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Full Length Research Paper

Contribution to the early diagnosis of HIV in children born to HIV-positive mothers in Bobo Dioulasso: Real-time PCR and quantitative p-24 assay

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Objective: To evaluate the diagnostic performance of real-time PCR and p24 antigen with children born to HIV-positive mothers. Method: It is about a prospective longitudinal study over a period of 14 months. PCR RNA of HIV-1 (HIV Charge Kit Generic Virale® ANRS G2) and P24 antigenemia (Biomerieux ®) were performed on blood samples of children from 6 weeks to 18 months. Children with viral load below 300 copies / ml (> 2.48 Log) and a title Ag P 24 less than 3 pg / ml were considered uncontaminated. All children were followed clinically and biologically up to 18 months when two serological tests (Determine® and Immunocoombs®) were performed on them for confirmation. Results: Were included 214 children born to HIV-positive mothers. The average age of children was 6 months (1.5 -18 months). The sex ratio was 0.79. The infection rate was 11.2% (24/214). The outcomes of the methods are given below: sensitivity and specificity were respectively 95.8% and 100% for PCR-RNA real-time and 83.3% and 98.4% for the quantitative p24 antigen. The correlation coefficient was r = 0 .37 which shows that there was not a good correlation between the two techniques. Conclusion: Both virologic and immunological tests have proven usefulness to the early diagnosis of HIV infection with children born to HIV infected mothers. Because of the low sensitivity of the P-24 assay in the early diagnosis of HIV infection with children born to HIV infected mothers, a popularization of this test in resource limited areas is not advised.

Key words: HIV, early diagnosis, PCR-RNA, P-24 assay, Burkina Faso.

INTRODUCTION

HIV is a retrovirus infecting humans and causes AIDS. AIDS is the final stage of HIV infection. AIDS remains a major global health priority. The number of people living with HIV worldwide has continued to increase in 2009, reaching a total of 33.3 million (Avettand-Fenoel et al., 2009). Of the thirty three (33) million people infected worldwide about nine (09) million are children and Africa is the biggest victim. Preventive measures already exist through prevention programs of Mother to Child Transmission (PMTCT). Despite these measures, 10 to 30% of children born to HIV positive mothers in developing countries are themselves infected and must be diagnosed during the perinatal period (Tersmette, 1989). Early molecular diagnosis of HIV with children born to HIV positive mothers confers enormous benefits to their families and to the PMTCT program for early confirmation of infection and initiation of ARV treatment that would reduce morbidity and HIV-related mortality. However, the immediate postnatal laboratory diagnosis of HIV is a major concern in Burkina Faso. Indeed, if rapid diagnostic tests are routinely used with adults, their performance is compromised in children born to HIV-positive mothers due

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to the presence in the serum of the newborn of maternal IgG that may result in the diagnose of "false positives" before 18 months. Very early diagnostic efficient tools have been developed in the world for this purpose, it is PCR, the viral culture, the quantitative research of ultrasensitive p24 Ag. Rouet in Côte d'Ivoire reported that the real-time PCR was good sensitivity and specificity in early laboratory diagnosis of HIV infection with children born to HIV positive mothers (Rouet et al., 2005). Thus, WHO recommends confirmation of laboratory diagnosis of HIV infection with vulnerable children aged 4-6 weeks. Otherwise, an immediate ARV treatment should be performed while waiting for the 18th month confirmation (WHO, 2009).

Unfortunately this confirmation through viral culture and PCR is less available in developing countries laboratories; so some children die or are declared lost before they are 18 months, thus the search for early, reliable and efficient alternative test is necessary. It is in this context that this work evaluates the diagnostic performance of real-time PCR and p-24 assay with children born to HIV-positive mothers.

METHODOLOGY

It is about a prospective longitudinal study over a period of 14 months. The recruitment and clinical monitoring of our patients was done in the Pediatrics Department of Centre Hospitalier Universitaire Souro SANOU (CHUSS). The biological investigations were performed in the laboratory of CHUSS and the real-time PCR HIV-1 RNA was done in the Virology Laboratory of Centre Muraz, Bobo-Dioulasso.

