Frequency of a *Toxoplasma* Circulating Antigen and Hepatitis C Virus Antigen in Patients with Hepatocellular Carcinoma

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Abstract: It is known that toxoplasmosis rarely leads to various liver pathologies. Hepatocellular carcinoma (HCC) patients are subject to a variety of cellular as well as humoral immunity disorders. Also, hepatitis C virus (HCV) infection is now recognized to be a major risk factor for HCC which triggers tolerance breakdown in specific conditions. It is aimed in this study to investigate the frequency of toxoplasmosis in patients with HCC. Serum samples from 134 HCC patients undergoing surgery and 205 samples from asymptomatic individuals were used for screening of HCV antigen and *Toxoplasma* antigen (Toxo-Ag) using ELISA. 96 out of 134 serum samples (72%) of HCC patients and 51 out of 205 serum samples of asymptomatic individuals (25%) were positive for HCV antigen. 36 KDa *Toxoplasma* antigen was purified from sera of HCC patients using electroelution technique and quantified in serum samples. A dose-response curve was constructed to estimate Toxo-Ag in serum samples and the cutoff level was set at 1.35 ng/ml. Of the 96 HCC patients associated with chronic HCV infection, 52 (54.2%) were Toxo-Ag positive. However, of the 38 HCC patients non-infected with HCV, 14 (36.8%) were positive for Toxo-Ag**.** 10 out of the 51 (19.7%) asymptomatic individuals positive for HCV-Ag were positive for Toxo-Ag . Also, 30 out of the 154 (19.5%) asymptomatic individuals negative for HCV-Ag were positive for Toxo-Ag. A positive significant correlation was found between the concentration of Toxo-Ag and HCV-Ag in patients with HCC. There is a new lethal coinfection of HCV and *Toxoplasma*; each may accelerates the course of the other.
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1. Introduction:

Chronic hepatitis C virus (HCV) infection provides clues to the possible role of viruses as triggers of autoimmunity. Autoimmune disorders may be due to dysfunction of both cellular and humoral immunity (**Vergani and Mieli-Vergani, 2007)**. HCV infection is now recognized to be a major risk factor for HCC, evidenced by finding both antibodies to HCV (anti-HCV) and HCV RNA in serum of a substantial proportion of patients with HCC around the world and by the progression of liver disease to cirrhosis and HCC in individual patients infected with HCV (**Di Bisceglie, 1997).** Egypt has possibly the highest HCV prevalence worldwide, estimated among the general population to be around 14%. HCC is a major cause of cancer death worldwide, accounting for over half a million deaths per year. The geographic pattern of HCC incidence is parallel to exposure to viral etiologic factors. Its incidence is increasing, ranging between 3% and 9% annually depending on the geographical location (**Anwar *et al.*, 2008)**. Patients who have HCC are subjected to a variety of cellular as well as humoral immunity disorders.

Toxoplasmosis is a disease of considerable public health impact. As the transmission, occurrence and phenotype of this disease are influenced in a complex way by host genetics, immunity, behaviour and by the agent characteristics, prevention will not be simple. It is a common opportunistic infection in patients with advanced HIV disease (**Elsheikha, 2008)**. *Toxoplasma gondii* (*T.gondii)* is an obligate intracellular protozoan of worldwide distribution. Development of cell-mediated immunity after acute infection with *T.gondii* results in control but not eradication of the infection. In most countries, seroprevalence of *Toxoplasma* ranges between 20% and 60%. The prevalence is quite low in extremely dry and cold regions. It has been reported that the prevalence is rather high in warm and humid areas (**Montoya and Remington, 2000)**.

Toxoplasmosis can vary from an asymptomatic, self-limiting infection to a fatal disease, as seen in patients with congenital infections. Immunocompromised hosts, especially those with deficient cellular immunity, are at risk of recrudescence of chronic infection and dissemination, with the occurrence of fulminating disease (**Yazar *et al.*, 2004).** Therefore, it is of

interest to investigate the frequency of toxoplasmosis in patients with HCC and to detect and identify a *Toxoplasma* circulating antigen in sera of HCC patients.

