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Comparison Study of Two Serological Commercial Assays with Quantitative Real-Time PCR for Diagnosis of Hepatitis B Virus among Hepatitis Patients at Al-Ramadi City.

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ABSTRACT

HBV DNA quantitation is used extensively worldwide for the diagnosis and monitoring of treatment of Hepatitis B virus (HBV) infection. The aim of this study was to quantitate HBV DNA by Real time – PCR technique in patients with hepatitis B infections. Also to compare the results with serological markers of HBV detection by dipstick assay and by ELISA test. One hundred plasma samples of Hepatitis B patients (positive for hepatitis B surface antigen) using dipstick assay confirmed by ELISA test) were categorized into different groups according to their serological profile and the subjects of study were conducted to detect the presence of HBV DNA in blood samples using RT-PCR. Twenty specimens were taken from apparently healthy persons served as a negative controls. The amplification detection was carried out by using Smart Cycler II analysis software version 2.0 - sequence detector. HBV DNA was detected upon amplification in 86/100 (47 males and 39 females) patients positive for HBsAg. HBsAg positive patients showed different viral load depending on the presence or absence of other serological marker (HBsAb, HBeAg, HBeAb and HBcAb) of HBV in their plasma samples. Detection of HBV DNA by Real-time PCR technique serves as an important tool besides serology in a number of clinical settings, especially in determining low levels of viremia in patients with non-replicative HBV disease and chronic hepatitis. HBeAg status did not necessarily reflect HBV-DNA level in the serum, as 76/90 of Hepatitis B group, were positive for HBV DNA but negative for HBeAg and three from ninety patients negative for HBeAg reported to have viral load of more than (100 million virion/ml).

Introduction

Hepatitis B virus (HBV) infection is an important global health problem and may cause both acute and chronic infection in man as more than 2 billion people alive today have been infected with HBV [(1)]. The clinical spectrum of HBV infection ranges from subclinical to acute symptomatic hepatitis or, rarely, fulminant hepatitis during the acute phase and from the inactive hepatitis B surface antigen (HBsAg) carrier state, chronic hepatitis of various degree of histologic severity to cirrhosis and its complications during the chronic phase [(2)].

Detection of serological markers is the mainstay of diagnosis of HBV infection and Hepatitis B surface antigen (HBsAg) is the hallmark of HBV infection [(3)]. HBeAg is generally used as secondary marker to indicate high levels of virus in the blood [(4)]. The monitoring of hepatitis B virus DNA in serum is as

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important as serological markers in predicting the clinical outcome of infection. More recently molecular diagnostic methods have been used to quantify the levels of HBV DNA in serum as a marker of viral replicative activity [5]. Quantification of HBV DNA can be useful to assess the efficacy of antiviral therapy as a more direct method of detecting viral replication than HBV serologic markers [6,7].

The introduction of PCR-based methods has resulted in a large increase in the sensitivity of HBV DNA detection [8]. Further the development of real time PCR methodology has been improved the ease with which HBV DNA levels can be monitored and has increased the range over which such levels can be accurately quantified [9,10]. In the present study, a sensitive real time PCR assay was used and validated for the detection and quantitation of HBV-DNA in certain clinical situations.

The detection occurred via the fluorescent labeling of oligonucleotide probes that bind specifically to the PCR amplicate. Detection of the fluorescence intensity during the course of real time PCR enables verification as well as quantification of the product. The aim of the study was firstly to quantitate HBV-DNA by real time PCR method in Hepatitis B patients, secondly to compare the results of HBV-DNA estimation with HBV serological markers.

Materials and Methods

Study patients:

One hundred HBsAg positive plasma samples were randomly collected from acute and chronic HBV infected patients with age range from (4-65) years old in Ramadi city. These patients attended Al-Ramadi Teaching Hospital and private laboratories during the period extended from November 2011 to August 2012. All these patients were initially positive for HBsAg, underwent for HBV DNA amplification and various serological markers. The results obtained categorized into different groups on the basis of serological markers.

Controls:

Twenty plasma samples were taken from apparently healthy persons served as negative controls for the study.

