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EVALUATION OF REAL-TIME RT-PCR QUANTITATION OF HCV RNA COMPARED TO OTHER DIAGNOSTIC METHODS

(With 3 Tables and 3 Figures)

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تقييم تفاعل البلمرة المتسلسل ذو الوقت الحقيقي في التقدير الكمي لفيروس الالتهاب الكبدي الوبائي ومقارنته بالوسائل التشخيصية الأخرى

مختار طـــــــــه ، محمد وائل عبد العظيم محمد

يعتـبر فـيروس الكـبد الوبائي آفة رئيسية في حالات نقل الدم و أمراض الالتهاب الكبدي الأخرى. من محددات البحث في مجال فيروس الالتهاب الكبدي الوبائي نقص في الاختبارات المتخصصـة والحساسـة في قياس كمية الفيروس في بلازما الدم وهذا قد يعزي إلي وجود الفـيروس بنسـخ قلـيلة في الدم و إن جينوم الفيروس مختلف المنشأ لدرجة كبيرة. استخدم العديد من الباحثين وسائل مختلفة لتقدير كمية الفيروس في مرض الالتهاب الكبدي الفيروسي وقـد أسـتخدم حديـثا اختبار تفاعل البلمرة المتسلسل ذو الوقت الحقيقي للبحث والتطبيقات الإكلينيكـية. في هذا البحث تم دراسة ٧٢ مريض تم تحويلهم إلي وحدة البيولوجيا الجزيئية بالجامعـة لـتقدير الفـيروس الكمي بواسطة تفاعل البلمرة المتسلسل ذو الوقت الحقيقي. تم فحص جميع الحالات بصفة مبدئية باستخدام اختبار الأليزا للكشف عن وجود أجسام مضادة للفيروس تمثلت النتائج في وجود ٦ (٣, ٨ %)حالات إيجابية و ٦٦ (٧, ٩١ %)حالة سلبية لوجود الأجسام المضادة في المصل. باستخدام تفاعل البلمرة المتسلسل لتقدير الكمي للفيروس فـي الست حالات الإيجابية للأجسام المضادة أثبتت وجود الحمض النووي للفيروس. بالنسبة لـــ ٦٦ حالة السلبية باختبار الإليزا وجد أن ٤٨(٧, ٧٢ %) حالة تحمل الحمض النووي للفيروس في المصل بكميات عالية ومتوسطة ومنخفضة و١٨ (٣, ٢٧ %)حالة سلبية. أكدت هـذه النـتائج حساسـية وتخصص تفاعل البلمرة المتسلسل ذو الوقت الحقيقي في اكتشاف وتقديـر فـيروس التهاب الكبد الوبائي في مصل الحالات السلبية للأجسام المضادة باستخدام الالـيزا. تـم اختـيار الحالات التي تحمل كمية فيروس منخفضة وعددها ٣٠ حالة للدراسة بواسطة تفاعل البلمرة المتسلسل التقليدي وأوضحت النتائج وجود أربع حالات (٣, ١٣ %) سـلبية بهذا الاختبار بالرغم من وجود الفيروس بكميات منخفضة كما بينت سابقا في تفاعل الـبلمرة المتسلسـل ذوا لوقت الحقيقي. أكدت هذه النتائج أهمية تفاعل البلمرة المتسلسل ذو الوقـت الحقيقي في اكتشاف فيروس التهاب الكبد الوبائي بمقارنته بتفاعل البلمرة المتسلسل

التقليدي ألقت الضوء على صالحة استعماله للتشخيص الدقيق لوجود الفيروس وكميته ومن ثم متابعة تأثير العقاقير على مرضى التهاب الكبدي الوبائي.

