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# A Simple and Rapid Method for the Detection of HIV-1/HCV in Co-Infected Patients

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## ABSTRACT

***Background:*** Due to some limitations of serological methods in diagnosis of patients infected with HIV-1 (human immunodeficiency virus) and HCV (hepatitis C virus), it is profoundly important to use molecular methods for the detecting of these infectious agents. However, the most significant problems are the exorbitant cost of these methods and the need of a thermocycler which is an expensive instrument.
***Objectives:*** The current research recruits a multiplex nucleic acid sequence base amplification (NASBA) in order to simultaneously detect HIV-1 and HCV genomes in patients' plasma samples. Sensitivity and specificity of this method have been evaluated using clinical samples.
***Materials and Methods:*** A multiplex NASBA assay for simultaneous detection of HCV and HIV-1 by the use of specific primers were designed and validated. A well-conserved region in the HIV-1 pol gene and 5'-NCR of HCV genome were used. A total of 40 samples of HIV-1 (20 samples) and HCV (20 samples) were used in the NASBA assay. The specificity and sensitivity of the assay were evaluated.
***Results:*** Our results have demonstrated that the primers used in the assay had no interrelation with each other and other possible interfering agents in the assay. The analytical sensitivity of the assay for both HIV-1 and HCV was determined to be 1000 copies/mL and the clinical sensitivity and specificity were 93.3% and 100%, respectively.
***Conclusions:*** By exploiting this multiplex NASBA assay, it is possible to detect HIV-1 and HCV infection/co-infection in patients' plasma with a suitable sensitivity and specificity. Furthermore, due to its simplicity and multiplexing feature, it could be used in limited access laboratories in a cost-effective manner.

*Keywords:* Nucleic Acid Sequence-Based Amplification; Human Immunodeficiency Virus -1; Hepatitis C Virus; Co-infection



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Implication for health policy/practice/research/medical education:
Diagnosis of HIV/HCV infections in Laboratories and Blood Banks.

