Research Article

BOTANY

Quantitative and Qualitative Evaluation of Hepatitis C Virus Core Antigen (HCVcAg) as an Alternative Diagnostic Marker in Chronic HCV Infection

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KEY WORDS	ABSTRACT
Hepatitis C virus; HCV Ab; qRT-PCR; HCVcAg.	This study compared routine analysis for diagnosing Hepatitis C infection by detecting HCV-Ab using ELISA and HCV RNA using qRT-PCR with the detection of Hepatitis C Virus core antigen (HCVcAg) using ELISA. A total of 40 patients and 10 normal individuals as control were investigated. The data were collected at the time of diagnosis and after the treatment course by Sovaldi, Daklinza, and Ribavirin. At diagnosis, all participants showed positive HCV-Ab and qRT-PCR tests, but about 90% (36 of 40) showed positive HCVcAg. After the treatment course for about six months, all participants showed negative RT-PCR and 73% (29 of 40) showed negative HCVcAg, while all subjects recorded positive HCV-Ab. Three months later, during the repetition of the PCR analysis strategy, nine patients who had received treatment had recovered and showed negative PCR results. Nevertheless, five of the subjects had negative results for HCVcAg, while the other four had positive results. Interestingly, after 6 months, two subjects became positive PCR (relapsed) and four (including the two relapsed patients) became positive core-Ag. Moreover, the correlation analysis showed a positive correlation between RT-PCR data and HCVcAg results. These results suggested a diagnostic marker for detecting HCV infection that might be as accurate as PCR analysis but cheaper, like ELISA.

Introduction

Hepatitis C (HCV) is a significant cause of liver inflammation, and its global prevalence poses a substantial public health threat. The virus belongs to the Flaviviridae family, characterized by its small size, enveloped structure, and positive-sense single-stranded RNA genome (**Ferri, 2015**). HCV is primarily transmitted through blood-to-blood contact, such as intravenous drug use, unsafe medical equipment, and transfusions, with a low risk of sexual or vertical transmission (**Shepard et al., 2005**). Egypt faces a particularly high burden of HCV infection, with one of the highest prevalence rates globally. The World Health Organization estimates that 10-15% of the Egyptian population is infected with HCV, predominantly caused by genotype 4 of the virus (**WHO, 2022**).

HCV is genetically diverse and can be classified into various genotypes and subtypes (**Oluwapelumi et al., 2023**). Different genotypes exhibit varying responses to interferon-based therapy, with genotype 1 generally being less responsive compared to genotypes 2 and 3 (**Simmonds et al., 2005**). As a result, treatment strategies may differ based on the HCV genotype, requiring extended therapy duration or the addition of other drugs like ribavirin or direct-acting antiviral agents (DAAs) to achieve sustained virologic response (SVR) (**Sikavi et al., 2018**). Genotyping also aids in predicting treatment success and guiding therapeutic decisions, as genotypes 2 and 3 often respond more favorably to interferon-based therapy alone (**Clark et al., 2011; Kohli et al., 2014**).

While highly effective antiviral medications have been developed to cure HCV infections and prevent liver damage and associated complications, access to diagnosis and treatment remains limited, particularly in low-income countries. The high cost of medications and inadequate healthcare infrastructure contribute to the global burden of the disease, emphasizing the importance of expanding access to hepatitis C diagnosis and treatment (**Anoushiravani et al., 2023**).

Currently, the two primary markers for diagnosing HCV infection are HCV antibody (HCV Ab) detection using enzyme-linked immunosorbent assay (ELISA) and viral RNA detection using reverse transcription-polymerase chain reaction (RT-PCR). However, both approaches have limitations and drawbacks. HCV Ab detection exhibits low sensitivity in early infection stages, cannot differentiate between recovered

patients and those with active infection, and may yield false negative results in certain conditions like hemodialysis and immunosuppression (**Lai, 2001; Medhi et al., 2008**). RT-PCR, although considered the gold standard for diagnosing HCV infection, is expensive and requires skilled operators to prevent contamination (**Aoyagi et al., 1999**).

