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Specific primer sets used to amplify by PCR the hepatitis B virus overlapping S/Pol region select different viral variants

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SUMMARY. PCR detection of viral genomes has provided new insights into viral diagnosis. Nowadays, it is the most frequently used nucleic acid testing (qualitative and quantitative) technique. The aim of this study was to analyse the major circulating hepatitis B virus (HBV) variants PCR-amplified by three sets of primers in a patient infected with genotype E. The HBV S/Pol overlapping genomic region was amplified from the serum of an infected child using three primer sets previously described. Sequence analysis corresponding to the HBV S/Pol region revealed the presence of different viral populations depending on the set of primers used. D144A S-escape mutant was detected with two of the primer sets, while the rtL217R mutant within the Pol – conferring resistance to Adefovir – could be picked up with a different pair of primer sets. This study undoubtedly implies that the description of viral polymorphisms should be stated together with the sequence of the primers used for PCR amplification when studies of escape and/or antiviral-resistant HBV mutants are carried out.

Keywords: hepatitis B virus, primer sets, variants.

INTRODUCTION

The unique genomic structure and the replication cycle of hepatitis B virus (HBV) together with the high error-prone of its viral polymerase provide much opportunity for mutations to occur in any of its genes. Emergence of different viral variants or 'escape' mutants is probable. Several reports have documented the appearance of S-escape mutants from protective anti-HBs antibodies, as well as the occurrence of Pol-associated drug-resistant, precore/core and X mutants. Because of the overlapping arrangement of the four open reading frames (ORFs) of the HBV genome, almost every single mutation may influence more than one function of the corresponding nucleotide sequence [1].

Although some initial PCR limitations had led to misdiagnosing some HBV infections owing to false-positive or false-negative results, at present, PCR and especially its high throughput quantitative version (qPCR) are regarded as the benchmark technology for the detection/characterization/quantification of nucleic acids in a research or diagnostic setting.

Abbreviations: HBV, hepatitis B virus; ORF, open reading frames.

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The aim of this study was to analyse the major circulating HBV variants amplified by three sets of primers corresponding to the S/Pol overlapping region in the serum of a child infected with genotype E, who was previously reported to exhibit an escape S-mutant without any detectable anti-HBs [2].

MATERIAL, PATIENT AND METHODS

DNA was extracted using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) from the serum sample obtained from patient (S1) of African descent chronically infected with HBV, genotype E [2]. The HBV S/Pol overlapping genomic region was PCR-amplified using *Taq* DNA polymerase (Life Technologies Corp., Carlsbad, CA, USA). The HBV S/Pol overlapping region was amplified by PCR, using different sets of primers:

- sense primer P7 and antisense P8 described by Lindh *et al.* [3];
- sense primer HB1F and antisense HB6R, followed by a nested PCR with primers HB2F and HB2R reported by Sugauchi *et al.* [4]; and
- sense primer HBs1 and antisense primer HBs2 described by Cuestas *et al.* [5].

Appropriate precautions were strictly followed to avoid cross-contamination [6]. The potential *Taq* DNA polymerase misincorporation rate was investigated by bidirectionally sequencing a GB virus C clone fragment [2].

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Amplicons obtained were bidirectionally sequenced as well. After visual inspection of the chromatograms, nucleotide alignment and phylogenetic analysis were performed using the neighbour-joining method included within the PHYLIP package version 3.5 (by Joseph Felsenstein, University of Washington, Seattle, WA, USA).

RESULTS

Sequence analysis corresponding to the HBV S/Pol genes obtained from PCR products revealed the presence of different viral populations depending on the set of primers used (Fig. 1).

Two important implications derive from these observations. First, – depending on the primer set used – not all S-escape mutants could be recognized, as shown with the reportedly known D144A S-mutant, picked up with P7 and P8 (Fig. 1a), as well as with HBs1 and HBs2 primer sets (Fig. 1c), but not with that from Sugauchi *et al.* [4] (Fig. 1b). In other words, Sugauchi's primers failed to pickup in the analysed sample those genomes harbouring the A585C nucleotide change and picked preferentially the wild-type A585.

Secondly, depending also on the usage of a given set of primers, not all the antiviral resistance mutants already present in the patient's serum could be detected. In this regard, the L209V substitution in the S protein resulted in an rtL217R substitution within Pol, thus conferring resistance to adefovir. This was detected with primers P7 and P8 (Fig. 1d), as well as with those from Sugauchi *et al.* [4] (Fig. 1e), but not with primers HBs1 and HBs2, which preferentially picked up the wild-type T880 (Fig. 1f).

DISCUSSION

This study provides new insights into a previous report from the authors showing the circulation of an escape S-mutant in a chronically HBV genotype E-infected patient (S1) without any detectable anti-HBs [2]. Data obtained with Sugauchi's primer set detected a population of wild-type virus (D144), while those reported by Lindh *et al.* [3] and by Cuestas *et al.* [5] picked up the mutant variant, D144A.

