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Methodology Article

Evaluating the HIV-1 Proviral DNA Detection by Use of Real Time PCR from Blood Samples and Dried Blood Spots

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Abstract: The aim of this study is to evaluate the HIV-1 Infection Examination by using Real time PCR method from blood samples and Dried Blood Spots (DBS) in order to evaluate the efficiency and readiness of the method. The experimental complies with the requirements of the quality testing standard ISO 15189 which includes the following: comparing the infection examination results of HIV-1 between Real time PCR method and Multiplex Nested PCR method from the blood samples collected from children during clinical routines. Comparing the infection examination results of HIV-1 using Real time PCR method from dried blood spots which already had test results from the Multiplex Nested PCR method and improve DNA extraction by dissolving blood with 5% Chelex-100 as well as DNA extraction by dissolving blood with MagNa Pure Compact kit. Comparing the test results with DNA extraction using QIAamp and test to find the lowest amount of HIV-1 DNA in DBS. This extraction is done by using an improved method which is Real time PCR. By using the Real Time PCR method provided positive Ct criteria of ≤ 37, it was discovered that 22 samples tested positive and 436 tested negative, and is in compliance with the Multiplex Nested PCR Method. DNA from 140 Dried Blood Spot Samples, which were extracted using an improved method, provided a positive Ct criteria of ≤ 37. It was discovered that only 26 out of 40 samples (75%) tested positive. As for the negative group sample, it was discovered that 100 samples (100%) were tested negative. When the deciding Ct criteria was adjusted to ≤ 40, it was discovered that all 40 samples (100%) were positive and 100 samples (100%) were negative. When comparing the examination results of these samples, which were extracted with QIAamp DNA Mini Kit, were 100% match. In addition the limit of detection of the improved method for HIV-1 DNA is ≥ 250 copies/ml. These results show that the Infection Examination HIV-1 from blood by using Real time PCR provides results that were not different from the Multiplex Nested PCR. As for the Exanimation using DBS, the criteria for deciding positive results using Real time PCR does have an effect on the speed and accuracy of the examination. Due to the fact that the samples began with different amounts, using different criteria for positive results should be considered.

Keywords: HIV-1, DNA Extraction, Multiplex PCR, Real Time PCR

1. Introduction

After the cooperation of the Public Health networks of Thailand in implementing the project to aggressively end the transmission of HIV from mother to child and begin antiretroviral treatment for infected infants as soon as possible. WHO guidelines recommend to use the HIV proviral DNA PCR for the diagnosis of a suspected HIV infection in infants who are less than 2 years [1]. Indeed, these suspected groups in Thailand are focusing on less than 18 months of age [2]. Department of Medical Sciences was given the responsibility in checking for any infections among infants using the PCR

technique. The original project for developing and validation methods for HIV-1 DNA detection was conducted by researchers from the Clinical Research Center, Medical life Science Institute and they have continually improved their techniques in detecting and examining HIV-1 infections[3]. The institute used Real time PCR which can examine blood specimens as well as Dried Blood Spots (DBS) and had evaluated the efficiency of the method in cooperation with the multisite of the method validation composing of seven laboratories from different areas of Regional Medical Sciences Center, before bringing in the improved method in diagnosing for infection of HIV-1 in blood samples examined during medical routines.

Regional Medical Sciences Center 7, Khon Kaen, (RMSc 7) along with the Advanced Molecular Detection Laboratory(AMD Lab) was one of multi-validated sites, had joined this evaluation in order to improve the potential in examining HIV-1 infection by using Real time PCR both in Whole Blood Specimen and Dried Blood Spots. The evaluated experimental was conducted from October, 2013 to 2015 by comparing the original method, which was Multiplex Nested PCR to Real time PCR. It was discovered that Real time PCR only takes 3 hours compared to the usual 6 hours in order to conduct tests [3-5]. The method also reduces risks of any personnel coming in contact with dangerous chemicals by using Electrophoresis.

Apart from this, delivering the DBS samples is convenient for hospitals that are far away [6-7] and evaluating the improved method before putting it to use is also in compliance with the requirements of the quality standards ISO 15189: 2012 [8].