This study aims to evaluate the potential of Hepatitis C Virus core antigen (HCVcAg) as a new routine diagnostic marker for HCV infection, comparing it to the current significant markers, HCV Ab and RT-PCR. The HCV core protein, a highly antigenic component of the virus, plays a crucial role in its pathogenicity and induces the production of antibodies early in the infection (**Jolivet-Reynaud et al., 1998; Lai, 2001**). Furthermore, the core protein is encoded by one of the most conserved regions of the viral genome (**Bukh et al., 1994; Komatsu and Takasaki, 1999**). The results obtained from both HCVcAg detection and RT-PCR analysis were found to be consistent. HCVcAg detection overcomes the aforementioned limitations and provides rapid, accurate results at a lower cost compared to the RT-PCR method. Therefore, this study provides valuable insights into improving the diagnosis and management of HCV infection.

Materials and Methods
Collection of clinical blood samples
Blood samples (3 ml) were collected into anticoagulant-treated tubes (EDTA-treated) from patients of El-Mahalla liver hospital under sterilized conditions (ethical approval code: 36264PR184/4/23). The samples were centrifuged at 3000 rpm for 10 min at 4°C (G.T. 10-1, Beijing Era Bili, China). The red blood cells free supernatant is designated as plasma. Plasma samples were immediately transferred to an icebox for immediate analysis or preserved for future analysis at -20°C (**Saldanha et al., 2005; Thavasu et al., 1992**).

Detection of HCV Ab using ELISA
The clinical blood samples were assayed for HCV-Ab using an ELISA complete kit (BIOREBA, Switzerland) according to the manufacturer's protocol. Plasma diluent (180 μL) was added into each well of the microtiter plate, and 20 μL of the sample was added to the wells; additionally, a negative and positive control were added to the designated wells after dispensing the samples. The samples and control were thoroughly mixed with the sample diluent. The wells were covered with a lid and incubated for 1 h at 37°C. At the end of the incubation period, the plate was washed using an automated strip washer (Biotech Instruments, Inc., USA). The

automated strip washer was adjusted, so the fill volume was 500 µL/well from washing buffer (phosphate-buffered saline [PBS]). Five wash cycles, using working strength wash fluid, were performed. The dispensed height was set to give a positive meniscus on each well (without causing overflow). Then 100 µL of the conjugate (HRP-Streptavidin Conjugate) was added immediately to each well. The wells were covered with a lid and incubated for 30 min at 37°C under humid conditions. At the end of the incubation period, the plate was washed as described above. Next, 100 µL of substrate solution (tetramethylbenzidine [TMB]) was added to each well immediately after washing the plate. The wells were covered with their lids and incubated for exactly 30 min at 37°C. The plate was kept away from sunlight. A purple color developed in the wells containing the positive sample. Then 50 µL stop solution (2 N Sulphuric acid) was added to each well. Within 15 min, the absorbance of each well was read at 450 nm (**Aach *et al*., 1991**) using the ELISA reader (Infinite 50, Tecan, Austria).

Determination of HCV RNA level using real-time PCR method

Extraction of total RNA from plasma; about 200 µL from each collected plasma sample was added to 1.5 ml nuclease-free Eppendorf tubes.

Next, 200 µL of freshly prepared working solution (carrier RNA-supplemented binding buffer) was added to each tube and mixed with 50 µl proteinase K solution immediately. The tubes were incubated for 10 min at 72°C. Subsequently, 100 µL of binding buffer was added to each tube and mixed well. The samples were pipetted into QIAprep Spin Miniprep Columns and centrifuged for 1 min at 8000 rpm. The filtrates were discarded, 500 µL of inhibitor removal buffer was added, and then the columns were centrifuged for 1 min at 8000 rpm. The filtrates were discarded, and 450 µL washing buffer was added to each column and centrifuged for 1 min at 8000 rpm. This step was repeated three times to ensure the purity of the extracted viral nucleic acid. The columns were centrifuged at 13000 rpm for 1 min to remove any residual washing buffer. The columns were then transferred to 1.5 ml nuclease-free Eppendorf tubes, and then 50 µL of elution buffer was added and centrifuged for 1 min at 8000 rpm to elute the viral nucleic acids. The eluted nucleic acids were used directly for qRT-PCR. The qRT-PCR was carried out using the Cobas Amplicor Auto-Analyzer (Roche Molecular Systems, USA) and the Qiagen one-step RT-PCR kit (Qiagen, Germany), following the manufacturer's instructions. The kit contains optimized components that

facilitate a one-step reaction for both reverse transcription and PCR amplification. The reaction employed specific primers, 5'-AGACGTATTGAGGTCCATGC-3' (sense) and 5'-CCGCAGCGACGGTGCTGATAG-3' (antisense), designed based on core region sequences of all HCV genotypes to amplify an NS5A region. Additionally, human GAPDH gene expression levels were measured as an internal control using the primers 5'-GCCATCAATGACCCCTTCATT-3' (sense) and 5'-TCTCGCTCCTGGAAGATGG-3'.

