High prevalence of occult hepatitis B virus genotype H infection among children with clinical hepatitis in west Mexico

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Studies on the prevalence of infection with hepatitis B virus (HBV) among children are scarce in Latin American countries, especially in Mexico. This study was aimed to investigate the prevalence of HBV infection, occult hepatitis B infection (OBI) and HBV genotypes among children with clinical hepatitis. In total, 215 children with clinical hepatitis were evaluated for HBV infection. HBV serological markers and HBV DNA were analysed. OBI diagnosis and HBV genotyping was performed. HBV infection was found in 11.2% of children with clinical hepatitis. Among these HBV DNA positive-infected children, OBI was identified in 87.5% (n = 21/24) of the cases and 12.5% (n = 3/24) were positive for both HBV DNA and hepatitis B surface antigen. OBI was more frequent among children who had not been vaccinated against hepatitis B ($p < 0.05$) than in those who had been vaccinated. HBV genotype H was prevalent in 71% of the children followed by genotype G (8%) and genotype A (4%). In conclusion, OBI is common among Mexican children with clinical hepatitis and is associated with HBV genotype H. The results show the importance of the molecular diagnosis of HBV infection in Mexican paediatric patients with clinical hepatitis and emphasise the necessity of reinforcing hepatitis B vaccination in children.

Key words: occult hepatitis B infection - clinical hepatitis - HBV genotype H - children - vaccination

Viral hepatitis is a leading cause of chronic liver disease and a serious public health problem in children worldwide. Hepatitis B virus (HBV) is one of the most frequent aetiological agents of viral hepatitis in paediatric patients who are infected primarily through mother-to-infant transmission (Kelly 2006, Yeung & Roberts 2010, Pol et al. 2011). In children, the age at which primary HBV infection is acquired is relevant because it may lead to chronic liver inflammation in 90% of the cases when acquired at birth and in 50% of those infected during childhood. Indeed, this may be of great concern in regions with a high prevalence of HBV infection (Broderick & Jonas 2003, Yen-Hsuan 2011).

Overt HBV infection is diagnosed by the presence of hepatitis B surface antigen (HBsAg) and/or HBV DNA in serum (Lok & McMahon 2009). In contrast, occult hepatitis B infection (OBI) presents low titres of HBV genomes (≈200 IU/mL) in serum or in liver tissue in subjects who are negative for HBsAg, but either positive or negative for serological markers of past HBV infection (Raimondo et al. 2008b, Hollinger & Sood 2010). OBI has significant clinical implications for patients who receive transplants or organ donations, blood transfusion or haemodialysis because it increases their risk of becoming infected by HBV, which may potentially contribute to the development of chronic liver disease, cirrhosis, acute liver failure (ALF) and hepatocellular carcinoma (Chemin & Trépo 2005, Pollicino et al. 2007, Romero et al. 2011). In the paediatric setting, OBI has been reported among recipients of blood transfusions (Liu et al. 2006), hepatitis B-vaccinated children (Mu et al. 2009, Utsumi et al. 2010), patients with haematological diseases and paediatric cancer patients (Said et al. 2009) in countries with a high prevalence of HBV infection. In these groups, the prevalence of OBI ranges from 0.6-21%.

HBV infection has distinct clinical outcomes based on the heterogeneous geographical distribution of HBV genotypes associated with distinct host populations (Roman et al. 2014). In Mexico, the endemicity of HBV infection is associated with the predominance of HBV genotype H (Panduro et al. 2013, Roman & Panduro 2013), consisting of a low HBsAg marker prevalence (< 0.3%) and a contrasting high anti-HBV core antigen (HBc) prevalence (2.6-15.9%) (Roman et al. 2009), a high OBI prevalence (14.2%) (Roman et al. 2010) and clinically low viral loads (VL) (Panduro et al. 2013). In addition, HBV genotypes D and A are reported among acutely or chronically infected adult Mexican patients (Panduro et al. 2013) and HBV genotype G has been detected among particular populations, such as patients infected with the human immunodeficiency virus (Roman & Panduro 2013).

Moreover, despite the low endemicity rate for HBV infection, it has been estimated that at least 15 million people among the Mexican population may have been exposed to HBV during their lifetime (Panduro et al. 2013)

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and several high-prevalence regions for HBV infection have been identified throughout the country (Roman et al. 2010). This epidaemiological pattern is the common scenario for OBI infection, which may be underestimated due to the immunological response of Mexicans to HBV genotype H (Fierro et al. 2011) or to a lack of sensitivity of the HBsAg assay (Roman et al. 2009). Additionally, OBI infection ranges from 2.4% among blood donors (Garcia-Montalvo & Ventura-Zapata 2011) to 14.2% among native Mexican communities located in regions of high endemicity for HBV infection (Roman et al. 2010).

