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Investigation of transfusion transmitted viruses in cases clinically suspected of posttransfusion hepatitis with undetermined ethiology

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Abstract

Transfusion transmitted viruses (TTV) were investigated in cardiac surgery cases who were previously transfused with blood and/or blood products and were suspected of having posttransfusion hepatitis (PTH) based on the results of physical examination, clinical findings, biochemical blood test results and in a smaller number, on radiological results. They were identified as having non-A–C hepatitis based on serological or molecular test methods.

In this study, out of 90 cases suspected for PTH and non-A–C, 78 (86.7%) were male, 12 (13.3%) were female and their ages were between 17 and 67. Ninety healthy blood donors, who donated blood for the first time and had never had a transfusion, were selected as the control group. They had alanine aminotransferase (ALT) levels < 40 U, were seronegative for hepatitis B virus (HBV) and hepatitis C virus (HCV). Seventy-seven were immune, and 13 were seronegative for hepatitis A virus (HAV). In this study, TTV-deoxyribonucleic acid (DNA) investigation was performed by the polymerase chain reaction (PCR) method suggested by Takahashi et al. with 5′ GCT ACG TCA CTA ACC ACG TG 3′ (T801) and 5′ CTG CGG TGT GTA AAC TCA CC 3′ (T935) primers.

TTV-DNA was found to be positive in 21 (23.3%) of the patient group and 4 (4.4%) of the control group ($p < 0.05$). In the patients determined to be TTV-DNA positive, the admission time following transfusion was a minimum of 3, and a maximum of 15 (average 7) weeks. The average ALT levels detected at the time of admission did not show a difference between TTV-DNA positive and negative cases ($p > 0.05$). However the ALT levels had a tendency to rise and reached their highest level nine weeks after transfusion in the TTV-DNA positive cases, although in two cases the ALT levels decreased to normal value after the 13th week. During the 24 month follow up of the TTV-DNA positives all cases except one were positive at the end of this period.

The results of this study are the same as those reported in the literature suggesting that TTV-DNA, excluding the main viral agents which are known to cause PTH, can be determined in transfused PTH or non-transfused asymptomatic patients in varying ratios.

In order to define the epidemiological properties and hepatic–extrahepatic pathologies more clearly we have looked for evidence of the viral agent, which probably contaminates both by transfusion and non-transfusion routes. It is suggested that, in addition to the case groups in this study, new clinical studies are necessary including transfused but non-PTH patients.

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1. Introduction

Viral hepatitis is caused by infection of the hepatocytes, either primarily by major hepatotrophic agents such as A, B, C, D, E, F, G or secondarily by non-hepatotrophic agents such as Cytomegalovirus (CMV), Varicella Zoster virus and Epstein–Barr virus (EBV). The clinical manifestations of viral hepatitis vary from acute to chronic hepatitis depending on both the quality of the agent and the immunity of the host. The existence of many hepatitis cases in which these known hepatotrophic and non-hepatotrophic viral agents were not demonstrated, the development of hepatitis after transfusion of blood products which did not contain HBV and HCV and an inability to determine the etiological factors in some acute, chronic and fulminant hepatitis and cirrhosis cases, caused the investigators to look for another virus [1]. In recent studies, hepatitis G virus (HGV) and then, TTV have been discovered as hepatitis-causing agents.

In this study, our aim was to investigate the presence of TTV in cardiac surgery cases, where patients were transfused with different numbers of blood and/or blood products and were suspected of PTH postoperatively because of the results of physical examination, clinical findings and laboratory data and were identified as having non-A–C hepatitis.

2. Materials and methods

The selection of the patient and the control group. Between January 1998 and June 2000, 5788 cases underwent open heart surgery. They were transfused an average of 7 units (5–39 U) of blood and blood products. After discharge from hospital, 133 of these cases returned to the hospital over an average of nine weeks (between the 3rd and the 17th week), with various clinical complaints such as: slight weakness, fatigue, lack of appetite, nausea (91 cases), abdominal pain (11 cases), fever $\geqslant 38$ °C (9 cases) and skin eruptions (1 case). The results of physical examination included: slight icterus (2 cases) and hepatomegaly (4 cases); and biochemical test results showed: ALT values >40 U/l (93 cases), total bilirubin >2 mg/dl (3 cases) and bilirubinuria (1 case) with slight hepatomegaly upon ultrasonography (4 cases). Ninety-three cases had clinically suspected PTH. (Patients whose ALT was high postoperatively and who had slight icterus were not included in the study.)

