

## Torque Teno Virus (TTV) Infection In Egyptian Patients with Chronic Liver Disease and Hepatocellular Carcinoma

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**Abstract:** TTV has been recently identified as a causative agent of non-A to non-G hepatitis. However, the exact role of this virus in the pathogenesis of chronic liver disease (CLD) and the development of hepatocellular carcinoma (HCC) remain controversial. To clarify these issues, the prevalence of this virus and its impact on the severity of liver disease and development of HCC among Egyptian patients were assessed. The study was conducted on 84 patients with CLD and 22 with HCC. They were diagnosed by clinical, biochemical, ultrasonographic and histopathologic findings. Chronic liver disease patients were classified into: chronic hepatitis (CH=48), compensated cirrhosis (CC=12) and decompensated cirrhosis (DC=24). Serum samples were collected from all patients and from 80 healthy volunteers as controls. All samples were serologically tested for markers of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. HBV DNA, HCV RNA and TTV DNA were detected either by nested or RT-PCR assays. HCV serotyping was performed for 60 out of 85 HCV positive samples using an enzyme immunoassay. Among CLD and HCC patients, 85 had chronic HCV, 5 had chronic HBV and 10 had dual HCV and HBV infections. Coinfection with TTV was detected in 27%, 0% and 20% of patients respectively. TTV DNA was detected in 23.8%, 31.8% and 25.0% of CLD, HCC patients and healthy volunteers respectively. The prevalence of TTV infection in CLD and HCC patients did not significantly differ from that of healthy volunteers, however TTV infection was significantly higher among patients with CC (41.7%) compared to those with DC (16.7%) ( $P < 0.05$ ). No significant differences were detected among TTV infected and non-infected patients concerning demographic, clinical and virologic data. Moreover, TTV viraemia was not significantly higher in HCC-positive than in HCC-negative patients. In conclusion, TTV viraemia is a common finding among adult Egyptian patients with CLD, HCC as well as in healthy volunteers. Infection with TTV does not contribute to the severity of liver disease nor to causation of HCC. [Nature and Science. 2006;4(2):38-45].

**Keywords:** Torque Teno Virus (TTV); Infection; Egyptian; Patients; Liver Disease; Hepatocellular Carcinoma

**Abbreviations:** CH: Chronic hepatitis; CC: Compensated cirrhosis; DC: Decompensated cirrhosis; HCC: Hepatocellular carcinoma.

### 1. Introduction

Chronic liver disease and hepatocellular carcinoma constitute a major health problem in Egypt and have been known to be commonly associated with HBV and HCV infections (Darwish *et al.*, 1993; Waked *et al.*, 1995). However, a significant proportion of cases is still of unknown aetiology, indicating the existence of additional causative viral agents (Tangkijvanich *et al.*, 2000).

A novel hepatitis virus candidate designated TTV was recently identified as possible causative agent of human viral hepatitis. It was first identified in a patient with post-transfusion hepatitis of unknown aetiology and named with the initials of the person in whose serum it was first detected (Nishizawa *et al.*, 1997). The virus was characterized as being non-enveloped with

circular, single-stranded DNA (Mushahwar *et al.*, 1999). Due to its genomic structure, the virus was placed tentatively within the *Circoviridae* family (Miyata *et al.*, 1999) or in a novel virus family; the *Circinoviridae* (Mushahwar, 2000) or the *Paracircoviridae* (Takahashi *et al.*, 2000).

TTV has an extremely wide range of sequence divergence for a DNA virus, and it has been proposed that it exists as a swarm of closely related but different viral species (Khudyakov *et al.*, 2000). Five major phylogenetic groups including at least 23 genotypes have been identified (Peng *et al.*, 2002). Multiple infection with different TTV genotypes has been detected in humans and evidence for TTV evolution in patients with persistent infection has also been recognized (Irving *et al.*, 1999; Takayama *et al.*, 1999).

