Abstract. Infection with hepatitis C virus (HCV) has become the most important public health problem in Egypt. In Egypt, viral hepatitis along with infection with Schistosoma mansoni is the major cause of chronic liver disease and liver cirrhosis. Although HCV infection is highly prevalent in Egypt, very little information is available on the distribution of the different genotypes of HCV. Our aims in this study were first to determine the prevalence of viral and parasitic infections in patients with chronic liver disease and then to assess the distribution of HCV genotypes in these patients. In the present study, 151 individuals (50 with chronic liver disease, 51 with chronic diseases of organs other than the liver, and 50 apparently healthy persons) were investigated. The last 2 groups served as control groups. These individuals were subjected to routine liver function tests and detection of serum antibodies to bilharziasis, hepatitis B surface antigen (HBsAg), and HCV. Furthermore, the presence of hepatitis G virus (HGV) and HCV in the serum samples were tested for by a reverse transcription polymerase chain reaction (RT-PCR). Prevalence of different genotypes of HCV in patients positive for HCV were determined by RT-PCR using type-specific primers. Results of the study revealed that 84, 74, 12, and 20% of patients with chronic liver disease were positive for Schistosoma mansoni, HCV, HBsAg, and HGV, respectively, as compared to 51, 43.1, 2, and 4% of patients with other chronic diseases and 22, 6, 0, and 0% of apparently healthy individuals. One hundred percent of patients with chronic liver disease, 72.5% of those with other diseases, and 26% of normal controls were shown to have at least one of the studied infectious agents. Two or more of the agents were highly coincident in patients with chronic liver disease. In Egypt, HCV genotype 4a is highly prevalent, where it contributed 85% of the tested samples in comparison to 10, 2.5, and 2.5% for subtypes 1b, 2a, and 3a, respectively. In conclusion, these results suggest that in Egypt, HCV along with schistosomal parasite infection is the major risk factor for chronic liver disease. In most Egyptian patients, HCV genotype 4 is highly prevalent.

Hepatitis C virus (HCV) has been identified as the major causative agent of posttransfusion and sporadic community-acquired non-A, non-B hepatitis (NANBH).1,2 Hepatitis C virus is a single-stranded RNA virus closely related to pestiviruses and flaviviruses. The viral RNA has 2 short nontranslational regions at the 5′ and 3′ ends enclosing a single large open reading frame. Individual proteins, the core (C), envelope (E1 and E2), and nonstructural proteins (NS1–NS5)3–9 are cleaved from the polyprotein by host and viral protease. Hepatitis C virus is highly prevalent in Egypt, where its antibodies were detected in 10–30% of the Egyptian population.5–8 Recently, HCV infection was concluded to be the main cause of chronic liver disease in Egypt, where this infection is largely associated with schistosomiasis (Gandin C and others, unpublished data).9 At present, 9 known HCV types composed of at least 28 subtypes are assumed to be prevalent in different part of the world.10 Various methods have been used for genotyping HCV, including genomic amplification and sequencing,11,12 These include reverse transcription polymerase chain reaction (RT-PCR) with genotype-specific primers,13,14 restriction fragment length polymorphism (RFLP) of RT-PCR–amplified DNA,15 hybridization of PCR product with specific probes by slot blot16 or with different probes immobilized on a membrane, which is called line probe assay,17 and serotyping neutralizing antibodies to each genotype.18

Little information is available on the distribution of different HCV genotypes in Egypt. This may explain the discrepancies in the RT-PCR results between different laboratories in Egypt, because they depend on primers purchased from different manufacturers all over the world. Through amplification and sequencing of 5′ noncoding region (NCR), Simmonds and his colleagues11 were able to distinguish a new HCV genotype termed type 4a, which was detected in sera from Egyptian patients. In another study, by amplification and sequencing of the NS5 region isolated from samples of 3 patients, the same group12 confirmed that the Egyptian genotype is 4a. By RFLP analysis of the 5′ NCR, 17 of 19 Egyptian patients were found to have HCV genotype 4a.15 More recently, by amplification and sequencing of the NS5 region, 8 residents of Germany, who were thought to have acquired their HCV infection in Egypt, were shown to have genotype 4a.19 By sequencing of the C and E1 regions, HCV genotype 4a was identified in 1 Egyptian patient and 1 Yemeni patient.20