In order to detect TTV carriers preoperatively (and also before transfusion), 1.5 ml of serum, from each patient, were stored at −30 °C for TTV-DNA investigation during routine serological tests (HbsAg, anti-HCV, anti-HIV 1/2, Rapid Plasma Reagin (RPR)) in 5788 cases. PCR study of the 93 cases PTH suspected was performed on the serum samples, which were kept at −30 °C. Of these, two cases were determined as TTV-DNA carriers.

Except for these two cases and another case of HCV positivity, 90 cases with non-A–C were included in the TTV-DNA investigation. Seventy-eight (86.7%) of these patients were male and 12 (13.3%) were female. When these patients, applied to the clinic, TTV-DNA investigation was performed by PCR using 10 cc of blood without anticoagulant at the 6th, 12th and 24th month. The ALT levels of the TTV-DNA positive cases were measured weekly until the values decreased below normal.

Ninety healthy blood donors who did not have a history of liver disease during the last one year were included as the control group. Seventy-nine (86.7%) were male, 12 (13.3%) female. Their ages ranged between 18 and 60. They donated blood for the first time and had never been previously transfused. They had ALT levels <40 U, and were seronegative for HBV and HCV, 77 were immune, and 13 were seronegative for HAV.

(A) Biochemical tests	Normal values
ALT	8–40 U
Aspartate aminotransferase (AST)	8–40 U
Total bilirubin	0.1–1 mg/dl
Gamma glutamil transferase (gamma-GT)	11–50 U/l (for male) and 7–32 U/l (for female)
Alkaline phosphatase	47–147 U

The samples were measured in the Opera device (Bayer, USA) from venous blood which was given by fasting patients.

(B) Hematologic tests: The hematological parameters (erythrocyte count, hematocrit, hemoglobin, leukocyte and platelet levels) were measured using the Cell Dyn 1700 device (Abbott, USA).

(C) Serologic tests	Normal values	Device	Method
Related to HAV			
Anti-HAV IgG	< 1 signal/cut off: S/CO positive	IMX (Abbott, USA)	Fluorescent polarisation
HAV IgM	> 1 S/CO positive	Access (Beckman, USA)	Chemiluminescence
Related to HBV			
HBsAg	> 1 S/CO positive	Access	Chemiluminescence
Anti-HBc	> 1 S/CO positive	Access	Chemiluminescence
Anti-HBs	> 10 ml U/ml immun	Access	Chemiluminescence
HBeAg	> 2.1 S/N positive	IMX	Fluorescent polarisation
Anti-HBe	< 1 S/CO positive	IMX	Fluorescent polarisation
Related to HCV			
Anti-HCV	> 1 S/CO positive	Access	Chemiluminescence

(D) Biochemical tests of urine: pH, urobilinogen, bilirubin were examined with the Multistick 10 SG (Bayer, USA) stripes.

(E) Radiologic tests: Liver ultrasonography.

(F) Nucleic acid tests: In our study, TTV-DNA was detected, as recommended by Takahashi and co-workers [2], with a PCR method by using the oligo sequences of the primer pairs which were: T 801(5′GCT ACG TCA CTA ACC ACG TG 3′) the sense primer nucleotide 6–25 and T935(5′ CTG CGG TGT GTA AAC TCA CC 3′) anti-sense nucleotide 204–185 primers.

Accordingly: "DNA was extracted with the phenol–chloroform method from the serum samples of the control and the patient group and also from the positive and the negative controls in our laboratory. The quality of the extracted DNAs were checked with the spectrophotometric methods. Later on, a PCR master mix was prepared for amplification of the nucleic acid. The prepared master mix was made of distilled water, 10 × buffer (100 ηM) Tris–HCl [pH 8.3] + 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dnTPs, 2 μl 10 mg/ml ethidium bromide, primers, and Taq polymerase. The extracted DNA samples were added to the prepared mixture and put into the thermal cycles. At the end, the amplification was checked by staining the PCR products with ethidium bromide and by 2% agarose gel electrophoresis. ϕX174/Hae III was used as the size marker. The results, with positive and negative controls, were evaluated by observation in the UV transilluminator, with the accompaniment of the size marker".

