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Comparison of Multiplex PCR-ELISA and conventional Multiplex PCR for detection of HIV-1/HCV co-infection

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ABSTRACT

Background and Objectives: PCR is a sensitive assay and could be used as an accurate diagnostic method for detecting various types of microorganisms' genome in low concentration in biological specimens. The demand for sensitive, rapid, safe and easy detection of PCR products has led researchers to a combination of this method with ELISA.

Materials and Methods: Conserved sequences were selected for design of primers. Samples were tested by ELISA (for detection of specific HIV and HCV proteins) and real- time PCR for detection of specific nucleic acid and viral genome respectively. Viral genome was extracted and reverse transcription was performed with M-Mulv and the cDNA kept at -80º C. The PCR products were labeled by DIG-dUTP. Diluted PCR products were analyzed with both electrophoresis and ELISA methods.

Results: Thirty-five samples were tested with the PCR-ELISA method. False positive or negative reactions were not observed. ELISA results of diluted products were compared with results obtained by electrophoresis. In gel electrophoresis, dilution of 1/10 was positive, but in ELISA, optical density of 1/100 dilution was much more than the cut-off value.

Conclusion: Detection limits for gel electrophoresis as well as ELISA have been evaluated. It was shown that the PCR-ELISA method is ten times more sensitive than conventional PCR.

Keywords: Multiplex, PCR- ELISA, HIV, HCV.

INTRODUCTION

The introduction of nucleic acid amplification strategies have found important applications in biomedicine, monitoring antiviral therapy, improving the safety of blood supplies and diagnostic techniques in clinical laboratories (1, 2). Study of viral infections has benefited much from these technologies. Detection of very low copy number of genome allows laboratories to make an early and rapid diagnosis of life threatening infectious agents (1, 3-6). For a long time, efforts have been made to employ multiplex-PCR techniques or reverse transcription (RT)- polymerase chain reaction (PCR) for detection of several infectious agents in co-infected patients simultaneously (7, 8). For this purpose, several pairs of primers or probes are designed to amplify specific target sequences simultaneously at a high speed and with high efficiency (9, 10). This allows visualization of amplicons routinely done by gel electrophoresis which differentiates products of different size by ethidium bromide staining and by hybridizing to specific DNA probes and other available techniques (9-12). A small but very important risk of pathogenic virus transmission exists due to the inability of routine serologic tests either to detect newly infected individuals in the window period of infection or to detect antigenic variants of these viruses (12). Development of nucleic acid amplification tests (NATs) have meaningfully reduced the window phase (2, 10, 13-16). However, a limitation is that the cost of NATs is 5–10 fold greater than that of the most expensive enzyme immunoassay. This has led to immunoassay use for large-scale routine screening and NATs remain limited and controversial considering poor cost effectiveness. To access a cost effective NAT, two approaches have been used in this study, namely examination of samples as pooled plasma testing for reducing in number of tests that we need to screen large numbers of samples. However, clinical trials have documented a lack of sensitivity

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Table 1. The sequences of primers and probed designed for detection of HCV and HIV in multiplex-RT PCR ELISA.

Virus	Primers and Probes	Sequence	Usage	Nucleotide
HCV	F1	ACTCCCCTGTGAGGAACTACTGTCT	Forward primer	39-63
HCV	R1	GCAAGCACCCTATCAGGCAGTACC	Reverse primer	308-285
HCV	zcv	Biotin-GCCATGGCGTTAGTAYGAGTGTC	Probe	
HIV-1	F2	CAGAAGGAGCCACCCCACAAGAT	Forward primer	862-884
HIV-1	R2	TTCCTGCTATGTCACTTCCCCTTGG	Reverse primer	1050-1026
HIV-1	ziv	Biotin-CTTGGTTCTCTCATYTGGCCT G	Probe	

due to dilution of samples with low viral load, and the use of multiplex instead of uniplex assays that can detect several viruses simultaneously (17, 18).

Cost-effective and time saving tests which do not compromise efficiency and feasibility are the most important advantages of multiplex assays (18). Although multiplex PCR simplifies the procedures and reduces the need for time, final analysis remains tiresome, expensive and time consuming (1, 19). To address this problem, we introduce here the use of an in-house multiplex-PCR-ELISA assay for the detection of multiplex RT-PCR products in a single tube for simultaneous detection of HIV and HCV as two important viral infections. This co- infection is an important factor in determination of AIDS patients' future and is responsible for elevated morbidity and mortality in these patients (21, 22). In recent years, several PCR-based multiplex techniques have been introduced by different laboratories (12).