The inclusion criteria were:

- be aged 6 weeks-18 months
-be born to HIV1 positive mothers under antiretroviral or not
-accept to be involved in the study

Sample Preparation

HIV RNA PCR (Kit Biocentric ®) and P24 antigenemia (Mini VIDAS ®) were performed on blood samples of 5 ml of children aged 6 weeks to 18 months at the crease of the elbow or on the heel per age. Serology at 18 months was chosen as the gold standard from the specimen. It was conducted using immunochromatography (Determine®) test and ELISA (Immunocoombs ®).

Analysis of samples in laboratory

Enumeration of CD4 + T lymphocytes

The analysis were performed on a flow cytometer (FacscountV1.5) from whole blood collected by venipuncture into tubes containing EDTA (ethylene diamine tetra acetic) as anticoagulant.

Measurement of plasma HIV-1 RNA plasma

We used an automated test RT-real time PCR (ANRS G2) applied to the quantification of plasma HIV-1 RNA. It also makes possible the diagnosis of HIV-1. The amplified target region was represented by the LTR gene. The plasma volume required was 200 uL. The RNA extraction was done manually using a QIAgen (viral RNA minikit Qiamp, QIAgen, Bandol, France) procedure. The sequences (A) and (B) were respectively as follows: 5'-GCCTCAATAAAGCTTGCCTTGA 3'et5'GGCGCCACTGCTGAGATTTT-3'. The internal probe hydrolysis TaqMan had the following sequence: 5'AAGTAGTGTGTGCCCGTCTGTTRTKTGACT3.

'This probe was fluorochromes: 5 'reporter (FAM) and 3' quencher (TAMRA). We performed all manipulations in a total volume of 25µL containing the RNA extract (10 ul) and (15µL) Mix: primers A and B (volume 1µL of each), probe C (volume 1µL) The 1X PCR buffer and RNase inhibitor mix MultiScribe 1X RT (one- step TaqMan RT-PCR Master Mix) (Applied Biosystems, Forster City, USA) 12µl. The controller to do this PCR test was the ABI. Applied Biosystems PRISM 7000 using a 96-well format. Children with viral load below 300 copies / mL (> 2.48 log) were considered uncontaminated.

Quantitative research of p24 antigen by the ELFA method.

We apportioned 200µL plasma of each child into the sample pit of the cartridges. All steps were then handled automatically by the tool mini VIDAS. The results were obtained in 1h 30mn and expressed in pg / mL Agp24.The threshold values recommended by the supplier of reagents for the interpretation of p24 bioclinical titles are:

<3.0 pg / ml of p24 Ag: negative;
≥ 3.0 pg / ml and <5.0 Pg / ml p24: equivocal results;
≥ 5.0 pg / ml p24: Positive resulting output.

All samples giving positive results were confirmed by neutralization with anti-p24 goat serum (VIDAS HIV p24 confirmation II) compared to the same sample incubated with goat serum devoid of anti-p24 antibodies. An applied formula was used to define the percentage of neutralization:

$$R = \frac{R2 - R1}{R2 - C2} \times 100$$

A: Percent neutralization.
C2: negative control VIDAS HIV P24 II cabinet.
R1: RFV value obtained with the R1 identified cartridge.
R2: RFV value obtained with the identified cartridge R2.

All values of R ≥ 60% confirmed the presence of p24 and those <60% did not confirm the presence of Agp24. Also, samples giving an equivocal result were retested. If the second result was equivocal or positive, the sample was confirmed by neutralization.

The samples for which the test was greater than 400 pg / mL p24 were diluted 1/10 with the negative control C2 cabinet VIDAS HIV p24 II (ref. 30117) and then tested again with the same case.

