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Vertically acquired occult hepatitis B virus infection may become overt after several years

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SUMMARY

Objectives: To study the frequency of vertically acquired occult hepatitis B virus (HBV) infection (OBI).
Methods: We investigated 44 children born to hepatitis B surface antigen (HBsAg) positive mothers. They received HBV vaccine directly after birth and at 2, 6 and 52 weeks of age; eight with HBeAg-positive mothers also received hepatitis B immunoglobulin (HBIG). HBV DNA was analyzed in blood collected at 6 weeks and 12 months of age, and HBV antibodies at 12 and 18 months of age.
Results: HBV DNA, but not HBsAg or anti-HBc, was detected at 12 months of age in three children. The viral sequences were almost identical with HBV DNA from their mothers who all were HBeAg-positive and had received tenofovir during pregnancy. Follow-up at 5–7 years age showed that one of the three children had become seropositive for HBsAg and anti-HBc. This child and one of the other two had detectable HBV DNA at the follow-up, with whole genome sequences identical to those in HBV from their mothers.
Conclusions: Mothers-to-child transmission of HBV can, despite adequate prophylaxis, lead to OBI which may later develop into overt HBV infection. Whether such infections are of clinical importance needs to be further investigated.

Introduction

Chronic hepatitis B virus (HBV) infection is a main cause of liver cirrhosis and hepatocellular carcinoma (HCC) world wide.[1] During the infection, viral particles containing HBV DNA are secreted into the blood. In addition, infected hepatocytes produce large numbers of subviral particles, covered with hepatitis B surface antigen (HBsAg), and therefore HBsAg can be detected in serum in essentially all forms of HBV infection. HBV DNA can however sometimes be detected in liver tissue, serum or peripheral blood mononuclear cells (PBMC) also when HBsAg is not detected in serum, a phenomenon called occult hepatitis B (OBI).[2] OBI is relatively common after resolution of acute or chronic hepatitis B, and in such seropositive OBI, antibodies to HBV (anti-HBc and/or anti-HBs) can be detected in serum.[3,4]

A different form, primary OBI, was observed in woodchucks that were injected with low amounts of woodchuck hepatitis virus (WHV). In these animals, surface antigen was not produced and not core or surface antibodies either, but WHV DNA was detected in PBMC.[5] Primary OBI might develop also in humans, but this is difficult to identify or study because human exposure to small amounts of HBV is rarely recognized. However, children born to HBsAg positive mothers are probably often exposed to low amounts of HBV, either because the HBV DNA level of the mother is low or because the majority of the viral particles are blocked by hepatitis B immunoglobulin (HBIG) given directly after birth. Administration of a vaccine composed of HBsAg directly after birth and in three additional doses also reduces the risk of vertical transmission.[6,7] To further reduce this risk, hepatitis B e antigen (HBeAg) positive pregnant women with high HBV DNA levels in serum may receive antiviral treatment in the third trimester of pregnancy.[8–11]

Although these preventive efforts in general are very effective in terms of preventing overt infection in the child, it is possible that primary OBI can develop in some children. Reports during recent years suggest that such OBI after mother to child transmission might be rather common. The frequency rates however differ greatly between the studies,[12–18] and the data are uncertain because most of the studies have not applied confirmatory HBV sequencing of samples from both mother and child.

In this prospective, observational study we investigated if children born to HBsAg positive women may develop primary occult

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hepatitis B, using highly sensitive PCR assays and confirmatory sequencing analyses.

Subjects and methods

Study population

All pregnant women in Sweden are offered HBsAg screening at the antenatal clinic, and those testing positive are referred to infectious diseases specialists for further evaluation and preventive recommendation. During the period September 2009–February 2012, 103 HBsAg-positive pregnant women that had been referred to the Infectious Diseases Clinic at the Sahlgrenska University Hospital, Gothenburg, Sweden, were invited to participate in the study. Exclusion criteria were co-infection with hepatitis C virus, hepatitis delta virus, HIV virus, or inability to understand written study information in Swedish. In total, 47 pregnant women were enrolled in the study (two of them during two pregnancies). One of them withdrew the consent and four were lost to follow-up, resulting in 42 mothers and 44 children being included in the study. The study was approved by the regional ethical review board in Gothenburg. All mothers gave written informed consent to be sampled, and both parents gave written informed consent for the unborn child's participation.

