Characteristics of hepatitis viruses among Egyptian children with acute hepatitis

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Abstract. Hepatitis viral infection is hyperendemic in Egypt, western Asia and Africa. However, little is known about the status of hepatitis viruses among rural Egyptian children. Therefore, this study sought to examine the prevalence and characteristics of hepatitis viruses among symptomatic Egyptian children. Serological and molecular analyses of hepatitis viral infection were conducted in 33 children hospitalised at Mansoura University with symptomatic hepatic dysfunction (mean ± standard deviation age, 9.7±3.4 years; alanine aminotransferase level, 130±68 IU/ml). Eleven children (33%) were positive for anti-haemagglutination-IgM and were diagnosed with acute hepatitis A. Hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) were detected in 9 (27%) and 7 (21%) children, respectively, indicating acute-on-chronic infection with hepatitis viruses. None of the children was positive for anti-hepatitis B core antigen-IgM. Phylogenetic analysis confirmed that all HBVs belonged to genotype D (subgenotype D1) and that HCV belonged to genotypes 4a and 1g. HBV-DNA was detected in 9 children (27%) in the pre-S/S region and in 16 children (48%) in the core promoter/precore region. The Y134F amino acid mutation in the 'α' determinant region was detected in all of the patients. The A1762T/G1764A double mutation, and the T1846A and G1896A single mutations were common in children with occult HBV infection. In conclusion, hepatitis viral infection, including acute-on-chronic infection with HCV and HBV, is common in Egyptian children hospitalised with acute hepatitis.

Introduction

Hepatitis virus infection is a major global health problem. Acute hepatitis is sometimes serious and may be fatal in children because of their immature immune system. Egypt has one of the highest prevalence rates of hepatitis C virus (HCV) infection owing to the vigorous public health campaigns conducted between the 1950s and 1982 to eradicate schistosomiasis (1). Hepatitis B virus (HBV)-related liver disease is also common in Egypt, like many other countries. Consequently, Egyptian children are at particularly high risk of HBV and HCV infection.

Egypt was one of the first countries to introduce universal HBV vaccination in 1992. The Ministry of Health and Population conducted a wide range of prophylactic strategies to control viral hepatitis. It was reported that the prevalence of hepatitis B surface antigen (HBsAg) positivity among healthy individuals decreased from 10.1% in 1985 to 1.18% in 2008, and the frequency of acute HBV infection as a cause of symptomatic hepatitis decreased significantly from 43.4% in 1983 to 28.5% in 2002 (2-4). Hepatitis virus infection, particularly HCV infection, was reported to be an important risk factor for acute hepatitis in Egyptian children (5). In Africa, acute hepatitis is still common and is sometimes fatal. However, the reason for this is unclear, and may be related to coinfection with *Leptospira* or Rift Valley fever virus, for example (6,7).

Hepatitis A virus (HAV) is also an important pathogen that is frequently associated with acute hepatitis. An Egyptian earlier survey examined more than 5,000 patients with acute hepatitis and showed that 40.2% of patients had HAV-related acute hepatitis (8). In addition, 94.4% of children aged >5 years were reportedly positive for anti-HAV IgG (9). These findings also suggest that most Egyptian children were exposed to HAV in their childhood.

In this study, we analysed the aetiology of hepatitis virus using serological and genetic methods in 33 Egyptian children hospitalised with acute hepatitis.

Materials and methods

Study subjects. This study was conducted at the Children's Hospital, Mansoura University, Mansoura, Egypt. Thirty-three children with acute hepatitis were identified and included in the study. The study subjects were mostly male (n=26), with a mean ± standard deviation (SD) age of 9.7±3.4 years. Overall,

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60.6% of the children lived in rural areas. All of the children enrolled in the study underwent thorough clinical examinations and their medical history was carefully reviewed. Acute hepatitis is defined as acute hepatic injury, manifested by the release of cytoplasmic enzymes, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In three of the patients, with mean ALT, AST and total bilirubin (T-Bil) levels of 130.1±68.3 IU/l, 146±68.3 IU/l and 2.9±1.2 mg/dl, respectively, increases in these enzymes was accompanied by symptoms such as fever, loss of appetite, abnormal bilirubin metabolism-related jaundice, dark urine and pale stools. All of the patients had AST and ALT levels over two times the upper limit of normal at acute onset. Informed written consent was obtained from the parents of all the children. The study was approved by the Ethics Committee of Mansoura University.

