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Original Article

Comparison of pyrosequencing and sanger sequencing for HBV DNA genotyping and resistance mutations

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Abstract: Drug resistance gene mutations in Hepatitis B virus (HBV) are the main reason for failure of currently used therapeutic nucleoside analogues. Two methods-Pyrosequencing and Sanger sequencing, are most commonly used for HBV genotyping and identification of its mutations, but their advantages of the two methods are undefined. Herein, the two methods were used to identify the HBV genotypes and drug-resistance mutations in the sera specimen of 138 HBV patients treated with nucleoside analogues. It had no significant difference in the detective rate of HBV genotypes B or C between the two methods, but the Pyrosequencing had an error rate of 7.25% for HBV genotyping but Sanger sequencing showed no mistakes. Sanger sequencing also had a lower failure rate and a significantly higher detection rate for the common drug-resistance mutations of HBV compared with the Pyrosequencing, and it could detect unknown new mutations in clinical samples. We also found that the Sanger sequencing had significant higher detection rate for single and multiple drug resistance mutations than the Pyrosequencing. In summary, the results indicated that the Sanger sequencing is a more reliable method with a lower failure rate and a higher detection rate for drug-resistance mutations in HBV patients' samples, particularly in that with long-term anti-virus treatment.

Keywords: HBV, genotype, resistance, pyrosequencing, sanger sequencing

Introduction

It is estimated that more than 350 million people are chronically infected with Hepatitis B virus (HBV), three-quarters of whom reside in the Asia Pacific region, particularly in China [1]. HBV infected patients are at risk for the development of cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC). Every year there are approximately 600,000HBY related deaths [2]. HBV infection is an important global public issue, and remains a disease of significant global health burden.

Antiviral therapy is the efficient way to prevent bad clinical outcomes of HBV infection. Currently, there are two types of anti-HBV drugs: interferon-alpha (IFN-α) and nucleoside analogs. IFN-α is highly efficient in preventing replication of the virus by stimulating leukocytes to secrete antiviral proteins, but has numerous side effects and the administration by injection is inconvenient. Nucleoside analogs include Lamivudine (LAM) [3-6], Telbivudine (LdT) [7], Entecavir (ETV) [8], Adefovir (ADV) [9, 10], Tenofovir (TDF) [9] and Emtricitabine (FTC) [11]. They are orally administered effective anti-HBV agents. They combine with HBV polymerase to prevent the replication of HBV DNA. These nucleoside analogs are well tolerated and easy to consume, and has reduced liver toxicity with fewer side effect profile. However, the big limitation is emerging drug resistance due to HBV DNA genetic mutations.

Hepatitis B virus (HBV) is a double-stranded DNA molecule with approximately 3200 base pairs (bp), consisting of S, C, P, X4 open reading

frames [12]. HBV lacks RNA polymerase and reverse transcriptase correction function, and is a highly variable virus. One or more nucleotides may incur reverse transcriptase mutation (s) during its replication [13]. The rate of nucleotide substitution per site is estimated to be 1.4 × 10^5-3.2 × 10^5 per year [14, 15]. This results in the emergence of HBV genotypes and subgenotypes. It has identified at least eight different genotypes (A-H) that differ in more than 8% of the genome [16]. It is reported that HBV genotypes affect clinical outcomes in chronic HBV patients [17]. Therefore, a reliable and applicable gold standard method for HBV genotyping is very important for HBV clinical application.

Moreover, HBV mutation can occur in any genotypes and also any one area of its four open reading frame, such as pre-c region, c gene promoter mutation and HBV polymerase gene region [18, 19]. The mutations of HBV can naturally occur in a chronic persistent infection, but also in the immune pressure, even during in the anti-viral drugs. Nucleoside analogues (NAs), such as Lamivudine, Adefovir, Entecavir, Tenofovir and Famciclovir, have been widely used in patients with chronic hepatitis B (CHB) as antiviral therapy, to inhibit HBV reverse transcriptase activity and prevent replication of viral nucleic acid. But long-term use can cause HBV P gene mutations which led to drug resistance [20, 21]. Therefore identifying the HBV genotypes and mutations could aid HBV diagnosis and direct clinical treatment.

