

Molecular Epidemiological Study of Hepatitis Viruses in Ismailia, Egypt

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Key Words

Hepatitis B virus · Hepatitis C virus · Epidemiology

Abstract

The hepatitis virus is hyperendemic in Egypt, western Asia and Africa. In Egypt, most studies have been carried out in the regions of the upper and lower Nile Delta, and so little is known about other parts of the country. Our project aimed to clarify the carrier rate of various hepatitis viruses in the northeastern province of Ismailia. A total of 214 patients with elevated liver enzymes were enrolled in this study. Sera were collected in Ismailia hospital. We conducted a serological and molecular-based survey of hepatitis viruses, including their genetic variability and genotype-related differences. There were 10 (4.7%) cases positive for hepatitis B surface antigen (HBsAg), and 156 (72.9%) positive for anti-hepatitis C virus antibody (HCV-Ab). Phylogenetic analysis showed that genotype C of HBV and genotype 4a of HCV were prevalent. Hepatitis D virus RNA was not detected in HBsAg carriers. Although anti-hepatitis E virus IgM antibody was positive in 5 cases (2.3%), no case was positive for its RNA. Among 54 cases negative for HBsAg and HCV-Ab, HBV-DNA was detected in 35 cases (65%). Our results

revealed that HBV and HCV, including occult HBV infection, are widespread and related to liver diseases in Ismailia province, Egypt.

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Introduction

Hepatitis virus infection is now a major health problem both in developed and developing countries. In addition, diseases related to hepatitis virus infection are widespread in the world. Although many studies have been reported so far in Egypt, there has been no detailed molecular-based epidemiological study regarding hepatitis viruses in the Suez Canal area and Sinai. The Ismailia governorate is located mostly on the west side of the Suez Canal in the northeastern part of the Nile Delta (fig. 1). Ismailia City is approximately 90 minutes by car from Cairo, and the geographic and demographic features are different from the prevailing pattern in the central, upper and lower parts of Egypt.

Hepatitis B virus (HBV) caused as much as 28.5% of symptomatic hepatitis in 2002 [1]. In Egypt, the prevalence of hepatitis B virus antigen (HBsAg) is around 1.2%

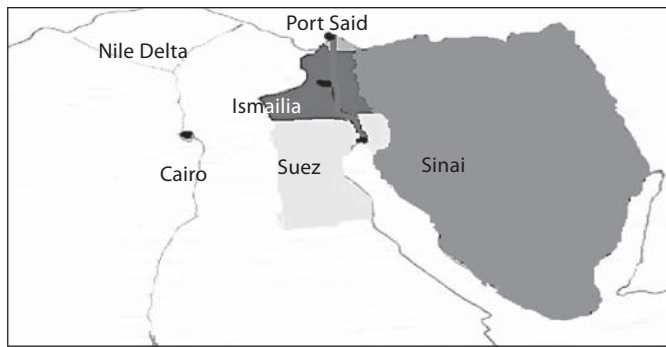


Fig. 1. Location of Ismailia province. The Ismailia governorate is mostly on the west side of the Suez Canal in the northeastern part of the Nile Delta.

in the healthy population, and this is slightly lower than the global average.

Many studies have revealed that occult HBV infection might be frequent and clinically important [2]. Occult HBV infection is diagnosed when HBV-DNA is detected in the plasma despite HBsAg being serologically negative [3]. Occult HBV infection has been described in acute and chronic hepatitis and also hepatocellular carcinoma. However, there has been no community- or hospital-based study of occult HBV infection in Egypt.

Egypt shows one of the highest prevalences of hepatitis C virus (HCV) in the world: 10–20% of the general population is infected, and HCV is the leading cause of hepatocellular carcinoma and chronic liver disease [4, 5]. The HCV epidemic appears to have been initiated by the vigorous public-health campaigns to eradicate schistosomiasis that were conducted from the 1950s until 1982. During these mass-treatment campaigns for the general population, tartar emetic (potassium antimony tartrate) was administered as a series of intravenous injections, using non-sterile injection equipment, with additional parenteral anti-*Schistosoma* therapy [6]. There is a correlation between the level of exposure to parenteral anti-*Schistosoma* therapy and HCV prevalence among different age groups and geographic regions [7–10].

