Journal of the Egyptian Society of Haematology & Research, Vol. 3, No. 1, March: 1-7, 2007

The Detection of Transfusion Transmitted Virus (TTV) by Polymerase Chain Reaction Among Cancer Patients and Apparently Healthy Individuals

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ABSTRACT

Background: TTV was first isolated in 1997 from patients with hepatic failure, despite many attempts, no clear evidence has been found to define TTV as a causative agent in disease. The high prevalence of TTV in healthy individuals suggests that most infections have no pathogenic importance; thus, it was proposed that certain genotypes might be more pathogenic than others or that TTV might play a role in accelerating the effects of other infectious agents. According to the published reports on this newly characterized virus, it is evident that TTV is prevalent in several countries of the world. As such, it is not involved in causation of a serious problem in the body and simply acts as a bystander without much impact of its single or co-infection with other viruses. Attempts are still going on to find out exact clinical implications of TTV infection. Much is already known about the molecular biology of the virus, yet there still remains a need to develop simple techniques based on molecular and immunodiagnostics to diagnose TTV infection.

The Aim: Of this study was to investigate the incidence and genotyping of the TT Virus infection among cancer patients and apparently healthy individuals.

Materials and Methods: This study included 200 cases divided into two groups, control group which included 100 apparently healthy individuals, and the patients group which included 100 patients.

Results: Of this study showed the prevalence of TTV DNA in the sera of normal healthy persons and patients. The incidence of TTV DNA in patients group was 43%. While incidence of TTV genotype 1a was 16%. The incidence of TTV genotype 1b was 20%. The incidence of TTV non-genotype 1a & 1b was 16%. In controls group the incidence of TTV DNA was 44%. The incidence of TTV genotype 1a was 25%. While incidence of TTV genotype 1b was 61%. The incidence of TTV non-genotype 1a & 1b was 13% with no difference between the two groups.

Conclusion: High incidence of TTV infection was found among cancer patients and healthy individuals in Egypt with no difference between the two groups. TTV infection is widely spread among all type of cancer patients due to damage of their immune system and exposure to risk factors for infection but there was no relation between the type of cancer and infection with TTV. High incidence of genotype G1 (1a and 1b) among patients and controls in addition to low incidence of non 1a non 1b genotypes. Co-infection of TTV with HCV and HBV in patient and control groups were found, but TTV infection may have no effect on HCV and HBV infection. TTV infection is symptom-free, and found in normal persons and patients with different types of cancer, without any symptoms or signs. From that we conclude that TTV may be a part of the normal flora in human body. More studies are needed and also follow-up of TTV infection in patients and controls.

Key Words: *Transfusion transmitted virus (TTV) – HCV – HBV – Cancer patients – PCR.*

INTRODUCTION

Transfusion transmitted virus (TTV) is a recently discovered virus, which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus, based on its genomic characteristics **[1]**.

Cloning of TTV provide the nucleotide sequences that allowed the development of methods for its detection by PCR. The original PCR method used primers from the N22 region (a part of the gene encoding the TTV structural protein) to investigate cases of post-transfusion

hepatitis and other liver diseases of unexplained etiology. The pathological role of this virus in liver disease is generally small or does not exist. High detection rates of this virus have been found in serum of healthy children **[2]**.

Hino and Miyata in 2007 reported a second virus distantly related to TTV and provisionally described as TTV-like Minivirus (TLMV) which was accidentally discovered by PCR with TTV primers that partially matched homologus sequences in TLMV. Even less is known about disease associations and epidemiology of TLMV, but it has been established that infection in human is common and that it shows genetic heterogeneity comparable to or even greater than that of TTV. TLMV was found to be with approximately 209 Kb genome **[1]**. Some TTV subtypes have less than 50% sequence identity. However, there are certain conserved regions. Primers were designed in such a way that most of the subtypes could be detected. Recently, real time PCR based methods with either SYBR Green or TaqMan Probe, designed to quantitate selectively TTV and TLMV, have been used.

