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Performance Evaluation of HBV-DNA Detection by ABI7500 Polmerase Chain Reactor and Analysis of Anti-interference Ability

Haiyan Zhang[1], Jian Dong[2,*]

[1]Research Center, Department of Basic Medicine, Zhangzhou Health Vocational College, Zhangzhou, Fujian, 363000, China
[2]Laboratory Department, 909th Hospital of Zhangzhou (Southeast Hospital Affiliated to Xiamen University), Zhangzhou, Fujian, 363000, China
996066270@qq.com
[*]Corresponding author

Abstract: *The aim of this paper is to evaluate the performance of ABI7500 HBV-DNA detecting system to determine whether the system meets the clinical detection requirements. Referring to the document CNAS-CL02-A009: 2018 of the National Accreditation Committee for Conformity Assessment, the application of the accreditation criteria for quality and competence of medical laboratories in the field of molecular diagnosis, the in-batch precision, inter-batch precision, accuracy, linear range and anti-interference ability of the detection system were evaluated. The precision of the system is less than 3/5TEa in batch and 4/5TEa between batches. Correctness is good. The linearity of the system is good in the range of 2.61×10^2-2.61×10^8 copies/ml. The results of high and median serum tests are not easily interfered by three substances: bilirubin, hemoglobin and triglyceride. The low-value serum tests are more susceptible to the interference of these three substances. The ABI7500 HBV-DNA detection system has good performance evaluation results and can meet the requirements of clinical detection.*

Keywords: *HBV-DNA; ABI7500; precision; correctness; linearity; interference test*

1. Introduction

Currently, about 240 million people worldwide are infected with hepatitis B, with approximately 30 million deaths related to hepatitis B infections[1-2]. HBV-DNA testing is the most direct, specific, and sensitive indicator of HBV infection. Higher levels of HBV-DNA indicate stronger viral replication and higher infectivity, making HBV-DNA one of the most critical indicators in the hepatitis B diagnosis and treatment system[3]. The ABI7500 Real-Time Fluorescent Quantitative PCR Instrument, manufactured by the American company ABI, is an integrated platform capable of rapidly and specifically detecting and quantifying target genes in real-time. This instrument is widely used in various molecular biology laboratories. This paper evaluates the performance of the ABI7500 Real-Time Fluorescent Quantitative PCR Instrument in detecting and quantifying HBV-DNA, in conjunction with the reagents used in the detection system.

2. Materials

2.1 Samples

Clinical specimens were sourced from outpatient patients at our hospital between January 2021 and July 2021. All serum samples were collected from patients in a fasting state by drawing 3~5ml of venous blood into vacuum blood collection tubes, centrifuged at 3000r/min for 10 minutes to separate the serum, and stored at 4~8℃ for 12 hours before testing.

2.2 Main Instruments and Reagents

- ABI7500 Real-Time Fluorescent Quantitative PCR Instrument
- Thermo PICO21 Benchtop High-Speed Centrifuge

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- BSC-1100IIB2-X Biological Safety Cabinet
- Thermo-shaker Dry Bath Incubator
- HBV-DNA Nucleic Acid Detection Kit (Zhongshan Daan Company)

3. Methods

According to the China National Accreditation Service for Conformity Assessment's relevant document CNAS-CL02-A009:2018 "Application Guidelines for Quality and Competence in Molecular Diagnostics in Medical Laboratories", performance verification of quantitative projects should include at least the following items: within-batch precision, between-batch precision, accuracy, linear range, and anti-interference capability. The DNA extraction method in this paper is based on the ♦*one-step method.

3.1 Within-Batch Precision

High concentration (7.98E+7) and low concentration (2.42E+3) clinical serum samples were collected, with 20 DNA extractions performed on the same day for each sample, and the results were analyzed after logarithmic transformation to calculate the coefficient of variation (CV). According to CNAS-CL02-A009:2018 Appendix A.2, the total allowable error (TEa) is the proficiency test/interlaboratory comparison evaluation limit (target value ±0.4 logarithmic values). If the within-batch precision is <3/5TEa, the evaluation is considered passed.

