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Article

Detection of Torque Teno virus in IRAQI hemodialysis patients

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ABSTRACT

Some data suggest that the liver is being affected by a new set of variables, and one of them is the Torque Teno virus, which is widespread in the liver other than the well-known hepatitis A-E viruses. Dialysis patients with hepatitis C virus (HCV) and those who are not HCV infected were compared to healthy persons using the nested PCR method to evaluate the extent of the viral dissemination in the Iraqi dialysis population and how it affects infection severity. The research began in 2021 until the end of 2022. Blood was drawn from 35 HCV-infected dialysis patients, 35 HCV-uninfected dialysis patients, and 20 healthy individuals. Immunological (ELISA), chemical and hematological testing findings were reported. In this study, molecular detection by nested PCR was performed to identify the Torque Teno virus. PCR identifies the Torque Teno virus in hemodialysis patient blood samples. The infection rate was 0% in healthy persons, 14.29% in dialysis patients without HCV (5 out of 13), and 20% in dialysis patients with HCV (7 out of 13). This research found a greater prevalence of the Torque Teno virus in HCV patients than in dialysis patients without HCV infection, and the detection of TTV by PCR technique was 100% for sensitivity specificity. This research demonstrated non-significant differences between Torque Teno virus infection and liver enzymes in hemodialysis patients.

Keywords: Torque Teno virus, hemodialysis, *Anelloviridae*

INTRODUCTION

Torque Teno virus (TTV) is a novel member of the recently discovered family Anelloviridae, a circular ssDNA virus only around 3.8kb in size [1,2]. It was considered to have a strong connection to hepatitis [3]. TTV is a particularly problematic disease because of its high incidence and a very high degree of genetic variability. In patients with unidentified hepatic diseases, it was found in the liver and blood of such patients. Tainted blood transfusion was once thought to be the primary route of viral transmission. Due to the virus's prevalence in various bodily fluids, including feces and saliva in addition to river water tainted by sewage, new transmission routes have emerged [4]. Infections with the TTV virus have been recorded in a wide spectrum of people, including those with liver illness, HIV-positive persons, intravenous drug users, thalassemia patients, and hemodialysis patients on maintenance [5]. Two features of TTV infection have emerged from early studies, making it a possible cause of liver illness. The

presence of TTV-DNA in the sera of patients with non-A-E hepatitis was first reported by [6], who found a strong association between ALT levels and the presence of TTV-DNA.

TTV is proposed to be implicated in numerous disorders such as acute respiratory diseases, liver diseases, AIDS, cancer, and autoimmune responses [7,8,9,10,11]. Nonetheless, substantial data supporting TTV's function in the described illnesses have not been documented. Since TTV has a vast diversity of genotypes [12], certain genotypes may be responsible for the pathogenesis of the virus.

MATERIALS AND METHODS

Hemodialysis patients (30 samples) were used in the research, 4 of them serving as a control, 13 patients who did not have HCV and 13 patients who did have HCV aged (18 to 55). Blood samples were taken randomly from the Medical Hospital, Al-Kindi Hospital in Baghdad, and Baquba Teaching Hospital in Diyala during November 2021 and January 2022.

Specimens' collection

Five milliliters of blood were drawn from each individual by puncturing each patient's vein using a disposable syringe. The blood sample was separated into two parts: the first part, which was 2 milliliters, was collected in a tube containing ethylene diamine tetra acidic acid (EDTA) as an anticoagulant with a low mix for hematological study, and the second part, which was 3 milliliters, of the blood sample was collected in a gel tube left about one hour to clot in room temperature then centrifuged for 15 minutes at 3000 revolutions per minute to separate the serum is then divided into three aliquots and stored at a temperature of -20°C: the first aliquot is used for the biochemical test, the second aliquot is used for the immunological test (ELISA), and the third aliquot is used for the viral DNA extraction.

Biochemical testing

The levels of alanine aminotransferase (ALT) and aspartate transferase (AST) were measured using the Kinetic (Biosystem bts350), following the kit provided by the manufacturer for the linear enzymatic approach (Spain). For ALT, abnormal readings were judged to be below 45 IU/L, while for AST, abnormal values were below 40 IU/L.

Hematology Tests

Hematology studies blood diseases. Hematological tests were performed on whole blood samples from patients and controls. Complete blood picture (CPC) HB and WBC alterations due to TTV infection.

