can be stored at room temperature, even in tropical areas, without loss of test sensitivity or mailed unrefrigerated in sealed envelopes. Measuring the HIV-1 burden from whole blood dried on filter paper provides a suitable alternative for low-technology settings with limited access to cool chain, as is in sub-Saharan Africa.

DBS use represents a paradigm shift in accessibility to virologic testing for HIV infection. In addition to antibodies, nucleic acids in DBS have been shown to be stable for several months at ambient temperatures, provided the DBS specimens have been thoroughly dried and are stored with desiccant. Thus DBS specimens can be collected at remote rural sites and transported to a central or regional testing laboratory, and its use is recommended also in resistance surveillance (Bertagnolio et al., 2010) and the evaluation of different RNA extraction methods and storage conditions of Dried Plasma or Blood Spots for Human Immunodeficiency Virus Type 1 RNA quantification is urgently needed in AIDS patients monitoring (Monleau et al., 2009).

Unlike the method with whole blood, that required sophisticated, expensive equipment, and the samples have to be sent to the laboratory within 24 hours under proper cold chain maintenance, DBS can be stored and shipped to testing facilities without refrigeration, so DBS is user friendly and cost-effective mode in resource-limited settings (Jain et al., 2011).

Moreover, DBS allows non-invasive sampling for newborns because few drops of blood are needed for each sample (Behets et al., 1992; Cassol et al., 1992; Uttayamakul et al., 2005; Patton et al., 2007; Rollins et al., 2007; Workenesh et al., 2007; Creek et al., 2008; Ngo-Giang-Huong et al., 2008; Andreotti et al., 2010).

Drug Resources Enhancement against AIDS and Malnutrition (DREAM) programme, providing HIV care in several Sub Saharan African Countries (WHO-Comunità di Sant'Egidio, 2005; Palombi et al., 2007; Marazzi et al., 2007; 2009) conducts also a Mother to Child Prevention and Care (MCPC) service in which the use of Dried Blood Spot (DBS) could be eligible as a simple and powerful approach for early infant diagnosis. In addition, DREAM has one of the biggest pediatric cohort, with 4.500 children under antiretroviral treatment, and an important part of them are aged less of 18 months. An early diagnosis is needed to guide therapy, determine prognosis and assess interventions.

The aim of this study was to find an alternative HIV-DNA extraction method from DBS affordable and reliable as the standard commercial protocols. Experimental extraction methods were performed in order to standardize reproducible and cheap testing procedures that could be implemented in Sub-Saharan Africa.

MATERIALS AND METHODS

Between 1st of September and 31st of December 2010 a total of 146 children ranging from 1 month to 18 months followed up at DREAM centres in Malawi and Mozambique were recruited into the study. Children were not included into the PMTCT program, but were screened after their arrival at the DREAM centre because an HIV infection status was suspected. This was a multicentre study involving DREAM centres of Maputo-Consolata and Benfica in Mozambique and DREAM centres of Blantyre, Balaka, Masuku, Kapire, Namandanje and Mntengo Wa Nthenga in Malawi. Patients were included randomly, following their access to the DREAM Centres in the considered range of time, and an informed consent was given to their relatives. The Malawian Ethical Committees approved with approval number NHSRC #486. Overall feature of the population are summarized in Table 1.

Sample collection for HIV-DNA testing

A whole blood sample (1 ml) in EDTA and a Dried Blood Spot (DBS, grade 903# filter cards, Roche DBS Kit, Roche Molecular Systems, Branchburg, NJ) were obtained from each patient for HIV-DNA testing. Ten blood spots were prepared for each patient by applying carefully a few drops of fresh blood (approximately a volume of 50 µl of fresh blood for each spot) drawn by heel-prick with a lancet, on the absorbent filter paper matrix. After the blood saturated the filter paper, each DBS was simply air-dried for 30 min and placed in a high-quality bond envelope. Whole blood samples stored at -20°C and DBS stored at room temperature were then shipped to DREAM reference laboratories, Maputo Criança in Mozambique and Blantyre in Malawi.

Table 1. Study population.