3.2 Between-Batch Precision

High and low control serum samples produced by Daan Company (batch number 2018002) were used. DNA was extracted and tested each working day, with data collected from April 1 to April 30, 2019 (29 control data points). Results were logarithmically transformed. According to CNAS-CL02-A009:2018 Appendix A.2, the total allowable error (TEa) is the proficiency test/interlaboratory comparison evaluation limit (target value ±0.4 logarithmic values). If the between-batch precision is <4/5TEa, the evaluation is considered passed.

3.3 Accuracy Evaluation

Ten HBV-DNA proficiency test samples provided by the National Center for Clinical Laboratories in 2018 were used. Each sample was tested three times, and the average value was compared with the target value and allowable range provided by the National Center for Clinical Laboratories. If the results are within the allowable range, the evaluation is considered passed. If the project proficiency test score is ≥80%, the accuracy evaluation is considered passed.

3.4 Linear Range

Negative serum was used to dilute a 2.61×10^8 copies/ml serum sample into seven concentrations: 2.61×10^8, 2.61×10^7 , 2.61×10^6 , 2.61×10^5, 2.61×10^4 , 2.61×10^3, 2.61×10^2copies/ml. Each serum sample was extracted and tested three times, and outliers were excluded. The logarithmic average of three tests was taken. Linear regression analysis was performed between the predicted and measured values, yielding the regression equation $Y=bX+a$ and the correlation coefficient R^2. The closer R^2 is to 1, the better the correlation.

3.5 Anti-Interference Capability

Clinical samples negative for HBV-DNA and containing high concentrations of hemoglobin, triglycerides, and bilirubin were selected for anti-interference tests. The interfering substances were diluted into high, medium, and low concentration levels using saline as a blank control. HBV-DNA positive samples were tested, and the logarithmic average of the results was taken. The deviation between the results of serum with interfering substances and the blank control was used to evaluate anti-interference capability. A deviation of ≤7.5% indicates that the interfering substance has no impact on the test results.

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3.5.1 Anti-Bilirubin Interference Test

Two high (1.0×10^8~1.0×10^6 copies/ml), medium (9.0×10^5~1.0×10^4 copies/ml), and low (9.0×10^3~5.0×10^2 copies/ml) serum samples were selected. Serum from a patient with a total bilirubin level of 320 μmol/L and HBV-DNA negative was diluted with saline to high, medium, and low bilirubin concentrations. These were added to high, medium, and low serum samples, respectively. The control was prepared by diluting the same HBV-DNA samples with saline instead of bilirubin-containing serum. The bias was calculated. If the bias is ≤7.5%, bilirubin is considered non-interfering; if the bias is >7.5%, bilirubin is considered interfering.

3.5.2 Anti-Hemoglobin Interference Test

HBV-DNA negative whole blood with EDTA-K2 as an anticoagulant was centrifuged at 3000 r/min for 10 minutes, and the red blood cell layer was washed with saline three times. Hemoglobin solutions with concentrations of 180g/L, 120g/L, and 60g/L were prepared. These were frozen at -20℃ for 4 hours, thawed to room temperature, and fully hemolyzed. These solutions were added to high (1.0×10^8~1.0×10^6 copies/ml), medium (9.0×10^5~1.0×10^4 copies/ml), and low (9.0×10^3~5.0×10^2 copies/ml) HBV-DNA serum samples. Saline was used to prepare control groups with different concentrations of HBV-DNA serum samples. DNA was extracted and tested.

3.5.3 Anti-Triglyceride Interference Test

HBV-DNA negative serum with high triglyceride content was centrifuged to extract the chylous layer. Triglyceride solutions with concentrations of 30mmol/L, 15mmol/L, and 5mmol/L were prepared, representing high, medium, and low triglyceride groups. These solutions were added to high (1.0×10^8~1.0×10^6 copies/ml), medium (9.0×10^5~1.0×10^4 copies/ml), and low (9.0×10^3~5.0×10^2 copies/ml) HBV-DNA serum samples. Saline was used to prepare control groups with different concentrations of HBV-DNA serum samples. DNA was extracted and tested.

4. Results

4.1 Intra-assay Precision

For the high concentration serum (7.98×10^7 copies/ml) tested 20 times, the mean was 7.95, the standard deviation was 0.065, and the coefficient of variation (CV) was 0.82%. For the low concentration serum (2.42×10^3 copies/ml) tested 20 times, the mean was 3.50, the standard deviation was 0.098, and the CV was 2.79%. The intra-assay precision for both high and low concentration serum samples was less than 3/5 of the total allowable error (TEa). The results are shown in Table 1.