Previously, we reported the presence of the HBsAg marker in 3.1% of cases among 215 children with clinical hepatitis from western Mexico, along with a prevalence of 13.9% of cases among this group without a known aetiological agent (Escobedo-Melendez et al. 2012). However, to date, information regarding the prevalence of OBI and the main HBV genotypes among Mexican paediatric patients with clinical hepatitis is unknown. Therefore, this study was aimed to analyse the clinical and molecular characteristics of HBV infection in children, which may contrast to what has been reported in adults.

SUBJECTS, MATERIALS AND METHODS

Study population - In the present study, serum samples from 215 paediatric patients were tested by molecular analysis to confirm the diagnosis of clinical hepatitis due to HBV infection. We previously reported the serological profile of these patients, who were admitted to the Paediatric Infectious Disease Department of the Civil Hospital of Guadalajara during a five-year period (2005-2009) (Escobedo-Melendez et al. 2012). This tertiary-level hospital provides medical attention to people from the rural towns and urban cities of western Mexico who have a low income and very limited access to social security hospital insurance.

Clinical hepatitis was defined as hepatomegaly, fever (> 38ºC) and/or jaundice with elevated levels of serum aspartate aminotransferase (AST) (> 38 UI/L) and alanine aminotransferase (ALT) (> 35 UI/L) (Escobedo-Melendez et al. 2012). Based on their molecular and HBV serological profiles, the patients were diagnosed with HBV infection when they tested positive for HBsAg and/or HBV DNA. OBI-infected patients were confirmed as positive for HBV infection if testing negative for HBsAg and positive for HBV DNA. HBV DNA detection was based first on the use of a diagnostic set of primers and a subsequent confirmatory PCR that consisted of the use of four sets of primers [1st-round and nested polymerase chain reaction (PCR)] that annealed within four different regions of the viral genome (Raimondo et al. 2008a). OBI samples were considered positive for HBV DNA when positive for at least three PCR assays (1 diagnostic and 2 confirmatory PCR reactions). Patients with an OBI diagnosis were further classified into OBI-seronegative (HBV DNA+/anti-HBc-) or OBI-seropositive (HBV DNA+/anti-HBc+) groups, as previously described (Raimondo et al. 2008a, Hollinger & Sood 2010).

Clinical and demographic data - All patients were evaluated by a trained paediatrician using a structured questionnaire to investigate clinical history and demographical data (Escobedo-Melendez et al. 2012). This information included age, gender and clinical features attributable to hepatic inflammation such as jaundice, hepatomegaly, nausea, vomiting, fever, abdominal pain, choluria, acholia and ALF. The child's medical history was registered to establish the time of onset of these clinical symptoms in months. Hepatitis A and B vaccinations were verified by the child's vaccination card. Vaccination was defined as complete if he/she had a two-dose schedule at six and 12 months of age for hepatitis A and a three-dose schedule at two, four and six months of age for hepatitis B. Risk factors known to be associated with HBV infection were investigated in both the children and the children's parents during the medical visit, as previously described (Sanchez et al. 2007). Hepatitis B infection was also investigated in the children's parents.

Serum samples and laboratory tests - Blood samples were collected from the 215 children with clinical hepatitis and stored as serum at -80ºC until use. ALT, AST, direct bilirubin (DB) and albumin levels were measured in the serum using an enzymatic method (Human, Germany) with an automatic analyser. Elevated levels of serum ALT and AST (> 35 UI/L and > 38 UI/L, respectively) were considered abnormal. Samples from the HBV DNA+ children were screened to detect HBsAg, anti-HBc IgM and total anti-HBc. HBsAg was analysed using a third-generation microparticle immunoenzymatic assay [AxSYM HBsAg (V2), Abbott Laboratories, USA] with the AxSYM analyser. Total anti-HBc (IgM and IgG) and anti-HBc IgM were assessed with an immunoenzymatic assay (MONOLISA Anti-HBc PLUS and anti-HBc IgM PLUS, Bio-Rad Laboratories, USA) and a PR 3100 TSC analyser. As previously reported, all serum samples were tested for anti-hepatitis A virus (HAV) and anti-hepatitis C virus (HCV) antibodies to test for the presence of these viruses (Escobedo-Melendez et al. 2012).

OBI diagnosis and genotyping - Diagnostic PCR assay - Briefly, DNA was extracted from a 100-µL aliquot of serum using a phenol-chloroform protocol, as described previously (Sanchez et al. 2002). All samples were analysed at least twice and in duplicate. The detection of HBV DNA was performed by a standardised first-round and nested PCR of the S gene using bidirectional primers to amplify a 418-nt and 232-nt fragment, respectively, as previously described (Sanchez et al. 2002) (Table IA). Standard precautions to avoid cross-contamination were exercised throughout both the extraction and amplification procedures. A negative serum sample was included in each run to ensure specificity.