DREAM centre	Patients (Number)	Age (mean range in months)	Gender (% male)	Patients under high active antiretroviral therapy (HAART) (Number)
Criança	17	4.5	34	1
Benfica	27	3.4	39	7
Blantyre	25	5.8	35	8
Balaka	20	6.9	38	4
Masuku	20	6.1	42	6
Kapire	14	7.8	44	5
Namandanje	14	5.3	37	3
Mtengo Wa Nthenga	9	6.8	39	1

LEGENDA: registry description of the studied sample, with ARV treatment status.

HIV-1 DNA extraction methods

Extraction from whole blood

An amount of 500 µl of whole blood was kept from each sample for HIV-DNA extraction following the Amplicor HIV-1 DNA Test procedures (Amplicor HIV-DNA assay, version 1.5-Roche Molecular Systems, Branchburg, NJ) (Fransen et al., 1995).

Extraction from dried blood spot

For each patient 10 DBS were used to perform HIV-DNA extraction with nine experimental methods and one commercial kit the QIamp DNA Mini kit (Qiagen ®, QIAGEN Strasse 140724 Hilden Germany). A 3 mm hole punch was used to punch out a whole blood spot from the filter paper, into a 1.5 ml Eppendorf tube. Due to the amount of blood available for each sample, it was not possible to repeat in double the tests. The hole punch was sterilized between each sample and then cleaned by repeatedly punching through clean filter paper. The nine experimental extraction methods are listed below:

A. Extraction with PBS/0.1% Tween20 – 5% Chelex 100: a solution composed by 5 gr of 5%Chelex 100 into 100 ml of sterile water was prepared, shaked for 1 hour and heated at 50°C for 10 min. A second solution containing 0.1 ml of 0.1% Tween 20 (Sigma) in 100 ml of PBS was produced. Afterwards 300 µl of PBS-0.1% Tween 20 solution were added into a tube containing one spot. The sample was incubated on a shaker running at 120 rpm for 10 min at 25°C. The supernatant was removed and 300 µl of PBS-0.1% Tween 20 solution were added for a second time. After vortexing samples were incubated as previously described. Supernatant was eliminated and 200 µl of 5% Chelex 100 solution were dispensed. Samples were then incubated at 60°C for 30 min and subsequently at 100°C for 30 min. After centrifugation at 14000 rpm for 3 min, the supernatant (Final Volume: 100 µl) was collected and stored (Fischer et al., 2004).

B. Thermal Shock with MEM (Minimum Eagle Medium): 2 disks (3 mm of diameter) were punched out into a tube containing 45 µl of MEM (Minimum Essential Medium Eagle (Sigma). Samples were spinned and incubated overnight at 4°C. The day after samples was incubated at 55°C for 1 h and at 100°C for 7 min. They were then centrifuged at 10000 rpm for 1 min, cooled in the fridge to reach a temperature of 4°C and centrifugated again at 10000 rpm for 1 min. The supernatant (Final Volume: 50 µl) was then transferred to a new tube and stored at -80° for 1 h (Barbi et al., 2000).

C. Extraction with sterile distilled water: 1 spot was cut into a tube containing 500 µl of sterile water. After vortexing for 5 s each sample, the supernatant was collected and eliminated. A spin step was executed in order to eliminate completely the supernatant. 100 µl of sterile water were then added and samples were incubated at 95-100°C for 30 min. Afterwards samples were vortexed for 1 min, spinned and the supernatant (final volume: 100 µl) was conserved (Jacob et al., 2008).

D. Extraction with Wash Buffer Solution Roche: 1 spot was cut and suspended into 1 ml of Specimen Wash Buffer Solution (Amplicor Whole Blood Specimen Preparation Kit; Roche Diagnostics Systems). Samples were shaked on a rotor for 1 h at room temperature and the centrifuged at 12000 rpm for 1 min. The supernatant was removed and 200 µl of 5%chelex-100 resin solution were added. After incubation at 100°C for 1 h with intermittent vortexing, samples were centrifuged at 13000 rpm for 1 min. Supernatant (final volume: 200 µl) was collected and stored (Cassol et al., 1994).

E. Thermal Shock with PBS: 1 spot was placed into 500 µl of PBS and placed 10 min at 90°C. The supernatant was collected into a new tube. The sample was centrifuged at 23000 rpm for 1 h, the supernatant was discharged and the pellet suspended in 20 µl of PBS. Thus further 80 µl of PBS were added. The tube was centrifuged at 7000 rpm for 10 min and the supernatant was cooled at -80°C ready to be used (Shan et al., 2008, modified).

