Pyrosequencing for Detection of Lamivudine-Resistant Hepatitis B Virus

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Chronic hepatitis B virus (HBV) infection can result in severe liver disease, including cirrhosis and hepatocellular carcinoma (10). The magnitude of this problem is very large because approximately 5% of the world’s population is chronically infected with HBV. Until relatively recently, alpha interferon was the only drug licensed for use for the treatment of HBV infection, but unfortunately, resistance to lamivudine commonly develops during monotherapy. Lamivudine-resistant HBV mutants display specific mutations in the YMDD (tyrosine, methionine, aspartate, aspartate) motif of the viral polymerase (reverse transcriptase [rt]), which is the catalytic site of the enzyme, i.e., methionine 204 to isoleucine (rtM204I) or valine (rtM204V). The latter mutation is often accompanied by a compensatory leucine-to-methionine change at codon 180 (rtL180M). In the present study, a novel sequencing method, pyrosequencing, was applied to the detection of lamivudine resistance mutations and was compared with direct Sanger sequencing. The new pyrosequencing method had advantages in terms of throughput. Experiments with mixtures of wild-type and resistant viruses indicated that pyrosequencing can detect minor sequence variants in heterogeneous virus populations. The new pyrosequencing method was evaluated with a small number of patient samples, and the results showed that the method could be a useful tool for the detection of lamivudine resistance in the clinical setting.

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### Materials and Methods

Reference material and patient samples. The initial optimization of the PCR protocol was performed with selected HBV DNA-positive serum samples that had undergone previous routine qualitative or quantitative HBV PCR at Clinical Virology, Huddinge Hospital. The samples were between 1 and 12 months old and had been stored at −20°C. The samples that were used for the optimization of extraction and PCR were subjected to repeated quantification by the Amplicor HBV Monitor assay (Roche Molecular Systems, Roche Diagnostics, Branchburg, N.J.).

For the mixing experiments described below we used plasma samples from one patient who had developed lamivudine resistance following liver transplantation. The sample (sample MUT) was kindly provided by Magnus Lindh at the Department of Virology, Swedish Institute for Infectious Disease Control, S-171 82 Solna, Sweden.
The principle and chemistry of pyrosequencing is based on real-time detection of PPi, as it is released when a DNA polymerase incorporates nucleotides into a growing primer-template complex. (A) The five steps of a single pyrosequencing cycle, which takes approximately 60 s to complete and involves the cooperative actions of four enzymes. The first step is the addition (dispensation) of a new nucleotide triphosphate (panels 1, right and left). If the nucleotide is complementary to the next position in the template (matching), it is incorporated and PPi is released (panel 2, left). If the nucleotide does not match the template, it is not incorporated and no PPi is released (panel 2, right). If PPi has been released, it is used to convert AMP into ATP (panel 3, left). ATP is used to produce visible light through the action of the firefly enzyme luciferase (panel 4). Thus, a light signal is generated if a complementary nucleotide is added, but no light signal is generated if a noncomplementary nucleotide is added. The cycle is completed when apyrase degrades unused dNTPs and ATP (panel 5). (B) Example of a theoretical pyrogram obtained by using a cyclic nucleotide dispensation order. The intensity of the light generated in each pyrosequencing cycle is visualized as peaks with heights that correspond to the number of incorporated nucleotides. The cyclic nucleotide dispensation order (Disp.:) is displayed directly under the pyrogram. The pyrosequencing result is displayed at the bottom of the panel. This type of cyclic nucleotide dispensation order is preferred if the sequence of interest is highly polymorphic. In our experiments we instead used a specific nucleotide dispensation order (C), because this allows the design of clearly distinguishable patterns for different genotypes (Fig. 2) and also reduces the number of pyrosequencing cycles. (C) Specific nucleotide dispensation order (Disp.:) displayed under the pyrogram. The pyrosequencing result is displayed at the bottom of the panel.
µL, which was the volume used in each PCR. The PCR results followed the Poisson distribution, and both strongly positive and negative results were observed with high dilutions, which indicates that the analytical sensitivity of the PCR was close to 1 HBV DNA copy.

