Validation of a solid-phase electrochemical array for genotyping hepatitis C virus

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A B S T R A C T

Hepatitis C viral infection is a major cause of progressive liver disease. HCV genotype is one of the most significant baseline predictors of response to HCV antiviral therapy. The objective was to evaluate an HCV genotyping method that targets the 5′-untranslated region (UTR) to detect genotypes/subtypes using the GenMark eSensor® XT-8 system. The HCV amplicon of major genotypes/subtypes from the Roche TaqMan® assay served as a template for the nested PCR followed by a direct analysis on the XT-8 detection system. The assay was validated for limit of detection (LOD), specificity, accuracy and precision. The LOD determined was below 175 IU/ml for all the subtypes except 6b. The genotypes detected using this assay were in concordance with the LiPA assay. The high performance characteristics (LOD, specificity, intra- and inter-assay precision, and accuracy), make this assay particularly well suited for clinical HCV genotyping in order to guide antiviral therapy.

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Introduction

Hepatitis C virus (HCV) infection is a major cause of liver disease and one of the most common chronic viral infections in the world (Chen and Morgan, 2006; Lavanchy, 2011). In the United States, 2.7–3.9 million people are currently living with this chronic blood-borne infection, representing approximately 1–2% of the nation’s population (Spradling et al., 2012). It is a major public health problem and a leading cause of chronic liver disease with a significant risk for the development of cirrhosis, end-stage liver disease, and hepatocellular carcinoma (HCC) (El-Serag, 2012; Yang et al., 2012). Furthermore, it is also a major cause of liver transplantation in this country (Akamatsu and Sugawara, 2012; Chak et al., 2011).

In a recent report that evaluated the cause of mortality associated with HCV, HBV and HIV, it was estimated that HCV has rapidly surpassed HIV as a cause of mortality in the US. The investigation of approximately 21.8 million death records over 9 years revealed that HCV infection was associated with a higher mortality rate than HBV and HIV infections. The mortality rates were 0.56, 4.16 and 4.58 deaths per 100,000 people per year for HBV, HIV and HCV infections, respectively (Ly et al., 2012).

HCV belongs to the family of flaviviruses and is an enveloped virus with a single-stranded RNA genome of approximately 9.4 kb in length. The viral genome harbors a single open reading frame which is flanked by 5′- and 3′-untranslated regions that are essential for polyprotein translation and genome replication. The open reading frame encodes a long polyprotein of approximately 3000 amino acids that produces three structural proteins (core, envelope E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Fig. 1). The structural proteins are cleaved by host endoplasmic reticulum signal peptidase(s) and released from the polyprotein whereas the non-structural proteins are cleaved by the viral proteases, thus, the proteolytic processing of the polyprotein yields ten mature viral proteins (Kupfer, 2012).

There exists a high degree of heterogeneity within the HCV RNA genome. Phylogenetic analyses have shown that HCV strains can be classified into at least 6 major genotypes and different subtypes (designated a, b, c, etc.) within each genotype. The genotypes differ in their nucleotide sequence by 30–35% and within a genotype, subtypes differ by 20–25% (Penin et al., 2004; Simmonds et al., 2005). Genotype 1 is by far the most common genotype in chronically infected patients worldwide accounting for about 60% of global infections. Although subtypes 1a and 1b represent the vast majority of circulating strains worldwide, distinct geographic distributions of the various genotypes have been noted with some rare genotypes commonly found in select populations (Jahan et al., 2011; Torres et al., 2012). In the United States, genotype 1 is the most common (57% and 17% of people with hepatitis C have 1a and 1b genotypes, respectively) followed by genotypes 2 and 3, which show better therapeutic response rates (Carcamo and Nguyen, 2012; Torres et al., 2012). Treatment of chronic hepatitis C is based on the combination of pegylated
interferon alpha (pegIFN) and the nucleoside analog ribavirin. Following treatment with pegIFN and ribavirin for 48 weeks, a sustained virologic response (SVR) occurs in 40%-50% of subjects infected with genotype 1 while 70%-80% of patients infected with genotype 2 or 3a will have a SVR with a reduced treatment regimen of only 24 weeks (Morgan and O’Brien, 2011). The addition of protease inhibitors such as boceprevir and telaprevir to the combination of pegIFN and ribavirin can improve outcomes and is becoming the new standard of care for HCV infection especially in the treatment of genotype 1 infection (Jacobson et al., 2011; Pacanowski et al., 2012; Pearlman, 2012; Poordad and Dieterich, 2012; Poordad et al., 2011). Hence the duration and response to therapy in HCV infected patients vary and can be predicted by the HCV genotype which is the most significant baseline predictor of response to therapy. The objective of this study was to validate a novel technology that targets the HCV 5′-UTR for identifying HCV genotypes using the GenMark eSensor® XT-8 system. The GenMark eSensor® HCV Genotyping Test utilizes a combination of steps during the process of detection including a) PCR amplification of target sequences, b) sequence-specific capture of target sequences by surface-bound oligonucleotide capture probes formed within a pre-assembled monolayer and c) electrochemical detection using ferrocene labeled oligonucleotide signal probes.