Confirmatory PCR assay - To confirm the positivity of the OBI samples, diagnostic PCR-positive samples were amplified by two subsequent rounds of PCR (1st-round and nested PCR) with four different sets of specific oligonucleotide primers (highly conserved nucleotide sequences) annealing at four different regions within the HBV genome. A positive sample for HBV DNA was confirmed if the corresponding fragment of at least two of the four primer sets was revealed, according to the Taormina meeting criteria (Raimondo et al. 2008a, Hollinger & Sood 2010) (Table IB).

TABLE I

Diagnostic, confirmatory and genotyping-polymerase chain reaction (PCR) assays for the molecular analysis of hepatitis B virus (HBV) genome

A: diagnostic assay

PCR primers	Primer sequence	Nucleotide position (HBV)	Fragment size (bp)	Tm (C)
S gene				
First round				
DS-7 (sense)	5'-TCCTGCTGGTGGCTCCAGTT-3'	55-74	418	60
DS-8 (antisense)	5'-CAAACGGGCAACATACCTTG-3'	474-455		
Nested				
MS-1 (sense)	5'-GGACCCCTGCTCGTGTTACA-3'	182-201	232	60
MS2 (antisense)	5'-CAGGATGAAGAGGAA(T/G)ATGA-3'	415-396		

final reaction volume: 25 µL. PCR buffer: 1X $MgCl_2$, 2 mM, dNTPs, 75 µM, *Taq* DNA polymerase 1 IU (Invitrogen™, Life Technologies™). Primers: 0.5 µM each, 5 µL of DNA sample. PCR cycle: 94ºC 3 min, 94ºC 30 s, 60ºC 30 s, 72ºC 30 s, 72ºC 5 min. Forty and 25 cycles, first and nested PCR, respectively. Analytical sensitivity: 1-10 copies HBV DNA (Sanchez et al. 2002). Tm: melting temperature.

B: confirmatory-PCR assay

PCR primers	Primer sequence	Nucleotide position (HBV)	Fragment size (bp)	Tm (C)
S gene				
First round				
S1-sense	5'-CATCAGGATTCCTAGGACCCCT-3'	168-189	290	55
S3-antisense	5'-AGGACAAACGGGCAACATAC-3'	478-458		
Nested				
S2-sense	5'-CTTGTTGACAAGAATCCTCACA-3'	214-235	228	55
S4-antisense	5'-CCAACAAGAAGATGAGGCATA-3'	442-420		
C gene				
First round				
C1-sense	5'-TCACCTCTGCCTAATCATC-3'	1825-1843	566	55
C3-antisense	5'-GAGGGAGTTCTTCTTCTAGG-3'	2391-2371		
Nested				
C2-sense	5'-TTCAAGCCTCCAAGCTGTGCC-3'	1862-1882	415	68
C4-antisense	5'-AGGAGTGCGAATCCACACTCC-3'	2277-2267		
P gene				
First round				
P1-sense	5'-CGTCGCAGAAGATCTCAATC-3'	2420-2439	425	60
P3-antisense	5'-TCTTGTTCCCAAGAATATGGT-3'	2845-2825		
Nested				
P2-sense	5´-CCTTGGACTCATAAGGT-3'	2463-2479	376	55
P4-antisense	5'-TCCCAAGAATATGGTGACCC-3'	2839-2820		
X gene				
First round				
X1-sense	5'-CGCCAACTTACAAGGCCTTTC-3'	1100-1120	528	60
X3-antisense	5'-GGCGTTCACGGTGGTCTCCAT-3'	1628-1608		
Nested				
X2-sense	5'-CCATACTGCGGAACTCCTAG-3'	1266-1685	274	55
X4-antisense	5'-CGTAAAGAGAGGTGCGCCCC-3'	1540-1521		

final reaction volume 50 µL. PCR buffer: 1X $MgCl_2$, 2 mM, dNTPs, 75 µM, *Taq* DNA polymerase 1 IU (Invitrogen™, Life Technologies™). Primers: 0.5 µM each, 5 µL of DNA sample. PCR cycle: 94ºC 3 min, 94ºC 30 s, 30 s, 72ºC 30 s, 72ºC 5 min. Forty and 25 cycles, first and nested PCR, respectively. The analytical sensitivity of OBI primer sets: 1-10 copies HBV-DNA. Tm: melting temperature.

C: HVB genotype H-specific PCR-assay

PCR primers	Primer sequence	Nucleotide position (HBV)	Fragment size (bp)	Tm (C)
S gene				
H1-sense	5'-CTACAGCATGGGAGCACCTCTCTC(A/C)ACGGC-3´	2844-2873	279	60
H2-antisense	5'-GTGGATC(G/T)GGTGGCGAGGTTGTCAGAATGC-3'	3123-3098		

final reaction volume 25 µL. PCR buffer: 1X $MgCl_2$, 1.5 mM, dNTPs, 200 µM, Hot Star *Taq* polymerase 2.5 IU (Qiagen). Primers: 0.2 µM each, 5 µL of DNA sample. PCR cycle: 95ºC 15 min, 94ºC 1 min, 60ºC 1 min, 72ºC 2 min. Single PCR, 35 cycles. Analytical sensitivity: 1-10 copies HBV-DNA. Tm: melting temperature.