Table 1: Intra-assay Precision Test Results for HBV-DNA

Serum Sample Concentration (copies/ml)	Number of Tests	Mean ± SD (logarithmic results)	CV (%)	Performance Requirement
High concentration (7.98×10^7)	20	7.95±0.065	0.82	<3/5TEa
Low concentration (2.42×10^3)	20	3.50±0.078	2.23	<3/5TEa

4.2 Inter-assay Precision

For the high-value control serum, 29 quality control data points were collected. The mean was 6.364, the standard deviation was 0.082, and the CV was 1.29%. For the low-value control serum, 29 quality control data points were collected. The mean was 3.436, the standard deviation was 0.112, and the CV was 3.19%. The inter-assay precision for both high and low-value control samples was less than 4/5 of the total allowable error (TEa). The results are shown in Table 2.

Table 2: Inter-assay Precision Test Results for HBV-DNA

Control Serum Concentration (copies/ml)	Number of Tests	Mean ± SD (logarithmic results)	CV (%)	Performance Requirement
High value (2.28×10^6)	29	6.364±0.082	1.29	<4/5TEa
Low value (2.33×10^3	29	3.441±0.110	3.19	<4/5TEa

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4.3 Accuracy

The Ministry of Health's external quality assessment specimens for the year 2018 were tested, with a total of 10 specimens: 5 in the first half of the year and 5 in the second half. The results were taken as the logarithmic mean values, all of which fell within the allowable range provided by the Ministry of Health's Clinical Laboratory Center. The proficiency testing (PT) score was 100%, and the accuracy evaluation passed. The results are shown in Table 3.

Table 3: HBV-DNA Accuracy Test Results

Sample ID	Test Result	Log Result	Target Value	Allowable Range	Evaluation Result
1811	1.27×10^4	4.10	4.5	4.10-4.90	Passed
1812	0	0	0	-1.00-1.00	Passed
1813	6.08×10^4	4.78	5.09	4.69-5.49	Passed
1814	0	0	0	-1.00-1.00	Passed
1815	3.30×10^4	4.52	4.61	4.21-5.01	Passed
1821	1.51×10^5	5.18	5.13	4.73-5.53	Passed
1822	0	0	0	-1.00-1.00	Passed
1823	0	0	0	-1.00-1.00	Passed
1824	4.43×10^4	4.65	4.79	4.39-5.19	Passed
1825	3.13×10^5	5.50	5.61	5.21-6.01	Passed

4.4 Linearity Range

A linear regression analysis was performed on the measured values and the predicted values, resulting in the regression equation Y=0.9751X+0.7054, with R^2=0.9949. The measured values were plotted on the X-axis and the predicted values on the Y-axis, as shown in Figure 1. According to the linear range analysis, the HBV-DNA assay demonstrated good linearity within the concentration range of 2.61×10^2-2.61×10^8 copies/ml.

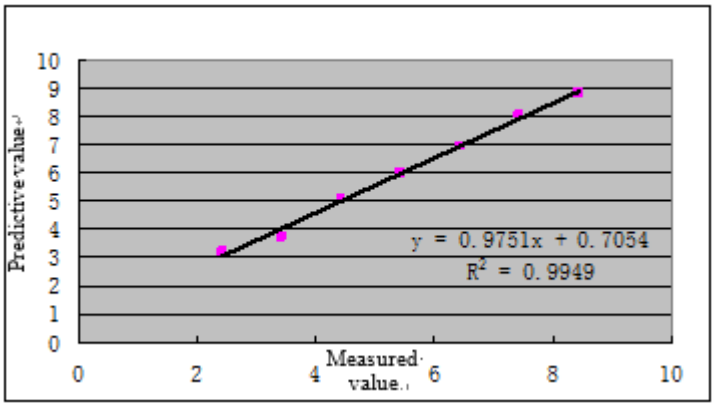

Figure 1: Linear Range Analysis Chart

4.5 Interference Resistance

4.5.1 Resistance to Bilirubin Interference

Bias was calculated between the experimental groups and the control group for high, medium, and low concentration serum samples. The bias for the high concentration serum group was ≤7.5%, indicating that high, medium, and low concentrations of bilirubin did not interfere with the detection of high concentration serum. For the medium concentration serum group, high and medium concentrations of bilirubin resulted in a bias >7.5%, while low concentrations of bilirubin resulted in a bias ≤7.5%, indicating that high and medium concentrations of bilirubin interfered with detection, but low concentrations did not. For the low concentration serum group, biases were >7.5% for high, medium, and low concentrations of bilirubin, indicating that all concentrations interfered with detection. The specific results are shown in Table 4.