Statistical analyzes of the data

Processing and data analysis were done using EPI-INFO V15 software. 01, Excel 2007 software for tables and graphs. The Wilcoxon test was used for comparison of rates. The percentages were compared using the Fisher test (t). Non-parametric tests (Kruskal Wallis and Mann-withney) were used when the distribution was not normal. Results were considered statistically significant when p values < 0.05.

Ethics and consent

This study complied with the recommendations on Ethics and Good Practice in Epidemiology adopted in 1999 by the Association of Epidemiologists French Language [ADELF 1998]. Furthermore, it was performed according to the protocol and recommendations of Good Practices and biomedical research laboratory and meets regulatory requirements in Burkina Faso; free and informed consent of the legal guardian was obtained before involvement.

RESULT

Sample Characteristics

During the study period, 214 mother-infant pairs were included. HIV status of 24 children were confirmed positive, 191 children were negative at 18 months of life and four dead children. The gynecological obstetric and socio-demographic characteristics were noticed in 208 HIV-positive women out of 214. The six serum samples from children born to non selected HIV-positive mothers were obtained without precise data in our study.
Female gender was the most represented with a sex ratio of 0.79 (95/119). The average duration for starting treatment of mothers was 3 ± 1 year.

The infection rate

The HIV status of the children was confirmed at 18 months. The results shown in Table I show that the transmission of HIV infection was not significantly related to child's sex (Chi2 = 1.89, p = 0.7).

Early immunological diagnosis

The proportions of infected children were inversely related to CD4 count. The infection rate decreases when the CD4 count is high. Table II shows the distribution of infected children based on immunological stage.
Yate corrected Khi2 = 175.72; the Relative Risk = 96.0 (24.18-381.03) Fischer's exact test p = 0.0.

The viral load of infected children

The average viral load of infected children under 18 months was 5.86 Log. Table III reports the distribution of viral load of infected children under 18 month.

The performance of diagnostic methods

The sensitivity and specificity were respectively 95.83% and 100% for the real-time PCR by relating the number of positive serological tests at 18 months. Table IV shows the sensitivity and specificity of early diagnosis methods in case of antiretroviral treatment of mother and child.
Table VI reports the sensitivity and specificity of p24 antigenemia to more than 6 months.The sensitivity and specificity of more than 6 months were 62.5% and 98.95% respectively for the quantitative research of p24 Ag and 100% for real-time PCR.
Diagnostic Performance of P24 antigenemia
The method of p24 antigenemia gave 1.4% (3/214) false positive and 0.9% false negative.

DISCUSSION

The overall infection rate was 11.2%. This result is higher than that reported in Uganda Kekitiinwa: 6.8% (Kekitiinwa, 2008). This high rate in our sample would be justified by the difficulties of coordination between PMTCT services and early management of antenatal HIV / AIDS infection.
The prevalence of infection among female subjects we report (14.3% vs 7.4%) is shared by several authors who would mention a female predominance in their series (Kekitiinwa, 2008; Taha et al., 2005). However, the pathophysiological mechanisms remain poorly understood. It appears from our study that an average rate of CD4 is higher among HIV-positive mothers and children than those of HIV-negative children respectively 623 ± 234,7cellules / microL and 341 ± 112.4 cells / microL. These apparently conflicting results, especially when we know that the risk of mother to child transmission is associated with a high viremia with a low CD4 count; but is explained by the initiation of ART in these mothers with an average CD4 341 ± 112.4. Indeed according to WHO recommendations in Casablanca (Morocco, 2010), these infected parturients were eligible for triple therapy throughout pregnancy and should be continued after delivery. Thus, this treatment has helped reduce mother to child transmission in this group of HIV positive women.

Early immunological diagnosis

The average rate of CD4 T lymphocytes of the children was 30.7% .That of infected and uninfected children was 9.5% and 36.9% respectively. It is clear from our work that the best threshold to differentiate children infected and not infected with HIV was 15% of CD4 + T lymphocytes. These results are shared by Rouet in Côte d'Ivoire (Rouet, 2006) who reported an average rate of CD4 + T lymphocytes of 17.7% and 34.8% respectively for the infected

Table I. HIV infection rates per gender with children.

