Implementation of a Classic Nested PCR DNA for HIV Diagnosis in Kinshasa

Erick Kamangu Ntambwe [1*], Christian Mayemba [2], Samclide Mbikayi [2], Adolphe Ndarabu [2], Richard Kalala Lunganza [1], Georges Mvumbi Lelo [1], Dolores Vaira [3]

[1] Unit of Molecular Biology, Department of Basic Sciences, Faculty of Medicine, University of Kinshasa (UNIKIN), Kinshasa, Democratic Republic of Congo (DRC)
[2] Department of Infectious Diseases, Monkole-3, Monkole Hospital, Kinshasa, Democratic Republic of Congo (DRC)
[3] Laboratoire Reference SIDA (LRS), Centre Hospitalier Universitaire, University of Liège (CHU-Lg), Liège, Belgium

*** Corresponding Author:** Erick KAMANGU N.
Telephone: +243 (0) 81 54 01 600, | Email: erick.kamangu@unikin.ac.cd

Abstract

Background: The interest in the technique of HIV diagnosis by Polymerase Chain Reaction (PCR) is in: (i) primary infection by the Human Immunodeficiency Virus (HIV), where it is necessary to identify the patient in time, (ii) screening of newborns for HIV-positive mothers, and (iii) the accuracy and reliability of the technique.

Objective: This study aims to assess the feasibility and the performance of the Nested PCR of Deoxyribose Nucleic Acid (DNA) for HIV diagnosis in Kinshasa in order to improve the detection and management of HIV infected patients.

Methods: The present study is a cross-sectional study in collaboration with 3 centers in Kinshasa. Our sample consisted of 171 individuals who have made voluntary testing for HIV. 5.0ml of blood was collected in tubes with anticoagulant, centrifuged for separation into 3 phases. 500µl of buffy coat was collected in a pre-labeled tube. The DNA extraction was made from 200µl of buffy coat using the QIAamp DNA Mini Kit from QIAGEN ® for DNA extraction. Classical HLA PCR and Nested PCR *gag* and *pol* were performed to determine the proviral DNA. Nested PCR on *env* was performed in cases of discordance of the results of *gag* and *pol*. The revelation was made under UV after electrophoretic migration on 1% agarose gel. The data were collected in confidentiality in the forms developed and pre-tested for the study, entered on Windows Excel 2007 and analyzed using SPSS 17.0 for Windows. The statistical significance was set at $p < 0.050$.

Results: For Monkole, 22 samples were positive, 18 negative and 6 indeterminate for HIV. For Organization for Disease Prevention and Health Promotion (OPPS), we received 55 samples positive for HIV who served as a control for the study. For Foundation "La Grâce", 30 samples were positive, 25 negative and 15 indeterminate for HIV. This gives a total of 107 samples positive, 43 negative and 21 indeterminate for HIV. After amplification by Nested PCR DNA, we have a total of 112 samples positive and 59 HIV-negative. Of the 21

indeterminate samples, 3 were positive and 18 negative by PCR. 2 samples were negative by RDT but positive by PCR.

Conclusion: The Rapid Diagnostic Test (RDT) results and those of PCR DNA Nestled allowed us to evaluate and validate the technic of diagnosis of HIV in Kinshasa by this new technic. This method provides a more reliable alternative for the diagnosis of HIV Kinshasa.

Key words: diagnosis, HIV, Nested PCR, Kinshasa

Introduction

The technique of amplification by Polymerase Chain Reaction (PCR) generated a lot of enthusiasm. Its "relatively simple" realization has led many teams to try its realization. Its principle is attractive since the shortcomings of previous methods can be related to the mode of virus replication, with a possible eclipse phase and especially the need for integration in the cell genome without the virus or its antigens are expressed necessarily continuously. It was extremely tempting to go "dig out" the provirus where he was hiding in the Deoxyribose Nucleic Acid (DNA), within the genome of the infected cell. But the relative ease of technical execution, are increasing difficulties of interpretation and training of appropriate staff.[1]

The medical and scientific interest in the technique of HIV diagnosis by PCR lies in: (i) primary infection, where it is necessary to identify the patient in time, (ii) screening of newborns of mothers HIV-positive, and (iii) the accuracy and reliability of the technique.[2-5]

This study aims to implement a Classic Nested PCR DNA for HIV diagnosis in Kinshasa in order to improve the detection and management of HIV-infected patients.