Serological markers of HBV infection. HBsAg was assessed using a reversed passive hemagglutination (R-PHA) test (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan). Anti-HCV antibody (HCV-Ab) was examined using the passive Ortho HCV-Ab PA Test II (Fujirebio Inc., Tokyo, Japan). Anti-hemagglutination (HA)-IgM, anti-hepatitis B surface (HBs) and anti-hepatitis B core antigen (HBc)-IgM antibodies were assessed using radioimmunoassays (SRL Inc., Tokyo, Japan). Laboratory investigations, including liver function tests, were performed using a Synchron autoanalyser (Beckman Coulter, Fullerton, CA, USA). ALT, AST, albumin and T-Bil levels were measured in all serum samples.

DNA/RNA extraction and viral load of HBV. Viral DNA was extracted from 200 μl of serum using a QIAamp DNA Blood mini kit and a QIAamp viral RNA kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. The viral load was assessed by real-time PCR using an ABI Prism 7700 analyser (Applied Biosystems, Foster City, CA, USA). HBV was amplified with a primer and probe set, as previously described (10).

Amplification of the HBV/HCV genome and identification of mutations. The sequence of the core promoter/precore (CP/PC) region was amplified by PCR with nested primers (11). The amplified fragments were directly sequenced and the G→A substitution at nucleotide (nt) 1,896 in the PC, A→T at nt 1,762, G→A at nt 1,764 in the basal CP, and the Kozak sequence (CCACC; nt 1,809-1,813) were analysed.

The complete nucleotide sequences of HBV from two samples were sequenced using two overlapping amplicons with specific primers (12). A second PCR was performed to detect the full genome sequence in two virus isolates and for pre-S1/S2/S gene detection using the previously reported primers and PCR conditions (13).

The extracted RNA was reverse-transcribed to cDNA using a Sensiscript RT kit (Qiagen GmbH) with oligo dT primers (Promega, Madison, WI, USA). The transcribed cDNA was used for HCV amplification by nested PCR. The 5'-non-coding regions of HCV-RNA were amplified (14,15).

Genotyping of hepatitis virus by phylogenetic analysis. The amplified products of the second PCR were directly sequenced using the Taq Dye Deoxy Terminator cycle sequencing kit with a 3100-*Avant* genetic analyser (Applied Biosystems).

The two full-genome and S gene sequences of the HBV strains determined in this study were compared with those of 20 reference sequences retrieved from the DDBJ/EMBL/GenBank database. The subtypes of the strains used for comparison were obtained from published articles (16).

The sequences were aligned using CLUSTAL X software and the phylogenetic trees were constructed by the neighbour-joining method (17). To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1,000 times. These analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software program (available at http://www.megasoftware.net) (18).

Table I. Serological and clinical characteristics of the patients.

	HA-IgM	HBsAg	anti-HCV
Positive number (%)	11 (33%)	9 (27%)	7 (21%)
Male/female	8/3	7/2	5/2
Age (years)	8.0±3.3	10.8±2.9	9.3±3.6
ALT (IU/l)	137±89	134±34	131±64
T-Bil (mg/dl)	3.0±1.2	2.8±1.0	2.0±0.4

Results

Serological markers and laboratory characteristics. Serological data are summarized in Table I. Overall, 11 (33%) and 7 (21%) children were positive for HA-IgM and anti-HCV antibodies, respectively. HBsAg was detected in 9 children (27%) while the other 24 (73%) were negative. There were no significant clinical differences among children according to the type of hepatitis. Of the HBsAg-positive children, one (case 4) was coinfected with HAV and three (cases 9, 16 and 19) had HCV. On the other hand, only three children were positive for anti-HBs antibodies and none was positive for anti-HBc-IgM antibodies. HBV-DNA corresponding to the pre-S/S and CP/PC regions was detected in all nine HBsAg-positive children. HBV-DNA corresponding to the CP/PC region was detected in 7/24 HBsAg-negative children. There were no clinical differences between the HBsAg-positive and HBsAg-negative children (Table II).

