TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2013) 43: 294-298
© TÜBİTAK
doi:10.3906/sag-1204-81

Anti-HDV enzyme immunoassay index in predicting HDV viremia in serum samples

Sibel ÖZSU CAYMAZ[1], Ayça Arzu SAYINER[2], Ayşe Aydan ÖZKÜTÜK[2,*], Yusuf Hakan ABACIOĞLU[2]

[1]Department of Clinical Microbiology, İzmir Training and Research Hospital of the Ministry of Health, Bozyaka, İzmir, Turkey
[2]Department of Medical Microbiology, Dokuz Eylül University, Faculty of Medicine, İnciraltı, İzmir, Turkey

Received: 18.04.2012 • **Accepted:** 24.07.2012 • **Published Online:** 15.03.2013 • **Printed:** 15.04.2013

Aim: To determine whether the total anti-delta antibody enzyme immunoassay (EIA) test results can be used to predict viremia.

Materials and methods: The EIA index value of an anti-delta total antibody assay was determined in 76 serum samples obtained from 67 patients positive for the anti-hepatitis delta virus (HDV) antibody. The presence of HDV RNA was investigated by reverse transcriptase polymerase chain reaction (RT-PCR). The relation between the RT-PCR result and EIA index was evaluated by ROC analysis.

Results: Of the 76 total samples, 56 (74%) anti-HDV–positive samples were found to be PCR-positive. The samples with EIA index values below 75.75 were PCR-negative, while samples with index values above 102.55 were PCR-positive. When HDV RNA presence is considered as the gold standard, the sensitivity, specificity, and positive and negative predictive values of the chosen EIA index (100.10) in predicting viremia were 93%, 80%, 93%, and 76%, respectively.

Conclusion: HDV RNA is the most reliable marker for the diagnosis of active HDV infection, and EIA index value is closely related to viral RNA presence.

Key words: Anti-HDV enzyme immunoassay, ROC analysis, HDV RNA, reverse transcriptase polymerase chain reaction

1. Introduction

Hepatitis delta virus (HDV) infection is an important health problem causing fulminant hepatitis, chronic liver disease, cirrhosis, and hepatocellular cancer. HDV is an enveloped, negative-sense, single-stranded RNA virus. It is a satellite of the hepatitis B virus (HBV) and requires a surface antigen of hepatitis B virus (HBsAg) for its assembly and transmission (1,2). Simultaneous infection with HDV and HBV results in coinfection in an HBV-naïve individual, whereas HDV infection in a patient who is already chronically infected with HBV causes superinfection. HDV superinfection frequently leads to chronic hepatitis D, while a minority of coinfected cases may progress to chronicity (1,3).

It is estimated that approximately 5% of global HBsAg carriers (approximately 15 million people) are infected with HDV (1). Although HDV infection is distributed worldwide, its prevalence shows geographic differences. It is endemic in the Mediterranean countries, including Turkey. While anti-HDV seroprevalence showed a significant reduction in Turkey since the 1980s, the studies done in the 2000s give a mean rate of 2.9% for asymptomatic HBsAg carriers, 11% for chronic hepatitis B, and 24% for liver cirrhosis patients (4). Presence of anti-HDV may rise up to 45.5% among chronic HBV patients in eastern regions of Turkey (5). Although overall HDV incidence in West Europe might be decreasing, studies done in some countries show that HDV is not a vanishing disease due to migration from regions with a higher prevalence such as East Europe or Africa (6). Nucleotide sequence analysis of several isolates allowed classification of HDV into at least 8 genotypes, which also shows a geographic distribution (7). Genotype 1 is dominant in Turkey (8,9).