Limiting dilution was also used to amplify single HBV molecules of lamivudine-resistant (MUT) and WT HBV variants for the mixing experiments described below. Serum samples that had been shown to contain primarily lamivudine-resistant or WT virus by direct population sequencing were diluted to contain, on average, less than 1 HBV DNA copy/µL. Single HBV molecules were amplified from the diluted material by the nested PCR described above. The PCR products were sequenced on an ABI310 instrument to verify that these "clones" had the expected sequence, i.e., 100% lamivudine resistant and 100% WT, respectively. The PCR products obtained from single HBV molecules (clones) were used in the mixing experiments described below.

### Sanger sequencing

The PCR product (45 µL) was purified with a QiAquick PCR purification kit (Qiagen, Hilden, Germany), according to the recommendations of the manufacturer. The purified amplicons were sequenced with an ABI PRISM BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase (FS: Perkin-Elmer). The sequencing reaction mixture contained 2 µL of Terminator Ready Reaction Mix, 6 µL of 2.5× Sequencing Buffer, 3 µL of template, 8 µL of deionized water, and 1 µL of one of the two inner PCR primers, primers JA225 and JA226. The cycle sequencing profile was 25 cycles of 96°C for 0.5 s, 50°C for 5 s, and 60°C for 4 min, followed by incubation at 4°C. The sequencing fragments were purified with 70% ethanol, 95% ethanol, and sodium acetate. Sequencing was performed on an ABI Prism 310 Genetic Analyzer with ABI Prism 310 Collection and Sequencing Analysis software. The sequences generated by the forward and reverse sequencing primers were assembled and analyzed with the software program Sequencer (Gene Codes Corporation, Ann Arbor, Mich.). The resulting complete sequences were translated into amino acid sequences.

### Pyrosequencing

The principle of pyrosequencing is illustrated in Fig. 1. Two primers were designed to anneal adjacent to the two resistance sites of interest, i.e., positions 180 and 204. The primer sequences and their positions in the HBV genome are provided (i.e., positions 180 and 204). The primer sequences and their positions in the HBV genome (positions 180 and 204). This was possible because the sequence of the HBV genome is not a cyclic order rather than a cyclic nucleotide dispensation order (Fig. 1B and C).

Pyrosequencing was performed at 25°C in a total volume of 50 µL in the automated 96-well pyrosequencer, according to the instructions of the manufacturer, with the PSQ SNP 96 reagent kit (Pyrosequencing AB, Uppsala, Sweden). We used a specific rather than a cyclic nucleotide dispensation order because pyrosequencing is performed at room temperature, which increases the risk of mispriming, which can generate a background sequence. We decided to add the nucleotides in a specific order rather than a cyclic order (Fig. 1B and C), and this was possible because the sequence of the HBV genome is not a cyclic order rather than a cyclic nucleotide dispensation order (Fig. 1B and C).

The nucleotide dispensation order was designed on the basis of our prior knowledge about the WT and MUT HBV sequence variants. An additional advantage of the specific nucleotide dispensation strategy is that it reduces the number of pyrosequencing cycles and thereby increases the read length and sequence quality. It was possible to use a specific nucleotide dispensation strategy because lamivudine resistance involves only a few sequence variants. If the sequence of interest had been highly polymorphic, we would have been forced to choose the less attractive cyclic nucleotide dispensation strategy.

