RT-PCR DETECTION OF HIV IN REPUBLIC OF MACEDONIA

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Abstract

The aim of the study was to detect HIV RNA in seropositive patients using RT-PCR method and thus, to establish PCR methodology in the routine laboratory works.
The total of 33 examined persons were divided in two groups: 1) 13 persons seropositive for HIV; and 2) 20 healthy persons - randomly selected blood donors that made the case control group. The subjects age was between 25 and 52 years (average 38,5).
ELFA test for combined detection of HIV p24 antigen and anti HIV-1+2 IgG and ELISA test for detection of antibodies against HIV-1 and HIV-2, were performed for each examined person. RNA from the whole blood was extracted using a commercial kit based on salt precipitation. Detection of HIV RNA was performed using RT-PCR kit. Following nested PCR, the product was separated by electrophoresis in 1,5 % agarose gel. The result was scored positive if the band of 210bp was visible regardless of intensity. Measures of precaution were taken during all the steps of the work and HIV infected materials were disposed of accordingly.
In the group of blood donors ELFA, ELISA and RT-PCR were negative. Assuming that prevalence of HIV infection is zero, the clinical specificity of RT-PCR is 100 %. The analytical specificity of RT-PCR method was tested against *Hepatitis C and B, Human Papiloma Virus, Cytomegalovirus, Herpes Simplex Virus, Rubella Virus, Mycobacterium tuberculosis, Chlamydia trachomatis*. None of these templates yielded amplicon. In the group of 13 seropositive persons, 33 samples were analyzed. HIV RNA was detected in 15 samples. ELISA and ELFA test were positive in all samples. Different aliquots of the samples were tested independently and showed the same results. After different periods of storing the RNA samples at –70°C, RT-PCR reaction was identical to the one performed initially. The obtained amplicons were maintained frozen at –20°C for a week and the subsequently performed electrophoresis was identical to the previous one. The reaction is fast, simple for manipulation; with low detection level of 60 IU/ml. RT-PCR needs a small amount of RNA, as well as a small volume of sample. HIV RNA was detected in different periods of time with different clinical presentations in patients, with or without antiretroviral therapy.
RT-PCR method gives the opportunity for reliable determination of HIV-1 RNA with border of detection of 60 IU/ml. The test is reproducible and has high analytical and clinical specificity.

KEY WORDS: HIV, RT-PCR

Introduction

Three crucial questions regarding HIV diagnostics have been addressed in medicine worldwide over the last years: whether the subject was infected, how active HIV replication was, and how sensitive to retroviral therapy the virus was? Detection of virus genome made by *in situ* hybridization or Polymerase Chain Reaction (PCR) is an attempt to provide answers to all of the three questions. Two methods for detection of HIV RNA are available: Target amplification with modalities of Reverse transcriptase (RT) PCR and Nucleic acid sequence-based amplification assay (NASBA), and Signal amplification, as branch DNA test (1-10). Clinical relevance of those assays dependens on their reproducibility and amplification dynamics for different genotypes. Some studies addressed comparisons of the results obtained from different assay systems (11-15). The aims of the study are to introduce PCR methodology in HIV diagnostics in our country and to detect HIV RNA with nested RT-PCR in seropositive subjects in order to evaluate analytical specificity and reproducibility of this method.

Patients and Methods

The case control study involved 33 subjects, divided in two groups:
a) HIV seropositive subjects (13 patients), and
b) Sedentary healthy subjects (20 blood donors).

Seropositive patients, according to the HIV transmission, were divided in three groups: heterosexuals (10 patients); intravenous drug users (1 patient), and homosexuals (2 patients). 10 of seropositive patients were men and 3 women, with mean age of 38,5 years (range from 25 to 52 years old). The mean age of control group was 40,5 years (range from 19 to 62 years old). In all patients, the following tests were done: ELFA test (BioMerieux - HIV duo) for combined detection of HIV-1 p24 and anti-HIV-1 and anti-HIV-2 IgG and ELISA (NeoDin ™) for detection of HIV-1 and HIV-2 antibodies. Examined material was whole blood, taken in 10 ml Vacutainer tube with EDTA as anticoagulant. RNA was isolated from the plasma sample using Purescript RNA Isolation kit (Gentra systems). The procedure is based on salt precipitation with highly effective inhibitors of RNA activity which allows isolation of RNA from cells and body fluids. The first step is cell lysis using anionic detergent. DNA and proteins are separated with salt precipitation. RNA is isolated with alcohol precipitation and dissolved in RNAse free water. After the isolation RNA yield was verified in 1,5% agarose gel (Figure 1). HIV detection was performed with qualitative RT-PCR in one step with NeoDin kit. The kit consists of: 1) RT–PCR mix of: four nucleotides (dNTPs) - dATP–deoxyadenine triphosphate; dGTP–deoxyguanine triphosphate; dTTP–deoxythymine triphosphate; dCTP–deoxycytosine triphosphate; 10 X reaction buffer (Tris – HCl, KCl and $MgCl_2$); dithiotreitol (DTT); 5' and 3' primers designed for reverse transcriptase gene detection and deionized water. The same primers are used in reverse transcription and first amplification of cDNA. 2) RT–Enzyme 1 – with a function of reverse transcriptase 3) RT–Enzyme 2 – with a polymerase activity,

