

• VIRAL HEPATITIS •

Follow up of infection of chacma baboons with inoculum containing a and non-a genotypes of hepatitis B virus

Marina Baptista, Anna Kramvis, Saffie Jammeh, Jocelyn Naicker, Jacqueline S. Galpin, Michael C. Kew

Marina Baptista, Anna Kramvis, Saffie Jammeh, Jocelyn Naicker, Michael C. Kew, University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa

Jacqueline S. Galpin, School of Statistics and Actuarial Sciences, University of the Witwatersrand, Johannesburg, South Africa

Correspondence to: Professor Michael C. Kew, Department of Medicine, Witwatersrand University Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa. kewmc@medicine.wits.ac.za

Telephone: +27-11-4883628 **Fax:** +27-11-6434318

Received: 2002-12-05 **Accepted:** 2002-12-22

Abstract

AIM: To determine whether one genotype (A or non-A genotypes of HBV) predominated over the other during the course of HBV infection.

METHODS: Four baboons were inoculated with HBV. DNA was extracted from serum obtained at monthly intervals post-inoculation for 52 weeks and HBV DNA was amplified using primers specific for the core region containing an insert characteristic of genotype A (nt 2 354-2 359, numbering from the *EcoRI* site). The amplicons were cloned into PCR-Script™ and a minimum of 15 clones per time point were sequenced in both directions.

RESULTS: Both genotypes persisted for the entire follow-up period of 52 weeks. Genotype non-A predominated in two baboons and genotype A in one baboon. Neither genotype predominated in the fourth baboon, as shown at a 5 % level of testing.

CONCLUSION: No conclusions concerning the dominance of either genotype or the natural progression or replication rates of HBV could be drawn because the pattern of the genotypes found may have been caused by sampling fluctuations at the time of DNA extraction and cloning as a result of the very low viral loads in the baboon sera.

Baptista M, Kramvis A, Jammeh S, Naicker J, Galpin JS, Kew MC. Follow up of infection of chacma baboons with inoculum containing a and non-a genotypes of hepatitis B virus. *World J Gastroenterol* 2003; 9(4): 731-735

<http://www.wjgnet.com/1007-9327/9/731.htm>

INTRODUCTION

Xenografts from closely related non-human primates or pigs have been suggested as one way to alleviate the chronic shortage of donor organs for human liver transplantation. Baboons (*Papio* species) which are phylogenetically close to humans, have reasonably large livers, and are not endangered. They have already been the source of xenografts for two humans undergoing liver transplantation^[1-3] (as well as for a few patients receiving kidney or heart transplants^[2, 4-6]). The use of liver xenografts would, however, be precluded if they

were infected by zoonotic pathogens. In addition, because chronic hepatitis C and B virus infections are now the most frequent causes of end-stage liver disease requiring transplantation in humans^[7], the donor livers should be resistant to infection with these viruses. Our study has previously shown that Chacma baboons (*Papio ursinus orientalis*) are resistant to infection of hepatitis C virus^[8] but are susceptible to infection of hepatitis B virus (HBV)^[9].

In the latter study^[9], pooled serum from three patients with acute hepatitis B (the serum of all three was HBV surface (HBsAg)- and e antigen (HBeAg)-positive and had high titers of HBV DNA) had been injected into the baboons. Direct sequencing of HBV DNA amplified at various times post-inoculation indicated that the baboons had been inoculated with a mixed population of HBV. Cloning of the HBV DNA amplified from the inoculum revealed that it fortuitously contained approximately equal proportions of A and non-A genotypes of HBV. HBV has been classified into genotypes A-H, with an intergenotypic diversity of at least 8 %^[10-13]. Genotype A accounts for 80 % and genotype D for 10 % of the genotypes found in southern Africa, with the other genotypes being either absent or present in very few isolates^[14]. HBV genotypes have a characteristic geographical distribution^[15], which help in tracing the route of HBV infection^[16], and may influence the severity and outcome of infection with this virus^[17]. However, little is known about the natural progression and severity of the infection when more than one genotype is present^[18].

