CONFERENCE PROCEEDINGS

A new enzyme immunoassay for the detection of antibody to hepatitis E virus

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Abstract

Background and Aim: The purpose of the present study was to develop enzyme immunoassay (EIA) for the detection of IgG anti-hepatitis E virus (HEV) activity using two new recombinant proteins as antigenic targets, and to evaluate these EIA with the aid of statistical methods.

Methods: Two proteins, a mosaic protein and pB166 containing region 452–617 aa of the ORF2 of the HEV Burma strain, were used to develop the new HEV EIA. This EIA was evaluated using several panels of serum specimens obtained from: (i) acutely HEV-infected patients; (ii) patients with non-A, non-C hepatitis; (iii) normal blood donors (NBD) from non-endemic countries; and (iv) experimentally infected chimpanzees.

Results: A new HEV EIA was developed using two new recombinant proteins. This assay was able to detect anti-HEV activity in all specimens from acutely HEV-infected patients. When NBD were tested, more than 15% of specimens were found to be IgG anti-HEV positive. All NBD anti-HEV-positive specimens were tested with overlapping synthetic peptides spanning the entire HEV ORF2-encoded protein. More than 90% of the anti-HEV-positive NBD specimens immunoreacted with an average of 15 synthetic peptides derived from different regions of the HEV ORF2 protein. These data suggest that the HEV EIA is at least 90% specific in detecting remote HEV infections.

Conclusion: The new HEV EIA developed in the present study is a highly specific diagnostic assay for the detection of anti-HEV activity in serum specimens obtained from different epidemiologic settings.

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Key words: enzyme immunoassay, hepatitis E virus, recombinant proteins, synthetic peptides.

INTRODUCTION

Hepatitis E virus (HEV) is a common etiologic agent of enterically transmitted non-A, non-B hepatitis. Outbreaks of HEV infection have occurred predominantly in developing countries of Asia and Africa, the central Asian republics of the former Soviet Union, and in the Middle East.1–4

Hepatitis E virus is a currently unclassified non-enveloped virus5 containing a single-stranded positive-sense RNA molecule of approximately 7.5 kb6,7 Three open reading frames (ORF) were identified within the HEV genome: ORF1, which encodes for non-structural proteins; ORF2, which encodes for a putative structural protein(s);7,8 and ORF3, which encodes for a small protein with unknown function. The antigenic structure of this virus has been thoroughly studied. Several antigenic regions of diagnostic relevance were found within proteins encoded by ORF1, ORF2 and ORF3 by using overlapping synthetic peptides of different sizes9–12 and recombinant proteins.13–16

The cloning of the HEV genome and expression of recombinant proteins have allowed the development of enzyme immunoassays (EIA) for serological diagnosis of HEV.11–15,17–22 The first experiments with recombinant proteins, however, demonstrated that not all HEV recombinant antigens are equally suitable for this purpose. It was found that synthetic peptides and some

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ORF2- and ORF3-derived recombinant proteins expressed in *Escherichia coli* strongly immunoreacted with acute phase sera, but showed diminished immunoreactivity with convalescent sera, whereas the ORF2-derived protein expressed in insect cells in a form of virus-like particles and some other ORF2-derived proteins expressed in *E. coli* demonstrated a strong and broad immunoreactivity with both acute and convalescent serum specimens.13,14,18–20 Earlier, two new HEV recombinant antigens were obtained in the Centers for Disease Control and Prevention. One antigen, pB166, consisting of the region of the HEV Burma strain ORF2-encoded protein at position 452–617 aa, was shown to efficiently model the conformation-dependent HEV neutralizing antigenic epitope.22 The other protein, MP-II, was composed of short diagnostically relevant regions derived from the HEV Burma ORF2-protein and from the HEV Burma and Mexico strain ORF3-proteins. The MP-II was expressed in *E. coli* using a synthetic gene (T. Ulanova unpubl. data 2001). Both proteins demonstrated a very efficient detection of the HEV-specific antibodies, as in convalescent so in acute phase serum specimens.23 The present paper describes the development of a diagnostic assay for the detection of anti-HEV activity using these two new recombinant antigens and the evaluation of this new assay.

