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Influence of Delta Virus Infection on the Virologic Status in Egyptian Patients With Chronic Hepatitis B Virus Genotype D

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Hepatitis delta virus (HDV) usually have an unfavorable clinical outcome in chronic hepatitis B virus (HBV) patients. In Egypt, data about epidemiology, the spectrum of disease, and impact of HDV on HBV infection are rare. To assess the prevalence, clinical and virological characteristics of HDV infection among Egyptian patients with chronic HBV. Adult patients with Hepatitis B surface antigen (HBsAg)-positive were evaluated for the presence of HDV using anti HDV-IgG and HDV RNA by RT-PCR. Routine laboratory investigations, genotypes and subtypes for both HBV and HDV, abdominal sonography, and transient elastography (TE) were done. Liver biopsy was performed only in whenever indicated. One hundred and twenty-one treatment-naïve chronic HBV patients were included. Wild HBV genotype-D2 was found in 98.2% and 81.9% were HBeAg negative. Prevalence of HDV was 8.3% by anti-HDV IgG and 9.9% by RT-PCR. Wild HDV genotype-IIb was reported in 83.3%. HDV infection was more common in males, 90.9% of delta patients were HBeAg negative. Compared to the mono-infected HBV, concomitant HBV/HDV infection was not associated with more derangment in ALT nor advanced stage of fibrosis. 66.7% of HDV patients had significantly lower HBV-DNA level compared to the non-delta patients ($P < 0.001$). HDV is not uncommon in Egypt. HBV genotype-D was associated with HDV genotype-IIb. Delta infection was associated with negative HBeAg status, reduction of HBV replication, but neither influenced the clinical course nor increased significant liver damage risk. ***J. Med. Virol. 88:837–842, 2016***. © 2015 Wiley Periodicals, Inc.

KEY WORDS: HDV; HBV; genotype; Egypt

INTRODUCTION

Hepatitis D virus (HDV) infection is the most severe form of viral hepatitis in humans. HDV is a defective RNA viroid which depends on HBsAg for transmission, that is why Hepatitis D occurs only in subjects positive for the HBV surface antigen (HBsAg) through either coinfection or superinfection [Wedemeyer and Manns, 2010].

Epidemiological investigations carried out in 1980 showed that HDV infection was endemic worldwide and prevalence varied among different regions, regardless of the prevalence of the concomitant HBV infection [Smedile and Rizzetto, 2011].

It was proved worldwide that the percentage of HBV patients co-infected with HDV is 5% [Ataei et al., 2011; Latika and Summaiya, 2012].

Prevalence of HDV in Egypt as assessed by anti-HDV Ab was 4.7% among 170 HBsAg positive patients at Ismailia Governorate [Gomaa et al., 2013]. In Egypt, data about the epidemiology, the influence of HDV on HBV infection, as well as the spectrum of disease in presence of both infections is rare. In this study, we aimed to assess the prevalence, clinical and virological characteristics of HDV infection among Egyptian patients with chronic HBV.

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MATERIALS AND METHODS

Our study is a cross-sectional one that was conducted on 121 chronic HBV patients, attending the outpatients' clinics of the Endemic Medicine Department, Faculty of Medicine, Cairo University and Cairo Fatemic Hospital, Ministry of Health and Population (MOHP) over the period from January 2013 to December 2013. The study had been approved by the institution ethical committee. An informed consent was obtained from all patients.

Inclusion criteria: Adult ($\geq$18 years old) symptomatic or asymptomatic chronic HBV patients (positive for HBsAg $>$ 6 months) of both genders within the clinical spectrum of chronic HBV infection.

Exclusion criteria: HCV Co-infection, HBV patients who had received or currently under anti-viral therapy (interferon or nucleos (t) ide analogues), alcohol, or illicit drug abuse.

All patients included in this study were subjected to complete clinical evaluation and routine laboratory investigations including CBC, complete liver biochemical profile (serum bilirubin, SGOT, SGPT, ALP, albumin, and INR) in addition to kidney function tests, blood sugar assessment, and Alpha fetoprotein (AFP). Abdominal ultrasound and Transient elastography (FibroScan®; Echosens, Paris, France) were done by a single operator for all patients. Liver biopsy was done whenever indicated according to [EASL Guidelines, 2012].