To date, there are over ten codons associated with primary antiviral drug resistance in CHB [22-24], which map to five of the functional domains of polymerase (Pol) gene: A-domain at codons rtL80, rtV84, and rtS85A; B-domain at codons rtI169, rtV173, rtL180, rtA181, and rtT184; C-domain at codons rtS202, rtM204 and rtV/L/M207; D-domain at codon rtV214, rtQ215S and rtN236; and E-domain at codon rtM250. It is reported that different types of anti-HBV nucleoside analogues induce mutations on the specific codons. Lamivudine-resistant amino acids have been described at positions rtA181T and rtM204V/I/S [3, 25], Adefovir at positions rtV84M, rtS85A, rtL80V/I, rtA181V/T, rtV214A, rtQ215S and rtN236T [26-28], Entecavir at positions I169T, V173L, rtS202I, rtL180M, rtT184S, and rtM204V/I [29-31], Tenofovir at positions rtL180M, rtA181IV, rtA194T, rtM204V, rtV214A and rtQ215S [32-34], Famciclovir at positions rtG173L, rtL180M, rtV/L/M207I [35, 36]. Therefore identifying the HBV mutation in patients' sample could help understand drug resistance of the patients and direct clinical therapy.

Many methods have been used to detect HBV drug resistance and genotype in recent years, such as direct sequencing, gene ChIP, real-time PCR et al [37-39]. Recently even it is reported to use the HiSeq sequencing to identify the genotyping and mutations of HBV [40]. Comparison of these methods, HiSeq sequencing could obtain thousands of replicates for every nucleotide and help to detect very low frequency HBV mutation variants, but this method has drawbacks of high cost, complex for data analysis and difficulty for data explanation. The most common method is direct PCR sequencing. This method can provide nucleotide sequences to identify the virus genotype and variants intuitively. Both Sanger and Pyrosequencing are direct PCR sequencing methods, which has high-throughput, simple operation and high detection sensitivity [41]. However, there are a lot of test fails using pyrosequencing to detect HBV mutations. Here, we examined the HBV genotypes and drug-resistance mutations in 138 HBV patients with Sanger and pyrophosphate direct PCR sequencing, and compared the advantage of the two methods, and found that Sanger sequencing is more reliable for HBV genotyping, has high sensitivity and low failure rate for identify the drug-resistance mutations in clinical samples.

Materials and methods

Ethics statement

This study was approved by the Institutional Review Board of Renmin Hospital, Wuhan University School of Medicine. A written informed consent was obtained from each participant in accordance with the Ethics Committee of the Renmin Hospital of the Wuhan University.

Patients and plasma preparations

A total of 138 samples each 3-5 ml in volume (anticoagulated with EDTA) was obtained from the peripheral blood. All individuals had quanti-

tative real-time PCR (qPCR) confirmed HBV infection at the Department of Infectious Diseases, Renmin Hospital of Wuhan University, and were treated with nucleoside analogs(NAs), such as Lamivudine, Adefovir, Entecavir, Tenofovir or/and Famciclovirfor over one year duration. The HBV DNA titers of all patients were ≥ 10^3 IU/ml in the peripheral blood. The patients ages ranged from 22-67 years (median, 42.9 years), and 43 patients had a history of interferon therapy of more than 6 months. All samples were centrifuged for 5 min at 3,000 g, and the supernatants were collected and stored at -70°C.

DNA extraction and PCR

DNA was extracted from each plasma sample with the UltraSens Virus Kit (QIAamp, German), according to the recommendations of the manufacturer. Briefly, 0.8 ml Buffer AC and 5.6 μl carrier RNA solution was pipetted on top of 1 ml plasma. After mixing and incubating at room temperature for 10 min, it was centrifuged and the supernatant was discarded. Then 300 μl Buffer AR and 20 μl proteinase K was added and vortexed. Afterwards, 300 μl Buffer AB was added and mixed thoroughly by vortexing and the sample was transferred to a QIAamp spin column. The column was centrifuged and the flow through was discarded, the silica pellet was washed with 500 μl Buffer AW1 and 500 μl Buffer AW2. The nucleic acids were eluted in 30 μl Buffer AVE and stored at -70°C. The PCR was designed to amplify the DNA fragment of the full-length sequences of RT (aa 1-344), wherein the primer sequence of the upstream primer was 5'-CCAGAGTGAGGGGCCTATATT-3' (F1), and the downstream primer sequence was 5'-GCGAGCAAAACAAGCTGCTA-3' (R1), the amplification length was 1270 bp. The PCR reaction was done as follows: 94°C (3 min); 94°C (30 sec), 56°C (50 sec), 72°C (120 sec)- for 35 cycles; then 72°C for 10 min. The PCR products were electrophoresed on a 1.2% agarose gel for gel purification and stored at -70°C.