Methods

Framework

The present study is a cross-sectional study that was conducted in collaboration with the Department of Infectious Diseases (Monkole-3) *Centre Hospitalier Monkole* (CHM) which is a screening service and support for People Living with HIV (PLHIV)[6], the non-profit Organization for Disease Prevention and Health Promotion (OPPS) and Foundation "*La Grâce*" which are associations that participate in voluntary HIV testing in the community, all in the city of Kinshasa. This study took place from 01 August to 27 October 2012.

Patients

Only people willing to voluntary testing for HIV were selected for this study. Our sample consisted of 171 individuals who have made voluntary testing for HIV by Rapid Diagnosis Test (RDT). Seventy (70) persons were screened through the Foundation "*La Grâce*", 55 through the OPPS and 46 through the CHM. All were willing and signed consent for screening with the respective institutions. No results for RDTs had been revealed before the PCR results.

Blood samples

A minimum of 5.0 ml of blood was collected into tubes containing EDTA from the respective testing centers. The collected blood was centrifuged at 3000 rotation per minute (rpm) for 10 minutes to obtain a separation into 3 phases: the supernatant (plasma), the intermediate zone (buffy coat) and the pellet. Five hundred microliters (500µl) of buffy coat was collected in a pre-labeled tube and stored at 4 ° C before being sent to the laboratory of Molecular Biology of the Faculty of Medicine for analysis.

Extraction and DNA amplification

The DNA extraction was made from 200µl of buffy coat using the QIAamp DNA Mini Kit from QIAGEN ® for DNA extraction.[7] The DNA extract was stored at -20 ° C.

After extraction, a Classic PCR on HLA and Nested PCR on *gag* and *pol* region were performed to determine the proviral DNA. Nested PCR on *env* region was performed about in cases of discordant results of *gag* and *pol*. The primers for amplification were HLA: GH26 and GH27 (Table 1).[8] The primers for the first stage of the HIV PCR were: GAG1 and GAG4 for *gag* amplification [**9**], POLITG1 and POLITG4 for *pol* [10], and ENV1 and ENV4 for *env* (Table 1).[9] The primers for PCR were Nestled: GAG2 and GAG3 for *gag* [9], POLITG2 and POLITG3 for *pol* [10], and ENV2 and ENV3 for *env* (Table 1).[9] PCR assays were carried out under the conditions described in Table 2.

Interpretation of Results

The revelation was made under UV after electrophoretic migration on agarose gel 1% prepared in 1X TAE (Tris / acetate / EDTA). For a sample to be counted positive for HIV by PCR, it should be positive for HLA amplification and positive amplifications for two pro-viral (*gag / pol, gag / env* and/or *pol / env*).

Statistical Analysis

The data were collected in confidentiality in the data developed and pre-tested to work. They were transcribed on Windows Excel version 2007 and analyzed on SPSS version 17.0 for Windows. The statistical significance was set at $p < 0.050$.

Results

A total of 171 samples were received for Nested PCR DNA: 46 from CHM, 55 from OPPS and 70 from Foundation "*La Grâce*."

RDT's status

For CHM, 22 samples were positive, 18 negative and 6 indeterminate for HIV. For the OPPS, we received 55 samples positive for HIV who served as a control for the study. For Foundation "*La Grâce*", 30 samples were positive, 25 negative and 15 indeterminate for HIV.

This gives a total of 107 samples positive, 43 negative and 21 indeterminate for HIV (Tables 3 and 4).

DNA amplification

After PCR amplification Nestled DNA, we have a total of 112 samples positive and 59 HIV-negative. Of the 21 indeterminate samples, 3 were positive and 18 negative by PCR. Two (2) samples were negative by RDT but positive PCR (Tables 3 and 4).