		Serological results		
		Positive	**Negative**	
Sex	Female	17 (14.3%)	102 (88.7%)	119 (100%)
	Male	7 (7.4%)	88 (92.6%)	95 (100%)
Total		24 (11.2%)	190 (88.8%)	214 (100%)

Table II. Distribution of infected children based on immunological stage.

CD4 in %(n =208)	**Size**	**Infected children**	**Percentage**
≥25	182	2	1.1%
15 to 24	8	4	50%
<15	18	18	100%
Total	208	24	

Table III. Viral load distribution of infected children under 18 month.

Viral load	**Size**	**Percentage**
>6Log	12	52.2%
4 Log to 6Log	10	43.5%
< 4 Log	1	4.3%
Total	23	100%

and uninfected children and a sensitivity and specificity compared with the real-time PCR respectively 87.1% and 78.3%. This performance is less sensitive and specific, but laboratories in developing countries without molecular testing could use the measure of the rate of CD4 + T lymphocytes as an early diagnostic marker of HIV infection in children born to HIV positive mothers, Pending confirmation by molecular tests.

The viral load of infected children

In our study, the majority of infected children (52.2%) had a high viral load greater than 1 million copies / mL or 6 log copies / mL. Burgard in France reported among 29% of infected children a viral load below 1000 copies / mL or 3 log copies / mL. This disparity is due to early detection of infected mothers followed by early treatment initiation in the series of Burgard (Burgard, 2005).

Performance of diagnostic methods

Serological results at 18 months were our gold standard. About the technique for early diagnosis of HIV-1 infection with children born to HIV-positive mother the real-time PCR HIV-1 RNA had a sensitivity and specificity generally satisfactory with respective rates of 95.83% and

Table IV. Sensitivity and specificity of methods for early diagnosis in case of antiretroviral treatment of mother and child.

Test	Sensitivity (N=24) n/N (%)*	Specificity (N=190) (n/N) ‡
PCR in real time	23/24(95.83%)	190/190(100%)
Ag P24	20/24(83.33%)	187/190(98.42%)
Lymphatic classification (CD 4+)	21/24 (87.5%)	189/190(99.47%)

*Number of Ag P24 tests or positive PCR / number of HIV positive tests at 18 months.
‡ Number of Ag P24 tests or negative PCR / number of serological negative tests at 18 months

To improve the diagnostic performance by targeting the most favorable time to affirm the HIV status of children born to HIV-positive mother, we compared the tests by dividing the children into two groups: group of children from 6 weeks to 6 months and that of children aged 6 months and older. Table V tells us the sensitivity and specificity of the two methods of diagnosis between 6 weeks and 6 months.

Table V. Sensitivity and specificity of diagnostic methods between 6 weeks and 6 months.

Test	Sensitivity n/N (%)*	Specificity (n/N) ‡
PCR in real time	15/16(93.75%)	189/189 (100%)
Ag P24	14/16 (87.5%)	189/189(100%)

100%. On the contrary the research of Ag P24 had respective rates of sensitivity and specificity of 83.33% and 98.42%, and lymphocyte counts gave respectively a sensitivity and specificity rates of 87.5% and 99.47%.

If the research performance of Ag P24 in our study are lower than those from Beats in the Democratic Republic of Congo (De Baets et al., 2005) who reported respective rates of 92.3% and 100% for sensitivity and specificity, they are similar to those found by Panakitsuwan Thailand who reported respectively a sensitivity and a specificity rate of 87.8% and 100% (Panakitsuwan et al., 1997). Before 6 months of life, sensitivity and specificity of the tests were:

- For real-time PCR HIV-1 RNA (ANRS G2) 93.75% and 100% respectively;
- For the research of Ag P24 87.5% and 100% respectively.