Simultaneous detection of several organisms in one reaction with minimal cost is important (23, 24). PCR would be an impractical approach for rapid screening of hundreds of specimens per day for a range of transfusion transmitted viruses (24). The aim of the current study was to design a more sensitive method for detection of PCR products and diagnosis of HIV-1/ HCV co-infection accurately and also compare sensitivity of PCR-ELISA to conventional PCR. The multiplex-PCR and/or PCR-ELISA assay has not been available as a commercial diagnostic kit for detecting HIV-1 and HCV infection till now.

MATERIALS AND METHODS

Viral standards and clinical specimens. Plasma samples were collected from AIDS Research Center at Imam Khomeini Hospital and Digestive Disease Research Center (DDRC) at Dr. Shariati Hospital. All the samples had previously been analyzed by Real-time PCR and sequencing method in which 9 cases were HIV (+), 8 were HCV (+), and 10 were positive for both HIV and HCV. Ten cases were negative for both HIV and HCV and used as negative control. Positive samples were collected from volunteer infected patients. Samples were transferred to the Laboratory of Molecular Virology, Department of Virology at University of Tarbiat Modares, for further molecular investigations. Samples were then aliquoted and stored in -80°C for future analysis. For sensitivity analysis of the multiplex PCR- ELISA detection system, we used HCV standard panel with certain concentration of viral genome according to Table 2 for later analysis.

Isolation of viral RNA. HCV and HIV-1 RNA were isolated from 140 µl of plasma samples by the use of a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. Viral RNA was eluted from the filter column with 50 µl of nuclease-free elution buffer and stored at -80°C until analysis.

Probe and Primer design. For performance, target RNA sequences were selected within the regions of the respective virus genomes showing maximum sequence conservation for the ability of the test to detect all known genotypes and subtypes of both HCV and HIV-1. In this study we used HIV-1 gag and HCV 5'-NCR region specific primers to avoid major mismatches due to HIV-1 and HCV genome variability. Primer specificity was tested using the Mega 4 software (National Center for Biotechnology Information: NCBI,) against the collected genome sequences (available in the GenBank-EMBL database, www.EBI.ac.uk): HCV complete genome genotypes, HIV-1 complete genome group M. Based on insufficient data about circulating subtype of HIV in Iran, probes were designed with degenerate mode. Gene Runner, NCBI Blast, Oligo analyzer3, Mega4 and Oligo6 were employed in all steps of the design. The primers and probes selected are shown in Table 1. Depending on the location of the designed primer, the length of HIV and HCV amplicons were 189 and 270 nucleotides respectively. Probes were biotin labeled. The

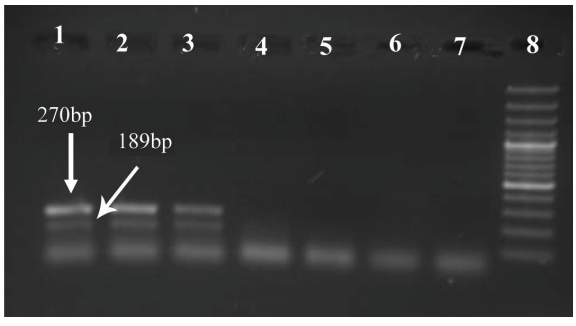

Fig. 1. Multiplex PCR
Lanes: 1-3; positive samples for both HIV and HCV, 4-7; negative samples, 8; 100 base pair ladder.

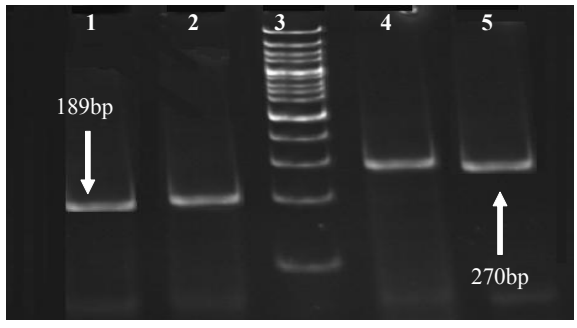

Fig. 2. Lanes: 2 &4, HIV & HCV labeled products,1&5; HIV & HCV unlabeled products.

oligonucleotide primers and probes were synthesized by Fazapajooh Biotech Company (Fazapajooh, Tehran, Iran).

cDNA synthesis. RNA was thawed on ice before amplification. Complementary DNA (cDNA) was synthesized and amplified using a reaction mix single tube in RT-PCR assay.