In Egypt, although no major epidemiological study on hepatitis E virus (HEV) has been conducted, HEV is widely endemic, with 17.2–60% of adults being serologically positive [11–14]. Recent studies have described the high prevalence of anti-HEV antibody among healthy adults and pregnant females in rural areas of Egypt (67.7 and 84.3%, respectively) [15, 16], and the rate of anti-HEV antibody positivity is as high as 85.1% among acute hepatitis cases in rural villages of the Nile Delta [17].

Based on previous data, we focused on the prevalence and diversity of hepatitis virus in Ismailia province, Egypt.

Materials and Methods

Sample Collection

We collected serum samples in the General Hospital of Ismailia University from 214 patients (157 males and 57 females, median age 42.2 ± 8.6 years) who had liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) over 2 times the upper limits of the normal range. Patients were inpatients during medical therapy and outpatients who were healthy viral carriers. The ranges were as follows: AST 51–4,970 (median 244 ± 550); ALT 50–5,013 (median 293 ± 594). Collected samples were stored at -80° until the analysis of hepatitis markers as well as for RNA and DNA extraction. This study was approved by the ethics committee of Kobe University.

Serological Marker Testing

Serum samples were assessed for HBsAg by reverse passive hemagglutination (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan), anti-HCV antibody (HCV-Ab) by passive Ortho HCV-Ab PA Test II (Fujirebio Inc., Tokyo, Japan), and IgM-HEV antibody (IgM-HEV-Ab) by enzyme-linked immunosorbent assay (ELISA; Viragent HEV-Ab human IgM; Cosmic Corp., Tokyo, Japan).

Detection of Viral DNA/RNA

Viral DNA/RNA was extracted from 200 μ l 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 extracted RNA was reverse-transcribed to cDNA using a Sensiscript RT kit (Qiagen GmbH) and oligo dT primers (Promega, Madison, Wisc., USA). The transcribed cDNA was used for amplification of HCV and HEV-RNA by nested PCR. The detection of HBV-DNA was carried out by nested PCR for core promoter/pre-core regions and HCV-RNA was amplified for 5'-non-coding regions [18, 19]. HEV-RNA was also amplified by nested PCR using universal primers for a part of the ORF2/ORF3 overlapping region [20]. HDV-RNA presence was examined by PCR for amplification of the HDV genomic nucleotide positions from 855–1,287 using primers 853P (5'CGGATGCCCGAGGTCGGACC3') and 1267N (5'GAAGGAAGGCCCTGGAGAACAAGA3') [21].

Determination of Genotyping by Phylogenetic Analysis

Genotyping was determined by PCR and the direct sequencing method for the S region of HBV and NS5B region of HCV [22–24]. Amplified second PCR products were sequenced directly by dideoxy sequencing using the Taq Dye Deoxy Terminator cycle sequencing kit with a 3100-Avant genetic analyzer (Applied Biosystems, Foster City, Calif., USA).

The S gene sequences of HBV strains from 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 [25].

NS5B gene sequences for HCV strains from this study were compared with those of 86 reference sequences retrieved from the

