IDENTIFICATION OF HEPATITIS B VIRUS VERTICAL TRANSMISSION FROM FATHER TO FETUS BY DIRECT SEQUENCING

Shanshan Wang, Guifu Peng, Mimin Li, Hong Xiao, Pulin Jiang, Nianhua Zeng and Zhibing Wang

Department of Epidemiology, Medical Institute of Guangzhou Command PLA, Guangzhou, People's Republic of China

Abstract. To identify the possibility of hepatitis B virus (HBV) vertical transmission from father to fetus, eight male HBV carriers whose wives were negative for any HBV marker and their eight aborted fetuses who had been infected with HBV *in utero*, were studied. S gene 451 ~ 660 nucleotide sequence of HBV in 6 cases of father/fetus pairs and C gene 2022 ~ 2321 nucleotide sequence in the other 2 cases of father/fetus pairs were amplified by nested polymerase chain reaction (NPCR), and sequenced. HBV DNA was detected in the semen and spermatid of male HBV carriers. The homologies of HBV sequences between father and fetus were very high. Six father/fetus pairs had the same subtype *adw*. The sequences of the fragment were identical between father and fetus in 4 cases. Especially in case 3, both father and fetus had the same point mutation, which caused an amino acid substitution at codon 126. The other two cases had point mutations in the fetus at nucleotide positions 491, 494, 546, 581 resulting in amino acid substitution at codons 113, 114, 131, 143. The C gene 2022 ~ 2321 nucleotide sequences in two cases were identical. There were eleven common point mutations between father and fetus, but those mutations did not cause phenotypic changes. Our finding suggested that HBV vertical transmission from father to fetus was present. A HBV carrier father may transmit the virus to his fetus by spermatid.

INTRODUCTION

The modes of transmission of HBV differ in various parts of the world. In the United States, sexual activity and intravenous drug use are important risk factors (Hollinger *et al*, 1990), whereas perinatal HBV transmission is common in Southeast Asia (Ip *et al*, 1989). Studies of HBV transmission have been based largely on epidemiologic surveys, serologic tests and clinical histories. In addition, intrafamilial transmission among Chinese has been demonstrated by serotyping (Stevens *et al*, 1975) and the clustering of infected individuals in certain families (Lok *et al*, 1987). Such studies can show the prevailing mode of transmission among various populations, but generally do not provide sufficient detail to confirm or exclude the occurrence of HBV transmission between specific individuals.

The finding of mutant HBVs in two Chinese families by DNA sequence analyses showed the most probable routes of transmission within each family (Lin *et al*, 1990). Siblings within each family had the same variants. The degrees of homology between the variant sequences that differed within a given family fell into two groups (Lin *et al*, 1991). The mothers positive for HBsAg can transmit the virus to their offspring *in utero* (Badur *et al*, 1994; Zhong *et al*, 1996). If HBV infection occurred *in utero*, the effect of the HBsAg-positive father was also important. HBV DNA was found in human semen and spermatid (Xu *et al*, 1992; Zhao *et al*, 1998). According to this finding, it has been speculated that the vertical transmission of HBV from a HBsAg-positive father

Correspondence: Dr Shan-shan Wang, Medical Institute of Guangzhou Command PLA, 91 Dong Guan Zhuang Road, Shahe, Yanling, Guangzhou 510507, People's Republic of China.
Tel: 020-87165241-2021, 020-87165241-2103, 020-87796684
E-mail: doctorxiao@163.net

to his infant is also possible. But HBV cannot be isolated and cultured, to confirm the route is difficult. At present, the gene detection technique (Norder *et al*, 1994, 1996; Blitz *et al*, 1998) has provided a useful method for establishing the route of transmission by the detection of HBV DNA and the comparison of the degrees of homology of HBV sequences. In the present study, HBV nucleotide sequences from 8 cases of father/fetus pairs were amplified and sequenced. By identifying HBV DNA sequences between fathers and fetuses, we try to establish the route of transmission from father to offspring.

