

Limit of Detection of HEV RNA for Diagnosis of HEV Infection

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ABSTRACT

Background and Objectives: Diagnosis of hepatitis E virus (HEV) infection could be missed in some cases if serological tests are used solely. Molecular characterization of HEV is essential for diagnosis of acute and chronic HEV infections, and evaluating the chronic HEV infection status in immunocompromised patients. The aim of this study was to prepare a suitable HEV positive control, determine the limit of detection (LOD) of HEV RNA for a specific molecular test, and evaluate the efficiency and precision of the test .

Methods: Genomic region of HEV NCBI reference sequence was constructed. LOD, intra-assay precision, and inter-assay precision were calculated to evaluate the efficiency and precision of the test. Then, tenfold serial dilutions of the HEV positive control were prepared. Real time PCR was performed three times for each dilution. Mean, standard deviation, and coefficient of variation of cycle thresholds obtained in three independent and simultaneous tests were calculated, and the results were analyzed.

Results: The LOD of this test was determined as 1.4×10^4 copy/ml or 42 copy/reaction or 14 copy/ μ l. Intra-assay precision and inter-assay precision for all assays were lower than 2.5% and 10%, respectively.

Conclusion: We propose that the real time PCR assay targeting the ORF2/3 overlapping conserved region is suitable for detection of a wide range of different HEV genotypes found in acute and chronic HEV infections. However, the precision of the test should be improved for detecting HEV RNA lower than 103 copy/ml .

Keywords: Hepatitis E virus, Limit of Detection, Real Time PCR.

INTRODUCTION

Hepatitis E virus (HEV) is a single stranded RNA virus with icosahedral symmetry (1). The HEV genome contains three open reading frames (ORFs)(2). It has one serotype with four genotypes that causes mild symptoms in immunocompetent patients, and hepatic failure and death in pregnant women (3). In our previous studies, seroprevalence of HEV in Gorgan was 6.3% among women of childbearing age and 7.3% among pregnant women (4,5). Recently, several studies have reported chronic HEV infections in immunosuppressed patients such as HIV patients, patients with hematological disease and solid organ transplant recipients (6). Diagnosis of HEV infections could be missed in some cases if serological tests are used solely (7). Immunocompromised patients have impaired immune system and delayed antibody response, which may lead to false-negative results in the HEV antibody test. Therefore, molecular diagnosis of acute HEV infections could be useful for this group of patients (6). Molecular testing is also essential for diagnosing chronic HEV infection. Chronic hepatitis E is considered when HEV RNA persisted for at least six months in the serum (6). HEV RNA testing is also suitable for evaluating chronic HEV infection status after reduction of immunosuppression or initiation of antiviral therapy (6). This further highlights the need for molecular analysis when assessing HEV infections. This study aimed to evaluate the prevalence of HEV in 149 hemodialysis patients and 102 HIV-positive patients by designing a molecular test. Since we were unable to provide a biological HEV-positive sample, it was necessary to set up a positive control for the molecular test. We also aimed to prepare a suitable HEV-positive control and a specific HEV molecular test by evaluating the limit of detection (LOD) and intra- and inter-assay precisions.

MATERIAL AND METHODS

Positive control

Genomic sequence at positions 5256 to 5334 was constructed from the HEV NCBI reference sequence (Accession number: NC_001434.13)(Macrogen, Korea), and cloned into the T/A plasmid by a commercially available kit (RBC T/A Cloning Kit, Taiwan) according to the manufacturer's protocol. The construct was transformed into

Escherichia coli strain DH5alpha. Colony PCR was done to determine the accuracy of cloning and transforming process. Plasmid was extracted using a kit (Qiagen, Germany). The recombinant plasmid stock was quantified using the NanoDrop spectrophotometer (Thermo Scientific, UK) according to the manufacturer's instructions. It was later converted into genome copy numbers based on the following formula: copy number = [(concentration of linearized plasmid)/(molar mass)] × (6.023 × 10²³)(8).

Real time PCR

In the HEV genome, ORF2/3 overlapping region is described as more conserved compared to ORF2 (7). Since the HEV genotype is unknown in Iran, we performed real time PCR using TaqMan probe and primers targeting the conserved overlapping region ORF2/3 (nt 5261– 5330 of the HEV reference gene). The specific primers used in the real time PCR experiment were as follows: F, 5'-GGTGGTTTCTGGGGTGAC-3', R, 5'/AGGGGTTGGTTGGATGAA-3and 5'-FAM-TGATTCTCAGCCCTTCGC-BHQ-3' (7). PCR was carried out in 25 µl reactions containing 22 µl of master mix (Ampliqon, Germany) with 20 pm concentration of primers and probe, and 3 µl of sample. The assay was performed using an Applied Biosystems 7300 Real-Time PCR System in a 96-well format. Cycling conditions were as follows: initial denaturation and Taq polymerase activation at 95 oC for 15 min, 35 cycles of denaturation at 95 oC for 15 s, annealing at 60 oC for 20 s, and extension at 72 oC for 30 s. Distilled water and HEV RNA/HEV antibody negative sample were used as negative controls. A test was considered positive if the cycle threshold (CT) was lower than 33 amplification cycles.