The diagnosis of HDV infection is made by detecting anti-HDV antibodies and/or viral components, i.e. HDV RNA or HDVAg. Serum HDVAg detection does not provide high diagnostic sensitivity since the amount of detectable viral antigen varies according to the stage of infection and to the titer of circulating antibodies that may sequester the antigen. There is also broad sensitivity variability among the commercial HDVAg assays (10). Total anti-HDV antibody detection by enzyme immunoassay (EIA) is generally the preferred method for the initial screening for HDV infection among HBV-

* Correspondence: aydan.ozkutuk@deu.edu.tr

infected patients (6). If the patient is anti-HDV positive, the ongoing replication is evaluated by the presence of serum HDV RNA, since only a part of the seropositive patients are viremic and viremia indicates potential for liver damage (11–13). Reverse transcriptase polymerase chain reaction (RT-PCR) is the most frequently selected method for viral RNA detection due to its high sensitivity (14). However, HDV RNA detection assays are mostly homemade assays that lack standardization and are labor-intensive and costly compared to EIAs (15,16). Although screening anti-HDV EIAs gives a qualitative result, further information can be gained from the strength of the reaction signal. In the present study, the relationship between anti-HDV EIA index value and HDV RT-PCR is examined in order to determine whether EIA results can be used to predict viremia.

2. Materials and methods

2.1. Patients

A total of 76 serum samples from 67 patients with a positive total anti-HDV antibody test result were evaluated. The anti-HCV and anti-HIV antibodies were negative while HBsAg and total anti-HBc antibody were positive in all of the patients. The serum samples were collected from patients who were being followed by 2 different medical centers (30 patients from the hospital of the İstanbul University School of Medicine and 37 patients from the hospital of Dokuz Eylül University). They were aliquoted and stored at –70 °C.

2.2. Detecting anti-HDV antibody by EIA

Anti-HDV antibody status of the sera was tested by a commercial anti-delta total antibody EIA kit (Hepanostika HDV, Organon Teknika, the Netherlands; the assay is currently not commercially available). The assay and calculation of the EIA index with absorbance values were performed with the TekTime instrument according to the manufacturer's instructions. EIA index value was automatically determined by dividing (the mean of the negative control's absorbance – sample absorbance) by (the mean of the negative control's absorbance – mean of the positive control's absorbance) and then multiplying by 100.

2.3. HDV RNA RT-PCR

The presence of HDV RNA in serum samples was studied by RT-PCR using a set of primers targeting the delta antigen gene (Table 1). The nucleic acid was extracted from 200 µL of the patient's serum using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany) according to the manufacturer's instructions.

For cDNA synthesis, 5 µL of extracted RNA was mixed with 50 pmol of antisense primer (P-1291). The reaction tube was held 5 min at 65 °C and then cooled on ice. The 14-µL reaction mixture containing 5X buffer, 10 U RNase inhibitor (Roche Applied Science), 10 U AMV reverse transcriptase (Roche Applied Science), and 200 µM dNTP mix (PCR Nucleotide Mix, Roche Applied Science) was added to each reaction tube. Reverse transcription was performed by holding the tubes at 42 °C for 60 min and then at 72 °C for 10 min.

cDNA amplification was carried out in a 50-µL PCR mixture containing 5 µL cDNA, 1.5 mM $MgCl_2$, 200 µM dNTP mix, 25 pmol sense primer (P-889), 12.5 pmol antisense primer (P-1291), 2.5 µL hot start DNA polymerase (Fast Start Taq DNA Polymerase, Fermentas, Lithuania), and 10X buffer. The tubes were placed in a thermal cycler (PerkinElmer GeneAmp 9600). Primary denaturation was performed for 6 min at 95 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The final extension step was at 72 °C for 10 min. In order to prevent contamination during PCR, all the necessary measures were strictly taken. In each PCR run, 1 positive and 1 negative control were used along with the patient samples. The PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and visualized under UV light. EIA and RT-PCR tests were performed twice for each sample.

2.4. Statistical analysis

Receiver operating characteristic (ROC) analysis (SPSS 11.0) was used to examine the correlation between the RT-PCR results and the EIA index.

3. Results

Fifty-six of the 76 anti-HDV–positive samples (73.6%) had detectable HDV RNA (Figure 1). All of the samples with an anti-HDV EIA index value below 75.75 were HDV RNA negative, while samples with an index value above 102.55 were HDV RNA positive. The EIA index of 100.10 was chosen as the threshold value for predicting HDV viremia according to ROC analysis. Fifty-two of the 56 HDV RNA–positive samples (92.8%) had an EIA index of >100.10.

