Phylogenetic Analysis of New Hepatitis B Virus Isolates From Nigeria Supports Endemicity of Genotype E in West Africa

Solomon O. Odemuyiwa,[1,2] Mick N. Mulders,[1] Oluseyi I. Oyedele,[2] Samuel O. Ola,[3] Georgina N. Odaibo,[2] David O. Olaleye,[2] and Claude P. Muller[1]*

[1]*Department of Immunology, Laboratoire Nationale de Santé, Luxembourg, Luxembourg*
[2]*Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria*
[3]*Department of Medicine, University College Hospital, Ibadan, Nigeria*

Isolates of hepatitis B viruses were collected from 20 acute and chronic hepatitis patients in a highly endemic region of Nigeria. Sequencing classified the isolates to the *ayw4*, as they all contained the amino acid variations characteristic for that serotype. In the pre-S2 region of five isolates, three to seven amino acids were deleted, suggesting that immune escape mutations previously associated only with chronic HBV infection may be observed also in acute disease. Phylogenetic analysis of the complete pre-S2/S (large S) genes (831 nt) demonstrated that all the viruses belonged to the same genotype E. So far, no isolates of genotype E have been found in any other region of the world, including the Americas. This may suggest a relatively recent introduction of this genotype into humans and would explain the relatively low genetic diversity of viruses belonging to this genotype. One genotype E virus had been found previously in a chimpanzee, and viruses belonging to the CHIMP genotype are related to other genotype E viruses. These findings are compatible with a transmission of genotype E viruses from chimpanzees to humans. **J. Med. Virol. 65:463–469, 2001.**
© 2001 Wiley-Liss, Inc.

KEY WORDS: HBV genotypes; "*a*" determinant; S protein mutation

INTRODUCTION

Hepatitis B virus (HBV) uses a complex overlapping coding strategy to produce proteins essential for viral replication and maintenance of the structural integrity of the virion. The virion envelope surface protein consists of three polypeptides of 226 (major S), 281 (middle S), and 400 (large S) amino acids [Seeger and Mason, 2000]. Variations within the "*a*" determinant and other parts of the major S protein have led to the

recognition of nine different serotypes. Specifically, amino acid changes at residues 122 and 160 are responsible for the expression of two *d/y* and *w/r* subdeterminants, respectively [Okamoto et al., 1988]. The *d* and *y* or *w* and *r* determinants are mutually exclusive.

Although the overlapping coding pattern puts a substantial constraint on the susceptibility of HBV genomes to variation, involvement of the error-prone reverse transcriptase enzyme, which lacks proof-reading activity in the viral replication cycle, has lead to a high degree of genetic variability among HBV strains circulating worldwide [Boyer et al., 1992]. With a distinct geographic distribution, HBV strains are traditionally classified into six genotypes, A to F, on the basis of genomic diversity [Norder et al., 1993]. Strains belonging to genotype A are found mainly in Northwestern Europe, Central Africa, and North America [Norder et al., 1993]. Members of genotypes B and C predominate in Asia [Okamoto et al., 1988]. Most HBV strains found in the Americas belong to genotype F [Magnius and Norder, 1995]. HBV isolates from the Mediterranean area and the Middle East belong to genotype D. The recently characterised genotype G has been found in the United States and France [Stuyver et al., 2000].

In Africa, viruses belonging to five genotypes A to E have been found. An extensive study of genotypes A, B, C, and D circulating in South Africa has been reported [Bowyer et al., 1997]. Genotype D was also recently reported from Tunisia [Borchani-Chabchoub et al.,

2000]. The description of members of genotype E has hitherto been limited to viruses recovered from human and chimpanzees in Angola, Liberia, Senegal, and Central Africa [Norder et al., 1994]. Apart from these reports, however, information on the genotypes circulating in Africa is very scanty.

The nucleotide sequences of twenty HBV strains obtained from Nigeria are described. Even in this most populous country in Africa, an important centre of commerce and population mixes with a prevalence of hepatitis B virus carriers as high as 10–40% [Olubuyide et al., 1997], there is no information on the genotype of HBV circulating in the country. This information may be important in predicting the efficacy of subunit vaccines and sensitivity of diagnostic reagents for the detection of hepatitis B surface antigen (HBsAg) in clinical samples. Partial sequence analysis of HBV isolates may also contribute to a better understanding of the evolutionary history of HBV strains among peoples indigenous to different parts of the world.