METHODS

Recombinant proteins

A combination of two proteins, pB166 and MP-II, was used for the development of the anti-HEV assay. Both pB166 and MP-II were expressed in *E. coli* as hybrid proteins with glutathione S-transferase (GST) and were purified from bacteria as described previously.26 The HEV MP-II was designed from the ORF2 antigenic regions at positions 31–66aa, 85–114aa, 95–119aa, 398–427aa, 614–638aa, 626–655aa, 631–660aa and 635–660aa, and two sequence variants of the region at position 91–123 aa from the ORF3 protein, with one variant being selected from the Burmese strain and the other from the Mexican strain. This protein was expressed and purified as described previously.26

Serum specimens

One panel of serum specimens was composed of serum specimens from patients acutely infected with HEV (*n* = 66) residing in Vietnam (*n* = 33), China (*n* = 30), and India (*n* = 3). The other panel contained specimens obtained from normal blood donors residing in the USA (*n* = 367; Boston Biomedica, West Bridgewater, MA, *n* = 292; American Red Cross, *n* = 75) and Russia (*n* = 185). Serum specimens from acute non-A, non-C acute hepatitis patients were collected from China (*n* = 30), from the border between Mexico and the USA through the Border Infectious Disease Surveillance (BIDS) program at CDC (*n* = 54), and from Brazil (*n* = 115). A panel of BIDS specimens obtained from patients with acute hepatitis of various viral etiologies (*n* = 57) was also used in the present study. Additionally, serial specimens were obtained from four experimentally HEV-infected chimpanzees (*n* = 32).13 Another panel was composed of specimens obtained from 1–3-month-old healthy infants (*n* = 21) residing in Russia and from naive chimpanzees (*n* = 11). These specimens were used as known anti-HEV-negative controls.

Synthetic peptides

A set of 71 overlapping synthetic 30-mer peptides spanning the entire ORF2-encoded protein used in the present study was described previously.12 Thirty-two randomly selected irrelevant peptides derived from various proteins of hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis A virus (HAV), virus GB-A (GBVA) and GB-B (GBVB) were used as negative control.

Enzyme immunoassay

The EIA for anti-HEV was performed as described previously,27 except that the antibodies bound to the antigens were detected by adding 100 µL of horseradish peroxidase or alkaline phosphatase-labeled monoclonal antihuman IgG (Pierce, USA) appropriately diluted in PBST with 5% NGS and 1% non-fat milk, and incubated for 30 min at 37°C. The antibody complexes were detected by the addition 100 µL of orthophenylendiamine (Abbott, USA) for 10 min for HPRO-labeled antibodies or ELISA amplification system (Life Technologies, USA) for AP-labeled antibodies in accordance with the manufacturer’s instructions. The cut-off was statistically established as the mean of the optical density (OD) value at 492 nm of known negative samples plus 4.0 standard deviations of the mean.

The EIA protocol for the detection of anti-HEV by using synthetic peptides was previously described.12 An additional criterion established as the ratio between the OD for each of the HEV peptides and the mean OD for irrelevant peptides was applied to discriminate between positive and negative results. The serum specimen was considered immunoreactive with HEV peptides when this ratio was greater than 2.

All testing with commercially available assays for the detection of anti-HEV activity (Abbott HEV EIA, Abbott Diagnostics Division, USA; and HEV ELISA, Genelabs™ Diagnostics, Singapore) were performed in Hepatitis Reference Laboratory according to the manufacturer’s instructions.

RESULTS

Assay optimization

All optimization studies were performed using known anti-HEV-negative and -positive sera. The optimal con-
centration of the pB166 protein was found to be 1.2–1.4 μg/mL and of the MPII 1.1–1.2 μg/mL. The application of the ELISA amplification system was shown to increase the average signal to cut-off ratio for 31.2% (data not shown).