Co-infection with two or more genotypes may be the consequence of multiple exposures to infection at an early age when immune responses are immature or in older individuals with immune disorders^[19], or the result from genotype changes during seroconversion from e antigen positivity to negativity^[20]. Documented cases of co-infection with more than one genotype of HBV were rare^[18, 19, 21, 22], and the natural progression of HBV infection in this circumstance has not been thoroughly evaluated. In one patient studied recently, infection with genotypes D and A (with D predominant) was serologically "silent" (HBsAg-negative but HBV DNA-positive), although with pathological consequences because the patient was cirrhotic and died of liver failure^[18]. This patient may be of particular interest in view of our failure to detect HBsAg in the serum of our HBV DNA-positive inoculated baboons^[9]. Therefore, the opportunity was taken in the present study to monitor the changes over time in the relative proportions of genotypes A and non-A in the inoculated baboons and to ascertain if the two genotypes differed in their rate of replication or in their ability to persist in the inoculated baboons.

MATERIALS AND METHODS

Samples

For this study, serum samples which were collected at 4-weekly intervals from 4 baboons infected by inoculated HBV^[9] were analyzed which was started at week 8,. Because the initial phases of the study included the housing and inoculation of the baboons, the collection of serum samples were carried out at the Medical University of Southern Africa, this study was

undertook with the permission given by the Animal Ethics Committee of that institution. The Committee approved the procedures, and the care of the baboons according to its guidelines and those of the South African Medical Research Council. Each of the baboons had received intravenous injection of 1 ml pooled serum obtained from 3 HBV surface antigen (HBsAg) and HBV e antigen (HBeAg)-positive patients with clinically-overt acute hepatitis B. The concentration of HBV DNA in the pooled sera was 2 133 pg/ml (which was 2 982 pg/ml in another laboratory) using the Digene Hybrid Capture System (Digene Diagnosis Inc., Beltsville, MD, USA); the values in the individual isolates were 2 870 pg/ml, 6 660 pg/ml and 692 pg/ml, respectively. Using methods of amplification and cloning (see below), the HBV DNA amplified from the pooled inoculum was shown to contain equal proportions of genotype A and non-A of HBV. All of the baboon sera were tested for HBsAg, anti-HBc, and anti-HBs using commercially available assays (Abbott Laboratories, Chicago, IL, USA). All serum samples were stored at -20 °C.

PCR assay of HBV

HBV DNA levels were assessed with a quantitative PCR assay (Amplicor™ HBV monitor test, Roche Diagnostics). Briefly, 50 µl of serum were prepared with pre-treatment with polyethylene glycol, alkaline lysis of the pelleted viral particles, and neutralization of the lysate. After adding a fixed amount of internal standard and a PCR mix, 30 cycles of PCR amplification were performed according to the manufacturer's instructions. Biotinylated amplicons were captured on streptavidin-coated microwells and hybridized with specific dinitrophenyl-labelled oligonucleotide probes. It was incubated with alkaline phosphatase conjugated anti-DNP antibodies and a colorimetric substrate, then a kinetic of O.D. determination of HBV DNA levels was performed. The limit of detection of this PCR assay was 400 copies of viral genome per ml, and quantitation was linear up to 4×10^7 copies per ml^[23,24].

DNA extraction

DNA was extracted from serum using the QIAamp blood kit (Qiagen Inc., Hilden Germany), according to the manufacturer's instructions and as previously described^[25]. Known positive and negative sera, as well as best quality water were used as controls for the extraction procedure.

PCR of HBV DNA

HBV DNA in the core region was amplified using primers designed to amplify all the HBV genotypes (Table 1A and 1B). PCR was performed in 25 µL and 50 µL final reaction volumes for the first and second rounds, respectively. The reaction for the first round of the PCR consisted of 0.02 U/µL Dynazyme™ Taq DNA polymerase (version 2.0, Finnzymes OY, Espoo, Finland), 200 µmol/L of each of the nucleotide triphosphates, 1 µmol/L of each of the primers, 4 mmol/L MgCl₂ and 10 mmol/L Tris-HCl (pH 8.8 at 25 °C), 50 mmol/L KCl, and 0.1 % Triton® X-100. The reaction mixture for the second round of the PCR was the same as for the first round except that 1.5 mmol/L MgCl₂ was used. A third round of PCR was used on the serum from those time points that were negative after 2 rounds of PCR. The reaction mixture was the same as for the second round of PCR except that concentrations of MgCl₂ of 1.0 mmol/L, 1.5 mmol/L, and 1.5 mmol/L, were used for the first, second, and third rounds of PCR, respectively. All PCR assays were performed in a programmable thermal cycler (Perkin Elmer, Norwalk, CT, USA) with the 3-step cycling profile shown in Table 1B. Sera positive for HBsAg, HBeAg, and HBV DNA detected by slot-blot hybridization were used as positive controls and best quality water instead

of DNA as negative controls. To avoid cross-contamination and false-positive results, the precautions and procedures recommended by Kwok and Higuchi^[26] were strictly adhered to DNA extraction, the various stages of PCR amplification, and gel electrophoresis were performed in physically separate venues.