F. Extraction with Sterile Water and Chelex 100: 1 spot was punched out into 1 ml of sterile water. Sample was vortexed for 10 s and put on a shaker for 15 min. Samples were then centrifuges at 125000 rounds for 3 min. Supernatant was removed and 50 µl of sample were left. This step of washing was then repeated. 400 µl of a 5% Chelex 100 solution were added and after mixing and spinning samples were transferred in the incubator at 56°C for 2 h. Samples were vortexed and incubated at 100°C for 8 min, and later centrifuged at 100000 rounds for 1 min. 300 µl of liquid containing DNA were collected (Schleicher and Schuell protocols).

G. Extraction with Blood Spot Solution: 1 spot was punched out into 200 µl of Blood Spot Solution composed by: 1 ml Tris 1 M pH 8, 3, 0, 37 gr KCl (SIGMA cod P5405), 0.05 gr $MgCl_2$ (SIGMA cod M0250), 0.1 ml Triton X-100 (SIGMA T9284), 0.1 gr Gel (SIGMA G1890) and Sterile Water to reach the volume of 10 ml. The solution was incubated at 60°C for 20 min for am optimal mixing of reagents. Samples were vortexed and incubated at 100°C for 10 min. After centrifugation at 2400 rounds for 5 min the supernatant was collected with a final volume of extraction of 200 µl (Whatman protocols).

H. Extraction overnight with PBS: 1 spot was placed in 1 ml of PBS and was incubated at 4°C overnight. The supernatant was placed at -80°C, ready to be used (Fischer et al., 2004, modified).

I. Extraction of DBS in Master Mix: 1 spot was placed directly into

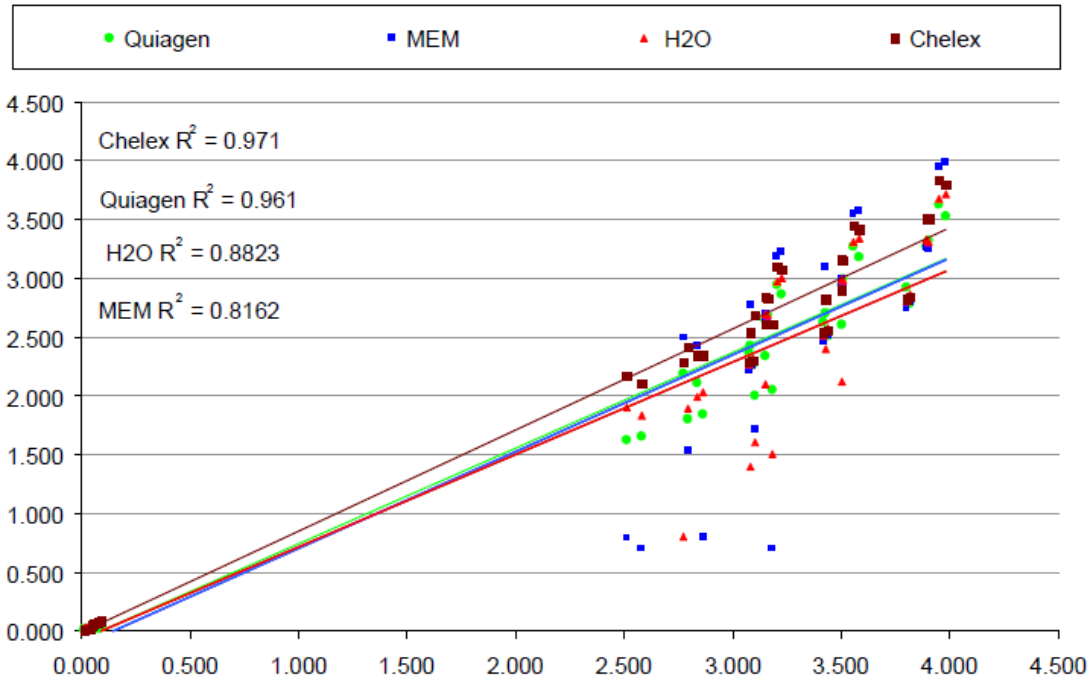

Figure 1. Optical density (O.D) correlation between the golden standard and 4 different extraction methods (Qiagen, A-CHELEX, B-MEM, C-H_2O). LEGENDA: this graphic shows the correlation between the 3 home – made methods with best sensitivity and specify, and the Qiagen one, considered the gold standard in DBS extraction.

the Amplicor HIV-1 DNA test Master Mix and the sample was processed as the Roche protocol (Fransen et al., 1995, modified).