### Mixing experiments

Mixtures of WT and resistant virus were created to evaluate the abilities of the Sanger and pyrosequencing methods to accurately detect and quantify minor sequence variants. PCR products from single HBV molecules (clones) representing WT and lamivudine-resistant (MUT) variants were prepared by limiting dilution as described above. The two viruses were both of genotype A, and apart from the two resistance mutations at positions 180 and 204, the two viruses had identical sequences in the region analyzed. The PCR products were quantified with a DNA 7500 Labchip kit (Agilent Technologies), according to the recommendations of the manufacturer. Briefly, a gel-dye mixture was prepared from a gel-matrix mixture and a gel-dye mixture. The gel-dye mixture was then added to the DNA chip, together with a donor marker, a DNA 7500 ladder for measurement of size, and the samples. The chip was inserted in a 2100 Bioanalyzer (Agilent Technologies). The DNA content in each sample was analyzed three times, and the mean of the three runs was calculated. The quantified PCR products were diluted to the same concentration, and mixtures ranging from 100% WT-0% MUT to 0% WT-100% MUT were prepared at increments of 10%. The WT-MUT mixtures were analyzed by traditional Sanger sequencing and pyrosequencing.

### RESULTS

#### Optimization of PCR and pyrosequencing

The PCR system was optimized by using selected serum samples from HBV-infected patients. The final optimized PCR protocol for amplification of the two positions in the HBV rt that are involved in development of resistance to lamivudine (positions 180 and 204) is described in Materials and Methods. The sensitivity of the optimized PCR was investigated by limiting dilution experiments and calculations performed by using the Poisson distribution formula. The results indicated that the analytical sensitivity of the PCR system was close to 1 HBV DNA copy per PCR (data not shown). Thus, it should be possible to perform the resistance test with samples with as little as 20 to 50 HBV DNA copies/ml if 500 µL of serum is used for sample preparation. This includes all samples for which testing for lamivudine resistance may be clinically relevant because patients infected with lamivudine-resistant HBV generally have much higher virus titers.

The principle of pyrosequencing is illustrated in Fig. 1. For pyrosequencing, two primers were designed for sequence analysis for mutations at positions 180 and 204. The primers were designed to anneal closely upstream of the positions of interest since pyrosequencing has a read length of approximately 40 bp. Special attention was paid to the specificity of primer annealing because pyrosequencing is performed at room temperature, which increases the risk of mispriming, which can generate a background sequence. We decided to add the nucleotides in a specific order rather than a cyclic order (Fig. 1B and C), and this was possible because the sequence of the HBV genome is not a cyclic order rather than a cyclic nucleotide dispensation order (Fig. 1B and C). The pyrogram patterns for the mutant and WT viruses at positions 180 and 204. As expected, the signal-to-noise ratio decreased toward the end of the sequence, but the read length is therefore limited to approximately 40 nucleotides. A specific nucleotide dispensation order reduces the number of pyrosequencing cycles required and thereby increases the pyrosequencing quality (Fig. 1B and C). Figure 2 shows that the actual pyrosequencing results displayed relatively high, distinguishable peaks that agreed well with the expected pyrogram patterns for both the WT and MUT viruses at positions 180 and 204. As expected, the signal-to-noise ratio decreased toward the end of the sequence, but the pyrogram patterns for the mutant and WT viruses at the two sites were clearly distinguishable.

### Detection of mixtures of resistant and WT virus

To test the abilities of pyrosequencing and Sanger sequencing to detect and quantify minor sequence variants, we prepared artificial mixtures of lamivudine-resistant (MUT) and WT viruses that
contained 100 to 0% resistant virus in increments of 10%. Figure 3A shows the theoretical pyrogram patterns for mixtures of different proportions. These patterns were used as templates for interpretation of the results from the mixing experiments. Figure 3B shows representative results for the detection of mutations at position rt180 by pyrosequencing and Sanger sequencing. The preparation and analysis of the mixtures were repeated three times, with similar results each time. Both sequencing methods could detect minor variants in the mixtures made from WT and MUT viruses. The results obtained with 100% WT virus and 100% mutant virus were correct by both methods. In the mixture with 50% WT virus and 50% MUT virus, the pyrogram indicated the presence of both WT and MUT viruses in similar amounts. In contrast, by