In our investigation, positive and negative control serum samples two each were used, at the

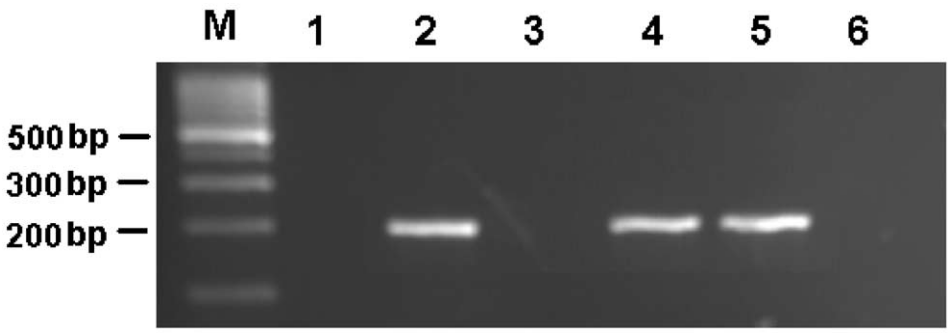

Fig. 1. The results of TTV-DNA positive and negative cases; and the positive and negative controls in agarose gel electrophoresis. M: marker (Promega 100 bp ladder); 1: negative control; 2: positive control; 3: negative patient; 4: positive patient; 5: positive patient; 6: negative patient.

extraction phase, in order to determine and confirm the true positivity of the serum samples. Also, at the master mix phase, the known positive and negative DNAs, were added to the study. In addition, each of the samples, both the control and the patient group, were examined twice. That is, the test was repeated twice from beginning to end. Also, the positive and negative controls were used in both the extraction and the master mix phases in each of the studies (Fig. 1).

The Chi-square test and the Mann–Whitney *U*-test, in the Epi Info Version 6.0 programme, were used for statistical calculations.

3. Results

TTV-DNA was found to be positive in 21 (23.3%) of 90 cases and 4 (4.4%) of the control group (x^2: 9.58, $p < 0.05$, OR 6.54; 95% CI 2.61–18.5) (Fig. 1). The TTV-DNA positive cases appeared at the hospital for the first time between 3 and 15 weeks with an average of 7 weeks after the transfusion. The average ALT level found at the first admission did not show any difference between the DNA positive and negative cases ($p > 0.05$). The average age was 50.1 in TTV-DNA positive cases and 48 in those that were negative ($p > 0.05$). The rate of TTV-DNA positivity did not show any significant difference between sexes ($p > 0.05$) (Table 1).

However, the ALT levels in TTV-DNA positive cases had a tendency to increase after the transfusion and reached the highest levels after the 9th week, except in two cases and the values decreased to normal after the 13th week except for one case (Fig. 2). In spite of this, TTV-DNA positivity continued to exist in the 24th month in 20 of 21 cases during follow up on the 6th, 12th and 24th month and was found to be negative in only one case (case number 8) (Table 2).

4. Discussion

In 1997 in Japan, TTV, an unenveloped, single stranded DNA virus was isolated from a patient with non-A–G hepatitis and the virus was named TT virus (TTV) after the initials of the patient. Studies on the epidemiological and ethiological effects of TTV were accelerated. The demonstration of TTV-DNA in the serums of 3 out of 5 PTH cases and the relationship of this with the ALT levels, suggested that this virus is responsible with PTH [3]. Subsequently, different TTV-DNA ratios were found in cases where the blood donors had liver diseases such as cryptogenic cirrhosis and idiopathic fulminant hepatic insufficiency [4–6]. The ratio of TTV-DNA in the liver is 10–100 fold the level in serum indicating that the replication and infection of TTV takes place in the liver [7]. Although the high ratio of TTV-DNA in blood donors who had no transfusion and the determination of infection or DNA in the stools of patients who have no risk of parenteral contamination, make us think that the contamination may