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## 1. Background

Infection with Human immunodeficiency virus-1 (HIV-1) and Hepatitis C virus (HCV) is a major health problem in the world. These viruses are among the foremost infectious agents transmitted by the blood and about 10 million people are assumed to be co-infected with these viruses all over the world (1). Infection with these viruses leads to a progressive chronic disease with a long common period. HCV infection in HIV-1 patients results in an increased risk of hepatotoxicity, a major cause of death in these patients which is a consequence of anti- HIV-1 drugs (2). In addition, HIV-1 infection intensifies the symptoms of HCV infected patients and decreases the self-clearance of HCV positive patients (2). These viruses have a similar transmission; sexual contact, blood and mother to fetus (3). Most HCV- infected population are Injecting drug users, but the transmission route of HIV-1 is variable (4). It has been estimated that if a mother is co-infected with HCV and HIV-1, transmission rate to the newborn will be 13.3% - 30% and 5.4% - 23% for HIV-1 and HCV, respectively. The probability of transmission to the newborn from co-infected mother is much greater than mono-infected a mother (5). The most common and principle method of detecting HIV-1 and HCV infection is serological assays which detect antibodies in the patient's serum. However, serological methods have some limitations. Amongst the most important limitations is that these assays cannot detect infected individuals during the window period when the immunologic determinants have not increased enough to be detectable. The presence of maternal antibodies makes it impossible to detect infection in newborns (6). Furthermore, in HCV-affected patients, serological methods are not capable of differentiating individuals with active infection from patients recovered from the disease because of the presence of anti-HCV IgG (7). In these situations, molecular methods including RT-PCR and Real-time RT-PCR are appropriate for the detection of HIV-1 and HCV infections. Such methods are highly sensitive and specific as well as simple, automatable and high-throughput and therefore are widely acceptable and applicable (8, 9). Whenever amplification of nucleic acids depends on thermal cycles, an expensive instrument is required. In RNA template amplification, these methods can also amplify contaminating DNA which makes it difficult to conclude that the RT-PCR product is totally due to RNA amplification (10, 11). By using techniques such as NASBA which is isothermal, problems like unwilling DNA amplification will be overcome. Another advantage of NASBA over RT-PCR is that no thermo cycler is required; therefore, laboratories that cannot afford a thermo cycler can use NASBA to amplify nucleic acids. In addition, compared to PCR, in NASBA there are fewer amplification cycles which leads to reduce enzymatic errors. These advantages have made NASBA an accessible technique in detection of microorganisms. In NASBA, RNA is amplified in an isothermal condition using reverse transcriptase, T7 RNA polymerase and RNase H enzymes. The process is initiated by the annealing of an oligonucleotide primer (P1) to the RNA sequence. The 3' end of the P1 primer is complementary to the target sequence; the 5' end encodes the T7 RNA polymerase promoter. After annealing, reverse transcriptase enzyme, which is present in the reaction mixture, uses the deoxynucleoside triphosphates (also available in the mixture) to extend the 3' end of primer 1, thereby forming a cDNA copy of the template and resulting in an RNA:DNA hybrid. RNase H hydrolyses RNA from RNA:DNA hybrid. Consequently, the original RNA is destroyed, leaving a single strand of DNA to which the second primer (P2) anneals. Reverse transcriptase synthesizes the second DNA strand, rendering the promoter region double stranded. The third enzyme in the mixture, T7 RNA polymerase, transcribes RNA copies from functional T7 RNA polymerase promoter, generating a large amount (10 - 1000) of anti-sense, single stranded RNA transcripts corresponding to the original RNA target. Each new RNA molecule is now available as a template for reverse transcriptase in the cyclic phase of the NASBA process. The entire NASBA process is conducted at 41°C, and the amount of amplified sequence is at least a factor of $10^9$ (12). It is a perfectly appropriate method for the detection of viral genomes, because there is no need for a reverse transcription step (11, 13). As mentioned, because of some limitations in serological methods, exploitation of molecular methods like NASBA has an important role in the diagnosis of RNA viruses such as HIV-1 and HCV. Nonetheless, one major shortcoming of molecular methods which limit their use as a screening assay is their high cost, compared to serological methods (10). One significant solution that has been used to lower costs is multiplexing assays for detection of more than one infectious agent in a single reaction. In multiplex assays by using the reactants for a single assay and simply by adding more oligonucleotides, it is possible to detect more than one infectious agent; therefore, it is more cost and time effective (14). So far some studies about multiplexing NASBA have been reported. Among these, are a multiplex NASBA for the detection of the expression of pp67 and Immediate- Early1 (IE1) coded by cytomegalovirus (CMV) (15) and also simultaneous detection of Mycoplasma pneumonia, Chlamydia pneumonia and Legionella species in respiratory system samples (10).

## 2. Objectives

The aim of the present study was to develop and exploit a multiplex NASBA assay for simultaneous detection of HIV-1 and HCV in plasma samples.

## 3. Materials and Methods

### 3.1. Samples

HIV-1 and HCV infected blood samples were collected in EDTA containing tubes. Samples were centrifuged at 2500 rpm for 20 minutes and plasmas were stored at -80°C until use.

### 3.2. Investigation of the Genomic Sequence of HIV-1 and HCV in Order to Choose Highly Conserved Regions for Primer Design

The genomic sequences of each virus were retrieved from GenBank, NCBI (http://www.ncbi.nlm.nih.gov/genbank) and each set of viral sequences were aligned with Mega 4 software (www.megasoftware.net/mega4/mega.html) in order to locate the conserved region of each viral genome. The chosen sequences were then analyzed for designing primers using Beacon Designer 7 software. Finally, 5'NCR of HCV and pol gene of HIV-1 were chosen as highly conserved regions of viral genomes. Each primer pair spans a 179 bp and a 241 bp of HIV-1 and HCV genomes, respectively (*Table 1*).

**Table 1.** Primer Sequences for HIV-1 and HCV

| Primer | Sequence[a] |
|---|---|
| **HIV-1 Forward** | 5'*GTA CAG TGC AGG GGA AAG*3' |
| **HIV-1 Reverse** | 5'*AAT TCT AAT ACG ACT CAC TAT AGG GCC AGA GIA GYT TTG CTG GTC*3' |
| **HCV Forward** | 5'*CAT GGC GTT AGT AYG AGT G*3' |
| **HCV Reverse** | 5'*AAT TCT AAT ACG ACT CAC TAT AGG GCT ATC AGG CAG TAC CAC AAG*3' |

[a] The part of the sequences in Italic is the T7 promoter sequence.

