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Serotype Analysis of Hepatitis C Virus in Patients with Liver Cirrhosis Positive and Negative for HCV RNA Using Enzyme Immunoassay

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Abstract: Hepatitis C virus serotype analysis of selected specimens from patients with liver cirrhosis positive and negative for HCV RNA was determined. Twenty four serum specimens were evaluated and stored at -20°C. The specimens all tested positive for HCV antibodies on a screening enzyme immunoassay, with confirmation on a recombinant immunoblot assay (RIBA). All specimens were also tested for HCV RNA by the reverse transcription polymerase chain reaction (RT-PCR). The sera were submitted to ELISA, modified, for the identification of antibodies against HCV serotypes 1, 2, 3, 4, 5 and 6 (Murex HCV serotyping 1-6 assay). Out of 24 serum samples, 3 samples were undetectable. Type 4 predominated (81%), followed by type 2 (19%), types 1, 3, 5 and 6 were did not detected in the tested samples. Mixed HCV serotype 4 and 2 were detected in 3 patients (14.2%). All positive HCV antibodies sera were also positive by RIBA test. Of 21 serum samples positive for HCV antibodies, 13 samples (13/21, 61.9%) were positive for HCV RNA. HCV serotype 4 was found positive in 10/13 (76.9%) of positive HCV RNA. In negative HCV RNA serum samples (n = 8), HCV serotype 4 were found positive in 6/8 (75%). In conclusions, HCV Serotype 4 is the predominate type in the tested patients. Serotype 1, 3, 5 and 6 were not detected in the tested samples Analysis of HCV serotyping was possible in patients who had negative for HCV RNA from their serum.

Key words: Hepatitis C virus, liver cirrhosis, serotype, HCV RNA, recombinant immunoblot assay

Introduction

It is well established that hepatitis C develops into cirrhosis of the liver and hepatocellular carcinoma (HCC) both of which are fatal diseases. The World Health Organization estimates that there are at least 21.3 million hepatitis C virus (HCV) carriers in the Eastern Mediterranean countries, which is close to the number of carriers estimated in the Americas and Europe combined (Strickland, 2006; Meky et al., 2006; Ramia and Eid-Fares, 2006). Accumulated data show that there are two main patterns for the distribution of HCV genotypes in the Middle East: in the first pattern, genotype 4 is prevalent in most of the Arab countries and in the second pattern, genotype 1a or 1b predominates in the non-Arab countries (Ramia and Eid-Fares, 2006). Several investigators have suggested that certain HCV serotypes (serological genotypes) vary in their propensity to produce clinically relevant liver disease, but other clinical studies have recorded severe and progressive disease with all the genotypes, (Dusheiko et al., 1996; Martin, 1995; Manns et al., 2006). These serotyping methods could facilitate larger population based studies as well as contribute to the prognostic profile of eventual long term patient management. Additionally, the particular geographical distribution of each serotype can be determined. With such a high disease burden of HCV infection in this part of the world and in light of

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the new evidence that genotypes may influence the outcome of antiviral therapy. In the present study, we aimed to an assessment of a developed commercial enzyme immunoassay (EIA) for the serotyping of HCV, as well as noting the HCV serotype analysis in Egyptian patients with liver cirrhosis positive and negative for HCV RNA.

Materials and Methods

Patients

This study included 24 patients with liver cirrhosis, submitted to Gastroenterology Center, Mansoura University. Sera were tested for anti-HCV antibodies using HCV ELISA (Sorine, Biomedica, Itay). Positive HCV antibodies samples were confirmed by Recombinant Immuno-Blotting Assay (RIBA) (Innoriba-Innogenetics, Belgium).

