Comparison of polimerase chain reaction and serological methods in the diagnosis of hepatitis C virus infection

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Ozet

Hepatit C Virus infeksiyonu tanısında polimeraz zincir reaksiyonu ve serolojik yöntemlerinin karşılaştırılması

Hepatit C virus (HCV) infeksiyonunun tanısı, serolojik olarak anti-HCV veya moleküler yöntemlerle HCV RNA'nın tespitiyle gerçekleştirilebilmektedir. Bu çalışmada HCV infeksiyonu tanısında polimeraz zincir reaksiyonu ve serolojik yöntemlerinin yerinin araştırılması amaçlanmıştır. Çalışmaya 87 hasta alındı. HCV RNA real time PCR (GeneAmp 5700 Sequence Detection System) ile tespit edildi. Seksenyedi hastanın 30 (%34.5)'unda HCV RNA ve 60 (%69)'ında anti-HCV pozitifliği tespit edildi. HCV antikorları pozitif hastaların 14'ünde antikor titreleri 2.5 U/ml'nin altında bulundu. Düşük titreli hastaların hiçbirinde HCV RNA pozitifliği saptanmadı. Antikoru negatif hastaların hepsinin HCV RNA'ları da negatifti. Ayrıca HCV RNA pozitif hastaların hepsinin HCV antikorları da pozitifti. HCV antikorları negatif tüm hastalarda HCV RNA'nın da negatif olması göz önünde bulundurulursa rutin tarama amaçlı işlemlerde güvenilir bir ELISA tekniği ile anti-HCV antikorunun araştırılmasının kişinin HCV virusu ile infekte olup olmadığını belirlemede yeterli olacağı düşünülebilir. Diğer yandan anti-HCV antikorları tespit edilen serumların öncelikle ELISA ile tekrar çalışılması ve hastalığın doğrulanması için HCV RNA testlerinin yapılması gerektiği düşünülmektedir. ELISA ve PCR metodlarının birlikte kullanılması pozitif sonuçların doğrulanmasına ve erken tanıya yardımcı olacaktır.

Anahtar kelimeler: Hepatit C virus, polimeraz zincir reaksiyonu, anti-HCV

Abstract

Diagnosis of HCV infection depends on detecting anti-HCV antibody (HCV-Ab) through serologic methods or on detecting of HCV RNA with molecular methods. The aim of this study was to evaluate the use of serological methods and polymerase chain reaction (PCR) for the diagnosis of HCV infection. A total of 87 patients were studied. HCV RNA was detected by real time PCR with GeneAmp 5700 Sequence Detection System. Thirty (34.5%) of 87 patients were HCV RNA positive and 60 (69%) were HCV-Ab positive. Fourteen of the HCV-Ab positive patients had low antibody titers (below 2.5 U/ml). None of these was found as HCV RNA positive. All of HCV-Ab negative patients were HCV RNA negative and all of the HCV RNA positive cases were HCV-Ab positive also. Since HCV RNA was negative in all of HCV Ab negative patients, it can be suggested that HCV-Ab detection by a reliable ELISA technique in the routine procedure is sufficient to determine HCV infection . On the other hand, low titer positive HCV-Ab cases must be restudied by ELISA, and PCR for HCV RNA confirmation. By using ELISA and PCR methods at the same time would help to confirm the positive results and early diagnosis.

Key words: Hepatitis C virus, polimerase chain reaction, anti-HCV

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Introduction

Hepatitis C virus (HCV) is a blood borne pathogen that is endemic in most parts of the world, with an estimated overall prevalence of nearly 3% (1,2). Approximately 80% patients with hepatitis C virus develop chronic infection, and progression to cirrhosis occurs in nearly 20% of these subjects. Moreover, patients with HCV-related cirrhosis are at an increased risk of developing hepatocellular carcinoma, which is estimated to occur at the rate of 1.5% to 4% per year (3-5). HCV transmission by blood and blood derivatives can always occur and in viral terms there is no safe blood if not the autologous one. HCV transmission, even by repeat donors is possible due

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to laboratory mistakes or to the 10–20 week window period separating anti-HCV detectability from infectivity (6,7). This risk has been estimated as 1 in more than 200 000 in UK (8), and 1 in 103 000 in the USA (9), and in Italy as of 1997 is 1 in 160 000 prior to the advent of HCV-RNA testing which in the future will contribute to a further reduction of HCV infectivity by blood transfusion (7). Diagnosis of HCV infection depends on detecting anti-HCV antibody through serologic methods or on detecting of HCV-RNA with molecular methods. In recent years, the development and widespread use of techniques for detection and quantitation of HCV RNA has provided useful information on viral dynamics during natural history of the infection and antiviral treatment, and has permitted the identification of predictors of response to therapy (genotype, viral load and quasiespecies) (5). The aim of this study was to evaluate the use of serological methods and polymerase chain reaction (PCR) for the diagnosis of HCV infection.