Mixture contained the thermo stable M-Mulv polymerase enzyme (Fermentas, Vilnius, Lithuania) and antisense primers for both HCV and HIV targets. Briefly, 5 µl of extracted RNA was added to 30 µl of RT-PCR mix in a single tube containing 4 µl Buffer (Fermentas, Vilnius, Lithuania), 2 µl of 10 mM dNTPs (Cinna Gene, Iran), 2 µl (20 pM) primer R1, 3 µl (30 pM) primer R2 (Table 1) and 200 U of M-Mulv. Five micro liters of extracted RNA was added to 3 µL of HIV R2 (30 pM), 2 µl HCVR1 (20 pM), and up to 11µl with DEPC treated water. The mixture was incubated in 70 ºC for 5 min. and chilled on ice. 4 µl 5× buffer, 2 µl 4 dNTP mix (10mM) and 20U riobonuclease inhibitor (Fermentas) was added and incubated at 37 °C for 5 min. The samples were chilled on ice and 200 unit M-MuLV (Fermentas) reverse transcriptase was added. The cDNA synthesis was carried out at 42 °C for 60 min and then at 70 °C for 10 min. The cDNA was stored at -80 °C until use.

HIV and HCV Uniplex-PCR. The HIV and HCV PCR reactions contained 5 µl template cDNA, as well as 0.5µl or 0.3µ l (10pM stock) of each amplification primer for HIV or HCV respectively, 0.5 µl dNTP (10 mM stock), 2.5 units Taq DNA polymerase (Cinnagen, Tehran, Iran), 0.5mM $MgCl_2$, 2.5 µl 10× buffer (500mM KCl and Tris-HCl pH 8.4). PCR amplifications were performed as follows: initial denaturation at 95 °C, 5 min and 35 cycles of 50 sec each at 94 °C, 40 sec at 65 °C, and 35 sec at 75 °C with a final 3 min at 72 °C. The PCR products were separated by electrophoresis in 2% agarose gels and visualized after staining with ethidium bromide.

Multiplex PCR. The HIV-HCV multiplex PCR mixture consists of 7 µl HIV and HCV template cDNA, 0.5 and 0.3 µl of HIV and HCV primer respectively and 0.5µl dNTPs, 2.5 Unit Taq DNA polymerase, 5mM $MgCl_2$, 2.5 µl 10X buffer (500mM KCl and Tris–HCl pH 8.4). Thermal cycler temperature program for amplifications and visualization were performed as HCV single PCR protocol above.

Product labeling. This step was performed by addition of Dig-dUTP containing dNTP into reaction mix. The dNTP mix consists of 2mM of dATP, dCTP, dGTP , 1.9mM dTTP and 0.1mM DIG-dUTP. Labeled product is slightly heavier than common product because of their digoxigenin content. This feature can be used for detection of labeled products. Due to high sensitivity of SDS page, 29% polyacrylamide gel was used for labeling verification.

ELISA assay. Labeled product was denatured in alkali condition and was hybridized to biotin labeled probe in temperature matching with product length and GC content. Achieved hybrid was affixed to strep avidin coated micro plate. Peroxidase labeled Anti-digoxigenin antibody was added to wells in the next step. Detection process was performed in 405nm (ELISA reader Fax Stat 2100, USA) by surveying the change of color in the reaction mix due to addition of ABTS substrate using ELISA reader. Labeled amplicons were analyzed for the presence of HIV-1 and HCV amplified sequence with Roche PCR ELISA (DIG Detection) kit according to manufacturer instruction. Cut-off value was calculated from average of negative control (seronegative samples) optical density plus3 standard deviations (17).

Methods optimization. Primer pair was tested against positive and negative controls and for

Table 2. The results of multiplex-RT PCR ELISA assay for detection HIV-1 and HCV from sera samples of different patients groups.

	Number of samples	Status of contamination	Positive results	Negative results
Group 1	9	HCV positive	9	0
Group 2	8	HIV-1 positive	8	0
Group 3	8	co -infected samples	8	0
Group 4	10	Confirmed negative	0	10

nonspecific attachment to HIV, HCV, HBV and human genome. Other PCR components such as $MgCl_2$, probe and primer concentration, temperature and number of cycles were optimized by trial-and-error gradient of each component.