Polymerase Chain Reaction (PCR) of HCV RNA in Serum Samples

HCV RNA was extracted from 100 µL serum according to Boom *et al.* (1990). The oligonucleotide primers were selected according to the highly conserved 5ʻUTR region. The extracted RNA was reverse transcribed with 10 unites of avian myelobastosis virus reverse transcriptase, 40 µL RNAsin and 1 µM L^{-1} external antisense primer in a final volume of 20 µL which was incubated at 39°C for 60 min. The cDNA was first amplified in 100 µL volume containing 200 µM dNTP,, 0.5 µ ML^{-1} of external sense and antisense primers and 2.5 unit of taq DNA polymerase (Promega). After a 5 min denaturation at 94°C, 35 cycles were carried out as: 94°C for 1 min, 58°C for 1 min and 72°C for 2 min. This was followed by further 10 min incubation at 72°C. A 1/10 volume of the PCRproduct was reamplified for 35 cycles using internal sense and antisemse primers External primers: Sense (5ʻCCA TGG CGT TAG TAT GAG TG 3ʻ), antisense (5ʻTGC TCA TGG TGC ACG GTC TA 3ʻ). Internal Primers: sense (5ʻAGA GCC ATA GTG GTC TGC GG 3ʻ), antisense, (5ʻCTT TCG CGA CCC AAC ACT AC 3ʻ). The concentration of dNTP, primers and Taq polymerase and PCR program were the same as in the first PCR amplification. The expected amplification product from PCR was 278 bp. The products of the PCR reaction were visualized by 2% Agarose gel electrophoresis and ethidium bromide staining. Positive and negative controls were tested in each PCR run. All precaution were taken to avoid contamination including cottoned, tips for all steps and separate space and facilities for pre and post-PCR manipulations.

Serotyping Assay

HCV serotyping assay (MUREX HCV SEROTYPING 1-6 assay, Abbott Diagnostics) was used to detect antibodies to serotypes 1-6 of HCV in serum. This assay used synthetic peptides representing the variable antigenic regions from NS4 (nonstructural) protein of HCV types 1-6 on the solid phase. The partial cross-reaction between antibodies to one type of HCV and peptides from heterologous types means that competing heterologous peptides in the free solution are required to yield type-specific binding of antibody to the solid phase. The assay was carried out according to the manufacturer's instructions. Briefly, 10 µL of diluted samples were incubated in the presence of the serotype-specific competing peptides, with microwells coated with type-specific HCV antigens. The wells were incubated for 1 h at 37°C to allow the serotype-specific anti-HCV antibodies to bind to the immobilized antigens. After washing to remove unbound material; the captured antibodies were incubated with peroxidase-conjugated monoclonal antihuman IgG for 1 h at 37°C. After removal of excess conjugate, bound enzyme was detected by the addition of a solution containing 3,3',5,5'-tetramethyl benzidine and hydrogen peroxide. A purple color developed in the wells, which contained anti-HCV reactive samples. The enzyme reaction was stopped with sulfuric acid to give an orange color, which was read photometrically.

Results

Serum samples (n = 24) from liver cirrhosis patients were included in this study. All the tested serum samples were positive for HCV antibodies as measured by ELISA assay. RIBA test were used as confirmatory test. Of the 24 patients included in the study, 20 patients were evaluated by RIBA test for an individualized diagnosis of the presence of anti-NS4 HCV antibodies. HCV NS4 antibodies were detected in all tested sera as shown in Fig. 1. All successful serotype samples by Murex HCV serotyping 1-6 assay were also positive for NS4 antibody by RIBA assay. It was not possible to determine the HCV serotype in 3 patients out of 24 tested samples (12.5%) possibly due to antibody levels below the detection limits of the test being present in these samples. Of 21 serum samples positive for HCV antibodies, 13 (61.9%) were positive for HCV RNA and 8 (38.1%) samples were negative for HCV RNA as determined by RT-PCR (Fig. 2). Type 4 was predominated (81%), followed by type 2 (19%). Types 1, 3, 5 and 6 were not detected in the tested samples. Mixed HCV serotype 4 and 2 were detected in 3 patients (14.2%) (Fig. 3). Serotype 4 was predominant in HCV RNA positive samples where out of 13 samples positive for HCV RNA, 10 (76.9%) samples were HCV serotype 4. In negative HCV RNA serum samples (n = 8), HCV serotype 4 were found positive in 6/8 (75%).
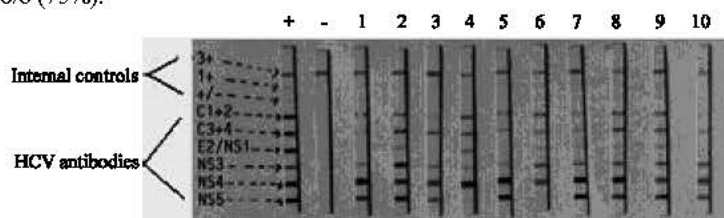