2. Materials and Methods

This research was a retrospective study which consists of 3 experimental steps as follows:

2.1. Evaluation of HIV-1 DNA Detection from Whole Blood by Using the Multiplex Nested PCR and Real Time PCR

Comparing the evaluation of the HIV-1 infection examination between Real time PCR and Multiplex Nested PCR from EDTA Blood of infants whose mothers are infected with HIV. Four hundred and fifty eight EDTA samples were collected from the AMD Lab's routine examination sent by hospitals in Health area 7 of Thailand from October 2012 to September 2015. As for DNA samples, 400 µl of each sample was pipetted into a 2.0 ml sample tube and taken into processes of the DNA extraction by using the MagNa Pure Compact nucleic acid isolation kit I with the automatic machine' program for DNA Blood (Roche Diagnostic GmbH Mannheim, Germany). For all DNA samples, HIV-1 proviral DNA were determined by using the routine method, Multiplex Nested PCR [1-2] as well as the improved method, Real time PCR method [3] which. Both the PCR and Real time PCR reagent kits were prepared by the Clinical Research Center, Medical life Science Institute, Department of Medical Sciences, Ministry of Public Health, Thailand. The target gene detection of the improved method of Real time PCR technique based on the study of Wei and colleagues in 2005 was composed of HIV Long Terminal Repeat (LTR) Gene Testing, and RNase P Gene Testing in order to control the quality of the samples.

The Primer and Probe that were used for testing HIV-LTR are LTR Primer-F; TGCTTAAGCCTCAATAAAGCTTGCCTTGA, LTR Primer-R; TCTGAGGGATCTCTAGTTACCAG, LTR Probe; FAM-AAGTAGTGTGTGCCCGTCTGT-BHQ1. As for the Primer and Probe that were used for testing RNase P are RNase P Primer-F; AGATTTGGACCTGCGAGCG, RNase P Primer-R; GAGCGGCTGTCTCCACAAGT, RNase P Probe; Hex-TTCTGACCTGAAGGCTCTGCGC-BHQ1. Real time PCR reactions was performed by using 7500 Real time PCR Systems (Applied Biosystems, USA). Every mixture volume of 20 µl per sample was composed of DNA extracted from sample 5 µl, 2x KAPA Master mix (KAPA Biosystems, USA) 10 µl, 10 µM LTR Primer-F 0.6 µl, 10 µM LTR Primer-R 1.0 µl, 10 µM LTR Probe 0.8 µl, 10 µM RNase P Primer-F 0.2 µl, 10 µM RNase P Primer-R 0.2 µl, 5 µM RNase P Probe 1.6 µl, DEPC-treated- water (Bioline Reagents Ltd, UK) 0.4 µl. The program for testing Real time PCR was the following: 50°C 2 minutes, 95°C 2 minutes, one round each, 95°C 15 minutes, 52°C 30 minutes with a total amount of 45 rounds by defining the fluorescent signal for every round at the temperature of 52°C. The test results of this sample group was compared with the Multiplex Nested PCR method which had already conducted its tests according to the standard operation procedure of RMSc 7, which also refer to standard method of the Clinical Research Center, Medical life Science Institute, and based on the study of Jan Albert and Eva Maria Fenyo in 1990.

2.2. Evaluation of HIV-1 DNA Detection from Dried Blood Spots by Using Real Time PCR

There are totally 140 EDTA blood samples in this experiment and these blood samples has been tested for HIV-1 DNA by using Multiplex Nested PCR method previously. Samples were divided into two groups: The first group has composed of 40 blood samples of HIV-1 DNA positive results collected from the clinical laboratory of Kalasin hospital, Kalasin Province, and another group has consisted of 100 blood samples of HIV-1 DNA negative results taken from the routine tests of AMD Lab. 100 µl whole blood aliquots from each sample were applied into a 2.0 ml sample collection tube. Then DNA extraction was performed by using the MagNa Pure Compact nucleic acid isolation kit I with the automatic DNA extraction machine using program DNA blood. These DNA samples were detected for HIV-1 DNA by the Real time PCR method. For DBS preparation, 50 µl of each sample was spotted onto a *903* filter paper card and did repeatedly to make six spots blood per card. The spots were allowed to dry at ambient temperature for more than 24 hours. 2 spots of each sample (total 100 µl) were cut with the sterile scissors and transferred to a 2 ml sterile tube with 600 µl of 5% Chelex-100 resin solution (BIO-RAD,USA) [9-10]. This solution was mixed thoroughly by using a vortex and incubated at 56°C of thermo-shaker for 20 minutes. After the solution tube was centrifuged at 2,500 rpm for 5 minutes, the dissolving blood