Table 1A Oligonucleotide primers

	Primer	Position ^a	Sequence
Double-round PCR	1687(+)	1687-1706	5' CGA CCG ACC TTG AGG CAT AC 3'
	2498(-)	2498-2477	5' AAG CCC AGT AAA GTT TCC CACC 3'
	2267(+)	2267-2284	5' GGA GTG TGG ATT CGC ACT 3'
	2436(-)	2436-2419	5' TGA GAT CTT CTG CGA CGC 3'
	1687(+)	1687-1706	5' CGA CCG ACC TTG AGG CAT AC 3'
	2498(-)	2498-2477	5' AAG CCC AGT AAA GTT TCC CACC 3'
Triple-round PCR	1795(+)	1795-1812	5' TGG TCT GTC GAC CAG CAC 3'
	2436(-)	2436-2419	5' TGA GAT CTT CTG CGA CGC 3'
	2267(+)	2267-2284	5' GGA GTG TGG ATT CGC ACT 3'
	2400(-)	2400-2382	5' CTG CGA GGC GAG GGA GTT 3'
	2400(-)	2400-2382	5' CTG CGA GGC GAG GGA GTT 3'
	2400(-)	2400-2382	5' CTG CGA GGC GAG GGA GTT 3'

Table 1B Polymerase chain reaction cycling profiles

		Amplification conditions			Size ^b
		Denaturation	Annealing	Extension	
Double-round PCR	PCR1	94°C 30 sec	62°C 40 sec	72°C 80 sec	812bp
	PCR2	94°C 30 sec	57°C 50 sec	72°C 50 sec	170bp
Triple-round PCR	PCR1	94°C 30 sec	62°C 40 sec	72°C 80 sec	812bp
	PCR2	94°C 30 sec	48°C 60 sec	72°C 50 sec	642bp
	PCR3	94°C 30 sec	50°C 60 sec	72°C 50 sec	134bp

Notes: PCR1: the first round polymerase chain reaction; PCR2: the second round polymerase chain reaction; PCR3: the triple round polymerase chain reaction. (+) sense; (-) anti-sense. ^aDenotes the nucleotide position of hepatitis B virus *adv* genome (GenBank accession #V00866) where the *EcoRI* cleavage site is position 1. ^bSize of the amplicons in base pairs.

Detection of amplified products

A 5 µl aliquot of the amplified PCR product was electrophoresed in a 2 % agarose gel. Bands of the appropriate size (Table 1A and 1B) were visualised under ultraviolet light after ethidium bromide staining.

Cloning and nucleotide sequencing

Following the double or triple round PCR, amplicons were cloned using PCR-Script (Stratagene, La Jolla, CA, USA). Plasmid DNA was extracted using the QIAprep spin miniprep kit (Qiagen Inc., Hilden, Germany) and restricted with *Pvu II* to confirm the presence of the correct insert (Table 1A and 1B). Sequencing of positive clones was performed using the T7 Sequenase Version 2.0 DNA sequencing kit (Amersham Life Science Inc., Cleveland, OH, USA). Sequences were analyzed in both forward and reverse directions with primers T3 and T7 on 8 % glycerol-tolerant acrylamide gels and autoradiographed. The number of clones of A and non-A genotypes obtained at each time point was used as a measure of the relative proportions of the two genotypes in the serum at that time.

Statistical analysis

Fisher's Exact test and the Chi-squared tests were used for statistical analysis, where appropriate.

RESULTS

HBV DNA was detected in the serum using either double or triple round PCR in the four inoculated baboons at various time points during the 52-week follow up period. For baboons 1 and 13 HBV DNA concentration was determined at various time of post-inoculation using the Amplicor HBV Monitor™ Test (Table 2). When serum was available for further analysis and HBV DNA was successfully amplified, the amplicons were cloned and sequenced. Genotype A was distinguished from the other genotypes by the sequence 5' CGGGAC3' (nt 2 354-nt 2 359, numbering from the Eco R1 site) that is specific to this genotype (Figure 1). Depletion of inoculum serum prevented us from amplifying the S region and carrying out restriction fragment length polymorphisms in order to assign the non-A genotype to one of genotypes B to H. Although we could not preclude the possibility that the non-A isolates were comprised of more than one genotype, the most likely genotype would be genotype D, the genotype besides A commonly found in South Africa^[14].