Study design

Immunoprophylaxis against hepatitis B was given to babies with HBV infected mothers according to the general Swedish recommendations. Within 12 h of delivery, all infants were given a dose of hepatitis B vaccine (ENGERIX-B, 10 µg recombinant HBsAg protein; Glaxo SmithKline). In addition, children born to women that were HBeAg positive were given one dose of HBIG at birth (UMANBIG, 180 IU; Scandinavian Biopharma). HBIG was not given to children born to HBeAg negative women, unless otherwise decided. Additional vaccine doses were given at 2, 6 and 52 weeks of age. Women with a high viral load, defined as HBV DNA > 7.5 log IU/mL, were offered antiviral treatment with nucleotide analogues (NA) from gestation week 32 until delivery.

At birth, cord blood was collected for HBsAg testing. At 6 weeks and 12 months of age blood was collected in a Ficoll-Paque test tube (Amersham Pharmacia Biotech AB). The tube was immediately centrifuged and peripheral blood mononuclear cells (PBMC) and plasma were separately collected and stored in −70°C for future analysis. All samples collected from the children (umbilical cord blood, PBMC and plasma collected at 6 weeks and at 12 months of age, and serum collected at 12 months of age) were analyzed for HBV DNA by PCR assays targeting the X and S regions (see below). All children were examined for HBsAg positivity at 6 weeks of age and hepatitis B serology (HBsAg, anti-HBs and anti-HBc IgG) was assessed at the age of 18 months.

HBV serology and HBV DNA amplification

HBsAg, HBeAg, anti-HBc and anti-HBs were analyzed by chemiluminiscent microparticle immunoassay with the ARCHITECT system (Abbot Diagnostics, Chicago, Ill.). Quantification of HBV DNA in serum from the mothers was performed with the Cobas AmpliPrep/Taqman HBV Test, v2.0 (Roche Diagnostics, Mannheim, Germany). Presence of HBV DNA in the children was investigated in serum/plasma and PBMC samples obtained at 6 and 52 weeks of age. The DNA in these samples was purified either by phenol-chloroform extraction or using QIAamp UltraSens Virus Kit (Qiagen, Hilden, Germany). Nested PCR was then performed using primers targeting conserved segments of the X and S regions of the HBV genome (Table 1).

Table 1
Primers for amplification and sequencing of HBV.

S/pol region	
First PCR	
55F	CCTCCTGCTGGTGGCTCCAGTTC
1192R	CGTCAGCAAACACTTG
Second PCR	
252F	CTCGTGGTGGACTTCTCTC
1019R	GCAAAGCCCAAAAGACCC
X region	
First PCR	
1266F	CCATACTGCGGAACTCCTAGC
1802R	ACAGACCAATTTATGCCTACAGCC
Second PCR	
1310F	CTGGAGCAAACATTATCGGG
1770R	CAAAGACCTTTAACCTGATCTCC
Cycle sequencing	
1383F	GGCTGTACTGCCAACTGGAT
1750R	TCCTCCCCCAACTCCTCC

Genotyping of HBV from mothers

The HBV genotype in serum samples from antenatal screening, or in some cases from an earlier time point, was determined by a real-time PCR based assay.[19]

Sequencing and phylogenetic analysis

Direct sequencing was performed on samples from children in which HBV DNA was detected, and also on a sample from the mother of that child in order to verify transmission by identification of the same HBV strain in the mother and the child. For this purpose, primers targeting the S and X regions were used (Table 1).

A different sequencing technique was applied on follow-up samples taken at 5–7 years of age. Ten partially overlapping amplifications (covering the whole HBV genome) were performed by primers that target conserved regions (Supplementary Table 1), followed by Ion Torrent sequencing in an Ion S5 instrument according to the instructions by the manufacturer (Thermo Fisher Scientific, Waltham, MA), and analysis of the data was performed with the CLC Genomic Workbench software (Qiagen). Maximum Likelihood phylogenetic analysis based on the Tamura–Nei model was performed using the MEGA7 software.[20]

Statistical analysis

Anti-HBs levels were compared by Mann-Whitney U test, the increase of anti-HBs by paired Wilcoxon's test, and group distribution by Fisher's Exact test, using SPSS, version 21 (IBM).

Results

Baseline characteristics of the mothers and children

Table 2 shows the baseline characteristics of the study participants. Forty mothers gave birth to one child and two mothers to two children each, in separate pregnancies. Thirty-six of the mothers (82%) were HBeAg negative. All children completed the scheduled vaccination series. HBIG was administered to the eight children born to HBeAg-positive women and to one child born to an HBeAg-negative woman with a high viral load.