Serological markers :

The plasma of HBsAg positive patients confirmed by ELISA test, by used HBsAg ELISA kit of Biotech company. 50 µL of HBsAg Positive and Negative Control added into the designated control wells and 50µL of test specimens into each test well then 50 µL of the HRP-HBsAb conjugates added to each well, except the blank well. Incubated at 37°C for to 60 minutes. The incubation mixture was removed carefully and each well filled with diluted wash buffer and shaked gently for 20-30 second.

The wash solution discarded completely. This step repeated 4 more times. 50 µL of TMB substrate A and 50 µL of TMB substrate B added into each well. The wells incubated at 37 ºc in dark for 15 minutes. The reaction stopped by added 50 µL of stop buffer and mixed for 30 sec. The microplate reader wavelength set at 450 nm.

HBV serological marker detection by Chromatographic immunoassay (A rapid, one step test for the qualitative detection of HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb in serum or plasma by using HBV combo test device). 3 full drops of serum or plasma (approx. 75 µL) transferred to each specimen well(s) of the test device respectively. The positive result was reflected by the appearance of red line in the test line for (HBsAg, HBeAg and HBsAb) and disappearance of red line in the test line for (HBeAb and HBcAb).

Molecular study:-

DNA extraction:

DNA was extracted from 150 µl of plasma with DNA extraction kit (Ribo-Sorb kit, Sacace, Italy) using the silica based technology according to the manufacturer's instructions, using fluorescent reporter dye probes specific for HBV and HBV Internal Control. Briefly, patient's plasma were subjected to lysis at 70^{o}C with 600µL lysis buffer and 20µL of protease reagent. The DNA was extracted from the lysate using 600µL absolute ethanol and subsequently purified using spin columns. Finally purified DNA was eluted from the spin columns using 50 µl RNase-free H_2O. The extracted DNA was subjected to amplification.

Amplification and quantitation:

Amplification was carried out using Real Time kit for the quantitative detection of hepatitis B virus in human plasma (Sacace biotechnologies, Italy). Amplification mixtures comprised of 300µL RT-PCR-mix-1-TM; 200µL of RT-PCR-mix-2-TM and 20µL of Hot Start Taq Polymerase. 16 PCR tubes Prepared, Two for Positive Controls, one for negative control, three for HBV Standard (QS1 HBV, QS2 HBV, QS3 HBV), three Internal Control Standard (QS1 IC, QS2 IC, QS3 IC) and seven for test samples. 12,5 µl of Reaction Mixure added into each tube. 12,5 µl of extracted DNA sample added to the appropriate tube with Reaction Mixture and mixed by pipetting. 12,5 µl of controls and standards added to the appropriate tube with Reaction Mixture. The tubes closed and transferred into the thermalcycler. DNA amplification was carried out in Smart Cycler II instrument (Cepheid). The amplification was performed as follows: initial hot start denaturation at 95°C for 15 min, followed by 42 cycles of denaturation at 95°C for 20 sec., annealing and extension at 60°C for 40 sec. Real-time monitoring was achieved by measuring the fluorescence at the end of the extension phase for each cycle. The quantitative analyses were conducted by using Smart Cycler II analysis software version 2.0 following the manufacturer's instructions (Cepheid). The concentration of HBV DNA for each control and patient specimen, calculated using the following formula:

HBV DNA IC DNA × coefficient*=copies HBV/ml

***** coefficient for this kit = 4.5×10^5.

RESULTS:

Males showed high rates of HBV infections (54/100) than females (46/100) and the higher HBV infections in patients within middle age group (25-44) years old while the lowest rate of infected persons was in the age group (1-14) years (see table 1 and Figure 1).

Serologic testing for the diagnosis of hepatitis B virus infection involves measurement of a panel of distinct HBV-specific antigens and host antibodies that react to these antigens. The interpretation of these tests can be complicated, and multiple possibilities exist based on the overall panel of responses. In general, the panel of responses can determine whether a patient is susceptible to infection, immune as a result of resolved infection, immune as a result of vaccination, acutely infected, or chronically infected (table 2).

Regarding Real Time PCR the standard curve was generated by using the Smart Cycler II software and serial dilution of know DNA concentration for HBV DNA and HBV Internal Control (IC) using fluorescent reporter dye probes specific for HBV and HBV IC in order to quantitatively measure the number of viral DNA (copy number/ml) of the patients' blood (fig.2 and fig. 3).