TTV is widely distributed throughout the world (Abe *et al.*, 1999). Both acute resolving and chronic persistent hepatitis infection have been identified among TTV-infected humans (Luo *et al.*, 2002). Furthermore, TTV has been detected in a variety of liver disease including non A-G post-transfusion hepatitis, fulminant hepatic failure, chronic persistent hepatitis, cryptogenic liver disease and hepatocellular carcinoma (Charlton *et al.*, 1998; Okamoto *et al.*, 1998; Yamamoto *et al.*, 1998; Orii *et al.*, 1999). Evidence of potential hepatotropism of TTV was reported with TTV DNA titres shown to be 10-100 folds greater in liver tissue than in serum (Okamoto *et al.*, 1998).

The significance and interaction of TTV with other hepatitis viruses remain controversial. Several studies on HCV patients coinfecting with TTV suggested that the association of both viruses increased severity of liver damage (Okamoto *et al.*, 1998; Zein *et al.*, 1999). Other studies found no such correlation and reported that the dynamics of ALT were unrelated to TTV viraemia (Gimenez-Barcons *et al.*, 1999; Irving *et al.*, 1999). However, it has been recently reported that TTV viral load was independently associated with HCC among patients with HCV infection (Tokita *et al.*, 2002). The levels of TTV vary in patients co-infected with other viruses and there has been considerable speculation as to whether TTV contributes to pathogenesis by other viruses or if the varying levels might be related to immune activation in the host (Fabrizio *et al.*, 2005).

Despite the worldwide distribution and intensive studies of TTV, the association of this virus with liver disease is still questionable. Its effect on severity of liver disease, aggravation of disease condition and progress to complications as cirrhosis and HCC has not yet been clearly defined. The aim of the present study was to assess the prevalence of TTV infection among Egyptian patients with CLD and HCC compared with healthy volunteers. The impact of this virus on the severity of liver disease and its association with the development of HCC were also investigated.

## 2. Patients and Methods

### 2.1. Patients

The present study was conducted on 84 patients with CLD and 22 with HCC who were examined and followed up at Theodore Bilharz Research Institute inpatient clinic between August 2001 and September 2003. Patients were diagnosed as CLD cases based on elevated serum ALT and AST for 6 months or longer, ultrasonographic evidence of liver disease or cirrhosis and histopathologic findings indicating the presence of chronic hepatitis or cirrhosis. The diagnosis of HCC was based on histopathology and/or a combination of mass lesions in the liver by

ultrasonographic imaging. Eighty serum samples were collected from healthy volunteers to serve as controls.

### 2.2. Methods

Patients' characteristics, personal history and clinical examination with special stress on manifestations of liver disease and decompensation were recorded. Patients were also examined by ultrasonography and upper endoscopy. Liver biopsy was done when indicated for 50 patients and diagnosed histopathologically. Blood samples were collected and subjected to complete blood picture and liver function tests (serum bilirubin, ALT, AST, alkaline phosphatase, albumin, globulins, prothrombin time and concentration) and alpha-feto protein. Serum samples were stored in several aliquots at -70°C until tested for viral markers of HBV, HCV, and PCR for HCV RNA, HBV DNA and TTV DNA.

Based on clinical, laboratory, ultrasonographic and histopathologic diagnosis, CLD cases were classified into: chronic hepatitis (CH=48), compensated cirrhosis (CC=12) and decompensated cirrhosis (DC=24). Decompensated cirrhosis was defined as the presence of complications related to portal hypertension such as ascites, encephalopathy, decreased hepatic synthetic function reflected by decreased albumin concentration and prolonged prothrombin time.

According to virological features, our patients were diagnosed as chronic HCV or chronic HBV or non-B non-C hepatitis on the basis of HCV antibody, HCV RNA, HBsAg, HBcAb and HBV DNA testing.

### 2.4. Serologic Assays

Serum HBsAg and HBcAb were measured using enzyme immunoassay kits (EIA Dia-Sorin, Italy). HCV antibody was detected using third generation EIA kit (Murex-Biotech Ltd., UK).