LOD

The LOD of this molecular test was defined as the lowest concentration of DNA that could be measured (8). In order to calculate the LOD, tenfold serial dilutions (1.5×10⁻¹–41.5×10⁻¹⁰) were prepared for the HEV-positive control. The real time PCR experiment was performed three times for all dilutions. The mean of CTs obtained after each experiment was calculated, and the results were analyzed.

Precision

Intra- and inter-assay precisions were assessed to determine repeatability (test–retest

reliability) and accuracy of the test. Inter-assay precision was assessed by calculating the standard deviation (SD) and coefficient of variation (CV) of the CT obtained for each dilution tested in three independent runs. Intra-assay precision was assessed by calculating the SD and CV of the CT obtained in triplicate assays of each dilution (8).

Statistical analysis

LOD was determined by calculating the mean of obtained CTs in triplicates. Inter- and intra-assay precisions were computed using the CT values, SDs, and CVs of the standard curves.

Ethical approval

The study has been approved by the Ethics Committee of the Golestan University of Medical Sciences, Gorgan, Iran (IR.goums.REC.1394.25).

RESULTS

The mean concentration of extracted plasmid was 215 ng / μ l. Length of the recombinant T/A plasmid was 2808 bp. The total number of genome copies in the extracted plasmid stock was calculated as follows: $[(215 \times 10^{-9}) / (2809 \times 650)] \times (6.023 \times 10^{23}) = 7 \times 10^{10}$ copy/ μ l. DNA copy number of each dilution was also determined (Table 1). Tables 1 and 2 show the mean, SDs and CVs of the CTs obtained for each dilution tested in three simultaneous and separate assays. The LOD of the test was determined as 1.4×10^4 copy/ml or 42 copy/reaction or 14 copy/ μ l. Intra-assay precision and inter-assay precision for all assays were found to be lower than 2.5% and 10%, respectively.

Table 1- Results of detection of 10-fold serial dilutions of the positive *HEV* control in three simultaneous real time PCR assays.

ID	Copy number/ μ l	Detected sample	Mean CT	SD	CV%
1	1.4×10^6	3/3	15.80	0.175	1.10
2	1.4×10^5	3/3	18.49	0.127	0.68
3	1.4×10^4	3/3	21.88	0.291	1.32
4	1.4×10^3	3/3	25.00	0.504	2.01
5	1.4×10^2	3/3	28.77	0.326	1.13
6	1.4×10^1	3/3	31.82	0.763	2.39
7	1.4	3/3	33.34	0.450	1.34
Negative control	-	-	-	-	-
Negative sample	-	-	-	-	-

Table 2- Results of detection of 10-fold serial dilutions of the positive *HEV* control in three independent real time PCR

ID	Copy number/ μ l	Detected sample	Mean CT	SD	CV%
1	1.4×10^6	3/3	16.35	1.252	7.65
2	1.4×10^5	3/3	21.63	2.165	10.00
3	1.4×10^4	3/3	24.29	1.181	4.86
4	1.4×10^3	3/3	27.60	1.234	4.47
5	1.4×10^2	3/3	30.53	2.491	8.15
6	1.4×10^1	3/3	31.19	1.089	3.49
7	1.4	3/3	31.33	1.872	5.96
Negative control	-	-	-	-	-
Negative sample	-	-	-	-	-

DISCUSSION

Molecular characterization of *HEV* is essential for diagnosis of acute and chronic *HEV* infections, and evaluation of chronic *HEV* infection status after reduction of immunosuppression and initiation of antiviral therapy in immunocompromised patients (6). Serological and molecular testing is necessary to investigate all aspects of *HEV* infection. In this study, we prepared a specific molecular test for detection of *HEV* RNA, and evaluated the test's efficiency and precision. The LOD of real time PCR test was 1.4×10^4 copies/ml. A

recent review on the detection of *HEV* RNA in immunocompetent individuals reported that the range of *HEV* RNA concentration was 126 to $>10^7$ copy/ml in acute phase, and 501 to $>10^7$ copies/ml in solid organ transplant recipients (6). Although we detected the presence of *HEV* in a wide range, detection of *HEV* RNA in a sample with less than 10^4 copies/ml seems impossible. Moreover, the type of kit used for molecular testing may influence the quality of molecular detection. Some studies on the incidence of *HEV* have

determined LOD using the World Health Organization (WHO) international standard for *HEV* RNA detection, and reported the values in international units (7,9–11). Currently, there is no standard formula for converting the amount of *HEV* RNA reported in copies/ml to the amount reported in International Units. Therefore, we are unable to compare our results with other studies that have reported the LOD values based on the WHO standards (12).