Serological, Virological Assay, and Genotyping for HBV and HDV

Five milliliter of venous blood was taken on EDTA vaccutainer tube, centifuged at 3,500 x*g* to separate plasma that was stored at −20°C for further laboratory assessment of the following:

- HBV Special Tests:

1. HBsAg for confirmation (ELISA), HBeAg (ELISA), HBeAb, HBc Ab (IgM, IgG).
2. HBV DNA qPCR (real time PCR): The work was done using real time PCR device; Step one (applied biosystem Foster city) using QIAamp MinElute Virus Spin protocol.

HBV PCR DNA Sequencing

Using Nucleospin Extract II columns (Centri-sep), HBV PCR products were purified then sequenced by using the bigdye terminator v3.1 kit (Life technologies). Sequences of amplified nucleic acids were determined using an ABI 310 sequencer (Life technologies). Then, the nucleotide sequences were analyzed with SeqScape v2.6 software (Life technologies). The Polymerase region nucleotide sequences were compared with reference strains, obtained from GenBank, representing all of the different genotypes (A–H). Blast analysis was used to determine genotyping of HBV and full genotypes were used as references.

- HDV special tests

1. Anti HDV IgG: using ELISA technique.
2. HDV RNA qPCR (real time PCR):

   - RNA Extraction:
     Five hundred microliter of *Lysis Buffer* were added to 100 µl of blood placed into a microcentrifuge tube then vortex at 10,000*g* for 10 sec.

Column Loading

- Three hundred microliter Isopropanol (or 0.6× volume of the cell lysate) were added to the prepared lysate then vortex.
- The mixture was transferred directly into the spin column.
- Centrifuged for 30 sec at 10,000*g* and then the flow-through was dicarded.

Primary Column Washing

- Seven hundred microliter of ethanol (primary washing buffer) were added to the Spin Column. Centrifuge at 10,000*g* for 30 sec.

Secondary Column Washing

- Seven hundred microliter of ethanol (secondary washing buffer) were added to the spin column and then centrifuge at 10,000*g* for 30 sec. The flow-through was discarded. To remove any residual ethanol, centrifuge again for 2 min at 10,000*g*.

Elution of RNA

- After, the spin column was placed into an RNase-free microcentrifuge tube, 40–50 µl of Elution Buffer were added to the column membrane. Then, incubated for 1 min at room temperature, centrifuged at 10,000*g* for 1 min to elute the RNA which was stored at −80°C.

HDV RNA RT-PCR detection

- Primers *900s* and *1280as* were used to detect HDV-RNA by PCR. This encompass a region conserved in all HDV genotypes, i.e., the "R0 region" (400 nt) covering the 3′end of the HDV gene as previously reported by Ivaniushina et al. [2001].
- For reverse transcription, 5 µl of extracted RNA mixed with random primers (0.4 pM) and dNTP (0.5 mM) then denatured for 3 min at 95°C. Denatured RNA was added to the reverse transcription mixture (total volume 25 µl; containing 20 U RNasin (Promega) and 100 U SuperScript reverse transcriptase (Life Technologies) in the buffer supplied by the manufacturer).

This reaction was incubated for 45 min at 42°C, then stopped by another incubation for 5 min at 94°C. The PCR reaction mixture (total volume 40 μl), containing 0.25 pmol/μl of primers and 1 U Ampli*Taq* Gold polymerase (Applied Biosystems) in the buffer included by the manufacturer, was added to 10 μl of the reverse transcription reaction.

- PCR was carried out in a thermocycler (Biometra, Germany) under the following conditions: 9 min at 94°C, then 40 cycles of 45 s at 94°C, 30 sec at 58°C, and 45 s at 72°C with a final extension step of 5 min at 72°C. Positive and negative controls were included in each set of reactions.
- PCR products of the R0 region were used to determine HDV genotype by sequencing.

HDV Genotyping by Sequencing:

Region R0, represents the 3′terminal part of the HD gene. It is flagged with primers 900s and 1280as, as was proposed by Casey et al. [1993].

PCR products were purified through mini-columns (Centrisep). Purified DNA parts were sequenced directly on both strands on an ABI Prism 310 automated sequencer (Applied Biosystems) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Statistical Analysis

Complete pre-coded data were filled in using "Microsoft Office Excel Software" program (2010) for windows. Data transferred for statistical analysis to the Statistical Package of Social Science Software program (SPSS), version 21.