The HBV DNA levels in the mothers are presented in Fig. 1. Out of the eight HBeAg-positive women, six met the criteria for tenofovir preventive treatment (HBV DNA > 7.5 $\log_{10}$ IU/mL) from gestation week 32 to reduce the viral load. Five women accepted

Table 2
Baseline characteristics of 44 children and 42 HBsAg-positive mothers.

	HBeAg-positive $n=8$	HBeAg-negative $n=36$
Mothers		
Age (years)[a]	28.4 ± 4.7	33.2 ± 5.3
ALT (x ULN)[a]	0.45 ± 0.23	0.43 ± 0.22
Antenatal HBV DNA (log_{10} IU/mL)[b]	8.29 (7.25–8.99)	2.43 (1.61–3.25)
HBV genotype[c]	3B, 3C, 1I	9A, 4B, 5C, 11D, 1F
Children		
Gestational age, weeks[a]	38.2 ± 1.2	39.9 ± 1.4
Cesarean section	12.5%	19.4%
Height, cm[a]	49.8 ± 1.5	50.5 ± 2.4
Weight, kg[a]	3.5 ± 0.3	3.5 ± 0.4
Apgar score (1 min)[a]	9.1 ± 0.6	9.2 ± 0.5

ALT, alanine amino transferase, ULN, upper limit of normal.
Two mothers (one with genotype C, the other with genotype D) gave birth to two children each.
[a] Mean ± standard deviation.
[b] Median (interquartile range).
[c] Genotype could not be determined in 5 HBeAg-negative cases.

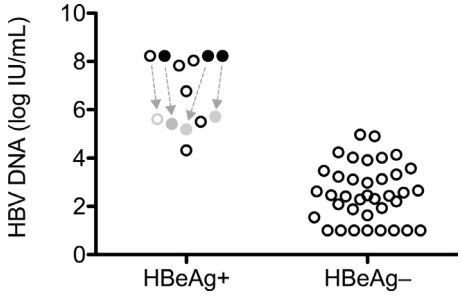

Fig. 1. HBV DNA levels in 8 HBeAg-positive and 36 HBeAg-negative mothers in samples taken at the antenatal clinic at week 21 (median; IQR 17-25) of pregnancy. The levels in the mothers to three children that developed OBI are indicated as filled circles. The gray open/filled circles show levels that were analyzed at the time of delivery in four of the five mothers that received tenofovir during the third trimester.

treatment and in that group there was at least a 2.5 log_{10} reduction in HBV DNA between inclusion in the study and birth of their infants. The five women discontinued treatment directly after delivery.

HBV markers in the children

No child was HBsAg-positive at 6 weeks or 12 months of age, or had developed anti-HBc IgG at the age of 12 or 18 months. Six children were HBsAg positive in cord blood at birth. All children were assessed for HBV DNA in both plasma and PBMC at 6 weeks and 12 months of age; one was positive in plasma at 6 weeks of age, and two in plasma or PBMC at 12 months of age. In all these three cases, HBV DNA was found to have the same sequence in the X region of the HBV genome as HBV DNA in plasma samples from the children's mothers taken at the time of delivery or on a previous occasion (Fig. 2). From two of the children, HBV DNA was also tested in extra serum samples that had been taken at 12 months of age for routine diagnostics (HBsAg and anti-HBs). HBV DNA was detected in both these cases, and direct sequencing showed the same X region sequence as in a plasma sample obtained at 6 weeks of age (OBIN-25) or in a PBMC sample obtained at 12 months of age (OBIN-86). In addition to the amplification of the X region, nested PCR was also performed with primers targeting the S region, but was negative for all three children.

As shown in Table 3, the mothers to these three children were all HBeAg positive, and had very high HBV DNA levels (>8 log_{10} IU/mL) in samples taken during the antenatal evaluation. All three mothers had been given antiviral treatment (tenofovir) from gestational week 32 and the HBV DNA levels at the time of delivery had declined to below 6.0 log_{10} IU/mL. The association between OBI and the HBeAg status (38% (3/8) vs. 0% (0/36)) or the antenatal HBV DNA level of the mother, was statistically significant ($p=0.0042$ and $p=0.006$, respectively).

Among the eight children born to HBeAg-positive mothers, the antenatal HBV DNA levels tended to be higher in the mothers to

Table 3
Characteristics of three children with occult hepatitis B virus infection and their mothers.