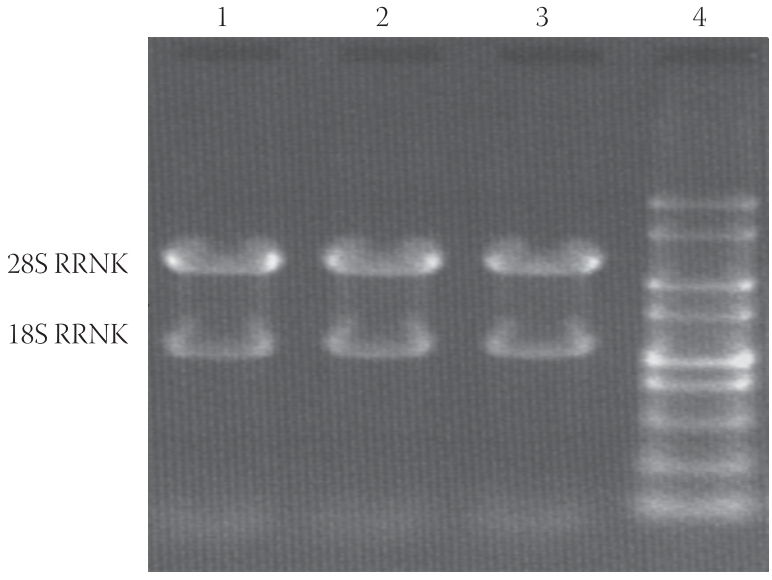

FIGURE 1. RNA estimation using agar electrophoresis: lane 1–ladder; lanes 2–4 RNA samples.

4) PCR mix for nested PCR of: four dNTPs, 10 X reaction buffer, primers for nested amplification, cresol red, glycerol and deionizated water; 5) PCR Enzyme – with polymerase activity; 6) Positive control; 7) Mineral oil. Deoxyuracil triphosphate (dUTP) and uracil-N-glycosylase are added to the reaction (11-15).

The method is based on HIV RNA transcription into complementary DNA copy (cDNA). Amplification was conducted under following conditions: for reverse transcription 3 min at 57°C, 30 min at 42°C, 3 min. at 95°C- one cycle; and for amplification 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C –35 cycles and one cycle at 72°C for 5 min. The conditions of the nested PCR were: 5 min at 95°C one cycle; 30 sec at 94°C, 30 sec at 68°C, 30 sec at 72°C for 30 cycles; and one cycle at 72°C for 5 min (16-19). PTC-100™ Thermal Cycler (MJ Research, inc.) was used. PCR products were separated using 1,5% agarose gel electrophoresis. Ethidium bromide was added (22). UV transiluminator was used for visualization of the bands. The result was scored positive if band of 210 bp was present regardless of intensity. The procedures were performed in Virology Laboratory in Republic Institute for Health Protection, Skopje, Macedonia, with biological protection class II. One-way process (pre-amplification to post-amplification) was used.

Results

HIV RNA was not detected in any of the case control patients: examples were not reactive to RT PCR kit. Supposing that prevalence of HIV-1 infection in seronegative blood donors is zero, we obtained test specificity 100%. To estimate analytical specificity of the test, we attempted amplification of nucleic acids pf *Hepatitis C* and *B, Human Papiloma Virus, Cytomegalovirus, Herpes Simplex, Rubella, Mycobacterium tuberculosis, Chlamydia trachomatis*. All the test reactions were negative. HIV RNA was detected in 15 samples (Table 1).