Quantitative variables were expressed as mean and standard deviation, whereas qualitative ones were expressed as frequency and percentage. Comparison between groups was done using independent sample *t*-test (if parametric); Mann–Whitney test for quantitative variables and Chi square or Fisher's exact test for qualitative ones. P values ≤ 0.05 considered statistically significant, and ≤ 0.01 were considered highly significant.

RESULTS

This study was conducted on 121 adult treatment-naïve patients with chronic HBV. The studied population showed a male predominance (89/121 = 73.6%), with mean age of 33.7 ± 10.6 years and mean BMI of $28.1 \pm 6.2\,Kg/m^2$.

Most of chronic HBV (102/121 = 84.3%) were asymptomatic with normal ALT and AST in 66.9%, 76%, respectively. Positive HbeAg status was found in only 18.1%.

Mean HBV viral load was $1.3 \pm 2.6 \times 10^6$ IU/ml (Median 2.4×10^6, IQR 0.36–1.2×10^6 IU/ml).

Out of 121 patients, 90 patients had high viraemia ($>20 \times 10^3$ IU/ml), while 10 patients (8.3%) had undetectable HBV DNA by PCR. The majority (65.1%) of the whole studied group had non-significant fibrosis (F0-1) by Fibroscan (Tables I and II).

According to our study, HDV prevalence was found to be 8.3% by HDV IgG and increased to 9.9% using PCR as shown in Table III.

In the current study, HDV patients were in the fourth decade of life and obese with BMI $31.4 \pm 11.2\,Kg/cm^2$ with male predominance (83.3%).

All delta patients diagnosed in the current study were asymptomatic with normal levels of ALT and AST (83.3% and 91.7%, respectively) without significant difference between HDV positive and HDV negative patients (Table IV).

The current study showed that 90.9% of HDV patients were HBeAg negative, 63.6 % were HBeAb positive. None of HDV infected patients were HBcIgM (Table V).

Among HDV infected patients, 66.7% had undetectable HBV-DNA by PCR with high significant difference between HDV positive and negative patients ($P < 0.001$).

In the current study, different stages of fibrosis were tested by fibroscan and/or liver biopsy among HBV patients whenever indicated (according to EASL guidelines). So liver biopsy was done for only 10 patients. All of them were A1 (100%) and seven were F1 (70%).

Fibroscan results were not in accordance with those of liver biopsy in 10 HBV patients who were indicated for biopsy (i.e., four patients with fibroscan F2-F3 appeared to be F1 by liver biopsy). However, none of the delta patients had undergone liver biopsy.

Fibroscan detected non significant fibrosis (F0 in 20% and F1 in 70%) in 10 HDV positive patients. However, when comparing stage F1 in HDV positive versus 19.2% in HDV negative patients there was a significant difference (P value = 0.004) (Table VI).

Genotype sequence analysis for HBV showed that HBV-genotype D2 was present in 109 patients and

TABLE I. Demographic, Clinical Features, and Transient Elastography Results of the Whole Group Studied

	Frequency (n = 121) and percentage
Demographic features	
Gender	
Male	89 (73.6)
Female	32 (26.4)
Age (mean ± SD)	33.7 ± 10.6
BMI (mean ± SD)	28.1 ± 6.2
Clinical picture	
Asymptomatic	102 (84.3)
Symptomatic	19 (15.7)
[a]Transient elastography: (KPa)	
F0-1	71 (65.1)
≥F2	38 (34.9)

[a]It was done for 109 patients only, 7 patients failed because of their obesity, 1 patient was contraindicated because of being pregnant, and 4 patients were missing.

TABLE II. Laboratory, Serological, and Virologic Features of the Whole Group Studied

	Frequency and percentage (number = 121 patients)
Transaminases	
ALT	81 (66.9%)
Normal	40 (33.1%)
Elevated	
AST	
Normal	92 (76 %)
Elevated	29 (24%)
Seromarkers[a]	
HBeAg	21 (18.1%)
Positive	95 (81.9%)
Negative	
HBeAb	
Positive	89 (76.7%)
Negative	27 (23.3%)
HBcIgG	
Positive	104 (89.7%)
Negative	12 (10.3%)
HBcIgM	
Positive	0 (0%)
Negative	116 (100%)
HBV viral load (PCR):	
Positive	111 (91.7%)
$<2 \times 103$ IU/ml	8
2×103– 20×103 IU/ml	13
$>20 \times 103$ IU/ml	90
Negative	10 (8.3%)

[a]Missing data of five patients.

two patients had HBV-genotype D2 mutant with pre S1/pre S2 sequence. Among HBV patients with mutant form, one was infected with delta virus genotype IIb. Genotype sequence analysis of HDV resulted in 10 patients having genotype IIb and two patients with genotype IIb mutant.