MATERIALS AND METHODS

Patients

Sera were collected from cases referred to the department of Virology, University College Hospital, Ibadan, Nigeria, between June 1996 and September 1998. Most of the sera used in the study (NIE001, NIE003, NIE004, NIE006, NIE007, NIE009, NIE013, NIE014, NIE017, NIE021, NIE022, NIE023, and NIE026) were from cases of acute hepatitis (SGOT: 80-1084 i.u./l, SGPT: 44-392 i.u./l; bilirubin: 4.1-25 mg/ml). The remaining sera (NIE002, NIE005, NIE010, NIE012, NIE024, NIE030, and NIE031) were obtained from cases referred to the department after recovering from acute hepatitis as well as from asymptomatic HBV carriers. Sera positive for hepatitis B surface antigen (HBsAg) by a commercial Enzyme Immunoassay (EIA) kit (Murex, Dartford, UK) were stored at −20°C. Cases of hepatitis were regarded as unrelated when the possibility of transmission among the cases could not be inferred from the case history.

DNA Isolation, Polymerase Chain Reaction, and Cycle Sequencing

HBV DNA was isolated from serum using a modification of the method of Boom et al. [1990]. Briefly, 150 μl of serum was mixed with lysis buffer and 10 μl of DNA binding resin. After short vortexing, the mixture was incubated for 1 hr at 37°C and centrifuged at 13,000 rpm to pellet the resin. The pellet was washed three times with washing buffer, and subsequently with 70% ethanol and acetone. After drying the pellet at 56°C, DNA was eluted from the resin with TE buffer and immediately used for polymerase chain reaction (PCR).

The complete pre-S/S gene was amplified to yield a product of approximately 1.4 kb with sense primers 2810 and antisense primer 979 (Table I) [Bowyer et al., 1997]. Nested primers were then used to amplify a fragment encompassing the 3'-end of the pre-S1 gene, the complete pre-S2 region and the S gene (Table I). PCR products were purified using the Concert™ Rapid PCR Purification System (Gibco Life Technologies). The products were sequenced in both directions using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems [ABI], Nieuwerkerk, The Netherlands) using the PCR primers as sequencing primers. Sequences were analysed using the Sequencing Analysis program (version 3.0, ABI). The Sequence Navigator program (version 1.0.1; ABI) was used to align the forward and reverse sequences to ensure reliability of the generated sequences and to resolve possible ambiguous nucleotides.

Data Analysis

Sequence alignments were made using ClustalX [Thompson et al., 1997]. The obtained results were used for further analysis using the Phylogenetic Inference Package (PHYLIP) version 3.5 [Felsenstein, 1993]. Distance calculations were made using the neighbour joining method with the Kimura-2-parameter model of nucleotide substitution (transition/transversion ratio set at 2.0) as implemented in DNAdist component of PHYLIP. Apart from this distance matrix method, the sequences were also analysed by maximum likelihood method using the

TABLE I. PCR and Sequencing Primers

Primer name	Primer sequence	Position*
2810[a]	CACGTAGCGCCTCATTTTCTGGGTCACCATATTCT	2794–2828
979[a]	CAAAAGACCCACAATTCTTTGACATACTTTCCAAT	1010–977
McIntosh-F[b]	TCGGATCCGGTATGTTGCCCGTTTGTCC	450–477
PF	ACATTCCACCAAGCTCTGCA	8–27
PR	AGGTTCCTTGAGCAAGAG	547–531
PRES2-F	CAACAAGGTAGGAGTGGGAGCAT	3009–3031
PRES-R	TCAACAAGAAAAACMCCGCCTGT	221–199
S-F	GTGTCTTGGCCAAAATTCGCAGT	295–317
S-R	GGAAGATGATAAAACGCCGCAGAC	404–381

*Numbering is according to the full-length HBV genome of HHVBBAS; accession number X75657.
[a]McIntosh et al. [1998].
[b]Bowyer et al. [1997].

DNAML and DNAPARS programmes in the PHYLIP package. Bootstrap trees (100 replicates) were generated using both the ClustalX and PHYLIP and visualised using the TREEVIEW program.