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Table 4: Results of Bilirubin Interference Test

Sample concentration	High Bilirubin			Medium Bilirubin			Low Bilirubin			Performance Requirement
	Test Group	Control Group	CV%	Test Group	Control Group	CV%	Test Group	Control Group	CV%	
High-value serum	8.65×10^7	8.32×10^7	0.21	6.24×10^7	5.94×10^7	0.30	4.31×10^7	5.13×10^7	0.99	≤7.5%
	1.07×10^6	2.85×10^6	6.59	1.50×10^6	2.49×10^6	3.56	3.39×10^6	4.88×10^6	2.42	≤7.5%
Medium value serum	1.30×10^5	1.96×10^5	3.49	1.13×10^5	1.82×10^5	4.10	3.33×10^5	4.02×10^5	1.48	≤7.5%
	0	1.07×10^4	100	1.03×10^3	8.74×10^4	39.0	1.77×10^4	3.37×10^4	6.58	≤7.5%
Low value serum	0	4.90×10^3	100	0	1.56×10^3	100	1.04×10^3	1.66×10^3	6.73	≤7.5%
	0	5.00×10^3	100	8.31×10^2	4.76×10^3	20.6	1.59×10^3	3.60×10^3	9.98	≤7.5%

Note: Concentrations in the table are in copies/ml.

4.5.2 Resistance to Hemoglobin Interference

For the high concentration serum experimental group, testing with high, medium, and low concentrations of hemoglobin showed that biases compared to the control group were all ≤7.5%, indicating no interference. For the medium concentration serum group, high concentration hemoglobin resulted in a bias of 10.16% (which is >7.5%), indicating interference, while medium and low concentrations did not interfere. For the low concentration serum group, biases for high, medium, and low concentrations of hemoglobin were all >7.5%, indicating interference. The specific results are shown in Table 5.

Table 5: Results of Hemoglobin Interference Test

Sample concentration	Sample Concentration			High Hemoglobin			Medium Hemoglobin			Performance Requirement
	Test Group	Control Group	CV%	Test Group	Control Group	CV%	Test Group	Control Group	CV%	
High-value serum	1.20×10^7	3.22×10^7	6.06	1.17×10^7	3.40×10^7	6.55	1.27×10^7	1.45×10^7	0.08	≤7.5%
	2.12×10^6	6.18×10^6	7.34	3.46×10^6	7.62×10^6	5.24	5.23×10^6	6.46×10^6	1.37	≤7.5%
Medium value serum	2.38×10^5	5.83×10^5	7.24	3.54×10^5	5.72×10^5	3.76	3.60×10^5	4.98×10^5	2.54	≤7.5%
	2.25×10^4	6.23×10^4	10.16	4.76×10^4	7.81×10^4	4.60	2.91×10^4	3.49×10^4	4.66	≤7.5%
Low value serum	0	2.35×10^3	35.23	5.06×10^2	2.56×10^3	26.04	8.19×10^2	1.98×10^3	13.16	≤7.5%
	0	3.79×10^3	100	0	1.92×10^3	100	9.12×10^2	1.73×10^3	9.39	≤7.5%

Note: Concentrations in the table are in copies/ml.

4.5.3 Resistance to Triglyceride Interference

For high and medium concentration serum samples, the biases between the experimental groups and the control group were all ≤7.5%, indicating that high, medium, and low concentrations of triglycerides did not interfere with the detection of high and medium concentration serum. For the low concentration serum samples, the biases were >7.5%, indicating that high, medium, and low concentrations of triglycerides interfered with detection. The specific results are shown in Table 6.