2. Subjects and Methods

Serum was prepared from blood samples of 134 patients with HCC attending to the outpatient hospital, Gastroenterology Unit, Mansoura University. The age range of the patients was 20-80 and was that of the asymptomatic (n=205) individuals. All patients were notified that the blood samples taken from them were for search purposes and they gave consent. All patients were diagnosed by computed tomography (CT) scan of the abdomen using intravenous contrast agent and three-phase scanning. In most of patients there was a mass shown on abdominal CT scan. All patients showed increased serum levels of liver enzymes, reduced serum albumin, and increased serum billirubin. Examination of the liver biopsies showed HCC.

Detection of HCV antigen:

HCV antigen in serum samples was detected using a specific anti-HCV antibody at dilution 1:200 which was kindly provided by Prof. Dr. Abdelfattah M.Attallah. according to the method of Crowther (1998).The cut-off value according to this method is 41 µg/ml.

Detection of *T. gondii* antigen:

A specific polyclonal antibody for *Toxoplasma* was used for the detection of *T.gondii* in serum samples according to the method of Crowther (1998). The cut-off O.D for ELISA positivity was set as mean O.D plus three S.D. for the sera from healthy individuals and it is 0.3 O.D.

***Toxoplasma gondii* antigen isolation:**

Serum samples from HCC patients were resolved using SDS-PAGE according to the method of Laemmli (1970), then electro-transferred onto nitrocellulose membrane (NC) according to the method of Towbin *et al.* (1970). The NC membrane was blocked using 3 % (w/v) bovine serum albumin (BSA) dissolved in 0.05% M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with specific polyclonal antibodies to *T.gondii antigen* (1 : 75) in 1% BSA dissolved in TBS with constant shaking. The NC membrane was washed three times, 15 min each, in TBS followed by incubation for 2 h with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1: 50 in TBS. After washing 3 times with TBS (15 min each), the NC membrane was exposed to alkaline phosphatase substrate [5-bromo-4-chloro-3-indolyl phosphate] (BCIP)/ nitroblue tetrazolium (NBT) in 0.1 M Tris buffer, pH 9.6 (Sigma) for 10 min and the reaction was stopped using distilled water. The specific band of target antigen appeared at 36 kDa.

Gel electroelution:

The target *Toxoplasma* antigen band was cut and electroeluted from preparative polyacrylamide gels at 200 volts for 4 h in a dialysis bag (Sigma). After dialysis, the electroeluted antigen band was concentrated using polyethylene glycol and 40% trichloroacetic acid, then centrifuged at 6500 × g for 15 min. The precipitate was washed twice using diethyl ether. The excess diethyl ether was removed by gentle drying and the pellet was reconstituted in phosphate buffered saline (PBS), pH 7.2. The protein content of the purified *Toxoplasma* antigen band was determined according to the method of Lowry *et al*. (1951).

Dose-response curve of isolated *Toxoplasma* antigen:

Serial Concentration of isolated *Toxoplasma* band (0.20 – 8.8 ng/ml) were tested in parallel to establish a dose-response curve using *Toxoplasma* polyclonal antibodies. The concentrations of the unknown samples were determined by interpolation from the dose-response curve (Fig. 1). Serum samples from 8 positive controls, 66 out of 134 HCC patients (49.2%) and 40 out of 205 asymptomatic individuals (19.5%) showed concentration above the cut off level of 1.35 ng/ml.

3. Results

96 out of 134 (72%) serum samples of HCC patients and 51 out of 205 (25%) serum samples of asymptomatic individuals were positive for HCV antigen. The mean serum HCV concentration in patients with HCC was 137.82±16.13 µg/ml. while, that of the asymptomatic individuals was 22.07±4.18 µg/ml. A highly significant ($P<0.0001$) difference exists between both values (Table 1).

Of the 96 HCC patients associated with chronic HCV infection, 52 (54.2%) were Toxo-Ag positive. However, of the 38 HCC patients non-infected with HCV, 14 (36.8%) were positive for Toxo-Ag. 10 out of the 51 (19.7%) asymptomatic individuals positive for HCV-Ag were positive for Toxo-Ag. Also, 30 out of the 154 (19.5%) asymptomatic individuals negative for HCV-Ag were positive for Toxo-Ag (Table 2).