Pyrosequencing

The second PCR was performed with the PyroMark PCR Master Mix kit (Qiagen, German) using the purified PCR products from above, following the instructions of the manufacturer. For the second PCR, the primer sequence of the upstream primer was 5'-TATTCCCATCCCATCRTCYTG-3' (F2) and the downstream primer sequence was 5'-GCATATAAAGGCATCARRGCA-3' (R2). The primer sequences used for pyrosequencing common NAs resistant mutant HBV detection, wherein the primer sequence is: sequencing primer 1, 5'-CRTCTTGGGCTTTMGS-3' (for detecting rtI169, rtV173); sequencing primer 2, 5'-AGTGGGCCTCAGYCCGTTTC-3' (for detecting rtL180, rtA181, rtT184); sequencing primer 3, 5'-CATTTGTTCAGTGGTTCGYMG-3' (for detecting rtA194, rtS202, rtM204); sequencing primer 4, 5'-TACCAATTTTCTKTTRTC-3' (for detecting rtN236, rtN250). The PCR primer F2 was biotinylated to allow immobilization of the PCR product on streptavidin-coated beads and preparation of single-stranded DNA for pyrosequencing. After PCR, sample preparation was done with PyroMark PCR Kit (Qiagen, German) according to the instructions of the manufacturer. When finished the optimal sample preparation using the PyroMark Q24 kit (Qiagen, German) to pyrosequencing analyze. The resulting complete sequences were analyzed for the HBV DNA mutation.

Sanger sequencing

The purified amplification products (PCR with F1 and R1) were sequenced with an ABI PRISM Big Dye 3.1 terminator cycle sequencing kit (ABI, USA). The sequencing primer of the upstream primer was same as F1, downstream of the primer sequence was same as R1.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 either of the two PCR primers, primers F1 and R1. The cycle sequencing profile was 25 cycles of 96°C for 10 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 3 M sodium acetate. Sequencing was performed on an ABI Prism 3130 Genetic Analyzer with ABI Prism 3130 Collection and Sequencing Analysis software. The sequences generated by the forward and reverse sequencing primers were assembled and analyzed with the software program Sequencher 5.2.3 (Gene Codes Corporation, USA). The resulting complete sequences were translated into amino acid sequences to analyze the HBV DNA mutation.