HBV/H-specific genotyping PCR assay - We have observed that during the course of an acute HBV genotype H infection, VL are low, perhaps due to an immune response that rapidly decreases HBsAg in conjunction with a gradual decline in HBV DNA. This leads to a wide window of occult infection that makes detection of HBV DNA by standard procedures difficult. This, together with the fact that serum is not available in large amounts, did not enable the sequencing of these samples. Thus, genotyping of the HBV DNA+ samples was carried out by a PCR assay using genotype H-specific primers under previously validated conditions (Roman et al. 2010, Panduro et al. 2013) (Table IC).

For this assay, a full-length HBV DNA genotype H genome that had been previously sequenced was used as a positive control in each run to avoid false-positives. Samples that were positive for the diagnostic and confirmatory PCR were only then genotyped by this method. Non-typeable samples were designated as probable genotype H in cases that had a low VL, but insufficient serum to reproduce the genotyping.

Samples that were negative for genotype H were subjected to bidirectional direct sequencing with the corresponding PCR amplification primers using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and a GeneAmp PCRsystem 2700 thermal cycler (Applied Biosystems). The sequences were determined using an automated fluorescent DNA analyser (ABI 3130, Applied Biosystems); sequence alignment was performed by MEGA software v.4.0 (megasoftware.net) and confirmed by visual inspection. The genetic distances were estimated using the six-parameter method and a neighbour-joining phylogenetic tree was built to identify the HBV genotypes.

Statistical analyses - The data for continuous variables are reported as the mean, median and standard deviation (SD). The clinical data, demographics and risk factors are provided as simple frequencies and proportions. HBV infection, OBI cases and HBV genotypes were analysed by comparing the children's age groups. Statistical associations of a hepatitis B-positive status were conducted by Student's *t* test, the chi-square test or Fisher's exact test when appropriate. The odds ratio with 95% confidence interval was estimated for risk factors. A $p < 0.05$ was considered as significant.

Ethics - Informed consent was obtained from the children's parents at the time of clinical evaluation. The local Ethical Committee of the Civil Hospital of Guadalajara approved the study protocol. The protocol was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 1983.

TABLE II

Polymerase chain reaction (PCR) results for hepatitis B virus (HBV)-DNA positivity among the 215 children with clinical hepatitis

PCR assay	Samples (n)	PCR results			
Diagnostic-PCR	215	170 -ve		45 +ve	
Confirmatory OBI-PCR	45	21 -ve (samples)			
		24 +ve[a]			
		S	C	P	X
		21	10	5	6
H-specific-PCR	24	H	Non-H	H[b]	
		17	3	4	

a: three samples were hepatitis B surface antigen (HBsAg) positive/HBV-DNA +ve; *b*: there was not sufficient volume of serum to perform DNA extraction once more to confirm genotype H; OBI: occult hepatitis B infection; -ve: negative samples; +ve: positive samples.

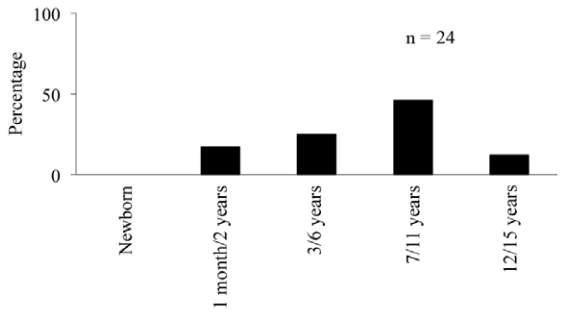
RESULTS

Prevalence of HBV infection in children with clinical hepatitis - Data regarding the results of the HBV DNA-PCR assays among the 215 children are depicted in Table II. From 215 children with clinical hepatitis included in this study, 45 cases (21%) were re-tested by the confirmatory PCR assay to confirm HBV infection. Among these cases, 24 cases were confirmed positive for HBV DNA, with 87.5% (21/24) being positive for HBV DNA and negative for HBsAg (OBI) and 12.5% (3/24) being positive for both HBV DNA and HBsAg (HBV DNA+/HBsAg+).

Table III shows the demographical and clinical data of the study adjusted by the HBV DNA+ (n = 24) and HBV DNA- children (n = 191). Among the HBV DNA+ patients, 13 were males and 11 were females (age range, 1-15 years; mean, 6.8; SD, 4.1 years). Additionally, values of the median ALT and AST were 629 UI/L and 461 UI/L, respectively, the median DB value was 3.2 mg/dL and the median albumin value was 4.4 mg/dL. Within the HBV DNA+ group, 70% (17/24) had less than six months of clinical symptoms. However, the frequency of HBV DNA+ children with more than six months of clinical symptoms was significantly higher than in the group of HBV-DNA- children (30% vs. 7%) ($p < 0.001$). Among the HBV DNA+ children, 54% were positive for anti-HAV IgM antibody, though none were positive for anti-HCV antibody.