SUMMARY

Hepatitis C virus (HCV), a single-stranded RNA virus belonging to the Flaviviridae family, has been identified as a major pathogen of post transfusion and community- transmitted non-A, non-B hepatitis. One limitation in HCV research is the lack of a highly sensitive and specific assay to measure viral loads in plasma or serum. HCV circulates in the blood at a low copy number and its genome is extremely heterogeneous. Many investigators used different methods to quantify the viral load in HCV-infected patients. Recently, a real-time RT-PCR analysis has been employed successfully for both basic research and clinical applications. In the present work, 72 patients were studied on referral to the molecular biology research unit of Assuit university, Egypt for HCV quantification by real-time PCR. Initially, all patients were screened by enzyme linked immunosorbent assay (ELISA) for the presence of anti-HCV antibodies. Six cases (8.3%) were anti- HCV positive and 66 cases (91.7%) were non-reactive for HCV- antibodies in their sera. Real-time RT-polymerase chain reaction assay for quantification of hepatitis C virus (HCV) RNA in the sera of all patients was carried out using a pair of primers and Taq-man probe that are specific for recognsion of highly conservative 5\- non coding region (5\- NCR) of HCV genome. The real-time RT-PCR assay on the HCV 6 seropositive samples yielded reproducible positive data. Real-time PCR quantification analysis of 66 seronegative patients demonstrated 48 positive cases for HCV genome (72.7%) and 18 cases (27.3%) were negative. The results confirmed the sensitivity and specificity of real-time RT-PCR compared to serodiagnostic ELISA technique. Among the 48 positive cases by real-time PCR, 30 cases showed minimal viral load ($< 10^5$ IU/ml), 8 cases showed moderate quantity (up to10^6 IU/ml) and 10 cases showed strong viral load ($>10^6$ IU/ml). Furthermore, the cases with low viral load were selected for qualitative HCV detection by conventional RT-PCR and agarose gel electrophoresis. The results showed 4 (13.3%) negative cases and 26 (86.7%) positive cases. These results also reassured the importance of real-time PCR technology for HCV-detection compared to the conventional RT-PCR and highlighted the validity of applying real-time PCR for the accurate diagnosis of the viral load, hence monitoring the efficiency of therapeutic drugs in hepatitis patients.

Key words: Real-time PCR, ELISA, HCV.

INTRODUCTION

Virological diagnosis and monitoring of HCV detection is based on two categories of laboratory tests, namely serologic assays detecting specific antibodies to HCV (anti-HCV) and assays that can detect quantity or characterize the components of HCV viral particles such as HCV RNA (Pawlotsky, 2002). Early stages of the infection are missed because the antibodies develop only after one and half months of infection and the test for anti-HCV antibody may be negative in the initial period before the seroconversion phase (Lai, 2001).

HCV RNA detection by polymerase chain reaction (RT-PCR) is highly sensitive and is a reliable test in the early diagnosis of HCV infection (Tanaka *et al.,* 2003). Many investigators used different methods to quantify the viral load in HCV–infected patients (Pawlotsky *et al.,* 1996 and Reichard *et al.,* 1998). However most of these methods can produce conflicting results with considerable differences (Raeymaekers, 1993).

Recently, a real-time RT-PCR analysis has been employed successfully for both basic research and clinical application (Heid *et al.,* 1996 and Tyagi and Kramer, 1996). The real-time PCR method solved these Problems with real-time monitoring of the PCR amplification process and greatly improved the PCR- based quantitation of HCV RNA.

The objective of the present investigation is to compare the sensitivity and specificity of real-time PCR quantitative analysis of HCV- RNA in seronegative as well as seropositive ELISA patients. Moreover, conventional RT-PCR was carried out to assess its validity of detecting cases with week viral load on agarose gel electrophoreses.

MATERIALS and METHODS

Samples

Blood samples were collected from 72 patients referred to the Molecular Biology Research Unit of Assuit University, Egypt, for the routine HCV detection by PCR.The samples were collected in clean sterile test tube, left in room temperature for clotting, and then centrifuged at 8000 rpm for 10 minutes. Sera were aspirated and stored at -20C°, for ELISA and RNA extraction.

ELISA

Sera from all patients were screened in duplicates for the presence of anti-HCV antibodies using commercially available ELISA kit (Biokit S.A., Barcelona-Spain). The presence or absence of anti-HCV antibodies in the samples analyzed was determined by relating the absorbance value of each sample to the cut-off value according to the instruction manual.

RNA extraction

Total RNA from the collected sera was extracted using QIAamp Viral RNA Mini Kit (QIAGEN) following the instruction manual. Viral RNA was stored at -20 C^o until used for both qualitative and quantitative analysis.