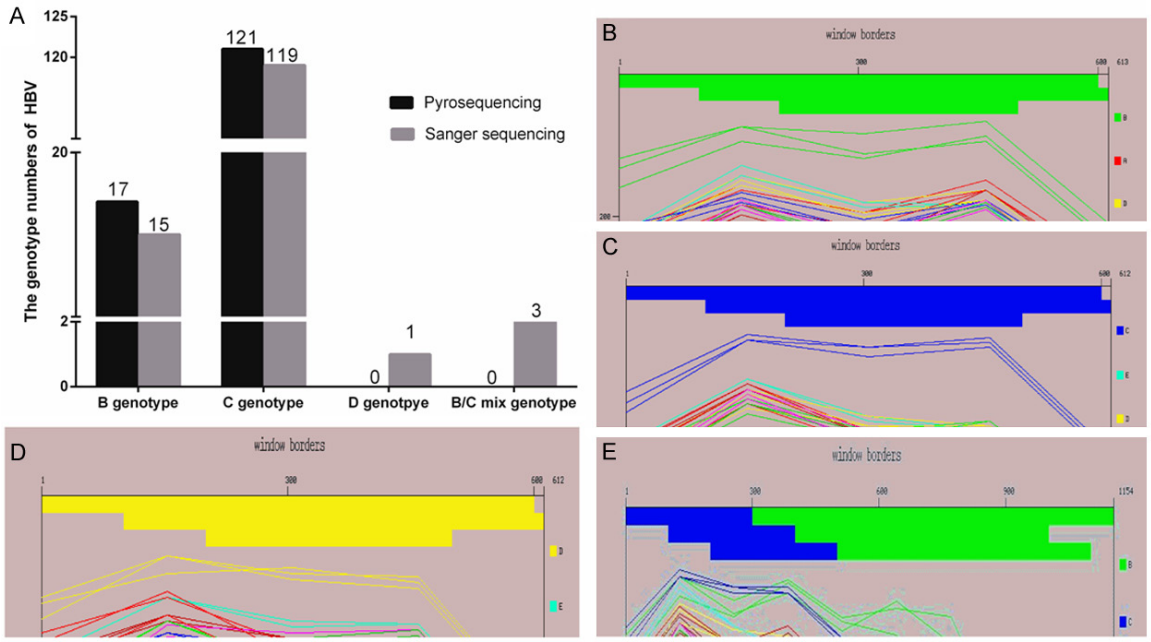

Figure 1. Results of HBV genotyping. A. The statistical data for HBV genotypes identified by Pyrosequencing and Sanger sequencing methods; B. HBV genotype B identified by alignment of Sanger DNA sequences of the patients' samples with that of HBV database; C. HBV genotype C identified by alignment of Sanger DNA sequences of the patients' samples with that of HBV database; D. HBV genotype D identified by alignment of Sanger DNA sequences of the patients' samples with that of HBV database; E. HBV genotype B/C hybrid type identified by alignment of Sanger DNA sequences of the patients' samples with that of HBV database.

HBV genotype and RT region mutation analyses

The genotypes and RT region mutation were analyzed using the HBV sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov/projects/genotyping), which contains 23 HBV DNA reference sequence for 8 HBV subtypes, including Subtype A (Accession No. X02763, X51970, AF090842), Subtype B (Accession No. D00329, AF100309, AB033554), Subtype C (Accession No. X04615, M12906, AB014381), Subtype D (Accession No. X65259, M32138, X85254), Subtype E (Accession No. X75657, AB032431), Subtype F (Accession No. X69798, AB036910, AF223965), Subtype G (Accession No. AF160501, AB064310, AF405706), and Subtype H (Accession No. AY090454, AY090457, AY090460).

Statistical analysis

Statistical analysis was performed with the IBM SPSS 20 (SPSS Inc., Chicago, USA). Distributions of continuous variables were analyzed by the Kruskal-Wallis test. For qualitative parameters, the difference for two groups was analyzed using a χ^2 test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

Results

Sanger sequencing is better for HBV genotyping than pyrosequencing

The pyrosequencing method only read about 60 bp short DNA, and we used multiple primers to sequence the HBV RT regions, and then used the resulted multiple short HBV DNA sequences in RT regions to do HBV genotyping by aligning with the 23 HBV DNA reference sequences in database (http://www.ncbi.nlm.nih.gov/projects/genotyping). With this method, we found that HBV genotype B and C were detected in 12.32% (17 cases) and 87.68% (121 cases), respectively in the 138 samples (**Figure 1A**).

Sanger sequencing method could read approximately 1100 bp long DNA sequences for the sequence primer, with which the DNA sequence for the full-length of HBV RT region could be obtained. Therefore the full length DNA

Table 1. The different results of the genotype of HBV detected by pyrosequencing and by Sanger sequencing

Sample	Pyrosequencing		Sanger sequencing		Error rate
	Genotype	Accession No.	Genotype	Accession No.	
4	C	---	B	AF100309	7.25%
28	B	---	B/C	AB014381	
30	B	---	B/C	X04615	
49	B	---	D	X65259	
67	B	---	C	AB014381	
82	C	---	B	AF100309	
101	C	---	B/C	AF100309	
105	B	---	C	AB014381	
117	B	---	C	X04615	
122	C	---	B	AF100309	

sequence of the RT region was aligned with the reference HBV nucleic acid database for genotyping (**Figure 1B-E**). With this method, we found HBV genotype B was detected 10.87% (15/138) in the cohort, and also 86.23% (119/138) C, 0.72% (1/138) D, 2.17% (3/138) B/C hybrid were detected. Statistical analysis showed that there was no difference in the detection rate of HBV genotypes B or C between the Sanger and pyrosequencing methods.