Table 1
Characteristics of the cases in which clinically suspected PTH and the TTV-DNA was also positive and negative in the control group

Properties	TTV-DNA		p
	Positive	Negative	
The group of the PTH-suspected cases			
Prevalence (%)	21 (23.3)	69 (76.7)	
Age (mean)	50.6	47.6	$p > 0.05$
Sex – male:female (%)	18:3 (23:25)	60:9 (69:75)	$p > 0.05$
Blood and blood product (average in units)	8.4	7.8	$p > 0.05$
1st application (minimum and maximum – as weeks)	3–15	4–8	
Mean	7	6.4	$p > 0.05$
The ALT levels at the 1st application, as U/l (min–max)	53–165	47–167	
Mean	84	79	$p > 0.05$
The control group			
Prevalence (%)	4 (4.4)	86 (95.6)	
Age (mean)	39.5	41.8	$p > 0.05$
Sex – male:female (%)	4:0 (5:0)	75:11 (95:100)	$p > 0.05$

be by non-parenteral routes. There are an increasing number of studies on patient groups who had previous blood and blood product transfusions [7,8]. A study from Japan, indicated that TTV-DNA was not detected with PCR in blood and blood products, especially in Factor VIII–IX or immunoglobulin preparates. In nine patients out of 50 with chronic liver disease and in four patients out of 21 with fulminant hepatitis, TTV infection was detected and this ratio was 7% for

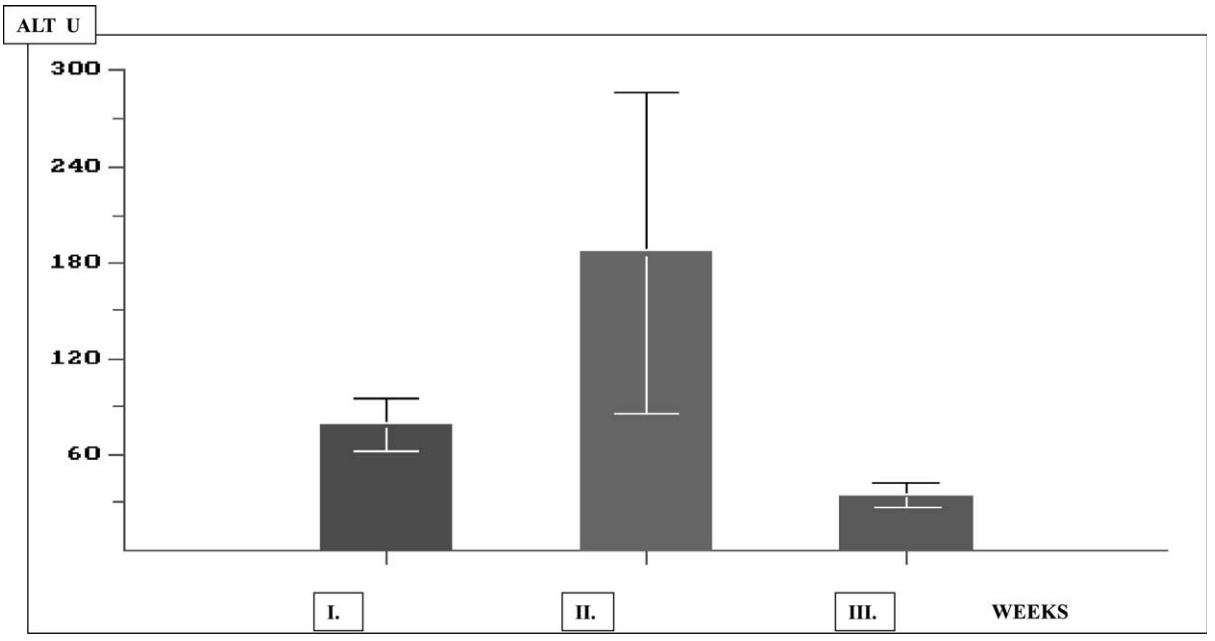

Fig. 2. The distribution of ALT values related to the weeks of admission, maximum level and normal, during the follow-up period of the cases suspected for PTH. 1st measurement: average application week (6.5th week); 2nd measurement: average week in which the ALT levels were determined as maximum (9th week); 3rd measurement: average week in which the ALT levels were decreased to normal (13th week).