HCV RNA Quantiation by real-time RT-PCR assay

HCV RNA was quantitated in sera of all patients included in the study using HCV protocol by Brilliant QRT-PCR Master kit, 1-Step on Mx 3000P instrument from Stratagene. A known amount of HCV quantification standard RNA was introduced into each specimen as internal positive control beside the no template, negative control to insure accurate viral load quantitation and absence of sample specific PCR inhibitors. Reverse transcription and amplification were carried out using primers that bind within a highly conserved 5′-non translated region of HCV. For detection of amplification products, the assay utilize real-time PCR technology with two different dual-labeled fluorescent oligonucleotide probes, which are able to bind HCV target amplicon and quantification standard amplicon, respectively, within the regions spanned by the primers. The two different probes for the HCV target and the quantification standard are labeled with two different fluorescent reporter dyes. The reporter fluorescence is suppressed in the intact probe by the proximity of the quencher dye due to inductive-resonance-based energy transfer (Förster-type energy transfer). During elongation, the hybridized dual-labeled oligonucleotide probe is cleaved by the 5′-3′ exonuclease activity of polymerase, leading to the separation of reporter and quencher dyes. Within each cycle during the annealing and elongation phase of PCR, the increasing emission of fluorescence light from such cleaved dual-labeled oligonucleotides is collected independently for the HCV target and quantification standard at different wavelengths. The larger the original HCV RNA amount of a specimen, the earlier the fluorescence of the reporter dye rises above certain assigned fluorescence levels (the critical-threshold value), whereas for the constant titer of quantification standard RNA, the fluorescence of the

reporter dye should appear at the same cycle for all specimens. By comparison of critical-threshold values (CT) obtained for the target HCV RNA and the quantification standard RNA, the original HCV RNA concentration (log quantity) of the specimen is calculated. Thermal cycler profile for real-time PCR is described in Table (1). At the end of each PCR run, data was automatically analyzed by the system and the amplification plots were generated.

Table 1: Thermal cycler profile

Steps	RT Incubation	Taq Activation	PCR (45 cycles)	
	Hold	Hold	Denaturation	Annealing & Extenation
Temperature	50C°	95 C°	95 C°	60 C°
Duration	30:00 min	10:00 min	00:15 min	01:00 min

Qualitative detection of HCV RNA by conventional RT-PCR

This technique was carried out on cases with week viral load quantified by real-time PCR assay using RT-PCR kit from Stratagene that included the following steps according to the instruction manual.

Reverse transcription of viral RNA into cDNA

Briefly 28 ul of extracted RNA was mixed with 3ul random primer (Stratagene), 5ul first strand buffer, 1ul RNase block, 2 ul dNTPs (100mM), 1ul MMLV-RT (20 U/ul) and 10ul d.w. The reaction mixture was incubated at 65C° for 5 minutes then cooled slowly at room temperature. The RT thermal cycler profile was 37C° for 1 hour, 90C° for 5 minutes then hold at 4C°.

Amplification of reverase-transcrptase viral RNA (cDNA)

Five ul of cDNA was used for the first amplification in a reaction volume of 95 ul containing 10 ul 10x buffer, 0.5 ul Taq-polymerase (5 U/ul), 5 ul of each gene- specific primer (10 pmol/ul), that amplify 390 bp of the genome, 0.8 ul dNTPs (100mM) and 73.3 ul d.w. After the first step of amplification consisting of one cycle of 4 minutes at 94 C°, 1 minute at 50 C°, and 2 minutes at 72 C°, we sat up 34 cycles at 94 C° for 1 minute, 50 C° for 1 minute, and 72 C° for 2 minutes. For the second round of amplification 24 cycles of the above conditions were performed. The amplified products were visualized by ethidum bromide staining after electrophoreses on 1.5% agarose gel. Negative and positive controls were included in the PCR reaction.

RESULTS

ELISA

Screening of sera of all tested samples (72 cases) demonstrated 6 out of 72 cases were anti-HCV antibodies positive (8.3%) where the remaining 66 cases (91.7%) were negative for anti-HCV antibodies (Table: 2).

Table 2: Showing numbers and percentages of serodiagnosis.

ELISA	Reactive	Non-reactive	Total
No.	6	66	72
%	8.3	91.7	100

HCV –RNA quantification by real-time PCR

A- ELISA positive cases

The 6 seropositive samples detected by ELISA showed high viral load (>10^6 IU/ml) by real –time PCR assay.