MATERIALS AND METHODS

Patients

Pregnant women and their husbands who wanted to terminate pregnancy for medical reasons were screened by hepatitis Bs antigen (HBsAg) in the hospital of a county in Hunan Province between March 1995 and March 1996. Eight male HBV carriers and their infectious fetuses were selected in 506 pairs of pregnant women and their husbands. The male HBV carriers were identified by five HBV serological markers (described below) determined by ELISA methods and HBV DNA by PCR. They were between 25 and 35 years of age and had normal ALT levels. They also had no history of hepatitis or prior injection of hepatitis B vaccine. Their wives, aged between 23 and 33, were negative for all HBV serologic markers (described below) and HBV DNA. The sera, leukocytes of the couples and sperm from the male HBV carriers were taken during hospitalization. Their aborted fetuses aged 3 months and over *in utero*, were also studied. The blood of the fetus was taken at the time the fetus was born. Sera were isolated and stored at low temperature. Leukocytes were isolated at the same time. The liver tissues of fetuses were taken. Fetuses positive for HBV DNA in any sample of serum, leukocyte and liver were taken as having contracted HBV infection *in utero*. We also selected 5 father/mother/fetus pairs, who were negative for all HBV markers, as control.

Materials

Primers were synthesized in the Shanghai Institute of Cell. Taq polymerase, DNA markers, proteinase K were purchased from the Sino-American Biotechnology Company. The Fmol DNA Cycle Sequencing System was from Promega. γ-^{32}P-ATP was purchased from Beijing Yahui Company. The digitoxin labeled HBV DNA probe was purchased from Shanghai Medical University.

Determination of HBV serological markers

Five HBV serological markers, HBsAg, HBeAg (hepatitis B e antigen), anti-HBs (hepatitis B s antibody), anti-HBe (hepatitis B e antibody), anti-HBc (hepatitis B c antibody) were determined using commercially available ELISA kits.

HBV DNA extraction

Leukocytes were isolated from anti-coagulative blood using leukocyte separating liquid. After washing three times with phosphate buffered saline solution (PBS), the volume of the leukocytes was restored by adding PBS and then stored at a low temperature. The liver specimens were obtained by adding 2ml PBS to 2g liver tissue in a grinding-bowel and grinding under sterile conditions. After washing three times with PBS, 2 ml PBS were added to the livers specimens and then stored at a low temperature. Sperm was centrifuged to isolate spermatids and semen. After washing three times with PBS, the volume of the spermatids was restored by adding PBS and the sample was stored at a low temperature. All of final the washes in PBS should be negative for HBV DNA.

Fifty microliters of the samples mentioned above and sera were, respectively, digested in the presence of 0.5% sodium dodecyl sulfate, 25 mmol/l sodium acetate, 2.5 mmol/l ethylenediaminetetraacetic acid, 1 μg/ml proteinase K in a volume of 200 μl for 2 hours at 68ºC. Two phenol-chloroform extractions were followed by two chloroform extractions and HBV DNA was then precipitated with ethanol. After washing twice with 70% etha-

nol, the DNA pellets were suspended in 10 μl water (Hou *et al*, 1995).

Detection of HBV DNA by nested PCR

Five microliters of the extracted HBV DNA were used as a template for nested PCR amplification. The oligonucleotide primers used in this study are presented in Table 1. The primers were selected from the S and C regions of the HBV genome. A pair of nested primers was designed for each region. In the S region, the outer primers were S1R, BS1, amplified a 787 bp fragment; the inner primers were S3, B3, amplified a 261 bp fragment. In the C region, the outer primers were C1, C3, amplified a 665 bp fragment; the inner primers were C2, C6, amplified a 394 bp fragment. The PCR conditions were as described before (Hou *et al*, 1995). After PCR, the positive sample was identified by Southern blot hybridization. The hybridization was performed according to the manufacturer's recommendations.

Direct PCR sequencing

Ninety microliters of second stage PCR products of S and C regions were purified with an equal volume of 4 mol/l sodium acetate and 2 volumes of isopropanol for 10 minutes at room temperature, pelleted by centrifugation for 10 minutes, washed two times with 70% ethanol, then suspended in 20 μl water for sequencing. Primers BS3 and C6 were respectively end-labeled with 25 μ Ci of γ-^{32}P-ATP in a volume of 10 μl using 10 units of polynucleotide kinase for 10 minutes at 37ºC. 1.5 μl of the reaction mix was used directly in the sequencing reaction. Briefly, 9.5 μl purified DNA was added to the reaction buffer with the end-labeled primer and 5 units of Taq enzyme. Dideoxynucleotide termination sequencing was performed according to the manufacture's instructions for the Fmol DNA Cycle System (Promega) (Hou *et al*, 1995). The sequencing results were handled by DNASIS software and compared with the corresponding sequences in Genebank.