Patient	1 analysis	2 analysis	3 analysis	4 analysis	5 analysis	6 analysis	7 analysis
1	+	-					
2	+	-	+	-	-	+	-
3	+						
4	+	-	-	+	-	-	+
5	+	+	+				
6	-	+	-				
7	-	-					
8	+	-	-				
9	-						
10	+						
11	-						
12	-						
13	+						

Legend: (+) = present, (-) = absent.

TABLE 1. Presence of HIV RNA in patients

In 13 patients, ELFA HIV duo was also positive for anti-HIV IgG and p24 antigen. ELISA test was positive which indicated presence of HIV antibodies (Table 2). In 8 patients HIV RNA was detected during the first visit. Six patients came to see the doctor only once. HIV RNA was detected in three of them. Patient No.7 showed undetectable level of HIV RNA in two tests. In patient No.1 HIV RNA was detected during the first visit, but it was undetectable during the second one. One patient was identifi ed as HIV infected based on the presence of HIV antibodies. Infection was confirmed by HIV RNA detection in three consecutive tests. In patients No. 2 and 4 fluctuations in HIV RNA test results were detected following seven series of tests. In order to estimate reproducibility, different aliquots of the samples were tested independently. All of them showed the same results. After different periods of storage at -70°C, 13 samples were resubmitted to reverse transcription and amplification. In all samples the results were identical to the first round of test. Also, amplicons

Patient	1 analysis	2 analysis	3 analysis	4 analysis	5 analysis	6 analysis	7 analysis
1	3,218 (12,43)	3,339 (21,50)					
2	2,985 (15,06)	3,017 (16,32)	3,295 (20,78)	3,122 (18,04)	3,200 (15,24)	3,917 (15,27)	3,401 (16,17)
3	3,394 (18,38)						
4	3,105 (15,18)	2,989 (23,03)	3,095 (13,23)	3,046 (11,39)	3,897 (19,44)	3,748 (16,42)	3,900 (20,71)
5	3,804 (24,01)	3,350 (24,96)	3,302 (16,08)				
6	3,366 (13,76)	3,608 (15,51)	3,539 (15,39)				
7	2,974 (24,73)	3,082 (14,02)					
8	3,339 (19,74)	3,097 (23,88)	3,218 (15,24)				
9	3,699 (15,93)						
10	3,673 (16,99)						
11	3,870 (20,80)						
12	2,719 (20,53)						
13	3,910 (20,50)						

Legend: ELFA test results are in brackets

TABLE 2. Results of ELISA and ELFA duo tests

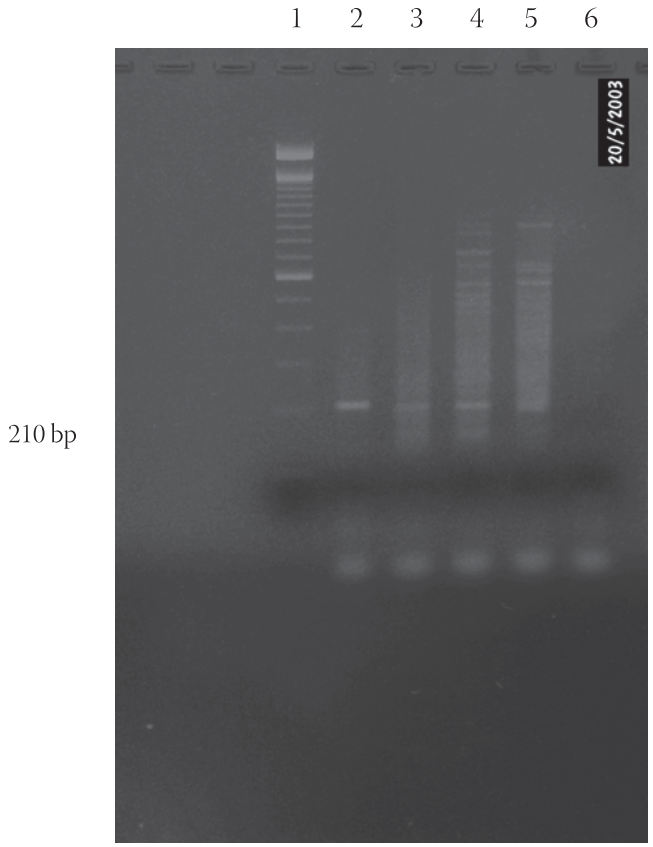

FIGURE 2. Electrophoretic analysis of amplified samples.

were frozen at –20°C, and reanalyzed by electrophoresis. The results did not differ from the first electrophoresis. The results of electrophoresis are shown in Figure 2.

