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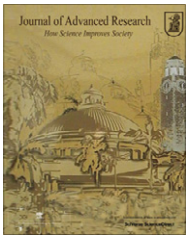

ORIGINAL ARTICLE

Impact of *Helicobacter pylori* infection on liver fibrosis in Egyptian patients with chronic hepatitis C

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Abstract Both *Helicobacter pylori* (HP) and hepatitis C virus (HCV) infections are endemic in Egypt. This work aimed to investigate the presence of HP in the liver of patients with chronic hepatitis C (CHC) and explore the relation between HP infection, liver histopathology and HCV viral load. The study included 60 patients with CHC. Virological, biochemical, liver biopsy and testing for anti-Hp and anti-schistosomal antibodies in serum were done. Liver tissues were examined for histopathological and presence of Hp by detection of HP 16S rRNA gene by PCR and sequence analysis. Anti-schistosomal and anti HP antibody was found in 45% and 61.7%, respectively. Low stages of fibrosis (F0–F3) were found in 73.3% and advanced fibrosis (F4–F6) in 26.7%. HP DNA was found in 10% of the liver specimens. Although the frequency HP antibodies was equally high in patients with advanced and low fibrosis (68.8% and 59.1%, $P > 0.05$), the HP DNA in liver tissue was significantly more frequent in patients with advanced fibrosis (31.25% vs. 2.7%, $P = 0.004$). Meanwhile, the median viral load of HCV was higher in patients with HP DNA in liver tissue compared to patients with no HP DNA in liver tissue (337.000 vs. 165.000,

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$P = 0.3491$). HCV RNA titer, fibrosis score and history of blood transfusion, are independent factors associated with HP DNA in liver tissue. In conclusion, the presence of HP in liver tissue of patients with advanced fibrosis suggests a potential relation between HP infection and progression of liver fibrosis due to HCV.

Introduction

Hepatitis C virus (HCV) is the major agent in non-A non-B hepatitis with serious complications ranging from chronic inflammatory disease to hepatic cirrhosis and end-stage liver failure or hepatocellular carcinoma (HCC) [1]. Egypt has high prevalence of hepatitis C, resulting in high morbidity and mortality from liver disease. Approximately 12% of blood donors are seropositive for HCV antibodies [2]. In a recent community-based study, El-Zanaty and Way, reported positive HCV RNA in sera of 9.8% of 1126 representative Egyptian citizens [2].

The course of HCV related hepatic disease varies markedly from one patient to another. Several factors including age at exposure, duration of infection, alcohol intake, male gender, viral immune response and steatosis have been shown to be associated with fibrosis progression [3].

However, even in the absence of these factors, disease progression may be observed in some patients, suggesting the role of other factors. Host genetic factors or environmental factors, such as a bacterial co-infection, could be involved [4]. It has been observed that *Helicobacter* species were associated with the pathogenesis of human enterohepatic diseases [5] The discovery of the presence of *Helicobacter* species DNA in liver material from patients with liver disease has led to the challenging hypothesis that these bacteria may play a role in the evolution of hepatic lesions from chronic viral hepatitis to cirrhosis and HCC. Determinants of this evolution are not yet fully understood, including those occurring in HCV positive patients [6].

Meyer-ter-Vehn et al. documented that several *Helicobacter* spp. could secrete a liver specific toxin that causes hepatocyte necrosis in cell culture, and might therefore also be involved in damaging liver parenchyma *in vivo* [7].

Concerning HCV liver diseases, HP and *H. pullorum* DNA have been detected in the liver tissue of patients with chronic hepatitis C (CHC) and HCC, suggesting that these bacteria could be implicated in the progression of CHC to cirrhosis and HCC [8].

Infection with HP is common in Egypt and acquisition of infection occurs at a very young age [9]. A study carried on Egyptian patients found that HP antibodies were found in 55.6% of HCV-infected patients vs. 39.4% of the healthy controls. Moreover, the prevalence of HP infection was increased significantly from chronic active hepatitis to cirrhosis [10].

The association between HP infection and severity of chronic liver diseases in patients with hepatitis C virus has been documented in different parts of the world. However, no conclusive data is available in Egypt till now. These observations promoted us to seek out the possible occurrence and association of HP DNA with the pathological stages in liver among CHC Egyptian patients.