RESULTS

Twenty new isolates of HBV from Nigeria were aligned with 49 reference isolates representing all the six known genotypes of HBV across a 831-nt interval encompassing the complete pre-S2 region and the S gene. Following comparison of the complete pre-S/S gene sequences of these new HBV isolates from Nigeria with the genotype reference sequences, a phylogenetic tree was produced (Fig. 1). All the 20 Nigerian isolates from the present study were shown to belong to genotype E although none was completely identical to any other sequence. The Nigerian HBV strains showed a mean diversity of 1.5% while a mean diversity of less than 0.4% was seen among other genotype E viruses

(distance matrix not shown). Members of genotype E, including the Nigerian isolates, were related most closely to viruses indigenous to chimpanzees (CHIMP genotype, mean divergence of 5.7%), members of genotype D (6.0%), and members of genotype C (6.4%). While the tree showed distinct sub-clusters within the branches of genotype D and CHIMP, no such pattern was found among genotype E.

In addition, in-frame deletions ranging from 12 to 21 nucleotides or four to seven amino acids in length were found in five of the Nigerian HBV isolates (Fig. 2). One of the isolates (NIE010) was also found to contain a single amino acid codon deletion at the 3'-end of the pre-S1 gene. Analysis of subtype-specific mutations within the sequences of the S protein showed the conservation of the lysine at residue 160 while the lysine at residue 122 was mutated to arginine (L122R) in all the twenty isolates. The leucine at residue 127 was also conserved. This pattern of mutation classified the isolates as subtype *ayw4*. Of all the twenty isolates, only one
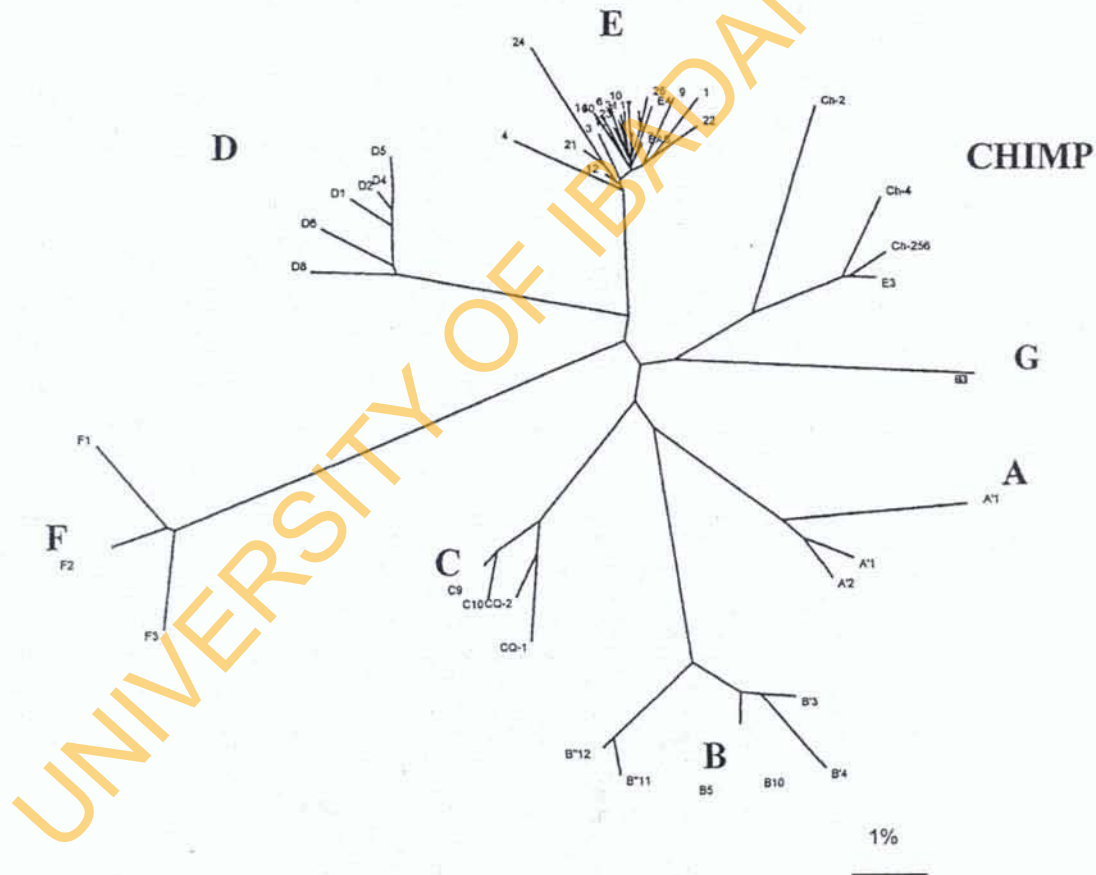

