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Diverse Patterns of Recognition of Hepatitis C Virus Core and Nonstructural Antigens by Antibodies Present in Egyptian Cancer Patients and Blood Donors

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Serum samples from 429 cancer patients, 82 unpaid blood donors, and 74 paid blood donors were tested for hepatitis C virus (HCV) markers in two commercially available enzyme immunoassays (EIAs). A total of 229 of 429 (53.4%) cancer patients were positive by the two EIAs. A total of 34 of 156 (21.8%) of the blood donors were positive by the EIAs, with a higher prevalence among paid blood donors (20/74; 27%) compared with that among the unpaid blood donors (14 of 82; 17%). EIA-positive sera were tested for confirmation of the results in an immunoblot assay (LiaTek) in which reactivities to four synthetic peptides representing the HCV core protein and two synthetic peptides representing nonstructural proteins 4 and 5 were measured. Of 243 first and/or second EIA-positive samples from cancer patients, 188 (77.2%) were confirmed to be positive in the synthetic peptide immunoblot. A total of 33 of 35 (94.3%) blood donor samples were confirmed to be positive. A great diversity in reactivity patterns was seen. However, all sera from the group of paid blood donors were exclusively reactive to core peptides 1 and 2. A subset of LiaTek assay-positive samples were tested by the four-antigen RIBA-2 assay. The sera from the paid blood donors were all nonreactive. A subset of the LiaTek-positive sera was analyzed for the presence of the HCV genome by reverse transcriptase-PCR. Eleven of the 20 serum samples with reactivity to LiaTek core peptides 1 and 2 only were HCV reverse transcriptase-PCR positive, as were the majority of the sera with other reactivity patterns by the LiaTek assay. The results confirm the very high prevalence of HCV infection in Egypt. Furthermore, the results indicate that there is circulating in Egypt, particularly in the group of blood donors paid for their donation, an HCV variant which elicits an immune response that is not detected by the RIBA-2 assay.

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis worldwide (5). It has a positive-strand RNA genome of approximately 9,500 nucleotides which encodes a polyprotein of ±3,000 amino acids that is processed into structural and nonstructural (NS) proteins (4). The prevalences of HCV antibodies among blood donors have been reported in several studies and appear to differ geographically. Prevalences are lower (0.2 to 0.8%) in Northern Europe and the United States (7, 12) and higher in the Mediterranean countries and Japan (10, 19). Among apparently healthy volunteer blood donors from Egypt, very high (11 to 22%) HCV antibody prevalences have been described (6, 11, 15). These are substantially higher than the prevalences among blood donors from Saudi Arabia (1.3%), Sudan (1.9%), and Yemen (2.4%) (15). In the present study, serum samples from Egyptian cancer patients and blood donors were tested for HCV antibodies. For this, anti-HCV enzyme immunoassays (EIAs; Organon Teknika, Boxtel, The Netherlands, and Abbott Diagnostic Division, North Chicago, Ill.) as well as a synthetic peptide immunoblot assay (LiaTek; Organon Teknika) were used, and a high prevalence of HCV antibodies was found among individuals from Egypt, particularly among cancer patients. The LiaTek synthetic peptide immunoblot assay revealed a great diversity in reactivity patterns among serum samples from cancer patients and unpaid blood donors (UBDs). However, serum samples from all of the paid blood donors (PBDs) revealed the same LiaTek assay reactivity pattern. Samples representative of different LiaTek assay reactivity patterns were also tested by a recombinant immunoblot assay (RIBA-2; Chiron Corporation, Emeryville, Calif.) and by HCV reverse transcriptase-PCR (RT-PCR). The results suggest that distinct HCV variants are circulating in Egypt among possibly distinct risk groups.

MATERIALS AND METHODS

Patient groups. Serum samples from 429 cancer patients collected at the hospital of the National Cancer Institute of the University of Cairo were used in the study. The various diagnoses are depicted in Table 1. Serum samples from 156 blood donors were used: 74 of them were PBDs and the remaining 82 were UBDs. For comparison, we used sequential serum samples from 10 intravenous drug users from Amsterdam seroconverting for HCV antibodies during a prospective study on the prevalence and incidence of human immunodeficiency virus (HIV) infection and AIDS (20).