The development of sensitive and reliable polymerase chain reaction (PCR) protocols allowed the detection of TTV DNA at a very high prevalence in sera of healthy populations around the world. Currently, the heteroduplex mobility assay to detect multiple infections with isolates of TTV belonging to different genotypes or subtypes has also been developed. In the simplest application of heteroduplex mobility assay, heteroduplexes are formed by denaturing and reannealing mixtures of PCR amplified DNA fragments from divergent isolates of the same virus. When these products are separated on polyacrylamide gels; a homoduplex band plus two slow moving heteroduplex bands are observed **[3]**.

MATERIAL AND METHODS

This study was performed on 200 cases which are divided into two groups, control group which included 100 apparently healthy individuals, and the patients group which included 100 patients, attending the out patient clinics of the medical and surgical oncology departments at the Egyptian national Cancer Institute, Cairo University, during the period form May 2004 to December 2005, all cases were subjected to the following:

Sample collection:

Samples were collected in 10 ml sterile tubes, centrifuged within half an hour of collection using a low speed centrifuge and separated to avoid hemolysis. Serum was divided into three 0.5 ml aliquots and immediately stored at -70°C under complete sterile conditions.

I- *Clinical assessment:*

Full history of cancer patients (including history of jaundice, blood and blood products transfusion and surgical intervention) was collected from patients' files and from apparently healthy individuals.

II- *Laboratory investigations:*

All patients and apparently healthy individuals were subjected to the following:

A- *Serological tests for viruses:*

1- Detection of hepatitis B surface antigen by ELISA technique; kits supplied by dialab system.

2- Detection of hepatitis C virus antibodies by ELISA technique; kits supplied by diasorin system.

B- *Molecular biology technique:*

Nucleic acid extraction from serum: As described by Boom et al., 1990 **[4]**.

1- Detection of hepatitis C virus RNA by polymerase Chain Reaction (PCR) for all samples (whether positive or negative for HCV-Ab). HCV-RNA can be reverse transcribed into cDNA then amplified using PCR technique (RT-PCR). PCR is a cDNA amplification technique in which template cDNA is amplified using a thermal cycler with three different temperature steps: Denaturation, primer annealing and primer extension. The one-step RT-PCR was performed in a 50ul volume containing 1x buffer containing (50mM. Tris-HCl (pH 8.3), 20mM. KCl, 0.2mM. $MgCl_2$, 0.2ml. (each) dNTPs (Sigma), 100ng RB-6A (Sense primer 5' GTG AGG AAC TAC TGT CTT CAC G 3' [nt 47 to 68]), 100ng RB-6B (antisense primer 5' ACT CGC AAG CAC CCT ATC AGG 3' [nt 292 to 312]) and 10ul of RNA **[4]**.

2- Detection of hepatitis B virus DNA for HBsAg positive samples using PCR technique. PCR amplification was performed using a published oligonucleotide primer Set selected from the highly conserved HBV core gene. 10µl of DNA was added to 90µl of reaction mixture containing 2.5 unit Taq polymerase, 100 ng of each primer, 0.2 mM. of dNTps, 1x PCR- buffer of reaction buffer (50 mM. KCl, 10 mM. Tris-HCl, PH = 8.3) and 1.5 mM. $MgCl_2$. Samples were denatured for 5 minutes at 95ºC and then subjected to 40 cycles of 1 min. at 95ºC, 1 min. at 55ºC and 2 min. 72ºC in an Eppendorf thermal cycler (Master cycler 5330 Germany) **[4]**.

3- Detection of TTV virus DNA by PCR. TTV DNA was amplified using nested PCR which used two set of primers; one set external and the other internal. All experiments included TTV positive control and negative control. Four primers sequance were used. for the 1st round PCR, 1st primers pair used were (T45as, 5' GAA GAT AAA GGC CTT ATG GCG 3') antisence (T1 5' AGT GCA CTT CCG AAT GGC TG 3'). For the nested PCR internal primers pairs used were (T2 5' -GAG TTTT CCA CG CCC GTCCG 3') and (T46as 5' GTCTGG CCCC ACT CAC TTT CG 3'). The 1st PCR round were performed in a 50µl reaction volume containing 10µl of nucleic acid, 2.5U recombinant Taq DNA polymerase (promiga, Madison, WI), 0.2 mM. of deoxyribonuclease triphosphates, 100ng of each primer pairs, 1.5 mM. $MgCL_2$, 1x buffer, The PCR was performed in 37 cycles as follow: 1 cycle at 95°C for 5 minutes, 1 cycle at 72°C for 5 minutes, 35 cycles each consisting of 30 sec. at 95°C (denaturation) and 45 sec. at 55°C (annealing) and 1 minute at 72°C (extension); 2µl of PCR products were subjected to an additional 37 cycles of amplification with the nested set of primers by using the same ingredients and condition as described above except that the volume of water was increased to 36.5µL **[5]**.