The mean of serum Toxo-Ag concentrations in HCC patients with HCV infection and those with non-infection were 2.69±0.05 and 2.09±0.5 ng/ml respectively, while its concentrations in HCV

infected and non-infected asymptomatic individuals were 0.71±0.1 and 0.70±0.08 ng/ml respectively (Table 3).

A positive significant correlation was shown between the concentrations of Toxo-Ag and HCV antigen in patients with HCC (Fig. 2) while no significant correlation was shown between both parameters in asymptomatic individuals (Fig. 2).

Fig. 3 shows box plot representing the relation between the pathological diagnosis and Toxo-Ag concentration in different groups. The median of serum Toxo-Ag levels were 0.67, 0.69, 1.6 and 2.1 in asymptomatic individuals non-infected with HCV, asymptomatic individuals infected with HCV, HCC patients non-infected with HCV, and HCC patients infected with HCV respectively.

The statistical significances for the differences each 2 mean serum toxoplasma levels are shown in Table 4.

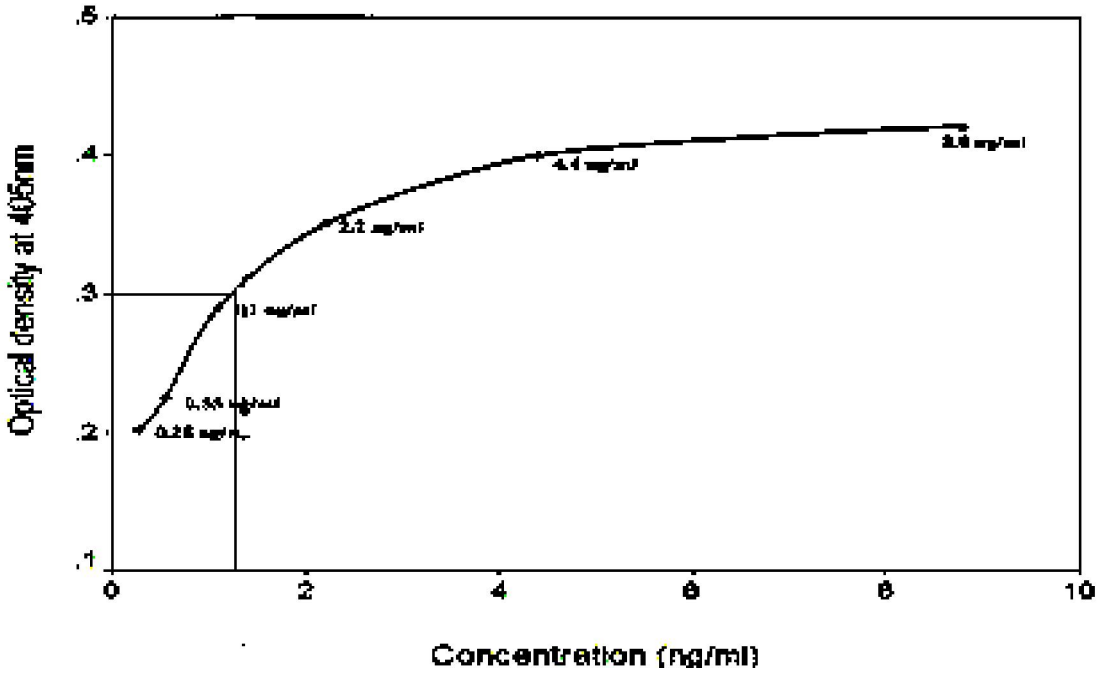

Figure 1. Dose-response curve for purified 36 KDa *Toxoplasma* antigen in the ELISA showing optical densities (OD) at 405 nm. Cut off level was set at 1.35 ng/ml.

Table 1. Detection of serum HCV antigen concentration in HCC patients and asymptomatic individuals:

Group	HCV antigen mean (µg/ml)	Range (µg/ml)	HCV-Ag using ELISA		Detection rate %	P *
			+ ve	-ve		
HCC (n=134)	137.82±16.13	2-900	**96 (72%)**	**38 (28%)**	**72 %**	**<0.0001**
Asymptomatic individuals (n=205)	22.07±4.18	0-169	**51 (25%)**	**154 (75%)**	**25 %**	

* Highly significant (P<0.0001) difference in the detection rate of HCV antigen between the two groups.