### 2.5. HCV Serotyping

Serotyping (1-6) of HCV positive samples was performed using rapid EIA (Murex-Biotech Ltd., UK). Typable results were recorded according to pattern of response and interpreted as single or mixed serotype.

### 2.5. Detection of HCV RNA by Nested RT-PCR

RNA extraction was performed by the acid guanidinium thiocyanate and phenol-chloroform single-step method (Chomczynski and Sacchi, 1987). HCV RNA was detected by qualitative nested RT-PCR using 2 sets of primers within the 5' non-coding region (Van Doorn 1994). The PCR products were analyzed using 2% agarose gel electrophoresis.

### 2.6. Detection of HBV DNA by Nested PCR

HBV genomic DNA was extracted from serum using guanidinium isothiocyanate phenol chloroform

method and dissolved in EDTA. HBV DNA was detected by nested PCR using a set of primers designed for the core/precore region. Amplification products (approximately 0.23 kb band) were visualized using 1.5% agarose gel electrophoresis. The detection limit of this system was 200 DNA copies/reaction (Saber et al., 1996).

**2.7. Detection of TT Virus DNA by Nested PCR**

DNA was extracted from 100 µL serum by serum by guanidinium isothiocyanate phenol chloroform method and dissolved in EDTA (Saber et al., 1996). Nested PCR was performed according to Takahashi *et al.*, (1998a). Thermal cycler was programmed first to preheat the sample at 95°C for 10 min to activate Taq

DNA polymerase. Samples were then subjected to 55 cycles of 94°C for 20s and 72°C for 30s and finally one cycle at 72°C for 10 min using Perkin Elmer, Norwalk, C.T. thermal cycler. The sequences of TT virus-specific primers were: 5'-GCT ACG TCA CTA ACC ACG-3' ( T801, sense primer, nucleotides 6 to 25) and 5'-CTB CGC TGT GTA AAC TCA CC-3' ( T 935, antisense primer, nucleotide 185 to 204, B=G, C, or T) as designed by Takahashi *et al.*, (1998a), in the 5'-end region of the TA278 isolate.

**2.8. Statistical analysis**

Test of proportion and Chi square analysis were used for statistical analysis as appropriate. P value <0.05 was considered significant.

Table 1. Prevalence of TTV infection among patients coinfecting with other hepatitis viruses

Group(n)	TTV +ve	
	n	%
HCV (85)	23	27.0
HBV (5)	0	0
HCV+HBV (10)	2	20.0
Non B non C (6)	2	33.3

Table 2. Prevalence of TTV DNA among the different studied groups

Group (n)	TTV +ve	
	n	%
CLD (84)	20	23.8
HCC (22)	7	31.8
Healthy volunteers (80)	20	25.0

CLD: Chronic liver disease.

HCC: Hepatocellular carcinoma.

Table 3. Prevalence of TTV infection among different studied patients according to the severity of liver disease

Stage of Liver Disease (n)	TTV +ve	
	n	%
CH (48)	11	23.0
CC (12)	5	41.7*
DC (24)	4	16.7
HCC (22)	7	31.8

CH: Chronic hepatitis.

CC: Compensated cirrhosis.

DC: Decompensated cirrhosis

HCC: Hepatocellular carcinoma.

\* P value <0.05 relative to DC group.

Table 4. Demographic, virological and clinical data of patients with chronic liver disease and hepatocellular carcinoma relative to TTV DNA viraemia

Characteristic	TTV +ve (n=27)		TTV -ve (n=79)		P value
	n	%	n	%	
Age (mean±SD)	42.22±9.22		43.56±10.8		NS
Sex (M:F)	20:7		60:19		NS
Mean ALT level (IU/L)	53±12		62±32		NS
<b>Virological features</b>					
HBV markers +ve	0	0%	5	6.3%	NS
HCV markers +ve	23	85.2%	62	78.5%	NS
HBV and HCV +ve	2	7.4%	8	10.1%	NS
HBV and HCV -ve	2	7.4%	4	5.1%	NS
<b>Disease categories</b>					
Chronic hepatitis	11	40.7%	37	46.8%	NS
Compensated cirrhosis	5	18.5%	7	8.9%	NS
Decompenstated cirrhosis	4	14.8%	20	25.3%	NS
Hepatocellular carcinoma	7	25.9%	15	19.0%	NS