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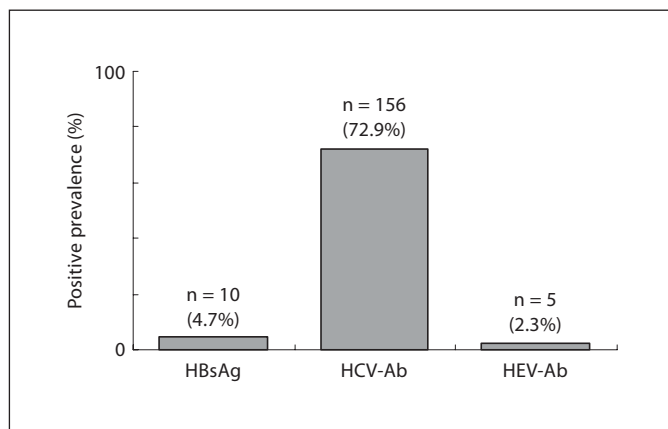
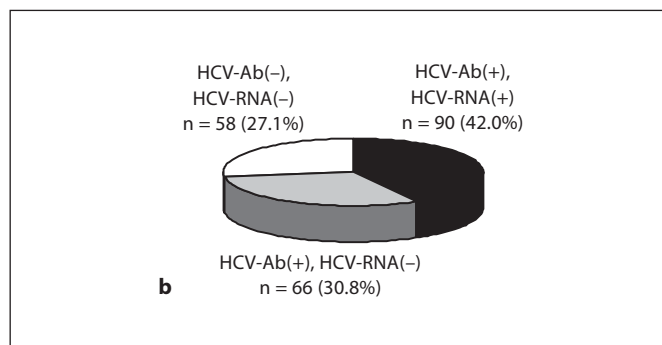
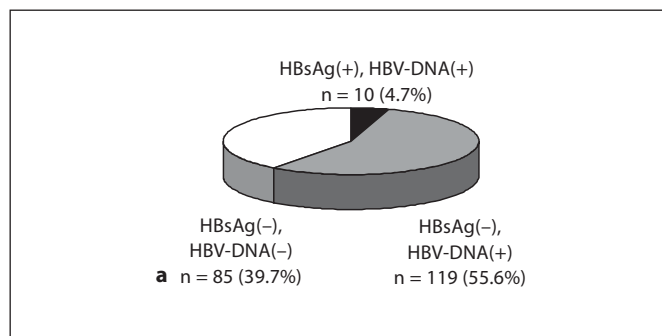


Fig. 2. Prevalence of HBsAg, HCV-Ab and IgM-HEV-Ab. Overall, HBsAg, HCV-Ab and IgM-HEV-Ab were positive in 4.7, 72.9 and 2.3% of cases, respectively. No difference was shown in the positive rate among generations.

Fig. 3. Serological and molecular-based analyses of HBV and HCV. Although HBV-DNA was positive in 129 cases (60.2%), only 10 cases were positive for HBsAg. A total of 119 cases out of 129 were thought to represent occult HBV infection. HCV-RNA was detected in 90 cases out of 156 HCV-Ab-positive carriers.



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Table 1. Comparison among HBsAg and HCV-Ab carriers

Category	Cases, n	Age	AST (IU/l)	ALT (IU/l)
HBsAg(+), HCV-Ab(-)	4	43.5 ± 5.1	844 ± 983	1,230.3 ± 1,471.5
HBsAg(+), HCV-Ab(+)	6	39.6 ± 10.7	259.2 ± 409.6	477.6 ± 898.9
HBsAg(-), HCV-Ab(-)	54	42.1 ± 8.6	273.6 ± 680.8	394.1 ± 733
HBsAg(-), HCV-Ab(+)	150	42.2 ± 8.7	217.2 ± 481.2	223.6 ± 457.6

Los Alamos HCV database (<http://hcv.lanl.gov/content/hcv-index>) [24].

The sequences were aligned using Clustal X software and phylogenetic trees were constructed by the neighbor-joining method [26]. To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1,000 times. These analyses were carried out using the Molecular Evolutionary Genetics Analysis software program (www.megasoftware.net) [27].