**Antibody detection in anti-HEV-positive specimens**

Serum specimens obtained from acutely HEV-infected patients (n = 66) and from experimentally HEV-infected chimpanzees (n = 32) have been tested using the newly developed assay. When serial specimens from four HEV-infected chimpanzees were tested, all animals were found to become positive 35–100 days after inoculation and remained anti-HEV positive for up to 4 years, representing the entire observation period. The HEV acute phase serum specimens were collected from different parts of the world, from patients infected with geographically distinct HEV strains. All human specimens were tested by this new EIA. In addition, these specimens were tested by pB166 and MPII separately. The pB166 detected more anti-HEV-positive specimens than MPII (97.1% and 98.6%, respectively). However, when the proteins were combined the assay detected all anti-HEV-positive specimens.

**Assay specificity**

Because the seroprevalence rate of HEV infection in different human populations is unknown and because of the absence of a gold standard EIA for the detection of anti-HEV activity, which would establish the identification of a true anti-HEV state of serum specimens, there exists a problem with the selection of specimens used in the evaluation of specificity of a new assay. In the present study, specimens obtained from naive chimpanzees (n = 11) and from healthy 1–3-month-old infants (n = 21) were tested to evaluate the specificity of this new assay because these specimens are less likely to be anti-HEV positive. All specimens were tested negative by the new anti-HEV assay developed in the present study, strongly suggesting a high specificity of this assay.

**Detection of anti-HEV activity in normal blood donors and non-A, non-C acute hepatitis patients**

To further characterize the new EIA two additional panels of serum specimens obtained from normal blood donors (NBD; n = 552) and from patients with acute non-A, non-C hepatitis (China, BIDS; n = 84) were investigated. The rate of anti-HEV activity in different NBD collections ranged from 11.9% to 17.8% with an average of 15.8% (Table 1). The rate of anti-HEV activity in specimens from non-A, non-C hepatitis patients was significantly greater and varied from 25.9% in specimens obtained from the border between Mexico and the USA through the BIDS program, to 43.3% in specimens obtained from China (Table 1). It is important to note that eight out of 13 immunoreactive specimens from non-A, non-C patients residing in China were found to be HEV PCR-positive using a set of universal HEV primers. This last observation suggests that a significant portion of the anti-HEV-positive specimens detected by the new anti-HEV EIA is from patients who were unequivocally HEV infected.

**Immunoreactivity of serum specimens with synthetic peptides**

As was shown in the previous section, approximately 16% of the NBD specimens were found to be anti-HEV positive by the new EIA. One potential explanation for these data could be low specificity of this assay. To address this issue, 48 out of 87 anti-HEV-positive NBD specimens were randomly selected for testing against a set of 71 overlapping synthetic 30-mer peptides spanning the entire ORF2-encoded protein. The immunoreactivity of all these peptides has been studied using confirmed anti-HEV-positive serum specimens to identify six antigenic domains within the ORF2-protein. The testing performed in the present study demonstrated that more than 90% of NBD specimens immunoreacted with at least two or as many as 44 peptides. On average each immunoreactive specimen immunoreacted with 15.1 peptides. For comparison, an average of 18.8 peptides immunoreacted with specimens obtained from acutely HEV-infected patients. These findings suggest that the NBD serum specimens, which were found

<table>
<thead>
<tr>
<th>Collection ID</th>
<th>Total sera</th>
<th>HEV EIA positive n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD collections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBI 99†</td>
<td>92</td>
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<tr>
<td>BBI 00‡</td>
<td>200</td>
<td>30 (15)</td>
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<tr>
<td>Red Cross</td>
<td>75</td>
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<tr>
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<tr>
<td>Total (%)</td>
<td>552 (100)</td>
<td>87 (15.8)</td>
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<tr>
<td>Average signal:cut-off</td>
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<td>5.9</td>
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<tr>
<td>Non-A-non-C acute hepatitis collections</td>
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<tr>
<td>Non-A-non-C, China</td>
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<td>13§ (43.3)</td>
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<tr>
<td>Non-A-non-C, BIDS¶</td>
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</tr>
<tr>
<td>Total (%)</td>
<td>84 (100)</td>
<td>27 (32.1)</td>
</tr>
<tr>
<td>Average signal:cut-off</td>
<td>–</td>
<td>9.8</td>
</tr>
</tbody>
</table>