RESULTS

The HBV serological markers and HBV DNA of eight father/fetus pairs are presented in Table 2. Among the eight HBV carrier fathers, six were positive for HBsAg and HBeAg, the other two were positive for HBsAg and negative for HBeAg. Only one fetus (12.5%) in the eight HBV infectious fetuses had HBV serological markers. None of the samples from the five control fetuses had HBV markers.

We have sequenced S gene 451 ~ 660 nucleotide sequence of HBV of six father/fetus pairs and C gene 2022 ~ 2321 sequence in HBV of the other two father/fetus pairs by direct PCR sequencing. The nucleotides were numbered from the EcoRI restirction site of the HBV genome (Galibert *et al*, 1979). The part S gene sequences are shown in Fig 1. The homology of the sequences from father and fetus are 98 ~ 100%. It is known that HBV serological subtypes are determined by the amino

Table 1
NPCR primers used for HBV gene amplification.

Primer	Position	Nucleotide sequence
S1R	842 ~ 822	5′-TTAGGGTTTAAATGTATACCC-3′
BS1	56 ~ 76	5′-CCTGCTGGTGGCTCCAGTTCC-3′
S3	427 ~ 448	5′-CATCTTCTTGTTGGTTCTTCTG-3′
BS3	687 ~ 668	5′-GGCACTAGTAAACTGAGCCA-3′
C1	2394 ~ 2370	5′-GGCGAGGGAGTTCTTCTTCTAGGGG-3′
C3	1730 ~ 1754	5′-CTGGGAGGAGTTGGGGGAGGACATT-3′
C2	1955 ~ 1974	5′-TTGCCTTCTGACTTTCTTTCC-3′
C6	2348 ~ 2330	5′-TAAACAACAGTAGTTTCCGG-3′

Table 2
HBV markers of father/fetus pairs.

Case no.	Father's HBV-DNA				Fetus' serological markers				Fetus' HBV-DNA		
	Serum	Leukocyte	Semen	Spermatid	HBsAg	HBeAg	Anti-HBc	Anti-HBe	Serum	Leukocyte	Liver
1	+	+	+	+	+	+	-	-	+	+	+
2	+	+	+	+	-	-	-	-	+	+	+
3	+	+	-	-	-	-	-	-	+	+	+
4	+	+	+	+	-	-	-	-	+	+	+
5	+	+	-	-	-	-	-	-	+	+	+
6	+	+	/	/	-	-	-	-	+	+	+
7	+	-	+	+	-	-	-	-	+	-	+
8	+	+	+	+	-	-	-	-	+	+	+

/ Sperm not obtained.

acid at the 122 and 160 codon of the S gene (Norder *et al*, 1992). Lysine or arginine at the 122 codon determines the subtype of *d* or *y*, while that at the 160 codon determines the subtype of *w* or *r*. In six father/fetus pairs, the triplets of the 122 and 160 codon were AAA (lysine), so all of them belonged to the subtype *adw*. The results of direct sequencing showed that the sequences of cases 3, 4, 5, 6 were identical. Fig 2 shows the part sequences of HBV DNA from case 4. The sequences of the HBV DNA from the serum and spermatid of the father and the serum of the fetus were identical. Compared with the sequence in Genebank (Norder *et al*, 1992), the case 4 sequence had two point mutations at positions 499 and 508. Nucleotide C was mutated to A at positions 499, while C was mutated to G at position 508. The two point mutations did not result in phenotypic changes. Some nucleotides of the fetus in case 1 were different from those of the father. The nucleotides at positions 491, 494, 505, 544, 546, 581, 586, 592 were T, T, C, A, C, A, C, C in the father, while A, A, T, C, A, T, T, T in the fetus. The changes of nucleotides 544, 546 in case 2 were the same as that in case 1. Mutation of T-to-A at nucleotides 491 and 494 resulted in amino acid substitutions from serine to threonine at the 113 and 114 codon. Mutation of C-to-A at nucleotide 546 resulted in amino acid substitution from threonine to asparagine at codon 131. Mutation of A-to-T at nucleotides 581 resulted in amino acid substitution from threonine to serine at codon 143. The other point mutations did not result in phenotypic changes. There were five common point mutations in this sequence of father/fetus pairs. They occurred at nucleotide positions 493, 499, 508, 529 and 530. Only the point mutation of A-to-G at nucleotide position 530 in case 3 resulted in amino acid substitution from threonine to alanine at codon 126. The others did not result in phenotypic changes.