Discussion

We tested a commercial test, applicable for extraction, reverse transcription and amplification of HIV RNA. In routine work time consumed is a very important thing. Period of 6 hours that elapsed during our analysis (from samples received to the final results) is relatively short. We isolated total RNA from whole blood, with ready for use kit based on salt precipitation according Nelson and Kim recomendations (23). Low amount of required material is of utmost importance. Our method required 300 µl of whole blood to yield 10 µl of isolated RNA. Cunningham et al. compared RT PCR and DNA PCR tests for the detection of HIV and concluded that the former is more sensitive (96% to 100%). Sensitivity of qualitative RNA test is more pronounced than of the quantitative one (24). The test that we used did have performance of RNA detection of 60 IU/ml as the lowest limit. Using the primers located for pol gene sequence variability has been avoided and bigger sensitivity got (25). We used the method that Roche Molecular Biochemicals did for determination of clinical and analytical specificity of Amplicor HIV-1 Monitor test v.1.5, which is the oldest PCR method certified by Food and Drug Administration. Clinical specificity of the test is determined when in healthy blood donors: anti HIV antibodies and p24 antigen, as weel as HIV RNA were not detected. Thus, we got clinical specificity of 100%. Analytical specify is detected when nucleic acids are used to test their reactivity to HIV-1 primers. None of those showed any reactivity, which indicates high analytical specificity of the test. In order to test the reproducibility different aliquots of the same sample were tested independently. All of these concurred with the earlier result and confirmed the test as reproducible. The suspended RNA was preserved at temperature 4°C max for one week. When the analysis is postponed for a long time freezing at –70°C is recommended (23). In prolonged storage RNA degradation is expectable. We maintained RNA frozen at -70 for one year, but we did not find any RNA degradation. Sometimes it is necessary to postpone electrophoresis and store amplicons at -20°C. After amplicons were stored at–20°C for one week and rerun in aga-

rose gel, the results were identical to the previous run. Nelson and Kim consider that material may evaporate when maintained at room temperature, which results in little and invisible gel product (23). Incorporation of positive and negative controls is necessary for correct interpretation of the test. Positive control consists of RNA molecule with high level of transcription. We used deionized sterile water as negative control (26). False positive or negative results may result from primers mispriming or non-optimized temperature of annealing. We used hot start technique with thermostable polymerase to increase the sensitivity of PCR reaction. With aim of reducing the risk of false positive results the kits incorporated uracil–N–glykosilase (27,28). Nested PCR is a procedure that increased specificity of the test and has the potential to detect very little amounts of DNA/RNA. Amplification of the grade 107 is gained after the first round of PCR consisting of 25-35 cycles. Nested PCR results in amplification of the grade 1012 (20, 21). Nested PCR was important in the detection of very small amounts of HIV RNA in the serum of asymptomatic HIV positive patients that were negative for p24 antigen in the study of Munoz et al. (34). Optimization of PCR reaction is very important. Electrophoretic analysis should reveal only one band. Undetectable HIV RNA means either that RNA is bellow the low border of detection or that the virus subtype is not detectable with this test. In order to improve the reliability of those molecular assays better standardization is important. Universal precaution and elimination of RNAase activity is provided in the lab, during the process (29,30). One way (preamplification to post-amplification) method is used. Safety action is maintained in order to prevent contamination and ensure reliable results. We used class II biosafety with HEPA-high-efficiency particulate air (31,32). This qualitative test, introduced in our country for the first time, can gain results when serology cannot because of the ELISA limits of detection, as in cases of possible HIV exposure, person with clinical symptoms of acute retroviral syndrome, or newborns of HIV infected mothers. Since quantitative tests for measuring of viral load are still not introduced, this test can also be used in monitoring the efficacy of antiretroviral therapy.

Conclusion

According to our data we can conclude that RT-PCR method enables reliable determination of HIV-1 RNA with limit of detection at 60 IU/ml. The test is reproducible and has high analytical and clinical specificity. Qualitative RT-PCR method for the detection of HIV RNA is recommended for use in routine laboratory work for documentation of HIV infection, as well as for the clinical management of HIV infected patients and monitoring of antiretroviral therapy efficacy.

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Acknowledgment

I would like to thank Academician Professor Georgi Efremov, from Macedonian Academy of Science and Arts for initial idea for this thesis.