DISCUSSION

Delta virus is a defective RNA viroid that occurs in individuals positive for HBsAg [Wedemeyer and Manns, 2010]. In the current, we aimed to evaluate the prevalence of HDV among a cohort of Egyptian patients infected with chronic HBV and its impact on clinical, laboratory, serological, and virological features.

In the current study, there was male predominance (73.6%) among chronic HBV patients and consequently delta patients demonstrated (83.3%) males as well with no gender difference between HDV positive and negative patient. Similar findings were reported before by Reinheimer et al. [2012] as well as Gish et al. [2013] which may be due to increased risk factors for viral acquisition among them. Delta infection was more common in the fourth decade of life as supported by Ziaee and Azarkar [2013], whose patients were in fourth decade as well and Zaidi et al. [2010] who reported patients in fifth decade.

HBV-genotype D2 was the most prevalent genotype in our patients (98.2%) and this is consistent with

TABLE III. Prevalence of HDV Among the Studied Patients

	Number and percentage
HDV IgG	
Positive	10 (8.3%)
Negative	111 (91.7%)
HDV DNA BY PCR	
Positive	12 (9.9%)
Negative	109 (90.1%)

previous data obtained in Egypt [Habil et al., 2013; Zaky et al., 2010]. As regards, the serological and virological markers of HBV in the whole studied group, HBeAg-negative and HBeAb positive patients were 81.9% and 76.7%, respectively. Habil et al. and Zaky et al. reported that more than 80% and 94% of the studied subjects were associated with HBeAg-negative status in Egypt, respectively.

In the current study, HDV prevalence was found to be 8.3% by HDV IgG and increased to 9.9% using PCR which could be explained by false negative antibody test confirming that PCR is a more sensitive and useful method for the detection of viremic patients.

The predominant HDV-genotype was IIb except for two patients with HDV-genotype IIb mutant. This is not in agreement with the data obtained from a study done by Saudy et al. [2003] who reported that HDV-genotype I was the most prevalent genotype with HBV-genotype D among Egyptian patients.

Delta infection did not affect the clinical or laboratory features of HBV patients. However, among HDV-coinfected patients, the HBV-DNA was negative

TABLE IV. Demographic, Routine Laboratory, and Serum AFP of HDV Positive Patients in Comparison to HDV Negative Patients

	HDV PCR positive (n = 12)	Negative (n = 109)	*P*-value
Demographic features			
Gender			
Male	10 (83.3%)	79 (72.5%)	0.5
Female	2 (16.7%)	30 (27.5%)	
Age (Mean ± SD)	35.8 ± 11.2	33.5 ±10.6	0.5
BMI (Mean ± SD)	31.4 ± 6.3	27.7 ± 6.1	0.1
Hb (gm/dL)	14.1 ± 1.9	13.6 ± 1.9	0.4
TLC ($x10^3$)	6.4 ± 2.6	6.8 ± 3.4	0.7
PLT ($x10^3$)	238.3 ± 51.2	224.4 ± 69.2	0.5
INR	1.12 ± 0.24	1.12 ± 0.18	0.9
Albumin (g/dl)	4.5 ± 0.3	4.2 ± 0.6	0.07
T. Bilirubin (mg/dl)	0.7 ± 0.44	0.96 ± 2.39	0.7
Creatinine (mg/dl)	0.9 ± 0.2	1 ± 0.8	0.7
AFP (ng/ml)	8.2 ± 5.5	5.9 ± 4.7	0.1

TABLE V. Comparative Features Between HBV/HDV Co-infected and HBV-Monoinfected Patients