The sequences located at the nucleotide positions 2022 ~ 2321 in the HBV C gene of the fathers and fetuses in cases 7 and 8 were identical, with a homology of 100% (Fig 3). The number of common point mutations in the sequence of this fragment in the father and fetus was eleven. None of the mutations resulted in phenotype changes.

DISCUSSION

The possibility of father-infant transmission of HBV has been discussed for a long time. HBV markers, including antigens, antibodies, HBV DNA were detected in semen and spermatids by ELISA and dot hybridization (Xu *et al*, 1992; Zhao *et al*, 1998). After using the gene detection technique, the rate of detected HBV DNA in sperm increased. It was

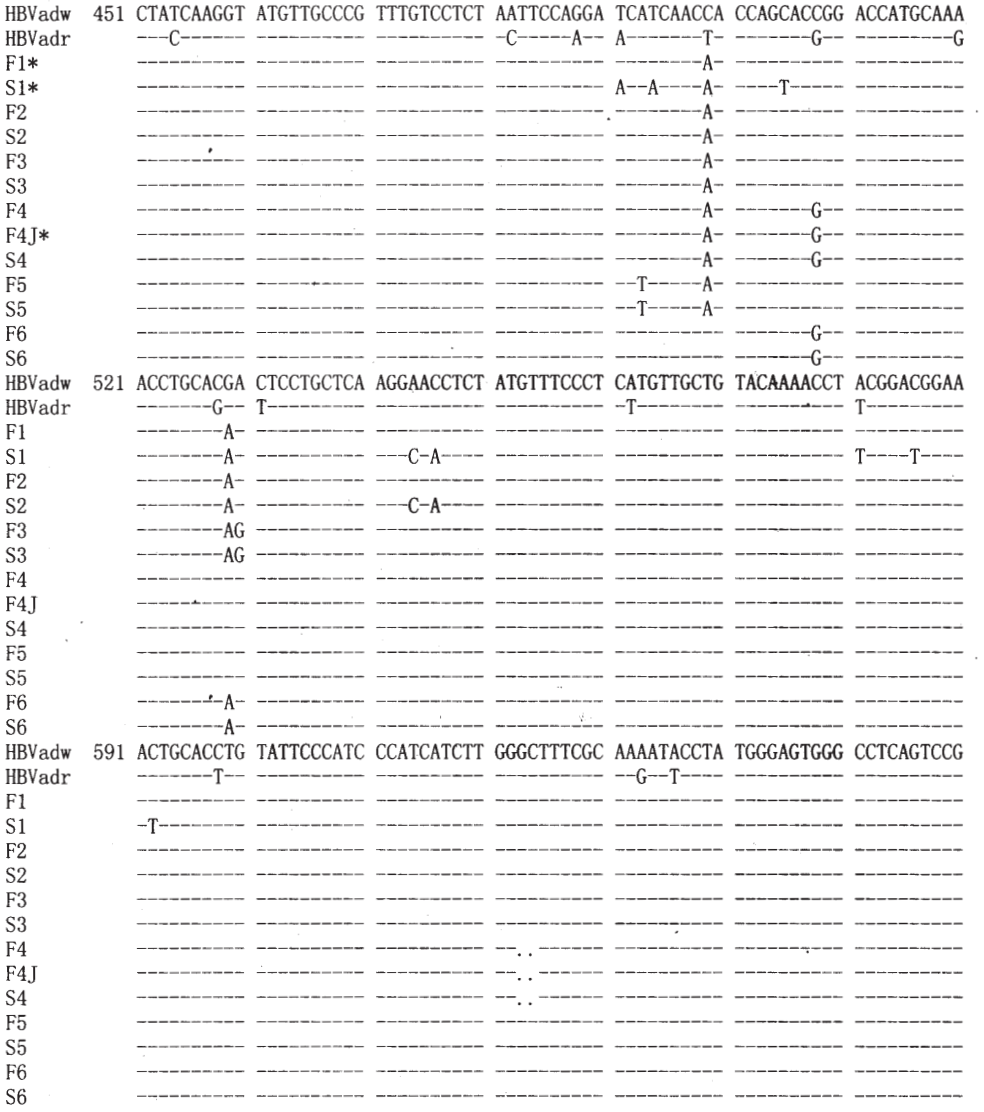

Fig 1–Sequences of HBV S gene nucleotides 451~660 from 6 pairs of father/fetus compared with those of HBVadw and HBVadr (Norder *et al*, 1992).
*F: HBV from father's serum; J: HBV from father's spermatid; S: HBV from fetus' serum; - nucleotide is consistent with that of HBV adw; .. nucleotide overlapping (without reading frame shift).

reported that HBV infection was detected in neonates born to mothers with negative HBV serological markers was detected (Zhang *et al*, 1994). It was inferred that there might exist the possibility of father-neonate transmission of HBV. Our results proved HBV transmission from father to fetus before birth. The HBV carrier father can transmit the virus to his fetus via the spermatid. In the present study, HBV DNA was not detected in the semen or spermatids of 2 HBV carrier fathers. Perhaps the occurrence of HBV in semen, especially in the

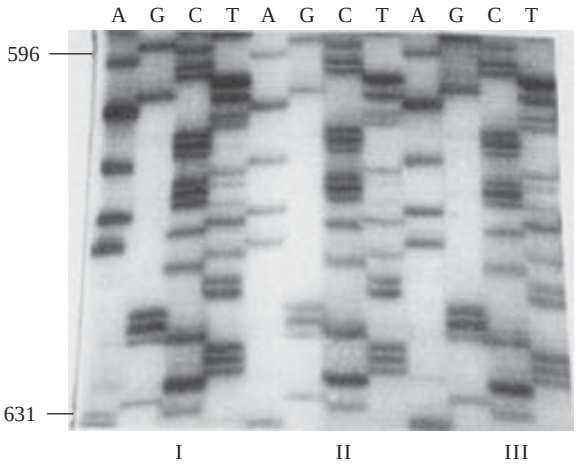

Fig 2–Direct sequencing of HBV S gene nucleotides 596 ~ 631 from father and fetus in case 4. Sample I: serum from father; Sample II: spermatid from father; Sample III: serum from fetus.

spermatid, is not persistent, but intermittent. The time difference between sperm collected and the production of a fertilized ovum may have been the cause of the negative results in the 2 cases. Factors influencing the occurence of HBV in the spermatid needs further study. The homology of the HBV gene between the father and his fetus is high.