Moreover, with pyrosequencing, 10 samples were shown to be wrongly genotyped, the error rate was 7.25% (10/138), in which the 3 patients with genotype B was classified as C, and 3 with genotype C classified as B, 2 with genotype B/C as B, 1 with genotype B/C as C and 1 with genotype B as D (**Table 1**). However, no mistakes for genotyping were found with Sanger sequencing. Furthermore, Sanger sequencing could also read the full length of the HBV S region. By aligning the database with full-length of S region, we could obtain more accurate reference sequences (**Table 1**), which benefit the analysis of drug-resistance mutations in the HBV samples.

Pyrosequencing has higher failure rate for detection of drug-resistance mutations

Both Pyrophosphate and Sanger methods can fail to detect the drug resistant mutation in HBV patients' samples. We compared the failure rate of the methods at 10 common mutation sites: rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236 and rtM250. The failure rate for pyrophosphate method was 2.90%, 4.35%, 5.07%, 2.90%, 2.17%, 2.90%, 3.62%, 2.90%, 2.90% and 0%, respectively; for Sanger method, they were 0.72%, 0.72%, 0, 0, 0, 0, 1.45%, 0.72%, 0.72% and 0%, respectively (**Figure 2A**). The failure rate for pyrophosphate method was significantly higher than that of Sanger method except for the rsM250 site where the failure rate was 0% for both methods.

Moreover, with Sanger sequencing method, many mutations un-related to nucleoside analog drug resistance, were detected in samples where the pyrophosphate method could not detect any mutations (**Figure 2B**). Moreover, we found many samples where the drug-resistance mutations were not detected by pyrophosphate method were from the patients who had received interferon treatment for more than 6 months. We also found that a few samples were detected with low frequency of drug-resistance mutations using the pyrophosphate method but were negative with Sanger method (**Figure 2C**).

Sanger sequencing has higher rate for detection of HBV drug-resistance mutations in patients' samples

With pyrosequencing, the detection rate for the 10 common drug-resistance mutations of HBV was obviously lower than by Sanger sequencing. The detection rate for the mutations : rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236 and rtM250, with pyrophosphate method was 0.72%, 1.45%, 18.84%, 6.52%, 0.72%, 0.72%, 2.17%, 28.26%, 1.45% and 0.72%, respectively which was obviously lower than by the Sanger sequencing: 2.90%, 4.35%, 23.19%, 11.59%, 3.62%, 2.90%, 5.07%, 31.88%, 4.34% and 3.62%, respectively (**Table 2**). Moreover, with Sanger sequencing, mutations in other drug-resistance sites such as rtL80, rtV84, rtS85, rtV214 and rtQ215 were also detected, and the frequency of the mutations was 9, 5, 1, 3 and 2, respectively, in the 138 patients' samples (**Table 2**). These five mutation sites are usually not analyzed by the Pyrophosphate method (**Table 2**). But Sanger sequencing covered full-length of RT region that includes those 5 sites. In our data we found that in up to 9 samples we