Polymerase chain reaction (PCR) amplification assay and HIV-1 DNA detection

Amplicor HIV-1 DNA Test, version 1.5 (Roche Molecular Systems, Branchburg, NJ) was used as HIV-DNA PCR assay, and amplification of a 155-bp fragment of the gag gene was performed.

Statistical methods

Data were evaluated with the Bland Altman correlation and linear regression. Calculations have been performed using Microsoft Office Excel 2003, Microsoft Corporation.

RESULTS

In total, 146 samples were tested using a commercial kit, the Amplicor HIV-1 DNA Test, version 1.5 (Roche Molecular Systems, Branchburg, NJ). This method was used for extraction, amplification and detection of HIV-DNA from whole blood samples and considered for this study as gold standard.

Step A: the first step of the study aimed at comparing the Amplicor HIV-DNA extraction method from 500 µl of whole blood, the extraction by Qiagen HIV-DNA kit and the nine experimental extraction methods from DBS of the same patient. In this first step, a total of 41 whole blood samples were processed by the Amplicor HIV-1 DNA PCR assay. Overall, 30 samples resulted in been HIV positive and 11 resulted HIV negative. From each of the 41 samples, 10 DBS have been collected and DNA was extracted using the Qiagen kit plus the other nine methods.

DNA amplification was performed using Amplicor HIV-1 DNA PCR assay. The results were expressed as optical density as recommended in the Roche Amplicor kit. Comparative analysis of the experimental methods showed that only Qiagen, Methods A, B and C yielded results comparable to the gold standard (extraction from whole blood) (Figure 1) (Qiagen R^2 = 0.961, Chelex R^2 = 0.971, MEM R^2 = 0.8162, H_2O R^2 = 0.8823).

The Amplicor Roche results' detection gives a negative value if Optical Density (O.D.) is <0.2, uncertain whether O.D. is > 0.2 and <0.8 and positive if O. D. is > 0.8. Figure 1 shows that four methods (Qiagen, A, B, C) give a reading, while the other six methods always resulted under the 0.2 O.D. threshold and were not included in further analysis.

Qiagen had been used to have a further point of comparison for the extraction methods tested. Given the excellent correlation obtained by the three methods A, B and C, and considered the cost of Qiagen reagents, it is preferred from this point of the study, only to continue the comparison between the whole blood and the three methods showing good results.

Step B: Thus, 105 remaining DBS were extracted only with Methods A, B, and C, and compared with whole blood extraction. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) has been calculated (Table 2).

Table 2. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of "A", "B" and "C" DNA DBS extraction methods.

n=105		Whole blood					
		Pos.	Neg.	sensitivity	specificity	PPV	NPV
A	Pos.	73	0	1.00	1.00	1.00	1.00
	Neg.	0	18				
B	Pos.	87	0	0.86	1.00	1.00	0.56
	Neg.	14	18				
C	Pos.	83	0	0.95	1.00	1.00	0.82
	Neg.	4	18				

LEGENDA: The table shows the 3 home – made methods - "A" Extraction with PBS/0.1% Tween20 – 5% Chelex 100, "B" Thermal Shock with MEM (Minimum Eagle Medium), and "C" Extraction with sterile distilled water - that better correlated with the gold standard (whole blood extraction with Amplicor HIV-1 DNA Test).

The method A, showed 100% concordance with the gold standard, as shown in Table 2, where sensitivity was 1.0. The results obtained with the method B revealed that there was an incomplete agreement with the golden standard method (Table 2). This methodology produced 14 false negative results and had the lowest sensitivity (0.86). Method C, showed 4 false negatives and a sensitivity of 0.95. Bland-Altman analysis has been performed to compare the DNA extraction from whole blood with method A, B, C respectively.