Fig. 1. Phylogenetic analysis of HBV isolates from Nigeria. Reference strains representing genotypes A, B, C, D, and F were named according to Bowyer and Sim [2000]. Genotype E; BAS, X75657; E4, X75664; Genotype G: IG, AF160501.

```
Consensus   VGVGAFGPGF TPPHGGLLGW SPQAQGMLKT LPADPPPAST NRQSGRQPTP ITPPLRDTHP
26          .......... .......... .......... .......... .......... ..........
5           ....L.... ........S  .......... .......... .......... ..........
1           .......... .......... .......... .......... .Y........ ..........
14          .......... .......... .......... .......... .......... ..........
9           .......... .......... ....S..... .......... .......... ..........
3           .......... .......... ..H....... .......... .......... ..........
6           .......... .......... .......... .......... .......... ..........
13          .......... .......... .......... .......... .......... ..........
7           .......... .......... .......... ....I..... .......... ..........
12          .......... .......... .......... .......... ...L...... ..........
4           .......... .......... .......... .......... ...L...... ..........
21          .......... .......... .......... .......... ...L...... ..........
30          .......... .......... .......... .......... .......... ..........
2           .......... ...L..... .......... ........Q. .......... ..........
31          .......... ...L.....S ....L..... .......... .......... ..........
23          .......... .......... .......... .......... .......... ..........
22          .......... .......... .......... .......... .......... ..........
10          .......... ...Q...... .......... .......... .......... ..........
24          .......... .......... .......... .......... ...L...... ..........
17          .......... .......... .......T.T .......... .......... ..........
```

```
Consensus   QAMQWNSTTF HQALQDPRVR GLYFPAGGSS SGTVNPVPTT ASLISSIFSR IGDLAPN
26          .......K.. .......... .......... .......... .......... .......
5           .......K.. .......... .......... .......... .......... .......
1           .......... .......I-  ---IR..... .......... .......K.. .......
14          .......... .......--- ---I...... .......... .......... .......
9           .......... .......--- ---P-..... .......... .......... .......
3           .......... .......... .......... .......... .......... .......
6           .......... .......... .......... .......... .......... ....V..
13          .......... .......... .......... .......... .......... .......
7           .......... .......... .......... .......... .......... .......
12          .......... .......... .......... .......... .......... .......
4           .......... .......... .......... .......... .......... .......
21          .......... .......... .L........ .......... .......... .......
30          .......... .......... .L........ .......... .......... .......
2           .......... .......K.. .......... .......... .......... ...L...
31          .......... .......K.. .......... .......... .......... ...L...
23          .......... .......K.. .......... .......... .......... ...L...
22          .......... .......... .V........ .......... .......... ....Q.
10          -H.....K.. ......-- --VS...... .......... .......... .......
24          ..IK..YK.. ......-- --VI...... .......... ....T..... .......
17          .......K.. .......... .......... .......... .......... ...L...
```

Fig. 2. Alignment of the amino acid sequences of the carboxy half of preS1 and complete preS2 regions of Nigerian HBV isolates. (−) indicate aa deletions.

(NIE003) showed a mutation (T131N) within the "a" determinant of the HBsAg sequence. Examination of amino acid changes in other regions of the HBsAg sequence apart from the "a" determinant showed mutations that are unique to three Nigerian isolates (F80S on isolate NIE014; G43R, H60Y, and Q101H on NIE009; I68N, C69W, and I92N on NIE004).