Real-time PCR was performed on Ampliprep real-time [(CAP-CTM), Qiagen, Germany] in a thermal cycling profile with 15°C for 15 s, 95°C for 10 min, then 45 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 10 s.

Determination of HCVcAg using ELISA

Inactivated sample (100 ul) or HCVcAg (Qualitative, MyBioSource, USA) standard was added to the microtiter plate, covered, and incubated at 37°C for 2 h. The wells were washed five times with 250 μL washing buffer/well. Next, 100 μL of the diluted HRP-Conjugated Anti-FITC monoclonal antibody was added to all wells, covered, and then incubated at room temperature (23 – 27°C) for 1 h on an orbital shaker. After the covers were removed, the wells were washed 5 times with 250 μL washing buffer/well. Then 100 μL of the substrate solution was added to each well, including the blank wells. The plate was incubated at room temperature on an orbital shaker for 10-20 min. Finally, 100 μL of stop solution was added to each well, including the blank wells and the result was detected on an ELISA reader at 450 nm.

Statistics

All statistical analyses were performed and plotted using GraphPad Prism® (Prism 6 for Windows) v6.01, 2012 (© 1992-2012 Graph Pad Software Inc., California, USA). (Cut off calculation = Mean of negative results in control group + 3 (standard deviation) = 0.13 + 3 (0.04) = 0.25).

Results

HCV diagnosis using ELISA, and RT-PCR

Traditionally, ELISA and RT-PCR were used to detect HCV antibody and HCV-RNA levels in the serum of the selected subjects. In addition, a possible trial for detecting HCVcAg in the serum of all participants was estimated as an alternative diagnostic marker for HCV infection. All subjects with chronic infection with HCV showed positive results with a highly significant level of both HCV-Ab and HCV-RNA compared to the normal subjects. HCVcAg was

detected in 36 patients out of the 40 selected patients with highly significant levels compared to the normal subjects. After the treatment course, the HCV-RNA level was not detected in all participants; however, HCV-Ab was still positive. Most of the core-Ag results, 29 of 40 patients, were negative, with a highly significant difference from the results before the treatment course. Results obtained from core-Ag mostly match PCR results but not with ELSA data **(Fig. 1, 2, and 3)**. After completing the treatment and during the repetition of the PCR analysis strategy, 3 months later, nine patients of the treated subjects recovered and had negative PCR data, but 5 subjects showed negative HCVcAg, and the other four showed a positive result. Interestingly, after 6 months, two of these four subjects became positive PCR (relapsed), data not included. These data suggest the high accuracy of using core-Ag as a diagnostic marker of HCV infection.
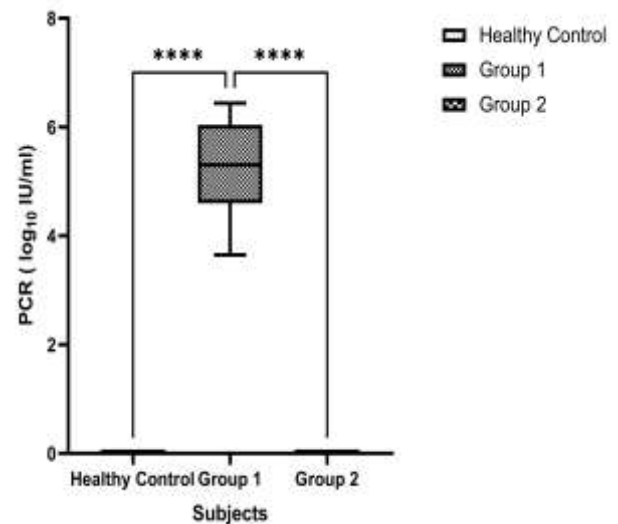