Age group-based analysis of HBV infection - The prevalence of HBV DNA+ children (24 cases) adjusted by age showed an upward trend that was not significant ($p = 0.096$), beginning at 0% in newborns, 17% among the infancy age group (1 month-2 years), 25% in the preschool-age group (age 3-6 years) and 46% in school children (7-11 years) (Figure). However, a reduction in the prevalence of HBV infection (12%) in the adolescent group (12-15 years) was detected. The HBV DNA- children with clinical hepatitis who were registered also followed the same pattern with respect to age distribution. However, the prevalence of HBV DNA- children among the total cases of clinical hepatitis was lower in the infancy age group (7%) and the distribution by age group was not significant ($p = 0.565$) (data not shown).

High prevalence of OBI genotype H patients - Among the HBV DNA+ children, 87.5% (21/24) were diagnosed with OBI. Based on their serological anti-HBc marker profile, 90.5% of the children with OBI were negative for the anti-HBc marker (OBI-seroneg-



Hepatitis B infection by age group in the 24 hepatitis B virus (HBV) DNA-positive children with clinical hepatitis (2005-2009). The data are presented as percentage of each children's age group who were positive for HBV DNA.

ative) and only 9.5% were positive (OBI-seropositive). When the HBV DNA+ children were analysed according to hepatitis B vaccination status, all of the non-vaccinated children had OBI ($p < 0.05$) and 93% of them were OBI seronegative (Tables IV, V).

HBV genotype H was the most frequent genotype in the HBV-infected children, appearing in 71% of the cases, followed by HBV genotype G in 8% of the cases and HBV genotype A in 4% of the cases. In four cases, the HBV genotype was designated as probable genotype H, as explained in the Subjects, Materials and Methods section (Tables II, IV, V). There was no significant difference related to age, sex or hepatic injury among the different HBV genotypes (H, G or A). Seventy-three per cent of the HBV genotype H cases and both cases of HBV genotype G were not vaccinated against hepatitis B. In contrast, only the HBV genotype A case had been vaccinated. There was no significant difference between HBV genotypes among the hepatitis B-vaccinated and non-vaccinated groups ($p = 0.469$) (Table IV).

Risk factors in children with HBV infection - History of hospitalisation and surgery were the most common risk factors found in the HBV DNA+ children, at 46% and 21% of the cases, respectively. Other risk factors found in these children with HBV infection were tattoos (4%), blood transfusion (4%), sex with a sex worker (4%), promiscuity (4%), acupuncture (4%) and inoculation with contaminated needles (4%). No male child reported sexual relationships with men. Only one of the HBV genotype G-infected children had a history of blood transfusion (data not shown). Vaccination against

TABLE III

Demographical and clinical data in hepatitis B virus (HBV)-positive and HBV-negative Mexican children with clinical hepatitis from 2005-2009

Subject data	HBV-positive[a]	HBV-negative[b]	p[c]
n (%)	24 (11.2)	191 (88.8)	-
Age (year) [mean ± SD (range)]	6.8 ± 4.1 (1-15)	7.3 ± 3.7 (1-15)	0.584
Sex [female/male (n/%)]	11/13 (46/54)	95/96 (50/50)	0.718
Clinical features [n (%)]			
Time of onset of clinical symptoms: </> six months	17/6 (70/30)	169/13 (93/7)	0.0004[d]
Jaundice	21 (95)	174 (97)	0.560
Hepatomegaly	20 (91)	176 (98)	0.130
Vomiting	17 (74)	145 (81)	0.558
Nauseas	17 (77)	144 (80)	0.884
Fever	16 (73)	129 (72)	0.917
Abdominal pain	15 (68)	127 (71)	0.818
Choluria	13 (60)	110 (61)	0.855
Acholia	6 (27)	53 (29)	0.833
Acute liver failure	1 (4)	5 (3)	0.664
ALT (UI/L) [mean ± SD (range)] (median)	1,881 ± 5,290 (76-25,823) [629]	1,272 ± 2,426 (40-25,894) [782]	0.375
AST (UI/L) [mean ± SD (range)] (median)	670 ± 740 (64-2,650) [461]	835 ± 955 (42-5,011) [415]	0.453
Direct bilirubin (mg/dL) [mean ± SD (range)] (median)	3.5 ± 3.2 (0.31-12.3) [3.2]	3.8 ± 4.3 (0.31-39) [3.2]	0.626
Albumin (mg/dL) [mean ± SD (range)] (median)	4.1 ± 1.2 (1.5-6.1) [4.4]	4.5 ± 1.1 (1.8-7.6) [4.6]	0.222
Anti-HAV IgM positive [n (%)]	13/24 (54)	92/113 (81)	0.630
Anti-HCV positive [n (%)]	0 (0)	2 (1)	0.605

a: positive for HBV DNA in at least two different regions within the HBV genome; *b*: negative for HBV DNA; *c*: by *t* test, chi-square test or Fisher's exact test; *d*: $p < 0.05$; ALT: alanine aminotransferase; AST: aspartate aminotransferase; SD: standard deviation.