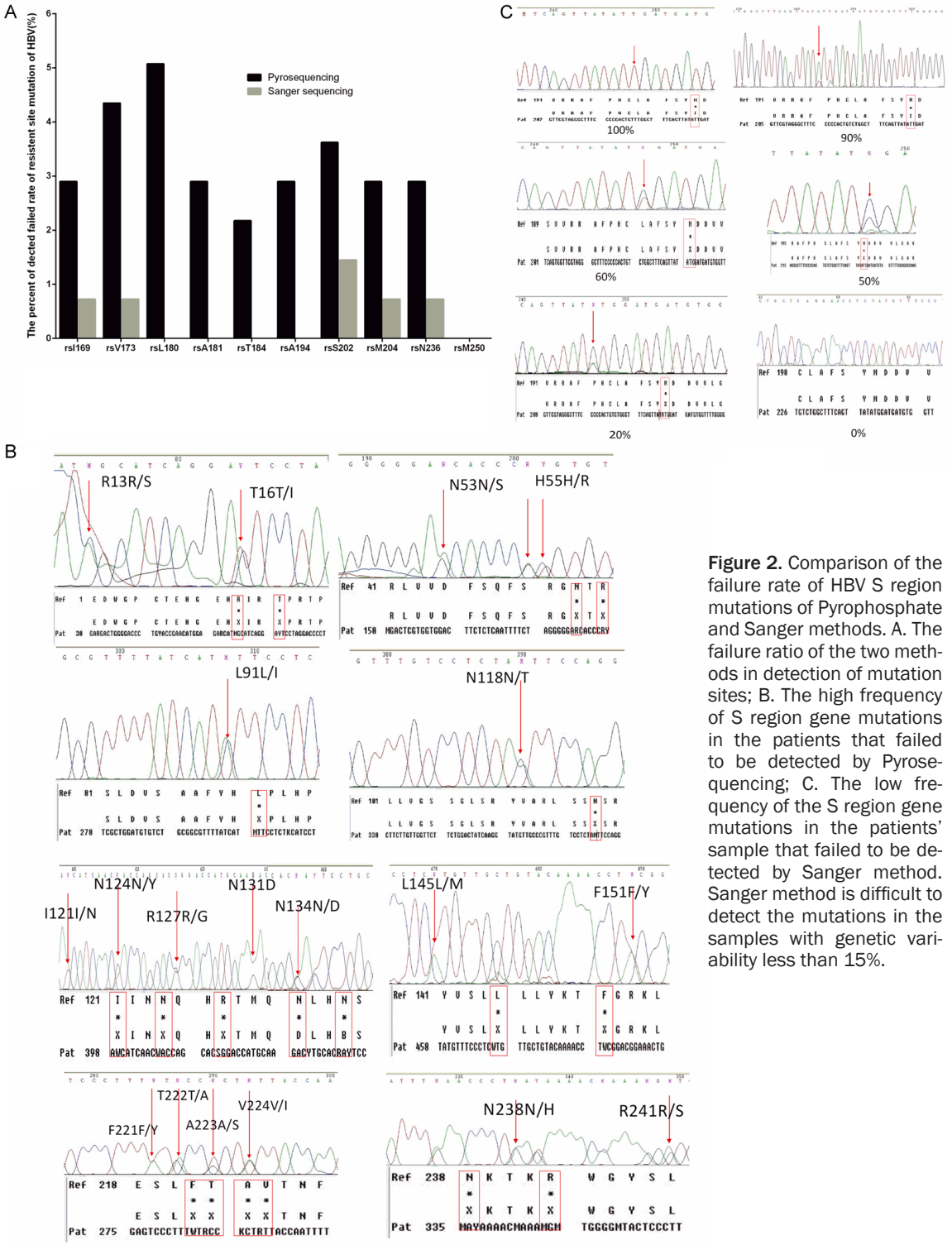

Figure 2. Comparison of the failure rate of HBV S region mutations of Pyrophosphate and Sanger methods. A. The failure ratio of the two methods in detection of mutation sites; B. The high frequency of S region gene mutations in the patients that failed to be detected by Pyrosequencing; C. The low frequency of the S region gene mutations in the patients' sample that failed to be detected by Sanger method. Sanger method is difficult to detect the mutations in the samples with genetic variability less than 15%.

detected the mutations in rsL80 site, 5 in rsV84, 3 in rs214, 2 in rsQ25 and 1 rsS85 (**Table 2**). We also analyzed the number of mutations in samples detected by the two methods and found Pyrophosphate method had high detection rate in the samples with 1-2 mutations, but Sanger method had a much higher detection rate in samples with 3 mutations, but in the samples with 4 and ≥ 5 mutations, they were only detected by Sanger meth-

Table 2. Detective rate of drug-resistance mutations in different gene sites identified by the two methods