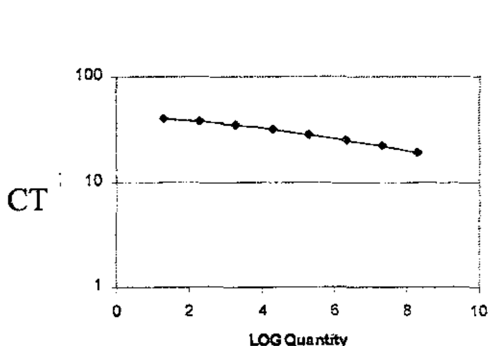
B- ELISA negative cases

Real-time PCR quantification assay of the 66 seronegative cases demonstrated 18 (27.3%) negative for viral HCV RNA and 48 cases (72.7%) were positive for HCV RNA Table(3).

Table 3: Correlation between seronegative cases and their respective real-time PCR results.

Non-reactive ELISA cases	Real-time PCR			
	Positive		Negative	
No.	No.	%	No.	%
66	48	72.7	18	27.3

According to the obtained CT value of the 48 HCV-RNA positive cases, the data was automatically analyzed via the standard curve that measures the log quantity versus the CT (Figure: 1) and categorized the cases into 30 cases of week viral load (< 10^5 IU/ml) 8 cases of moderate viral quantity (up to 10^6 IU/ml) and 10 cases showed high viral load (>10^6 IU/ml) (Figures: 2).



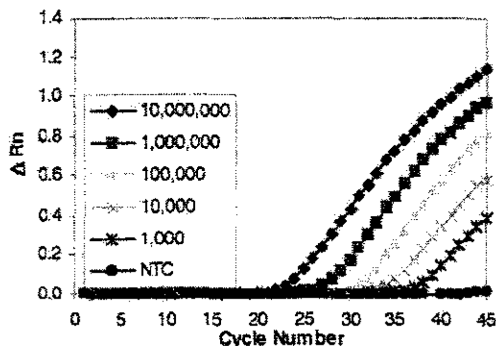


Fig. 1: Standard curve

Fig. 2: Amplification plot of representative samples showing variable amount of viral load

Conventional RT-PCR

Upon examination of agarose gels of 30 samples with low viral load (< 10^5 IU/ml), 4 cases showed no band representing the HCV RNA on the gel while the rest 26 cases showed positive band of 390 bp that represented the amplified PCR products of HCV genome (Figure: 3).

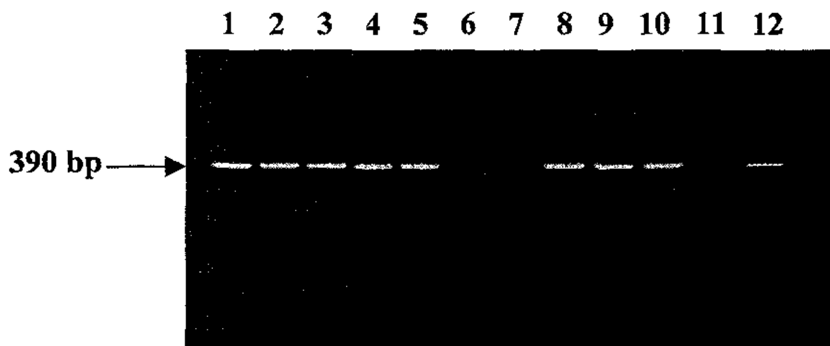


Fig. 3: Ethidium bromide stained agarose gel showing amplified Fragment of 390bp in some cases.
Lane 1-5& 8-10: Positive cases of 390 bp representing the amplified PCR products of HCV genome.
Lane 6&7: Negative PCR products for HCV genome.
Lane 11: Negative control (No template RNA).
Lane 12: Amplified fragments of 390 bp of positive control for HCV genome.

DISCUSSION

In the present work, employing ELISA technique as a serodiagnostic method for detecting anti-HCV antibodies, revealed positive results in 6 cases out of 72 tested samples. Although ELISA can detect mixture of antibodies directed agnist HCV epitobes, its sensitivity is difficult to be determined (Chevaliez and Pawlotsky, 2006).

In our findings, the high number of sreonegative ELISA patients my be attributed to failure to identify HCV infected cases before seroconvresion the specific antibodies have not yet been produced or are in low titer (Reddy, *et al.*, 2006). Ramalingam *et al.* (1998) mentioned that with the use of antibody test alone, a proportional of hepatitis C virus infected individuals is missed while testing of RNA additionally increases the sensitivity of detection. The authors added that the highly prevalence of infected individuals without HCV antibody underlines the need for using PCR on such patients. This may either be due to a low antibody response or early infections.

