

# A polymerase chain reaction-based assay to identify genotype F of hepatitis B virus

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## Abstract

We have developed a polymerase chain reaction (PCR) assay which distinguishes genotype F from the other genotypes of hepatitis B virus (HBV). The method was used to characterize HBV strains isolated in urban areas of the Brazilian Amazon. DNA was amplified in 54 of a total of 78 HBsAg-positive serum samples, using universal, non-genotype-specific primers. Only 4 (7.4%) were identified as genotype F by our genotype-specific PCR assay. This proportion is notably lower than that previously reported in Argentina, Venezuela, Peru, and Central America.

## Key words

- Hepatitis B virus
- Genotype F
- Subtype *adw4*
- Polymerase chain reaction
- Brazil

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Hepatitis B virus (HBV) is one of the most important agents of human liver disease, including acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HBV isolates have been classified into 9 serotypes, *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4*, *adrq*<sup>+</sup>, and *adrq*<sup>-</sup>, according to the antigenic determinants and subdeterminants of their surface antigen (HBsAg) (1). In all serotypes, the common "a" antigenic determinant is located within domains bordered by amino acids 120 to 147 of HBsAg. The expression of *d/y* and *w/r* determinants has been localized at residues 122 and 160, respectively. A shift from Lys to Arg at these sites is responsible for subtype changes (2). More recently, HBV has been divided into six genotypes, A to F, by comparison of complete nucleotide sequences (3). HBV serotypes and genotypes are not uniformly distributed around the world. Genotype A is commonly found in Europe, USA, and sub-Saharan Africa. Genotypes B and C seem to

be confined to original populations of the Far East, genotype D is the most widespread, and genotype E has only been found in Africa. In South America, a large diversity of HBV strains has been demonstrated (4), more frequently of genotypes A, B, D, and F (5,6) and more rarely of genotype C (7).

Genotype F, which contains all *adw4* isolates identified, possesses two unique characteristics. First, it is the most diverging branch of the HBV phylogenetic tree, with a divergence of 15% compared to 8-11% among the other genotypes (8). Second, it has been detected in aboriginal populations of the Americas, suggesting an origin in the New World (8,9).

A simple and efficient genotyping method, as described for hepatitis C virus (10), would greatly facilitate molecular epidemiology studies of HBV. We developed a polymerase chain reaction (PCR) assay which distinguished HBV strains of genotype F from the others, and used it to characterize

HBV strains from the Brazilian Amazon Basin.

A panel of ten HBV strains of known subtype and genotype was used to develop a PCR assay for specific detection of genotype F isolates. Strains *adw2* (BrA and BrL; genotype A), *adw4* (BrC and 7-1991; F), *ayw2* (BrF and BrN; D), and *ayw3* (BrI and BrJ; D) have been previously characterized (5,11). They represent the four subtypes and three genotypes most commonly found in Brazil. Two *adr* strains (genotype C) from Japan were included in the panel because this subtype has been shown to infect South American populations of Asian origin (7). Seventy-eight HBsAg-positive samples collected in the cities of Manaus, State of Amazonas, and Macapá, State of Amapá (Brazilian Amazon region), were studied to determine the frequency of genotype F.

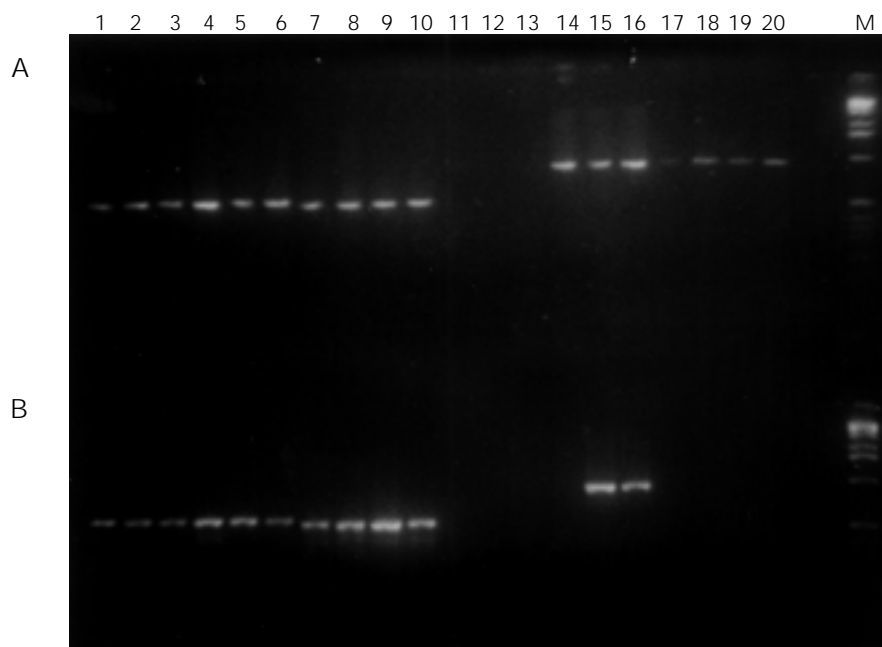
Two hundred microliters of serum were treated with 0.5 mg/ml of proteinase K in the presence of 0.2 M NaCl, 0.25% SDS, for 4 h at 37°C. HBV DNA was then extracted with phenol/chloroform and precipitated with ethanol (12). The pellet was dried and resuspended in 30 µl distilled water. PCR assays

were done with 1 µl of DNA in a volume of 50 µl.