HBV (recombinant vaccine) was not found in 62% of the HBV DNA+ children at two, four and six months of age and none of these children had received hepatitis A vaccination. Additionally, none of the children received hepatitis B vaccination at birth. Regarding the hepatitis B vaccination status and risk factors, there were no significant differences between HBV DNA+ children vs. HBV DNA- children with clinical hepatitis. A history of hepatitis B infection among the parents of four HBV DNA+ children and in 12 parents among the HBV DNA- children was not significant (p = 0.087) (Table VI).

DISCUSSION

HBV infection is the leading cause of chronic liver disease and a serious public health problem in children throughout the world. Further information regarding the prevalence of HBV infection, circulating genotypes and a reliable OBI diagnosis are required to have a deeper understanding of the role of HBV infection in the development of liver diseases among paediatric patients.

The prevalence of HBV infection among 215 children with clinical hepatitis was 11.2% after testing for HBV DNA. These data are consistent with the fact that OBI is common among the adult Mexican population (Roman et al. 2010, Garcia-Montalvo & Ventura-Zapata 2011, Panduro et al. 2013). In this study, the data support the notion that HBV infection is underestimated when viral DNA is not evaluated. This is particularly relevant because Mexico is classified as a low-prevalence region for HBV infection, but only by serological testing (HBsAg) (Panduro et al. 2011). Within the HBV DNA+ group, 70% (17/24) had less than six months of symptoms based only on clinical criteria. However, the serological, biochemical and molecular findings suggest that these patients were diagnosed at a late phase of an acute HBV infection, concordant with the reduced levels of serum HBV DNA and undetectable HBsAg, resulting in occult B infection. Therefore, it may be convenient to practice a follow-up clinical evaluation in such patients because most chronic infections in adults are acquired during childhood (Broderick & Jonas 2003, Yen-Hsuan 2011).

Most of the HBV-infected children were OBI seronegative (negative for anti-HBc). This finding contrasts with studies that report a high prevalence of OBI seropositivity (positive for anti-HBc) among adults, which may indicate past HBV infection (Raimondo et al. 2008a). However, several studies in children have reported OBI-seronegative cases more often than OBI-seropositive cases (Liu et al. 2006, Mu et al. 2009, Said et al. 2009, Utsumi et al. 2010). This may be explained by potential differences in the immune response of HBV-infected children and HBV-infected adults; thus, serological markers that indicate viral elimination may be undetectable (Rehermann 2003, Fierro et al. 2011).

With regard to HBsAg test sensitivity, the samples were tested with the AxSYM MEIA system because it was the most available kit at the time of the study. Regarding the plausibility that HBsAg mutations in the "a" determinant region may have given false-negatives using these kits, we have reported that mutations in the S and X regions are rare in OBI genotype H infection (Panduro et al. 2013). If S-mutations had been present, they would yield escape mutants with non-detectable HBsAg and higher VL, which are considered "false OBI" (Raimondo et al. 2008a). Nonetheless, we observed a high diversity of the S region, which may be related to the non-detection of HBsAg with the use of commercial kits that have been validated using non-H genotype samples.

Although we cannot discard the presence of specific mutations in some cases, it appears that the main cause of OBI among the Mexican population is due to an effective T cell response with a rapid decline of serum HBsAg,

TABLE IV

Hepatitis B virus (HBV) infection, occult hepatitis B infection (OBI) and HBV genotypes in Mexican children with clinical hepatitis from 2005-2009 according to vaccination status

	Total (n = 24)	Hepatitis B vaccinated[a] (n = 9)	Hepatitis B non-vaccinated (n = 15)	
HBV infection	n (%)	n (%)	n (%)	p[b]
HBV DNA+/HBsAg+	3 (12.5)	3 (33)	0 (0)	0.0415[c]
OBI (HBV DNA+/HBsAg-)	21 (87.5)	6 (67)	15 (100)	-
OBI-seropositive (anti-HBc+)	2/21 (9.5)	1/6 (17)	1/15 (7)	0.500
OBI-seronegative (anti-HBc-)	19/21 (90.5)	5/6 (83)	14/15 (93)	-
HBV-genotypes				
Genotype H	17 (71)	6 (67)	11 (73)	0.469
Genotype G	2 (8)	0 (0)	2 (13)	-
Genotype A	1 (4)	1 (11)	0 (0)	-
Probable genotype H[d]	4 (17)	2 (22)	2 (13)	-

a: hepatitis B vaccine were given at two, four and six months of age; *b*: by chi-square test or Fisher's exact test between hepatitis B-vaccinated and non-vaccinated subgroups of HBV DNA+/HBsAg+ and OBI (seropositive and seronegative) categories and HBV genotypes; *c*: $p < 0.05$; *d*: probable genotype H as defined in Subjects, Materials and Methods.