The final concentration of components in multiplex reaction were: The HIV-HCV multiplex PCR mixture consist of 7 µl HIV and HCV template cDNA, 0.5 and 0.3 µl of HIV and HCV primer respectively and 0.5µl dNTPs, 2.5U Taq DNA polymerase, 1.5mM $MgCl_2$, 2.5 µl 10× buffer containing, 500mM KCl and Tris-HCl pH 8.4. Also we used AMS buffer instead of KCl in PCR reaction.

The final cycling program employed was as follows: initial denaturation at 95 °C, 5 min and 35 cycles of 50 sec each at 94 °C, 40 sec at 65 °C, and 40 sec at 72 °C with a final 3 min at 72 °C.

In the ELISA step, the best concentrations for both probes were 20 picomol, and probe annealing was performed in 55 °C for 3 hours.

RESULTS

This method was optimized separately for HIV-1 and HCV with positive controls and serial dilutions and the multiplex reaction was optimized (data not shown). The PCR product sizes for HCV and HIV were 270 and 189 bps. After the labeling stage, polyacrylamide gel was used and labeling was verified. Then, using labeled products, ELISA was performed. 35 samples (4 groups) were tested with this method. Development of color shows the positive whereas no color shows negative result.

Positive controls that had been confirmed as well as negative control sera were used to assess the reproducibility. As expected, the developed method was able to detect major subtypes of HIV-1 in Iran (A and B) as well as genotypes of HCV (1a, 1b and 3a) that exist between Iranian patients. Also, primers were tested with the HBV genome and human genome (100 and 200 ng). Probes were also tested for non-specific cross reaction with amplicons.

This method detected HCV genomic RNA in 9 samples that were in group1 and 8 samples in group 3. HIV-1 genomic RNA was detected in 8 samples in group 2 and 8 samples in group 3. All samples in group 4 were negative. False negative or positive results have not been observed. The results are shown in Table 2.

Cut-off value was calculated from an analysis of optical density of seronegative samples (Group 4) as means of the values plus 3 standard deviations.

Evaluation and verification of method. After each examination, the test results for a given sample were evaluated. If result values correlated with observed values and fell within a certain deviation limit, the results of that detection were considered to be correct. Four measures were used to evaluate the test performance: Specificity (SP), sensitivity (SN), accuracy (AC) and correlation coefficient (cc) (19).

In the current study, false positive or negative reactions were not observed. It seems the sensitivity, as well as specificity, needs more positive and negative samples to improve precision.

DISCUSSION

Between 4 to 5 million people are co-infected with HIV and HCV worldwide (25). Despite the sensitivity of serological tests, infection can be transmitted during the phase in which viral specific antibodies are not detectable.

PCR, with its astonishing sensitivity, is the method of choice for the detection of nucleic acids present in very low concentrations in biological specimens (26). In view of the increasing number of multiple infections in patients and the interest in domains as different as blood banking and disease control, it has become vital to find optimal techniques for screening large numbers of samples (1). Despite the fact that multiplex NAT assays for the diagnosis of HBV, HCV, and HIV-1 have been introduced, these techniques need expensive material and apparatus. They are difficult and time

consuming procedures for the visualization of fluorescence or chemiluminescence signals (2, 12, 18, 27-29). AMPLINAT MPX test using TaqMan real-time PCR developed by ABI PRISM 7700 analyzer (12) and the Procleix Ultrio assay based on transcription-mediated amplification are two hemi automated multiplex assays commercially used for detection of HBV, HCV and HIV, but these techniques do not distinguish between the three viruses (29).

The need for a method to detect PCR-amplified products, which is able to offer sensitivity, specificity and an objective evaluation of results, has led to the coupling of PCR with ELISA (26). In most RT-PCR methods, final amplicon detection is performed using agarose gel electrophoresis that does not have sufficient sensitivity for very low viral loads and the results can be reported as false negative. The sensitivity of the RT-PCR was increased more than 10-fold via ELISA detection (30). PCR–ELISA shows similar sensitivity to the Real-time PCR and does not require the use of expensive equipment, as real-time PCR presently does, and as a replacement for basic instruments present in every diagnostic laboratory (26).