Use of sensitive serologic and RT-PCR assays to detect HCV infection revealed that a small proportion of transfusion-associated and community-acquired NANBH was not related to HCV infection.21 Two hepatitis agents, designated hepatitis GB virus C (HGBV-C)22 and hepatitis G virus (HGV),23 were reported. Sequence analysis revealed that these 2 agents are related to members of the family Flaviviridae.23,24 Although HGBV-C was suggested to induce fulminant hepatitis,25 no causal association between the virus and hepatitis was established.26–28 Therefore, in this report we have determined the incidences of HCV, HGV, and hepatitis B virus (HBV) in Egyptian patients with chronic liver disease where schistosomiasis is endemic. Genotyping of HCV in those patients was determined by RT-PCR using type-specific primers. A group of patients with chronic diseases other than liver disease and a group of apparently healthy individuals were included as controls.
Patients’ sera. Serum samples were collected from 151 individuals. Patients were diagnosed based on clinical examination, ultrasonography, computed tomography scan, and liver biopsy whenever possible. The 151 individuals were classified into 3 groups. The first group included 50 patients with chronic liver disease, 30 men and 20 women ranging in age between 23 and 72 years with a mean of 47 years. Of these patients, 28 had cirrhosis, 12 had periportal fibrosis, and 10 had chronic hepatitis. These patients were randomly chosen from those admitted to the Tropical Medicine Department, Al-Azhar University Hospital. They presented with ascites, lower limb edema, epistaxis, or complaints associated with chronic hepatitis. They were admitted for splenectomy, injection of esophageal varices, or aspiration of the ascitic fluid, or were admitted seeking treatment of chronic hepatitis. The second group consisted of 51 patients with other chronic diseases, 36 men and 15 women ranging in age between 33 and 65 years with a mean of 48 years. Of these patients, 19 had chronic urinary tract disease, 12 had chronic obstructive airway disease, mainly asthma, and 20 had various chronic gastrointestinal disorders including gastritis, peptic ulcer, and colitis. These patients were randomly selected from those visiting the Urology, Chest, or Medicine Departments, Al-Azhar University Hospital. The third group included 50 randomly selected apparently healthy individuals, 37 men and 13 women ranging in age between 27 and 68 years with a mean of 43 years. These apparently healthy controls were chosen from the workers and staff of Al-Azhar University, attempting to match the sex and age distributions as closely as possible to those of the 2 patient groups. Most of the individuals in the 3 groups lived in Cairo but had migrated there from various areas of Egypt.

Ten-milliliter blood samples were withdrawn from each individual and separated serum was aliquoted. A fresh aliquot was used to perform routine liver function tests, and the others were stored at -80°C to be used in the other tests. Routine liver function tests, such as serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), direct bilirubin (DB), total protein, and albumin, were performed using a Beckman Autoanalyzer (Synchron CX4; Beckman, Neon, Switzerland). Results of total protein and serum albumin were used to calculate the albumin to globulin ratio (A/G). Serum samples were tested for hepatitis B surface antigen (HBsAg) using an enzyme immunoassay kit supplied by Bionike, Inc. (San Fransisco, CA). This study was approved by the human experimentation committee of Al-Azhar University (protocol approval HCV/3/1996), and informed consent was obtained from all patients.

Schistosomiasis infection (bilharziasis). Serum antibodies to schistosomal parasite infection were detected using a commercially available indirect hemagglutination test (IHAT) kit supplied by Behring Institute (Marburg, Germany). The IHAT is a sensitive test to detect bilharziasis, but it does not differentiate between past and recent infection or between Schistosoma mansoni and Schistosoma hematobium.30