Oligonucleotides PS1 and PS2 (4) were used as generic primers to amplify DNAs of all HBV strains, since their sequence seems to be well conserved in strains from around the world. Primers PS42 (5' ACACACATC CACAGGCAATGCAG 3') and S22 (5' GTATTTAAATGGATACCCACAGA 3'), localized at genome positions 3194-3216 and 841-819, respectively, were used for the amplification of sequences from genotype F. PCRs were done in 0.2-ml tubes placed in a Perkin-Elmer 2400 thermocycler. After an initial denaturation of 2 min at 94°C, DNA was amplified for 35 cycles at 94°C for 30 s, variable annealing temperature (see below) for 1 min, and 72°C for 2 min, followed by a final elongation of 7 min at 72°C. Ten microliters of amplification products were electrophoresed on 2.0% agarose gel and stained with ethidium bromide.

For molecular cloning, DNA from the pre-S/S region was amplified using PS1 and S2 (5' GGGTTTAAATGTATACCCAAA GA 3', nt 841 to 819) oligonucleotides (PS1-S22 for strains belonging to genotype F).

Figure 1 - Agarose gel electrophoresis of PCR products. The annealing temperature of PCR was 55°C (A) or 61°C (B). PCR primer pairs were PS1-PS2 (lanes 1 to 10) and PS42-S22 (lanes 11 to 20). Lanes 1, 2, 11, 12: strains *adr*; lanes 3, 4, 13, 14: *adw2*; lanes 5, 6, 15, 16: *adw4*; lanes 7, 8, 17, 18: *ayw2*; lanes 9, 10, 19, 20: *ayw3*. The molecular weight marker (M) was a 1-Kb DNA Ladder (Life Technologies, Gaithersburg, MD).



PCR products were quantified by ethidium bromide staining using DNA standards as controls, and 5-10 ng were ligated into a 50-ng pCRII vector (TA cloning kit; Invitrogen, San Diego, CA). Recombinant plasmid DNA was purified for sequencing by ultracentrifugation in a CsCl gradient. Nucleotide sequences were determined with a Cy5 autoread sequencing system (Pharmacia Biotech, Uppsala, Sweden) using M13 universal and internal S4 (5) primers. Sequencing reactions were analyzed on an ALFexpress automated sequencer (Pharmacia).

Two series of PCR experiments were carried out in parallel with the PS42-S22 primer pair and with the universal, non-genotype-specific PS1-PS2 primer pair. In the first experiments, when the annealing temperature for PCR was 55°C, all 10 strains yielded visible bands in agarose gel after amplification with primers PS1-PS2 (Figure 1A, lanes 1 to 10), whereas seven strains yielded visible bands with primers PS42-S22 (lanes 11 to 20). To increase PCR specificity, the annealing temperature was elevated

in a stepwise way two degrees at a time. At 61°C, all strains continued to give a positive result with PS1-PS2 universal primers, while only the two *adw4* isolates were amplified with PS42-S22 primers (Figure 1B). In addition, we noted that the size of the PS1-PS2 amplification products from *ayw* strains was slightly lower than that from *ad* isolates (compare lanes 1-6 to lanes 7-10 in Figure 1A and B). This was probably due to a 33-nucleotide deletion at the 5' end of the pre-S1 region of *ayw* strains.

DNAs of 78 HBsAg-positive samples originating from the Brazilian Amazon region were extracted and submitted to PCR (annealing temperature 55°C). Fifty-four of them were successfully amplified by PS1-PS2 primers. Decreasing annealing temperature to 50°C had no effect on the number of PCR-positive samples. Of these, 37 (69%) showed a DNA band of a size compatible with the *ad* profile, whereas 17 (31%) seemed to be *ay*. Using the PCR assay described above, DNAs from only four (7.4%) strains were amplified by using genotype F (*adw4*)