Table 1. The primers used for HDV RNA RT-PCR.

	Primer		Location (nt)	Product length
P-889	Sense	5'-ATGCCATGCCGACCCGAAGAGGAA-3'	889–912	403 bp
P-1291	Antisense	5'-GAAGGAAGGCCCTCGAGAACAAGA-3'	1291–1268	

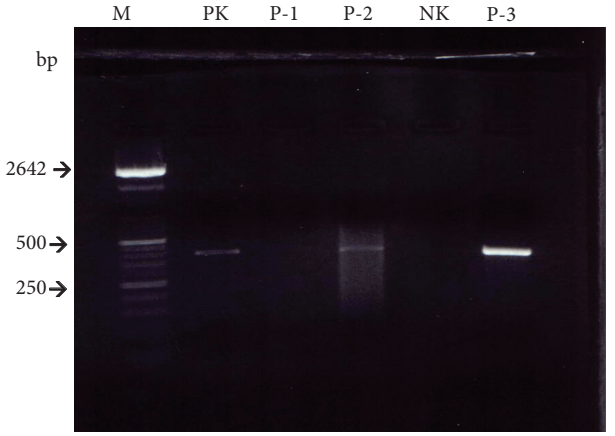

Figure 1. HDV RNA RT-PCR products of 3 patients' sera. Amplicons were visualized on a 2% agarose gel stained with ethidium bromide. M: Marker; PK: positive control; NK: negative control; P-1: HDV RNA-negative patient; P-2 and P-3: HDV RNA-positive patients with a specific product size of 403 bp.

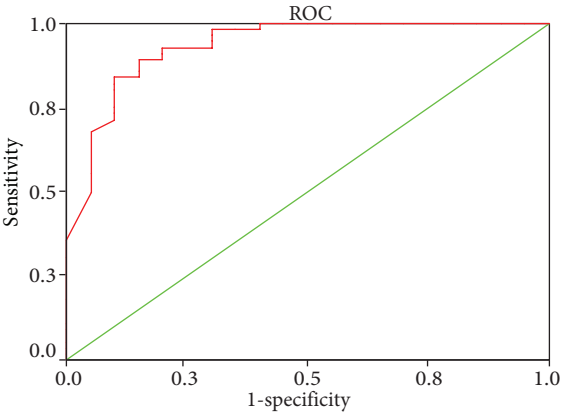

Figure 2. The sufficiency analysis of EIA index in predicting viremia (ROC area: 0.93).

Four of the 20 HDV RNA negative samples had an EIA index of ≥100.10 while the index values of the remaining 16 samples (80%) were below this threshold. When the presence of HDV RNA in the serum was accepted as the gold standard, the sensitivity, specificity, and positive and negative predictive values of the threshold EIA index (i.e. 100.10) for predicting viremia were 93%, 80%, 93%, and 76%, respectively (Table 2). ROC analysis showed that the EIA index value of a PCR-positive sample would have a higher result with a 93% probability than a PCR-negative sample (ROC area = 0.934) (Figure 2).

4. Discussion

Total anti-HDV antibody detection by EIA is widely used for the diagnosis of HDV infection. There are several commercial anti-HDV assays. They are relatively standardized, cost-effective, and easy to apply, and they can be automated for assaying a large number of samples (10). It is important to differentiate chronic HDV infection with an ongoing viral replication from a resolved infection in an anti-HDV–positive patient. Methods such as the titration of total anti-HDV and/or anti-HDV IgM and differentiation of monomeric anti-HDV IgM (7S) from the pentameric (19S) form have been used for identifying various clinical forms of delta infection (15,17,18). However, none of these procedures are suitable for the routine diagnostic laboratory. In an epidemiological study done with an in-house anti-HDV EIA, it was shown that the optical density (OD) value was significantly higher in the HDV RNA-positive individuals compared to HDV RNA-negative subjects, but determination of a cutoff value was not attempted (19). The aim of this study was to use the EIA index of the total anti-HDV assay to predict viremia. This can easily be calculated while performing the screening assay.