Method A (Figure 2) shows the points well clustered around the mean, with a bias of 0.5. Some outliners are present both in the top than in the bottom of the graph with a standard deviation of 0.54. Method B (Figure 3) displays a different pattern with three different populations, one of them outside the confidence interval. The bias is 1.25 and the standard deviation is 1.39. Method C (Figure 4) has a bias of 1.07 and a standard deviation of 0.99.

DISCUSSION

Results obtained from DBS tested with experimental methods were compared with those obtained with the whole blood as the gold standard, and results obtained with Qiagen extraction were compared with the nine experimental methods. After the first step, it was detected that the six methods, from D to I, were not able, in this study, to detect HIV-1 DNA in DBS collected from DREAM patients, and for this reason were suspended. The comparison was performed only on A, B and C methods *versus* Qiagen and whole blood.

Between the experimental methods, method A, demonstrated the best accuracy in detecting HIV-1 DNA from DBS compared to the gold standard. Sensitivity and specificity were excellent (1.00 and 1.00, respectively) as PPV (1.00) and NPV (1.00), confirmed in the second step of the study. Method B and method C give decreasing results in terms of sensitivity (0.86 and 0.95, respectively) and NPV (0.56 and 0.82 respectively) (Table 2). These results were particularly interesting also because the sample volume for DBS is significantly reduced than the whole blood (50 µl *versus* 500 µl). It seems from these data that 50 µl is enough to detect HIV-1 DNA.

Figure 1 shows the correlation of the Qiagen, method A, B and C against the golden standard. Qiagen, as expected, has a good coefficient (r^2 =0.961). Among the 3 experimental methods, method A (chelex) scores an excellent coefficient (r^2=0.971) suggesting that the resin is particularly suitable for nucleic acid binding within the range of viral particles presents in the randomly chosen samples.

Bland Altman analysis shows that a trend in the comparison of the three methods against the whole blood extraction is present. In Figure 2, the data of the method A against gold standard are well dispersed around the mean of the difference, meaning that the agreement between the two methods is conserved along all the range of absorbance. Even if a systematic underesti-mation of the DNA with the method A (bias=0.5) can be observed, there is not a DNA amount effect on the dispersion of the points, being them randomly dispersed from 2 to 4.5 O.D. Moreover, the underestimation doesn't affect the quality of the method (sensitivity= 1.0 NPV = 1.0).

In Figure 3, method B is compared with golden standard, a trend is evident. From 2.0 to 3.0 O.D the difference of the extracted DNA with the golden standard subtracted by the DNA extracted with the method B is bigger than the mean, whereas in the range from 3.0 to 4.5 O.D the results are lower than the mean. This trend can be explained by the effect of the total DNA amount on the extracting performance of the two methods. The less is the total amount of DNA on the spot the worse is the DNA extraction performance of the method B, infact increasing the value of the mean of the two methods the difference between them decrease. When the mean is around 4.0 the difference between the two methods is close to 0.

In Figure 4, the comparison of the method C with the whole blood extraction still shows a trend similar to above but less marked. There are some outliners and a trend in data is still evident even if not as with the method