Table 2. Relation between detection of Toxo-Ag and HCV-Ag in HCC patients and asymptomatic individuals:

HCV-Ag	Toxo-Ag					
	HCC patients			Asymptomatic individuals		
	Positive N(%)*	Negative N(%)*	Total	Positive N(%)*	Negative N(%)*	Total
Positive	52 (54.2%)	44 (45.8%)	96 (72%)	10 (19.7%)	41 (80.3%)	51 (25%)
Negative	14 (36.8%)	24 (63.2%)	38 (28%)	30 (19.5%)	124 (80.5%)	154 (75%)
Total	66 (49.3%)	68 (50.7%)	134	40 (19.5%)	165 (80.5%)	205
P value	0.0001**			0.78***		

* N(%)= number of samples (percentage).

** A highly significant difference was shown between the detection rates of Toxo-Ag in HCC patients non-infected HCV and HCC patients with HCV infection

*** No significant difference was shown between the detection rates of Toxo-Ag in asymptomatic individuals non-infected with HCV and asymptomatic individuals with HCV infection

Table 3. The mean concentration of Toxo-Ag concentration in sera of patients with HCC and asymptomatic individuals.

Group	Toxo-Ag (ng/ml)		T test
	Mean	Range	
HCC patients non-infected with HCV	2.09±0.5	0.01-10	P<0.05**
HCC patients with HCV infection	2.69±0.05	0.01-8	
Asymptomatic individuals non-infected with HCV	0.70±0.08	0.00-6	P>0.05*
Asymptomatic individuals with HCV infection	0.71±0.1	0.00-3.2	

* P > 0.05 not significant. **P < 0.05 significant.

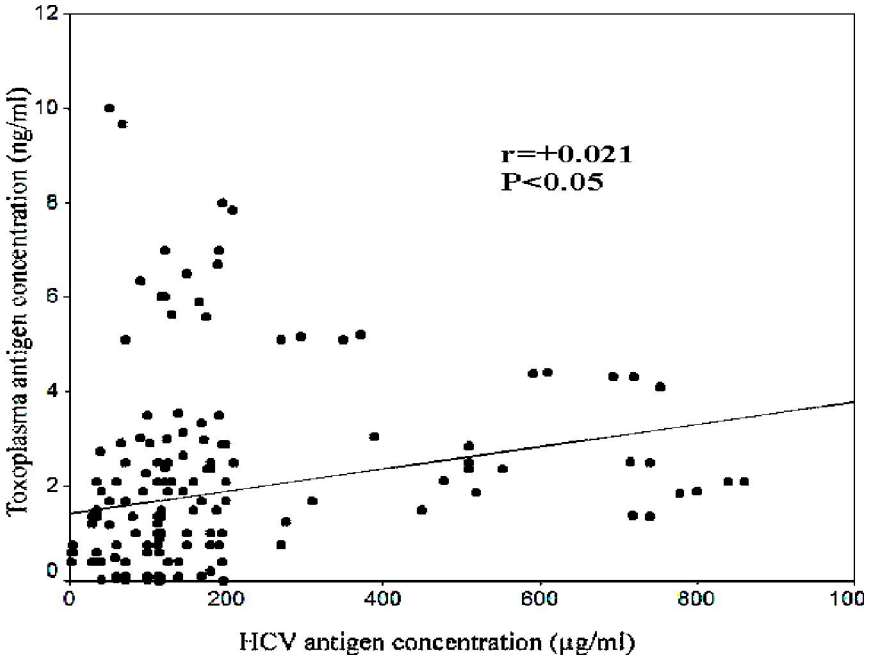

Figure 2: The correlation between the concentrations of Toxo-Ag and HCV-Ag in patients with HCC.