Materials and methods

The study group consisted of 87 patients (51 female and 36 male with a mean age of 42) with chronic liver disease. Anti-HCV was detected by a 3rd generation kit microparticle enzyme immunoassay technique (Axsym, Abbott Laboratories, USA). RNA extraction: Total RNA was extracted from 150 ml of serum using NucleoSpin RNA Virus kit (Macherey-Nagel, Düren-Germany) according to the manufacturer's instructions. Purified RNA was resuspanded in 50 ml of water and then, 5 ml of RNA solution was used for RT-PCR.

Amplification and detection (TaqMan PCR assay): The TaqMan methodology uses a real-time PCR technique to measure PCR product accumulation through a dual-labeled fluorogenic probe (TaqMan probe) (10). The fluorescent signal is generated by means of 5'-nuclease activity that separates a fluorescent reporter dye and quencher dye (11). Briefly, the method uses a dual-labeled fluorogenic hybridization probe that specifically anneals the template between the PCR primers. The probe contains a fluorescent reporter (6-carboxyfluorescein [FAM]) at the 5' end and a fluorescent quencher (6-carboxytetramethylrhodamine [TAMRA]) at the 3' end. When the probe is intact the emission spectrum of the reporter is suppressed by the quencher. The nuclease degradation of the hybridization probe releases the reporter, resulting in an increase in fluorescence emission. The use of a sequence detector (ABI Prism 5700) allows measurement of the amplified product in direct proportion to the increase in fluorescence emission continuously during the PCR amplification. The amplification plot is examined early in the reaction at a point that represents the logarithmic phase of product accumulation. The point representing the detection threshold of the increase in the fluorescent signal associated with the exponential growth of the PCR product for the sequence detector is defined as the cycle threshold (C_T). C_T values are predictive of the quantity of input target; that is, when the conditions of the PCR are the same, the larger the starting concentration of a template, the lower the C_T.

The reaction mixture for RT-PCR was prepared in a single tube as follows: 1× buffer A (50mM KCl, 10mM Tris-HCl, 0.01mM EDTA, 5mM $MgCl_2$, 20pmol of primer C-149, 20pmol of primer C-342, each deoxynucleoside triphosphate (Boehringer) at a concentration of 0.3mM, 0.4U of RNase inhibitor per µl, 0.4U of Moloney murine leukemia virus reverse transcriptase per µl, and 0.025U of *Taq* Gold Polymerase per µl (the enzymes and the buffer containing passive reference were from Perkin Elmer). Fluorogenic probe [FT-275 5'-(FAM)CACCCTATCAGGCAGTACCACAAGGC C(TAMRA)-3'] was added to the PCR mixture to a final concentration of 150nM. Forty microliters of the reaction mixture was added to the PCR tubes containing 10µl of RNA from serum or RNA from a diluted standard that had previously been denatured at 90°C for 90s. Revers transcription was performed at 45 ºC for 30 min followed by inactivation of UNG at 95 ºC for 2 min.

HCV RNA was reverse transcribed into cDNA (30min at 45°C) and amplified by PCR in a single tube for 45cycles (95 ºC for 15 s, at 52 ºC for 25 s and at 72 ºC for 30 s) with specific oligonucleotides ([sense primer], 5'-TGCGGAACCGGTGAGTACA-3' and [antisense primer], 5'-CTTAAGGTTTAGGATTCGTGCTCAT-3'). We used the program PrimerExpress (Perkin-Elmer) to design the primers and probes, following the guidelines for the best performance of the PCR. From among all the possibilities, we have selected the set that fits with the consensus sequences of published HCV 5' NCR and core sequences.

The analytical sensitivity of our method, i.e., the smallest amount of HCV RNA tested and reliably quantified, is 1,000Êcopies/reaction mixture. In

practice, however, we have detected up to 3.3×10^2 copies/ml, because the linearity of our standard curve permits us to interpolate values located below the lower limit of the dynamic range (1×10^3 copies/ml). On the other hand, the system can quantify the HCV RNA in samples with more than 10^7 copies/reaction mixture. This degree of sensitivity along with a considerably wide dynamic range allows the use of a single method for the detection of the wide range of loads found with HCV.

Results

Thirty (34.5%) of 87 patients were HCV RNA positive and 60 (69.0%) were HCV-Ab positive. Fourteen of the HCV-Ab positive patients had low antibody titers (below 2.5 U/ml). None of low titer HCV-Ab positive patients was HCV RNA positive. Thirty (65.2%) of 46 high titer HCV-Ab patients were HCV RNA positive but only 50% of all HCV-Ab positive patients were HCV RNA positive. All of HCV-Ab negative patients were HCV RNA negative. All of the HCV RNA positive cases were HCV-Ab positive also.