Anti-HCV EIA. The samples from both the group of cancer patients and the blood donor groups were tested twice by using two commercially available EIAs (UBI HCV EIA [Organon Teknika], and Abbott HCV EIA-2 [Abbott Diagnostic Division]).

Samples from the intravenous drug users were tested twice by the same commercially available EIA (Abbott HCV EIA-2; Abbott Diagnostic Division). HCV EIAs were performed according to the instructions of the manufacturers.

Anti-HCV immunoblot assay. Anti-HCV EIA-positive samples were tested by a synthetic peptide immunoblot assay (LiaTek; Organon Teknika) for confirmation of the results. In this sandwich-type line immunoassay, four bands encompassing synthetic peptides representing different epitopes on the HCV core (C), one band representing an epitope on NS protein 4 (NS4), and one band repre-

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TABLE 1. Anti-HCV antibodies in cancer patients and blood donors from Egypt

Patient group (total no. of patients)[a]	No. of patients positive by:		No. (%) positive by first and second EIAs	P value[b]
	First EIA[c]	Second EIA[d]		
NHL (127)	44	43	43 (33.9)	0.01
ALL (84)	47	46	46 (54.8)	$<10^{-6}$
AML (11)	9	9	9 (81.8)	0.00001
CML (14)	11	10	10 (71.4)	0.00006
BLCa (57)	42	41	41 (71.9)	$<10^{-6}$
HCC (68)	49	55	49 (72.1)	$<10^{-6}$
RM (18)	14	9	9 (50.0)	0.007
BrCa (12)	4	5	4 (33.3)	0.2
NeuBL (8)	6	4	4 (50.0)	0.07
Other (30)	18	14	14 (46.7)	0.003
PBD (74)	21	20	20 (27.0)	0.2
UBD (82)	14	14	14 (17.0)	

[a] Abbreviations: NHL, non-Hodgkin's lymphoma; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; BLCa, bladder cancer; HCC, hepatocellular carcinoma; RM, rhabdomyosarcoma; BrCa, breast cancer; NeuBL, neuroblastoma.

[b] Chi-square test; comparison of each of the group of cancer patients and the group of PBDs with the group of UBDs.

[c] Organon Teknika anti-HCV EIA.

[d] Abbott anti-HCV EIA.

senting an epitope on NS5 are coated on the test strip. In addition, human immunoglobulin G control and cutoff lines (low and high cutoffs) and an additional anti-human immunoglobulin G (sheep) sample control line are coated on the strip. The assay was performed according to the instructions of the manufacturer. A subset of 15 LiaTek assay-positive samples was tested by the commercially available four-antigen RIBA-2 assay (Chiron Corporation). Four HCV recombinant antigens are coated on the strips of this recombinant immunoblot: 5-1-1, C100, and C33c representing NS proteins (NS3 and NS4) and C22 representing the C protein of HCV. The assay was performed according to the instructions of the manufacturer.

Nucleic acid purification. HCV RNA was purified from 100 μl of serum by a method previously described by Boom et al. (2).

Primers and probe. The primer used for reverse transcription of HCV RNA was HCV-6: 5′-ACC.TCC-3′ (nucleotides [nt] 319 to 324; nt numbering is according to Choo et al. [3]). The antisense PCR primer for HCV was RB-6B (5′-ACT.CGC.AAG.CAC.CCT.ATC.AGG-3′ [nt 292 to 312]) and the sense PCR primer was RB-6A (5′-GTG.AGG.AAC.TAC.TGT.CTT.CAC.G-3′ [nt 47 to 68]). The oligonucleotide RB-6P (5′-TTG.GGT.CGC.GAA.AGG.CCT.TGT .GGT.ACT.G-3′ [nt 264 to 291]) was labelled at the 5′ end with digoxigenin and was used as a probe in hybridization experiments to determine the specificities of the PCR products. The HCV oligonucleotides are specific for the 5′ untranslated region of the HCV genome.