resulting in HBsAg negativity and a low VL (Fierro et al. 2011). On-going studies in our laboratory have repeatedly encountered low prevalence or null detection of HBsAg and positivity for HBV-DNA, even with commercial kits with higher sensitivity. In addition, with regard to the lack of sequence verification to preclude environmental contamination, we exerted additional precautions using a PCR confirmatory test, as recommended, before PCR genotyping (Raimondo et al. 2008a).

In accordance with the fact that the major circulating HBV genotype is H among distinct Mexican populations (Panduro et al. 2013, Roman & Panduro 2013), in the present study, HBV genotype H was predominant in both OBI and non-OBI infection, followed by HBV genotypes G and A among the OBI patients, but these differences were not significant. However, the trend of low VL among HBV genotype H-infected patients in Mexico is most likely to be associated with the finding of OBI, which requires further investigation. Interestingly, the children infected with HBV genotype G had a history of blood transfusions, but lacked a paternal history of sexual relationships with men. HBV infection with genotype G has been reported not only in patients who engage in homosexual relationships with men (Chudy et al. 2006, Sanchez et al. 2007, Dao et al. 2011), but also among blood donors and haemodialysis patients (Mora et al. 2011, Sayan & Dogan 2012). Because HBV genotype G infection may occur as OBI, the use of serological probes may be unsuitable for diagnosis (Zaaijer et al. 2011). Moreover, given that this genotype can cause liver fibrosis in immunocompromised individuals (Chudy et al. 2006) and in chimeric mice carrying human hepatocytes (Sugiyama et al. 2007), nucleic acid testing for HBV genotype G and clinical follow-up of infected patients may be required.

In 2000, the National Ministry of Health declared mandatory HBV vaccination for all children under five. A three-dose schedule at two, four and six months of age with the DPTw-HB/Hib pentavalent vaccine (diphtheria, tetanus and pertussis, whole-cell-hepatitis B/haemophilus influenza type-b and recombinant HBsAg) was indicated, but was not effectively given until mid-2001. In 2007, the recombinant antihepatitis B vaccine was indicated at birth and at two and six months of age,

TABLE V

Clinical and molecular description by case of the hepatitis B virus (HBV)-infected Mexican children with clinical hepatitis from 2005-2009

Case	Age	Sex	ALT (UI/L)	DB (mg/dL)	ALF	Hepatitis B vaccine[a] [doses (n)]	Anti-HAV IgM	HBsAg	Anti-HBc total	Anti-HBc IgM	HBV genotype
1	10	M	328	1.6	No	3	-	+	-	-	H
2	5	F	224	0.4	No	3	+	+	-	-	NG
3	6	M	689	4.3	No	3	-	+	-	-	NG
4	2	M	88	0.3	No	3	-	-	+	-	H
5	4	F	629	1.4	No	3	+	-	-	+	H
6	2	F	205	3.9	No	3	+	-	-	-	H
7	3	F	1,982	3.2	No	3	+	-	-	-	H
8	4	M	680	4.1	No	3	+	-	-	-	H
9	6	F	1,041	3.2	No	0	-	-	-	-	H
10	6	M	210	3.5	No	0	-	-	-	-	H
11	7	F	334	2.9	No	0	+	-	-	-	H
12	7	M	2,877	12.3	No	0	-	-	-	-	H
13	7	M	3,389	4.4	No	0	+	-	-	-	H
14	8	M	1,110	1.7	No	0	+	-	-	-	H
15	11	M	171	1.3	No	0	+	-	-	-	H
16	11	F	74	1.8	No	0	+	-	-	-	H
17	14	F	76	5.2	No	0	-	-	-	-	H
18	14	M	220	0.8	No	0	+	-	-	-	H
19	15	F	664	1.1	No	0	+	-	-	-	H
20	5	M	620	2.9	No	0	+	-	-	-	NG
21	5	M	107	0.7	No	0	-	-	-	-	NG
22	1	F	25,823	11.0	Yes	0	-	-	-	-	G
23	9	M	805	0.4	No	0	-	-	-	-	G
24	2	F	913	8.1	No	3	-	-	-	-	A

a: a child with a complete three-dose schedule at two, four and six months of age; ALF: acute liver failure; ALT: alanine aminotransferase; DB: direct bilirubin; F: female; HAV: hepatitis A virus; HBc: hepatitis B core antibody; HBsAg: hepatitis B surface antigen; M: male; NG: non-typeable genotype as defined in Table II.