Results

Prevalence of HBsAg, HCV-Ab and IgM-HEV-Ab

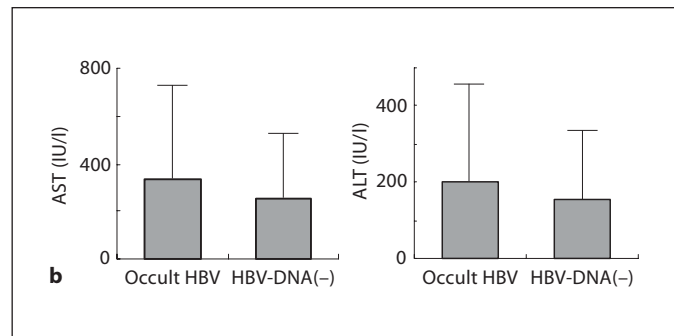
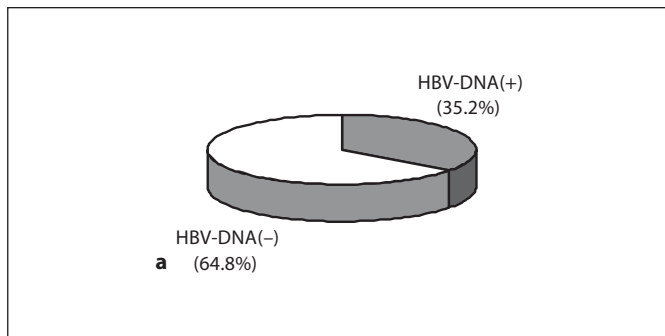
The prevalence of HBsAg and HCV-Ab is shown in figure 2. Overall, HBsAg and HCV-Ab were positive in 4.7 and 72.9%, respectively. No difference was shown in the positive rate among generations. Six cases (2.8%) were

positive for both HBsAg and HCV-Ab. A comparison of HBsAg and HCV-Ab carriers regarding age, serum transaminase and gender is summarized in table 1.

IgM-HEV was detected in 5 cases (2.3%), and their average age and mean liver enzyme levels are summarized in table 2.

Detection of HBV-DNA, HCV-RNA and HDV-RNA

HBV-DNA testing was positive in 129 cases (60.3%), although only 10 cases were positive for HBsAg. A total of 119 cases out of 129 were thought to represent occult HBV infection. HCV-RNA was detected in 90 cases out of 156 HCV-Ab carriers (fig. 3). As for the investigation of the significance of occult HBV infection, samples negative for both HBsAg and HCV-Ab were collected. Among 54 cases negative for HBsAg and HCV-Ab, HBV-DNA was detected in 35 (64.8%; fig. 4a).



	1,761	1,851	1,891
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Fig. 4. Characteristics of occult HBV infection. **a** The prevalence of occult HBV infection. Among 54 cases negative for HBsAg and HCV-Ab, HBV-DNA was detected in 35 (64.8%). **b** Serum AST/ALT levels. Serum AST/ALT levels were significantly higher in occult HBV cases. **c** Sequence data of the core promoter/pre-core region were aligned and compared. No specific mutation was found in either the HBsAg-positive carriers or occult HBV cases.

Average serum AST/ALT levels in occult HBV cases were higher than those in HBsAg-positive carriers (fig. 4b). Sequence data of core promoter/pre-core regions were aligned and compared (fig. 4c). A1762T and G1764A mutations were detected in 3 out of 7 HBsAg-positive carriers and 2 out of 10 occult HBV cases. G1896A mutation was found in 1 out of 7 HBsAg-positive carriers and 1 out of 10 occult HBV cases. HDV-RNA was not detected in either HBsAg-positive or occult HBV cases.

Genotypic Distribution and Phylogenetic Analysis

Direct sequencing and phylogenetic analysis were carried out based on the pre-S to S region of HBV and the NS5B region of HCV. Five samples were successfully sequenced: 4 samples were classified into genotype C (subgenotype C2), and 1 sample was classified into genotype D (subgenotype D1). The HCV genome was sequenced in 19 samples: 15 samples (78.9%) were subtype 4a, 2 (10.5%) were subtype 1g, and 1 (5.2%) was subtype 4m and 4o (fig. 5).