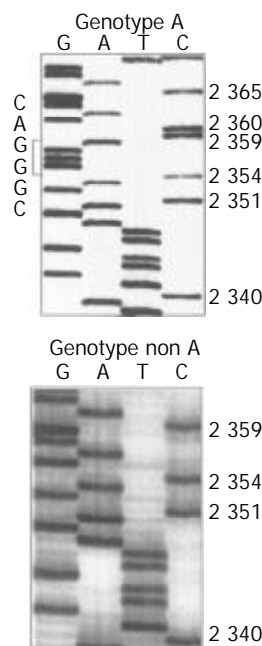


Figure 1 Sequence profiles of the core region (nucleotides 2 340-2 368 numbering from the *EcoRI* site) showing the insertion of 5' CGGGAC3' (nucleotide 2 354-2 359) found in genotype A and its absence in genotypes non-A. Tracks G, A, T and C are labelled.

Table 2 HBV DNA levels measured by the amplicor HBV monitor test

Time of post-inoculation (months)	HBV DNA Levels (genomes/ml)									
	2	3	4	5	6	7	8	9	10	10
Baboon 1	<400	<400	4 338	3 711	ns	ns	<400	ns	<400	<400
Baboon 13	<400	ns	ns	ns	ns	ns	2 6216	1 003	ns	ns

Notes: ns: no serum available.

Figure 2 showed the relative concentration of genotypes A and non-A at the various time points represented as a percentage of the total number of clones obtained and sequenced at that time point. The hypothesis of equal proportions of the genotypes over time was rejected for all four baboons ($P < 0.002$ in each instance). For each specific time/baboon combination, the hypothesis that the proportions of genotype A and non-A were equal was rejected in all but 4 cases, namely, at 10 months for baboons 2 and 12, and at 11 months for baboons 1 and 13.

Genotype non-A predominated in two baboons (baboon 2 and 13) and genotype A in one baboon (baboon 1); neither genotype predominated in the fourth baboon (baboon 12), as shown at a 5 % level of testing. In the three baboons in which one or other genotype predominated, there was at least one time-point at which the non-dominant genotype was present in the highest concentration at a proportion significantly different from zero.