Fig. (1): HCV-viral load (log value) in blood samples of HCV patients compared with the control group. Asterisks (***) denote high significant difference compared to the control group, ANOVA test, and P value < 0.001.
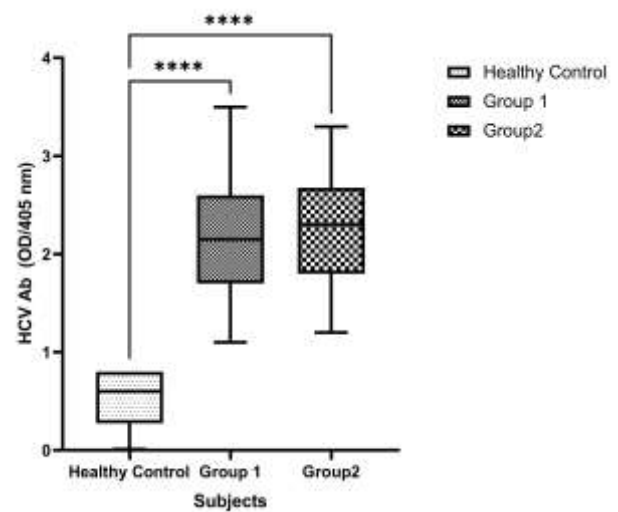

Fig. (2): HCV Ab in blood samples of HCV patients compared with the control group. Asterisks (***) denote high significant difference compared to the control group, ANOVA test, and P value < 0.001.
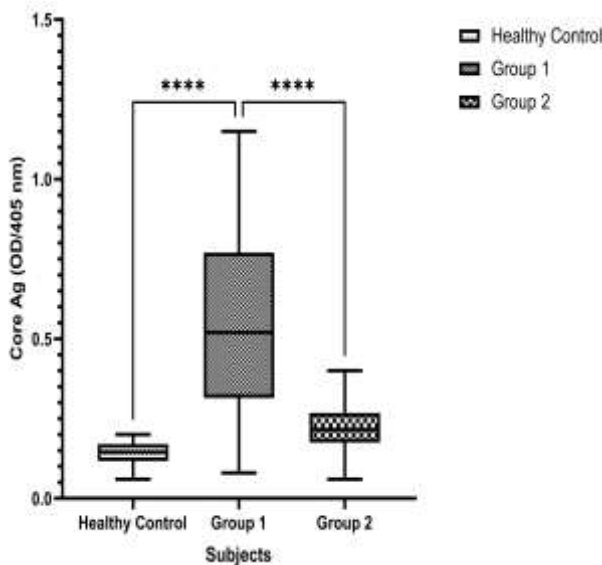
Fig. (3): HCV core Ag in blood samples of HCV patients compared with the control group. Asterisks (***) denote high significant difference compared to the control group, ANOVA test, and P value < 0.001.

Fig. (4): Heat map showing the correlation between different variables; Blue colors represent a positive correlation, and red colors indicate a negative correlation; the intensity of the colors indicates a strong correlation.

Correlation analysis among all detected parameters

The person's correlation coefficient analysis was done among all detected parameters. A significant positive correlation was observed between HCV-RNA, HCVcAg, and IL-10 levels (**Fig. 4, and 5**). This analysis supports the previous results, which suggested using the detection of HCVcAg as an alternative diagnostic marker with high accuracy.
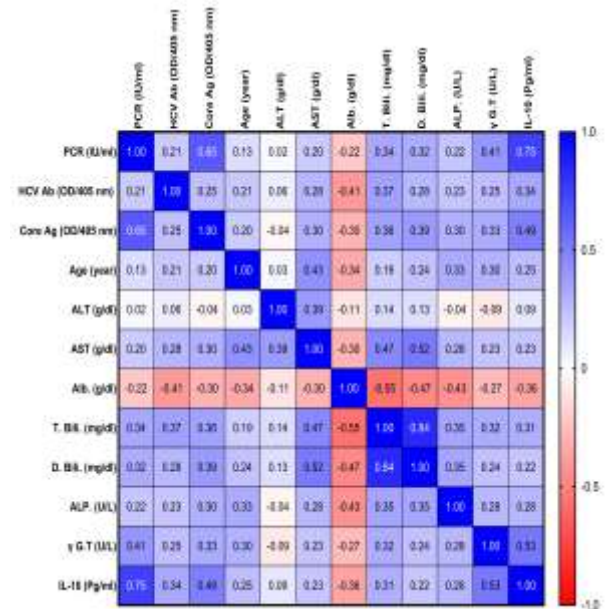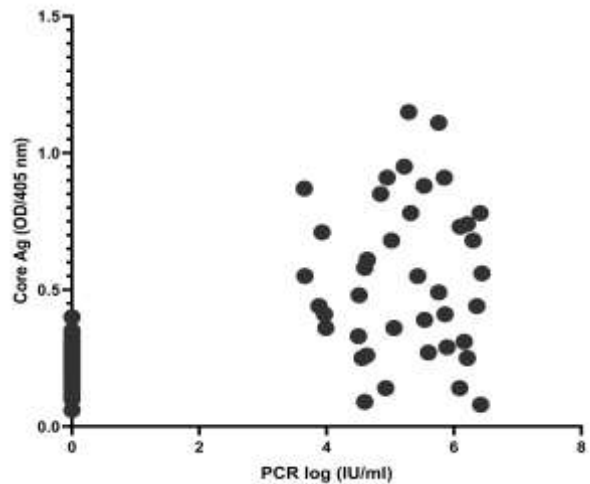