FIG. 2. Theoretical pyrogram patterns (top of each panel) and examples of raw data from pyrosequencing (bottom of each panel). (A) WT pattern (ATG; methionine) at position rt204; (B) valine mutation pattern at position rt204 (GTG; rtM204→V); (C) isoleucine mutation pattern at position rt204 (ATT; rtM204→I); this mutation is sometimes seen during the transition from the WT sequence to a sequence with a valine mutation; (D) WT pattern (TTG; leucine) at position rt180; (E) WT pattern (CTG; leucine) at position rt180; the patterns in both panels D and E are considered WT and encode a leucine; (F) methionine mutation pattern at position rt180 (ATG; rtL180→M); this mutation is often seen in combination with the more important mutation at position rt204. The letters under the black bars show the dispensation (Disp:) order. The actual sequence detected by pyrosequencing is indicated below the panels after “Seq:”; note that when the pyrogram has a relative peak high of 2.0, the sequence is interpreted as two consecutive identical nucleotides (e.g., the double G in panel B). The light gray area shows the pyrogram for the codon of interest. Note that the nucleotide dispensation order has been designed so that the pyrogram patterns for WT and resistant variants differ at several positions, even though the sequences differ by only a single point mutation.
Sanger sequencing the MUT sequence appeared to dominate and only traces of WT sequence could be detected. With the mixture with 80% WT virus and 20% MUT virus, both pyrosequencing and Sanger sequencing detected the presence of both virus variants, but the pyrosequencing results agreed better with the expected ratio between the WT and MUT sequences. With the mixture with 20% WT virus and 80% MUT virus, Sanger sequencing did not detect WT virus, but pyrosequencing still detected the WT virus. The artificial mixtures were also used to evaluate the abilities of the methods to detect sequence polymorphisms at position rt204. For this position, the results of pyrosequencing also agreed somewhat better.

FIG. 3. Detection of mixtures of WT and lamivudine-resistant (MUT) virus at position rtL180. (A) Theoretical pyrogram patterns for 100% WT (variant TTG), 100% MUT, and WT-MUT mixtures at position rtL180. Note that the 50% WT-50% MUT mixture is expected to create a pyrogram pattern that at each position represents an average for the WT and MUT patterns. The light gray area shows the critical positions for evaluation of the pyrosequencing and the Sanger sequencing results. (B) Results from actual sequence analysis of mixtures of WT and MUT HBV variants by Sanger sequencing (top of each panel) and pyrosequencing (bottom of each panel). The panels show 100, 80, 50, 20, and 0% WT. Mixtures were also made to contain 90, 70, 60, 40, 30, and 10% WT (data not shown). The “W” in the second panel of Fig. 3B is a International Union of Pure and Applied Chemistry code which signifies that the ABI310 software identified a mixture of the A and the T nucleotides at this position.
than those of Sanger sequencing with the expected ratio of the WT virus/MUT virus sequences (data not shown).

Note that small background peaks were observed at certain positions by both Sanger sequencing and pyrosequencing (Fig. 3B). By Sanger sequencing, a background G nucleotide was observed together with the C nucleotide at position 7 in all five panels in Fig. 3B. By pyrosequencing, background peaks were observed at positions 3 (T) and 7 (C) and were best visualized in the bottom panel of Fig. 3B (100% MUT). By pyrosequencing the distinction between background peaks and real polymorphisms was aided by the fact that several peaks in the pyrogram were used to evaluate a single polymorphic position. Thus, position 4 in the pyrogram for 100% MUT has no signal, which indicates that the peaks at positions 3 and 7 represent background noise.

