TT virus infection in acute and chronic liver diseases and in patients regularly receiving blood products in Belgium

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Abstract

Background : **TT viruses are single-stranded DNA viruses, suggested to be involved in non A-E hepatitis. We studied the prevalence of TTV infection in acute or chronic hepatitis in Belgium in comparison with that in blood donors and in patients regularly receiving blood products.**

Methods : **TTV-DNA was detected by PCR using the primer set of Takahashi et al (1998) or a nested-PCR specific for genotype-2, because it had been reported that this subtype might be more pathogenic (Tagger *et al.*1999).**

Results : **TTV-DNA was present in 49% of 128 patients with chronic hepatitis C, in 54% of 54 with chronic hepatitis B and in 54% of 24 with acute liver failure. This prevalence is similar to the 47% in 127 patients with clotting disorders, or the 64% in 103 undergoing chronic haemodialysis, but lower than the 29.7% found in 340 healthy blood donors. Significant differences in clinical or biochemical characteristics between TTV- positive or TTV-negative patients could not be substantiated. The genotype-2 subgroup comprised 3.9%, but they also did not differ from non genotype-2 patients.**

Conclusions : **The prevalence of TTV infection was higher in patients than in healthy blood donors. Its clinical significance remains questionable since clinical and biochemical characteristics were not different between TTV positive and TTV negative patients. The higher prevalence of TTV in patients might be related to parenteral transmission, but the relatively high prevalence in healthy blood donors points to an additional presumably faeco-oral infection. The presence of TTV in animals suggests that infection might also originate from food. Long term follow-up will have to define whether co-infection with TTV eventually alters the natural history of chronic hepatitis.** (Acta gastroenterol. belg., **2004**, 67, **161-165**).

Key words : TT virus (TTV), chronic hepatitis, fulminant hepatitis, haemodialysis, haemophylia.

Introduction

Despite extensive screening for markers of viral hepatitis A to E and exclusion of non-viral diseases, there are still patients with acute or chronic liver diseases presumably resulting from viral infection. Therefore, searches for other hepatotropic viruses continue. Using representational difference analysis, Nishizawa *et al.* in 1997 isolated a viral clone from serum of a patient (with initials TT) with post-transfusion non A to G hepatitis (1). Subsequent studies showed that this clone originated from a DNA virus (TT virus) (2). Initial studies were based on PCR detection using a primer set derived from the Open Reading Frame (ORF) 1 region but subsequent studies revealed that TTV has a very high sequence variability and that PCR with a primer set derived from the non-coding region and partly from ORF 2 has a much higher sensitivity (3). Further studies, carried out during the last 5 years, have shown that there are several genetically distinct but closely related TTV-like viruses, including TTV-like mini viruses, Sen virus etc (4-9). Most of them are picked up by the primers now in use in the PCR determination. TTV seems hepatotropic, since the titer found in liver tissue is 10-100 times higher than that of serum (2) ; however, it has been now found in other tissues as well (4). TT virus is a negative single stranded circular DNA virus. It has no envelope and has a genome of approximately 3.9 kb length with two possible large open reading frames, capable of encoding 770 and 202 amino acids respectively (2,10).

The aim of the present study was to determine the prevalence and the clinical significance of TTV infection and of its possibly more virulent (11) subgroup genotype-2 in patients with liver disease in Belgium.

Patients and methods

Patients

Serum or plasma of 128 patients with chronic hepatitis C, 54 with chronic hepatitis B and 24 with acute liver failure were analyzed. The samples were aliquotted directly after collection and stored at -20°C. For comparison, sera of 340 healthy regular blood donors from the local blood bank, of 127 patients with congenital clotting disorders and of 103 patients undergoing chronic hemodialysis were studied.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated by density gradient centrifugation using Ficoll/Isopaque solution as described by the manufacturer (Lymphoprep, Nycomed, Oslo, Norway). After isolation, the cells were washed with PBS, snap-frozen in liquid nitrogen and stored at –20°C.