Materials and methods

Sample processing

Blood samples were collected in sterile tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Plasma was separated from the whole blood within 6 h of collection by centrifugation and was stored at 2–8 °C for up to 3 days or frozen at −80 °C until analysis. Validation samples were selected based on previous HCV genotyping results using the Versant™ HCV genotyping LiPA assay (Siemens, Tarrytown, NY) (Maertens and Stuyver, 1999). These plasma specimens, representing the major subtypes of HCV (1a, 1b, 2a, 2a/c, 3, 4, and 6a/b), were stored at −80 °C and then subjected to quantitative RT-PCR using the Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test. HCV genotype 5 was not found in our patient population but plasma representing this genotype was procured from SeraCare Lifesciences, Inc., (Milford, MA) and stored at −80 °C until the analysis.

RNA extraction and quantification

The Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test is an in vitro nucleic acid amplification test for the quantitation of HCV RNA in human plasma or serum using the COBAS® Ampliprep Instrument for automated specimen processing and the COBAS® TaqMan®48 Analyzer for automated amplification and detection. Viral RNA was extracted using the COBAS® Ampliprep Instrument and subsequently the samples were manually transferred to the COBAS® TaqMan®48 Analyzer for RT-PCR and viral quantification (Sizmann et al., 2007). The resulting amplicon from this reaction was retained for the eSensor® HCV genotyping assay.

GenMark eSensor® HCV Genotyping Test

The GenMark eSensor® HCV Genotyping Test requires a PCR step, exonuclease digestion and a genotype detection step. The assay was carried out using the kit supplied by GenMark that contains eSensor® amplification reagents (PCR mix and Taq DNA polymerase), detection reagents (exonuclease, signal buffer, buffers 1 and 2) and genotyping test cartridges for the detection of the viral genotypes. The assay is designed to genotype a panel of 8 HCV genotypes/subtypes: 1a, 1b, 2a, 2a/c, 3, 4, 5, and 6a/b.

Amplicon obtained from the Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test was diluted and used as the template for the nested PCR with supplied reagents according to the manufacturer’s directions for the GenMark eSensor® HCV Genotyping Test. The PCR thermal cycling conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 10 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. Following the thermocycling, the samples were subjected to selective exonuclease digestion of one strand of the amplicon. For the final specimen processing and detection of genotypes, a hybridization solution mix was prepared and added to each digested PCR reaction before transferring to labeled eSensor® HCV genotyping cartridges, according to the manufacturer’s protocol. The cartridges were loaded into appropriate slots of the XT-8 instrument for automated hybridization, detection and genotype calling. In addition to the test samples, amplicon from a negative HCV control was included with each batch as a DNA contamination monitor for quality control purposes.