Table 5. Analysis of features of HCC among 85 patients with HCV-related liver disease

Features	Patients with HCV-related liver disease				P value
	With HCC (n=22)		Without HCC (n=63)		
Age (mean±SD)	50±6.2		43±6.2		0.05
Sex (M:F)	15:7		44:19		
Liver cirrhosis	17	77.3%	31	49.2%	0.01
Anti-HCV +ve	22	100.0%	41	65.0%	0.001
HCV RNA +ve	17	77.3%	28	44.4%	0.01
TTV DNA +ve	7	31.8%	18	28.5%	NS

Table 6. Distribution of HCV serotypes in 60 patients with chronic HCV relative to TTV viraemia

HCV Serotype (n)	TTV +ve	
	n	%
Type 4 (42)	21	50*
Mixed (14) (1+4 or 2+4 or 4+5)	2	14.2
Other serotypes (4) (1 or 3)	--	--

- P value <0.01 relative to mixed group.

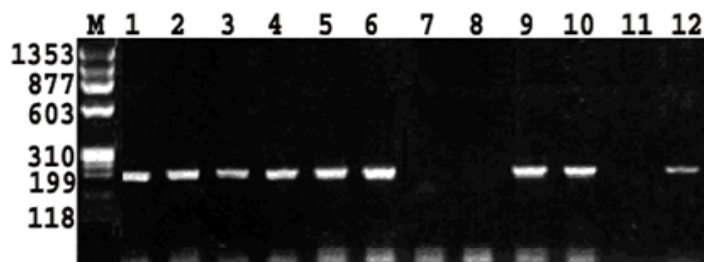


Figure 1. Detection of TTV-DNA by nested PCR. PCR products were separated on 1.5% agarose gel electrophoresis and were stained with ethidium bromide. Lanes 1,2,3,4,5,6,9,12, show positive TTV DNA viraemia at 199 bp. Lane 7,8 Negative TTV-DNA. Lane 10,11 positive and negative control respectively.

### 3. Results

Virologic marker testing of the patient groups showed that 85 had chronic HCV, 5 had chronic HBV, 10 had dual HCV and HBV infections and only 6 patients had non-B non-C. Coinfection with TTV was detected in 27.0%, 0%, 20.0% and 33.3% of patients respectively (Table 1).

TTV DNA was found in 23.8%, 31.8% and 25.0% of patients with CLD, HCC and healthy volunteers, respectively. Although a high prevalence of TTV viraemia was detected among all patient groups compared with the controls, no significant differences were observed (Table 2).

Positive results of nested PCR test for detection of TTV DNA are shown in Figure 1. The prevalence of TTV infection in relation to the severity of liver disease is shown in table 3. TTV infection was significantly higher (41.7%) among patients with CC compared to those with DC (16.7%) (P value <0.05).

Demographic, virologic and clinical data were compared according to the status of TTV infection in patients with CLD and HCC (Table 4). There were no significant differences between TTV-infected and non-infected patients regarding mean age, sex distribution, ALT serum levels, virologic features of HBV and HCV or category of liver disease. Histopathologic examination of the 50 cases in which liver biopsy was done revealed no variation in necro-inflammatory activity or fibrotic stage between cases between cases of dual TTV and HCV infections compared to isolated HCV infection.

Analysis of features of HCC among 85 patients with chronic HCV-related disease showed that HCC was significantly associated with higher age (P<0.05), liver cirrhosis (P<0.01) and HCV RNA positivity (P<0.01). TTV viraemia was not significantly higher in HCC-positive than in HCC-negative patients (Table 5).