Table 2. Characteristics of HEV-positive carriers

Case	Age/sex	AST (IU/l)	ALT (IU/l)
1	28/F	491	581
2	37/M	1,117	1,240
3	45/F	317	202
4	42/M	82	96
5	51/M	114	65

Discussion

The Ismailia governorate is located mostly on the west side of the Suez Canal in the northeastern part of the Nile Delta. Ismailia City is approximately 90 minutes by car from Cairo, and the geographic and demographic features of Ismailia are different from those in central, upper and lower Egypt. The Ismailia governorate has rural inhabitants, representing 50% of the total population of

835,000 individuals (according to 2003 census data from www.ismailia.gov.eg), being partly agricultural and partly desert, and lacks the usual compact village structure of the Nile valley.

This study showed that the HBsAg-positive prevalence was 4.3%, and this is consistent with a previous report that showed HBsAg was detected in 4.3% of subjects with an elevated ALT level among 20,000 inhabitants of rural villages in the Nile Delta [17]. A recent study showed that the prevalence of HBsAg was 1.18% among healthy Egyptian blood donors [28], being much lower than the previous results (from 36.3 to 54.5%) [13, 29]. The trend toward a decrease in HBV prevalence in Ismailia Province and the Suez Canal area seems to be a general observation in Egypt. The steep decrease in HBV infection is attributed to the universal HBV immunization of infants that was initiated in 1991. More than 90% of young Egyptians have been immunized, and a large proportion of older Egyptians are resistant to HBV infection because they have either been immunized or previously infected [17, 30].

In the present study, among 214 patients with elevated liver enzymes, HBV-DNA in the presence of HBsAg (active HBV infection) was detected in 10 (4.7%). However, HBV-DNA in the absence of HBsAg (occult HBV infection) was detected in 119 (55.6%). These results indicate that occult HBV infection is predominant in Egypt. A recent study including 760 Egyptian healthy blood donors showed that the prevalence of anti-HBc was 10.96%, and HBV-DNA was detected in 9 cases among those anti-HBc-antibody-negative [28]. It was interesting that the ALT level in occult cases tended to be higher than that of HBsAg-positive carriers, suggesting that occult HBV infection is clinically significant.

Only a few reports have described HBV genotypes in Egypt. A recent study of pediatric cancer patients showed that genotypes D (37.1%) and B (25.7%) were predominant and genotypes A (10%) and C (8.6%) were the least frequent [31]. In Egypt, it was reported that genotype D was the most prevalent, because HBV was vectored by many tourists and visitors from Mediterranean countries [32]. Our result that genotype C was the most prevalent differed from the previous report, possibly due to the different geographic distribution.

HDV-RNA was not detected in either HBsAg- or HBV-DNA-positive patients. The steep decline in HBV infection among Egyptians will consequently be accompanied by a decrease in HDV infection. Thus, molecular and serological studies on HDV in Egypt are needed to assess the current prevalence and effect of HDV on HBV-infected persons in Egypt.

Egypt is experiencing the most rapid spread of HCV in the world, and our results also showed a high prevalence of HCV-Ab positivity (72.9%). This result was similar to that of a recent study on elevated liver enzyme patients in the Nile Delta area, in which the prevalence of HCV-Ab was 78.7% [17]. So far, the reported prevalence of HCV-Ab positivity has been diverse: one study reported an incidence of 5% in healthy blood donors from various Egyptian governorates [28], with another study reporting positivity in 24.8% of blood donors [34]. In addition, the prevalence depends on the geographic distribution and general population, being 10.3% among residents of a newly reclaimed area of Sinai that is near our study area [34]. HCV prevalence is very high in the general population of the rural Nile Delta, being 11.8% overall and reaching 40% in aged males [35].