Analysis of clinical patient samples. The clinical utility of pyrosequencing was evaluated with serum samples from 20 HBV-infected patients that had been routinely analyzed for the presence of lamivudine-resistant virus at Clinical Virology, Huddinge Hospital. For most patients the resistance test was carried out to investigate lamivudine treatment failures, as evidenced by increasing HBV levels in serum. In addition, the HBV PCR was tested with routine samples from an additional 34 patients. All samples with detectable HBV (>400 HBV DNA copies/ml by the Amplicor HBV Monitor assay) were successfully analyzed. The pyrosequencing and Sanger sequencing results for five representative patients are shown in Fig. 4. For three patients whose viruses displayed lamivudine resistance, we also analyzed a sample obtained before treatment and a sample obtained earlier during treatment. The results of Sanger sequencing and pyrosequencing agreed for patient 1, with both methods detecting a WT pattern. The early treatment sample from patient 2 had a mixture of WT and mutant (rtM204→V) viruses. Sanger sequencing indicated that approximately 30% of the virus population was resistant, while pyrosequencing indicated that the proportion of resistant virus was smaller (approximately 10%). The late treatment sample from patient 2 showed mutant virus by both sequencing techniques. The last sample from patient 3 tested had a mixture of WT and resistant (rtM204→V) viruses. By pyrosequencing approximately 15% of the virus population appeared to be lamivudine resistant, whereas by Sanger sequencing a larger proportion (approximately 30%) appeared to be resistant. The two earlier samples from patient 3 had the WT sequence by both sequencing methods. The last sample from patient 4 showed a mixture of WT and resistant (rtM204→V) viruses by pyrosequencing but only resistant virus by Sanger sequencing. The two earlier samples showed WT virus by both methods. The virus from patient 5 displayed an rtM204→I mutation by both methods.

DISCUSSION

In this study we have designed a pyrosequencing method for the detection of lamivudine resistance in clinical samples from HBV-infected individuals. The method was shown to be at least as sensitive as traditional Sanger sequencing for the detection of minority virus populations and had advantages in terms of throughput.

Pyrosequencing and Sanger sequencing differ in several aspects. The most important difference is that pyrosequencing is faster than Sanger sequencing, even when the latter is done on an automated sequencing machine. An ABI310 machine has a throughput of approximately 1 sample per h, whereas the newer ABI3100 machine can analyze 16 samples per h. By pyrosequencing it is possible to sequence 96 samples in approximately 10 min. Another advantage with pyrosequencing compared with Sanger sequencing is that preparation of the PCR product before sequencing is easier and quicker. Cycle sequencing by the Sanger method involves purification of the PCR product, sequencing, and precipitation of DNA before the sample is loaded in the sequencing machine. By pyrosequencing all of these steps are replaced by a fully automated sample preparation step; thus, the time needed for presequencing preparation is approximately one-fifth of the time needed for that for conventional sequencing. Finally, pyrosequencing has the advantage that the nucleotide dispensation order can be designed so that sequence variants that differ by only a single nucleotide generate distinct pyrograms that differ at several peaks rather than at a single peak (Fig. 2). In addition, the use of a specific rather than a cyclic nucleotide dispensation strategy also reduces the number of pyrosequencing cycles and thereby increases sequence quality and the read length. In our experiments it was possible to use a specific nucleotide dispensation strategy because lamivudine resistance involves only a few sequence variants. If the sequence of interest had been highly polymorphic, we would have been forced to choose the less attractive cyclic nucleotide dispensation strategy.

One present limitation of pyrosequencing is that relatively few laboratories have access to pyrosequencing equipment, while many laboratories have automated Sanger sequencing machines. A limitation inherent to the pyrosequencing technique is that the read length is short compared to that of Sanger sequencing: approximately 40 bp versus more than 500 bp. However, this is not a problem during analysis of point mutations, such as the lamivudine resistance mutations in HBV described here. Thus, pyrosequencing is well suited for large-scale screening for known point mutations but not for scanning of larger regions in a viral genome.

Pyrosequencing, like other PCR and sequencing techniques, in this study we designed PCR, pyrosequencing, and Sanger sequencing primers that anneal to regions of the HBV genome that are highly conserved among HBV genotypes A to G. The HBV sequences in all 54 serum samples with detectable HBV levels that we have analyzed thus far have successfully been amplified by PCR. These samples included HBV isolates of genotypes A, B, C, and D (data not shown). This indicates that false-negative results due to primer-template mismatches should be rare, even though more extensive evaluations with samples representing all genotypes are required to formally prove that this is the case.