HCV serotyping was done in 60 of 85 (70%) of samples. Serotype 4 was detected in 42 (70%), mixed serotypes (1+4 or 2+4 or 4+5) were detected in 14 (23.3%), other serotypes (1 or 3) were detected in 4 (6.7%) of those samples. TTV infection was significantly associated with HCV serotype 4 (50%) compared to patients infected with mixed serotypes (14.2%) (P<0.01) (Table 6).

### 4. Discussion

Knowledge about novel hepatotropic virus TTV is growing fast, but some fundamental aspects remain to be elucidated. Its prevalence and clinical significance are being assessed worldwide, however its relationship with aggravation and progression to severe liver disease and HCC remain controversial (Tangijvanich et al., 2003; Tokita et al., 2002).

The present study attempted to clarify these issues in Egypt, a country known for its high endemicity of

liver disease and hepatotropic viruses (El-Gohary et al., 1995; Arthur et al., 1997).

The prevalence rates of TTV DNA in the sera of normal healthy persons were found to vary widely in different countries with a high frequency of viraemia in adults (Bendinelli et al., 2001). In the present study, the prevalence of TTV infection among Egyptian healthy volunteers was high (25%). This prevalence was higher than that detected in western population (1-13%) (Abe et al., 1999; Gallian et al., 2000), but lower than that found in Asian countries as Japan (92%), Thailand (62%), Korea (53%) and among nationals and non-nationals in United Arab Emirates (35% and 89% respectively) (Kato et al., 1999; Kobayashi et al., 1999; Nakano et al., 1999; Al-Moslih et al., 2004).

In the present study, the prevalence of TTV viraemia among Egyptian patients with CLD and HCC was not significantly higher than that found in healthy volunteers. This was consistent with other studies reporting that TTV viraemia was prevalent among patients with CLD, whether viral or cryptogenic, at similar rates to control groups with no liver disease (Gad et al., 2000; Berg et al., 1999; Nakano et al., 1999).

In contrast, the prevalence of TTV in patients with CLD from USA and Europe was significantly higher than that in blood donors (50-72.5% versus 1-13%) (Maggi et al., 1999; Mizokami et al., 2000). The difference in prevalence of TTV DNA carrier state found in our study compared to others may be attributed to the population density, lifestyle, standard of living and mode of transmission of the virus. It has been proved that TTV is not only parenterally transmitted but is also transmitted by faeco-oral route, saliva, breast milk and transplacentally (Inami et al., 2000; Lin et al., 2000; Schröter et al., 2000; Bendinelli et al., 2001). Moreover, the ubiquitous nature of the virus raises the speculation whether the virus is pathogenic, opportunistic, a cofactor of other infections or a modulator of immunity that can promote other microbes to be infectious (Maggi et al., 2003). The variability in prevalence of TTV in different studies may be also attributed to the extreme heterogeneity of its genome making detection dependent on the viral DNA segment targeted for amplification. This has an enormous impact on PCR sensitivity, the only available method for detection of the virus (Takahashi et al., 1998b; Salakova et al., 2004).

In the present study, TTV coinfection with HCV, HBV and dual HCV HBV infections was 27%, 0% and 20% respectively. This was lower than that detected in other studies by Savas et al., (2003) from Turkey (73% with HCV and 91% with HBV) and by Szenborn et al., (2003) from Poland (53.8% with HCV and 47.3% with HBV). The lower prevalence of TTV DNA in our

study may be due to regional differences of TTV genotypes and the variability in the primers used in PCR (Salakova et al., 2004; Itoh et al., 1999; Verdi et al., 2001). One study in Turkey detected TTV infection in 34% and 6% with two different primer sets in the same 50 chronic HBV patients, while the infection rate in 150 healthy blood donors was 40% and 8 % respectively (Verdi et al., 2001). Moreover, in our study estimation of TTV prevalence in the HBV patients cannot be judged due to the small number of patients identified with HBV markers.