	111	d/y 122	w2-4 127	w/r 160	170			
BrQ	PGSTTTTSTG	<u>P</u> <u>K</u> TCTT	<u>L</u> AQGTSMF	SCCCSKP	SDGNCTC	IPSSWALG	<u>K</u> YLWEWASARF	
BrR	-----	-----	-----	-----	-----	-----	-----	<i>adw4</i>
BrS	-----	-----	-----	-----	-----	-----	-----	
BrW	-----	-----	P-----	N-----	T--T-----	P--FA-----	V--	
BrZ	-----	-----	P-----	N-----	T--M-----	FA-----	V--	
BrAB	-----	-----	P-----	N-----	T--T-----	FA-----	V--	<i>ad</i>
BrAL	-----	-----	P-----	N-----	T--T-----	FA-----	V--	
BrAP	-----	-----	P-----	N-----	T--T-----	FA-----	V--	
BrAC	---S-----	R---P-----	Y-----	T-----	-----	F--F-----	-----	
BrAF	---S-----	R---P-----	Y-----	T-----	-----	F--F-----	-----	
BrAG	---S-----	R---P-----	Y-----	T-----	-----	F--F-----	-----	<i>ay</i>
BrAN	---S-----	R---P-----	Y-----	T-----	-----	F--F-----	-----	
BrAQ	---S-----	R---P-----	Y-----	T-----	-----	F--F-----	-----	

Figure 2 - Amino acid sequences deduced from partial nucleotide sequence of S genes of 13 HBV strains from the Amazon region. Asterisks indicate the positions of the antigenic determinants and subdeterminants. The HBsAg subtype of the strains was deduced from the substitutions of codons 122 and 160, which determine d/y and w/r, respectively. Antigenic subdeterminant w4 is defined by a Leu at codon 127 (9). Note that all strains other than *adw4* possess a Pro defining the subdeterminant w2 at position 127.

specific primers. This proportion was much lower than that reported in other studies performed with samples from the Amazon region (13,14). This suggested that the DNAs of some *adw4* strains were not amplified in the genotype-specific PCR assay (false negative samples). To rule out this hypothesis, 10 strains presumed not to be *adw4* (five *ad* and five *ay*) were selected and serotyped by nucleotide sequencing of the S gene region (codons 111 to 170). The same was done for three presumed *adw4* strains. Amino acid sequences were then deduced and are shown in Figure 2. Sequencing results corroborated the serotype of the *adw4* strains. Also, the identities of the five *ad* (not *adw4*) and five *ay* strains were confirmed. These ten last strains possessed the *w2* subdeterminant. As is common in Brazil when a small number of samples is analyzed, no strain presented the *r* determinant. The sequence variations observed among HBV isolates from the Brazilian Amazon were not different from those previously detected in the city of Rio de Janeiro (5).

Molecular epidemiology studies of HBV would be facilitated by simple and fast serotyping or genotyping methods. Although HBV serotyping into nine different serotypes was developed over twenty years ago, it has never been implemented as a routine diagnostic method due to the lack of reagents and requirement for expertise in techniques such as immunodiffusion and immunoelectroosmophoresis (9). On the other hand, PCR has been extensively used in the study of hepatitis viruses, notably for rapid diagnosis, antiviral studies, and pathogenesis. In the last few years, assays have been developed to differentiate HBV subtypes by PCR using specific primers (15-17). Genotyping has been devised based on restriction fragment length polymorphism analysis of amplicons (18). Due to the importance of genotype F in the New World, a method capable of discriminating it should be useful for HBV molecular epidemiology

studies in the Americas. Recently, it was shown that the presence of a *SmaI* restriction site could be used to recognize sequences from genotype F (19). Our method presents the advantage that is very simple and fast. It does not need nested PCR or restriction endonuclease digestion. All the *adw4* strains belong to genotype F. Reciprocally, the large majority of strains from genotype F has been subtyped as *adw4*, although non-conventional genotype-subtype associations, namely *adw2* and *ayw4* within genotype F, have been recently described (13).

In Central America (20) and Venezuela (13), 75-80% of the HBV strains studied were genotype F. In the Peruvian Amazon Basin, an outbreak of acute hepatitis was provoked by a genotype F HBV (19). In Buenos Aires, Argentina, 5 of 12 (42%) sequenced HBV samples were genotype F (6). Data obtained by serotyping samples collected from 1980 to 1983 showed that serotype *adw4* represented about 40% of HBV in the Northern region of Brazil (Amazon region) (14). The proportion of *adw4* reported here was notably lower (7.4%). This difference may be a consequence of the use of some monoclonal antibodies that resulted in an overestimation of the percent of *adw4* in previous studies. Indeed, we have found that some strains previously characterized as *adw4* by monoclonal antibodies were identified *adw2* or *ayw3* by nucleotide sequencing (data not shown). In the other regions of Brazil, the proportion of *adw4* strains has been reported to vary from 4 to 8%. Our results suggest that the epidemiological pattern of the urban areas of the Brazilian Amazon region is similar to that of other parts of Brazil, probably due to internal migration from other regions.

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