### 3.3. Viral RNA Extraction From Plasma

Viral RNA extractions were carried out (QIAamp Viral RNA Mini Kit, Qiagen, USA) according to the manufacturer's manual and eluted with 50µl of nuclease-free water and stored at −80°C until use.

### 3.4. Detection of HIV-1 and HCV Genomes Using Monoplex and Multiplex NASBA

The mono and multiplex assay was carried out with a final reaction mixture volume of 25 µL containing 40 mM Tris-HCl (pH 8.5), 50 mM KCl, 12 mM MgCl2, 1 mM of each of the dNTPs, 2 mM of each of the ribonucleoside 5'triphosphates, 10 mM dithiothreitol, and 15% (vol/vol) dimethyl sulfoxide; 0.4 µM of each primer was used for the mono and multiplex NASBA; 5 µL of purified RNA was added to 18 µL of amplification mixture in a 0.2 mL microcentrifuge tube, which was incubated for 5 min at 65°C in order to disrupt secondary structures in the target RNA. Each tube was immediately cooled to 41°C for 5 min, after which 2 µL of an enzyme mixture containing 2.6 µg of bovine serum albumin (in 50% glycerol; Roche Diagnostics Corp., Indianapolis, Ind.), 40U of T7 RNA polymerase, 8 U of avian myeloblastosis virus reverse transcriptase, 0.2 U of RNase H, and 12.5 U of RNasin were added (All enzymes were purchased from Fermentas, except AMV-reverse transcriptase from Roche). Finally, NASBA products were run on 2% agarose gel to ascertain the amplification of the target sequences.

### 3.5. Assay Sensitivity

#### 3.5.1. Construction of a Trustworthy Reference RNA as NASBA Control

In order to determine the assay sensitivity, PCR products were cloned in a T/A cloning vector. The RNA was transcribed using T7 RNA polymerase (Fermentas, Germany); in the presence of 2mM NTP mix; 10 µL transcription buffer; 50U RNase inhibitor and 30U T7 RNA Polymerase in a 50µL reaction.

### 3.6. Eliminating DNA From in-vitro Transcribed RNA

Since the recombinant plasmid DNA has the T7 promoter element, if the in-vitro transcribed RNA is used in NASBA, it leads to a false positive result. Therefore, DNA should be thoroughly removed from *in vitro* transcribed RNA. To do so, RNase free DNase I enzyme were used. Because of the intense activity of DNaseI, 4 - 5 units of the enzyme are sufficient. Furthermore, since DNase I is capable of digesting the dsDNA produced as an intermediate product during NASBA reaction, it should be thoroughly inactivated. To inactivate DNaseI, the reaction should be incubated at 70°C for 15 minutes. RNA was quantified based on the A260 value and the copy number of standard molecules was calculated using the following formula:

(X g.µL$^{-1}$ RNA/ [RNA length in nucleotides x 340]) x 6.022 x $10^{23}$ =Y molecules/µL

From a stock of $10^7$ copies/mL solution, a series of serial dilutions were prepared (10000, 5000, 1000 and 500 copies/mL).

### 3.7. Assay Specificity

In order to determine the analytical specificity, 20 samples including human genome samples and some viral samples transmitted by the blood were used. The analytical sensitivity was performed using NCBI Nucleotide BLAST software for evaluating designed primers.

## 4. Results

### 4.1. Specificity of Monoplex and Multiplex NASBA Primers

The specificity analysis done by performing a nucleotide BLAST showed no significant interaction between designed primers and irrelevant sequences in GenBank NCBI. Secondly, NASBA reaction by using viral samples including HTLV-1, HBV, CMV, HSV-1, HSV-2 and B19 showed no detectable amplification on gel electrophoresis. Therefore, the specificity of the assay was considered to be 100%.

### 4.2. Monoplex and Multiplex NASBA Sensitivity

Using serial dilutions with defined copy numbers, the sensitivity of the monoplex and multiplex NASBA were analyzed. Dilutions of 10000, 5000, 1000, 500 copies/mL of the in-vitro transcribed RNA were added to the healthy individuals' plasma. Following RNA extraction, five NASBA reactions of each standard were performed on two different days; the sensitivity of the assay was 1000 copies/mL (*Table 2*).