CP/PC mutations. Mutations in the pre-S/S and CP/PC regions were detected by the PCR-direct sequencing method. All of the children, except for cases 22 and 24, were double-wild for the A1762T/G1764A double mutation. The G1896A mutation was found in 3/7 (43%) HBsAg-negative children, compared with just 1/9 (11%) HBsAg-positive children. No specific mutations were found in C1653, T1753 or T1858.

Sequencing and phylogenetic analysis of the pre-S/S gene. The entire pre-S/S gene was sequenced in the nine HBsAg-positive children and was converted to the corresponding amino acid sequence to identify amino acid variations. Fig. 1 summarises the amino acid mutations/variations in the pre-S/S region. While A79T was found in the pre-S region in one child (case 9), the

Table II. Prevalence and characteristics of HBV carriers.

	HBV-DNA(+)			
	HBsAg(+)	HBsAg(-)	HBV-DNA(-)	Total
Positive number (%)	9 (27%)	7 (21%)	17 (52%)	33
Age (years)	10.8±2.9	9.1±3.7	9.4±3.5	9.7±3.4
Gender (male/female)	7/2	7/0	12/5	26/7
Residence (rural/urban)	5/4	5/2	10/9	20/13
ALT (IU/l)	134.4±34.7	127.9±47.6	128.9±88.6	130.2±68.3
T-Bil (mg/dl)	2.8±1.0	2.3±0.8	3.2±1.4	2.9±1.2

									Pre-S			S				CP/PC			
	Age	Sex	HBsAg	HBsAb	IgM-HBc	HBV-DNA	ALT	T-Bil	A79T	P149H	P160L	T115P	Y134F	A157D	S204R	G1757C	A1762T/G176A	T1846A	G1896A
Case 4	13	F	(+)	(+)	(–)	<2.6	120	2.8	T	H	L	T	F	A	S	G	A/G	T	G
Case 9	9	M	(+)	(–)	(–)	3.6	155	1.4	A	H	L	T	F	A	R	G	A/G	T	G
Case 10	10	M	(+)	(–)	(–)	2.8	130	2.4	T	H	L	T	F	A	S	G	A/G	T	G
Case 11	15	M	(+)	(–)	(–)	7.6	94	3.5	T	H	L	T	F	A	S	G	A/G	T	G
Case 14	11	M	(+)	(–)	(–)	4.1	170	4.0	T	H	L	T	F	D	S	G	A/G	T	G
Case 16	8	M	(+)	(–)	(–)	4.0	140	2.5	T	H	L	T	F	A	R	G	A/G	T	A
Case 18	8	M	(+)	(–)	(–)	8.2	170	4.5	T	H	L	T	F	A	S	C	A/G	T	G
Case 19	8	F	(+)	(–)	(–)	3.1	160	2.3	T	H	L	T	F	A	S	G	A/G	T	G
Case 29	15	M	(+)	(–)	(–)	4.1	70	2.0	T	H	L	P	F	A	S	G	A/G	T	G
Case 5	13	M	(–)	(–)	(–)	<2.6	130	1.6								G	A/G	A	G
Case 13	12	M	(–)	(–)	(–)	<2.6	150	3.5								G	A/G	T	G
Case 15	7	M	(–)	(–)	(–)	<2.6	150	3.5								G	A/G	T	A
Case 21	7	M	(–)	(+)	(–)	<2.6	100	2.0								G	A/G	T	G
Case 22	5	M	(–)	(–)	(–)	<2.6	250	1.5								G	T/A	A	A
Case 24	6	M	(–)	(–)	(–)	<2.6	75	2.1								G	T/A	T	G
Case 28	14	M	(–)	(–)	(–)	<2.6	60	1.9								G	A/G	T	A

Figure 1. Clinical data and mutations/variations in the pre-S/S and CP/PC regions of HBsAg-positive and occult HBV cases. The P149H and P160L variants in the pre-S region and the Y134F variant in the 'α' determinant region were detected in all of the children. A1762T/G1764A double mutations in the CP/PC region were detected in cases 22 and 24.

P149H and P160L variants were found in all of the children. The T115P and A157D mutations were detected in one child and S204R mutation was detected two children in the S region. The Y134F mutation in the 'α' determinant region was identified in all of the children, but no specific mutations, such as T131I, K141E or G145R, were found in the α loop (amino acids 111-156).