HDV RNA is an important tool to evaluate seropositive individuals since viremia is the best marker of viral replication and plays a major role in the pathogenesis of liver injury (11,17). Its detection requires a highly sensitive nucleic acid assay such as RT-PCR. HDV RNA was detected in 22%–80% of anti-HDV–positive patients, depending on the study group and the nucleic acid assay (8,19,20). The need for a specifically designed laboratory and educated personnel, the lack of standardization, and the use of homemade assays are the problems related to HDV RNA detection. Persistence of high titers of anti-HDV is consistent with chronic HDV infection and presence of HDV RNA (1,10,17). However, determination of antibody titer requires serial dilution of the serum sample. In the present study, EIA index was used instead of

Table 2. The sensitivity, specificity, and positive and negative predictive values of 3 different EIA index values in predicting viremia.

EIA index value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
75.75	100	60	88	100
100.10	93	80	93	76
102.55	36	100	100	36

anti-HDV titer. The results confirmed that the EIA index of the total anti-HDV assay was closely related to HDV RNA positivity. The ROC analysis showed that, for this EIA assay, when the threshold value of 100.10 was chosen for the EIA index to predict viremia, the specificity and sensitivity were 80% and 93%, respectively. Prediction of viremia by the EIA index can minimize the amount of nucleic acid testing needed while improving the clinical significance of the screening assay. A similar approach was recommended by the CDC for the diagnosis of HCV infection (21). This guideline suggests using the sample to cut-off OD ratio of the reactive anti-HCV test to determine the next step in the algorithm. As seen with HCV assays, EIA index values used for prediction of viremia depend on the antibody and nucleic acid detection tests used due to a lack of standardization between assays. This factor could be more important for HDV, since international standards for HDV RNA assays are not yet developed (22). Therefore, the EIA index chosen for the prediction of viremia in this study is only valid for the specified conditions.

The limitations of diagnosis by the detection of anti-HDV antibodies are the potential for false negative results in the early phase of the infection (before antibody seroconversion) and infection in immunosuppressed patients. HDV RNA detection has a crucial role in these circumstances (1). Viral load assessment also helps in monitoring the response to the therapy (23).

In conclusion, the search for a total anti-HDV antibody is widely used as the initial screening method for the diagnosis of HDV infection. As a second step, the presence of HDV RNA should be determined in an anti-HDV–positive patient, since liver injury is related to chronic viremia. The present study showed that the EIA index of the reactive total anti-HDV assay is highly correlated with the presence of HDV RNA detected by RT-PCR. It can be used as a predictor of viremia in a diagnostic microbiology laboratory. It might be necessary for each laboratory to determine their own EIA index due to lack of standardization between HDV RNA detection assays.

Acknowledgment
We thank Professor Selim Badur (İstanbul University School of Medicine) for providing some of the serum samples.