RT-PCR of HCV. Reverse transcription was performed in a 25-μl reaction volume containing 20 U of RNase inhibitor (Promega Biotec, Madison, Wis.), 67 mM Tris · HCl (pH 8.8), 17 mM ammonium sulfate, 1 mM β-mercaptoethanol, 6 μM EDTA (pH 8.0), 0.2 mg of bovine serum albumin (BSA; Boehringer) per ml, 6 mM $MgCl_2$, 25 ng of primer HCV-6, 0.6 μl of 25 mM (each) deoxynucleoside triphosphates, 11.5 μl of the nucleic acid eluate, and 200 U of superscript RT I (GIBCO-BRL, Gaithersburg, Md.). The mixture was incubated at room temperature for 5 min and then at 37°C for 60 min. RT was denatured by incubation for 5 min at 95°C. The PCR was performed in a 50-μl volume containing 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus), 50 mM Tris · HCl (pH 8.3), 20 mM KCl, 1.2 mM $MgCl_2$, 1 mg of BSA per ml, 12.5 μl of the RT reaction mixtures, 200 μM (each) deoxynucleoside triphosphate, and 100 ng each of primers RB-6A and RB-6B. The samples were denatured at 95°C for 5 min and were subjected to 35 rounds of thermal cycling in a DNA thermal cycler (type 480; Perkin-Elmer Cetus). A cycle consisted of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. After the cycling program, the samples were incubated for 10 min at 72°C. All samples were analyzed twice for HCV RNA by the RT-PCR on different days, with identical results.

RNA template production. To construct an HCV RNA transcription vector, HCV sequences from nt 47 to 1032 were cloned after RT-PCR into the pSP 64 [poly(A)] vector (Promega), resulting in plasmid PMOZ.1.HCV. The presence of the right insert was confirmed by DNA sequence analysis. HCV template RNA was transcribed in vitro from PMOZ.1.HCV. Briefly, 5 μg of plasmid DNA was linearized with *Eco*RI. The linear plasmid DNA was purified from an agarose gel and then incubated with 50 U of SP6 RNA polymerase for 2 h at 37°C in the presence of 500 μM (each) ribonucleoside triphosphates (GTP, ATP, UTP, and CTP), 100 U of RNAsin, 10 mM dithiothreitol, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl in a total reaction volume of 100 μl. After the transcription reaction, the DNA template was degraded by two rounds of digestion with RNase-free DNase (Boehringer) for 30 min at 37°C with 10 U of enzyme. Upon completion of the digestion, two rounds of extraction with phenol-chloroform-isopropyl alcohol and then ethanol precipitation were done. The HCV RNA transcripts, which contained a poly(A) tail, were further purified on an oligo(dT) cellulose column. The RNA concentration was determined spectrophotometrically by determination of the A_{260} with UV light. An aliquot was analyzed by agarose gel electrophoresis to assess its integrity.

Sensitivity of the RT-PCR assay. HCV RNA synthesized in vitro was diluted into TE (Tris-EDTA) buffer at a concentration of approximately 10^6 copies per μl and was stored at −20°C. Serial 10-fold dilutions of these stock solutions were made in H_2O just prior to the RT-PCRs. One hundred copies were routinely detected.

Southern hybridization and chemiluminescence detection. Electrophoresis of the PCR products, transfer of the DNA onto a positively charged nylon membrane (Boehringer Mannheim GmbH), and hybridization with the probe were done as described previously (1a). The detection of the hybridized digoxigenin-labelled probe was done with the DIG Luminescence Detection Kit by the protocols recommended by the supplier of the kit (Boehringer Mannheim GmbH).

RESULTS

HCV antibody prevalence measured by EIAs. Among the 429 serum samples from cancer patients, 244 were positive for HCV antibodies in the first EIA (Organon Teknika) and 236 were positive in the second EIA (Abbott). A total of 229 samples were positive in both EIAs, resulting in an overall prevalence of 53.4% among cancer patients. The HCV antibody prevalence per cancer diagnosis group is summarized in Table 1. High percentages of positive serum samples were seen in all groups, ranging from 33.3% in the breast cancer group to 81.8% in the group of patients with acute myeloid leukemia. Also, a high prevalence of anti-HCV antibodies was found in the group of patients suffering from hepatocellular carcinoma and the group with bladder cancer.

Thirty-five of the 156 samples from the blood donor group were positive in the first EIA and 34 were positive in the second EIA. Thirty-four of 156 (21.8%) samples were positive in both EIAs. Among the PBDs, HCV antibody prevalence was 10% higher compared with the prevalence among the NBD group, i.e., 27 versus 17%, respectively. This difference is marginally significant by Fisher's exact test.