was transferred to a 2.0 ml sample collection tube. Then the lysate volume of 400 µl was used for DNA extraction by using the MagNa Pure Compact nucleic acid isolation kit I with the automatic DNA extraction machine using program DNA blood according to the manufacturer's instructions. All DNA samples were tested for HIV-1 infection using the Real time PCR method. Furthermore, each remained DBS of 140 samples was cut to make six spots by a 3-mm-diameter puncher and transferred to a 1.5-ml sample collection tube. These punched DBS were brought to DNA extraction processes of the commercial DNA extraction kit by use of the QIAamp DNA Mini Kit (QIAGEN, Germany) to be as the DNA Extracted comparison method from DBS according to the manufacturer's recommendations. Lastly all DNA Extracts were also tested for HIV-1 infection using the Real time PCR method.

2.3. Determination of the Lowest HIV-1 DNA Amount Tested Positive by Using Real Time PCR

Ten diluted series of HIV-1 DNA spiked samples were prepared by using a ratio of two-fold dilution of the 2 copies/µl of 8E5 cells (ATCC number CRL-8993) supported by the Clinical Research Center with the HIV non-infected blood collected from the routine blood samples of AMD Lab. These diluted samples were provided 300 µl blood aliquots for DBS preparing and their DNA extracts were performed by 5% Chelex-100 resin solution plus MagNa Pure Compact nucleic acid isolation kit I as well as use of the QIAamp DNA Mini Kit. Furthermore, 100 and 400 µl blood aliquots of each dilution were transferred to a 2.0 ml sample collection tube. Then each DNA extraction was performed by using the MagNa Pure Compact nucleic acid isolation kit I with the automatic DNA extraction machine using program DNA blood. All DNA extracts of HIV-1 DNA spiked samples were determined for HIV-1 DNA by using the Real time PCR method.

3. Results

3.1. Comparing Results of HIV-1 DNA Detection from Whole Blood

The HIV-1 infection test results from WB 458 samples using the Real time PCR method with positive deciding criteria of the cycle threshold (Ct) ≤ 37 lead to the discovery of 22 samples testing positive and 436 samples testing for negative. The results are correlated with all the samples which are indicated on table 1.

Table 1. *Comparing the test results of HIV-1 infection from blood using the Multiplex Nested PCR and Real time PCR methods.*

		Multiplex Nested PCR method		
		positive	**negative**	**Total (samples)**
Real time PCR method	positive	22	0	22
(Positive cutoff, Ct ≤ 37)	negative	0	436	436
	Total (samples)	22	436	458

There is no significant difference between Multiplex Nested PCR method and Real time PCR method
(χ^2 = 436.4, p< 0.001, kappa correlation = 100%)

3.2. Comparing Results of HIV-1 DNA Detection from DBS

140 DBS samples consisted of 40 positive samples and 100 negative samples, which each sample was extracted with 3 different methods to prepare as DNA samples. From all DNA extracts determined for HIV-1 infection by Real time PCR, the extraction was done through improved methods with positive deciding criteria Ct of ≤ 37. It was discovered that only 27 were tested positive from 40 samples. But when the positive deciding criteria were adjusted to Ct ≤ 40, it was discovered that all 40 samples tested positive. As for the tested results of 100 DNA samples of DBS which were prepared from blood samples and tested negative for HIV-1 infection using Multiplex Nested PCR, it was discovered that all 100 samples tested negative with the positive criteria of ≤ 37 and 40 as shown in Table 2a and 2b.