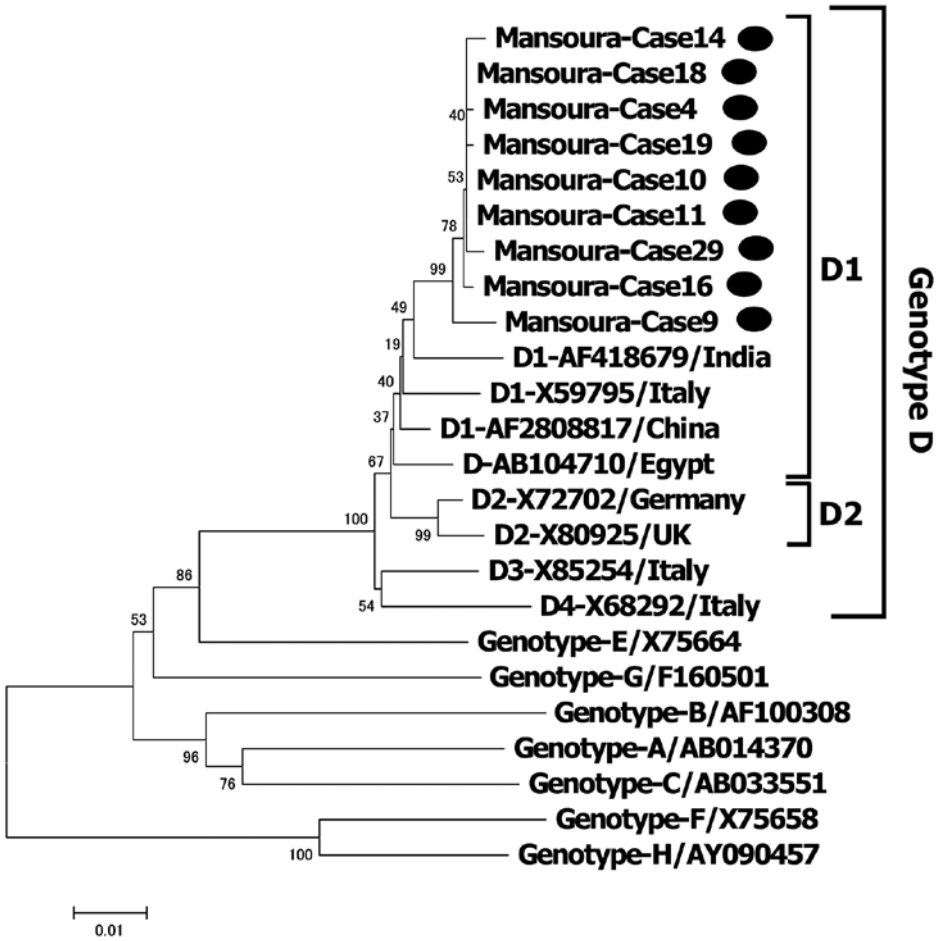

Figure 2. Phylogenetic analysis of the nine HBsAg-positive strains based on the pre-S/S region. A phylogenetic tree was constructed using 15 reference strains. The accession numbers and country of origin are indicated for the reference isolates. The length of the horizontal bars indicates the number of nt substitutions per site.