HCV antibody prevalence in the various cancer patient groups and the PBDs were compared with the prevalence in the NBDs. The prevalence was significantly higher when tested by the chi-square test for all groups except the groups of patients with breast cancer and neuroblastoma and the PBDs (Table 1).

HCV antibody reactivity as measured by immunoblot assay. Overall, 243 first and/or second EIA-positive serum samples from the cancer patient group and 35 samples from the blood donor group were tested by a synthetic peptide immunoblot assay (LiaTek; see Materials and Methods). A total of 188 of 243 (77.2%) serum samples from cancer patients and 33 of 35 (94.3%) serum samples from the group of blood donors were positive by the immunoblot assay (Table 2). Among the various groups of cancer patients, the synthetic peptide immunoblot assay confirmation percentages ranged from 30.8% for the group with rhabdomyosarcoma to 100% for the group with chronic myeloid leukemia. The confirmation rates in the UBD and the PBD groups were 100 and 90.5%, respectively.

HCV antibody reactivity patterns in the synthetic immunoblot assay. Remarkably, in the sera from the PBD group, only antibodies that reacted weakly with C peptide 1 and strongly with C peptide 2 on the LiaTek strips could be detected (Fig. 1). This reactivity pattern is hereafter referred to as pattern C1,C2. Pattern C1,C2 was seen only once in the group of 14

TABLE 2. Anti-HCV LiaTek immunoblot assay reactivity for EIA-positive samples from Egyptian cancer patients and blood donors

Patient group[a]	No. of samples tested	No. (%) of samples immunoblot positive	No. (%) of samples with pattern C1,C2[b]
NHL	44	34 (77.3)	4 (11.8)
ALL	47	33 (70.2)	8 (24.2)
AML	9	7 (77.8)	1 (14.3)
CML	8	8 (100)	0 (0.0)
BLCa	39	37 (94.9)	4 (10.8)
HCC	55	45 (81.8)	3 (6.7)
RM	13	4 (30.8)	3 (75.0)
BrCa	5	4 (80.0)	0 (0.0)
NeuBl	5	3 (60.0)	0 (0.0)
Other	18	13 (72.2)	2 (15.4)
All cancer patients	243	188 (77.2)	25 (13.3)
PBDs	21	19 (90.5)	19 (100)
UBDs	14	14 (100)	1 (7.1)
All blood donors	35	33 (94.3)	20 (60.6)

[a] See footnote a of Table 1 for abbreviations.
[b] C1,C2; weak reactivity with C peptide 1 and a strong reactivity with C peptide 2 but no reactivity with the other HCV core and NS peptides coated on the strip.

NBDs but was seen in 13.3% of the cancer patients, among whom it was most prevalent among the rhabdomyosarcoma patients (Table 2). The other reactivity patterns seen with the sera from the NBDs and the cancer patients were very diverse, and a specific pattern could not be detected for any subgroup. The patterns ranged from reactivity to one or two NS peptides either in combination or not in combination with reactivity to one or more C peptides. Also, reactivity with only the two NS peptides or only three or four C peptides was frequently observed. We refer to this heterogeneous group of reactivity patterns collectively as the E pattern.

To discern if the C1,C2 pattern reflects antibody production early in the course of HCV infection, we tested sequential serum samples from 10 Dutch intravenous drug users, seroconverting for HCV antibodies as measured by EIA, in the LiaTek synthetic peptide immunoblot assay as well. No C1,C2 reactivity pattern was found in sera from this group, and the reactivity patterns were as diverse as those in the E pattern group. Following HCV seroconversion, the reactivity patterns appeared to be constant during the 69-month follow-up period. A subset of LiaTek assay-positive samples showing reactivity patterns E and C1,C2 were tested by the four-antigen RIBA-2. Of nine reactive samples with LiaTek assay pattern E (five from UBDs and four from hepatocellular carcinoma patients), seven were RIBA-2 assay positive and two were indeterminate. The five reactive serum samples from PBDs with LiaTek assay pattern C1,C2 were negative by the RIBA-2 assay and the one serum sample from the patient with hepatocellular carcinoma and LiaTek assay pattern C1,C2 was indeterminate by the RIBA-2 assay. Although the number of serum samples examined is small, the difference in positive reactivities in the RIBA-2 assay between the LiaTek assay pattern E-positive samples and the LiaTek assay pattern C1,C2-positive samples is statistically very significant ($P = 0.007$ by the two-tailed Fisher exact test).