Although the anti-*Schistosoma* campaigns were terminated in the early 1980s, the prevalence and incidence of HCV remains high in Egypt. In 1983, non-A and non-B hepatitis virus infections caused acute viral hepatitis in 38.7% of cases, compared with 31% in 2002 [36]. This could be a result of several factors. First, it seems that the current status of HCV in Egypt is not only a consequence of the mass anti-*Schistosoma* therapy, but also due to new infections acquired beyond that era [36]. Second, initial exposure to the virus occurs most often in children. Initial exposure usually causes few or no acute symptoms, and is difficult to confirm with serological tests [37]. Lastly, HCV-related liver cirrhosis and cancer develops slowly, so the incidence of these diseases in Egypt may not yet have peaked. Even under the most optimistic scenario of zero contemporary transmission, a standard dynamic model of prevalence reduction indicates that HCV infection in Egypt will remain above 5% for at least the next 50 years [38]. Parenteral anti-*Schistosoma* therapy probably led to a massive increase in the reservoir for HCV and HBV in the general population. Because of the high rate (85%) of chronicity in HCV infection, this reservoir is responsible for the marked incidence of HCV today [38].

In this study, samples with elevated liver enzymes were collected. The negative rate for all hepatitis viral markers including occult HBV infection was relatively high, at 8.9%. This is because of several reasons. First, it is possible that infections of other forms of viral hepatitis are related. Secondly, other causes including parasitosis, alcohol abuse, and steatohepatitis are possibly related. *Schistosoma* infection is still a major problem in Egypt.

In this study, there was no association between age and HCV infection. This may be because our study included patients with elevated liver enzymes. In addition, the

prevalence of HCV-Ab in males was higher than that in females, being consistent with other studies in general populations: males 12.9% versus females 10.8% [35]. It was likely that boys were more often treated for schistosomiasis than girls [35].

HCV genotype 4 is the most common variant of HCV in the Middle East and Africa, particularly Egypt. This region has the highest prevalence of HCV worldwide, with more than 90% of infections due to genotype 4 [39]. There are many African countries where genotype 4 is prevalent, including Gabon, Tanzania, Libya and the Democratic Republic of the Congo, where seroprevalence can reach 8% [40]. Parenteral treatment for schistosomiasis was considered the primary risk factor for genotype 4 HCV infection in Egypt. In this study, the direct sequencing and phylogenetic analysis of the HCV NS5B gene were successful in 19 out of 30 positive NS5B samples: 15 (78.9%) were subtype 4a, 2 (10.5%) were subtype 1g, and 1 (5.2%) was related to subtype 4o. These results showed that genotype 4 was predominant, being consistent with a previous study [41]. This previous study was performed near the south Sinai and Suez governorates, and showed that the Egyptian HCV epidemic is com-

posed of multiple lineages of genotypes 1 and 4, including subgenotypes 4a, 4o and 1g [41]. Another study on the genotype 4 isolate from Alexandria district showed that 78% of isolates were of the 4a variant, whereas the remaining identified variants were 4m (11%), 4o (5.5%), 4n (2.7%) and 4p (2.7%) [42]. Careful observation is needed because subgenotype 4o was associated with hepatocellular carcinoma [43].

In this study, anti-HEV IgM antibody for HEV was detected in 5 cases (2.3%), but HEV-RNA was not detected in acute infected cases. This result was consistent with a recent study, in which the rate of anti-HEV IgM was 2.1% in HAV-infected patients in rural villages in Egypt [17]. The detection of anti-HEV antibody in the absence of HEV-RNA may be attributed to 2 reasons. Firstly, the result may be a false-positive. It was reported that after a child showing symptoms of acute liver infection with jaundice and having anti-HAV IgM and anti-HEV IgM was followed up, this child had not seroconverted for anti-HAV IgG or anti-HEV IgG by 2 or 6 months [17]. Moreover, the sensitivity, specificity and accuracy of HEV IgM were 26.7, 85.7 and 71.9%, respectively [44]. Secondly, the avirulent HEV genotype, possibly genotype 3, is endemic