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Table 1. — **Prevalence of TTV infection in our center**

	N	% TTV positive
Blood donors	340	30%
Patients with clotting disorders	127	47%*
Patients in chronic hemodialysis	103	64%**
Patients with chronic hepatitis C	128	49%**
Patients with chronic hepatitis B	54	54%*
Patients with fulminant hepatitis	24	54%
due to hepatitis A virus	4	0%
hepatitis B virus	2	100%
hepatitis C virus	3	66%
unknown origin	12	66%
toxic liver injury	3	33%

*p value < 0.05 ; **< 0.01 vs blood donors.

Sample Preparation

Total DNA was extracted from 150 µl sera or plasma using basic phenol followed by precipitation with absolute ethanol (4). The extracted DNA was dissolved in 10 µl TE buffer (10 mM Tris HCl, 1 mM EDTA) pH 8.0.

TTV detection by PCR assay

TTV-DNA was detected by a single round PCR assay using primers (T801 and T935) as described by Takahashi *et al.* (5). Briefly the PCR mixture contained 5 µl of DNA solution, 15 pmol of each primer, 0.2 mM of each dNTP and 1.25 unit of Taq DNA polymerase (Promega, Madison, USA) in a total volume of 50 µl PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100). Samples were overlaid with mineral oil and subjected to a 9-min denaturation step at 95°C, this was followed by 60 PCR cycles, each consisting of 95°C for 30s, 60°C for 30s and 72°C for 45s. After the last cycle, there was an additional 7 min at 72°C to complete elongation. Detection of TTV genotype-2 was performed by nested-PCR as described by Tagger et al (6) with one primer specific for genotype-2 (TR242). First round PCR was performed for 60 cycles using primer T50 and TR430 in the same conditions as PCR for TTV detection. 2 µl of the first-round PCR product was then subjected to additional 30 cycles amplification using primer T72 and TR 242 in the same conditions as described above. The PCR products were then subjected to electrophoresis on 2% agarose gel and ethidium bromide staining to detect the TTV-sequence specific band length of the PCR product.

HCV-RNA and GBV-C/HGV-RNA detection

These were performed as described previously (7).

Serum markers of HCV or HBV infection and determination of aminotransferase levels

HCV antibodies were detected by a second-generation anti-HCV ELISA from Ortho Diagnostic Systems (Raritan, NJ, USA) or the UBI assay (United Biomedical, Lake Success, NY, USA). Serological markers of HBV infection were assayed by commercial enzyme immunoassays (EIA) (Auszyme-II, AUSAB, Corab/ Corzyme, Abbott HBc, Abbott Laboratories, North Chicago, IL). Antibodies to HIV were determined using recombinant HIV-1/HIV-2 3.0 generation EIA (Abbott Laboratories).

Aminotransferases were determined using routine laboratory procedures.

Statistics

Results were expressed as mean ± SD. For statistical comparison, the chi-square test was used.

Results

The prevalence of TTV infection was 49% in patients with chronic hepatitis C, 54% in chronic hepatitis B and 54% in patients with acute hepatic failure. This was higher than the 29.7% observed in healthy blood donors, but comparable to the 47% detected in 127 patients with clotting disorders and the 64% in the 103 patients undergoing chronic haemodialysis (Table 1). The presence or absence of TTV in patients with chronic hepatitis B or C did not influence the ALT levels nor the gender and age distribution between the 2 groups (Table 2). In general, HCV infection is often associated with TTV infection, since overall 50% of HCV-positive patients were also positive for TTV (Table 3). The incidence of TTV was even slightly higher in those positive for GBV/C-HGV (71%). In the patients undergoing chronic hemodialysis, the overall prevalence of TTV was high (64% ; Table 3) but independent of the HCV or GBV-C/HGV status. This suggests that infection with one of these viruses did not occur simultaneously in this group of patients.

We could retrospectively perform a longitudinal study of TTV on stored samples of some patients with chronic hepatitis C. Spontaneous fluctuations of TTV viremia were observed. Four out of 10 patients had persistence of TTV infection, 2 became TTV positive during follow up, whereas TTV disappeared spontaneously in 2 and a transient TTV viremia was observed in another 2. In a similar way, the effect of interferon on TTV could be studied retrospectively in patients co-infected with HCV and TTV. Of 20 patients treated, 4 had persistent TTV infection, 6 had temporary but no sustained disappearance, while 4 had permanent disappearance of TTV following interferon treatment. In 6 patients, TTV-DNA was intermittently positive in serum.