DISCUSSION

HBV isolates obtained from 20 cases of hepatitis and chronic HBV carriers from Ibadan, Nigeria were analysed in the present study. Although the isolates were obtained in the same hospital from patients living in the same city, no epidemiological linkage could be established among cases. Therefore, these HBV strains were considered to be representative of the strains circulating in the community [Hardie and Williamson, 1997]. HBV isolates generally share at least 85% nucleotide sequence homology. Based on a complete genome alignment, the genotype demarcation varies between 8.5 and 10% [Okamoto et al., 1988]. The pre-S2/S nucleotide sequences described here showed a mean divergence of 6.4–10.4% with reference sequences belonging to genotype A, B, C, and F. The Nigerian isolates cluster closely with genotype E

viruses (1.5% nt divergence) and are, therefore, classified as genotye E viruses.

With respect to genotype D, genotype E isolates showed a divergence of only 6.0%. This observation is similar to that of Kidd-Ljunggren et al. [1995] who, on the basis of the nucleotide sequences of the X gene, questioned the differentiation of genotype E as a separate monophyletic group distinct from genotype D. These workers showed that members of genotype E consistently clustered with genotype D irrespective of the phylogenetic methods used to analyse the X gene. However, recent analysis of complete genome of hepatitis B viruses showed that they do form a separate genotype when the envelope gene was analysed, despite a greater similarity of other genomic regions with genotype D. This phenomenon was demonstrated to be due to genome recombination events [Bowyer and Sim, 2000].

Only a few isolates from West Africa have been described so far and all of these were assigned to genotype E [Norder et al., 1994]. So far, genotype E viruses have been found exclusively in human sera from Angola, Gambia, Liberia, and Senegal and in Chimpanzees originating from West and Central Africa [Norder et al., 1994; Kidd-Ljunggren et al., 1995; Takahashi et al., 2000]. In other parts of Africa, e.g., South Africa, only the genotypes A, B, C, and D were found [Rivkina et al., 1988; Chirara and Chestsanga, 1994; Bowyer et al., 1997].

Furthermore, no genotype E virus has been detected so far in the Americas or any other part of the world despite the forced migration of slaves from West Africa to the New World [Norder et al., 1994; Arauz-Ruiz et al., 1997]. This may suggest that West Africa was free of chronic HBV until genotype E emerged after the slave trade subsided in the mid to late nineteenth century.

In this context, it is interesting that genotype E viruses have been isolated from captive and wild-born chimpanzees [Takahashi et al., 2000]. Although it was suggested that this could also be explained by the practice of injecting human serum into chimpanzees after their capture in Africa [Hu et al., 2000], HBV from West and Central African chimpanzees are related closely to genotype E (5.7%) (and genotype D; 6.1%; Fig. 1). Similar to the limited geographic distribution of genotype E, genotype F was found only in the Americas [Bollyky et al., 1997]. On the basis of signature pattern analysis of amino acids, both genotype E and F viruses are related most closely to chimpanzee viruses [Takahashi et al., 2000]. Thus, chimpanzees may be a possible source of separate primate to human transmission events of HBV both in South America and West Africa.

Despite the different host species and geographic origins, a diversity of less than 1% was observed among earlier isolates of genotype E, suggesting that genotype E may be less variable than other genotypes. Other researchers [Bowyer and Sim, 2000] showed that members of genotypes A, B, C, and D are diverse enough to justify classification into subgroups with inter-subgroup divergence of up to 4%. This high level of intragenotypic diversity, even among isolates found within the same city [Bowyer et al., 1997; Mbayed et al., 1998], has been interpreted as an indication of endemicity and a long natural history of these genotypes within their human hosts. In our isolates, combined with earlier genotype E isolates, the diversity of genotype E reached only 1.5%. The low diversity of these isolates from a country for which a hepatitis B prevalence of up to 40% has been reported [Olubuyide et al., 1997] is therefore particularly striking, and may be an additional indication of a more recent introduction of genotype E viruses from a limited source.

HBV is well known to develop adaptive strategies to overcome host immune mechanisms. As a result, novel variants may evolve. Mutations within the "a" determinant of the major S protein have been described in surface antigen mutants following hepatitis B immunoglobulin treatment or vaccination [Zuckerman, 2000]. An analysis of the variability of the "a" determinant of HBsAg in the present study showed that none of the viruses carry mutations previously associated with escape from neutralising antibodies. However, one of the twenty isolates from Nigeria (NIE003) carries a mutation in this important antigenic determinant. The T131N mutation, within the major hydrophilic region of the "a" determinant, although a natural variation of the adw serotype, was recently found in Singaporean patients and vaccinees who tested negative for HBsAg, but positive for anti-HBV core antibodies (anti-HBc) [Chen and Oon, 2000]. These workers showed that this mutation resulted in failure to detect HBsAg in the sera of patients by antigen capture enzyme immunoassay (EIA) using monoclonal and polyclonal sera directed against the "a" determinant. These mutants were also not neutralised by levels of anti-HBs antibodies (10 mIU/ml) that are generally believed to be protective. This mutation did not interfere with the detection of HBsAg by Murex antigen capture EIA kit. The apparent discrepancy between both studies may be explained by differences in formats and antibodies in the in-house [Chen and Oon, 2000] and the commercial EIA.