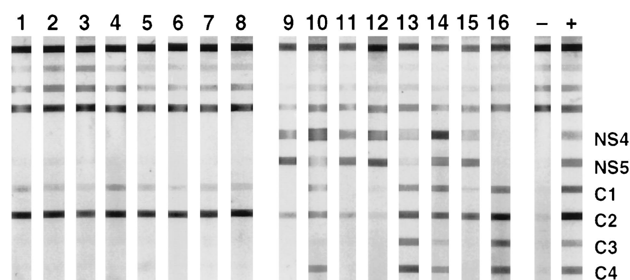

FIG. 1. Random selection of LiaTek immunoblot assay patterns observed with sera from PBDs (lanes 1 to 8) and from cancer patients or UBDs (lanes 9 to 16). NS4, peptide of NS protein 4; NS5, peptide of NS protein 5. C1, C2, C3, C4, four peptides specific for the HCV C protein. The upper four lines are immunoglobulin G control lines and low and high cutoff lines. The figure was generated with Adobe Photoshop 3.0 and MacDrawPro 1.5 (Claris Corporation).

TABLE 3. RIBA-2 assay and RT-PCR reactivities of sera reactive in the LiaTek immunoblot assay

LiaTek pattern	Group (no. of samples)	No. of samples (reactivity)	
		RIBA-2 assay	RT-PCR
E	HCC[a] (4)	4 (+)	3 (+)
	UBD (5)	3 (+), 2 (±[b])	4 (+)
C1, C2	PBD (5)	5 (−)	4 (+)
	HCC (1)	1 (±)	1 (+)
	PBD (14)	ND[c]	6 (+)

[a] HCC, hepatocellular carcinoma.
[b] ±, indeterminate reactivity.
[c] ND, not done.

The Egyptian sera tested by the RIBA-2 assay were also screened for the presence of the HCV genome by RT-PCR. Seven of the nine LiaTek assay pattern E-positive serum samples were positive by the HCV PCR. All 19 samples from the PBD group with reactivity pattern C1,C2 were tested by the HCV RT-PCR. Ten of 19 PBDs appeared to be HCV PCR positive. Of the five LiaTek assay pattern C1,C2-positive serum samples from PBDs that were tested in the RIBA-2 assay and found to be negative, four were positive in the RT-PCR. The serum sample from the patient with hepatocellular carcinoma, LiaTek assay pattern C1,C2, and an indeterminate RIBA-2 assay pattern was RT-PCR positive. The results of the serological assays and the RT-PCR are presented in Table 3.

DISCUSSION

In the present study, using two different EIAs, we found a high prevalence (17%) of antibodies to HCV in serum samples obtained from UBDs in Egypt. The prevalence (27%) among the PBDs appeared to be higher, and extremely high prevalences (34 to 82%) were found in the different groups of Egyptian cancer patients. By using a six-antigen synthetic peptide immunoblot assay for confirmation of the EIA reactivity, 77.2% of the EIA-positive serum samples from the cancer patients, 90.5% of the EIA-positive serum samples from the PBDs, and 100% of the EIA-positive serum samples from the UBDs were confirmed to be positive.

The 17% HCV antibody prevalence among the UBDs confirms the prevalences reported by Saeed et al. (15), Kamel et al. (11), and Darwish et al. (6). The high anti-HCV prevalence among Egyptian cancer patients can be explained in part as the consequence of frequent transfusions with contaminated blood in this group. This is probably the case for the groups with lymphoma or leukemia. Furthermore, 55 of 243 patients had hepatocellular carcinoma, which is associated with HCV infection (16). This still cannot fully explain the high prevalence that we observed. The high prevalence of HCV among patients with bladder cancer, which is related to chronic schistosomal infes-

tation (6, 9), indicates that environmental factors common for hepatocellular carcinoma and bladder cancer patients may be involved. Other factors must be considered, such as common environmental conditions, like water; the possibility of transmission of HCV through an animal vector, such as mosquitoes or schistosomes; or iatrogenic transmission, such as the use of more than once disposable syringes for bilharzia treatment. Further studies are urgently needed because it appears that Egypt is a region where HCV is highly endemic, and as was indicated recently, liver disease and hepatocellular carcinoma are on the rise there (1).