4- Identification of TTV genotype 1a and 1b using restriction enzymes. Positive samples for TTV infection obtained were used to study genotyping of TTV by using restriction enzymes. The genotyping study was performed in the following steps: The PCR products of the primer T1/T45 was subjected to another PCR round with a set of primers designed to be specific for genotype G1, the primer sequence was (T6 5'- AGCTCC CAC GCT GCT ATGT -3') anti sense (T11 5'- CGT CTA GCA GGT CTG CGT CT -3'). The PCR products then digested using restriction enzymes (Mbo1 and Ban1) to discriminate between the two subtypes of G1 (1a and 1b). the digestion performed by adding 10µL of PCR products, 30µL buffer and 1µL of restriction enzymes then incubate at 37°C for 1hr, then at 70°C for 10 minute, the products were visualized as follows: 5µl loading buffer (blue/orange loading dye, promega) was added to the digested products and mixed, then analysed by electrophoresis in 2% agarose gel, DNA species were visualized under UV light after staining with ethidium bromide **[6]**.

RESULTS

This study included 200 cases which are divided into two groups, control group included 100 apparently healthy individuals, patients' group included 100 patients, divided into two subgroups:

Subgroup I: 50 cases with solid tumors.

According to the data obtained from patients' files 16 patients with breast cancer, 15 patients with bladder cancer, 4 patients with rectum cancer, 3 patients with colon cancer, 13 patients with HCC cancer and only one case with abdominal mass.

Subgroup II: 50 cases with hematological tumors.

9 patients with AML, 9 patients with CML, 8 patients with ALL, 10 patients with CLL and 12 patients with NHL.

In patients' group, male to female ratio was 1.07:1 and their ages ranged from 35 to 75 with a mean of 55 years. Distribution of blood transfusion was 21% and surgical intervention was 15%. In control group male to female ratio 1.6:1 and their ages ranged from 21 to 49 years with mean of 35 years. A highly significant difference between patients' and control group in blood transfusion and surgical intervention was detected.

In the patients group 43/100 (43%) cases were positive for TTV DNA. While in the control group 44/100 (44%) individuals were positive for TTV DNA, Fig. (1).

Table (1): Clinical history of patients and control group.

	Patient (NO.100)	Control (NO.100)	Total	*p*-value
Blood transfusion	21 (21%)	0	21	0.000*
Surgical intervention	15 (15%)	0	15	0.000*
Mean age	55±20	35±14	—	—
Male	52 (52%)	62 (62%)	114	0.153
Female	48 (48%)	38 (38%)	86	0.153

* Significant *p*-value

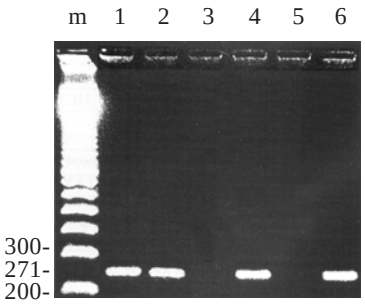

Fig. (1): Detection of TTV-CNA by PCR.
Lanes 1,2,4,6 represent positive samples.
Lanes 3,5 represent negativ samples.