Normal Hb: 12.2-16.1 g/dL

Normal WBC: 4.7-10.2 K/L

Detection of TTV

DNA extraction: According to the manufacturer's instructions, DNA was purified from whole blood using the High Pure Viral Nucleic Acid Kit (Maxwell® 16 Viral Total Nucleic Acid). DNA was stored at -20°C for further processing.

Detection of 5′-UTR region

Interestingly, noted that the conserved regions may be found in the genome's non-coding region (UTRs) [13], which is preserved sections and GC-rich regions.

Primers

Primers selected for this study were shown in (Table 1) according to [14]. These primers bind to 5′-UTR of TT virus. Regarding the locations of designed primers,

TTV amplicons were formed, having lengths of 220 nucleotides for 5′-UTR regions.

No	Primer Sequences 5′…….3′	primers	Company	Origin	Reference
1	TTTGCTACGTCACTAACCAC	NG054 F	Alpha DNA	United State	**Moghimi *et al.*, 2020**
2	GCCAGTCCCGAGCCCGAATTGCC	NG1471 R1 (external)	Alpha DNA	United State	
3	AGCCCGAATTGCCCCTTGAC	NG1321 R2 (inter-nal)	Alpha DNA	United State	

Table 1. The Primers Used in the Current Study

Nested PCR

Usually, nested PCR is programmed into two rounds. For the first round (external), the following primers were utilized: NG054 F(5' TTTGCTACGTCACTAACCAC 3') and NG1471 R1(5' GCCAGTCCCGAGCCCGAATTGCC 3'). The processes were carried out in 20 µl volumes and consisted of 10 µl of Master Mix, 1 µl of each primer, 8 µl of template DNA. Amplification consisted of 35 cycles, each of which consisted of 5 minutes of initial denaturation at 95°C, 40 seconds of denaturation at 95°C, 45 seconds of annealing at 60°C, and 50 seconds of extension at 72°C after a 7-minute final extension was performed at 72°C. The following sequences were used as primers for the second round of (internal) PCR: NG054 F(5' TTTGCTACGTCACTAACCAC 3') and NG1321 R2(5' AGCCCGAATTGCCCCTTGAC 3'). The reactions were carried out in 25 µl volumes, which included 12.5 µl of Master Mix, 1 µl of each primer, 2µl of template DNA (first round product), and 8.5µl of sterilizing nuclease-free water. The volumes were used to carry out the reactions. The amplification process consisted of 35 cycles, each of which consisted of 5 minutes of Initial Denaturation at 95°C, 50 seconds of denaturation at 95°C, 40 seconds of annealing at 65°C, and 40 seconds of extension at 72°C, followed by a final extension of 7 minutes at 72°C. The PCR products were separated by electrophoresis in 1.5% agarose gels and visualized after staining with ethidium bromide using ultraviolet (UV) light.

DNA sequencing

PCR products with a forward primer of six (6) samples were sent for Sanger sequencing utilizing ABI3730XL automated DNA sequences by Microgen Corporation – Korea. The results were received by email, then a homology search was conducted using Basic Local Alignment Search Tool (BLAST) program,

which is available at the National Center Biotechnology Information (NCBI) online then, used Molecular Evolutionary Genetics Analysis (MEGA) for sequences alignment.

Statistical analysis

SAS (2012) was used to determine the influence of study components. LSD test (ANOVA) was used to compare means. This research employed the Chi-square test to compare probabilities (0.05 and 0.01).

RESULTS

In this research, 30 samples were randomly selected, including 13 patients with HCV infection, 13 patients without HCV infection, and 4 healthy blood donors with an average age (18-55) years. Results showed that 12/30 (40%) of the investigated samples were positive using nested PCR technique, agarose gel electrophoresis of PCR products for the 5′-UTR region in which the amplified size was approximately 220 bp as compared with the molecular ladder in the second round(nested) as cleared in Figure 1.