**Table 2.** Determination the Sensitivity of Monoplex and Multiplex NASBA

| HIV-1, Copies/mL | Positive Monoplex | HCV, Copies/mL | Positive Monoplex | HIV Positive Multiplex | HCV Positive Multiplex |
|---|---|---|---|---|---|
| 10000 | 10/10 | 10000 | 10/10 | 10/10 | 10/10 |
| 5000 | 10/10 | 5000 | 10/10 | 10/10 | 10/10 |
| 1000 | 10/10 | 1000 | 10/10 | 10/10 | 10/10 |
| 500 | 6/10 | 500 | 8/10 | 5/10 | 10/10 |

10 Monoplex NASBA reaction for each of HIV-1 and HCV and 10 multiplex reactions were carried out. In the 1000 copies/mL standard, all the reactions for both viruses either in monoplexor multiplex NASBA were positive, therefore the sensitivity of the assay was considered to be 1000 copies/mL.

10 Monoplex NASBA reaction for each of HIV-1 and HCV and 10 multiplex reactions were carried out. In the 1000 copies/mL standard, all the reactions for both viruses either in monoplexor multiplex NASBA were positive, therefore the sensitivity of the assay was considered to be 1000 copies/mL.

### 4.3. Performing NASBA for the Detection of HIV-1 and HCV Genome Using Positive Samples

A total of 40 samples of HIV-1 (20 samples) and HCV (20 samples) were used in the NASBA assay which were previously reported as positive using Artus HIV-1 RG Kit (Qiagen) and Artus HCV RG Kit (Qiagen) system. The products were run on 2% agarose gel. The presences of 179 bp and 241 bp bands were indicative for amplification of HIV-1 and HCV genomes, respectively (Figure 1).

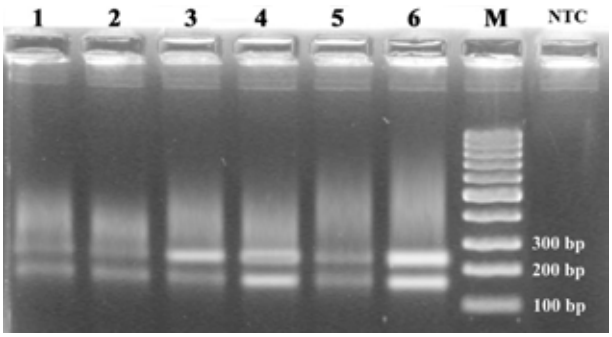
### 4.4. Multiplex NASBA for Simultaneous Detection of HIV-1 and HCV

Multiplex NASBA was performed on 30 co-infected samples. *Figure 2* shows a multiplex NASBA run on a 2% electrophoresis gel in which the amplification of HIV-1 and HCV is distinguishable based on the different sizes of the amplicons. 28 out of 30 samples showed positive results and two were negative. Therefore, the sensitivity of the assay was considered to be 93.3%. The positive predictive value (PPV) or precision rate is the proportion of positive test results that are true positives (such as correct diagnoses). It reflects the probability that a positive test reflects the underlying condition being tested for. It is calculable using this formula: PPV= number of True Positives/ (number of True Positives+ number of False Positives). So the PPV of the assay was 100%. The negative predictive value (NPV) is defined as the proportion of subjects with a negative test result who was correctly diagnosed. A high NPV for a given test means that when the test yields a negative result, it is most likely correct in its assessment. In familiar similar formula: NPV= number of True Negatives/ (number of True Negatives + number of False Negatives). The NPV of this assay was determined to be 90.91%.

**Figure 1.** Monoplex NASBA Band


Lanes 1 and 2 Show the Amplification of Target RNA in NASBA Reaction for HCV and HIV-1, Respectively. Lane 3 is a 100 bp RNA Marker (Fermentas, Germany. Catalog No. SM1831).

**Figure 2.** Multiplex NASBA Amplification




241 bp and 179 bp are bands in lanes 1 through 6 shows simultaneous amplification of HCV and HIV-1, respectively. The marker is a 100 bp RNA marker. NTC (Non Template Control) shows no amplification.