We have investigated the possibility of combining multiplex RT-PCR with an ELISA assay for coincident detection and identification of HCV and HIV-1. The primers used in this study had not been described befor Our primers can detect a large part of the HCV genotype that was registered in NCBI (using mega 4 software) and HIV-1 subtypes A, B, C, E, F1, G, H and I can also be detected. To set up of this method, we used samples with known viral loads from 10^2 to 10^6 that were confirmed with the real- time method. Our method can detect all of the samples as it should be, and false positive or negative reactions have not been observed.

To compare conventional PCR and PCR ELISA, labelled products were diluted and analyzed with both methods. In gel electrophoresis, dilution of 1/10 was positive and the expected band was observed, but in ELISA optical density of 1/100 was much more than cut off value. So the designed method is 10-fold as sensitive as the conventional PCR. When separate reactions were combined and multiplex reaction was created, we observed a decrease in sensitivity of HIV-1. This problem was solved with increasing the HIV-1 specific reverse primer in RT reaction and using AMS (200mM AMS + Tris-HCl [pH: 8.8]) buffer in PCR reaction instead of KCl (500mM KCl + Tris-HCl [pH: 8.4]) buffer. Decrease in number of samples and increase in sensitivity and specificity (because of using specific probes) are advantages of this method. We did not observe non-specific interactions between primers and the human genome, HBV or other pathogens. Also, probes attached only to desired sequences and non-specific attachment was not observed. Future efforts should be focused on large number of samples and samples which are in the window period. In comparison with other studies (17), our method can detect further genotypes of HCV including (1a, 1b, 1c, 2a, 2b, 2c, 2e, 2f, 2k,3a, 5a, 6a, 6b ,6c 6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l vs. 1b, 2a, 2b, 2a/c, 3a, 3b, 4, 6). We used separate enzymes for RT and PCR steps which are more cost effective than *rTth*, with acceptable sensitivity and specificity.

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REFERENCES

1. Defoort JP, Martin M, Casano B, Prato S, Camilla C, Fert V. Simultaneous detection of multiplex-amplified human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA using a flow cytometer microsphere-based hybridization assay. *J Clin Microbiol* 2000; 38: 1066-1071.
2. Candotti D, Fletcher-brown F, Pierre allain J, Leei H. Simultaneous visual detection of multiple viral amplicons by dipstick assay magda anastassova dineva. *J Clin Microbiol 2005*; 43: 4015-4021.
3. Guatelli JC, Gingeras TR, Richman DD. Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. *J Clin Microbiol* 1989; 2: 217-226.
4. Stoeckl E, Barrett N, Heinz FX, Banekovich M, Stingl G, Guggenberger K, et al. Efficiency of the polymerase chain reaction for the detection of human immunodeficiency virus type (HIV-1) DNA in the lymphocytes of infected persons: comparison to antigen-enzyme- linked immunosorbent assay and virus isolation. *J Med Virol* 1989; 29: 249-255.
5. Vyas GN, Yang G, Murphy E. Transfusion related transmissible diseases: Detection by polymerase chain reaction-amplified genes of the microbial agents. *Transfus Med* 1994; 8: 253-266.
6. Hart C, Schochetman G, Spira T, Lifson A, Moore J, Galphin J, et al. Direct detection of HIV RNA expression in seropositive subjects. *Lancet* 1988; 2: 596-599.

7. Mahony JB. Multiplex polymerase chain reaction for the diagnosis of sexually transmitted diseases. *Clin Lab Med* 1996; 16 :61-71.

8. Tenorio A, Echeverria JE, Echeverria ICJM, Tabares E. Detection and typing of human herpes viruses by multiplex polymerase chain reaction. *J Virol Methods* 1993; 44: 261-269.

9. Schoske R, Vallone PM, Ruitberg CM, Butler JM. Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci. *Anal Bioanal Chem* 2003; 75: 333-343.

10. Rachlin J, Ding C, Cantor C, Kasif S. MuPlex: multi-objective multiplex PCR assay design. *Nucleic Acids Res* 2005; 33. w544-w547.

11. Kox LFF, Noordhoek GT, Kunakorn M, Mulder S, Sterrenburg M, Kolk AHJ. Microwell Hybridization Assay for Detection of PCR Products from Mycobacterium tuberculosis Complex and the Recombinant Mycobacterium smegmatis Strain 1008 Used as an Internal Control. *J Clin Microbiol* 1996; 34: 2117-2120.