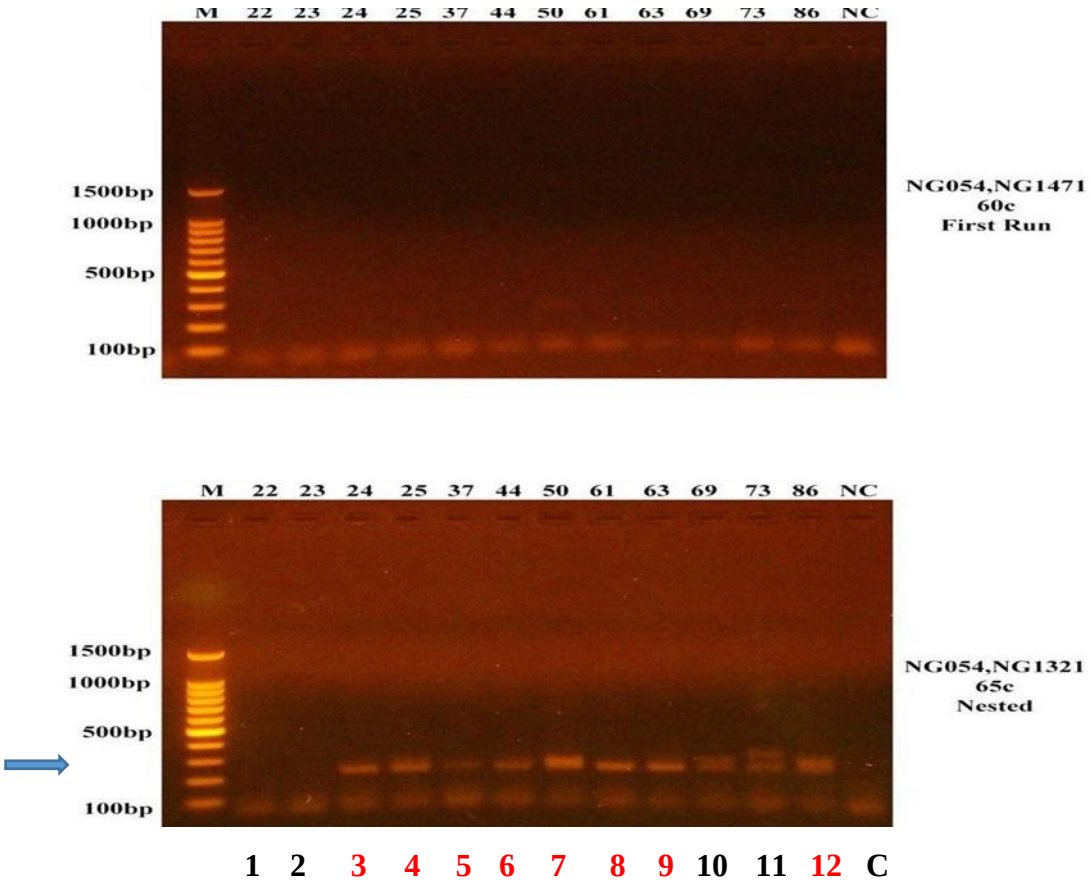

Figure 1. The amplification results of the first and second runs for the 5′-UTR region were fractionated on 1.5% agarose/100V/ 60min stained with Eth. Br.M:100bp ladder marker. Lanes 22-86 resemble PCR products. Lane 3,4,5,6,7,8,9,12 =positive with (220 pb), while Lane1,2,10,11= negative Lane 13= control

Regarding the sex distribution, the percentage of males was *20* (*66*%), while the percentage of females was *10* (*33*%) *as* ,shown in (Table 2).

Group		Control (No=4)	H.NO virus (No=13)	HCV (No=13)
Gender	Male	2(50%)	8(61.5%)	10(76.9%)
	Female	2(50%)	5(38.4%)	3(23.0%)
Age (year)	Mean ± SE	37.45 ±3.07	39.40 ±1.48	39.68 ±1.43

Table 2. Distribution of sample study according to Gender and Age in different groups

Biochemical test

The study results showed significant differences in liver enzymes AST and ALT between healthy subjects and between patients undergoing dialysis operations with and without hepatitis, as shown in *. Table 3*

Group	Mean ± SE	
	AST NV0- 40 (U/L)	ALT NV0- 40 (U/L)
Control	12.75 ±1.7 c	7.1 ±1.35 c
H.NO virus	29.69 ±4.83 b	16.54 ±5.17 b
HCV	43.31 ±4.29 a	29.6 ±4.46 a
LSD value	11.67 **	8.37 **
P-value	0.0001	0.0002

Data expressed as mean±SE. LSD test was used to calculate the significant differences between the tested mean, the letters (a, b, and c) LSD for columns represented the levels of significant, highly significant starting from the letter (a) and decreasing with the last one. Similar letters mean there are no significant differences between tested ** (P≤0.01).