## 5. Discussion

In recent decades, molecular diagnostic methods have greatly developed and more practical methods have come into existence for molecular diagnosis of diseases and research. Among these, Nucleic Acid Testing (NAT) has an important role. For instance, in 1999, in Germany, NATs became obligatory to screen Hepatitis C virus for blood donors via Paul- Ehrlich institute as part of the blood transfusion organization. By making it mandatory, the risk of blood donors being undetectable during the window period of HIV-1 and HCV was significantly reduced to 1 in 5000000 (16-18). Nonetheless, the application of these methods remains limited because of their high cost and time consuming process. In order to lower the cost of NAT, two solutions have been proposed. The first one was the usage of a pool of different donors' plasma. Clinical tests showed that sensitivity has decreased because of low copy number of virus; or false positive results have increased as a result of using ultracentrifuge to concentrate viral particles in the samples (19). In addition, if a pool of plasmas has a positive result, determining the infected individuals' plasma would be cumbersome and time-consuming, which delays the preparation of blood and blood products. The second one is the development of a multiplex assay for simultaneous detection of multiple viruses. This approach has lowered costs and uses less time for detection (20). NASBA, first used in mid-1999, was exploited as a qualitative and quantitative diagnostic method for RNA viruses especially HIV and HCV (12). Traditional methods which were based on antigen detection were less sensitive than molecular methods like PCR. PCR facilities are costly, need a skillful operator and cannot be available in all laboratories (21). However, NASBA is more sensitive, without the necessity of cDNA synthesis and usage of a thermocycler. There are several reports on multiplex detection of microorganisms using NASBA. In a research conducted by Jean et al. (22) multiplex NASBA had been used for simultaneous detection of intestinal viruses which cause food poisoning. In another investigation, Lau et al. (11) used this method for the detection of respiratory system infectious viruses. Furthermore, in a study, published by Loens et al. (10), Real-Time NASBA was used to detect Mycoplasma pneumonae, Chlamydia pneumonae and Legionella species in respiratory samples. In the present study, we have developed a multiplex NASBA for simultaneous detection of HIV-1 and HCV. This assay is capable of detecting and distinguishing HIV-1 and HCV on 2% agarose gel, with an appropriate sensitivity. To design the primers, genomic sequence of each virus was aligned to locate the highly conserved regions. Assessment of sensitivity and specificity of the designed primers demonstrated that they are capable of detecting only HIV-1 and HCV without interaction with irrelevant viral genomes. Detection of mentioned viruses with low copy number in patients is an important issue for treatment. Sensitivity of the assay for both viruses is equal and is considered to be 1000 copies/mL. Sensitivity and specificity of the assay were determined to be 93.3% and 100%, respectively. Predictive value of tests is the probability of a target condition (for example a disease) identified by the results of a test, often used in medical tests. In cases where binary classification can be applied to the test results (such as yes versus no, or either a positive or negative test), then each of the two outcomes has a separate predictive value. For example, for positive or negative test, the predictive values are termed positive predictive value or negative predictive value, respectively. The PPV and NPV of the developed assay were 100% and 90.91%, respectively. Because of low cost, simplicity, accessibility, no need for a skilled operator, high sensitivity and specificity, NASBA can be used as an appropriate method for simultaneous detection of several pathogens in various clinical samples and can effectively replace the traditional methods which are used for blood screening in blood transfusion organizations. In addition to high sensitivity and specificity, low cost and simplicity are among the major advantages of the assay. Moreover, it is a suitable and rapid method for detection of HIV-1 and HCV co-infection, screening of donated bloods in blood transfusion organization and in diagnostic laboratories.

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## Authors' Contribution

Mahdi Paryan: acquisition of data and drafting the article, Mahdi Forouzandeh Moghadam: the conception and design of the study and interpretation of data, Vahid Kia:

analysis and interpretation of data, Samira Mohammadi-Yeganeh: revising it critically for important intellectual content, Abbasali Raz: analysis and interpretation of data, Siamak Mirab Samiee: the conception and design of the study. All authors had finally approved the version to be submitted.

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## Ethical Approval

Approved by Tarbiat Modares University of Medical Sciences, Tehran, Iran.

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