Fig. 1: RIBA test for detection of HCV antibodies in patients with liver cirrhosis. Lane (+) for positive control strip showing all tested HCV antibodies (C1+2, C3+4, E2/NS1, NS3, NS4 and NS5) also showed the internal controls with different degree of stain intensity 3+, 2+, +/-). Lane (-) for negative control. Lanes 1-10 demonstrated serum from patients with liver cirrhosis positive for HCV antibodies. NS4 antibodies were detected with strong stain intensity in all tested patients
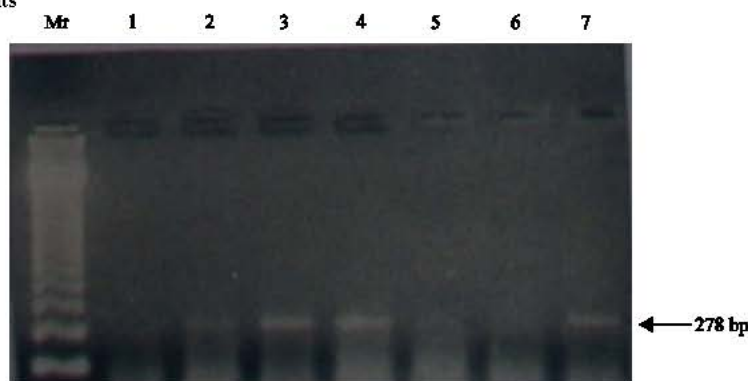

Fig. 2: Polymerase Chain Reaction (PCR) for HCV RNA for serum samples positive for HCV antibodies from patients with liver cirrhosis. Sera from 7 HCV positive antibodies patients (Lanes 1-7) (Lanes 2, 3, 4, 7 were positive for HCV RNA. Lanes 1, 5, 6 were negative for HCV RNA was detected in serum samples by nested HCV RT-PCR and the products were resolved on 2% agarose gel as described in materials and methods. The presence of a 278 bp band indicates presence of viral RNA while absence of the band indicates negative HCV RNA. Lane M shows φx Hae III digest as a molecular weight marker
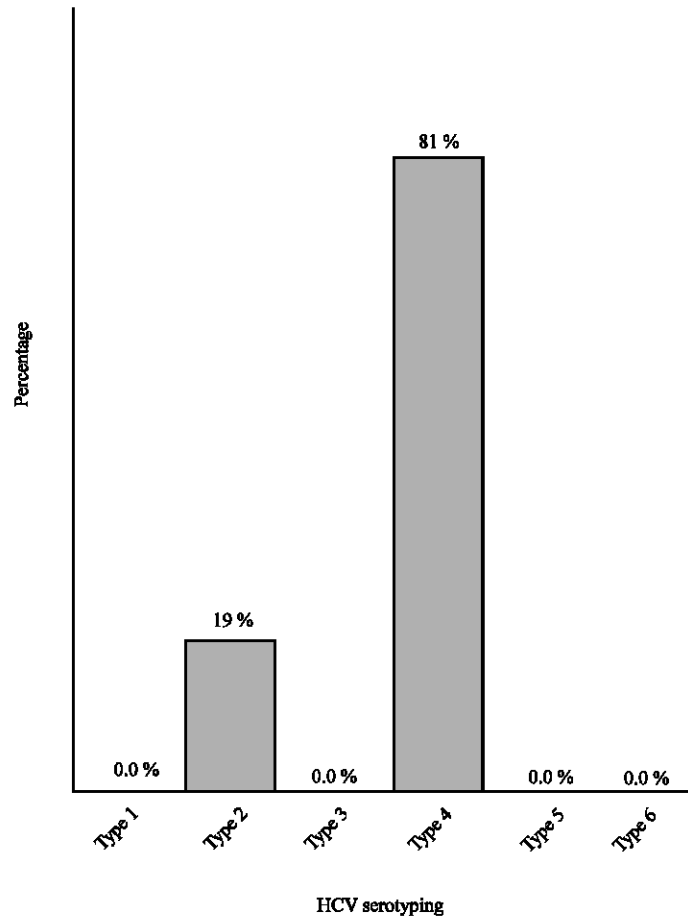
Fig. 3: Serotype prevalence of hepatitis C virus (HCV) in patients with liver cirrhosis using enzyme immunoassay