Table 3. Distribution of AST and ALT results in this study

Hematology Tests

This study was conducted to find out the difference in white blood cells ratio between patients and controls, as shown in Table 4 that there are significant differences in white blood cells between the control group and patients undergoing dialysis without infection HCV and patients with HCV infection, with means (8.00), (5.34), (4.27) respectively.

Table 4 showed small differences in hemoglobin ratios between the healthy group and the group of patients undergoing dialysis with HCV infection and without HCV infection.

Group	Mean ± SE	
	WBC NV(3.5-10.0)	Hb NV(11.0-16.0)
Control =4	10.48 ±0.82 a	10.85 ±0.9
H.NO virus=13	3.88 ±0.55 b	9.15 ±0.63
H.C.V=13	4.03 ±0.5 b	8.85 ±0.24
LSD value	1.15 **	0.98 NS
P-value	0.0001	0.21
Data expressed as mean±SE. LSD test was used to calculate the significant differences between the tested mean, the letters (a, b, and c) LSD for columns represented the levels of significant, highly significant starting from the letter (a) and decreasing with the last one. Similar letters mean no significant differences between tested ** (P≤0.01).		

Table 4. Distribution of HB and WBC results in this study

Detection of TTV

The study results showed that 40% of samples were infected with TTV as appeared shown in Figure 2.

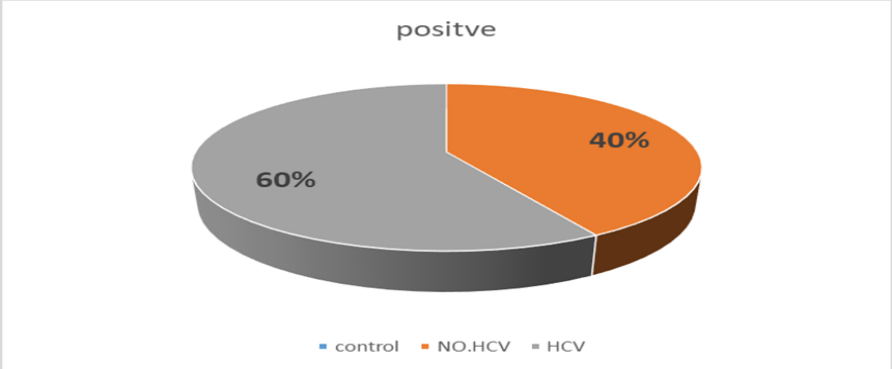

Figure 2. Distribution of positive TTV virus on study samples by PCR

In this study, seven (53.8%) of the 13 patients tested were found to have TTV infection with HCV infection, whereas five (38.46%) of the 13 patients tested were found without HCV infection with 5 (38 %) male and 2 (15 %) female in HCV patients, and only 5 (38 %) male in patients without HCV this is shown in Table 5.

Total no (30)			Control (No=4)	H.NO virus (No=13)	HCV (No=13)
TTV By PCR	Positive	Total	0 (0.00%)	5(38.46%)	7(53.8%)
		Female	0 (0.00%)	0(%0.00)	2(15.38
		Male	0 (0.00%)	5(38.46%)	5(38.46%)
	Negative	Total	4(100%)	8 (61.5%)	6(46.15%)
		Female	2(50%)	5(38.46%)	1(7.69)
		Male	2(50%)	3(23.07%)	5(%38.46)5

Table 5. Distribution of positive and negative results by Nested PCR

The results of our study indicate that there is no significant difference between liver enzymes and TTV positivity, as well as no significant differences between age, wbc, Hb, and TTV positivity, as indicated in Figure 3