In case 4, the sequences of the HBV gene fragment from the serum and spermatid of the father and the serum of the fetus were identical. Nucleotide mutations at position 508 were all from C to G. This finding explained the the molecular biological evidence of father-fetus transmission in detail. The DNA sequences in the S gene of HBV showed that six father/fetus pairs had the same subtype *adw*. This may be due to the fact that all of the cases came from the same place. In addition, amino acid substitutions were detected at codons 113, 114, 126, 131, and 143. Mutations at codons 126 and 131 are often seen in neonates who have received injections of HBV vaccine. Mutations at codons 113, 114 and 126 can be detected in hepatitis B patients negative for HBV serological markers (Luo, 1996). Hitherto, all of the mutations at codon 126 have had an amino acid substitution from threonine to isoleucine without exception. Interestingly, we found an amino acid substitution from theronine to alanine at codon 126. Similar studies on other condons were reported by other scholars (Fan *et al*, 1997). This variant needs to be studied further. The surface membrane proteins of HBV are related to cellular and humoral immunity. Since the main target epitope for conventional immunization is located in the middle fragment of the S gene of HBV, mutations in this fragment are directly responsible for the production of neutralizing antibodies. Infants infected with these HBV variants would fail to acquire conventional immunization against HBV. In another words, lack of response to vaccine resulting from immune tolerance can be seen in paternal-fetal transmission. The homology of the sequence of 2022 ~ 2321 fragment in the C region between the father and his fetus was 100%. In addition, none of eleven common mutations sites caused phenotypical changes. Perhaps this was due to the conservative character of the C gene.