A single infection with TTV in the absence of HCV or HBV infection is uncommon in patients with CLD (Abraham et al., 2003). In the present study, TTV was detected in 2 of 6 patients who had no viral markers for HBV or HCV and their ALT levels did not differ from TTV-negative patients of the same group. This is in accordance with Fiordalisi et al. (1996) who reported that the presence of TTV DNA in sera of patients with CLD of unknown aetiology does not prove a causal relation of liver disease. Also, Itoh et al., (1999) suggested that TTV of restricted genotypes may be associated with liver disease. 1- HBV and HCV are major causes of CLD and cirrhosis which may ultimately lead to liver transplantation. Identification of factors that modulate the progression of liver disease is therefore potentially of great importance. Our results showed that TTV did not appear to aggravate the severity of liver disease among Egyptian patients with CLD resulting from HBV and/or HCV infections. Analysis of TTV-positive versus negative cases showed no association between the presence of this virus and severity of liver disease in terms of serum ALT levels, ultrasonographic and histopathology findings. These data agree with the reports of other investigators (Nakano et al., 1999; Maggi et al., 2003; Salakova et al., 2004; Maggi et al. 2003). Moreover, our finding that TTV viraemia was significantly higher in patients with compensated than in those with decompensated cirrhosis may further deny the role of this virus in the advancement of cirrhosis. However, evidence on the non- pathogenic role of TTV in CLD is not totally unequivocal, since data from other reports support a possible role of TTV in development of severe liver disease (El-Gohary et al., 1995). Several studies on HCV patients, coinfecting with TTV appeared to be associated with increased severity of biochemical and histologic parameters of liver diseases (Yamamoto, et al., 1998; Abraham et al., 2003; Fiordalisi et al., 1996). Also, the ability of TTV to replicate in the liver makes it likely that TTV may cause occasional liver damage (Tuveri et al., 2000). Many speculations regarding the conditions upon which TTV can produce liver damage have been suggested. First, TTV might cause disease only when activated by superinfection with other viruses Van Doorn (1994). Second, liver damage might

become evident only when virus replication is above a certain threshold. Third, certain TTV types or variants might be especially hepatopathogenic Okamoto et al., (1999). 2- The high prevalence of TTV in general population, may complicate linking TTV to hepatic disease and other pathologic states Maggi et al. (2003). This unusual feature among viruses aroused the proposal that TTV might be a commensal virus or part of human microflora Simmonds P (2002). Another major complication is the extreme heterogeneity of TTV genome, its divergent genogroups (1-5), and genotypes (23) each of which possesses distinct biologic properties and pathogenic potentials Peng et al. (2002); Maggi et al. (2003).

The development of HCC is a major problem in chronic HCV infection, and persistent infection has proved to be an independent risk factor for HCC development in Egyptian population Hassan et al. (2001). Other risk factors for HCC development in HCV-related CLD have been reported as HBV or HIV infection, heavy alcohol intake and liver cirrhosis Colombo M (1999). Moreover, Tokita et al. (2002) suggested that a high TTV viral load is an independent factor associated with occurrence of HCC in HCV patients. The high prevalence of TTV infection in patients with HCC found in this study as well as other studies (Nakagawa et al., 1999; Tagger et al., 1999; Pineau et al., 2000; Hassabo et al., 2003) is intriguing. This may suggest a potential pathogenic association between TTV and HCC in HCV-related disease. However, as we could not detect a significant difference in terms of TTV prevalence among HCC- positive and negative cases; nor in terms of HCC prevalence among TTV-positive and negative persons. Therefore, TTV might be a coincidental agent rather than a cause in the development of HCC.

A possible explanation for the high prevalence of TTV viraemia with HCC may be related to the immunosuppressed status in these patients. Another explanation is the higher exposure rate of HCC patients to TTV. Those patients are more likely to have undergone multiple medical or radiological interventions such as paracentesis or angiography which may be associated with increased risk of exposure (Zein et al, 1999).

In conclusion, the results of the study indicate that TTV is commonly present in adult patients with CLD and HCC as well as in healthy volunteers at comparable rates. It seems that the infection does not contribute to the severity of liver disease nor in causation of HCC.

Attempts to correlate TTV with liver disease need further studies that deal with viral load quantification, genetic characterization and detection of the virus in liver tissue.

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