Peripheral Blood Mononuclear Cells (PBMC) were isolated from 10 TTV-DNA positive patients with chronic hepatitis B and from 10 with chronic hepatitis C. For comparison 12 TTV-DNA negative patients were also tested, 6 with chronic hepatitis B and 6 with chronic hepatitis C. Sixteen of 20 (80%) TTV-DNA positive cases were tested positive for the presence of TTV-DNA in their PBMC. None of 12 TTV-DNA negative cases had TTV-DNA sequences in their PBMC.

Table 2. — **Liver disease activity in relation to TTV infection**

	TTV-DNA pos	TTV-DNA neg
Chronic hepatitis C	N = 63 (49%)	N = 65
Male : Female	42 : 21	34 : 31
Mean age in years	48 ± 15	47 ± 17
Co-infected with GBV-C/HGV	11	5
Median ALT (25%-75% range)	72 (28-166)	53 (27-112)
% elevated ALT	67%	65%
Chronic hepatitis B	N = 29 (54%)	N = 25
Male : Female	21 : 8	19 : 6
Mean age in years	47 ± 9	46 ± 17
Co-infected with GBV-C/HGV	0	2%
Median ALT (25%-75% range)	47 (16-84)	51 (19-108)
Acute Fulminant Hepatitis	N = 13 (54%)	N = 11
Male : Female	6 : 7	4 : 7
Mean age in years	39 ± 17	41 ± 18
Co-infected with GBV-C/HGV	1	1
Median ALT (25%-75% range)	1315 (663-2520)	1656 (741-3315)

Table 3. — **Association between HCV, GBV-C/HGV and TTV in the patient population**

	HCV		GBV-C/HGV	
	pos	neg	pos	neg
Chronic hepatitis C				
N = 128	128	0	16	112
TTV pos	49%		69%	46%
Clotting disorders				
N = 127	101	26	15	112
TTV pos	50%	10%	73%	47%
Chron Hemodialysis				
N = 103	4	99	17	86
TTV pos	75%	63%	69%	64%

Sera of 24 patients with acute fulminant hepatitis were also examined. The causes of acute fulminant hepatitis and the TTV-DNA positivity are shown in Table 1. Thirteen of 24 patients (54%) were TTV-DNA positive ; 6 males and 7 females, with a mean age of 38.5±17.1 year. This prevalence was higher than that observed in the blood donors (29.7%), but the group is relatively small. No significant differences were present between the TTV-pos and TTV-neg patients with regard to age, gender, ALT or cause (Table 2).

At present, TTV is known to exhibit a great variability in its genomic sequence. Until now, at least 28 genotypes have been identified (8). These genotypes were classified mostly using phylogenetic analysis of the sequence obtained by PCR products. Tagger *et al.* (6) introduced a genotype-2 specific primer TR242 derived from ORF1 to determine the prevalence of genotype 2 TTV. We have used this primer set for the specific detection of genotype 2 in all samples that were positive for TTV-DNA. Thirteen of 331 (3.9%) samples analyzed were positive for genotype 2. The patients with genotype-2 TTV were not different from the non genotype-2 TTV patients with regard to type of hepatitis, age, gender or ALT levels.

Discussion

In 1997, Nishizawa *et al.* detected a novel DNA virus in the serum of a patient with post-transfusion hepatitis of unknown etiology and named it TTV (1). Since this discovery, many new TT viruses have been identified and sequence information of isolates has rapidly documented several complete sequences (2,4-11,14-19). TTV is at present considered to exist as a swarm of related but distinct viruses (4-9). TTV was considered to be hepatotropic (2), but it was found in high titer also in other tissues (4,20) suggesting that viral replication may take place in multiple tissues at distinct levels in infected individuals. In addition, TTV is present in the faeces (21,22) and can thus also be transmitted through the faecal-oral route. It appears to be an ubiquitous virus, being also present in various animal species from chicken to primates (14,23,24). Using a primer set derived from a more conserved region (3), we have documented a high prevalence of TTV infection even among the healthy blood donor population (29.7%) (25). A much higher infection rate was found in patients with liver diseases or those regularly receiving blood products or haemodialysis (Table 1), but it should be realized that healthy blood donors are a select population. As such the prevalence in the overall population might be higher than in the blood donors and might not be that different from the patients group. When first reported, TTV was thought to represent a cause of non A-G hepatitis (1). However, in the present study, as in others (26-28), there are no significant differences between TTV-positive and TTV-negative patients in ALT levels, age or gender distribution nor in causes or evolution of the liver disease. It is therefore suggested that TTV is not involved in the pathogenesis of hepatitis. Although it is rather unlikely that TTV would change the natural course of chronic hepatitis B or C, long-term evaluation remains required since it was e.g. recently suggested that another non-pathogenic virus GBV/C-HGV may have altered the natural history of HIV-patients (29). An intriguing observation was recently made by Cacoub *et al.* (30) who observed that TTV-DNA was more frequently present in HCV-patients with type II mixed cyroglobulinemi vasculitis than in control HCV patients (37.5% vs 9.5%). This certainly needs confirmation.