Our data also demonstrated that only the minor strain of the father's virus pool was transmitted to his fetus. Most of the fathers transmitted their advantageous strain to their fetuses. For example, fathers in cases 4, 5 and 6 transmitted their wild-type strains to their fetuses; the father in case 3 transmitted his variant strain. In contrast, a small proportion of the fathers, such as the fathers in cases 1 and 2, transmitted their minor strains from their virus pool to their fetuses. The fathers in cases 1 and 2 had wild-type strains, while the fetuses had variant strains. It is, therefore, possible that an undetectable minor HBV strain was transmitted from a carrier father to his fetus. This finding is consistent with current opinion on the vertical transmission from mother to infant (Luo, 1996).

Because infants who have been infected with HBV from their fathers *in utero* will suffer from a lot of differing factors after birth, such as immune pressure in their own bodies, injection of HBV vaccine, etc, outcomes might also differ. It is assumed that some infants may

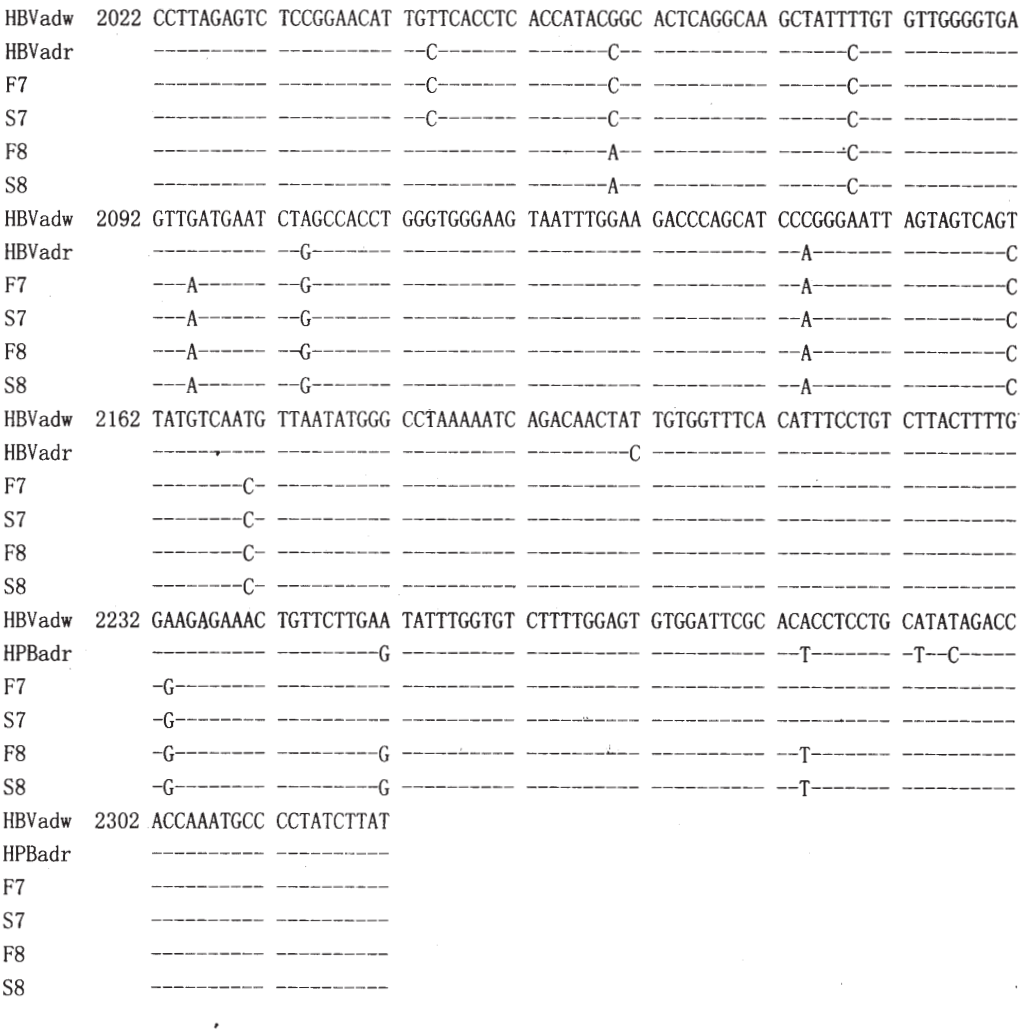

Fig 3–Sequences of HBV C gene nucleotides 2022~2321 from 2 pairs of father and fetus compared with those of HBVadw and HBVadr (Noder *et al*, 1992).

develop a persistent, stable HBV infection and form an advantageous HBV strain. Further studies are required to provide evidence for this proposition.