Discussion

As early diagnosis of HCV relies on the detection of HCV RNA by nucleic acid testing and as these tests are relatively expensive and not available yet in many routine laboratories, the diagnosis of HCV is usually delayed until antibodies to the virus have appeared. This is on average 70 days after exposure, whereas the viral RNA is detectable in the serum after 2–5 weeks. Furthermore, serological diagnosis in some immunocompromised patients may be complicated, as they are not able to mount an antibody response to the virus (5-7). Detection and quantitation of HCV RNA has become an essential tool for clinical investigation. However, interpretation of clinical trial data may not be accurate without a previous knowledge of technical limitations of the HCV RNA test used. Methods for HCV RNA detection and quantitation should be sensitive, specific, reproducible and accurate (3,12).

The earliest anti-HCV assays had important limitations, notably, a high rate of false-positive and false-negative results (13). To increase both sensitivity and specificity, in these diagnostic assays a greater number of HCV-encoded antigens are now included, allowing more specific antibody detection. Second-generation and now third-generation enzyme-linked immunosorbent assays (ELISAs) (ELISA-2 and ELISA-3, respectively) are also available. The anti-HCV ELISA-3 includes antigens coded by the putative core and NS3, NS4, and NS5 regions of the HCV genome. ELISA-3 is widely used to screen donor blood (14,15), and despite its high specificity (99.7%), false-positive results may occur (16). The positive predictive value of the test depends on the prevalence of HCV antibodies in the donor population, which is very low. Medina et al. were explanied that the HCV EIA 3.0 test with the supplemental confirmatory RIBA 3.0 test may improve the sensitivity for the detection of anti-HCV. Nevertheless, in potentially immunocompromised patients undergoing dialysis, PCR continues to be the only reliable test for detecting viremia (17). In view of this, anti-HCV ELISA reactivity should be tested with a supplemental assay. Recombinant immunoblot assays have been developed as supplemental tests for discriminating between true- and false-positive results for samples repeatedly reactive by ELISAs (13). At present, the serological diagnosis of antibody against HCV remains the most common method of assessing HCV infection. Although a positive result by ELISA and RIBA HCV indicates active HCV infection, it does not distinguish between current infection and previous exposure. Direct detection of HCV would be more useful. However, methodologies aimed at the direct detection of the HCV genome, such as PCR, are expensive and laborious and need appropriate standardization (18,19). It is now accepted that qualitative and quantitative HCV RNA tests will soon find a place in the routine management of HCV-infected individuals. Assays available worldwide, easy to carry out and highly standardized are needed (3).

Although ELISA screening of blood donors is considered and is in the reality highly effective in reducing the risk of HCV transmission by blood transfusion, HCV can be transmitted by seronegative ALT normal blood donors during the so-called window period which may last from ten weeks to several months. To reduce this period testing for HCV-RNA by molecular amplification techniques has been proposed and used in preliminary studies (7). The ABI Prism 5700Êquantitation methodology is used in our laboratory to follow the kinetics of the decrease in viral load in patients treated with alpha interferon, as well as in comparative studies of viral load in serial samples from serum (12). In present study, 30 (34.5%) of our patients were HCV RNA positive and 60 (69.0%) were HCV-Ab positive. All of HCV-Ab negative patients were HCV RNA negative. All of the HCV RNA positive cases

were HCV-Ab positive also. According to our results, 3rd generation ELISA, used for HCV-Ab testing, was very sensitive. But on the other hand, since HCV RNA positivity was detected in none of 14 low titer HCV-Ab positive patients' sera, and furthermore, 14 (34.8%) of 46 high titer HCV-Ab patients were HCV RNA negative, specificity of HCV-Ab test was considered to be low. Sayan et al. indicated that HCV RNA was not detected in samples with low titer positive HCV-Ab. This discrepancy was noted previously as absence of HCV RNA in a proportion of anti HCV positive cirrhotics (5). A fluctuation of HCV RNA, presence of a mutant HCV, or a past HCV infection could explain this occurrence of anti-HCV alone. It is possible that false positive reactions for anti-HCV antibody may occur due to multiple infections encountered in the tropical countries producing hypergammaglobulinemia (5). In addition, false positivity may lead to the unnecessary use of expensive tests like HCV RNA for confirmation (20). As a result, for the confirmation of samples yielding low anti-HCV levels, immunoblot methods, retesting of same sample with another ELISA or testing of a new sample, would be appropriate and economical prior to use of HCV RNA tests.

Although it is infrequent, post-transfusion HCV infection may occur if the donors blood is collected in the window period between exposure and anti-HCV detectability by ELISA testing (3). In routine laboratories RT-PCR seems to be a valuable assay for HCV RNA determination for diagnosis and also following up therapies. It was suggested that by using ELISA and RT-PCR methods at the same time would help to confirm the positive results and early diagnosis. HCV-Ab detection by a reliable ELISA technique only which is the routine procedure is reliable for a safe transfusion that all of HCV-Ab negative patients were HCV RNA negative also. Low titer positive HCV-Ab cases must be first restudied by ELISA and then PCR for HCV RNA confirmation.

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