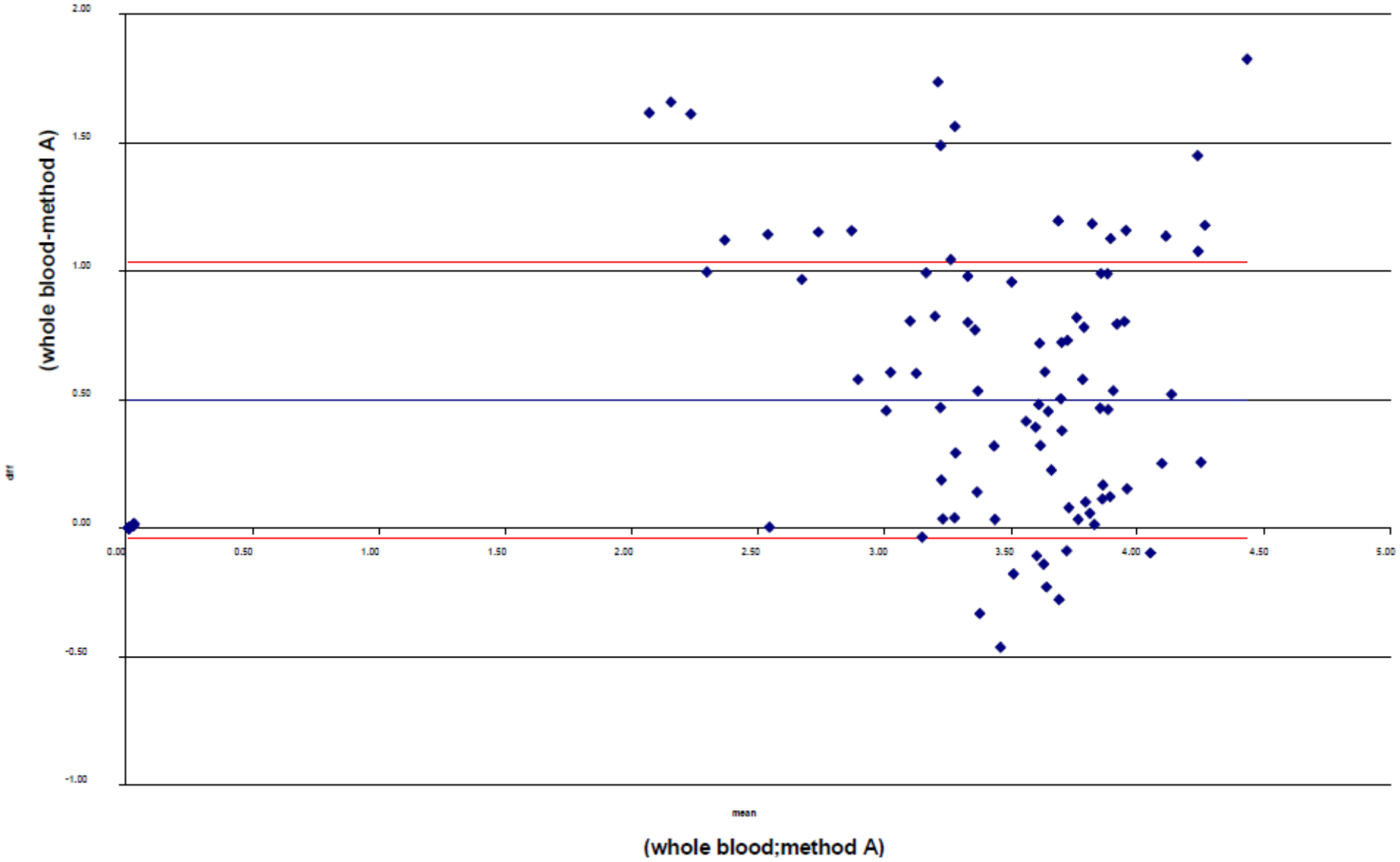

Figure 2. Mean of whole blood and method A. LEGENDA: this graphic show a good correlation between the home – made “A” method in DBS extraction versus the whole blood (assumed as gold-standard).