	OBIN-86	OBIN-49	OBIN-25
Mothers			
Age (years)	34.4	27.5	30.4
Previous pregnancies (deliveries)	2	2	0
ALT/ULN	0.36	0.48	0.52
Antenatal HBV DNA (log_{10} IU/mL)	8.62	8.40	8.32
HBV DNA at delivery (log_{10} IU/mL)	5.53	5.19[a]	5.53
HBV genotype	C2	B4	C2
Origin	Korea	Vietnam	Korea
Children			
Gestational age, weeks	36.3	39.3	39.3
Apgar score (1 min)	9	10	10
HBV DNA positive sample	Umbilical cord PBMC 12 months Serum 12 months Serum 5 years	Plasma 12 months Serum 7 years	Plasma 6 weeks Serum 12 months
HBV subgenotype	C2	B4	C2
Anti-HBc at 18 months age	Negative	Negative	Negative[b]
Anti-HBs at 18 months age (IU/L)	16	44	56[b]
Anti-HBs at 5–7 years age (IU/L)	1.7	4.9	27
Anti-HBc at 5–7 years age	Negative	Positive	Negative
HBsAg at 5–7 years age	Negative	Positive[c]	Negative
HBV DNA at 5–7 years age	Positive[d]	Positive[e]	Negative

ALT, alanine amino transferase, ULN, upper limit of normal.
[a] Taken 4 weeks prior to delivery (sample from date of delivery was missing).
[b] Taken at 12 months of age (sample from 18 months of age was missing).
[c] 0.82 IU/mL.
[d] Positive by PCR combined with Ion Torrent sequencing.
[e] 1.81 log IU/mL.