TABLE VI

Frequency of risk factors related to hepatitis B virus (HBV) infection in HBV-infected Mexican children with hepatitis from 2005-2009

	HBV-positive[a] (n = 24)	HBV-negative[b] (n = 191)	HBV-positive vs. HBV-negative		
Risk factors	n (%)	n (%)	OR	95% CI	p[c]
Hospitalisations	11 (46)	83 (43)	1.10	0.47-2.58	0.825
Surgery	5 (21)	25 (13)	1.74	0.60-5.10	0.302
Tattoos	1 (4)	3 (1)	2.72	0.27-27.29	0.379
Blood transfusion	1 (4)	6 (3)	1.34	0.15-11.63	0.569
Sex with a sex worker	1 (4)	2 (2)	4.11	0.36-47.10	0.300
Promiscuity	1 (4)	3 (2)	2.74	0.27-27.29	0.379
Acupuncture	1 (4)	1 (0.5)	8.26	0.50-136.59	0.211
Injection with contaminated needles	1 (4)	1 (0.5)	8.26	0.50-136.59	0.211
Hepatitis B non-vaccinated	15 (62)	123 (64)	0.92	0.38-2.22	0.855
History of HBV infection in children's parents	4 (17)	12 (6)	2.98	0.88-10.13	0.087

a: positive for HBV DNA by two sets of primers within the HBV genome; *b*: negative for HBV DNA by two sets of primers within the HBV genome; *c*: by chi-square test or Fisher's exact test; CI: confidence interval; OR: odds ratio.

even though it only became effective in mid-2008. Notably, among the 24 cases of HBV DNA+ children, 62% of them were not vaccinated against hepatitis B. In all of these cases, the children had OBI and 93% of them were OBI seronegative. A possible reason why these children missed their vaccination schedule may be explained by the age range of the 15 non-vaccinated children (1-15 years) at the time of the study. Those nine or older were born before the establishment of the mandatory universal vaccination programme that included the DPTw-HB/Hib vaccine. Those of nine years or younger (n = 9) may have not received the latest monovalent vaccine at birth because this 2007 vaccination scheme did not become effective until mid-2008-2009. Another possibility may be a low level of education among the children's parents, combined with several of the HBV-related risk factors that hinder a successful follow-up of vaccination care. These observations suggest that without a widespread hepatitis B vaccination programme, a large number of hepatitis B cases will occur, including OBI cases.

In contrast, the nine HBV DNA+ children, three of which were HBsAg-positive, reported a complete schedule of vaccination. These children received the recombinant hepatitis B vaccine (included in the DPTw-HB/Hib vaccine) at two, four and six months of age. Although the immunogenicity and efficacy of this hepatitis B vaccine was reported after three doses (Santos et al. 2002), this schedule might not protect against HBV infection acquired at birth.

The lack of hepatitis B vaccination among HBV DNA+ infected children and their parents indicates the necessity of focusing on thorough hepatitis B vaccination campaigns among high-risk children as well as in the general population. Vigilance strategies are required to ensure that hepatitis B vaccination occurs at birth (day 0) in Mexican children to avoid the dissemination of HBV infection and its clinical complications (CID 2012). Regarding the HAV, Mexico is a region of high endemicity for this infection because 80% of children below the age of 10 and 90% of adults in general are positive for anti-HAV IgG antibodies (Panduro et al. 2011). Thus, it is not surprising that in this study, 54% of the HBV DNA+ children, with most being OBI, had anti-HAV IgM antibodies. This fact also coincides with the finding that none of them had been vaccinated. Therefore, HAV-HBV co-infections among OBI cases may be more common than expected; moreover, they are not easily discriminated from each other by clinical evaluation only. The high prevalence of OBI found in this study suggests that nucleic acid testing for HBV may be a more objective diagnostic tool due to the misconception among most physicians that HAV is the only virus responsible for hepatitis in children. Both HAV and HAV-HBV co-infections can lead to ALF with high mortality (Squires et al. 2006); hence, vaccination against hepatitis A in children should be reinforced (AAP/CID 2007).

With regard to HBV-related risk factors, history of HBV infection in the children's parents was found to be more frequent among the HBV DNA+ children than the HBV DNA- children (17% vs. 6%). This finding is relevant because the main routes of transmission in children are either vertical or horizontal in their first years of life. However, in this study, vertical transmission was not confirmed due to the lack of serum samples from the children's parents. Therefore, case-control studies related to risk factors for HBV infection and vertical transmission are required to assess their impact on the transmission of HBV in paediatric clinical settings.

In conclusion, OBI is common in Mexican children with clinical hepatitis. Nucleic acid testing for HBV DNA should be considered for screening children with hepatic inflammation because this infection may be sub-

stantial in paediatric patients and the aetiological diagnosis has relevant clinical implications. HBV genotype H predominates in Mexico and is associated with OBI in children. The high prevalence of OBI among hepatitis B-non-vaccinated children observed in this study emphasises the importance of HBV vaccination in young children in Mexico.

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