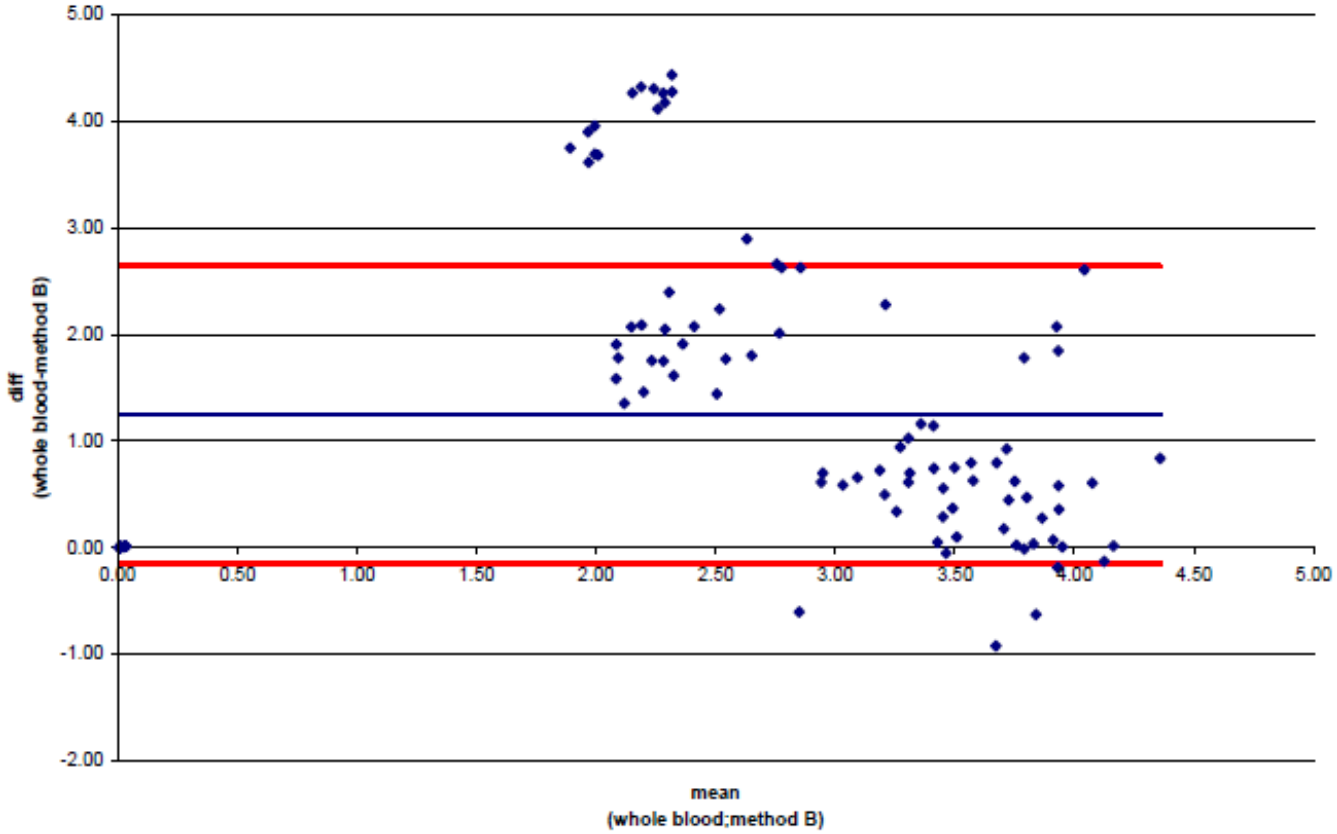

Figure 3. Mean of whole blood and method B. LEGENDA: this graphic show the correlation between the home – made "B" method in DBS extraction versus the whole blood (assumed as gold- standard). A too high dispersion of data is observed.