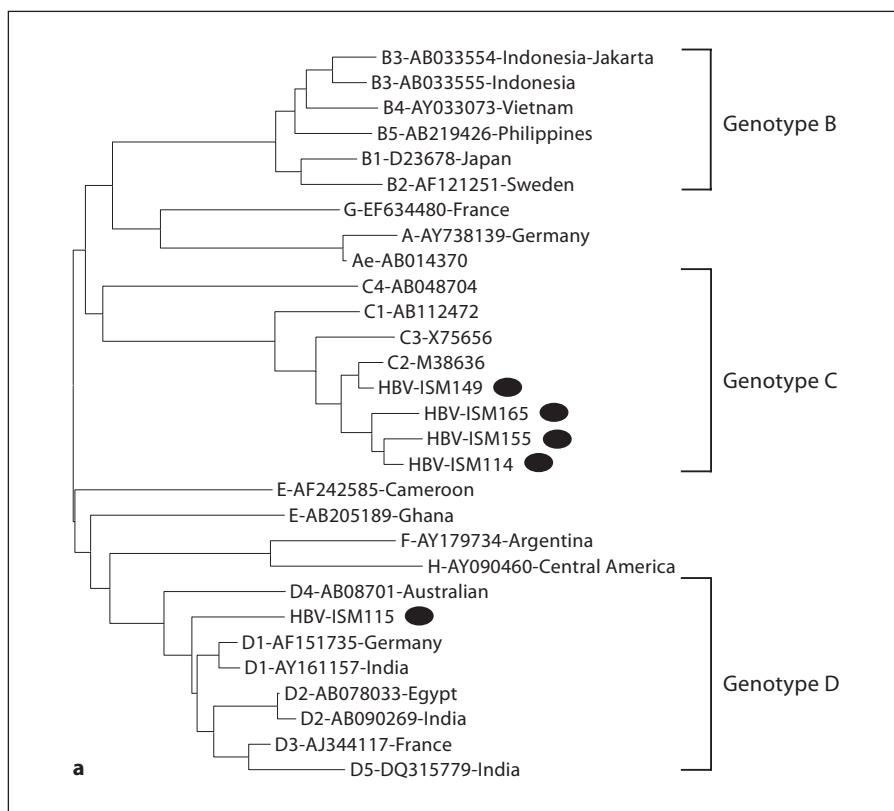
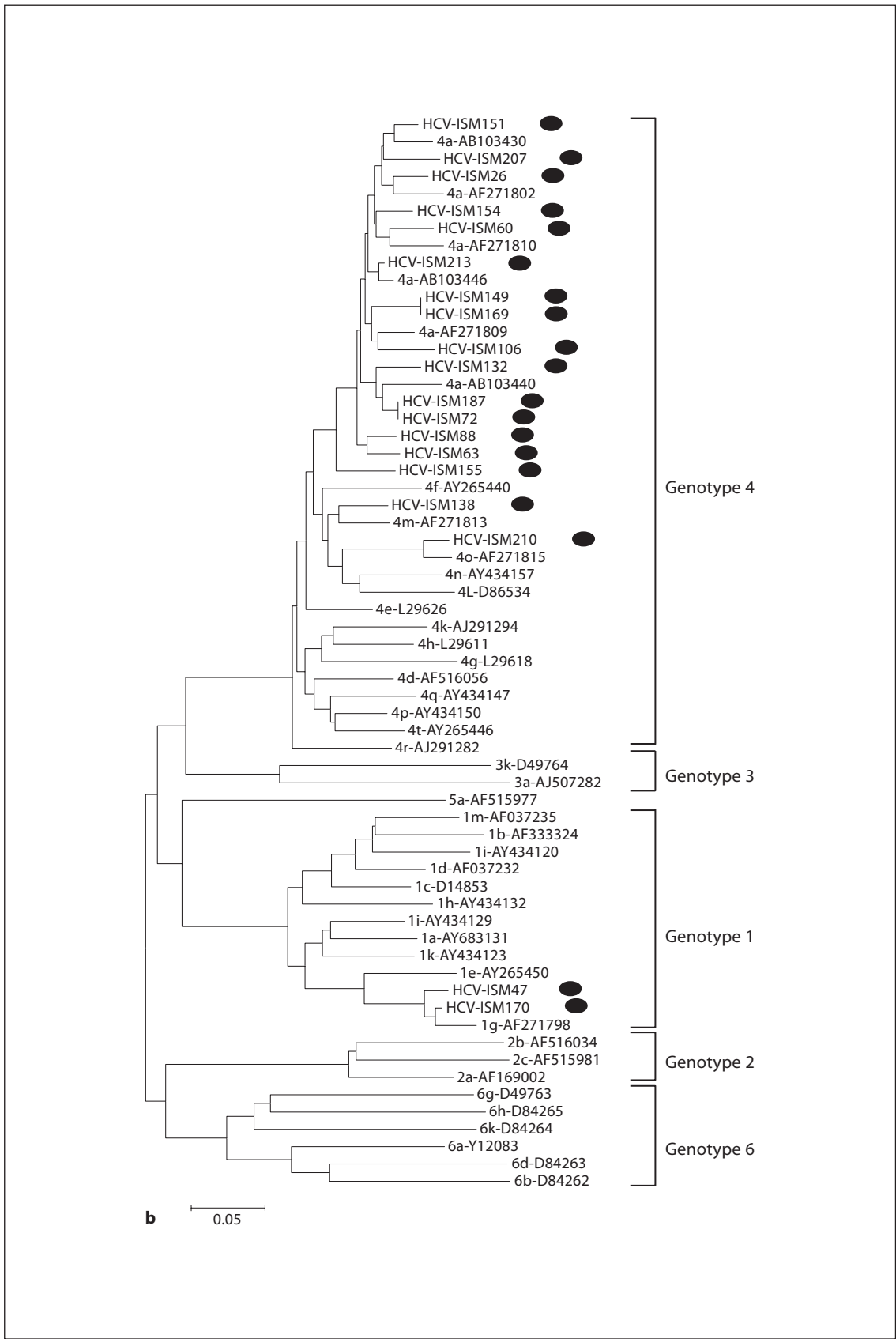