In our work, the seronegative ELISA cases were further assessed by real-time RT-PCR for the presence of viral RNA load in their sera. Sarrazine *et al.* (2006) mentioned that HCV RNA is the key parameter for management of acute and chronic hepatitis C. Different methods have been developed for commercially available assays for the measurement of HCV RNA in blood samples. Reddy *et al.* (2006) mentioned that polymerase chain reaction amplify HCV RNA before detection by colorimetric measurement using specifically labeled primers or DNA probs. However the different techniques have their restrictions, which have lead to diversification of assays suitable, either for sensitive qualitative or quantitative detection of HCV RNA. Real-time PCR technology has the potential to overcome these restrictions by linear online detection of HCV RNA from very low to extremely high concentrations.

Our results for viral quantitation by real-time PCR revealed that 18 cases (27.3%) were negative for the presence of viral RNA beside the absence of anti-HCV antibodies in their sera. Acute hepatitis C is very unlikely if both anti-HCV antibodies and HCV RNA are absent. It is also unlikely if anti-HCV antibodies are present without HCV-RNA. These patients should however be retested after a few weeks because HCV RNA can be temporary undetectable due to transit, partial control of viral replication by the immunoresponse before replication escapes and chronic infection established (Lavillet *et al.*, 2005).

In our findings we could quantify the viral load in 48 cases that showed no anti-HCV antibodies in their sera by the real-time RT-PCR assay. Thirty cases showed low viral load (<10^5 IU/ml) and 8 cases with moderate viral load (up to 10^6 IU/ml) and 10 cases with high viral load (>10^6 IU/ml). These results proved that HCV-RNA quantitation by real-time PCR is specific, sensitive, reproducible and accurate.

Real-time PCR has been described for viral load monitoring in serum and liver samples (Enomoto *et al.*, 2002 and White *et al.*, 2002) as it was shown that this technology could be used as a very reliable and highly sensitive method to quantify the viral load of HCV in serum (Komurain-Pradel *et al.*, 2001).

In our results, we have detected up to 30 IU/ml in one case because the linearity of our standard curve permits us to interpolate values located below the lower limit of the dynamic range ($1x10^3$ copies /ml). 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. As far as specificity is concerned, the fluorescence signal due to the cleavage of Taq-man florescence probe is generated only if the target sequence for the probe is amplified by the PCR. Therefore, no signal is generated by non-specific amplification. On other hand, the virtual absence of post-PCR manipulation prevents the carryover of amplification products that were synthesized during previous PCR. Real-time PCR is also reproducible.

Thirty cases with low viral load (<10^5 IU/ml) were further selected and screened by conventional RT-PCR for the presence of HCV-band on ethidum bromide-stained gels. Surprisingly, 4 cases out of 30 showed no band on gel representing the HCV amplified fragment. Such finding highlighted the restrictions and limitations of the end point detection of HCV by conventional PCR and agarose gel electrophoresis. It also, reassured that real-time PCR makes quantitation of DNA and RNA much more precise and reproducible because it relays on threshold cycle (CT) values determined during these exponential phase of PCR rather than the end point detection (Yaung ji-Hong. *et al.*, 2002). Therefore, real-time PCR assay is considered the technique of a choice for highly sensitive quantification of DNA or RNA targets (Germer *et al.*, 2005 and Konnick *et al.*, 2005). In real-time PCR, amplification of a PCR product is detected during each cycling rather than the final amount of PCR products accumulated at the end of PCR cycling. The release of a florescent reporter dye from hybridization probe in each cycle during

real-time PCR is proportional to the amount of the PCR product (Heid *et al.*, 1996 and Tyagi and Kramer, 1996).

In conclusion, serodiagnostic testing of HCV patients should be considered as a preliminary screening test for detecting anti-HCV antibodies. Real-time QRT-PCR proved high sensitivity, specificity and accuracy for quantification of HCV RNA in both seronegative and seropsitive patients. Nevertheless, it has the potential to quantify low copy number of the viral load in sera and should be adopted for monitoring the efficiency of antiviral drugs in both acute and chronic hepatitis patients. Also conventional RT-PCR is routinely used for qualitative HCV detection, caution should be undertaken in evaluating the negative cases of HCV RNA.

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