In 2014, Gerber et al. compared four different (A-D) *HEV* real time PCR tests by determining the LOD and precision for each test. The LOD of tests A and B was determined as 4×10^3 copy/ml and 4×10^4 copy/ml, respectively. However, the LOD of the other two tests was 4×10^6 copy/ml. They reported that tests A and B are appropriate for *HEV* diagnosis (8). The LOD we found in our study (1.4×10^4 copy/ml) was close to the result of test B test in the mentioned study. Thus, the LOD reported in the present study could also be suggested suitable for the *HEV* detection. In addition, comparison of the intra- and inter-

REFERENCES

- Hosseini-Moghaddam SM, Zarei A, Alavian SM, Mansouri M. *Hepatitis E virus infection: a general review with a focus on hemodialysis and kidney transplant patients*. Am J Nephrol. 2010; 31(5): 398-407. doi: 10.1159/000294505.
- Ahmad I, Holla RP, Jameel S. *Molecular virology of hepatitis E virus*. Virus Res. 2011; 161(1): 47-58. doi: 10.1016/j.virusres.2011.02.011.
- Pérez-Gracia MT, García M, Suay B, Mateos-Lindemann ML. *Current Knowledge on Hepatitis E*. J Clin Transl Hepatol. 2015; 3(2): 117-26.
- Moradi A, Besharat S, Minaiifar M, Roshandel G, Tabaraei A. *Seroepidemiologic assessment of Hepatitis E virus in women of reproductive age, Gorgan*. Zahedan J Res Med Sci. 2010; 12(1).
- Tabarraei A (last), Moradi A, Rodgari D, Javid N, Bakhshandeh Nosrat. *Anti-Hepatitis E Virus seroprevalence in pregnant women, in Gorgan, Iran, North East of Caspian Sea*. International Conference on life science and Technology. 2011; 3: 162-4.
- Kamar N, Dalton HR, Abravanel F, Izopet J. *Hepatitis E virus infection*. Clin Microbiol Rev. 2014; 27(1): 116-38.
- Mokhtari C, Marchadier E, Haïm-Boukoba S, assay precisions found in our study (<2.5% and <10%) and study of Gerber et al. (<4% and <7%) indicates that our assay has favorable precision (8).

CONCLUSION

We suggest a real time PCR test that targets the ORF2/3 overlapping conserved region is suitable for detection of a wide range of different *HEV* genotypes present in acute and chronic *HEV* infection. However, there is a need to improve its precision for detection of *HEV* RNA lower than 103 copy/ml. Further studies are required to compare the values found in the present study with WHO *HEV* standard and commercial kits.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

Jeblaoui A, Tessé S, Savary J, et al. *Comparison of real time RT-PCR assays for hepatitis E virus RNA detection*. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2013; 58(1): 36-40.

8. Gerber PF, Xiao C-T, Cao D, Meng X-J, Opriessnig T. *Comparison of real-time reverse transcriptase PCR assays for detection of swine hepatitis E virus in fecal samples*. J Clin Microbiol. 2014; 52(4): 1045-51.

9. Baylis SA, Blümel J, Mizusawa S, Matsubayashi K, Sakata H, Okada Y, et al. *World Health Organization International Standard to Harmonize Assays for Detection of Hepatitis E Virus RNA*. Emerg Infect Dis. 2013; 19(5): 729-35.

10. Hassing RJ, van der Eijk AA, Lopes VB, Snijdewind IJ, de Man RA, Pas SD, et al. *Hepatitis E prevalence among HIV infected patients with elevated liver enzymes in the Netherlands*. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2014; 60(4): 408-10.

11. Feldt T, Sarfo FS, Zoufaly A, Phillips RO, Burchard G, van Lunzen J, et al. *Hepatitis E virus infections in HIV-infected patients in Ghana and Cameroon*. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2013; 58(1): 18-23.

12. Franciscus A. *HCV Viral load tests*. Hepat C Basics. 2006; 2: 1-3.