Patients and methods

This cross sectional descriptive study included 60 patients with CHC, referred to the liver unit of Suez Canal University Hospital to have a percutaneous liver biopsy, to evaluate suitability for antiviral therapy with pegylated interferon/ribavirin. Their ages ranged from 26 to 58 years.

Diagnosis of CHC was based on positivity to anti-HCV antibodies, HCV RNA, either elevated or fluctuating ALT for more than 6 months, and/or bright liver by abdominal ultrasonography. The study excluded patients co-infected with HBV or HIV and patients with clinical or ultrasonographic evidence of cirrhosis.

Sera were collected from each individual and stored immediately at −20 °C until use. Liver function tests, alfa-fetoprotein (AFP), and anti-schistosomal antibodies were measured using commercially available indirect haemagglutination assays (IHA) kits. The HCV RNA viral load was quantified using Real Time PCR technique in an ABI PRISM® 7000 thermocycler (Applied Biosystems, Foster City, CA). The serological and biochemical tests were done in clinical pathology department and the molecular analysis was performed at oncology diagnostic unit of Suez Canal University Hospital. The study was approved by the Research Ethics Committee of the Faculty of Medicine and informed consents were obtained from each participant.

Processing of liver tissues

Liver tissues were cut into two parts: one was formalin fixed and paraffin embedded for histo-pathological examination and the second was immediately stored at −20 °C until further molecular analysis. Histo-pathological examination was performed by faculty staff of pathology. Hepatic fibrosis staging was made according to Ishak scoring system [11]. Accordingly patients were divided a group of low fibrosis including F0–F3 and a group of advanced fibrosis including F4, F5 (incomplete cirrhosis) and F6 (complete cirrhosis). According to the histological activity index patients were divided into a group of minimal to mild activity (grades from 1 to 8) and a group of moderate to severe activity (grades from 9 to18) [12].

Detection of anti-HP antibody

Plasma samples were tested for anti-HP IgG antibody using a commercial test kit, AccuBind™ ELISA Microwells (Monobind Inc., Lake Forest, USA) according to the manufacturer's instruction. Results were considered positive when higher than 20 U/ml.

Detection of *Helicobacter* DNA from liver biopsy.

DNA extraction

Genomic DNA was extracted from liver biopsy using Wizard®SV Genomic DNA Purification System (Promega Corporation, Madison, USA). DNA quantitation was performed using the NanoDrop® (ND)-1000 Spectrophotometer (NanoDrop Technologies Inc., Washington, USA). The extracted DNA was stored in −20 °C until used.

PCR amplification

Nested PCR was performed with *Helicobacter* genus-specific 16S ribosomal RNA gene (16S rDNA) primers (Helinest-S & R, Heli-S & R) which reported to amplify 26 species of *Helicobacter* genus [13].

First amplification

The amplification was carried out in a final volume of 50 µl reaction mixture containing: 1 µg DNA, 25 µl DreamTaq™Green PCR Master Mix (Fermentas, CA, USA), 50pM Heli-nest-S primer: 5′-ATTAGTGGCGCACGGGTGAGTAA-3′, 50pM Heli-nest-R primer: 5′-TTTAGCATCCCGACTTAAGGC-3′.

The reaction mixture was initially denaturated at 94 °C for 2 min, then amplified for 35 cycles as follow: Denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C, extension at 72 °C for $1^1/_2$ min and final extension at 72 °C for 5 min in Robocycler Gradiant 96 Thermo cycler (STRATAGEN®, LA, USA).

Second amplification

5µl of the first amplification product, 25 µl DreamTaq™Green PCR Master Mix (Fermentas, CA, USA), 50pM Heli-S primer: 5′-GAACCTTACCTAGGCTTGACATTG-3′, 50pM Heli-R primer 5′-GGTGAGTACAAGACCCGGGAA-3′ was amplified by following the same PCR condition as first amplification step.

The amplified products were visualized on 2% ethidium bromide stained agarose gel electrophoresis, the expected size of product from second amplification step was approximately 480 bp.

DNA sequencing

PCR products were sequenced as described [13]. The sequencing results were aligned and compared with known *Helicobacter* species using Basic Local Alignment Search tool (BLAST; National Center for Biotechnology Information).

Results

Demographic and laboratory data of 60 patients included in this study were summarized in Table 1. Their mean age was 42.98 ± 7.6 and 56.7% of patients were between 41 and 50 years. The majority of patients were males (45/60).