A great diversity in immunoblot reactivity patterns was found in the anti-HCV EIA-positive sera by using an HCV immunoblot assay (the LiaTek assay), in which synthetic peptides representing four epitopes on the HCV C protein, one epitope on NS4, and one epitope on NS5 are coated as discrete lines on the test strip. Remarkably, all sera from a group of PBDs showed the same immunoblot pattern. Only a strong reactivity with C peptide 2 and a weak reactivity with C peptide 1 was detected (C1,C2 pattern; Fig. 1). This pattern was never found in sequential sera from HCV-seroconverting Dutch intravenous drug users. The reactivity patterns had not changed in this group after follow-up for up to 69 months after seroconversion. Therefore, pattern C1,C2 does not seem to be a reflection of antibody reactivity to HCV antigens early or late in infection.

Sera with pattern C1,C2 reactivity in the six-antigen synthetic peptide immunoblot assay were either negative or indeterminate by the four-antigen recombinant immunoblot assay (the RIBA-2 assay). The recombinant C22 protein present on the RIBA-2 assay strip covers a large part of the C protein of HCV genotype 1. On the C1 to C4 lines of the LiaTek assay strip, synthetic peptides 19 to 21 amino acids long, on the basis of the genotype 1 sequence, are bound, and except for the amino-terminal amino acid of peptide C1, all are a part of the C22 protein. It could be that the epitopes recognized by antibodies present in the sera from the PBD group are buried in the bound C22 recombinant protein structure but are well exposed by the bound synthetic peptides. In a study on the geographical distribution of HCV genotypes in blood donors, 33 EIA-positive blood samples from Egyptian donors were examined by the RIBA-2 assay (13). Of these 33 samples, 23 were confirmed to be positive by the RIBA-2 assay, and of these, 19 were RT-PCR positive. The 10 indeterminate samples were all RT-PCR negative. In the present study, the results obtained by RT-PCR, which also used the conserved 5′ untranslated region of the HCV genome as the template, gave positive HCV signals for 7 of 9 samples with LiaTek assay reactivity pattern E as well as 11 of 20 samples with LiaTek assay reactivity pattern C1,C2. Five samples from PBDs with a LiaTek assay C1,C2 pattern were tested by the RIBA-2 assay, and all were negative, but four of these five samples were HCV RT-PCR positive (Table 3). It therefore appears that, especially in the group of PBDs examined, there is circulating an HCV variant which elicits an immune response not detectable by the RIBA-2 assay. A substantial proportion (13.3%) of the sera from cancer patients contained HCV antibodies reacting only with the C1 and C2 epitopes on the six-antigen immunoblot. Unfortunately, it is impossible to determine in retrospect if and to whom blood derived from the group of PBDs examined has been transfused. It is noteworthy that the group of PBDs that we examined is not necessarily typical for the Cairo area. Several coexisting groups of PBDs live in the metropolis. Each group has its own leader. The leader recruits the men from whom blood will be taken. It has recently been described that in French intravenous drug users, genotype 3a is much more prevalent than in patients infected by blood transfusion, in which genotype 1b is most prevalent (14). We do not know by which route the group of PBDs examined in the present were infected. It could be, however, one that differs from the still largely unknown routes by which the majority of the Egyptian HCV carriers without a history of blood transfusion are infected. It has been described that the majority of Egyptian HCV isolates belong to genotype 4 and that a few isolates could not be classified and were designated genotype U (8, 17, 18). In the study of McOmish and colleagues (13), it was reported that plasma samples from two blood donors harboring the Egyptian type U genotypes were serologically reactive in the RIBA-2 assay. The serum samples from five Egyptian PBDs with LiaTek pattern C1,C2 tested in the present study were RIBA-2 assay negative (Table 3). It remains to be determined by molecular analyses if in the group of PBDs an HCV genotype other than type 4 or type U is circulating. In addition, larger groups of donors should be studied to assess how generalized the distinction in viral seroreactivity is among Egyptian blood donors.

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