Hepatitis C virus. The presence of HCV RNA was tested by RT-PCR for the highly conserved 5’ NCR. The RNA was extracted from 100 μl of serum by the phenol–guanidium–thiocyanate method,30 and the RNA pellet was resuspended in 20 μl of distilled water. Amplification of HCV sequences by RT-PCR was performed using a standard procedure described previously.31 One microgram of RNA extract was used along with 250 ng of outer antisense (OAS) primer (5’-CACTACTGGCTAGC-3’) for cDNA synthesis for the detection of positive-strand HCV RNA. For RT, 10 μl of Avian Myeloblastosis Virus (AMV) RT enzyme was added, and cDNA synthesis was performed for 1 hr at 42°C. The resulting cDNA was amplified by PCR after adding 250 ng of outer sense (OS) primer (5’-CTGTGAGAACTACTGTC-3’) to 50 μl of PCR reaction mixture containing Taq DNA polymerase. A second PCR was carried out with an inner sense (IS) primer (5’-CACCGAGAAAGCCTAG-3’) and inner antisense (IAS) primer (5’-TTGATCCAAGAAAGGACC-3’) using one-tenth of the first PCR product as DNA template. In all experiments, RNA extracted from a liver specimen known to contain HCV was included as positive control and RNA extracted from a normal liver was used as negative control. The amplification products were electrophoresed on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide. The specificity of the amplicons was confirmed by Southern blotting. The sequence of primers and probe used in the RT-nested PCR have been described previously.31

Hepatitis G virus. For the detection of HGBV-C RNA sequences, RNA extract was subjected to RT followed by nested PCR using degenerate primers directed to NS3 sequences, as published by Yoshihisa and his colleagues.25 The RNA extract obtained from 100 μl of serum sample was subjected to the action of 10 U of AMV reverse transcriptase in the presence of 250 ng of OAS primer G9 (5’-TCYTGTGATGDGAACCTGTC-3’), 1 mM deoxynucleoside triphosphates (dNTPs), 1.5 mM MgCl2, and 1× RT transcription buffer in a final volume of 20 μl. The reaction was conducted for 1 hr at 42°C. One-half of this RT reaction product (cDNA) was amplified by 35 cycles of PCR after adding 250 ng of OS primer G8 (5’TATGGGGCATTGHA-THCYCT-3’) in a 50 μl-reaction mix containing 200 μM dNTPs, 2.5 μl Taq DNA polymerase, 1.5 mM MgCl2, and 1× Taq polymerase buffer. Five microliters of the first PCR product was run into 35 cycles of PCR in a 50-μl reaction mix containing 250 ng of IS primer G10 (5’-TTCYGGCA-YTCAAGGCAGGTGC-3’) and 250 ng of IAS primer G11 (5’-TCYTATTGCCCTRTAATAGGC-3’), 200 μM dNTPs, 1.5 μM MgCl2, 2.5 μl Taq DNA polymerase, and 1× buffer. Each PCR cycle was kept at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec. Twenty microliters of the second PCR product was separated on 2% agarose gel containing ethidium bromide followed by Southern blot analysis using radiolabeled oligonucleotides G12 (5’-TRGCYGGYCA-GATTATCCCTGCT-3’) as a probe. For these primers and probe, R = A or G; Y = T or C; D = A, G, or T; H = A, C, or T; and V = A, C, or G.

Genotyping of HCV. Twenty samples from patients with chronic liver disease and 20 from those with other chronic diseases who were positive for HCV using primers to 5’ NCR were randomly selected for HCV genotyping. The HCV genotyping was done by PCR using type-specific sense
primers directed to the NS5 region. This technique depends on applying universal antisense primer and a mixture of type-specific primers to amplify sequences with different lengths from different subtypes of the HCV genome, which can be differentiated by gel electrophoresis. The general antisense and sense primers specific to subtypes 1a, 1b, 2a, 2b, 3a, and 4a were reported by Chayama and his colleagues, whereas the primer specific to genotype 4a was reported by Simmonds and others. The nucleotide sequence and target position of the primer as well as the size of PCR products are listed in Table 1. The RNA extract was prepared from 100 μl of serum and the pellet was resuspended in 5 μl of distilled water. The entire volume was used for the RT reaction, as described by Enomoto and others, applying the universal antisense primer. Single PCR was performed using a mixture of the 6 type-specific sense primers as described by Chayama and others. The amplified product was subjected to electrophoresis on 3% agarose gel containing ethidium bromide. The subtype of each sample was determined by comparing the size of the amplified DNA fragment with the bands of a 100–base pair DNA marker.