TTV DNA was detected in 40.38% of solid tumor cases (21/50). The incidence of TTV DNA was 31.25% (7/14) in breast cancer cases, 46.6% (7/14) in bladder cancer cases, 50% (2/4) of rectum cancer cases, 33% (1/3) of colon cancer, 41.6% (5/12) in HCC cases and the patient with abdominal mass was found to be positive for TTV infection. TTV DNA was detected in 44% of hematological malignancies (22/50). The incidence of TTV DNA was 33.3% of AML cases (3/9), 66.6% of CML cases (6/9), 62.5% of ALL cases (5/8), 41.6% of CLL cases (5/12), 25% of NHL cases (3/12).

Regarding Hepatitis viral markers; in the solid tumor subgroup: 12/50 (24%) cases were positive for HCV-Ab with a significant *p*-value when compared with the control group. While 7/50 (14%) cases were positive for HCV-RNA; 1/50 (2%) cases were positive for HBs-Ag while 2/50 (4%) cases were positive for HBV-DNA. In the Haematological malignancies subgroup: 12/50 (24%) cases were positive for HCV-Ab while 9/50 (18%) cases were positive for HCV-RNA; 5/50 (10%) cases were positive for HBs-Ag while 5/50 (10%) cases were positive for HBV-DNA.

The solid tumor subgroup included 3/50 (6%) cases with history of blood transfusion and 14/50 (28%) with history of surgical intervention. In the Haematological malignancies subgroup: 18/50 (36%) cases with history of blood transfusion and 1/50 (2%) with history of surgical intervention. A highly statistically significant difference was detected between the solid tumor subgroup and the Hematological malignancies subgroup in blood transfusion and surgical intervention.

By studying the genotyping in our study groups; we found that: In the patients group 43/100 (43%) cases were positive for TTV DNA; genotype 1a was detected in 16/43 cases (37%), genotype 1b was detected in 20/43 cases (47%) and non genotype 1a or 1b was detected in 7/43 cases (16%). While in the control group 44/100 (44%) individuals were positive for TTV DNA; genotype 1a was detected in 11/44 cases (25%), genotype 1b was detected in 27/44 cases (61%) and non genotype 1a or 1b was detected in 6/44 cases (13%).

Table (2): The incidence of TTV DNA among patients' subgroups.

	No. of cases	No. of TTV positive cases	Percent of TTV positive cases
Breast cancer	16	5	31.25%
Bladder cancer	14	7	50%
Rectum cancer	4	2	50%
Colon cancer	3	1	33%
HCC	12	5	41.6%
Abdominal mass	1	1	100%
~~Total~~	~~50~~	~~21~~	~~40.38%~~
~~AML~~	~~9~~	~~3~~	~~33.3%~~
CML	9	6	66.6%
ALL	8	5	62.5%
CLL	12	5	41.6%
NHL	12	3	25%
Total	50	22	44%

Table (3): TTV and viral hepatitis co-infection among patients (solid and hematological tumour subgroups) and control groups.

	Positive blood transfusion	Surgical intervention	Positive HCV-RNA	Positive HCV-Ab.	Positive HBV-DNA	Positive HBs-Ag.	TTV DNA
Solid tumor (no. 50)	3 (6.0%)	14 (28%)	7 (14%)	12 (24%)	2 (4.0%)	1 (2.0%)	21 (40.38%)
Hematological tumor (no. 50)	18 (36.0%)	1 (2.0%)	9 (18%)	12 (24%)	5 (10.0%)	5 (10.0%)	22 (44%)
Total	21	15	16	24	7	6	43 (43%)
p-value	0.000*	0.000*	0.585	1.0	0.436	0.204	
Control (no. 100)	NA	NA	9 (9%)	9 (9%)	4 (4%)	4 (4%)	44 (44%)
p-value			0.134	0.004*	0.352	0.516	0.887

* Significant *p*-value NA: Not applicable

Table (4): Prevalence of TTV infection and its genotypes in patients and control groups.

	TTV DNA	Genotype 1a	Genotype 1b	Non 1a and 1b genotype
Patient (no. 100)	43 (43%)	16 (37%)	20 (47%)	7 (16%)
Control (no. 100)	44 (44%)	11 (25%)	27 (61%)	6 (13%)
p-value	0.887	0.191	0.201	0.812

DISCUSSION

Transfusion transmitted virus (TTV) is a recently discovered virus, which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus, based on its genomic characteristics **[1,7]**.