Table 2
Characteristics of the PTH cases in which the TTV was determined as positive

	Age	Sex	The number of transfused blood or blood products – as units	ALT level (U/l)/the determination period (in weeks)						TTV existence (in months)			
				1st application		Maximum ALT level		Decreased to normal (ALT)		0	6	12	24
				Period	ALT	Period	ALT	Period	ALT				
1	44	M	6	4	63	6	115	13	10	Positive	Positive	Positive	Positive
2	48	M	7	5	102	7	141	13	22	Positive	Positive	Positive	Positive
3	51	M	6	3	68	6	187	8	38	Positive	Positive	Positive	Positive
4	38	M	8	6	67	8	194	13	33	Positive	Positive	Positive	Positive
5	60	F	15	7	80	9	101	14	30	Positive	Positive	Positive	Positive
6	56	M	9	8	96	10	210	15	36	Positive	Positive	Positive	Positive
7	48	M	8	15	65	17	434	16	40	Positive	Positive	Positive	Positive
8	38	M	8	8	53	10	80	14	15	Positive	Positive	Positive	Negative
9	50	M	8	11	83	13	115	15	25	Positive	Positive	Positive	Positive
10	46	M	7	6	77	7	190	15	32	Positive	Positive	Positive	Positive
11	46	F	11	7	95	8	125	13	35	Positive	Positive	Positive	Positive
12	54	M	13	8	87	11	102	14	38	Positive	Positive	Positive	Positive
13	61	M	9	6	60	12	108	13	34	Positive	Positive	Positive	Positive
14	54	M	10	7	88	9	220	14	39	Positive	Positive	Positive	Positive
15	43	F	9	5	94	7	219	13	40	Positive	Positive	Positive	Positive
16	49	M	7	6	108	9	254	14	40	Positive	Positive	Positive	Positive
17	51	M	6	5	55	7	90	15	25	Positive	Positive	Positive	Positive
18	56	M	6	6	61	11	193	14	37	Positive	Positive	Positive	Positive
19	59	M	10	4	59	10	397	15	41	Positive	Positive	Positive	Positive
20	60	M	8	7	100	9	337	15	39	Positive	Positive	Positive	Positive
21	50	M	7	6	86	8	112	14	23	Positive	Positive	Positive	Positive

volunteer donors [8]. Charlton et al. [4] have found that the TTV-DNA ratios in cirrhotic patients who had previous blood and blood product transfusions, in non-transfused patients, and in blood donors, were: 18%, 4%, 1%, respectively. Colombatto et al. [9] from Italy have determined that the TTV prevalence was very high among patients who had previous blood transfusions. The ratio was also high in patients who had no transfusions and they related this result to the possibility of contamination by some other route. In 2000, Sugiyama et al. [10], in their studies with Okamoto and Takahashi primers, found the TTV-DNA ratios to be: 31.6%, 78.9% and 6.7%, 60%, respectively in transfused and non-transfused children. Because the ratio of the non-transfused group is also high when it is compared to the transfused group in addition to parenteral contamination, enteral contamination is also considered among the possibilities. In two separate studies from France, in one of the studies, during the follow-up of 173 cases with multiple blood transfusions, TTV-DNA was positive in 48 (27.7%) patients and in the other study, TTV-DNA was positive in 28.3% of the group with blood transfusions. This percentage was determined as 5.3% for blood donors [11,12]. Gad et al. from Egypt [13] have emphasized in their study that there is no relationship between TTV infection and a history of blood transfusion. In Brazil, TTV-DNA positivity was found at similar ratios in the group with non-A–C hepatitis and blood donors and it was declared that TTV is a widespread infection in Brazil [14].

In our country, TTV-DNA positivity was found in 4.5% of blood donors [15]. In our study, for surgical cases in whom multiple blood and/or blood products were transfused, TTV-DNA was found to be significantly higher in comparison with the non-transfused control group and this was parallel to the literature indicating that blood and blood products are important means of contamination. We found TTV-DNA positivity to be 4.4% in the healthy control group with no transfusion history who were donating for the first time.