Mutation site	Pyrosequencing		Sanger sequencing		*P* value
	Number	% (n=138)	Number	% (n=138)	
rtI169	1	0.7	4	2.90	0.37
rtV173	2	1.45	6	4.35	0.28
rtL180	26	18.84	32	23.19	0.46
rtA181	9	6.52	16	11.59	0.21
rtT184	1	0.72	5	3.62	0.21
rtA1 94	1	0.72	4	2.90	0.37
rtS202	3	2.17	7	5.07	0.33
rtM204	39	28.26	44	31.88	0.60
rtN236	2	1.45	6	4.34	0.28
rtM250	1	0.72	5	3.62	0.21
rtL80	NA	NA	9	6.52	NA
rtV84	NA	NA	5	3.62	NA
rtS85	NA	NA	1	0.72	NA
rtV214	NA	NA	3	2.17	NA
rtQ215	NA	NA	2	1.45	NA

Table 3. Numbers of drug resistance mutations in each patients' samples detected by the two methods

Mutation numbers	Pyrosequencing		Sanger sequencing		*P* value
	Number	% (n=138)	Number	% (n=138)	
0	61	44.20	49	35.51	0.18
1	35	25.36	31	22.46	0.67
2	33	23.91	31	22.46	0.89
3	9	6.52	13	9.42	0.51
4	0	0	9	6.52	0.003
5	0	0	5	3.62	0.06

ods (**Table 3**). These results indicate that Pyrophosphate method is suitable for detection of mutations in samples with low genetic variability, but Sanger method has higher detection rate for the samples with high genetic variability (**Table 3**).

Sanger sequencing has higher detective rate for single and multi-drugs resistance mutations

It is reported that the specific mutations in HBV is related to resist the common nucleoside analogues. Therefore, based on the mutations detected by the two methods, we could identify which common nucleoside analogues the patient should be resistance to, and calculate the resistance ratio to the common nucleoside analogues for each sequencing method. With the pyrophosphate method, we found the resistance ratio for the five nucleoside analogues, Lamivudine, Adefovir, Entecavir, Tenofovir and Famciclovir were 40.58% (56/138), 18.12% (25/138), 0% (0/138), 0% (0/138) and 20.29% (28/138), respectively (**Table 4**). For Sanger method, the ratios were 59.42% (82/138), 28.26% (39/138), 2.17% (3/138), 7.97% (11/138) and 36.23% (50/138), respectively (**Table 4**). Both resistance ratio and the case numbers with the Sanger method were obviously and/or significantly higher than that of pyrosequencing (**Table 4**).

We also analyzed mutations with multi-drug resistance in patients and found that with the Sanger sequencing, 87 patients were found to have multi-drug resistance mutations, but with pyrosequencing 54 patients were detected the mutations. The difference between the two methods was very significant ($P < 0.001$). We further analyzed the patients with mutations detected for one drug resistance, two drugs resistance, and 3 drugs resistance. It was found that the patient numbers and percentage in total patients with mutations for 1 drug, 2 drugs or 3 drugs resistance detected by pyrosequencing were 25.93% (14/54), 50% (27/54) and 24.07% (13/54), respectively; while the number and percentage for Sanger sequencing were 19.04% (17/87), 46.32% (49/87), and 14.94% (13/87), respectively. We found that 9.20% (8/87) patients had mutations for 4 of the 5 drugs detected by Sanger sequencing (**Table 5**). The patient numbers and percentage in total patients with mutations for different numbers of drugs detected by Sanger method are obviously and/or significantly higher than that of Pyrosequencing.

Discussion

We examined the HBV genotype with Sanger and pyrophosphate methods and compared the detection rate of the two direct PCR sequencing methods for identification of HBV drug-resistance mutations in patients' samples, and found that Sanger method is more reliable for HBV genotype and had higher detec-

Table 4. Comparison the rate of the two methods for detection of the mutations responsible for the resistance of single nucleoside analogue

Drug resistance mutations	Pyrosequencing	Sanger sequencing	*P* value
Lamivudine	40.58% (56/138)	59.42% (82/138)	0.03
Adefovir	18.12% (25/138)	28.26% (39/138)	0.06
Entecavir	0% (0/138)	2.17% (3/138)	0.25
Tenofovir	0% (0/138)	7.97% (11/138)	<0.001
Famciclovir	20.29% (28/138)	36.23% (50/138)	0.005