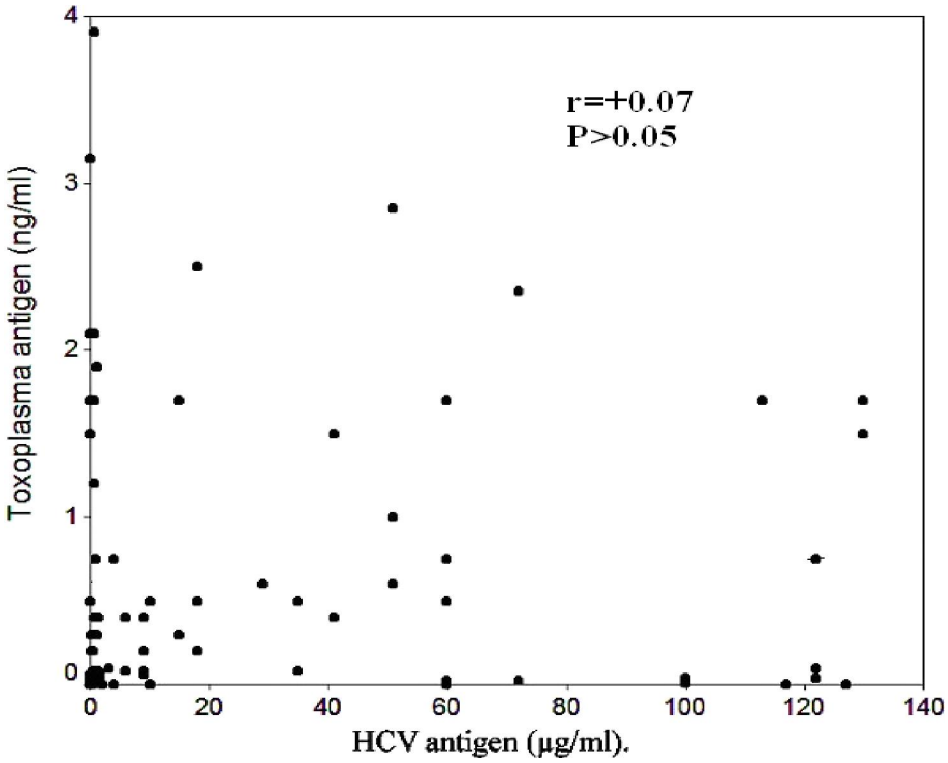

Figure3. Scattered diagram between the concentrations of Toxo-Ag and HCV-Ag in asymptomatic individuals.

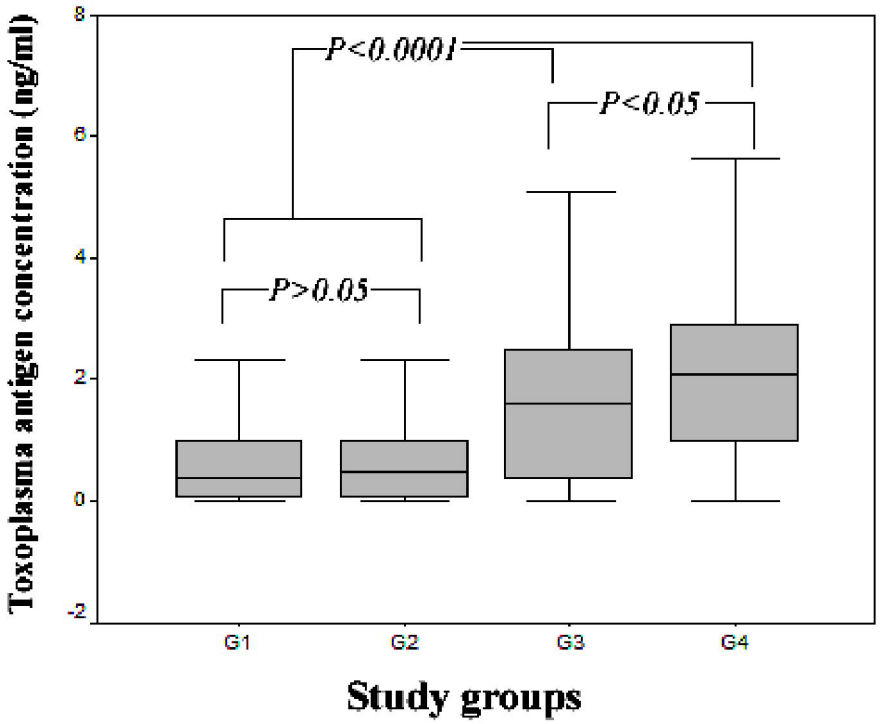

Figure 4: Box plots for Toxo-Ag in HCC patients and asymptomatic individuals.