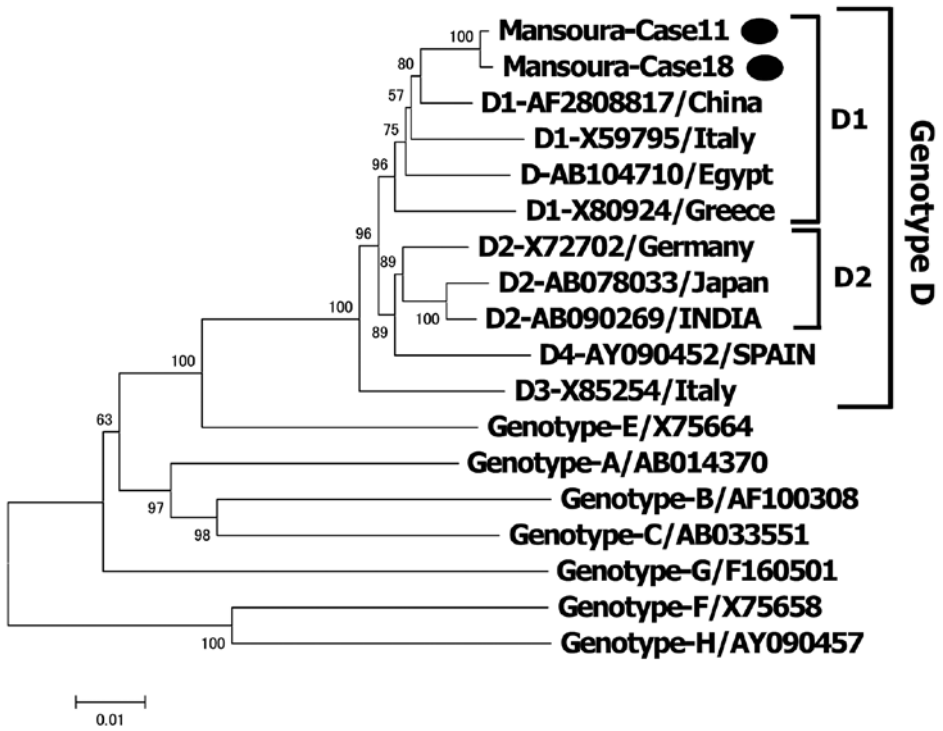

Figure 3. Phylogenetic analysis of two HBsAg-positive strains based on the complete genome. A phylogenetic tree was constructed using 15 reference strains.