Anti-HP antibody was present in 61.7% of patients, being equally high among male and females (62.3% and 60%, respectively). *Helicobacter* DNA was present in liver tissue of 6 out of 60 (10%) of studied patients, using *Helicobacter* genus specific 16S rRNA gene primers, Fig. 1. The PCR products

Table 1 Demographic and laboratory data of the studied population (n = 60).

Age mean age ± SD (range)	42.98 ± 7.6 (26–58)
Gender (male/female)	45/15
PLT mean ± SD (range)	207 ± 175.48 (105–1480)
ALT mean ± SD (range)	59.60 ± 36.922 (17–187)
AST mean ± SD (range)	51.64 ± 24.820 (18–159)
Total bilirubin mean ± SD (range)	0.72 ± 0.26 (0.3–1.8)
direct bilirubin mean ± SD (range)	0.27 ± 0.15 (0-.8)
HCV PCR median (range)	165000 (327–7520,000)
AFP median (range)	2.3 (0.1–209)
Anti-Bilharzial Ab no (%)	27 (45%)
Anti-HP Ab no (%)	37 (61.7%)
Grade of chronic hepatitis C activity no (%)	
Minimal to mild	49 (81.7%)
Moderate to severe	11 (18.3%).
Stage of fibrosis no (%)	
Low stage	44 (73.3%)
Advanced stage	16 (26.7%).

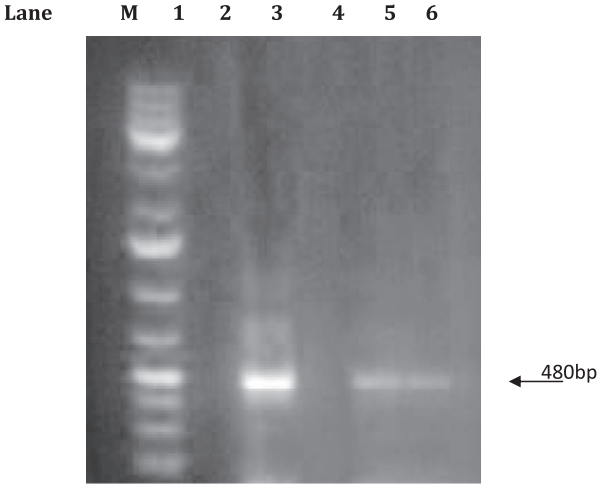

Fig. 1 Amplification of a 480-bp 16S rRNA DNA of *Helicobacter*. Lane M: molecular size marker (100–1000 bp); lane 2, 4, 5: positive samples; lane 6: negative control (double-distilled water).

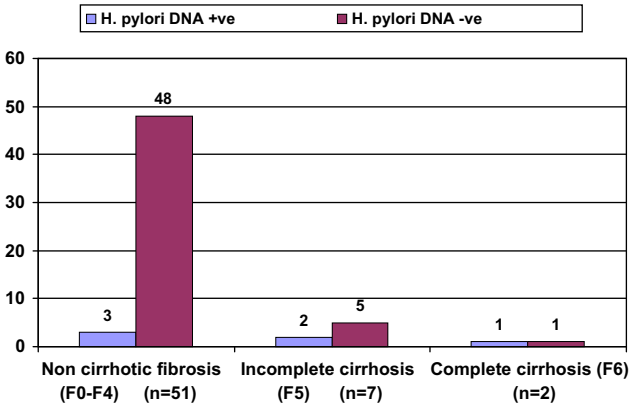

Fig. 2 HP DNA in liver tissues according to severity of fibrosis.

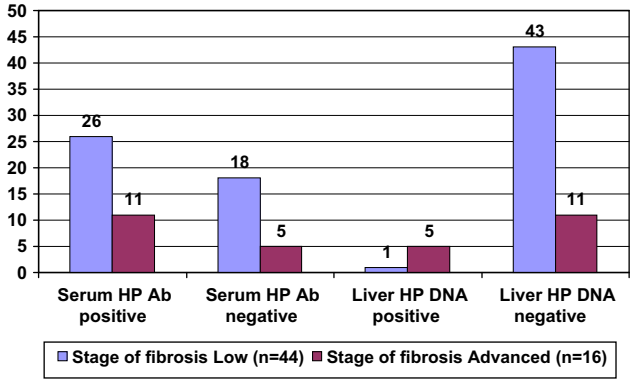

Fig. 3 HP Sero-reactivity and HP DNA in liver tissue of 60 patients in relation to fibrosis staging.

were sequenced and HP like organisms was identified. All HP DNA positive cases were anti-HP antibodies positive. Most of patients had low fibrosis (44 of 60, 73.7%) and minimal to mild activity of necroinflammation (49/60, 81.9%). Cirrhosis was found in nine patients; incomplete cirrhosis (F5) in seven and complete cirrhosis (F6) in two Table 3.