Table 2a. *Comparing the test results of HIV-1 infection using the Real time PCR method from DBS when the positive deciding criteria Ct ≤ 37.*

		specimens and extraction methods		
		Whole blood	**DBS**	
		MNP[a]	**5% Chelex-100 + MNP[a]**	**QIA[b]**
Real time PCR results	positive	40	26	26
(Positive cut off, Ct ≤ 37)	negative	100	114	114
	Total (samples)	140	140	140

[a] = MagNa Pure Compact nucleic acid isolation kit I, [b] = QIAamp DNA Mini Kit

There are some significant differences among specimens and extraction method when the positive samples decided criteria is Ct ≤ 37 (Cochran's Q = 28.0, p< 0.001)

Table 2b. *Comparing the test results of HIV-1 infection using the Real time PCR method from DBS when the positive deciding criteria Ct ≤ 40.*

		specimens and extraction methods		
		Whole blood	**DBS**	
		MNP[a]	**5% Chelex-100 + MNP[a]**	**QIA[b]**
Real time PCR results	positive	40	40	40
(Positive cut off, Ct ≤ 40)	negative	100	100	100
	Total (samples)	140	140	140

[a] = MagNa Pure Compact nucleic acid isolation kit I, [b] = QIAamp DNA Mini Kit

There are no significant differences among specimens and extraction methods when the positive samples decided criteria is Ct ≤ 40 (Cochran's Q = 0.00, p = 1.00)

When comparing the test results of all 140 DBS samples that were extracted with improved methods using QIAamp DNA mini Kit, all 140 samples were consistent. In addition, from comparing the average Ct values it was discovered that extracting using the MagNa Pure Compact nucleic acid isolation kit I, 5% Chelex-100 resin solution with the MagNa Pure Compact nucleic acid isolation kit I, and QIAamp DNA mini Kit, the average Ct values of LTR-HIV target were 33.7, 36.4, and 36.8 respectively and according to Figure 1a and 1b. For RNaseP gene, the average Ct values of each DNA extraction method were 20.9, 24.7, and 25.9 respectively and according to Figure 2a and 2b.

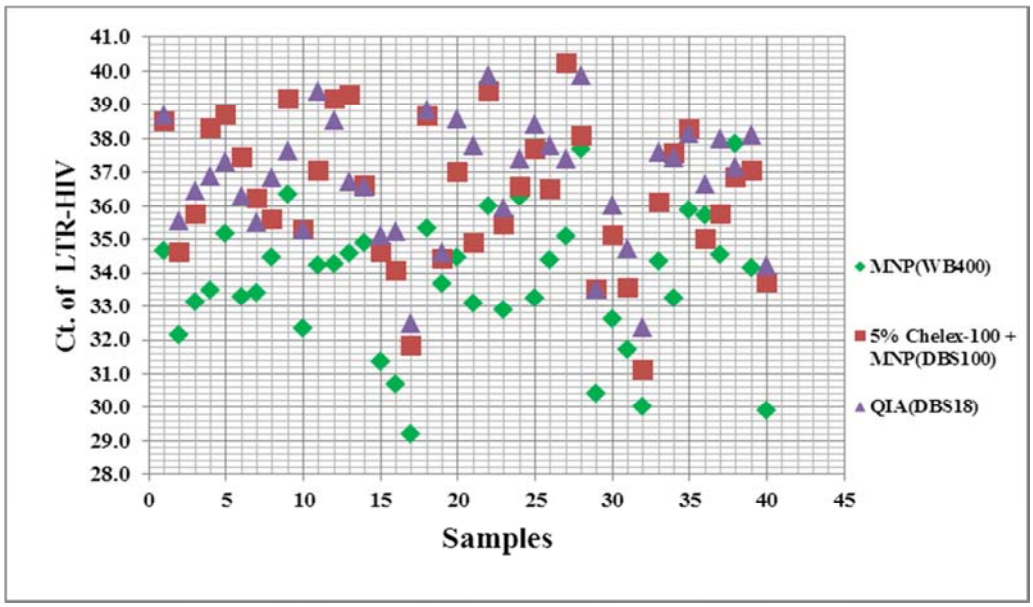

Figure 1a. *Comparing the Ct values of LTR-HIV gene among positive samples from whole blood and DBS which used different volumes.*