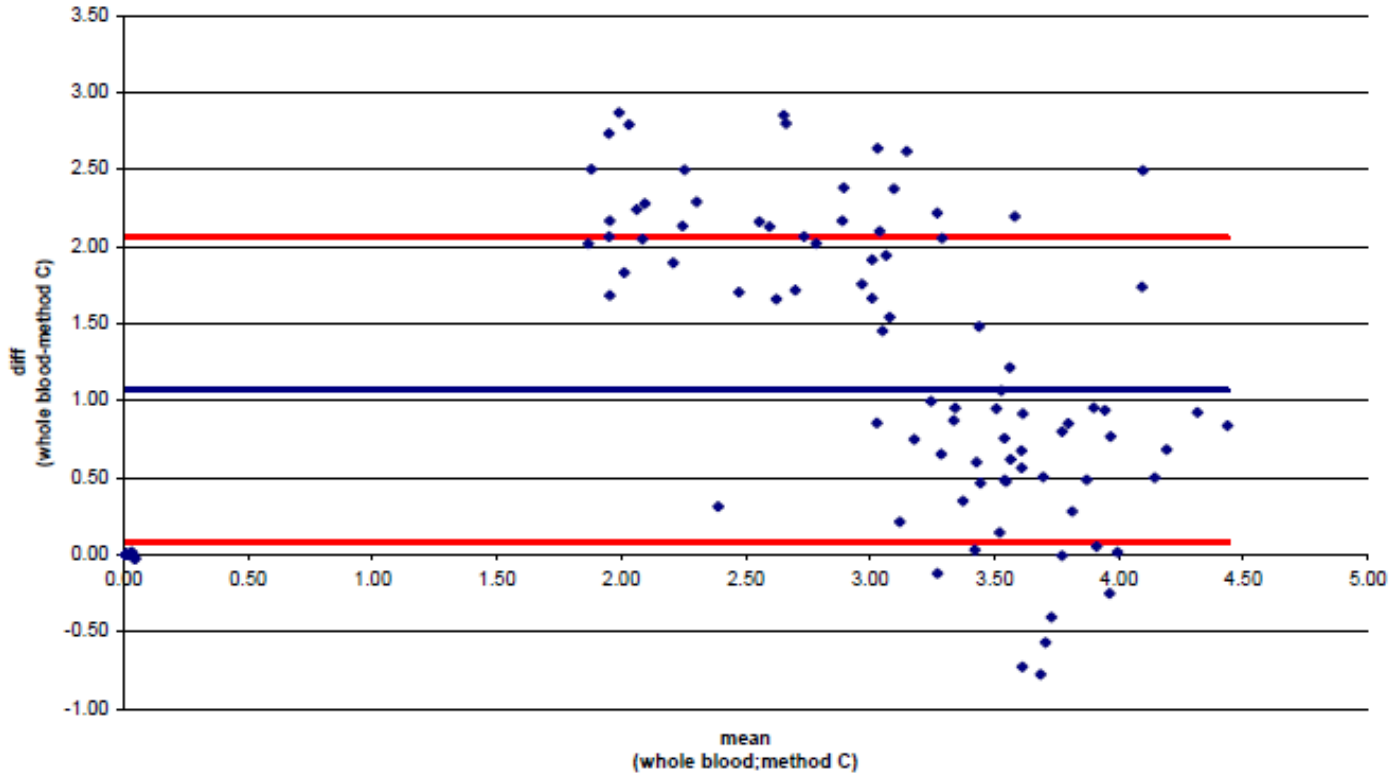

Figure 4. Mean of whole blood and method C. LEGENDA: this graphic shows the correlation between the home – made "C" method in DBS extraction *versus* the whole blood (assumed as gold-standard). The data are bad dispersed around the mean.

being the bias 0.5 (methods A), 1.2 (method B) and 1.1 (method C) respectively.

The decreasing NPV in methods C and B (0.82 and 0.56 respectively) can be explained with the reduced performance of those methods when the total amount of DNA of the samples decreases. The Qiamp Qiagen DNA mini kit is commercially available and developed for the extraction of DNA from DBS and, even if it is in excellent agreement with the gold-standard method, it is not affordable because of the cost for low income countries. In this study, the usage of the Qiagen extraction kit was intended only to confirm the good preparation of the DBS and that the overall process was performed properly. For that reason, it has been considered useless in the second step of the study.

Conclusions

HIV-1 DNA extraction from DBS is often the only opportunity for early detection by molecular diagnostic assays of infant infection in developing countries. There is an immediate need for simple, rapid and economic tests. DBS testing accompanied by easy, cheap extraction procedures is strongly recommended in rural areas without infrastructure, as this strategy facilitates the scaling up of paediatric treatment.

The obtained findings demonstrated that it is possible to extract DNA form DBS using a PBS/Tween 20/Chelex resin based extraction method with an efficiency comparable with a commercial standardized kit (Qiamp Qiagen DNA mini kit), decreasing the final test costs up to about 50%. Both methods, extracting HIV-1 DNA from DBS, performed well as compared to Amplicor HIV-1 DNA test, Roche, using whole blood extraction.

In addition, laboratories facilities and support required are the same for both methods, and specimen processing and handling with the method A are as easy as with commercial kits. Manipulation of specimens is minimal compared to the other in house methods tested, decreasing contamination risk. These data demonstrated that it is possible to extract proviral DNA form DBS using a Chelex resine with comparable efficiency to that of a commercial standardized kit (Qiagen). Both methods performed well as compared to a gold standard (Roche kit using whole blood). For these reasons, DBS could be used for specimen collection in HIV-1 early infant diagnosis and a cheap extraction could be applied using a PBS/Tween 20/Chelex resin with a standardized quail-tative PCR assay for HIV-1 DNA target amplification. So, in addition to the Roche kit for amplification, only about $0.20 is needed for extraction, reducing the final cost to about 50%. This affordable and high performing procedure could consequently be implemented in laboratories across African countries for the purposes of early diagnosis of HIV-infection in exposed infants.

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