The calibration curve was linear in a range of the six different DNA concentrations used to generate the standard curve (figure 4), with an R^2 value of {1}. Good correlation was found between the current study procedure and the standard SACACE HBV monitor test, with all 120 samples included in the study. Of the one hundred positive samples screened by ELISA of Hepatitis B group patients, HBV DNA detected in 86 (47 males and 39 females) using RT-PCR, whereas 14 (7 males and 7 females) were showed negative RT-PCR result (Fig. 5).

The mean concentration of HBV C/ml (plasma HB viral loads) in HBsAg +ve males plasma samples was (12391858.23) with Stander Deviation of (31336290.86), while the mean concentration of HBV C /ml in HBsAg +ve female plasma samples was (8653221.36) with Stander Deviation of (26942717.09) using RT-PCR (Table 3).

In this study the results of T test showed no significant difference was detected between RT-PCR results and the sex ;T test(0.69), ($P<0.01$).

Patients reveled different viral load according to their ages. The maximum mean of viral load (39338838.47 c/ml) was reported in group of patients between (15-24) years old, while the minimum mean of viral load (1107.60 c/ml) was reported in group of patients between (1-14) years old (fig.5).

HBsAg positive patients showed different viral load depending on the presence or absence of other serological marker (HBsAb, HBeAg, HBeAb and HBcAb) of HBV in their plasma samples. HBsAb was present in 5% of patients plasma samples, the mean of HBV load was (138630.40 c/ml) lower than the viral

load of HBsAb negative (13441749.17 c/ml) reported in 95% of patients plasma samples. HBeAg positive patients were 10% and they showed higher mean of viral load (86658616.70c/ml) than HBeAg negative patients (4567479.51 c/ml). The mean of HBV loads for HBeAb positive plasma samples was (4058827.92 c/ml) showed in 90% of patients, it was slightly lower than the mean of HBV loads for HBeAb negative plasma samples (40382850.04 c/ml). The absence of HBcAb was associated with high replicative rat of the virus and the mean of the viral load was more than 100 million copies/ml reported in 3% of patients, while the mean of viral load was (10078959 c/ml) in HBcAb positive plasma samples as shown in the (fig. 6).

Discussion

A number of sensitive and specific diagnostic tests have led to a deeper understanding of the natural history of HBV infection [(11)]. Serological markers are indispensable in the diagnosis of HBV infection. Hepatitis B surface antigen (HBsAg) is the hallmark of HBV infection and is the first serological marker to appear in acute hepatitis B, and persistence of HBsAg for more than 6 months suggests chronic HBV infection [(3)], but HBsAg does not provide information about active virus replication [(12)]. Moreover the presence of serum HBV DNA in chronic hepatitis patients indicates active virus replication. HBV DNA levels are detectable by 30 days following infection[(11)].

In our study, males showed the high rates of HBV infections than females and the higher HBV infections in patients within middle age group (25-44) years old was coincides with results obtained by Chu and Liaw., and Khan *et al*. [(13,14)]. This was probably due to occupational and other risk factors associated with the exposure of males.

HBV DNA was quantitated detected upon amplification in 86 from 100 patients positive for hepatitis B surface antigen using RT-PCR. The chronic hepatitis patients were divided into patients with replicative HBV disease (as determined by the presence of HBeAg) and patients with non-replicative HBV infection (defined by the absence of HBeAg). Patients positive for HBeAg or having replicative HBV disease tend to have more severe liver disease.

HBeAg was detected in 10% of HBsAg-positive individuals. This result was in agreement with Bonino *et al*. and Wu *et al*.[(15,16)] who found similar results. The presence of HBeAg is usually associated with the detection of HBV DNA in the sera of infected patients. Therefore, HBeAg is generally regarded as a marker of viral replication and greater infectiousness. At the same time we found that three from ninety patients negative for HBeAg included in our study reported to have viral load of more than (100 million virion/ml), these results might be due to infected with a pre-core mutant strain that prohibits the synthesis and secretion of HBeAg with high concentrations of HBsAg and HBV DNA [(17)]. We are aware that this needs to be further confirmed by DNA sequencing.