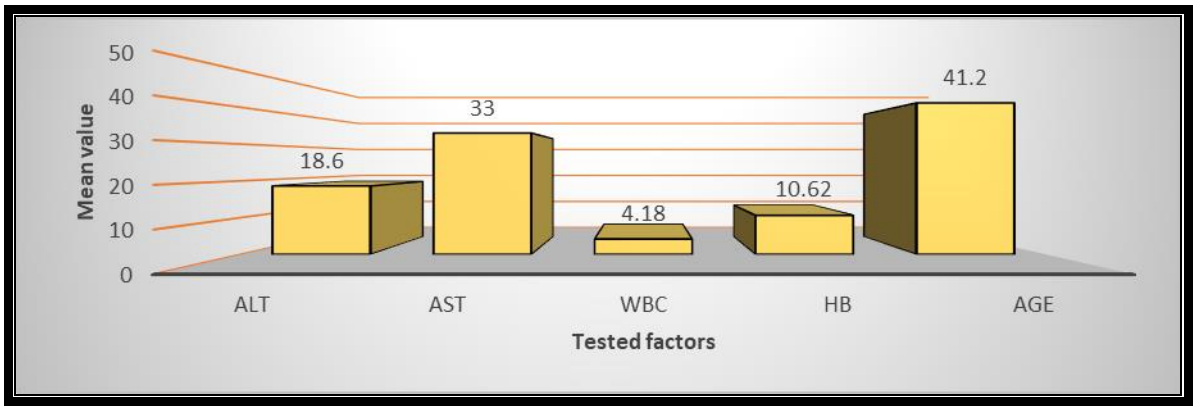

Figure 3. Relationship between the positivity of TTV and the parameter under study

NO	sex	Age	HB	ALT	AST	virus	W		BC TTV By PCR TTV By sequencing
24	Male	32	8.1	6	17	NOHCV	4.8	Positive	Positive
44	Male	48	10.7	7	18	NOHCV	3.2	Positive	Positive
50	Male	43	9.9	7	17	NO HCV	2.6	Positive	Positive
61	Male	45	10.8	31	41	HCV	3.5	Positive	Positive
69	Male	45	8.3	9	23	HCV	4.3	Negative	Negative
86	female	44	9.1	18	19	HCV	4.2	Positive	Positive

Table 6. Matching the positive results of TTV with different techniques

The second goal is to show the variables and mutations between detected sequences. Figure 4 illustrates the alignment of the five sequences with the TTV sequence as a control obtained from NCBI by using MEGA (Molecular Evolutionary Genetics Analysis). Also, some changes in the nucleotide sequencing took place in all isolates; such changes might alter the virus's interaction with the host and even its response to viral treatment.

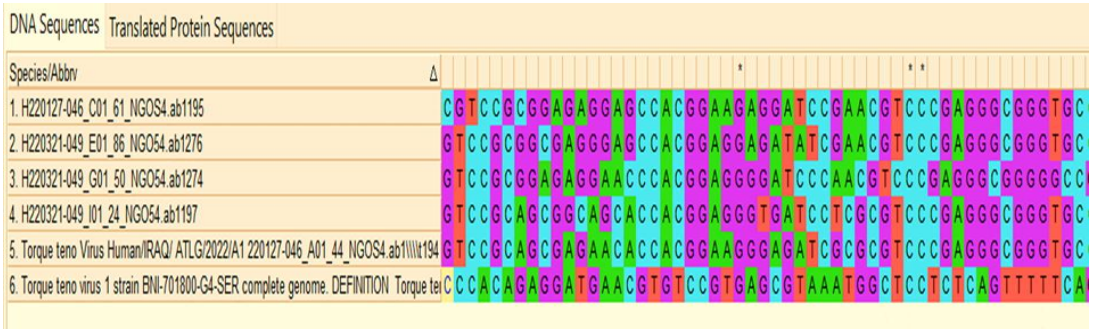

Figure 4. Partial alignment for DNA sequences (template was DNA) for 5´-UTR region by MEGA analysis program

DISCUSSION

TTV is found in the liver tissue of chronic hepatitis patients, particularly in the hepatic cells; the virus appears to be localized in the cell for unknown reasons [15]. DNA amplification by PCR is often used to detect the TTV genome using two regions: either (N22) coding region of the ORF1 or (UTR) non-coding region [16]. In this study, amplification of (UTR) non-coding region was performed as indicated in Figure 1

The incidence of TTV in HD in this study was 40.0. % as in Figure 2, which was near to study In Saudi Arabia, the prevalence of TTV was substantial and statistically significant among HD patients, coming in at 42.9 percent compared to 19 percent among the control group [17] same to results of another study showed that the Prevalence of TTV by 99.3% in HD patients in Iran [18]

Regarding gender distribution, as shown in Table 2, males are 20 (66%) and females 2 (33%), which is consistent with research that established that males are more likely than females [19] Furthermore, this study disagrees with studies that claimed that there was no difference in TTV infection between males and females [20]. This study is also in line with Takemoto et al. (2015), who demonstrated that there is no statistically significant difference between gender and TTV infection and also discovered that the majority of dialysis patients were men (55 %), with a mean age of (53.8) years old. This disparity in numbers between males and females may be the result of differences in pathophysiology, the physiological nature of work, the type of work performed, and nutrition, all of which may contribute to males having a greater likelihood than females of developing kidney disease or kidney failure [21]