	HDV PCR positive (number and percent)	Negative (number and percent)	*P*-value
Transaminases			
ALT			
Normal	10 (83.3%)	71 (65.1%)	0.3
Elevated	2 (16.7%)	38 (934.9%)	
AST			
Normal	11 (91.7%)	81 (74.3%)	0.3
Elevated	1 (8.3%)	28 (25.7%)	
Seromarkers			
HBeAg			
Positive	1 (9.1%)	20 (19%)	0.7
Negative	10 (90.9%)	85 (81%)	
HBeAb			
Positive	7 (63.6%)	82 (78.1%)	0.3
Negative	4 (36.4%)	23 (21.9%)	
HBcIgG			
Positive	11 (100%)	95 (90.5%)	0.6
Negative	0 (0%)	10 (9.5%)	
Viral load			
HBV DNA by PCR (IU/ML)			
Mean ± SD ($\times 10^3$)	87 ± 120	1,400 ± 2,600	0.06
Median(IQR) ($\times 10^3$)	39 (170–220)	250 (39–1,200)	
HBV DNA BY PCR			
Positive (n = 111)	4 (33.3%)	107 (98.2%)	
Negative (n = 10)	8 (66.7%)	2 (1.8%)	<0.001**

*$P < 0.05$ = significant, **$P < 0.01$ = highly significant.

TABLE VI. Transient Elastography Results of HBV-Mono-infected Versus Co-Infected HBV/HDV

	HDV positive	Negative	*P*-value
Fibroscan results			
Mean ± SD (Kpa)	6.3 ± 0.9	7.1 ± 3.3	0.5
Fibrosis stages			
F0 (n = 38)	2 (20%)	36 (36.4%)	0.3
F0-F1 (n = 7)	0 (0%)	7 (7.1%)	1
F1 (n = 26)	7 (70%)	19 (19.2%)	0.004**
F2 (n = 14)	1 (10%)	13 (13.1%)	1
F2-F3 (n = 15)	0 (0%)	15 (15.2%)	0.4
F3-F4 (n = 5)	0 (0%)	5 (5.1%)	1
F4 (n = 4)	0 (0%)	4 (4%)	1

*$P < 0.05$= significant, **$P < 0.01$= highly significant.

in 66.7% with median of 39×10^3 IU/ml and the majority (66.7%) were inactive carriers. The presence of delta infection caused suppression to HBV replication and this agreed with similar data obtained by Latika and Summaiya. Similarly, Gish et al. [2013] reported the presence of 56% of delta patients with undetectable HBV viraemia.

In our series, the application of HDV IgG demonstrated that HDV prevalence was 8.3% among patients with HBV infection similar to data obtained by El Zayadi et al. [1988] as well as Gish et al. [2013]. However, other reports demonstrated contradictory data. Lower prevalence by HDV IgG was reported by Gomaa and colleagues in 2013 and Roshandel et al. in 2008 [Roshandel et al., 2008] which was 4.7% among 170 HBV Egyptian patients and 5.8% among 139 HBV patients from Iran, respectively. Higher prevalence by HDV IgG was shown by Zaidi et al. in 2010 who reported the presence of HDV infection in 88.8% among 96 patients living in Pakistan, with only 70% having detectable HDV-RNA by PCR. The authors of these reports attributed these variations to the compulsory mass vaccination programs.

HDV-PCR was detected in 9.9% of the studied HBV/HDV patients meaning higher value than that detected by HDV-IgG (8.3%). This differs from Reinheimer et al. in 2012 who studied 2844 HBV patients in Germany and prevalence of HDV using HDV IgG was 7.4% while prevalence using HDV-RNA was 65% of HBV/HDV cases. This indicates the importance of screening by PCR rather that IgG alone.

In the current study, different stages of fibrosis were tested by fibroscan and/or liver biopsy among HBV patients whenever indicated. Fibroscan results were not in accordance with those of liver biopsy in 10 HBV patients who were indicated for biopsy (i.e., four patients with fibroscan F2–F3 appeared to be F1 by liver biopsy). However none of the delta patients had undergone liver biopsy.

Fibroscan detected F0 in 20% and F1 in 70% among 10 HDV positive patients. Owing to the fact that 70% of fibroscan results for HDV patients were F1 this reflects that HBV/HDV infection might not result in the presence of significant liver damage. Therefore, we were able to observe that fibroscan as one of the noninvasive techniques might not be so accurate in the assessment of liver fibrosis among this subset of patients. However, this finding needs further studies due to the small number of biopsies performed.

CONCLUSIONS

The prevalence of HDV superinfection was 8.3% by HDV IgG and 9.9% by HDV PCR. The most prevalent genotype in our study was HDV-genotype IIb with two patients having HDV-genotype IIb mutant. HDV-infected patients were more likely to be HBeAg negative (90.9%) and to have lower HBV viremia. The presence of delta virus infection was not associated with significant liver damage and the clinical course was not significantly affected compared to the mono-infected HBV patients.

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