ACKNOWLEDGEMENTS

The sequencing of PCR products was performed in the Laboratory of Department of Infectious Diseases, Nanfang Hospital, Guangzhou. The laboratory is a key one in PLA and Guangdong Province. We are grateful to Prof Kangxian Luo and the faculty members in the laboratory for excellent technical assistance.

REFERENCES

Badur S, Lazizi Y, Ugurlu M, *et al*. Transplacental passage of hepatitis B virus DNA from hepatitis B e antigen-negative mothers and delayed immune response in newborns. *J Infect Dis* 1994; 169: 704-6.

Blitz L, Pujol FH, Swenson PD, *et al*. Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. *J Clin Microbiol* 1998; 36: 648-51.

Fan JS, Zhuang H, Li YG, *et al*. Comparison of sequences of hepatitis B virus S gene fragment in eight cities of China. *Chin J Prev Med* 1997; 31:140-3.

Galibert F, Mandar E, Fitoussi F, *et al*. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli. Nature* 1979; 281: 646-50.

Hollinger FB. Controlling hepatitis B transmission in North America. North American Regional Study Group. *Vaccine* 1990; 8 (suppl): S122-8.

Hou JL, Karayiannis P, Waters J, *et al*. An unique insertion in the S gene of surface antigen negative hepatitis B virus Chinese carriers. *Hepatology* 1995; 21: 273-8.

Ip HM, Lelie PN, Wong VC, *et al*. Prevention of hepatitis B virus carrier state in infants according to maternal serum levels of HBV DNA. *Lancet* 1989; 1: 406-10.

Lin HJ, Lai CL, Lau JY, *et al*. Evidence for intrafamilial transmission of hepatitis B virus from sequence analysis of mutant HBV DNAs in two Chinese families. *Lancet* 1990; 336: 208-12.

Lin HJ, Lai CI, Lauder IJ, *et al*. Application of hepatitis B virus (HBV) DNA sequence polymorphisms to the study of HBV transmission. *J Infect Dis* 1991; 164: 284-8.

Lok AS, Lai CL, Wu PC, Wong VC, *et al*. Hepatitis B virus infection in Chinese families in Hong Kong. *Am J Epidemiol* 1987; 126: 492-9.

Luo KX. Hepatitis B: basic biology and clinical science. 1st ed. Beijing: People's Medical Publishing House, 1996: 42-3, 53-4.

Norder H, Ebert JW, Fields HA, *et al*. Complete sequencing of a gibbon hepatitis B virus genome reveals a unique genotype distantly related to the chimpanzee hepatitis B virus. *Virology* 1996; 218: 214-23.

Norder H, Courouce AM, Magnius LO. Molecular basis of hepatitis B virus serotype variations within the four major subtypes, *J Gen Virol* 1992; 73: 3141-5.

Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198: 489-503.

Norder H, Hammas B, Lofdahl S, *et al*. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; 73: 1201-8.

Stevens CE, Beasley RP, Tsui J, *et al*. Vertical transmission of hepatitis B antigen in Taiwan. *N Engl J Med* 1975; 292: 771-4.

Xu X, Wang L, Lin XM. The possible role of sperm in family HBV infection. *Chin J Epidemiol* 1992; 13: 337-9.

Zhang LY, Fang ZX, Zhen ZT, *et al*. Preliminary research on the paternal-fetal transmission of hepatitis B virus. *Chin J Infect Dis* 1994; 12:199-201.

Zhao LS, Liu XS, Zhang ZX, *et al*. Study on HBV vertical transmission via infected spermatozoa. *Chin J Dis* 1998; 16: 154-7.

Zhong M, Hou JL, Luo KX, *et al*. Identification of vertical transmission of hepatitis B virus from mother to child by direct sequencing a segment of the surface gene of the hepatitis B virus. *Natl Med J China* 1996; 76: 194-6.