References

1. Farci P. Delta hepatitis: an update. J Hepatol 2003; 39: 212–9.
2. Taylor JM. Hepatitis delta virus. Virology 2006; 344: 71–6.
3. Khalid M, Umair SA, Memon S, Khawaja A, Usmani MT, Moatter T et al. Virological and clinical characteristics of hepatitis delta virus in South Asia. Virol J 2011; 8: 312.
4. Değertekin H, Yalçın K, Yakut M. The prevalence of hepatitis delta virus infection in acute and chronic liver diseases in Turkey: an analysis of clinical studies. Turk J Gastroenterol 2006; 17: 25–34.
5. Bahcecioglu IH, Aygun C, Gozel N, Poyrazoglu OK, Bulut Y, Yalniz M. Prevalence of hepatitis delta virus (HDV) infection in chronic hepatitis B patients in eastern Turkey: still a serious problem to consider. J Viral Hepat 2011; 18: 518–24.
6. Wedemeyer H, Heidrich B, Manns MP. Hepatitis D virus infection not a vanishing disease in Europe. Hepatology 2007; 45: 1331–2.
7. Le Gal F, Gault E, Ripault MP, Serpaggi J, Trinchet JC, Gordien E et al. Eighth major clade for hepatitis delta virus. Emerg Infect Dis 2006; 12: 1447–50.
8. Altuğlu I, Özacar T, Sertoz RY, Erensoy S. Hepatitis delta virus (HDV) genotypes in patients with chronic hepatitis: molecular epidemiology of HDV in Turkey. Int J Infect Dis 2007; 11: 58–62.
9. Bozdayı AM, Aslan N, Bozdayı G, Türkyılmaz AR, Sengezer T, Wend U et al. Molecular epidemiology of hepatitis B, C and D viruses in Turkish patients. Arch Virol 2004; 149: 2115–29.
10. Shattock AG, Morris MC. Evaluation of commercial enzyme immunoassays for detection of hepatitis delta antigen and anti-hepatitis delta virus (HDV) and immunoglobulin M anti-HDV antibodies. J Clin Microbiol 1991; 29: 1873–6.
11. Yamashiro T, Nagayama K, Enomoto N, Watanabe H, Miyagi T, Nakasone H et al. Quantitation of the level of hepatitis delta virus RNA in serum, by real-time polymerase chain reaction and its possible correlation with the clinical stage of liver disease. J Infec Dis 2004; 89: 1151–7.
12. Sakugawa H, Nakasone H, Kawakami Y, Adaniya H, Mizushima T, Nakayoshi T et al. Determination of hepatitis delta virus (HDV) RNA in asymptomatic cases of HDV infection. Am J Gastroenterol 1997; 92: 2232–6.
13. Mansour W, Bollahi MA, Hamed CT, Brichler S, Le Gal F, Ducancelle A et al. Virological and epidemiological features of hepatitis delta infection among blood donors in Nouakchott, Mauritania. J Clin Virol 2012; 55: 12–6.
14. Tang JR, Cova L, Lamelin JP, Baginski I, Vitvitski L, Gaudin JL et al. Clinical relevance of the detection of hepatitis delta virus RNA in serum by RNA hybridization and polymerase chain reaction. J Hepatol 1994; 21: 953–60.
15. Niro GA, Gioffreda D, Fontana R. Hepatitis delta virus infection: open issues. Dig Liver Dis 2011; 43: 19–24.
16. Ferns RB, Nastouli E, Garson JA. Quantitation of hepatitis delta virus using a single-step internally controlled real-time RT-qPCR and a full-length genomic RNA calibration standard. J Virol Methods 2012; 179: 189–94.

17. Jardi R, Buti M, Rodriguez-Frias F, Garcia-Lafuente A, Sjogren MH, Esteban R et al. Clinical significance of two forms of IgM antibody to hepatitis delta virus. Hepatology 1991; 14: 25–8.

18. Huang YH, Wu JC, Sheng WY, Huo TI, Chang FY, Lee SD. Diagnostic value of anti-hepatitis D virus (HDV) antibodies revisited: a study of total and IgM anti-HDV compared with detection of HDV-RNA by polymerase chain reaction. J Gastroenterol Hepatol 1998; 13: 57–61.

19. Inoue J, Takahashi M, Nishizawa T, Narantuya L, Sakuma M, Kagawa Y et al. High prevalence of hepatitis delta virus infection detectable by enzyme immunoassay among apparently healthy individuals in Mongolia. J Med Virol 2005; 76: 333–40.

20. Özekinci T, Atmaca S, Akpolat N, Temiz H, Arıkan E. Short communication: evaluation of the correlation between hepatitis D virus (HDV) RNA positivity and HDV antibodies. Mikrobiyol Bul 2005; 39: 345–9.

21. Centers for Disease Control and Prevention. Guidelines for laboratory testing and result reporting of antibody to Hepatitis C virus. MMWR Morb Mortal Wkly Rep 2003; 52(RR03): 1–16.

22. Wedemeyer H. Re-emerging interest in hepatitis delta: new insights into the dynamic interplay between HBV and HDV. J Hepatol 2010; 52: 627–9.

23. Le Gal F, Gordien E, Affolabi D, Hanslik T, Alloui C, Deny P et al. Quantification of hepatitis delta virus RNA in serum by consensus real-time PCR indicates different patterns of virological response to interferon therapy in chronically infected patients. J Clin Microbiol 2005; 43: 2363–9.