The results of this study in Table 3 noted significant differences in AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) enzymes between the healthy group and patients undergoing HD with and without hepatitis, and the highest significant difference was in patients infected with viral hepatitis showed that ALT, AST values were higher in hepatitis than in healthy blood donors this is what agreed with the study was found that the activity of the amino group transporting enzymes (ALT & AST) is higher in hemodialysis patients with hepatitis virus compared to those without, which indicates liver injury [22,23] but does not agree with the results of a study showed that there were no significant differences between dialysis patients with HCV and without HCV infection in terms of liver enzymes [24]

Bone marrow produces white blood cells which then move into the bloodstream to begin their function in facing various types of infections, including viruses, bacteria, and germs, as they are an important part of immunity the results of this study as in Table 4 showed a decrease in the number of white blood cells in the

final stages of dialysis This is agreement with [25], also, Table 4 showed differences in hemoglobin ratios between the healthy group and the group of patients undergoing dialysis with HCV infection and without HCV infection because of anemia associated with renal failure due to relative deficiency in erythropoietin as a result of a defect in its production, severe deficiency in iron and foliate, shortening the lifespan of red blood cells with an increase in the rate of Its breakdown and a decrease in the rate of production in the bone marrow, as well as blood loss due to hemorrhage through the gastrointestinal tract and blood in the urine as a result of poisoning resulting from the accumulation of nitrogenous waste in the blood, and poisoning with heavy metals [26].

In Table 5, TTV-DNA was identified using a 5 -UTR primer-based PCR technique. Of 13 patients, 5 (38%) had TTV infection from a group free of HCV infection, and 7 (53%) had HCV infection. These findings agree with other scientific investigations on the TTV virus's potential hepatotropism and its coinfection with recognized hepatitis viruses. TTV infection has been demonstrated to be diagnosed in 40–60% of persons with HCV [27], and there is a marginally higher detection rate of TTV DNA in patients with known-etiology hepatitis compared to healthy donors [28]. Also, in IRAN [29] discovered that when compared to patients from other nations, the prevalence of TTV-DNA in Iranian patients with hepatitis B and C was quite high

Correlations between PCR positive results of TTV with parameters under study in general

The results of our study found that there are no significant differences between liver enzymes and TTV infection, as in Figure 3, and this is in agreement with a study that showed no significant association between liver enzymes and TTV infection[30] and this is in agreement with another study that showed no association between TTV positivity and laboratory factors such as liver enzymes AST, ALT. [31] In addition to, another study that showed No significant difference was detected in either AST or ALT levels in any of the groups[32], which concurs with the postulation that TTV is a commensal virus and only certain genotypes and Geno groups are associated with liver pathology [33]. Also, there are no significant differences between TTV positivity and age, and this is what agreed with the results of a study that found no correlation between age and TTV [34] while not agree with a study that showed significant differences between age and TTV positivity [35] Non-significant association of age in this study could be explained by the fact that this kind of viral infection could occur in people with any age [34]. On the other hand, our study's results showed no relationship between TTV infection and hemoglobin, as well as the number of white blood cells. But in general, WBC and granulocyte counts were higher in patients with bacterial infection than in those with viral infection, as mentioned by [36].

DNA Sequencing

After the PCR product was submitted for sequencing, a homology search was performed using the Basic Local Alignment Tool (BLAST) software available at the National Center for Biotechnology Information (NCBI) online for the number of nucleotides in our study. DNA sequencing was performed for two targets, the first to confirm the positive results for PCR, as shown in Table 6. All samples positive by PCR belonged to TTV after alignment using the Basic Local Alignment Search Tool (BLAST) software. Only sample no. 69 is negative by PCR and DNA sequencing, which improves and confirms the accuracy and specificity of PCR technology for TTV detection.

CONCLUSIONS

TTV may have a function in hepatitis since its presence was related to biochemical markers of liver damage and persistent HBV or HCV infection in individuals with a percentage of males more than the percentage of females. Also, this study confirmed that the PCR technique is more accurate and the best test with 100% accuracy compared to the ELISA technique for TTV detection.

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