The results of the present study have shown that from eleven patients with viral load more than (100 million virion/ml), eight of them positive for HBeAg, and all patient with undetectable HBV DNA were negative for HBeAg. In spite of being potentially infectious, some individuals remain HBeAg negative. Our study have been showed that the patients with non-replicative HBV disease (as determined by the absence of HBeAg) the positive rate of HBV DNA as amplification by Real Time PCR was 88% (78/86). Baker *et al*. [(18)] was reported that 78% positive rate of HBV DNA by PCR in non-replicative HBV disease.

In this study, there was a significant difference ($P<0.01$) between the viral load of HBeAg positive patients with mean of (86658616.70 virions/ml), and the viral load of HBeAg negative patients with mean of (4567479.51 virions/ml). Furthermore from 90 patients with HBeAg negative, HBV DNA was not detected in15.5% (14/90).

The proportion of anti-HBe positive was 76% from the patients included in this study and (1:3) from the controls whom have previously infected. All patients have anti-HBe positive were negative for HBeAg and Vice versa, because anti-HBe was became detectable soon after the time that HBeAg disappears [(19)].

In our studies 83% of anti-HBe patients have showed detectable DNA using PCR, but about 93% of undetectable DNA patients have anti-HBe in their blood. Previously, investigators believed that HBV DNA disappeared in all patients with the onset of anti-HBe,

but older studies had used the less sensitive HBV DNA hybridization assays [20,21]. Newer, more sensitive PCR assays have shown that greater than 70% of persons who develop anti-HBe have persistent HBV DNA [21,22].

The coexistence of HBsAg and anti-HBs has been reported to occur in about 5% only of HBsAg-positive individuals during present study. The mechanism underlying the presence of both HBsAg and anti-HBs despite viral replication is unknown. Early reports indicated that anti-HBs in such patients may be specifically directed to HBsAg subtypes different from the coexisting HBsAg. Because of technical limitations, no sequence analysis of HBV isolates in patients was performed in these early studies [23]. Later reports suggested that the presence of anti-HBs may drive the selection of HBsAg escape mutants in chronic hepatitis B virus carriers due to the sole pressure of the host immune system. It has been shown that amino acid substitutions may occur in the a-determinant of HBsAg of HBV isolates and reduce or abolish the binding of anti-HBs to HBsAg, leading to immune escape [24].

Anti-HBc is the first detectable antibody in the course of HBV disease. Anti-HBc was detected in 97% of HBsAg-positive individuals with varying frequencies dependent on the prevalence of HBV infection. IgM anti-HBc indicates acute infection and is the only serologic marker detectable during the "window period," when neither HbsAg nor anti-HBs is detectable. Patients with resolved infection have persistence of anti-HBc for life, but about 4-6 months after the appearance of anti-HBc, the total anti-HBc predominantly consists of IgG[25].

Finding in the present study was showed that (3%) of anti-HBc negative patients have viral load more than 100 million copies per ml, and other serological marker reported with those patients were HBsAg positive and HBeAg positive suggested that those patients were with acute infection and highly replicative viruses because anti-HBc is the first antibody appear after HBV infection as previous mention. On the other hand undetectable DNA was associated with anti-HBc detection. In the present study the anti-HBc was detected within three HBsAg control negative but HBV DNA was not detected among the negative controls, suggested prior infection with HBV.

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Table (1): Distribution of patients and controls according to age.

Age groups (Years)	patients		Controls	
	No	%	No	%
1-14	1	1	4	20
15-24	13	13	4	20
25-34	37	37	4	20
35-44	31	31	5	25
45-54	9	9	2	10
55-65	9	9	1	5
Total	100	100	20	100
Mean age (years)	35.6		29.12	

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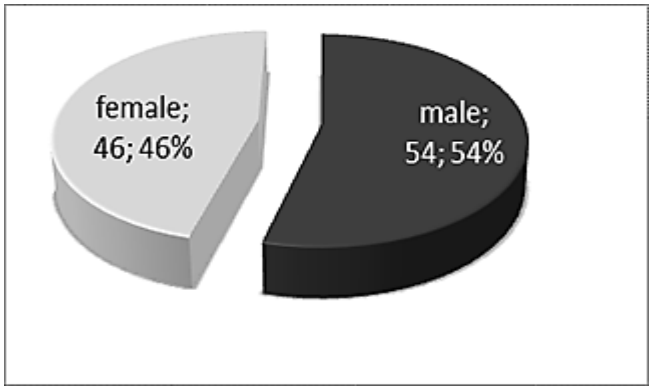

Figure (1): Prevalence of HBV Infection among patients by gender

Table (2): Showing the correlation between HBV serological markers in the HBsAg positive patients and control groups with the gender.