Although mutations within the "a" determinant often lead to the emergence of mutants that escape from vaccine induced neutralising antibodies, mutations outside the "a" determinant have also been found to be very important, especially in mutants escaping from antibodies resulting from natural infection [Moriyama et al., 1991]. Other researchers [Gerken et al., 1991; Santantonio et al., 1992; Yamamoto et al., 1994] have consistently associated mutants showing deletions of 39–60 nucleotides in the pre-S2 region with chronic HBV infections. Two viruses (NIE010 and NIE024) that were collected from patients almost one year after onset of acute hepatitis, had a four-amino acid deletion in the pre-S2 regions. Isolate NIE024 had a mutation that abolished the AUG initiation codon, in the pre-S2 region, for the translation of the middle S protein. This is in agreement with the finding of a high level of

rearrangements and inactivating mutations in the pre-S2 region during progression from acute to chronic HBV infection [Gerner et al., 1998]. Surprisingly, however, a four- and a seven-amino-acid deletion was also found in NIE001 and NIE009, two viruses associated with cases of acute hepatitis with high levels of serum transaminases and bilirubin. The amino terminal end of pre-S2, represented by residues 1–24, has been shown to contain potent HLA-1 and HLA-2 restricted immunodominant epitopes important in the primary response to HBV infection [Meisel et al., 1994; Chisari, 1995]. It is, therefore, conceivable that the occurrence of deletions and mutations in this region will confer an advantage on the HBV present in the patients even during the acute phase of the infection. Thus, deletion mutants may evolve early during the disease and predominate during chronic infection. The finding of such mutants in acute hepatitis may suggest that viruses carrying immune-induced "escape mutations" that are generally associated with symptom-free chronic HBV infection in non-endemic areas may be found also in acute cases of hepatitis in endemic regions.

A detailed knowledge of the variability within the antigenic determinants of HBV is important in the design of subunit vaccines and diagnostic reagents for use in the endemic areas of the world. Furthermore, phylogenetic studies may contribute to a better understanding of the natural history of HBV strains circulating in West Africa and beyond.

ACKNOWLEDGMENTS

REFERENCES

Arauz-Ruiz P, Norder H, Visona KA, Magnius LO. 1997. Molecular epidemiology of hepatitis B virus in Central America reflected in the genetic variability of the small S gene. J Infect Dis 176:851–858.

Bollyky PL, Rambaut A, Grassly N, Carman WF, Holmes EC. 1997. Hepatitis B virus has a New World evolutionary origin. Hepatology 26:765.

Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim van Dillen PM, van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin Microbil 28:495–503.

Borchani-Chabchoub I, Gargouri A, Mokdad-Gargouri R. 2000. Genotyping of Tunisian hepatitis B virus isolates based on the sequencing of pre-S2 and S regions. Microbes Infect 2:607–612.

Bowyer SM, Sim JGM. 2000. Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates. J Gen Virol 81:371–392.

Bowyer SM, Van Staden L, Kew MC, Sim JGM. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 78:1719–1729.

Boyer JC, Bebenek K, Kunkel TA. 1992. Unequal HIV-1 reverse transcriptase error rates with RNA and DNA templates. Proc Natl Acad Sci USA 89:6919–6923.

Chen WN, Oon CJ. 2000. Hepatitis B virus Surface Antigen (HBsAg) mutants in Singapore Adults and vaccinated children with high anti-hepatitis B virus antibody levels but negative for HBsAg. J Clin Microbiol 38:2793–2794.

Chirara MM, Chestsanga CJ. 1994. Variant of hepatitis B virus isolated in Zimbabwe. J Med Virol 42:73–78.