Today pyrosequencing is mainly used for the detection of SNPs in the human genome. In most cases SNP analysis can have three outcomes: homozygous nonmutant, heterozygous, and homozygous mutant. Analysis of viral genomes is more complicated because polymorphic nucleotide positions can contain mixtures of anything from 0 to 100% of the variant
sequence. Sanger-based cycle sequencing is known to generate uneven peak heights, and therefore, quantification of mixed nucleotide positions may be difficult. This has been shown for HIV type 1 resistance testing (7). Pyrosequencing has a theoretical advantage in such an analysis because the nucleotide dispensation order can be designed so that several peaks are used to evaluate a single polymorphic nucleotide position. This should allow identification of polymorphic positions that is more reliable than that which can be obtained by traditional sequencing, in which quantification is based on only one peak or nucleotide position in a chromatogram. Background noise or peaks occur by both pyrosequencing and Sanger sequencing.

FIG. 4. Comparison of Sanger sequencing and pyrosequencing for detection of mutations at position rtM204 in clinical serum samples from five patients who failed lamivudine treatment. For patients 2 to 4, the results of analysis of three samples obtained before the start of treatment and early and late during treatment are shown. For patients 1 and 5, only the results from analysis of the last available on-treatment samples are shown. “R” is an International Union of Pure and Applied Chemistry code which signifies that the ABI310 software identified a mixture of the A and the G nucleotides at this position.
but they are usually easier to distinguish from true sequence polymorphisms by pyrosequencing because more than one peak is evaluated. The background in pyrosequencing can be due to a low signal level, which can be caused, for example, by a suboptimal PCR and thereby low levels of pyrosequencing template. By pyrosequencing a cutoff level for minimal peak height is objectively calculated to avoid the interpretation of baseline flutter as a minor peak. Background can also appear at specific nucleotide positions as a result of sequence-dependent nucleotide misincorporations or small impurities in the dNTP reagent mixture. This type of background is often reproducible within runs and is directly analogous to similar problems that occur during Sanger sequencing. Because it is reproducible, it can be distinguished from true sequence polymorphisms by the inclusion of proper controls. However, interpretation of pyrosequencing results requires some training, just like the interpretation of results of Sanger sequencing. We are working on developing automated methods for pattern recognition that could facilitate a more rapid and reliable identification of polymorphic positions. In our experiments with artificial mixtures of WT and MUT variants, the results of pyrosequencing agreed somewhat better with the expected results than the results of Sanger sequencing did. More experiments with other HBV genotypes and clinical specimens are needed to definitively establish if one method is superior to the other, but our data and the theoretical considerations described above suggest that pyrosequencing is at least as efficient as traditional sequencing for the detection of minor sequence variants. Our conclusion that minor viral variants can be detected by pyrosequencing is supported by a previous study in which pyrosequencing was used to detect resistance to antiretroviral drugs in patients infected with HIV type 1 (4). However, it should be stressed that it remains to be shown if it is clinically relevant to be able to accurately quantify the proportion of resistant virus to WT virus in HBV-infected patients. The mixing experiments were done with clones generated by limiting dilution rather than conventional molecular cloning. Even though these two procedures differ, we feel confident that our results and conclusions would have been very similar if traditional cloning had been used; because both Sanger and pyrosequencing indicated that the clones were pure (within the limits of detection of the two sequencing methods).

Other simple methods for the detection of drug-resistant mutations in HBV have been described, e.g., the INNO-LiPA HBV DR line probe assay (LiPA). This method is based on reverse hybridization of amplified HBV DNA fragments with specific nucleotide probes immobilized on nitrocellulose strips. It has been shown that this method can detect resistance in some patient samples earlier than Sanger sequencing can (3) and can also detect mixtures of WT and resistant virus before viral breakthrough. We plan to compare our pyrosequencing method with LiPA.

In conclusion, we have developed a new assay for the identification of lamivudine resistance in clinical samples from HBV-infected patients. We show that the method has a high throughput and can detect minor sequence variants. Our pyrosequencing method should be useful for the analysis of clinical specimens for the presence of virus variants with lamivudine resistance.

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