Discussion

Molecular biology-based genotyping methods have been widely used for research purposes and are not adapted to large-scale HCV genotype determination. This emphasizes the need for more practicable typing technologies and several investigators have proposed serological techniques of HCV genotype determination (so-called serotyping techniques) based on the detection of genotype-specific antibodies by immunoenzymatic methods (Mondelli *et al.*, 1994; Bhattacherjee *et al.*, 1995; Dixit *et al.*, 1995). Anti-HCV antibody reactivities have been shown to be influenced by HCV genotypes. In particular, epitopes encoded by the NS4 region of the HCV genome emerged as candidates for serological discrimination among patients infected with a different HCV genotype (Pawlotsky *et al.*, 1995). A serotyping assay based on the use of branched synthetic peptides and competition enzyme-linked immunosorbent assay has been developed to detect genotype-specific anti-NS4 antibodies directed to HCV genotypes 1-6. Recently, Elsawy *et al.* (2005) reported that serological typing method may be of great value in microbiology laboratories that require a simple assay for identification of HCV genotypes, although the sensitivity of this assay may be limited by the immunocompetence of infected hemodialysis patients. Also comparative data reported in the

literature for genotyping and serotyping suggest that the sensitivity of serotyping methods is significantly lower than that of molecular biology techniques (Weck, 2005). The purposes of this study were to investigate the analysis of HCV serotypes in HCV-positive antibodies liver cirrhotic patients positive and negative for HCV RNA. In the present study, in relation to the samples (3 out of 24) for which it was not possible to determine the HCV serotype, there were frequently extremely low optical density (OD) readings for all HCV types tested, including the controls. Thus, we may assume that the volume of anti-NS4 or HCV type-specific antibodies may have been very low during that phase of infection, impairing the performance of this serologic test (Van Doorn *et al.*, 1996). The results obtained revealed a predominance of type 4, in agreement with the literature surveyed (Strickland, 2006; Meky *et al.*, 2006; Ramia and Eid-Fares, 2006). It should be emphasized the studies citing Egyptian samples used genotyping techniques, showing that the results of this new method for the diagnosis of HCV types agree with the results reported here (Elsawy *et al.*, 2005). In the present study, mixed HCV serotypes (2 and 4) were found in 3 cases out of 21 (14.2%). The mixed infection results may be explained by the serologic detection of previous infections with different HCV types. However, the presence of cross-reactions should not be ruled out and would probably explain most of these results and therefore these diagnoses. This may represent the presence of excess antibodies in the sample and consequently positive results for more than one HCV type. (Leruez-Ville *et al.*, 1998). Similar results were reported by Mizoguchi *et al.* (1996) indicating that serotyping is more sensitive than genotyping in detecting mixed infections. This could be attributed to the ability of serological methods to detect past infection. However, the presence of cross-reactive antibodies can not be excluded as a cause of mistyping and antibodies to NS4 epitopes do not persist for a long time after clearance of the virus, rendering the hypothesis of past infection unlikely (Yuki *et al.*, 1993). Alternatively, the untypeable samples may represent new genotypes which have not yet been identified (Zekri *et al.*, 2001). In the present study we found HCV RNA positive in 61.9% of cases positive HCV antibodies. The variation between the presence of anti-HCV and presence of HCV RNA in the sera of examined patients were found by many investigators (Sheu *et al.*, 1992; Carreno *et al.*, 2006). Sheu *et al.* (1992) and Gretch *et al.* (1992) detected HCV RNA by PCR in 66% and in 73% of anti-HCV positive cases, respectively. However, recently, Carreno *et al.* (2006) concluded that HCV persist and replicate in the liver and PBMCs of healthy, anti-HCV antibody-positive, serum HCV RNA-negative patients who have persistently normal ALT levels and they added that these patients should be followed up, because they have an ongoing viral infection. In the present study we found that HCV serotype 4 was found positive in 76.9% of HCV RNA positive samples and positive in 75% in samples negative for HCV RNA. This data is in agreement with Sakugawa *et al.* (1999) who concluded that with the serological typing ELISA, the retrospective typing of HCV was possible in the majority of the 43 patients who had cleared an HCV RNA from their serum. The discussion about the sensitivity of the Murex test is not really relevant, as this test is not an HCV diagnostic test. This is a subtyping test applied to samples already shown by other more sensitive tests to be infected with HCV, so it is not expected to have the levels of sensitivity that PCR or second/third-generation ELISA tests have to diagnose HCV infection. Despite the limitations pointed out in the present report and in view of the results obtained, which were compatible with those of other studies based on molecular biology for the investigation of HCV types, we conclude that, HCV Serotype 4 is the predominate type in the tested cirrhotic patients. Serotype 1, 3, 5 and 6 were not detected in the tested samples Analysis of HCV serotyping was possible in patients who had negative for HCV RNA from their serum

Acknowledgments

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