Chisari FV. 1995. Hepatitis B virus immunopathogenesis. Ann Rev Immunol 13:29–60.

Gerken G, Kremsdor D, Capel F, Petit MA, Daugnet C, Manns MP, Meyer zum Büschenfelde RK, Brechot C. 1991. Hepatitis B defective virus with rearrangements in the pre-S gene during chronic HBV infection. Virology 183:555–565.

Felsenstein J. 1993. PHYLIP: phylogeny inference package (version 3.5c). Distributed by the author. Department of Genetics University of Washington. Seattle, Washington.

Gerner PR, Friedt M, Oettinger R, Lausch E, Wirth S. 1998. The hepatitis B virus seroconversion to Anti-Hbe is frequently associated with HBV genotype changes and selection of pre-S2-defective particles in chronically infected children. Virology 245:163–172.

Hardie DR, Williamson C. 1997. Analysis of the pre-S1 gene of hepatitis B virus (HBV) to define epidemiologically linked and un-linked infections in South Africa. Arch Virol 142:1829–1841.

Hu X, Margolis HS, Purcell RH, Ebert J, Robertson BH. 2000. Identification of hepatitis B virus indigenous to chimpanzees. Proc Natl Acad Sci USA 97:1661–1664.

Kidd-Ljunggren K, Oberg M, Kidd AH. 1995. The hepatitis B virus X gene: analysis of functional domain variation and gene phylogeny using multiple sequences. J Gen Virol 76:2119–2130.

Magnius LO, Norder H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirol 38:24–34.

Mbayed VA, Lopez JL, Telenta PF, Palacios G, Badia I, Ferro A, Galoppo C, Campos R. 1998. Distribution of hepatitis B virus genotypes in two different pediatric populations from Argentina. J Clin Microbiol 36:3362–3365.

McIntosh ED, Givney R, Zhang SS, Courouce AM, Burgess M, Cossart YE. 1998. Molecular epidemiology and variation of hepatitis B in recent immigrant families to Australia. J Med Virol 56:10–7.

Meisel H, Sominskaya I, Pumpen P, Pushko P, Borisove G, Deepen R, Lu P, Spiller GH, Kruger DH, Grens E, Gerlich W. 1994. Fine mapping and functional characterization of two immunodominant regions from the pre-S2 sequence of hepatitis B virus. Intervirol 37:330–339.

Moriyama K, Nakajima E, Hohjoh H, Asayama R, Okochi K. 1991. Immunoselected hepatitis B virus mutant. Lancet 337:125.

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

Norder H, Hammas B, Lee SD, Bile K, Courouc AM, Mushahwar IK, Magnius LO. 1993. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. J Gen Virol 73:1201–1208.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Maumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence comparison of surface andtigen subtypes. J Gen Virol 69:2575–2583.

Olubuyide IO, Ola SO, Aliyu B, Dosumu OO, Arotiba JT, Olaleye DO, Odaibo GN, Odemuyiwa SO, Olawuyi F. 1997. Hepatitis B and C in doctors and dentists in Nigeria. Q J Med 90:417–422.

Rivkina MB, Lunin VG, Mahov AM, Tikchonenko TI, Kukain RA. 1988. Nucleotide sequence of integrated hepatitis B virus DNA and human flanking regions in the genome of the PLC/PRF/5 cell line. Gene 64:285–296.

Santantonio T, Jung MC, Schneider R, Fernholz D, Milella M, Monno L, Pastore G, Pape GR, Will H. 1992. Hepatitis B virus genomes that cannot synthesize pre-S2 proteins occur frequently and as dominant virus populations in chronic carriers in Italy. Virology 188:948–952.

Seeger C, Mason WS. 2000. Hepatitis B virus biology. Microbiol Mol Biol Rev 64:51–68.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, SchinaziRF, Rossau R. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 81:67–74.

Takahashi K, Brotman B, Usuda S, Mishiro S, Prince AM. 2000. Full-genome sequence analyses of hepatitis B virus (HBV) strains recovered from chimpanzees infected in the wild: implications for an origin of HBV. Virology 267:58–64.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL:X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, Okamoto H, Miyakawa Y, Mayumi M. 1994. Natuarlly occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 68:2671–2676.

Zuckerman A. 2000. Effect of hepatitis B virus mutants on efficacy of vaccination. Lancet 335:1382–1383.