Group1 (G1): Asymptomatic individuals non-infected with HCV. G2: Asymptomatic individuals with HCV infection. G3: HCC patients non-infected with HCV and G4: HCC patients with HCV infection.

Table 4. Differences between mean serum concentrations (ng/ml) of Toxo-Ag in HCC patients and asymptomatic individuals.

Comparison	Difference	t	P value
HCC patients non-infected with HCV vs asymptomatic individuals non-infected with HCV	**1.39**	**5.097**	**<0.0001*** **
HCC patients non-infected with HCV vs asymptomatic individuals with HCV infection	**1.38**	**3.84**	**<0.0001*** **
HCC patients with HCV infection vs asymptomatic individuals non-infected with HCV	**1.8**	**8.648**	**<0.0001*** **
HCC patients with HCV infection vs asymptomatic individuals with HCV infection	**1.79**	**6.005**	**<0.0001*** **
HCC patients with HCV infection vs HCC patients non-infected with HCV	**0.41**	**0.813**	**<0.05** **
asymptomatic individuals with HCV infection vs asymptomatic individuals non-infected with HCV	**0.01**	**0.109**	**>0.05***

* P > 0.05 not significant. ** P < 0.05 significant. ***P < 0.0001 highly significant.

4. Discussion

The present results indicate a strong causal association between HCV and HCC, as shown by the raised prevalence of HCV antigen in patients with HCC (Table 1). HCV antigenemia-based ELISA test can serve as a useful addition to HCV diagnostic methods since when ELISA was used for the detection of positivity rate of HCV antigen it gave 72% in HCC patients and 25% in asymptomatic individuals. When HCV antigen was quantitated in serum of HCC patients and asymptomatic individuals, the mean concentrations were 137.82±16.13 and 22.07±4.18 µg/ml respectively in patients with HCC and asymptomatic individuals and the difference between both values is highly significant (P < 0.0001).

It is known that toxoplasmosis rarely leads to various liver pathologies, most common of which is granulomatose hepatitis in patients having normal immune systems (Yazar *et al.*, 2004). When ELISA technique was employed for the detection of 36 KDa Toxo-Ag in HCC patients and the asymptomatic subjects total seropositivity rates were 49.3% and 19.5%; respectively. These results agree well with recent studies showing significantly higher seropositivity rate for Toxo-Ag in patients with neoplasia than the control subjects (Yuan *et al.*, 2007;Khabaz *et al.*, 2010). In addition other previous studies indicated that the seropositivity for IgG and IgM of patients with cirrhosis was 68.5% (Ustun *et al.*, 2004). These immunological changes may reveal that the immune system functions are disturbed in patients with neoplasias.

Toxoplasmosis has been most often described in association with some specific malignancies such as Hodgkin's disease, lymphoma, acute and chronic leukaemias or multiple myeloma. These diseases are associated with defects in cell-mediated immunity, and it is clear that T-cell dysfunction, when augmented by the use of immuno-suppressive therapies, predisposes to the development of toxoplasmosis (Ghasemian *et al.*, 2007). Thus, the patients with neoplasia may undergo acute reactivation. In the routine serological survey of cancer patients, results compatible with reactivation or acute infection could influence the treatment protocol.

The results of the present study indicate that the detection of *T.gondii* circulating antigen appears to be promising alternative approach for laboratory diagnosis of toxoplasma infection. Also, there was a significant (P<0.05) correlation between the concentration of Toxo-Ag and HCV antigen in patients with HCC. It may be considered that toxoplasmosis can cause more frequent and more severe diseases in patient with HCC and is capable of

changing the course of the disease or the contrary. Therefore, patients with neoplasias should be screened for *T.gondii* routinely to prevent the possibility of severe toxoplasmosis. Also, HCC patients with HCV and *Toxoplasma* might be seriously taken into account since each may accelerate the course of the other.

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