Our study results are concordant with the literature in emphasizing that TTV contaminates not only by transfusion but also contaminates by the enteral route and at the same time, a TTV carrier can be asymptomatic. But, both our study and the literatures revealed that because TTV is extremely variable and the viral population of the host is complex; the primers selected for the study or the amount of the nucleic acid which participate in the reaction and the methods related DNA extraction, demonstrate differences resulting in extremely variable TTV-DNA results. These results revealed that more standardized primers and methods are needed in respect to sensibility and specificity in order to be able to determine genomic DNA-related TTV infections more clearly. Depending on this, investigations will continue to resolve these problems for some time.

When we emphasize the properties of the TTV-DNA positive and negative cases in our study, we found no difference in age, sex and ALT levels with TTV viremia diagnosed at the same ratio in both male and female cases. Similar results on age were also seen in healthy blood donors and TTV viremia was seen only in males. In USA in 1998, a study [4] with 180 cases consisting of 100 blood donors, 33 patients with cryptogenic cirrhosis, 11 with idiopathic fullminant hepatic insufficiency, 36 postliver transplantation and an other study by Tanaka et al. [16] with 127 patients with chronic liver disease and 100 healthy donors, both reported that there is no difference between the TTV positive and TTV negative cases with respect to age and sex. Pincau et al. [17] have reported, in studies on the ethiopathogenesis of TTV in 293 hepatocellular carcinoma cases, that TTV viremia showed no distinction between age and sex. In a donor study in our country TTV positivity was seen only in males [15]. In 1999–2000, Takayama et al. [18], Kanda et al. [19], Gad et al. [13] and Prati et al. [20] have stated, as a result of studies with different patient groups and blood donors, that there is no significant difference in ALT levels between the TTV positive and negative cases. In contrast Lefrere et al. [11] have reported that TTV-DNA positive cases have high ALT levels. In a study on the ALT level with TTV and the relationship to HCV, Watanabe et al. [21] have shown that, among the TTV positive and negative cases the frequency of a high level of ALT and minor histopathological values did not show any difference and HCV co-infection and high level ALT

frequency increased statistically. Nizhizawa et al. [3] reported, in a study including 5 PTH cases who had different numbers of transfusions, that the highest level of ALT is between 80 and 434 U/l and this level is reached between 6 and 25 weeks.

In our study, the highest level of ALT in the TTV-DNA positive cases is between 108 and 385 U/l and this value was reached at 8–12 weeks and decreased to normal after the 13th week except in one case. The symptomatic and supportive therapies which are applied in hepatitis caused by other major viral agents were used with all but one of our TTV positive cases. This therapy was carried-out out of the hospital when there were no existing intensive histopathological changes in the liver (degenerative and regenerative changes, necrosis, hypertrophia, hyperplasia, etc.). The exception was our 7th case, who had clinical symptoms of loss of appetite, nausea, vomiting, abdominal pain, etc., related to the high level of ALT; his treatment was continued at the hospital. In 20 of the 21 cases that were followed up for 24 months and clinically recovered completely, TTV-DNA positivity continued in the 24th month except in one case (number 8) in whom TTV-DNA was negative in the 24th month by PCR study.

In most of the investigations, it was claimed that there were no differences between the TTV-DNA positive and negative cases with respect to ALT levels or the histopathologic activity of the liver; that is, in comparison with the TTV negative cases TTV positive cases do not have biochemical and histopathologic findings which indicate significant liver damage. With our results, which are similar, it is considered that further inclusive clinical investigations are necessary on the pathogenesis and the clinical importance of TTV in liver which have not yet been clearly defined.

The results of this study were the same as those in the literature suggesting that TTV-DNA, excluding the main viral agents which are known to cause PTH, could be determined in transfused PTH or non-transfused asymptomatic cases in varying ratios.

In order to define the epidemiological properties and hepatic–extrahepatic pathologies of the viral agent which probably contaminates by both transfusion and non-transfusion routes, more clearly the data indicate that in addition to the case groups of this study, new clinical studies are necessary which would include transfused but non-PTH patients.

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