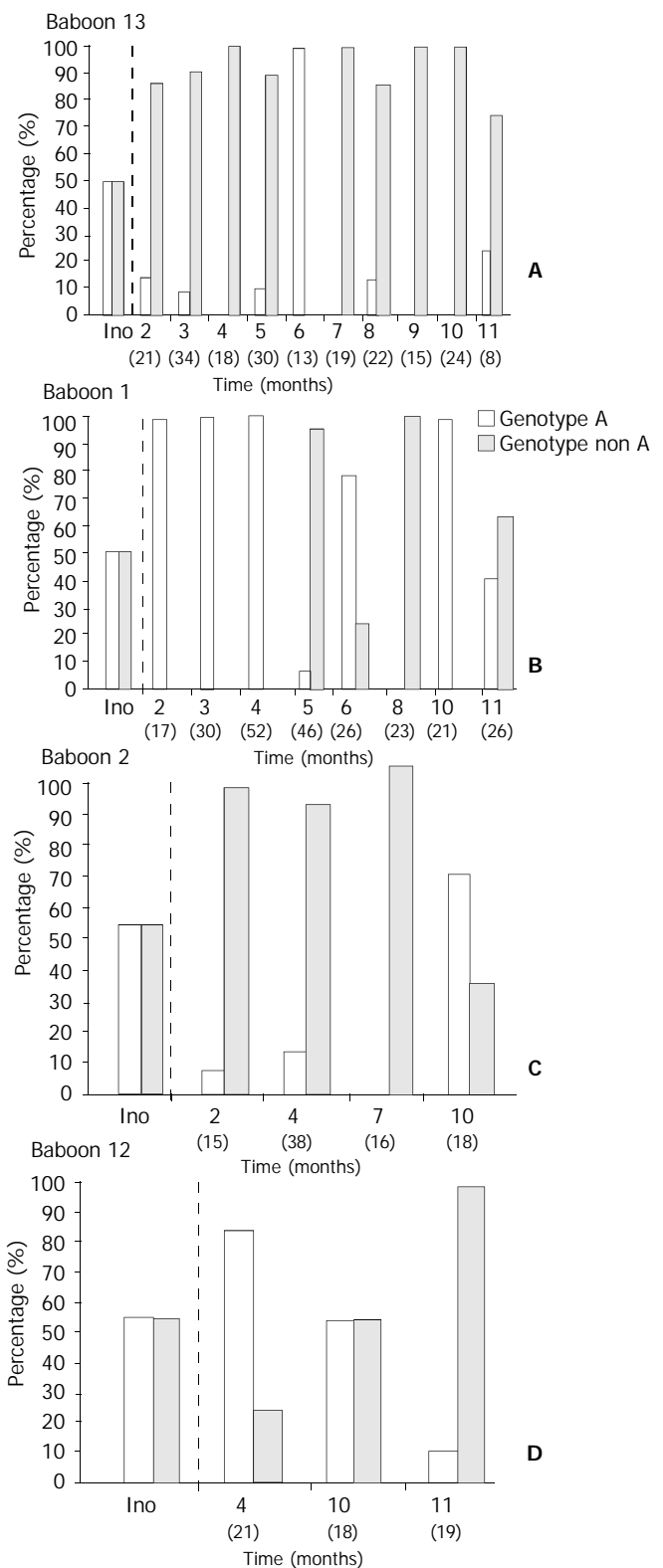


Figure 2 The change of genotype of hepatitis B virus at various time of post-infection which was represented as a percentage. The number of clones sequenced at each time point was showed in brackets. *ino: inoculum.

DISCUSSION

Neither HBsAg nor antibody to HBsAg (anti-HBs) was detected by conventional tests in the serum of the four inoculated baboons at any time during the 52 weeks they were monitored^[9]. HBV DNA identical to either the A or non-A HBV genotypes inoculated was, however, detected in each baboon throughout the follow-up period, and the presence of anti-HBc and Dane particles, small spherical particles and tubular particles was demonstrated in the serum at 16 weeks^[9]. The viral titers in the baboons were low (Table 2), as shown by the need to use nested PCR amplification to detect HBV DNA at all time points and three rounds of amplification at some time-points. No biochemical evidence of liver injury was evident at any stage and liver histology was normal at 52 weeks. These findings together suggested that the animals had become chronic asymptomatic carriers of the virus.

We had hoped that a clear pattern of different rates of replication of the two genotypes would be evident. However, no uniform pattern could be discerned in the relative concentration of genotypes A and non-A during the 52 weeks (Figure 2). Other explanations of a technical nature for the varying concentrations of the genotypes were needed therefore to be considered. One possibility was that the number of clones sampled at each time-point was too small to accurately assess the relative proportions of the genotypes in the serum at that time. This explanation was not supported by statistical analysis of the numbers of clones involved at each time point. A more likely explanation was that the low copy number of the genotypes resulted in sampling error at the time of HBV DNA extraction and cloning. This explanation was particularly apposite for the genotype pattern in baboon 13, in which genotype A alone was found in a single sample, whereas at all other time-points on either side of this sample, genotype non-A either predominated or was the sole genotype cloned (Figure 2A). It was also relevant that the conditions created in the study were artificial on two counts: the approximately equal concentrations of the two genotypes inoculated into the baboons resulted from the pooling of three serum samples from different patients, and we were assessing the effects of a human virus injected into a non-human primate.

Jeantet and co-workers were able to clone and sequence entire HBV genomes and found a number of mutations in the surface, precore and other regions affecting expression of the surface gene in both genotypes^[18]. The A genotype was fully replication competent, although, surprisingly, this was not true of the predominant D genotype. Sequencing of the subgenomic amplicons of HBV from our infected baboons did not reveal any mutations in the core region of the HBV isolates. Full genome analysis was impossible in our study because the study was carried out retrospectively. Either the serum samples were depleted or when serum was available full genome amplification did not work, possibly because of the low viral load.

The very low concentrations of HBV in the serum of the infected baboons (Table 2) and the resulting likelihood of sampling error during viral DNA extraction and cloning prevented us from drawing firm conclusions about the natural progression over time of genotypes A and non-A replication in baboons. The study did however show that both genotypes persisted for the entire period of 52-week of follow-up.

ACKNOWLEDGEMENTS

The authors thank Professor F. Chisari, The Scripps Research Institute for his helpful comments. The H.E. Griffin Cancer Trust supported this work.