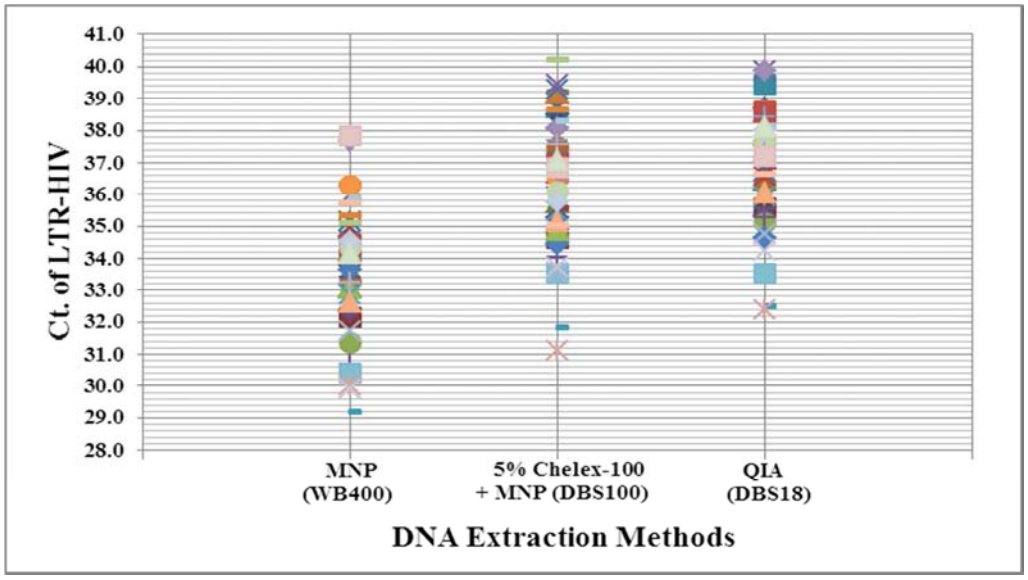

Figure 1b. *Comparing the Ct values of LTR-HIV gene among three different methods of DNA extractions from whole blood and DBS.*

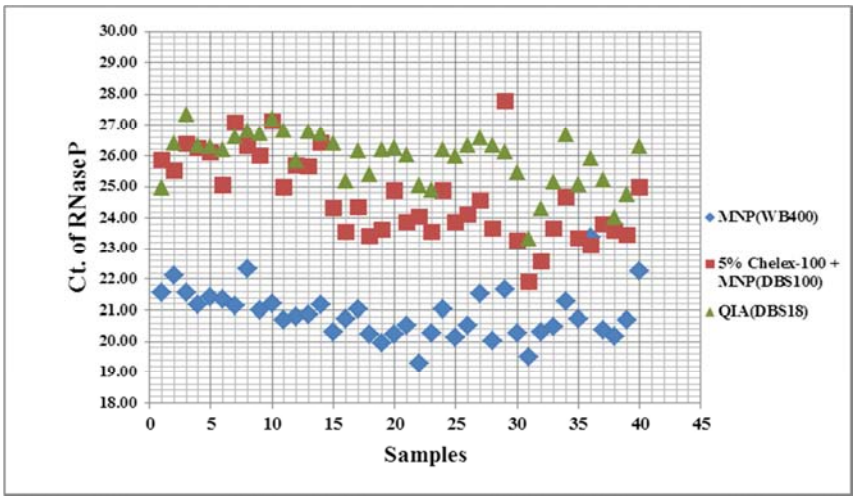

Figure 2a. *Comparing the Ct values of RNaseP gene among positive samples from whole blood and DBS which used different volumes.*

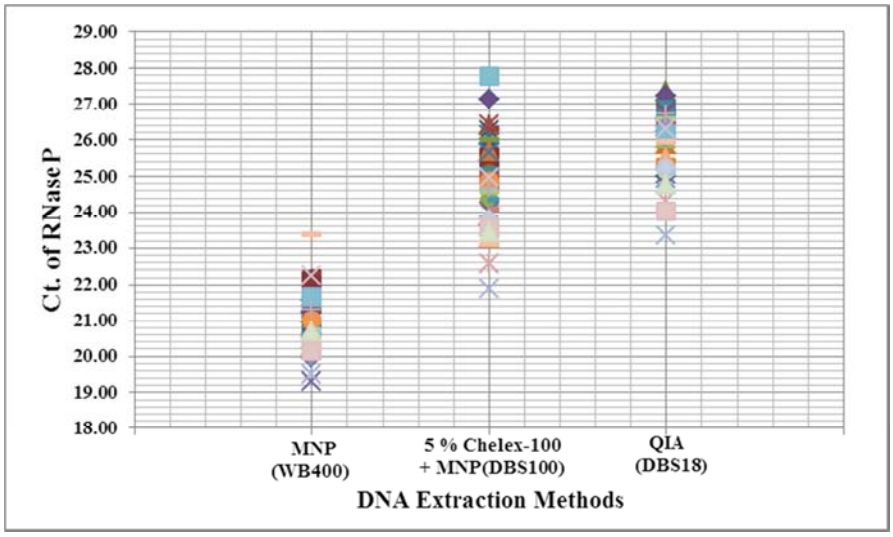

Figure 2b. *Comparing the Ct values of RNaseP gene among three different methods of DNA extractions from whole blood and DBS.*