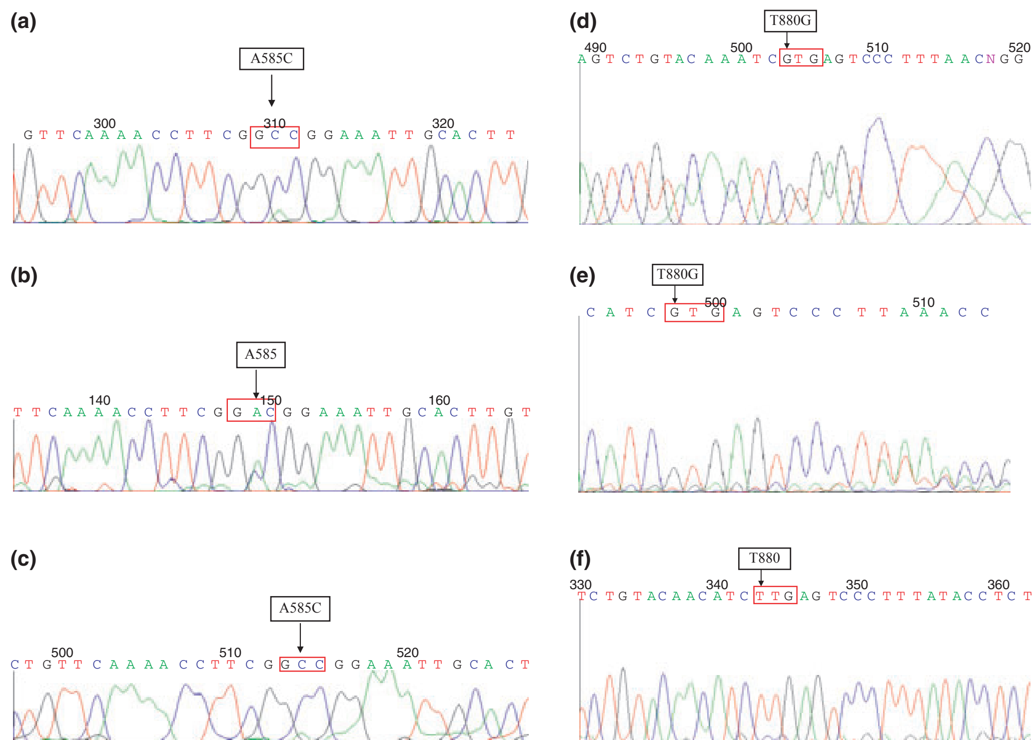

Fig. 1 Nucleotide sequences obtained after using different primer sets. (a, d) With P7 and P8 primers; (b, e) with primers used by Sugauchi *et al.*; (c, f) with HBs1 and HBs2 primers. The S-mutant D144A is observed in (a) and (c), whereas in (b), only wild-type virus is present. The L209V S-mutant (corresponding to the L217R antiviral-resistant mutant in the overlapping Pol gene) is shown in (d) and (e). The wild-type L209 viral population is represented in F. The numbers shown in the sets of 10 on top of the sequences refer to numbers assigned by the software to determine the position of nucleotides in the sequencing results. The letter and number inside each square above the sequence refer to the nucleotide and its position in the gene sequence. The arrow pointing to the nucleotide refers to the wild-type or the mutant nucleotide. The letters and numbers in the legend indicating the mutant, D144A or L209V refer to amino acid residues and their position in the protein sequence, respectively.

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Unexpectedly, this untreated patient showed also a natural antiviral-resistant rtL217R mutant within Pol, thus conferring resistance to adefovir, which was detected with 2 of the 3 primer sets mentioned previously.

Primer-related variant detection might be critical when mixed infections with distinct genotypes are involved. In this regard, although RFLP is a highly sensitive and reliable technique able to detect mixed infections after viral genome PCR amplification [7], the restriction enzymes may cut or not cut at the specific site, depending on the virus variant selected by the primers, potentially resulting in biased results. Furthermore, and even more crucial, the reliability of antiviral resistance studies should be cautiously interpreted, because some variants could not be detected in the analysis carried out with any given set of primers, as depicted in this study.

Given the potential presence of dissimilar viral variants in a given patient serum sample, results obtained in this study undoubtedly imply that the description of viral polymorphisms should be stated together with the sequence of the primers used (as should also be the case for qPCR). As most but not all peer-reviewed published research fulfil this requirement [8], data reported in this study strongly suggest to do so. This seems to be crucial because it is well known that an amplification performed with a single pair of primers or with a pair of primers followed by a nested reaction with internal primers may or may not result in the identification of known or new viral variants as demonstrated by Sugauchi *et al.* [4], as well as by the whole set of primers used in this research.

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CONFLICT OF INTEREST

None declared.

ETHICAL APPROVAL

This study was part of a Project, which had been approved by an Ethics Committee from the University of Buenos Aires. Daughter (S1)'s mother provided informed written consent to perform all analyses described in this study.

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