Although anti-HP was equally high in patients with low and advanced fibrosis with no statistically significant difference (59.1% and 68.8%, $P = 0.496$) Fig. 3, HP DNA in liver tissue was significantly more frequent in advanced fibrosis (5/16, 31.25%) compared to low fibrosis (1/44, 2.27%) ($P = 0.004$), Tables 4 and 5 and Fig. 2.

Patients with HP DNA in liver tissue showed higher median value of HCV RNA compared to patients with no HP DNA ($P = 0.3491$), Table 2. Meanwhile the median viral load was higher in patients with moderate to severe activity and patients with advanced fibrosis (286,000 and 192,500, respectively) compared to patients with minimal to mild activity and patients with low fibrosis (108,000 and 135,000, respectively), Table 6. Although of no significance, higher values of ALT, AST and AFP were found in patients with HP DNA in liver tissue compared to the other group, Table 2. Interestingly, independent factors associated with positive HP DNA (as dependent factor) in liver tissues included history of blood transfusion (OR = 100.5, 95% C.I. = 1.6–6176.8, $P = 0.028$), advanced fibrosis score (OR = 4.19, 95% C.I. = 1.4–12.52, $P = 0.01$) and high HCV RNA viral titer (OR = 1.0, 95% C.I. = 1.0–1.0, $P = 0.044$) Table 7.

Table 3 Distribution of HP DNA in liver tissues according to stage of fibrosis.

Stage of fibrosis	HP DNA +ve (no = 6)
Complete cirrhosis (F6)	1 (50%)
Incomplete cirrhosis (F5)	2 (28%)
Chronic hepatitis without cirrhosis (F0–F4)	3 (6%)

Anti-schistosomal antibodies were found in 45% of patients, 66.7% of them had anti-HP antibodies and only 3.7% had HP DNA in liver tissues Table 8.

Discussion

Previous studies showed that DNA from HP – and *H. pullorum*-like organisms were present in the liver of cirrhotic patients with or without HCC due to HCV, suggesting that *Helicobacter* species could be a co-morbid factor for disease progression [6]. In this study, we demonstrated the presence, *Helicobacter DNA* using genus-specific 16S rRNA gene primers in liver tissue of 10% of the studied patients.

Interestingly, the gene sequence obtained from positive *Helicobacter* species specific 16S rRNA PCR was analogous to HP and not similar to *H. hepaticus*, found in mouse liver tumors [14], or to species previously found in the biliary tract of humans, such as *H. pullorum*, *H. bilis*, and *H. Rappini* [15]. This finding encourages the speculation that the presence of *Helicobacter* DNA in human liver tissue might reflect the transport of HP of gastric origin or its DNA to the liver [13] and that intestinal *Helicobacter* might be implicated in hepatobiliary disease [16].

In this study, although anti HP was equally high in patients with low and advanced fibrosis with no significant difference, HP DNA in liver tissue was significantly associated with HCV related advanced hepatic fibrosis. It was present in 33.3% of patients with cirrhosis and 5.9% with no cirrhosis. This finding is comparable to 41.6% and 17% as reported by Castéra et al. [4] and 68% and 3.5% as reported by Rocha et al. [6] respectively in cirrhotic and non cirrhotic patients. Other similar studies have reported the association between HP DNA in liver tissue and

Table 2 Comparison of laboratory data regarding presence of HP DNA in liver tissue.

Laboratory data	HP DNA (in liver tissue)		*P*-value
	Positive (no = 6) Median (range)	Negative (no = 54) Median (range)	
HCV RNA titre	337,000 (51,200–7520,000)	165,000 (327–7,060,000)	0.3491[a]
Platelets	168,500 (11,000–236,000)	170,000 (105,000–336,000)	0.9803[a]
ALT	57 (17–143)	47 (22–187)	0.5962[a]
AST	58 (22–90)	42 (18–159)	0.4159[a]
Albumin	4.6 (4.1–5.1)	4.3 (3–5.1)	0.0979[a]
PT	12.7 (12.1–15.2)	12.8 (11.2–15.5)	0.9409[a]
T. Bilirubin	0.75 (0.5–1.8)	0.7 (0.3–1.3)	0.3974[a]
D. Bilirubin	0.25 (0.1–0.4)	0.28 (0.02–0.8)	0.9388[a]
AFP	3.5 (0.5–32.9)	2.2 (0.1–33.7)	0.7115[a]

Statistically significant ($P < 0.05$).
[a] Kruskal-Wallis test.