3.3. Limit of Detection of HIV-1 DNA by Real Time PCR from DBS

As for the Limit of Detection (LOD) test from the improved DNA extraction method DBS, HIV-1 DNA was ≥ 250 copies/ml. For DNA extraction from DBS using the QIAamp DNA Mini Kit the LOD value was ≥ 500 copies/ml. For the DNA extraction using the MagNa Pure Compact nucleic acid isolation kit I, which all processes were run by the MagNa Pure machine with the volume of blood being 100 and 400 µl the LOD values were > 31 copies/ml and > 15 copies/ml as show on Table 3.

Table 3. *Test to find the Limit of Detection value of the Real time PCR method for detecting HIV-1 infection from blood and DBS.*

Samples)HIV-1; copies/ml)	Test Results of HIV-1 DNA detection by Real time PCR							
	MNP[a] (WB400)		MNP (WB100)		5% Chelex-100 + MNP(DBS)		QIA[b] (DBS)	
	Ct of RnaseP	Ct of LTR HIV-1	Ct of RnaseP	Ct of LTR HIV-1	Ct of RnaseP	Ct of LTR HIV-1	Ct of RnaseP	Ct of LTR HIV-1
2000	20.32	32.8	22.21	35.47	22.99	35.37	24.19	36.21
1000	21.04	33.57	23.15	34.47	23.67	35.5	24.19	37.94
500	20.42	33.81	22.71	36.83	23.52	36.67	24.35	38.49
250	20.79	36.37	23.75	38.08	23.86	37.75	25.08	Undet.
125	20.17	36.36	22.75	37.49	23.43	Undet.	25.25	Undet.
> 62	20.95	37.29	23.68	36.75	23.59	Undet.	25.06	Undet.
> 31	20.83	37.99	22.17	37.96	23.39	Undet.	25.09	Undet.
> 15	21.12	38.39	22.25	Undet.	23.75	Undet.	24.59	Undet.
> 7	20.99	Undet.[c]	22.38	Undet.	23.62	Undet.	25.08	Undet.
> 3	20.83	Undet.	22.39	Undet.	23.48	Undet.	25.3	Undet.

[a] = MagNa Pure Compact nucleic acid isolation kit I, [b] = QIAamp DNA Mini Kit, [c] = Undetermined

4. Discussion

With the test results of the HIV-1 infection from 458 EDTA blood samples using Real time PCR with the deciding positive criteria of Ct ≤ 37, it was discovered that the results were 100% consistent with the Multiplex Nested PCR examining method. Nevertheless, examining time for HIV-1 infection with Real time PCR only took 3 hours compared to Multiplex Nested PCR which took 6 hours. In addition, examining 140 DNA samples from DBS using the Real time PCR method which were extracted using improved methods, when the deciding positive criteria Ct was set at ≤ 37, it was discovered that there were only 75% that tested positive. When the deciding positive criteria were adjusted to Ct ≤ 40, it was discovered that all 40 samples (100%) were tested positive. As for examining the DNA samples of DBS that were prepared from 100 blood samples and tested negative for HIV-1 infection with the deciding positive criteria Ct ≤ 37 and 40, was discovered that all samples tested negative which is considered 100%. At times, comparing DBS test results of all 140 samples which were extracted using improved methods with QIAamp DNA Mini Kit, there was 100% consistency. And with the test results of finding the Limit of Detection (LOD) using improved elution method for DBS by 5% Chelex-100 resin solution and the MagNa Pure Compact nucleic acid isolation kit I as the DNA extraction method, it was discovered that the volume of HIV-1 DNA was ≥ 250 copies/ml. These results indicated the LOD of HIV-1 DNA was not only depended on the specimen types and extracted techniques, but also depended on the sample volume used for the DNA extraction [11].