Discussion

This study was to assess the feasibility and the performance of the Nested PCR DNA for HIV diagnosis in Kinshasa. We had a total of 171 blood samples for this work from three different centers: *Centre Hospitalier Monkole* (CHM), which contributed 46 samples, the Foundation "*La Grâce*" with 70 samples and OPPS with 55 samples. All samples provided were previously tested for HIV using Rapid Diagnostic Tests (RDTs). The tests used, as by national recommendations, are: Determine ½, Uni-Gold and Double Check.[11]

For CHM, we received on 46 samples, 22 were positive, 18 negative and 6 indeterminate by RDTs. After Nested PCR diagnosis, we had 22 positive and 24 negative samples. The six samples that were indeterminate with RDTs were clearly negative by PCR.

For Foundation "*La Grâce*", out of the 70 samples received, 30 were positive, 25 negative and 15 indeterminate. After Nested PCR diagnosis, we had 35 positive samples and 35 negative for HIV. Two samples that were negative with RDTs were positive by PCR. Of the 15 indeterminate samples by RDTs, 3 were positive and 12 were negative by Nested PCR. The two discordant samples as well as indeterminate by RDTs that turned positive by Nested PCR were tested by ELISA, as 3rd diagnostic method to confirm the results, as these were positive by Nested PCR.

For OPPS, the 55 samples received were all positive tests for HIV by RDTs. After Nested PCR, all 55 samples were confirmed positive for HIV.

This shows us how much the Nested PCR is more specific and more accurate than RDTs. The Indeterminate results with RDTs were classified positive or negative, and then confirmed by ELISA. Nested PCR gives us much more accurate results than the diagnostic techniques used so far in our centers and laboratories. ELISA p24 antigen reduces the diagnostic gap to four weeks after infection, but in some cases it may remain negative for more than 3 months.[12-15] Commercial standardized tests, or RDTs, facilitated the diagnosis of HIV in their simplicity.[16] However, they are still shadows hanging over the specificity and accuracy of these since they were designed based on specific markers for certain types of HIV.[12] This confirms some of the deficiencies identified in our results RDTs and by Kleinman and colleagues.[17] The short-comings of the RDTs are mostly the causes of the false results in the diagnosis of the infection.

In case of indeterminate results, the patients are recommended to come back in 3 weeks to get tested again to see if there would be a more precise result.[11] However, it is very unlikely for a indeterminate-patient to come back for testing afterward fearing the unpleasant result. This

implies that, in our case, 14.29% of this class would have gone with the infection without coming back for their result.

The principle of the Nested PCR DNA is that the product of the first PCR is used for the second amplification. Thus giving a much more precise result for a qualitative PCR. The *env*, *gag* and *pol* regions are used for the amplification because they are the common regions for the virus genome.[1-5,8-10] Every mature and functional virus does have these regions. Therefore, they are present at the integrated proviral DNA. Even at different period of the virus cycle, these regions can be found.[8-10] These are some of the advantages of the technique; knowing that at any stage of the cycle, we can dig-out the virus from where it is hiding.

Although laborious, the Nested PCR technique has proven to be an accurate tool for diagnosing HIV.[1,12,18] The feasibility of this PCR does not required very sophisticated material. It requires a classic thermocycleur for the amplification, a kit for the migration and an UV table for the visualization; most material that could be found in developing country.

HIV diagnosis by Nested PCR is a useful tool for management accurately and early. Especially in cases that are indeterminate or doubtful.

Conclusion

The RDTs results and those of Nested PCR DNA allowed us to evaluate the performance of this diagnosis method for HIV in Kinshasa. Discrepancies were confirmed by ELISA for the Nested PCR results. This method provides a more reliable alternative for the diagnosis of HIV Kinshasa. It should be recommended for the general laboratory to strengthen HIV-infected patients diagnosis.