Table 4 HP sero-reactivity and HP DNA in liver tissue of patients with low and advanced stage of fibrosis.

HP infection	Stage of fibrosis		*P*-value
	Low (no = 44) No (%)	Advanced (no = 16) No (%)	
HP Ab sero-positive	26 (59.1)	11 (68.8)	(0.496)[c]
HP Ab sero-negative	18 (40.9)	5 (31.2)	
HP DNA positive in liver	1 (2.3)	5 (31.2)	(0.004)[ab]
HP DNA negative in liver	43 (97.7)	11 (68.8)	

[a] Statistically significant ($P < 0.05$).
[b] Fisher's exact test.
[c] Chi-square test.

Table 5 Relation between the grades of chronic hepatitis C and HP DNA in liver tissue.

HP DNA	HAI scoring grade		*P*-value
	Minimal/mild (49)	Moderate/severe (11)	
	No (%)	No (%)	
Positive	3 (6.1%)	3 (27.3)	0.068[a]
Negative	46 (93.9%)	8 (72.7)	

Statistically significant ($P < 0.05$).
[a] Fisher's exact test.

cirrhosis in patients with chronic liver disease related to HCV [10–19]. This association might be explained by increased colonization of HP in the liver of patients with chronic hepatitis C and advanced fibrosis. Otherwise, infection of the liver with HP acts as a co-factor in promoting fibrogenesis [6] particularly when the HCV RNA load is high. This hypothesis agreed with that of Fagoonee et al. who supposed that co-infection with HP or *Helicobacter* species might amplify the chronic inflammation of liver parenchyma, thereby leading to cirrhosis and HCC [20].

Chronic hepatitis is an inflammatory disease, characterized by increased levels of pro-inflammatory cytokines such as interleukins 1, 6 (IL-1, IL-6), tumor necrosis factor (TNF) and by the presence of lympho-mono cellular infiltrate and lymphoid follicle formation [21]. Viruses such as HCV are only capable of limited inflammation, due to shedding of IL-1 receptor in circulation, thereby limiting the possibility of IL-1 binding to cellular receptors [22]. Helicobacters, on the other hand, are strong inducers of the inflammatory cascade [23] infection with them could lead to the accumulation of extraordinary number of lymphocytes and polymorphonuclear cells in the infected tissue [24].

It is worth noting that the lower prevalence of HP DNA in liver specimens of cirrhotic patients in this study compared to Rocha et al. [6] is possibly due to the difference in the severity of liver disease in both studies. The cohort of Rocha and colleges included patients with chronic hepatitis and cirrhosis

Table 6 HCV RNA viral load in the studied patients according to the stages of fibrosis and grades of chronic hepatitis C.

	HCV RNA range	Median	*P*-value
Min to mild activity (no = 49)	327–7520,000	108,000	0.1906[a]
Mod to severe activity (no = 11)	51,200–2320,634	286,000	
Low fibrosis (no = 44)	560–7520,000	135,500	0.5418[a]
Advanced fibrosis (no = 16)	327–2320,436	192,500	

Statistically significant ($P < 0.05$).
[a] Kruskal-Wallis test.

Table 7 Multiple logistic regression analysis for independent factors associated with detection of HP DNA in liver tissues of 60 patients with chronic hepatitis C.

	Beta	Standard error	*P*-value	Odds ratio	95.0% C.I. for odds ratio	
					Lower	Upper
Constant	0.237	3.295	0.943	1.268		
Blood transfusion (reference: no)	4.61	2.101	0.028[a]	100.5	1.6	6176.8
HCV RNR titre (reference: low viral load < 400,000)	0.006	0.003	0.044[a]	1.000	1.000	1.000
Stages of fibrosis (reference: low fibrosis)	1.434	0.558	0.010[a]	4.194	1.404	12.528

Dependent variable: (HP DNA +ve = 1, HP DNA −ve = 0).
Excluded variables: Gender, residence, surgery, dental extraction, smoking, DM, HTN, schistosomal titre, platelet count, ALT, prothrombin time, T. Bilirubin, D. Bilirubin, AFP. HP antibody, HP PCR, degree of cirrhosis.
[a] Statistically significant ($P < 0.05$).