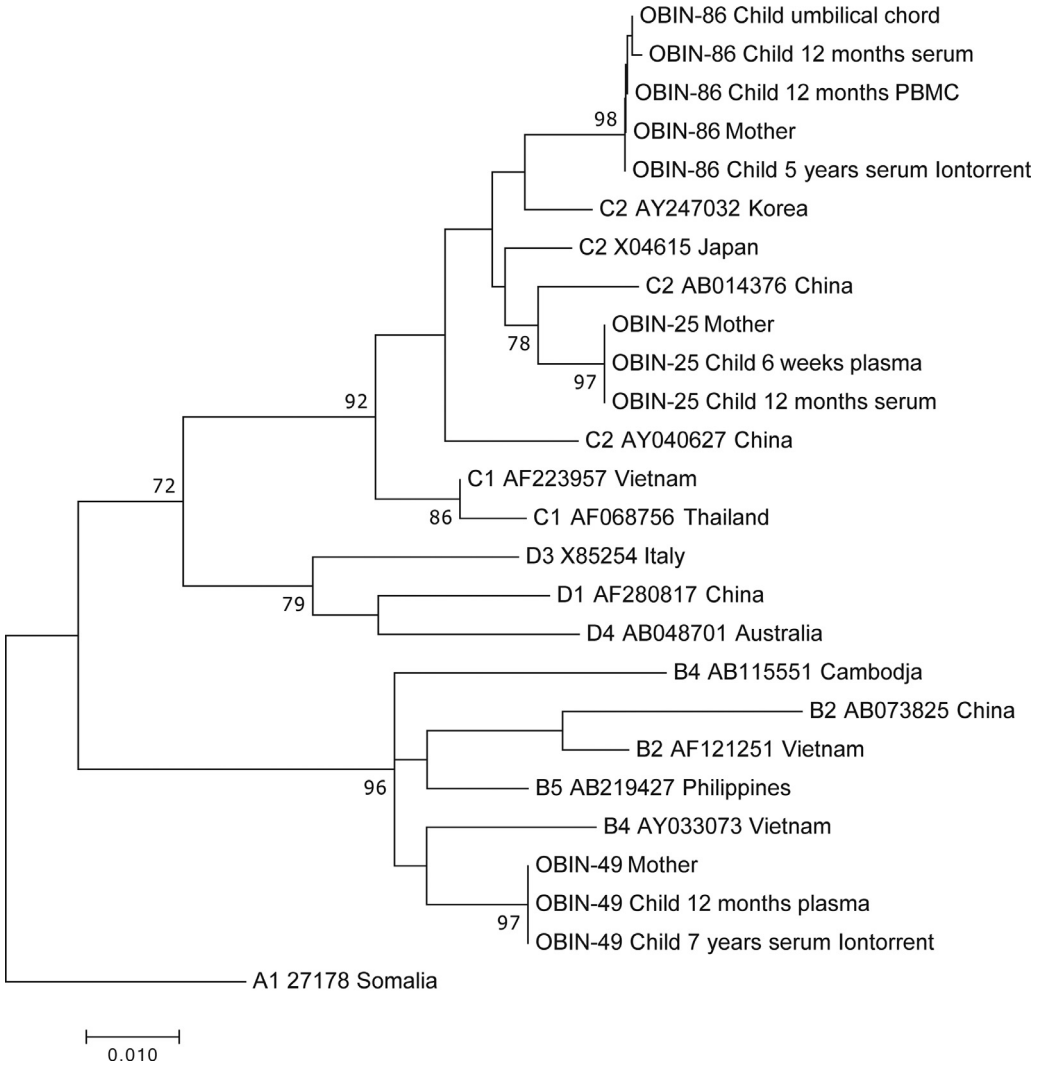

Fig. 2. Maximum likelihood tree of a 273nt segment of the X region, showing the sequences from three mother-and-child pairs (OBIN-49, OBIN-25, OBIN-86) and database sequences representing relevant genotypes.

the three that developed OBI than in mothers to the five that did not (median 8.40 vs. 7.86 log IU/mL, $p = 0.12$). There was no difference in postnatal prophylaxis given to the babies (all received both HBIG and full vaccination).

Because of the identification of OBI, a follow-up serum sample was taken from the three children at 5–7 years of age. As shown in Table 3, one of them had become positive for HBsAg (0.82 IU/ml), HBV DNA (1.81 log IU/ml) and anti-HBc antibody, indicating a shift from primary OBI to low-active chronic HBV infection. A recently developed PCR assay for whole genome deep sequencing was applied on the follow-up samples from all three children. In two of the cases, OBIN-49 and OBIN-86, it produced amplicons, which were subjected to Ion Torrent sequencing. The sequences obtained were of genotype B4 and C2, and were identical in the X region with the sequences in samples that were obtained from the two children at one year of age. They were also 99.9% and 100% similar to the whole genome sequences obtained from the respective mother.

Fig. 3 shows the anti-HBs levels before and after the fourth vaccination against HBV. Children born to HBeAg-negative women had significantly higher anti-HBs levels than children born to HBeAg-positive women at 12 ($p = 0.03$) and in particular at 18 month's ($p < 0.0001$) of age, and they also responded with a greater increase in anti-HBs levels after the fourth vaccination ($p < 0.0001$).

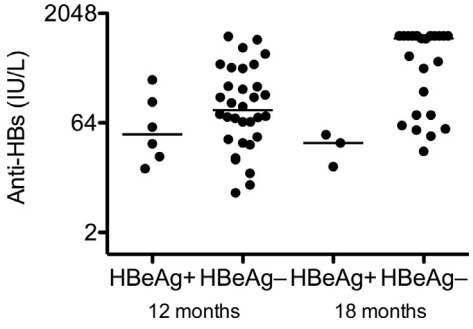

Fig. 3. Anti-HBs levels at the age of 12 and 18 months (i.e. before and after the fourth hepatitis B vaccination) in children born to mothers who were HBeAg positive or negative. Titres above 1000 IU/L are shown as 1000 IU/L. Samples from 18 months were lacking in 16 cases.

Discussion

In this study, primary OBI was detected in 3 out of 44 children born to HBsAg positive women, including one child who at 7 years of age had developed a low-active but overt HBV infection. These 3 OBI were found in children whose mothers were HBeAg-positive

with HBV DNA levels above 8 $\log_{10}$ IU/mL at antenatal screening and had received tenofovir treatment during the third trimester of pregnancy. Mother-to-child transmission was confirmed by DNA sequencing of the X region of HBV from the children and their respective mothers.

Only a few previous studies have investigated OBI in children after putative mother-to-child transmission, and with very different findings, as summarized in Supplementary Table 2. The reported OBI rates vary between 28% in Iranian children aged 10–128 months,[12] 5% in Chinese children aged 1.5–50 months,[13] 64% and 42% in Indian children aged 18 weeks and 24 months,[17] 0% in Chinese children aged 2.6 years,[14] 20% and 4% in Chinese children aged 7 and 24 months,[16] and 36% in Chinese children aged 3–36 months.[18] To some extent these variations may reflect differences in sampling age, assay sensitivity or viral loads in the mothers, but the lack of convincing sequence data is an overall limitation in these studies. One study did not perform any sequencing,[17] some studies sequenced HBV from the children but not the mothers,[12,13] and others applied sequencing on HBV from both mothers and children but obtained incomplete or conflicting results.[14,16,18] The purpose of sequencing is to reveal false positive results due to contamination of samples from other samples or from amplified HBV DNA, a risk that may be significant even if precautions are made to avoid contamination.