Acknowledgement: The authors thank the managers and staff of *Centre Hospitalier Monkole* (CHM), the Organization for Disease Prevention and Health Promotion (OPPS-non-profit organization), the Foundation "*La Grace*" and the Veterinary Laboratory of Kinshasa for their open collaboration.

EK thanks the University Cooperation for Development of Belgium (CUD) for the grant that allowed the training program, and the AIDS Reference Laboratory of the University Hospital of Liège (CHU-Lg LRS) for training and learning new techniques that are routinely used for the diagnosis of patients.

Conflicts of Interest: The authors declare that they have no competing interests.

References

1. Floch JJ. Diagnostic Biologique de l'Infection à VIH en Afrique : Mise en place d'une nouvelle technique localement. *Médecine d'Afrique Noire*. 1990 ; 37 (10) : 574-82.
2. Bertisch B and Vernazza P. Infection par le VIH – le dépistage précoce limite les dommages pour l'individu et la société. *Forum Med Suisse*. 2010 ; 10 (46) : 798–801.
3. Wolbers M, Bucher H, Furrer H, Rickenbach M, Cavassini M, Weber R, Schmid P, Bernasconi E, Hirschel B, Battegay M, Swiss HIV Cohort Study. Delayed diagnosis of

HIV infection and late initiation of antiretroviral therapy in the Swiss HIV Cohort Study. *HIV Medicine*. 2008; 9 (6): 397–405.
4. Hecht FM, Busch MP, Rawal B, Webb M, Rosenberg E, Swanson M, Chesney M, Anderson J, Levy J, Kahn JO. Use of laboratory tests and clinical symptoms for identification of primary HIV infection. *AIDS*. 2002; 16 (8): 1119–29.
5. Kahn JO, Walker BD. Acute human immunodeficiency virus type 1 infection. *N Engl J Med*. 1998; 339 (1): 33–9.
6. Kamangu EN, Kalala HN and Mesia GK. Profile of Antiretroviral Treatment Centers in Kinshasa, Democratic Republic of Congo [Poster 388]. *In proceedings of the First International African Society of Laboratory Medicine (ASLM) Conference*. 1-7 December 2012; Cape Town, South Africa. 2012: 377.
7. QIAGEN. *QIAamp® DNA Mini and Blood Mini Handbook*. 3rd Edition. April 2010: 27-9
8. Gyllensten UB and Erlich HA. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DA locus. *Proc Natl Acad Sci USA*. October 1998; 85: 7652-6.
9. Simmonds P, Balfe P, Peutherer JF, Ludlam CA, Bishop JO and Leigh Brown AJ. Human Immunodeficiency Virus-Infected Individuals Contain Provirus in small Numbers of Peripherial Mononuclear Cells and at Low Copy Numbers. *J. Virol*. Feb 1990: 864-72.
10. Fransen K, Zhong P, Beenhouwer H, Carpels G, Peeters M, Louwagie J, Jassens W, Piot P and Van G. Design and evaluation of new, highly sensitive and specific primers for polymerase chain reaction detection of HIV-1 infected primary lymphocytes. *Molecular and Cellular Probes*. 1994; 8: 317-22.
11. Programme National de la Lutte contre le Sida et les IST (PNLS), Ministère de la Santé Publique, République Démocratique du Congo : Guide National de Traitement de l'infection à VIH chez l'adolescent et l'adulte ; Mai 2008.
12. Klimkait T. Tests VIH. *NOVA, Forum Med Suisse*. 2008 ; 8 (15) : 278-81.
13. Daar ES, Moudgil T, Meyer RD and Ho DD. Transient high levels of viremia in patients with primary HIV Type 1 infection. *N Engl J Med*. 1991 ; 324 : 961-4.
14. Schaker TW, Hughes JP, Shea T, Coombs RW and Corey L. Biological and virological characteristics of primary HIV infection. *Ann Intern Med*. 1998 ; 128 : 613-20.
15. Greenwald JL, Burstein GR, Pincus J and Branson B. A rapid review of rapid HIV antibody tests. *Cur Infect Disease Reports*. 2006 ; 8 : 125-31.
16. Guidelines for the Use of Antiretroviral Agents in HIV-infected Adults and Adolescents. Bethesda, MD, Department of Health and Human service, 1999.
17. Kleinman S, Bush MP, Hall L, Thomson R, Glynn S, Gallahan D, Ownby HE and Williams AE, for the Retrovirus Epidemiology Donor Study: False-Positive HIV-1 Test Results in a Low-Risk Screening Setting of Voluntary Blood Donation. *JAMA*. 1998; 280: 1080-5.
18. Sagna T, Bisseye C, Sanou DS, Djigma F, Ouermi D, Zeba M, Pietra V, Pignatelli S, Gnoula C, Sia JD, Nikiema JB and Simpore J. Diagnostic précoce, par RT/PCR, du VIH-1 chez les enfants nés des mères séropositives. *Science de la Santé*. 2008 ; 31 (1) : 29-36.