Fig. (5): Correlation analysis between HCV viral load (PCR log values) and Core Ag levels in blood samples of all participants showed a significant positive correlation using Pearson r analysis; r value equal 0.6471, 95% confidence interval is 0.5081 to 0.7533, and R squared value is 0.4188.

Discussion

Early diagnosis of HCV infection is important to prevent disease progression and transmission to others. One approach to diagnosing HCV infection is to detect the presence of viral antigens or antibodies in a patient's blood sample **(Gretch, 1997)**. Enzyme-linked immunosorbent assay (ELISA) is a commonly used method for detecting HCV antibodies **(Swellam et al., 2011)**. However, antibodies can take several weeks to develop after infection, so ELISA may not detect early-stage infections. In this study, we investigate a possible newer approach to detect the presence of HCV core antigen (HCVcAg) in chronic hepatitis C patients using ELISA. HCVcAg is a viral protein that is present in infected cells and can be detected in patient blood samples within a few days of infection **(Chakravarti et al., 2013)**.

Identifying HCVcAg using ELISA as a new diagnostic marker for HCV infection was compared to routine analysis for diagnosing hepatitis C infection by detecting HCV-Ab using ELISA and HCV RNA using RT-PCR. We investigated 40 hepatitis C patients and 10 normal controls: with negative RT-PCR and HCV Ab results. The data were collected during and after the combination treatment with Sovaldi,

Daklinza, and Ribavirin. All subjects had positive RT-PCR and HCV-Ab tests at the time of diagnosis, however, only 90% (36 of 40) had positive HCVcAg results. After undergoing treatment for six months, all patients had negative RT-PCR results, and only 73% (29 of 40) had negative HCVcAg results, whereas all subjects showed positive HCV-Ab **(Fig. 2)**. Data obtained from detecting HCV-Ab is not accurate as using HCVcAg. This is because HCV Ab has high false positive results and cannot differentiate between recovered patients and those with an active infection **(Alzahrani, 2008)**. In addition, HCV Ab analysis gives false negative results in the case of some diseases, such as hemodialysis and immunodeficient patients **(Aoyagi et al., 1999)**. During the routine repetition of PCR analysis after 12 months of treatment, HCVcAg was also repeated for nine patients. Two out of nine patients had positive PCR (relapsed), and the other seven patients still had negative PCR. On the other hand, four out of nine patients (including the two relapsed patients) had positive core-Ag. These results proposed high accuracy of core Ag in detecting HCV infection. An earlier study found that in the early stage of HCV infection, the patients may have low viral load by PCR (<104), and HCVcAg may not be detected **(Blatt et al., 2000; Reddy et al.,**