Fig. 5. Phylogenetic analysis of HBV and HCV. Phylogenetic analysis was carried out based on the pre-S to S region of HBV (a) and NS5B region of HCV (b). HBV genotype C and HCV genotype 4a were prevalent.



to Egypt, being spread both as a zoonosis between animals and humans, and as an anthroponosis between persons. Finally, an alternative explanation for the lack of morbidity among anti-HEV incident cases could be that initial asymptomatic infections occur during early childhood with subsequent antibody titer boosting without illness upon re-exposure to the virus [15, 16]. However, more virulent genotypes of HEV, mostly genotype 1, could be a cause of sporadic cases of HAV in Egypt [44–47].

Zoonotic transmission of HEV has been suggested. HEV was first isolated from swine in the United States [48], then from a rat in Nepal, and then a wild boar, deer and mongoose in Japan [49–51]. HEV was firstly documented to cause infection and viremia in horses in Egypt, and it may have a possible role as a reservoir or it could incidentally infect humans [52]. There has been considerable interest in swine as a zoonotic reservoir for human HEV [53]. However, swine could not be the reservoir for human HEV in our predominately Muslim communities since they are not present. Moreover, anti-HEV antibodies have been detected in swine, wild ro-

dents, dogs, cattle, sheep and poultry [54]. Other animal reservoirs of HEV infection in Egypt should be investigated.

Conclusion

Our results suggest that HBV and HCV are widespread in Ismailia province, Egypt, leading to a high incidence of liver diseases. Moreover, there exists a genotypic variability that might be characteristic of the Suez Canal area or even the northeastern part of Egypt, possibly differing from other areas of the country.

Acknowledgments

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