Statistical analysis. Student’s t-test was applied to compare the mean values of liver function tests in the investigated groups. The chi-square test was used to evaluate the difference in the incidence rates of the studied infectious agents.

RESULTS

Results of biochemical analysis of sera from individuals included in this study are shown in Table 2. Liver enzymes, TB, and DB were significantly elevated in patients with chronic liver disease, followed by those with other chronic diseases. The A/G seems to be the most useful biochemical parameter to differentiate patients with established chronic liver disease from other individuals. The A/G was markedly decreased in patients with liver disease compared to patients with other chronic diseases or normal controls. Sixty-six percent of patients with chronic liver disease and 37.3% of those with other diseases had elevated serum AST or ALT, whereas 98% of the first group and 50.9% of the second had abnormal values of at least one of AST, ALT, or A/G. One hundred percent of patients with chronic liver disease and 52.9% of patients with other diseases had an abnormality in at least one of the applied biochemical tests. Combination of these laboratory tests added no additional information to that provided by the measurement of AST alone in the apparently healthy control group, where 8% had abnormal levels (Table 2).

The result of the analysis of the 151 blood samples for schistosomiasis infection, HBV, HCV, and HGV are shown in Table 3. Serologic analysis revealed a high incidence of schistosomal antibodies, HCV, HBV, and HGV in patients with chronic liver disease. These agents are more common in patients with chronic diseases other than that of the liver in comparison to normal controls. Thirty-five patients (70%) with chronic liver disease were shown to have at least 2 of the 4 infectious agents, as compared to 23.5% of patients with other chronic diseases and normal controls, respectively. Hepatitis G virus did not occur individually in the investigated groups. In the group with chronic liver disease, association between schistosomiasis and viral agents,

<table>
<thead>
<tr>
<th>Nucleotide sequence</th>
<th>Nucleotide position</th>
<th>Target subtype</th>
<th>PCR product (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTTGCTATACCCCTCTGAA</td>
<td>8,601–8,621</td>
<td>Universal</td>
<td></td>
</tr>
<tr>
<td>TGATCTCACCCCACAGGCGGT</td>
<td>8,315–8,338</td>
<td>1a</td>
<td>307</td>
</tr>
<tr>
<td>AGCTGACAGTGTAGACAGCCAC</td>
<td>8,460–8,485</td>
<td>1b</td>
<td>162</td>
</tr>
<tr>
<td>GCCGCTGGAGCCTGGAAGCCAC</td>
<td>8,487–8,515</td>
<td>2a</td>
<td>135</td>
</tr>
<tr>
<td>GCCCATCAGAACCAGGGAAGCAT</td>
<td>8,378–8,403</td>
<td>2b</td>
<td>245</td>
</tr>
<tr>
<td>CCGCGCTAGCGCGCTTTGC</td>
<td>8,423–8,442</td>
<td>3a</td>
<td>199</td>
</tr>
<tr>
<td>AGCTGCAAGTTATACCTGCC</td>
<td>8,004–8,025</td>
<td>4a</td>
<td>289</td>
</tr>
</tbody>
</table>

Table 1

EFFECT OF SCHISTOSOMIASIS AND HEPATITIS ON LIVER DISEASE

Table 2

Liver function tests in patients with chronic liver disease, patients with other chronic diseases, and apparently healthy normal controls*