Between 6 and 18 months of life, we found in our study a very good sensitivity and specificity of the real-time PCR (100%) and low sensitivity of the search technique of Ag P24 (62.5%) with a good specificity: 98.95%. This decrease in the sensitivity of the search of P24 antigenemia after 6 months was reported by several authors such as Zijenah in Zimbabwe who reported with children before 6 months of life a sensitivity and specificity rate of 98% and 90% respectively and between 6 and 18 months respective rates of 97% and 91% (Zijenah et al., 2005) our results are corroborated by Sherman in South Africa (Sherman et al., 2004). This decrease in the sensitivity of the search for antigenemia with age could be explained by the fact that the Ag P24 is found in pre-signing serological early phase of viral replication phase of the waning of the infection and its disappearance in a few months to reappear only in case

Table V: Sensitivity and specificity of diagnostic methods between 6 weeks and 6 months.

Test	Sensitivity n/N (%)*	Specificity (n/N) ‡
PCR in real time	15/16(93.75%)	189/189 (100%)
Ag P24	14/16 (87.5%)	189/189(100%)

of decrease in antibody due to immunosuppression induced by HIV. So the AgP24 test done during the first months of life allows early diagnosis of HIV infection with children born to HIV positive mothers in resource-limited areas. Regarding our real-time PCR, little work has been devoted to it; but if the RT-PCR tests in real time are singled, they will have similar or better performance in comparison with those marketed by companies (Abbott, Biomerieux and Roche). Our results for the real-time PCR HIV-1 RNA are also reported by Rouet in Abidjan (Rouet, 2007; Rouet et al., 2007) even if it is in its series sensitivity and specificity of 100%, whatever the age group. From what emerges from our results a single real-time PCR test is not sufficient for the early diagnosis of HIV infection with children born to HIV positive mothers, but repeated at least twice, it would be an efficient and reliable technique that is to say sensitive and reproducible for the diagnosis of infection with the newborn.

Diagnostic difficulties

Of the 214 reviews, the real-time PCR RNA gave one false negative (1/214) and no false positive. The search for AgP24 gave 3 false negatives (3/214) and 2 false positives (2/214). This false negative for PCR could be explained by the impact of genetic diversity. The very rapid change and continuous sequences of the viral genome HIV-1 requires continuous monitoring of the reliability of the quantification results of viral RNA load plasma HIV-1 compared to the multiplicity of recombination points and thus to the emergence of complex recombinant viruses. It could be an RNA extraction error of the virus due to the technician because we used a manual extraction method according to QIAGEN procedure. The P24 technique is free from the sensitivity to genetic diversity of HIV-1 that can impact the performance of molecular tests. However the quality of search results of Ag P24 may vary from one subtype to another according to some authors, due to the quantification of the epitopes of some subtypes. Indeed, the majority of studies using this test were performed with samples containing only subtype B. Studies from samples containing non-B strains specific to our own country are few and are the subject of controversial results. Burgisses reported lower correlation coefficients for the non-B strains as compared to the B strains (Bürgisser et al., 2000). Some authors (Maniez 1997; Janin et al., 2006) have speculated that several non-B epitopes Ag P24 may be poorly recognized by the anti-P24-Ac used in the kit.

Other authors (Tersmette, 1989; Bürgisser et al., 2000; Scarlatti, 1991) conversely showed through 18 samples of subjects of Malawi (subtype C) and a panel represented by subtypes A to F that the different epitopes were well recognized and quantified by the Ag P24 test.

CONCLUSION

Both virologic and immunological tests have proven usefulness to the early diagnosis of HIV infection with children born to HIV infected mothers. However, each test has shown its limits. Because of the low sensitivity of the P-24 assay in the early diagnosis of HIV infection with children born to HIV infected mothers, a popularization of this test in resource limited areas is not advised. It would be appropriate to direct researches towards other types of early diagnosis of HIV infection with children born to HIV infected mothers.

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Conflict of interest:

Authors declared no conflict of interest opposing them.

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