† Specimens obtained from Boston Biomedical in 1999; ‡ specimens obtained from Boston Biomedical in 2000; § non-A, non-C specimens obtained through Border Infectious Disease Surveillance (BIDS) program at the border of Mexico and USA. ¶ Eight specimens were tested and found to be HEV-PCR positive. NBD, normal blood donor.
to be anti-HEV positive by the new EIA, contain antibodies specific to HEV ORF2-protein.

DISCUSSION

A new EIA for the detection of antibodies against HEV was developed in the present study. This new assay is based on the use of two new HEV antigens. One antigen, protein pB166, efficiently models the HEV neutralizing antigenic epitope. The other antigen, MPII, is an artificial polypeptide constructed from several diagnostically relevant regions of the HEV ORF2 and ORF3 proteins. Both proteins are very potent diagnostic reagents. As was shown in the present study, the combination of these two antigens represents a very efficient diagnostic target for the detection of HEV-specific antibodies in serum specimens obtained from patients acutely infected with HEV and from serum specimens collected during the convalescent phase of hepatitis E. The performance of this new EIA was evaluated using serum specimens obtained from different countries of the world such as China, Vietnam, India, Brazil, Russia, and the USA.

Recently, the performance of a number of assays for the detection of anti-HEV activity in serum specimens was compared. All compared assays were carried out using recombinant proteins or synthetic peptides derived from different HEV proteins. This evaluation yielded several important conclusions on the performance of these assays: (i) poor concordance between assays; (ii) poor sensitivity of some assays, especially those based on synthetic peptides or recombinant proteins derived from the HEV ORF3 proteins, when used to detect antibody in convalescent specimens; and (iii) variable efficiency, with which some assays detect antibody against different HEV strains. The last conclusion was later strengthened by the observation that EIA based on the US HEV isolate specific peptides showed enhanced immunoreactivity with serum specimens from patients infected with a novel HEV variant found in Austria, whereas EIA based on recombinant proteins derived from the Burma and Mexico HEV strains were unable to detect anti-HEV activity in these specimens.

In concert with this observation, the EIA based on protein derived from the HEV genotype 4 was found to detect more cases of acute hepatitis E in China than a commercial assay based on genotype 1 HEV proteins. Two proteins used in the new EIA developed in the present study were designed to address this problem of variable assay sensitivity for the detection of antibodies to different geographic HEV variants. One protein, pB166, contains the conserved HEV neutralization antigenic epitope, antibodies against which were shown to cross-neutralize different geographic HEV strains. The other protein, MPII, is composed of strongly and broadly immunoreactive antigenic epitopes, some of which (ORF3 epitopes) are derived from two different HEV genotypes: the Mexico and Burma HEV strains. As was shown in the present study, the combination of these two antigens can be used as a very efficient diagnostic target suitable for the reliable detection of HEV-specific antibodies in different epidemiological settings.

Recent research revealed a high rate of anti-HEV in NBD from some industrialized countries such as the USA. For example, Thomas et al. reported that IgG anti-HEV could be detected in approximately 21% of NBD. This observation was recently extended by the finding of an average IgG anti-HEV prevalence of 18.3% in different populations in many US regions. In Japan, another country where HEV is not endemic, the prevalence of IgG anti-HEV in healthy individuals was found to range from 1.9 to 14.1%. The data obtained in the present study strongly confirm these observations by demonstrating the anti-HEV activity in NBD residing in Russia and US. This finding is not only supported by the detection of HEV antibodies by the new EIA but is also significantly substantiated by the identification of numerous ORF2- and ORF3-derived peptides strongly immunoreactive with NBD serum specimens.

In conclusion, the new anti-HEV detection assay using two new recombinant proteins was developed. The new assay may be considered as a prototype diagnostic assay for the reliable detection of anti HEV activity in serum specimens.

REFERENCES