<table>
<thead>
<tr>
<th>Liver function parameter</th>
<th>Chronic liver disease (n = 50)</th>
<th>Other chronic disease (n = 51)</th>
<th>Normal controls (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE % Abnormal</td>
<td>Mean ± SE % Abnormal</td>
<td>Mean ± SE % Abnormal</td>
</tr>
<tr>
<td>AST (U/liter)</td>
<td>62 ± 6.5 bC 60</td>
<td>41 ± 2.4 C 27.5</td>
<td>26 ± 1.5 8</td>
</tr>
<tr>
<td>ALT (U/liter)</td>
<td>47 ± 7.8 aC 24</td>
<td>31 ± 3.3 15.7</td>
<td>29 ± 1.9 2</td>
</tr>
<tr>
<td>ALP (U/liter)</td>
<td>106 ± 17.3 aA 18</td>
<td>74 ± 4 5.9</td>
<td>67 ± 2.7 0</td>
</tr>
<tr>
<td>TB (mg/dl)</td>
<td>2.36 ± 0.7 aC 34</td>
<td>0.76 ± 0.07 B 9.8</td>
<td>0.53 ± 0.03 0</td>
</tr>
<tr>
<td>DB (mg/dl)</td>
<td>1.28 ± 0.48 aC 52</td>
<td>0.19 ± 0.02 B 13.7</td>
<td>0.1 ± 0.01 0</td>
</tr>
<tr>
<td>A/G</td>
<td>0.61 ± 0.03 cC 94</td>
<td>1.16 ± 0.04 C 21.6</td>
<td>1.85 ± 0.06 0</td>
</tr>
<tr>
<td>AST and/or ALT</td>
<td>66</td>
<td>37.3</td>
<td>8</td>
</tr>
<tr>
<td>AST, ALT, and/or A/G</td>
<td>98</td>
<td>50.9</td>
<td>8</td>
</tr>
<tr>
<td>Any of the 6 tests</td>
<td>100</td>
<td>52.9</td>
<td>8</td>
</tr>
</tbody>
</table>

*SE = standard error; AST = aspartate transaminase; ALT = alanine transaminase; ALP = alkaline phosphatase; TB = total bilirubin; DB = direct bilirubin; A/G = albumin to globulin ratio. Different lowercase letters indicate significant difference from other chronic diseases at P = 0.05 (a), P = 0.005 (b), and P = 0.001 (c). Different uppercase letters indicate significant difference from normal controls at P < 0.05 (A), P < 0.005 (B), and P < 0.001 (C).
especially with HCV, was obvious. This association was manifested in 60, 10, and 20% of these patients for HCV, HBV, and HGV, respectively, in comparison to 21.6, 2, and 7% among normal controls (Table 3).

All patients with chronic liver disease could be distinguished by the criterion of abnormality in at least one of the studied liver function tests, as shown in Table 2. In the other 2 groups, all individuals with HCV had at least 1 abnormal liver test in comparison to 28.6 and 10% of bilharzial patients in the group with other chronic diseases and the normal controls, respectively (Table 4). The impact of HGV on the pathogenesis of chronic liver disease or its effect on liver function tests could not be investigated as no patient was found to have HGV as the sole infective agent.

By PCR with type-specific primers, 4 subtypes were detected according to the expected size of the amplified product. Results of genotyping revealed the high prevalence of subtype 4a, which was detected in 85% of the tested samples. Ten, 2.5, and 2.5% of the samples had subtypes 1b, 2a, and 3a, respectively (Table 5).

**Discussion**

Although schistosomiasis was the major public health problem in Egypt in the past, HCV currently is the major problem.7-9 Co-occurrence of these 2 infectious agents was previously reported.7,33,34 In the present study, a high predominance of bilharziasis and HCV was encountered among patients with chronic liver disease, followed by those with other chronic diseases, in comparison to normal controls.

One hundred percent of patients with liver disease were positive for at least one of the studied infectious agents. Concurrent infection with 2 or 3 of the studied agents was statistically higher in patients with liver disease than other individuals. An obvious association was found between the studied viruses and bilharziasis, which is in agreement with a recently published report.9 This high association could be attributed to the transmission of the viruses via sharing contaminated syringes used to inject tarter emetic, which was prescribed to treat patients with bilharziasis about 25 years ago. Also, bilharziasis may potentiate viral hepatitis caused by HBV and NANBH virus.7,35 A marked association between bilharziasis and HCV was noted in patients with chronic liver disease compared to the other 2 groups. Although the total prevalence rate of schistosomiasis was significantly higher in patients with chronic liver disease, the number of patients having parasite antibodies as the only positive test was markedly lower than that of patients with other chronic diseases. The concomitant infection or over-infection with HCV in patients with bilharziasis could be the cause of aggravation of liver damage. Other studies have shown that the 2 infectious agents have similar adverse effects on the immune system, as manifested by their action on cytokine production by T-helper 1 and T-helper 2 cells.36,37 Periportal fibrosis was found earlier to be a typical pathologic feature in advanced schistosomiasis,38,39 but bilharzial hepatic changes were suggested to be partly reversible.40 Infection with HCV was found to cause a more severe and irreversible form of liver damage than schistosomal infection. Therefore, HCV over-infection may explain the rapid progress toward end-stage liver disease and the early death of some patients with bilharziasis.9