Table 1: Primes and sequences for the PCR reactions

PCR	Primers	Sequences
HLA	GH26 forward	5'-GTGCTGCAGGTGTAAACT-3'
	GH27 reverse	5'-CACGGATCCGGT-3'
Gag	GAG1 forward	5'-GGTACATCAGGCCATATCACC-3'

	GAG4 reverse	5'-ACCGGTCTACATAGTCTC-3'
Pol	POLITG1 forward	5'-CCCTACAATCCCCAAAGTCAAGG-3'
	POLITG4 reverse	5'-TACTGCCCCTTCACCTTTCCA-3'
Env	ENV1 forward	5'-GAGGATATAATCAGTTTATGG-3'
	ENV4 reverse	5'-AATTCCATGTGTACATTGTACTG-3'
Nested gag	GAG2 forward	5'-GAGGAAGCTGCAGAATGGG-3'
	GAG3 reverse	5'-GGTCCTTGTCTTATGTCC-3'
Nested pol	POLITG2 forward	5'-TAAGACAGCAGACAAATGGCAG-3'
	POLITG3 reverse	5'-GCTGTCCCTGTAATAAACCCG-3'
Nested env	ENV2 forward	5'-GATCAAAGCCTAAAGCCATG-3'
	ENV3 reverse	5'-CAATAATGTATGGGAATTGG-3'

Table 2: PCR Cycle (temperature per cycle)

	gag and pol	env and Nested pol	HLA and Nested gag	Nested env
Initial Denaturation	95°C : 9min	95°C : 9min	95°C : 9min	95°C : 9min
Denaturation per cycle	94°C : 1min	94°C : 1min	94°C : 1min	94°C : 1min
Hybridation par cycle	50°C : 1min	50°C : 1min	55°C : 1min	45°C : 1min
Elongation par cycle	72°C : 1min	72°C : 1min	72°C : 1min	72°C : 1min
Elongation finale	72°C : 10min	72°C : 10min	72°C : 10min	72°C : 10min
Hold	4°C	4°C	4°C	4°C
Number of cycle	35	30	25	25

Table 3: Confirmation Diagnostic VIH RDT and PCR

	TDR	HLA		*gag*		*pol*		*env**	
		+	-	+	-	+	-	+	-
Positive	107	106	1	105	2	107	0	2	--
Negative	43	40	3	2	41	2	41	--	--
Indeterminate	21	20	1	3	18	3	18	--	--
Total	**171**	**166**	**5**	**110**	**61**	**112**	**59**	**2**	**--**

*PCR *env* to confirm status after difference of results with *gag* and *pol*

Table 4: Confirmation of results per center

	Centre Hospitalier Monkole	Foundation «*La Grâce*»	OPPS	Total
TDR+ => PCR+	22	30	55	107
TDR+ => PCR-	0	0	0	0
TDR- => PCR+	0	2	0	2
TDR- => PCR-	18	23	0	41
TDR± => PCR+	0	3	0	3
TDR± => PCR-	6	12	0	18
Total	**46**	**70**	**55**	**171**

°TDR± = Result Indeterminate with RDT