In order to avoid false positive results in the diagnosis of OBI, a general recommendation is to use primer sets that target four regions of the HBV genome, and to request that at least two of these reactions need to be positive.[4] This strategy reduces the risk of false positive results from contamination by amplicons, but has no or little effect if the sample is contaminated with whole HBV particles. Fortunately, in the identification of OBI after vertical transmission, the risk of false positive results can be further reduced by comparing the HBV sequences from mothers and children, with the request that they should be identical or very similar to each other but distinct from sequences representing HBV that circulate in the population. This important option and criterion has not been sufficiently applied in previous studies, and therefore the frequency of OBI due to vertical transmission has remained obscure.

When analyzing a 273-nucleotide (nt) sequence of the X region we observed almost identical sequences in mother and child in all three OBI cases, with sequences representing genotypes C2 in two cases and genotype B4 in one case. In all the three children the same sequence was identified in HBV from different samples, which essentially excludes that the findings were caused by contamination. One child developed overt HBV infection at the age of 7 years, and another still had OBI at the age of 5 years. At that time point, HBV whole genome sequences (3215 nt) that were identical to the HBV sequences from the mothers were identified by Ion Torrent deep sequencing. These findings confirm that OBI from mother-to child transmission had occurred, and also show that such occult infections can persist several years and even progress to overt infection, The fact that genotypes B and C are relatively rare among HBV infected in our population, but fit well with the origin of the mothers to the children (Korea and Vietnam), further supports that mother-to-child transmission had occurred.

In woodchucks, primary OBI has been observed in animals that were exposed to low infectious doses.[5] In these cases, woodchuck hepatitis virus was detected only in lymphatic cells, not in the liver, and neither surface antigen nor antibodies were detected. In humans, children born to infected mothers may similarly be exposed to low levels of HBV. A difference is that in the children, vaccine induced anti-HBs likely serves to block the spread of infection from hepatocytes that might be infected. Still, it seems reasonable to classify this type of infection as primary OBI if HBV DNA but neither HBsAg nor anti-HBc is detected.

In the woodchucks, overt infection may develop later on, and integration of viral DNA into chromosomal DNA seems to be a frequent event, which can promote hepatocellular carcinoma (HCC).[5] It is not known if primary OBI after vertical transmission in humans is of clinical importance or if such infections might become activated later on. Therefore, a main finding in our study was that one of the three children with OBI had become seropositive also for HBsAg and anti-HBc at 7 years of age, demonstrating that OBI can develop in to overt HBV infection. In this case the chronic HBV infection was low-active, but it cannot be excluded that the infection might become more active later in life, for example if immunosuppressive treatment is given. This finding underlines that long-term effects of vertically transmitted primary OBI should be investigated in additional studies.

In the present study, anti-HBs was assessed at both 12 and 18 months of age, i.e. before and after the fourth vaccination against HBV. The anti-HBs levels in children born to HBeAg-negative women (children who did not receive HBIG) were significantly higher than the levels in children born to HBeAg-positive women, in agreement with a previous Chinese study.[21] Interestingly, whereas there was a significant increase of anti-HBs levels after the fourth vaccination in children born to HBeAg-negative mothers, this was not observed in children born to HBeAg-positive women. One of the three children with OBI even had lower levels of anti-HBs after than before the fourth vaccination. The association might reflect that children born to HBeAg-positive mothers (who receive HBIG) respond less well to vaccination, or that a poor vaccine response might increase the risk of developing OBI. Alternatively, a lower anti-HBs levels could be the result of OBI, if occult infection produces HBsAg that forms immune complexes with anti-HBs. Larger studies, preferably including analysis of HBsAg/anti-HBs immune complexes, are warranted to confirm and explain differences in anti-HBs levels and booster vaccination response between HBeAg-positive and HBeAg-negative mothers.

In summary, this is to our knowledge the first report of primary OBI after mother-to-child transmissions verified by indisputable sequence data, and the first observation that such infections may later transform into overt hepatitis B virus infection. The potential clinical relevance of primary OBI after vertical transmission should be addressed in future studies.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.01.002.

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