The aim of this study is to investigate the incidence and genotyping of the TTV infection among cancer patients and apparently healthy individuals. The incidence of TTV DNA in the sera of normal healthy individuals and blood donors was found to vary widely in different countries **[8,9,10]**. In the current study the incidence of TTV DNA in healthy individuals (control group) was 44% of all cases. Our results were in agreement with other study carried in Egypt. The prevalence of TTV DNA in blood donors was 35.5% **[11]**.

Our results were in disagreement with other studies which found that the prevalence of TTV DNA in healthy individuals in Eygpt was 9%, **[12]**, in Western population was 13% **[13,14]**, in Asian countries as Japan 94% **[1,6,15]**, in Thailand 62%, Koria 53% **[16]**, in national and non national United Arab Emirates 40% and 89% respectively, in Saudi arabia, using primers derived from ORFI and 5UTR, TTV DNA was detected in 5.5% and 50.5% respectively in blood donors **[17]**.

The most probable explanation for the variation in the incidence of TTV DNA among healthy individuals and blood donors, that it may be due to the difference in the geographic area, the level of health care and the primers sets.

The high prevalence of TTV in general population, may reflect its high incidence in patients and may complicate linking TTV to any pathologic states. This unusual feature among viruses aroused the proposal that TTV might be a commensal virus or part of human microflora **[2]**.

A significant difference was found between control group and cancer patient in blood transfusion and surgical intervention; this result is in agreement with other authors' findings **[18, 19]**.

Although TTV was known to be transmitted mainly by blood transfusion; from our results we found high incidence of TTV in healthy individuals, all of them have no history of blood transfusion. So the relatively high prevalence of TTV in healthy individuals and patients with no history of transfusion of blood and blood

products led to the suggestion that alternative routes of transmission of TTV infection may exist. TTV DNA has also been detected in saliva, throat swabs, breast milk, semen and vaginal fluid thus, supporting routes of transmission other than blood and blood products **[5,20,21]**. In addition the children of TTV-infected mothers apparently tend to get infected more often and earlier after birth than children of TTV negative mothers, the role of postnatal transmission of TTV is being considered. Postnatal route of transmission from mother to child and infection via frequent social contacts seem to be very important modes of transmission in children. **[22,23,24]**. We could conclude that TTV is not only parenterally transmitted. This result was in agreement with another author who reported that following implementation of viral inactivation methods in the process of clotting factors concentrates production, the risk for HCV transmission was significantly reduced and to less extent for TTV DNA **[25]**. This conclusion was supported also by other researches that prove that TTV is also transmitted by faeco-oral route, saliva, breast milk and transplacentally **[26]**.

In the current study, TTV infection was detected in different types of cancer patients with no significant difference from its prevalence among the control group. Recently, Camci et al. has reported the high prevalence of TTV in patients with various malignancies. The viral load in cancer patients was extremely high. It might result from the impaired immune reaction. Further studies are needed to explain whether the impairment is caused by the neoplasm or the virus itself **[1,18,27]**.

Our results revealed that TTV is frequently detected in patients with other types of viral infection. TTV co-infection was noted with HCV and HBV in patient and control groups. We found that TTV co-infection had no effect on HCV and HBV infection; this was in agreement with other authors **[11,28]**. TTV did not change the results of chronic hepatitis B therapy with lamivudine and TTV genome was not integrated into the host hepatocyte DNA; which is probably necessary to initiate potential neoplasm development **[29]**.

Though the role of TTV on the cancer patients is not clear. The high incidence of genotype G1 (1a and 1b) among patients and controls in addition to low incidence of non 1a non 1b genotypes may suggested G1 as a candidate for pathogenicity of TTV strains in Egypt. Co-infection of TTV with HCV and HBV in patient and control groups were found, but TTV infection may had no effect on HCV and HBV infection. TTV infection is symptom-free, and found in normal persons and patients with different types of disease not only cancer, without any effects. From that we concluded that TTV may be a part of the normal flora in human body. More studies are needed and also follow-up of TTV infection in patient and controls.

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