The prevalence of TTV infection in patients with chronic hepatitis B or C was similar to that of individuals regularly receiving blood products, suggesting that TTV co-infection is rather a consequence of a common parenteral route. TTV is spread through blood products, but the high prevalence of TTV among the healthy blood donors shows that spreading via other routes such as the faeco-oral is possible. This is also supported by the finding of TTV in body fluid such as bile (21), feces (22), saliva (5,32), nasal and respiratory tract aspirates (32), semen (31), cervical swabs (33) and breast milk (34). Vertical transmission (33), sexual infection (29,35,36) as well as infection via breast milk is thus also possible.

The high prevalence observed in the present study is in accordance with data from Italy (37) and Japan (26,38). and can in part be ascribed to the high sensitivity of the PCR method used. Indeed, as shown in our previous investigation (25) and by others (24), PCR using the primer set as described by Takahashi *et al.* (3) is far more sensitive than the original one developed by Okamoto *et al.* (2).

TTV-viremia showed a spontaneous fluctuation in several cases. Whether this fluctuation is due to temporary disappearance of the virus out of the serum into tissue pools, such as bone marrow or peripheral blood lymphocytes (39-41) or whether re-infection occurs, remains unanswered. We could detect TTV in PBMCs in our study in 16 out of 20 TTV positive patients analysed. The presence of TTV-DNA in PBMC could be the result of contamination from serum, not being discarded by the washing procedure, but Okamoto *et al.* (20,40) and Kanda *et al.* (41) reported the presence of the TTV replicative form in bone marrow cells. Interferon treatment of hepatitis C patients clears TV from serum in one third to half of the patients (23,42), but loss of serum TTV-DNA does not affect the course of patients with chronic hepatitis B or C (43).

We observed a prevalence of TTV of 54% in non A-G fulminant hepatitis, in agreement with 47% in Japan (2) and 39.6% in Spain (26). Despite the high prevalence of TTV among patients with acute fulminant hepatitis of unknown etiology, TTV can not be credited as the cause of fulminant hepatitis, since the age, gender, ALT levels and etiology of TTV positive or negative patients was not significantly different. Moreover, the prevalence of TTV in the non-A to E fulminant hepatitis (66.7%) was not different from that in the fulminant hepatitis C (2/3, 66.7%) or B (2/2, 100%) patients, though our sample size is very small. From these data, it can be concluded that TTV co-infection is not the factor promoting a fulminant course in hepatitis B or C, nor does it cause hepatic failure itself. The high prevalence in these patients might be a coincidence due to a common transmission route of an etiologic agent of acute fulminant hepatitis.

TTV is now known to exhibit a great variability in its genomic sequence. Until now, more than 28 genotypes have been identified (44). Using phylogenetic tree analysis, genotype-2 TTV was reported from around the world. In our study, a prevalence of only 3.9% for genotype-2 in all our TTV positive cases was observed. This result is much lower than data reported from Japan : 15% of HCC patients (3), 45% in acute viral hepatitis (26) or from Korea 34.6% in blood donors and liver disease patients (45). Although we could detect genotype-2 in some of our patients, this is not the major genotype, and these patients did not differ from the non-genotype 2 patients. We could thus not confirm the initial suggestion that this subtype was more virulent (11).

In general, we may conclude from the present study that TTV is often present in blood, but that its clinical significance with regard to liver disease remains negligeable till now. Further evaluation remains necessary to observe possible effects on the long run.

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