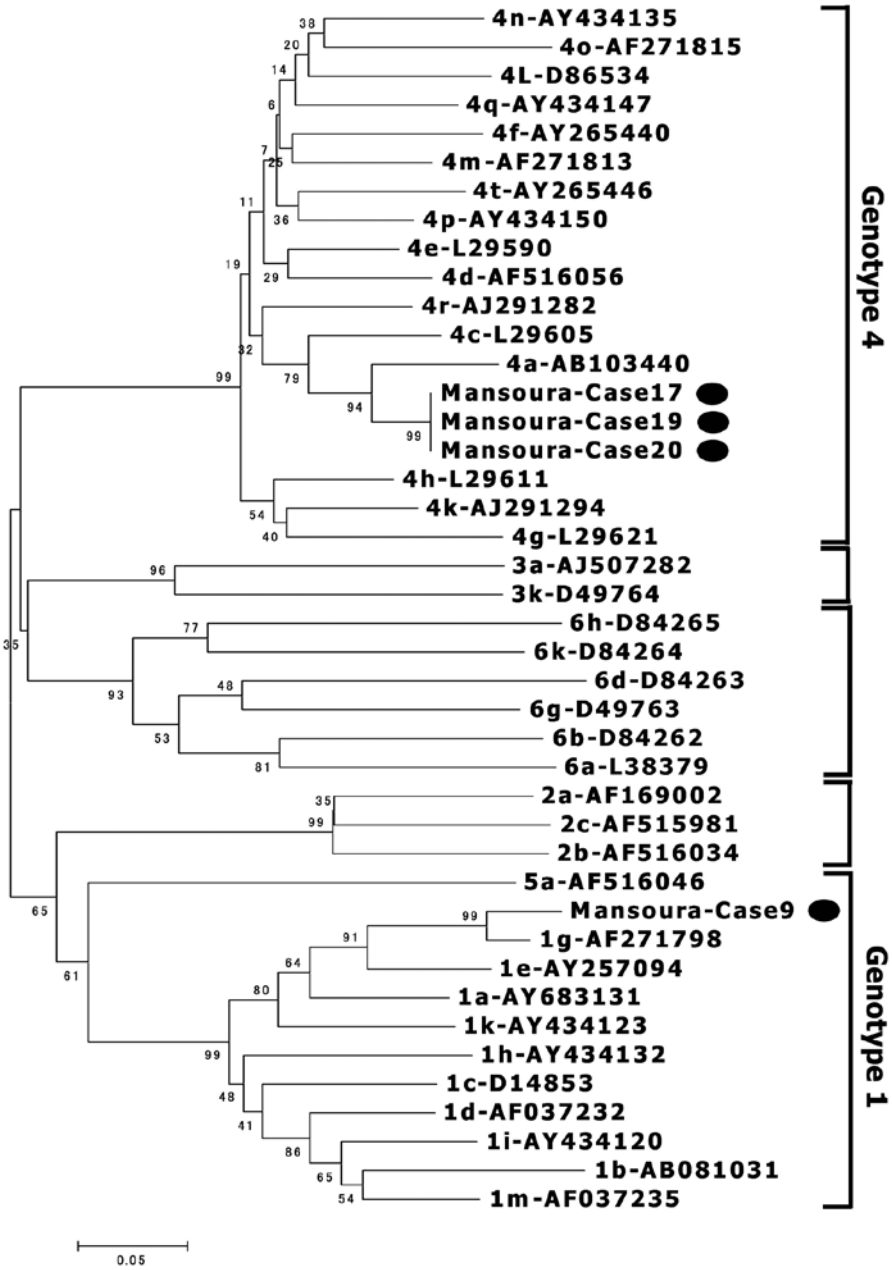

Figure 4. Phylogenetic analysis of four anti-HCV antibody-positive strains based on the NS5a region. Three genotypes were classified as subgenotype 4a and one was classified as subgenotype 1g.

A phylogenetic tree was constructed for the 9 children using 15 reference sequences of HBV isolates from various countries. HBV was classified as genotype D1 in all of the children (Fig. 2).

HBV genotypes and complete nucleotide sequence of HBV. To confirm the genotyping, a phylogenetic tree was constructed using two complete genome sequences with 16 reference sequences of HBV isolates derived from various countries. The two complete genomes were 3,182 bp long with a common deletion of 33 nucleotides in the pre-S1 region. Both genotypes were classified as genotype D (subgenotype D1) (Fig. 3).

Detection of HCV-RNA and phylogenetic analysis. HCV-RNA was detected in 4/7 children with anti-HCV antibodies. The HCV genomes from four children were amplified and sequenced, and three were classified as subgenotype 4a and one as subgenotype 1g (Fig. 4).

Discussion

The cause of acute hepatitis usually shows geographic differences. In particular, environmental factors (e.g., aflatoxin) and endemic infections (e.g., schistotomiasis) are associated with acute and chronic liver diseases in Egypt (19). Regarding viral hepatitis, it was reported that HCV infection is an important predictor of acute hepatitis among Egyptian children (5). Although HCV infection was relatively common, HAV infection was more prevalent in this study. Although the seroprevalence

of HAV in children is associated with socioeconomic status in Egypt, the location of Mansoura may also partly explain the high prevalence in this study (20). El Mansoura is a city in Egypt with a population of 420,000. It is the capital of the Ad-Daqahliyah Governate. Mansoura lies on the east bank of the Damietta branch of the River Nile, in the delta region, about 120 km northeast of Cairo. Acute HAV infection is usually curable and its clinical course differs from those of HBV and HCV infections. This also explains why long-term follow-up is necessary for HBV and HCV infections.

HBV infection is a significant global health problem and may cause both acute and chronic infection in humans (21). The World Health Organization recently estimated that there are at least 350 million individuals worldwide with HBV infection (22). Infection with HBV can lead to progressive liver disease, including liver cirrhosis and hepatocellular carcinoma (HCC), and approximately 1 million people with HBV die from HCC annually. HBV is associated with socioeconomic conditions, and Southeast Asia, China and Africa have the highest rates of infection (23).

The age at which HBV infection occurs influences the long-term outcomes and determines the primary targets of vaccination programmes. Perinatal transmission from a mother to child at or soon after birth occurs in about 90% of children, with long-term complications of chronic hepatitis, cirrhosis and hepatocellular carcinoma, leading to death in middle age, particularly in men. This has serious economic consequences for both the family and country as a whole. In 1991, the Child Survival Project/Expanded Program on Immunization implemented a nationwide plan to support immunization of all infants against HBV. In the Expanded Program of Immunization, infants were vaccinated with a 2.5 μg dose of a recombinant vaccine, together with a combined vaccine for diphtheria, tetanus and pertussis, at 2, 4 and 6 months of age. This recommended series of 3 intramuscular doses of the HBV vaccine induces a protective antibody response (i.e., anti-HBs antibody) in 90% of healthy adults and 95% of infants, children and adolescents (24). However, despite the introduction of successful infant and adolescent immunisation programs in many countries, the burden of HBV-related disease remains high. More than 90% of young Egyptians have been immunised and a large proportion of older Egyptians are resistant to HBV infection because they have either been immunised or were previously infected (4,5). In this study, 3 children (9%) were positive for anti-HBs antibodies, suggesting incomplete protection against HBV infection. On the other hand, the prevalence of HBV infection among children is rapidly decreasing, with a significantly lower frequency of acute HBV infection in 2002 (5%) than in 1983 (11.9%) among those aged 12-19 years. It is probable that the level of immunity against HBsAg is so low that anti-HBs could be diminished soon after infancy, although the HBV vaccine is useful to protect against HBV infection in early life. This may explain why the prevalence of acute HBV infection among adults aged 20-39 years was higher in 2002 (20.5%), compared with the same age group in 1983 (16.2%).