2006). These findings matched with the results of this study, where some patients showed low viral load by PCR, less than (<104), at the time of diagnosis and represented non-detectable results of core Ag. Noteworthy, in contrast to the previous findings, two patients showed low viral load (<104) three months after completing the treatment course, but core Ag was detected. Interestingly, these two patients showed positive PCR and positive core Ag during the routine repetition of PCR after 12 months of treatment. These results could give a prospect to relying on HCVcAg for detecting HCV infection in an early stage. HCVcAg assay helps monitor immunocompromised patients and those with hemodialysis (**Medhi *et al.*, 2008**). In 1997, during a study on transgenic mice in Japan, the researchers demonstrated that the core protein of HCV has been suggested to have a transcriptional regulatory function and, thereby, to be involved in inducing phenotypic changes in hepatocytes. Two independent transgenic mice lines carrying the HCV core gene were established by microinjection of the core gene into mice. These two mice developed progressive hepatic steatosis (fatty change of hepatocytes), which characterizes HCV infection (**Moriya *et al.*, 1997**). These results support the need to use HCVcAg as a routine method in the detection of HCV infection. The correlation analysis also revealed a direct association between the RT-PCR data and the HCVcAg outcomes. Therefore, a direct association between the RT-PCR data and the HCVcAg outcomes means that when the RT-PCR test results show a high level of HCV RNA, there is a higher chance of detecting HCVcAg protein in the blood. This correlation could be useful in monitoring the progression of HCV infection and evaluating the effectiveness of treatments. Generally, the results of this study showed high sensitivity and specificity of HCVcAg in the detection of hepatitis C infection; it also confirmed that HCVcAg could differentiate between the recovered subjects and those with an active infection. These findings raised the possibility of a novel diagnostic marker for HCV infection detection that might be as precise as PCR analysis but less expensive, like ELISA.

Conclusions

HCVcAg analysis using ELISA is an accurate test in the diagnosis of HCV infection and for recognizing an active infection in a patient who has recovered. It is also helpful in monitoring immunocompromised patients and those with hemodialysis, unlike the HCV Ab test, which gives high false positive and

negative results. HCVcAg detection using ELISA is a rapid method as well. It does not need a lot of training. In addition, it is less expensive than PCR. In the future, the detection of Hepatitis C infection using HCVcAg would likely be established in clinical laboratories as a routine analysis instead of HCV Ab, especially for hepatitis infection in countries like Egypt.

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التقييم الكمي والنوعي للمستضد الأساسي لفيروس التهاب الكبد الوبائي سي (HCVcAg) في العدوى المزمنة بفيروس التهاب الكبد الوبائي سي

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هدفت هذه الدراسة إلى مقارنة التحليل الروتيني لتشخيص الإصابة بفيروس التهاب الكبد الوبائي سي عن طريق كشف HCV-Abباستخدام ELISA وكشف HCV RNA باستخدام RT-PCR مع تحليل المستضد الأساسي لفيروس التهاب الكبد الوبائي سي (HCVcAg) باستخدام ELISA كعلامة تشخيصية جديدة محتملة. تمت دراسة أربعين مريضًا وعشرة أفراد أصحاء الذين أظهروا اختبار سلبي لـ HCV Ab واختبار سلبي لتحليل RT-PCR. تم جمع البيانات في وقت التشخيص وبعد العلاج باستخدام سوفالدي وداكلينزا وريبافيرين. في وقت التشخيص، أظهر جميع المشاركين اختبارات إيجابية لـ HCV-AbوRT-PCR، ولكن حوالي ٩٠٪ (٣٦ من ٤٠) أظهروا نتائج إيجابية لـ HCVcAg. بعد العلاج لمدة حوالي ستة أشهر، أظهر جميع المشاركين اختبارات RT-PCR سلبية وأظهر ٧٣٪ (٢٩ من ٤٠) اختبارات HCVcAg سلبية، في حين سجلت جميع الحالات اختبارات إيجابية لـ HCV-Ab. بعد ثلاثة أشهر، أثناء تكرار استراتيجية تحليلRT-PCR ، تعافى تسعة مرضى تلقوا العلاج وأظهروا نتائج سلبية لهذا التحليل، ولكن كانت نتائج اختبار HCVcAg سلبية لخمسة فقط من هؤلاء التسعة وإيجابية للأربعة الباقين. ومن المثير للاهتمام، بعد ٦ أشهر من هذا التحليل أصبح شخصان من الأربعة إيجابيان لـRT-PCR مع تسجيل نتائج إيجابية للأربعة لتحليل HCVcAg. علاوة على ذلك، أظهر تحليل الارتباط الإحصائي وجود ارتباط إيجابي بين بيانات RT-PCR ونتائج HCVcAg. تشير هذه النتائج إلى وجود علامة تشخيصية جديدة لاكتشاف الإصابة بفيروس التهاب الكبد الوبائي سي قد تكون متساوية الدقة مع تحليل RT-PCR ولكن أرخص تكلفة مثل تقنية ELISA.