Cases	Serological test					Mean of viral load c/ml	NO.	Male	female
	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb				
A	+	-	-	+	+	4552935.54	72	40	32
B	+	-	-	-	+	8661702.6	13	5	8
C	+	-	+	-	+	88083623.85	7	3	4
D	+	+	-	+	+	173288	4	3	1
E	+	-	+	-	-	*More than* 100000000	3	2	1
F	+	+	-	-	+	0	1	0	1
G	-	+	-	+	+	0	1	1	0
H	-	+	-	-	+	0	2	1	1
I	-	+	-	-	-	0	7	3	4
J	-	-	-	-	-	0	10	4	6

Table (3): showed the relationship between HBV viral load (c/ml) and sex of HBV infected patients.

sex	Mean	Std. Deviation	T test	p-value
male	12391858.23	31336290.1	0.69	0.480
female	8653221.36	26942717.1		

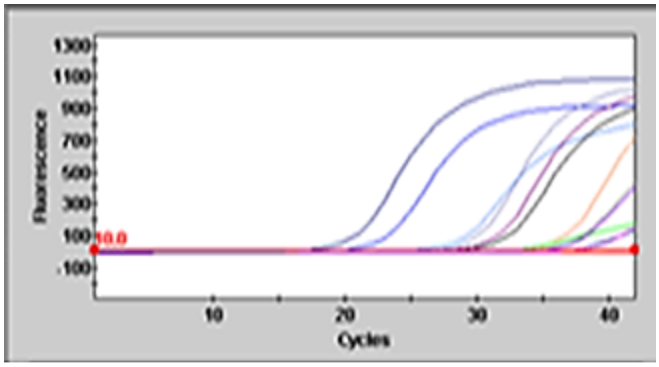

Figure (2): The graphs show fluorescence vs. the number of cycles for differing initial amounts of template copies of patients infected with hepatitis B virus.

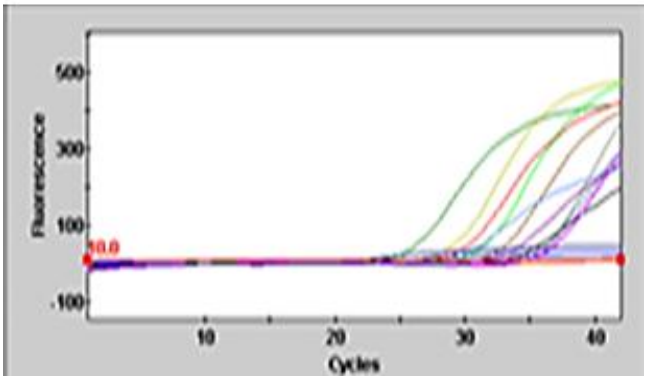

Figure (3): The graphs show fluorescence vs.the number of cycles of HBV IC.

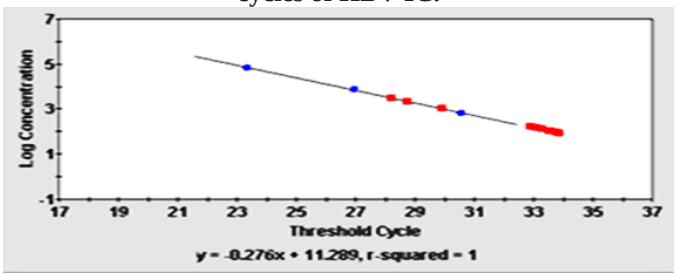

Fig. (4):Standard curve and R2 value used to evaluate the Real time PCR efficiency