REFERENCES

- 1 Lanford RE, Michaels MG, Chavez D, Brasky K, Fung J, Starzl TE. Persistence of extrahepatic hepatitis B virus DNA in the absence of detectable hepatic replication in patients with baboon liver transplants. *J Med Virol* 1995; **46**: 207-212
- 2 Starzl TE, Machioro TL, Peter GNH. Renal heterotransplantation from baboons to man: experience with 6 cases. *Transplantation* 1964; **2**: 752-756
- 3 Starzl TE, Fung J, Tzakis A, Todo S, Demetris AJ, Marino IR, Doyle H, Zeevi A, Warty V, Michaels M, Kusne S, Rudert WA, Trucco M. Baboon-to-human liver transplantation. *Lancet* 1993; **341**: 65-71
- 4 Bailey LL, Nehlsen-Cannarella SL, Concepcion W, Jolley WB. Baboon-to-human cardiac xenotransplantation in a neonate. *JAMA* 1985; **254**: 3321-3329
- 5 Barnard CN, Wolpowitz A, Losman JG. Heterotopic cardiac transplantation with a xenograft for assistance of the left heart in cardiogenic shock after cardiopulmonary bypass. *S Afr Med J* 1977; **52**: 1035-1038
- 6 Hitchcock CR, Kiser JC, Telander RL, Seljeskog EL. Baboon renal grafts. *J Am Med Assoc* 1964; **189**: 934-937
- 7 Sheiner PA. Hepatitis C after liver transplantation. *Semin Liver Dis* 2000; **20**: 201-209
- 8 Sithebe NP, Kew MC, Mphahlele MJ, Paterson AC, Lecatsas G, Kramvis A, de Klerk W. Lack of susceptibility of Chacma baboons (*Papio ursinus orientalis*) to hepatitis C virus infection. *J Med Virol* 2002; **66**: 468-471
- 9 Kedda MA, Kramvis A, Kew MC, Lecatsas G, Paterson AC, Aspinall S, Stark JH, De Klerk WA, Gridelli B. Susceptibility of chacma baboons (*Papio ursinus orientalis*) to infection by hepatitis B virus. *Transplantation* 2000; **69**: 1429-1434
- 10 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
- 11 Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69** (Pt 10): 2575-2583
- 12 Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67-74
- 13 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059-2073
- 14 Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; **78** (Pt 7): 1719-1729
- 15 Courouce-Pauty AM, Plancon A, Soulier JP. Distribution of HBsAg subtypes in the world. *Vox Sang* 1983; **44**: 197-211
- 16 Yamishita Y, Kurashina S, Miyakawa Y, Mayumi M. South-to-north gradient in distribution of the r determinant of hepatitis B surface antigen in Japan. *J Infect Dis* 1975; **131**: 567-569
- 17 Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 1999; **6**: 299-304
- 18 Jeantet D, Chemin I, Mandrand B, Zoulim F, Trepo C, Kay A. Characterization of two hepatitis B virus populations isolated from a hepatitis B surface antigen-negative patient. *Hepatology* 2002; **35**: 1215-1224
- 19 Yamanaka T, Akahane Y, Suzuki H, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M. Hepatitis B surface antigen particles with all four subtypic determinants: point mutations of hepatitis B virus DNA inducing phenotypic changes or double infection with viruses of different subtypes. *Mol Immunol* 1990; **27**: 443-449
- 20 Gerner PR, Friedt M, Oettinger R, Lausch E, Wirth S. The hepatitis B virus seroconversion to anti-HBe is frequently associated with HBV genotype changes and selection of preS2-defective particles in chronically infected children. *Virology* 1998; **245**: 163-172
- 21 Okamoto H, Imai M, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. Point mutation in the S gene of hepatitis B virus for a d/y or w/r subtypic change in two blood donors carrying a surface antigen of compound subtype adyr or adwr. *J Virol* 1987; **61**: 3030-3034
- 22 Paul DA, Purcell RH, Peterson DL. Use of monoclonal antibodies.

- ies to determine if HBsAg of mixed subtype is one particle or two. *J Virol Methods* 1986; **13**: 43-53
- 23 **Gerken G**, Gomes J, Lampertico P, Colombo M, Rothaar T, Trippler M, Colucci G. Clinical evaluation and applications of the Amplicor HBV Monitor test, a quantitative HBV DNA PCR assay. *J Virol Methods* 1998; **74**: 155-165
- 24 **Kessler HH**, Pierer K, Dragon E, Lackner H, Santner B, Stunzner D, Stelzl E, Waitzl B, Marth E. Evaluation of a new assay for HBV DNA quantitation in patients with chronic hepatitis B. *Clin Diagn Virol* 1998; **9**: 37-43
- 25 **Kramvis A**, Bukofzer S, Kew MC, Song E. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. *Hepatology* 1997; **25**: 235-240
- 26 **Kwok S**, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; **339**: 237-238

Edited by Xu XQ