In this study, HBsAg was detected in 9 children (27%). As none of the children was positive for anti-HBc-IgM, we think that these 9 children had acute-on-chronic HBV infection. The impact of HBV vaccination in Egyptian school children aged over 10 years in an endemic area of the Nile Delta was evaluated, but the prevalence of HBsAg did not change, even among vaccinated children (25). Therefore, the high prevalence of HBsAg in vaccinated and non-vaccinated children could be due to intrauterine HBV infection, a weak immune response, or infection with escape mutant variants (25).

In this cohort of patients with symptomatic HAV, acute HBV infection was not apparent in children at 9 years of age (i.e., children who had been vaccinated), compared with an infection rate of 6.8% in the same age group at the same hospital in 1983.

In this study, seven children were negative for HBsAg and positive for HBV-DNA and were therefore classified as having occult HBV infection. The prevalence of occult HBV varies considerably and greatly depends on the prevalence of HBV in the general population and the methods used to detect HBV DNA (26). Occult HBV infection has been reported in patients with resolved acute-on-chronic HBV infection and in patients lacking serological markers for past HBV infection (27). Two common findings and possible explanations for occult HBV are low levels of viral replicative activity and/or mutations in the 'α' epitope of the S gene encoding amino acid residues 100-160 of HBsAg (so-called S mutants or variants) (26). These seven children had low HBV-DNA levels, preventing us from amplifying the S region. The S gene of HBV has three open reading frames (i.e., the pre-S1, pre-S2 and S regions). The surface gene contains a neutralizing epitope, the 'α' determinant region, which is located at nt 124-147. Mutations in this region could alter the antigenicity of HBsAg, causing the failure of anti-HBs to neutralize HBsAg, allowing its escape from the host's immune system, resulting in active viral replication and liver disease (28). In this study, we found no specific mutation in the 'α' determinant region, such as T131I, K141E or G145R, although there were seven amino acid mutations in the pre-S and S regions in HBsAg-positive children. It is generally thought that the escape mutant is rare in Egypt. It was reported that the pre-S variant was associated with immune escape and mutations of some epitopes located downstream of the 'α' determinant region might affect the neutralisation domain (29). Although the A157D and S204R variants were detected in this study, they did not affect the production of HBsAg.

HBV is classified into seven genotypes, A-G, based on sequence divergence of the entire genome of >8% (30,31). An eighth genotype, designated H, was recently reported in Central America (32), but it has not been fully characterised. Therefore, eight genotypes of HBV (A-H) are currently recognized and subgenotypes, differing by ≥4%, have been described (30). A few reports have described the frequency of HBV genotypes in Egypt and revealed that HBV genotype D is the most prevalent. One explanation for this is that Egypt receives many tourists and visitors from countries where genotype D is prevalent, particularly other Mediterranean countries, with a high degree of nt homology (33). We recently reported that genotype D was prevalent among HBV carriers in Ismailia City (34). In the present study, all of the samples were classified as genotype D. A recent study showed that HBV infection exhibited some genotypic variation among children with cancer, and

genotypes B and D were more frequently associated with malignancies than were genotypes A and C (35). Because very few studies in Egypt have focused on children with cancer, we must carefully follow-up these patients.

In conclusion, hepatitis viral infection, including acute-on-chronic infection by HCV and HBV, is common among children hospitalised for acute hepatitis in Egypt. A large proportion of children were positive for HBV-DNA, possibly because of genetic variability and/or low-level immunity. Future studies should focus on improvements in immunisation programmes.

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