Table 5. Comparison of the two methods for the multiple drug resistance of the 5 nucleoside analogues

Number of resistance of drugs	Pyrosequencing	Sanger sequencing	*P* value
1	25.93% (14/54)	19.04% (17/87)	0.53
2	50% (27/54)	46.32% (49/87)	0.49
3	24.07% (13/54)	14.94% (13/87)	0.18
4	0% (0/54)	9.20% (8/87)	0.02

tive rate for HBV drug-resistance mutations. These results indicated that Sanger method is more reliable and sensitive for HBV genotype and identification of drug-resistance mutations.

The whole genome sequencing followed by phylogenetic analysis is the gold standard method for HBV genotyping [17, 42, 43]. It is highly sensitive and allows the detection of new and recombinant genotypes, however, this techniques is time-consuming, expensive and detects mainly the predominant genotype in genotype mixtures [42, 43]. Instead of whole genome sequencing, an alternative method is single gene sequencing as we did here [43]. The sensitivity of single gene sequencing depends on the degree of sequence homology as well as the sequence size [42, 43]. This method has time saving; also this method could detect both genotypes and HBV sequence mutations in plasma or serum specimens simultaneously. Usually the single gene of hepatitis B surface antigen (HBsAg) gene (s101-s237) or overlapping polymerase gene (rt99-rt280) are amplified by PCR and sequenced for genotyping and mutation detection. In this study the full-length of RT gene (rt1-rt344) was amplified, sequenced by Sanger sequencing and pyrosequencing. The resulting sequencings were compared to reference sequences for genotypes A-H to determine HBV genotype and mutational analysis. Comparison of the two methods, it is obviously the Sanger sequencing is more reliable for genotyping and has higher sensitivity for mutation detection than pyrosequencing. Therefore, we considered that Sanger sequencing is a gold standard applicable method for clinical HBV genotyping and mutation detection.

Pyrophosphate method can detect mutations with low frequency in the PCR products (≥ 5%), while Sanger method could detect the HBV mutations where the mutation frequency in the PCR amplified fragment is over 15%. Based on this, it is considered that the pyrophosphate method has higher sensitivity than the Sanger method. However, HBV often has spontaneous mutations at low frequency, and while this is less clinically significant, the Pyrophosphate method can detect these low frequency drug-resistance mutations. Importantly, the frequency of HBV mutations are dramatically increased in the patients treated with anti-HBV drugs such as interferon and nucleoside analogs, particularly in patients with long-term treatment. It is reported recently that drug treatment causes 63.7% genetic variability in A1 domain of HBV RT region [10]. We also found that HBV mutation detection rate reached up to 28.29% and also high multiple drug-resistance mutations in the patients. We further observed that Pyrophosphate method had a higher failure rate for detection of both the HBV single and multiple drug-resistance mutations compared to Sanger method, also it failed to detect the HBV mutations in the patients treated with Interferon for more than 1 year. This is because pyrophosphate method only reads about 80 bp DNA sequence and needs multiple primers to sequence just the hotspot RT region, therefore, high frequency of mutations in the patients' samples affect the binding of primers to the RT region which result in the higher failure rate and even failure to detect the mutations. Sanger method could read around 1100 bp DNA sequence and one pair of primer

could amplify and sequence full-length of RT region, the high frequency of the mutations barely affects the binding of the primers. Also, Sanger method could detect the mutations in the sites of rsL80, rsV84, rs214, rsQ25 and rsS85, which are not analyzed with Pyrophosphate sequencing method. Therefore, Sanger method is also a more reliable and applicable method for drug resistance mutation, particularly for the patients with long-term anti-virus treatment with Interferon and/or and nucleoside analogs

In summary, we examined the HBV genotypes and drug-resistance mutations in 138 HBV patients with Sanger and Pyrophosphate direct PCR sequencing. We found that that Pyrophosphate method is suitable for detecting mutations in HBV hotspot sites with low genetic variability, but Sanger method has higher detection rate and lower failure rate for drug-induced high frequency of HBV mutations in HBV patients' samples.

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Disclosure of conflict of interest

None.

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