Table 8 HCV RNA viral load and stage of fibrosis in patients positive and negative for anti-schistosomal antibody.

	Anti-schistosomal Ab		*P* value
	Positive (no = 27)	Negative (no = 33)	
HCV RNA (IU/ml)			
Range	377–7060,000	4320–7520,000	0.73[a]
Median	225,000	138,000	
Stage of fibrosis (F0–F6)			
Range	F0–F5	F1–F6	0.78[a]
Median	3	2	
HP DNA positive no (%)	1 (3.7)	5 (15.2)	0.15[b]
HP DNA negative no (%)	26 (96.3)	28 (84.8)	
HP Ab positive no (%)	18 (66.7)	14 (42.4)	0.47[c]
HP Ab negative no (%)	9 (33.3)	19 (57.6)	

Statistically significant ($P < 0.05$).
[a] Kruskal-Wallis test.
[b] Fisher's exact test.
[c] Chi-square test.

with and without hepatocellular carcinoma. In this study, all the studied patients were diagnosed clinically as chronic hepatitis and only 9 of them had cirrhosis (incomplete in 7 and complete in 2). In all there were no stigmata of portal hypertension, or decompensation.

The presence of HP in liver tissue could occur via a retrograde route from the duodenum or through the portal circulation. Rocha et al. suggested that the presence of *Helicobacter* could be the consequence of structural changes in the liver namely, intrahepatic shunts; when cirrhosis occurs [6]. However, this does not explain the existence of HP in liver specimens in 3 of 51 patients with non cirrhotic fibrosis and representing 50% of all patients with HP in liver tissue. In this setting, a retrograde route, from the duodenum to the liver might be the underlying mechanism for HP to colonize in liver tissue. It is worth noting that all patients with HP in liver were seropositive for anti-HP antibodies and patients negative for HP DNA in liver tissue were also negative to anti-HP antibodies. This result is similar to that reported by Petrenkienë et al. [25].

In this study, patients with HP DNA in liver tissue showed higher median value of HCV RNA compared to patients with no HP DNA. Meanwhile the median viral load was higher in patients with moderate to severe activity and patients with advanced fibrosis compared to patients with minimal to mild activity and patients with low fibrosis.

Although the explanation of these findings is difficult, the association of HP DNA in liver tissue with high serum HCV RNA load could play a synergistic role in enhancing cytotoxic immune response and promoting fibrosis in patients with CHC. This hypothesis is opposed by the absence of association between viral load and disease severity or progression in patients with chronic liver disease related to HCV in studies targeting the natural history of HCV infection [26–29].

It is worth noting that, history of blood transfusion, high HCV RNA viral load and advanced stage of fibrosis were significantly associated as independent risk factors with presence of HP DNA in liver tissues ($P = 0.028$, $P = 0.044$, $P = 0.01$, respectively). Up to our knowledge, no data concerning these factors and presence of HP DNA in liver tissues are available in the literature.

This study revealed a higher median stage of fibrosis and HCV viral load in patients positive for anti- schistosomal antibody compared to negative patients. However, the difference was statistically not significant. Although non significant, anti HP antibodies was more frequent in patients positive to anti-schistosomal Ab compared to negative patients ($P = 0.47$). This results are consistent with the results of El-Masry et al. study in which, In HCV-infected patients, the concurrent schistosoma infection was documented largely in anti-HP-positive patients [10]. On the other hand, in the anti- schistosomal antibody positive group only 1/27 patient was positive for HP DNA in liver tissue compared to 5/33 of the other group. The higher viremia and stage of fibrosis found in patients positive to anti-schistosomal antibody was associated with a low detection of HP DNA in liver tissue. Although the explanation is difficult, it is suggested that patients concomitantly infected with schistosomiasis and HCV may had an intense inflammatory reaction leading to less colonization of HP in liver tissue [30].

Limitation of this study is the small number of the study subjects and the inability to obtain normal liver tissue to examine for the presence of HP DNA. Therefore, further study on a larger sample size to validate the impact of infection with HP on the outcomes chronic hepatitis C and examine the possibility of finding HP DNA in normal liver tissues. Also to study the molecular similarity between hepatic and gastric HP in specimens from the gastric mucosa.

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