In the present study, because all patients with established chronic liver disease had abnormal liver function tests, we could not distinguish the impact of the studied infectious agents on liver pathogenesis. However, in patients with chronic diseases other than liver disease and apparently healthy individuals, HCV seems to be much more aggressive than bilharziasis. One hundred percent of patients with HCV...
or mixed infection with HCV and bilharziasis could be discriminated by laboratory findings, compared with 20.8% of patients with bilharziasis only.

Analysis of the present data revealed the prevalence of HGV RNA in 20 and 4% of patients with chronic liver disease and patients with other chronic diseases, respectively. In a recent study (Abdel-Hamid M and others, unpublished data) that included 50 Egyptians with hepatocellular carcinoma, 8% were positive for serum HGV RNA, whereas 26% had a history of bilharziasis. 20% were positive for HBV, and 78% were positive for HCV. Other investigators reported coinfection with HGV and HCV in 20–21% of patients with chronic hepatitis and 8% of transfusion recipients, reflecting the fact that both viruses can share the same portal route of transmission. Hepatic pathogenesis of HGV remains controversial. In the present study, we could not distinguish the pathogenic effect of HGV because no patients were shown to have HGV as the sole infectious agent. In 3 out of 6 Japanese patients with fulminant hepatitis, serum HGBV-C RNA was detected and this agent was suggested to be a frequent cause of the disease. Also, in patients receiving bone marrow transplantation in Taiwan, concurrent acute hepatitis episodes were higher in an HGV-infected group than in an HGV-negative one (Su WW and others, unpublished data). On the other hand, 75% of HGV-infected transfusion recipients were shown to have biochemical evidence of liver disease and coinfection with HGV had no apparent influence on the clinical outcome and ALT level in HCV-infected patients. Further confirmation of benign infection with HGV was found where none of 16 HGBV-C–infected dialysis patients had an elevated ALT level throughout 3–16 years of infection. More recently, 3 of 79 (4%) of transfusion recipients who had HGV RNA as the only viral marker were reported to have mild hepatitis (mild elevation of ALT and no jaundice) but no causal relation was established between HGV and hepatitis. In a series of transfusion recipients, persistent infection with HGV was common, but it did not lead to chronic disease during 1–9 years of follow-up and it did not affect the clinical course of patients with hepatitis A, B, or C.

Different genotypes of HCV may display significant biological and serologic differences and they may have distinguishing clinical manifestations and diversity of response to interferon therapy. Moreover, discrepancies between HCV genotypes may affect the results of PCR using a universal set of primers in the detection of HCV RNA. Different reports from a United Kingdom research team indicated that genotype 4a is the major HCV genotype in Egypt and other Middle Eastern countries. In the present study, genotyping using PCR depending on type-specific primers revealed that genotype 4a was prevalent in 85% of the investigated samples, whereas genotypes 1b, 2a, and 3a were detected in 10, 2.5, and 2.5% of samples, respectively. In a recent study applying line probe assay, 82% of Egyptian patients with chronic liver disease were shown to have genotype 4a, whereas the remaining 9 patients (18%) had single or mixed infection with genotypes 1a, 1b, 2a, and 2b.

In conclusion, bilharziasis, HCV, HBV, and HGV were associated with chronic liver disease in patients in Egypt, with HCV having the greatest impact. Coinfection with 2 or more of these infectious agents may potentiate pathogenesis of the liver disease. The HCV genotype 4a is the most common genotype in Egypt and type-specific PCR is a valid, simple technique for HCV genotyping. Another study including a large number of samples from different locations in Egypt is recommended to draw a map for the incidence of the different HCV genotypes in Egypt.

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