12. Meng Q, Wong C, Rangachari A, Tamatsukuri S, Sasaki M, Fiss E, et al. Automated multiplex assay system for *simultaneous* detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type 1 RNA. *J Clin Microbiol* 2001; 39: 2937-2945.

13. Forcic D, Mazuran R, Lupret L, Hecimovic A, Zgorelec R. Optimal pool size and window period. *Trans Apher Sci* 2001; 25: 153-155.

14. Ansaldi F, Bruzzone BM, Durando P, Lee S, De luigi C, Crovari P. Novel approach to reduce the hepatitis C virus (HCV) window period: clinical evaluation of a new enzyme-linked immunosorbent assay for HCV core antigen. *J Clin Microbiol* 2001; 39: 3110-3114.

15. Müller breitkreutz K, Baylis S, Allain J, sanguinis V. Nucleic acid amplification tests for the detection of blood-borne viruses. *Vox Sanguinis* 1999; 76: 194-200.

16. Chyang T. Blood screening for HBV DNA. *J Clin Virol* 2006; 36: 30-32.

17. Adami V, Falasca E, Dorotea L, Malangone1 W, Astori G, Marini L, et al. Qualitative multiplex RT-PCR for simultaneous detection of hepatitis C virus and human immunodeficiency virus in plasma samples. *Clin Microbiol Infect* 2004; 10: 1075-1080.

18. Candotti D, Temple J, Owusuofori S, Allain J. Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. *J Virol Methods* 2004; 118: 39-47.

19. Wang N, Gao X, Han J-X. Simultaneous detection of HBV and HCV by multiplex PCR normalization. *World J Gastroenterol* 2004; 10: 2439-2443.

20. Gibellini D, Gardini F, Vitone F, Schiavone P, Furlini G, Carla re M. Simultaneous detection of HCV and HIV-1 by sybr green real time multiplex RT-PCR technique in plasma samples. *Mol Cell probes* 2006; 10: 223-229.

21. Wellinghausen N, Wirths B, Essig E, Wassill L. Evaluation of the hyplex bloodscreen multiplex PCR-enzyme-linked immunosorbent assay system for direct identification of gram-positive cocci and gram-negative bacilli From positive blood cultures. *J Clin Microbiol* 2004; 42: 3147-3152.

22. Motta do canto L, Vallone PM, pannuti C, Cedenho A, Srougi M, Spaine D, *et al*. Detection of HIV and HCV RNA in semen from brazilian coinfected men using multiplex PCR before and after semen washing. *Rev Inst Med Trop S Paulo* 2006; 48: 201-206.

23. Puppe A, Weigl jai, Aronc G, Gröndahl B, Schmitt B, Niesters HGM, *et al*. Evaluationof a multiplex reverse transcriptase pcr elisa for the detection of nine respiratory tract pathogens. *J Clin Virol* 2004; 30: 165-174.

24. Elnifro L, Ahmed M, Ashshi L, Robert J, Cooper L, et al. Multiplex PCR: optimization and application in diagnostic virology. *J Clinic microbiol* 2000; 13: 559-570.

25. Lahijani S, Taylor L. HIV-HCV *Co infection Update.* November 2006; 1-6. www.hcvadvocate.org.

26. Musiani M, Venturoli S, Gallinella G, Zerbini M. Qualitative PCR-ELISA protocol for the detection and typing of viral genomes. *Nat Protoc* 2007; 2: 2502-2510.

27. Khanlari Z, Ravanshad M, Rasouli M, Ziyaeyan M. A novel sensitive multiplex RT-PCR ELISA method for detection of HIV-1/HCV co-infection. *Modares J Med Sci* 2008; 11: 57-63.

28. Hecht FM, Busch MP, Rawal B, Webb M, Rosenberg E, Swanson M, et al. Use of laboratory tests and clinical symptoms for identification of primary HIV infection. *AIDS* 2002; 16: 1119-1129.

29. Linnen J, Broulik A, Broulik A, Umali D, Kolk J, Dockter S, et al. Analytical sensitivity of the TMA-based Procleix Ultrio assay and effect of assay sensitivity on closing the HBV detection window. *Trans Apher Sci* 2002; 42: 8.

30. Wang E, Paessler S, Aguilar P, Carrara A, Greene I, Weaver S. Reverse Transcription -PCR-Enzyme Linked Immunosorbent Assay for Rapid Detection and Differentiation of Alphavirus Infections. *J Clinic Microbiol* 2006; 44: 4000-4008.