Table 6: Results of Triglyceride Interference Test

Sample concentration	High triglyceride group			Medium triglyceride group			Low triglyceride			Performance Requirement
	Test Group	Control Group	CV%	Test Group	Control Group	CV%	Test Group	Control Group	CV%	
High-value serum	5.95×10^7	8.74×10^7	2.15	2.05×10^7	5.09×10^7	5.40	2.69×10^7	3.86×10^7	2.11	≤7.5%
	9.84×10^7	6.74×10^7	2.06	9.94×10^7	8.23×10^7	1.03	4.15×10^7	36.77×10^7	2.79	≤7.5%
Medium value serum	3.48×10^5	5.63×10^5	3.77	2.55×10^5	4.03×10^5	3.68	2.08×10^5	4.35×10^5	6.03	≤7.5%
	5.33×10^4	9.63×10^4	5.44	4.75×10^4	9.83×10^4	6.75	4.87×10^4	8.95×10^4	5.64	≤7.5%
Low value serum	0	4.17×10^3	100	0	1.56×10^3	100	6.23×10^2	1.66×10^3	13.22	≤7.5%
	0	1.43×10^3	100	6.88×10^2	1.79×10^3	14.63	1.06×10^3	2.47×10^3	12.14	≤7.5%

Note: Concentrations in the table are in copies/ml.

5. Discussion

In recent years, due to the widespread use of antiviral drugs, HBeAg no longer accurately reflects viral replication in HBV patients[4]. As the most sensitive indicator in hepatitis B treatment, HBV-DNA has a significant correlation with hepatitis B serological markers and liver injury indicators. Monitoring

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changes in viral load can indirectly reflect disease progression and changes in other indicators of hepatitis B[3]. Thus, ensuring the accuracy of HBV-DNA test results is crucial for clinical diagnosis and treatment.

Real-time fluorescent quantitative PCR is the most commonly used method for detecting HBV-DNA. It has high sensitivity and specificity, playing a vital role in the diagnosis and treatment of hepatitis B. Clinical gene amplification laboratories must fully understand the performance of this method and take appropriate control measures to ensure the accuracy of test results[5]. Evaluating the performance of the detection system or methodology is essential for ensuring the quality of clinical testing[6]. Therefore, for HBV-DNA detection, validating the performance of the detection system is a prerequisite for effective laboratory quality control.

This study evaluates the performance of our laboratory's HBV-DNA detection system in terms of precision, accuracy, linear range, and interference resistance. Precision refers to the consistency between repeated test results; good precision and accuracy are prerequisites for accurate results and for conducting other performance validation tests[7]. In this study, intra-batch precision was <3/5 TEa, and inter-batch precision was <4/5 TEa, indicating good intra-batch and inter-batch precision of the detection system. Accuracy reflects the correctness of the detection system. Currently, using reference materials with assigned values in inter-laboratory quality assessment activities is a recommended method[8]. Our detection system monitored accuracy by participating in inter-laboratory quality assessments by the Ministry of Health, with all assessment results passing, indicating good accuracy. When the report range exceeds the concentration range of the calibrators, it is necessary to evaluate the linear range[9]. Through linear range experiments, we found that our detection system has good linearity in the range of 2.61×10^2 copies/ml ~2.61×10^8 copies/ml.

There are currently few reports on interference tests. We analyzed the interference resistance of the detection system by adding bilirubin, hemoglobin, and triglycerides, three common clinical interferents. We concluded that high and medium concentration serum test results are not easily affected by these three substances, while low concentration serum is easily affected. Qing Yu et al.'s study[10]found that the influence of bilirubin on HBV-DNA detection is related to both the serum HBV-DNA load and the bilirubin level. This is consistent with our results. Hemoglobin can irreversibly bind to Taq enzyme through its porphyrin ring, inhibiting Taq enzyme activity and reducing PCR amplification efficiency, ultimately lowering the HBV-DNA detection level[11]. The effect of triglycerides on HBV-DNA detection may be due to: (1) fluorescence quenching caused by triglycerides, reducing the fluorescence signal intensity; (2) inhibition of Taq enzyme activity by triglycerides, reducing PCR amplification efficiency[12].

In conclusion, the ABI7500 HBV-DNA detection system performs well in terms of precision, accuracy, linear range, and interference resistance, meeting clinical testing requirements.

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