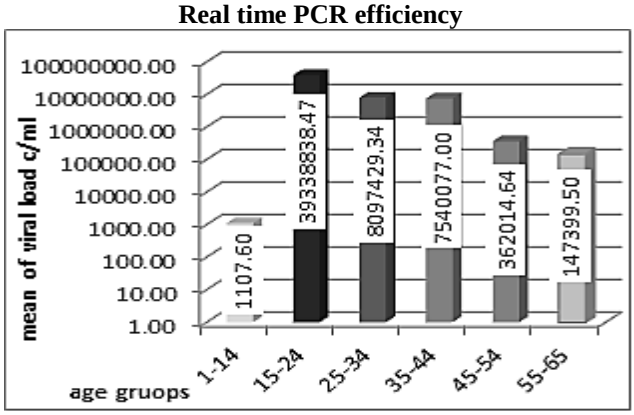

Fig. (5): Distribution of HBV patients viral load with the different age groups.

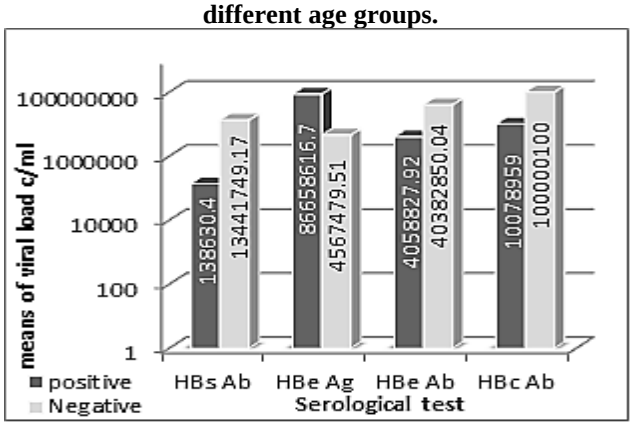

Fig. (6): The relationship between the viral load c/ml and Serological marker of HBV infected patients.

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مقارنة نوعين من الطرق المصلية التجارية مع طريقة التقدير الكمي RT-PCR للحامض النووي لفايروس التهاب الكبد نمط ب بين الاشخاص المصابين في مدينة الرمادي.

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الخلاصة:

التقدير الكمي للحامض النووي DNA لفايروس التهاب الكبد نمط ب HBV والذي يستخدم عالميا بشكل واسع في تشخيص الفايروس وكذلك تقدير فعالية العلاجات المستخدمة لعلاج الاصابة. الهدف من هذه الدراسة هو التقدير الكمي لفايروس HBV باستخدام تقنية RT–PCR في دم المرضى المصابين بهذا المرض ومقارنتها مع الفحوصات المصلية للفايروس والمقدرة باستخدام تقنية ELISA و الاختبار المناعي الكروماتوجرافي. شملت الدراسة 100 عينة بلازما لأشخاص مصابين (موجبة لاختبار المستضد السطحي الفايروسي HBsAg +ve) بالإضافة الى 20 عينة لأشخاص اصحاء (سالبة لاختبار المستضد السطحي الفايروسي HBsAg –ve). قسمت العينات الى مجموعات اعتمادا على الفحوص المصلية وكذلك اختبار الحامض النووي DNA للفايروس. عند اجراء الكشف عن الحامض النووي للفايروس تبين ان 86 (47 ذكور و 39 اناث) من عينات دم المصابين الئة كانت موجبة لهذا الفحص. لقد اظهرت الدراسة اختلاف اعداد الفايروسات (viral load) تبعا لنتائج الفحوصات المصلية الاخرى (HBsAb, HBeAg, HBeAb and HBcAb). كما اظهرت الدراسة اهمية استخدام تقنية RT–PCR الى جانب الفحوصات المصلية في تشخيص الاصابة كونها طريقة حساسة يمكنها الكشف عن النسب القليلة من الفايروس في دم المصابين. بينت الدراسة ان عدم وجود المستضد الغلافي HBeAg في دم الاشخاص لا يعكس بالضرورة (كما كان يعتقد سابقا) نسبة عدد الفايروسات في دم المصابين، حيث ان 90/76 من عينات دم المرضى كانت نتائج فحص الـ DNA لهم موجبة رغم ان اختبار المستضد الغلافي كان سالبا. كذلك فان 3 مرضى منهم كانت نسبة الحامض النووي الفايروسي لديهم اكثر من 100 مليون فايروس/مليلتر من الدم.