5. Conclusions

The aforementioned evaluation indicated that examining for HIV-1 infection from blood samples with the Real time PCR method provides results that were not any different from the Multiplex Nested PCR method. But from examining DBS, the deciding positive criteria using Real time PCR had an effect on the sensitivity and specificity of detection. This was due to both of the volume of the samples beginning differently and the technical limitation of individual method. Therefore, there should be a consideration when using different deciding positive criteria, or the Ct value should be set at > 37 up to 40 and not be a phase for reporting results, but rather a phase for retesting. Lastly, there should also be a consideration for requesting the blood samples to be reexamined in order to confirm the said results.

List of Abbreviations

HIV-1; Human Immunodeficiency Virus Type-1, Ct; Cycle threshold, EDTA; Ethylene Diamine Tetra Acetic Acid, DBS; Dried Blood Spots, PCR; Polymerase Chain Reaction, *LTR-HIV*; Long Terminal Repeat genome of Human Immunodeficiency Virus Type-1. RNase P; Ribonuclease P, LOD; Limit of Detection, AMD Lab; Advanced Molecular Detection Laboratory, RMSc 7; Regional Medical Sciences 7.

References

[1] WHO. Early detection of HIV infection in infants and children. Guidance note on the selection of technology for the ealy diagnosis of HIV in infants and children. WHO, Geneva, Switzerland, 2007. Available online at http://www.who.int/hiv/paediatric/EarlydiagnostictestingforHIVVer_Final_May07.pdf

[2] Department of Disease Control. Thailand National Guidelines on HIV/AIDS Treatment and Prevention 2014. Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand. Available online at http://www.silomclinic.in.th/file/2014ThailandHIVguidelines.pdf.

[3] Warachit P, Wongsiree S, Sanguanwong S, Ruchusatwat N, Uneklabh C. Detection for Proviral DNA of HIV-1 by Nested PCR. Bulltin of the Department of Medical Sciences.1994; 36: 11-18.

[4] Jan A, Eva MF. Simple, sensitive and specific detection of Human Immunodeficiency Virus Type 1 in clinical specimens by Polymerase Chain Reaction with Nested Primers. J Clin Microbiol. 1990; 28: 1560-1564.

[5] Wei L, Hua Y, Kimberly R, Chou-Pong Pau and Chin-Yih Ou. Detection of Human Immunodeficiency Virus type 1 DNA in dried blood spots by a Duplex Real-Time PCR assay. J Clin Microbiol. 2005; 43(4): 1851-1857.

[6] Sarah ML, Anne BM, Caroline CC, Anangisye IM, Sally E, Ben A, David JS, Lorenz VS, Werner S, Wendy SS, John AB, John AC. Evaluation of a dried blood spot HIV-1 RNA program for early infant diagnosis and viral load monitoring at rural and remote health care facilities. *AIDS*. 2009; 23(18): 2459-2466.

[7] Sujit RJ, Douglas HY, Sally Mm, David MK. Rapid, point-of-care extraction of Human Immunodeficiency Virus Type-1 proviral DNA from whole blood for detection by Real-Time PCR. J Clin Microbiol. 2009; 47(8): 2363-2368.

[8] International Laboratory Accreditation Cooperation (ILAC). ISO 15189 Medical Laboratory accreditation. The ILAC Secretariat, NSW 2128, Australia. 2011. Available online at https://www.ukas.com/services/accreditation-services/medical-laboratory-accreditation-iso-15189/

[9] Busarawan S, Nuanchawee W, Supaporn C, Noppavan J, Wiyada C. A study to implement early diagnosis of HIV infection in infants born to infected mothers. Southeast Asian Journal of Tropical Medicine and Public Health. 2003; 34(3): 221-226.

[10] Fischer A, Lejczak C, Lambert C, Servais J, Makombe N, Rusine J, Staub T, Hemmer R, Schneider F, Schmit JC, Arendt V. Simple DNA extraction method for dried blood spots and comparison of two PCR assays for diagnosis of vertical Human Immunodeficiency Virus type 1 transmission in Rwanda. Journal of Clinical Microbiology. 2004; 42(1): 16-20.

[11] Sally MM, Robin LW, Sujit RJ, Douglas HY, Diana H, David MK. A simple and rapid DNA extraction method from whole blood for highly sensitive detection and quantitation of HIV-1 proviral DNA by real-time PCR. Journal of Viral Methods. 2015; 214: 37-42.