Results

The validation of HCV genotyping using the GenMark eSensor® HCV Genotyping Test was carried out to assess the performance characteristics of the assay. The HCV amplicon obtained from the Roche assay served as a template for the genotyping analysis of 8 genotypes/subtypes (1a, 1b, 2a, 2a/c, 3, 4, 5, and 6a/b) on the eSensor® XT-8 detection system.

Limit of detection

To establish the limit of detection (LOD), plasma specimens harboring the 8 different genotypes/subtypes were diluted in negative human plasma matrix (Optimatrix, Life Technologies, Grand Island, NY) based on their HCV viral load as determined by the Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test as described earlier (Sizmann et al., 2007). The limit of detection was determined as the lowest...
concentration needed to provide a correct HCV genotype identification in at least 95% of the diluted samples tested. Following the dilution of two plasma specimens to approximately 1000, 500, 250 and 50, 25 IU/ml, a LOD below 175 IU/ml was evaluated for each subtype. Due to the variability inherent in quantitative PCR, the concentration of the diluted plasma as determined by the Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test varied slightly between genotypes as indicated in Table 1. HCV genotyping was performed in replicates of 20 for each of the eight genotypes/subtypes tested (1a, 1b, 2a/c, 3, 4, 5, 6a/b) using Roche HCV amplicon derived from the diluted plasma specimens. Overall, 96.8% of the 160 genotyping tests performed using plasma with HCV concentrations below 175 IU/ml resulted in correct genotype calls. Only five genotyping tests with expected genotype of 6a/b failed to produce genotype. No incorrect genotypes were observed. (Table 1).

**Precision**

In addition to establishing the LOD for this assay, the testing described above was also used to demonstrate the precision (reproducibility/ repeatability) of the assay. The 20 replicates that were performed for each genotype/subtype were performed by testing each sample in duplicate on 10 separate days over a five week span of time. Two days of testing was performed by a second operator. Since the precision was only 75% for genotype 6a/b with nominal concentration of 100 IU/ml and the viral load of 156 IU/ml, higher concentrations of the plasma were subsequently analyzed. A nominal concentration of 1500 IU/ml analyzed with the viral load detected as 4141 IU/ml gave an unfailed genotype call for all the 20 replicates tested. All genotypes obtained were identical to the expected genotypes demonstrating that identical results were obtained for all genotypes when the test was performed multiple times within the same run, within separate runs, and with separate operators.

**Analytical specificity**

Control plasma samples harboring human immunodeficiency virus-1 (6.21 log$_{10}$ IU/ml), hepatitis B virus (6.75 log$_{10}$ IU/ml) (Acrometrix-Life Technologies, Grand Island, NY), and West Nile virus (50,000 copies/ml) (Zeptometrix Corporation, Buffalo, NY) were tested with the eSensor HCV assay to assess the specificity of the assay. HIV and HBV were used for specificity testing since these viruses often infect a similar patient population and some patients may exhibit co-infections, while West Nile virus was tested since it is a human pathogen that, along with HCV, belongs to the Flaviviridae family of viruses. The eSensor HCV Genotyping did not detect the presence of any HCV genotypes when the HIV, HBV, and West Nile virus controls were tested, demonstrating specificity of the assay for the HCV genome.

### Table 1

<table>
<thead>
<tr>
<th>HCV Genotype</th>
<th>Nominal concentration (log$_{10}$ IU/ml)</th>
<th>Nominal diluted concentration (IU/ml)</th>
<th>Resulting Viral load (IU/ml)</th>
<th>Correct calls/total tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>4.64</td>
<td>50</td>
<td>104</td>
<td>20/20</td>
</tr>
<tr>
<td>1b</td>
<td>6.37</td>
<td>50</td>
<td>118</td>
<td>20/20</td>
</tr>
<tr>
<td>2b</td>
<td>5.86</td>
<td>50</td>
<td>65</td>
<td>20/20</td>
</tr>
<tr>
<td>2a/c</td>
<td>6.26</td>
<td>150</td>
<td>151</td>
<td>20/20</td>
</tr>
<tr>
<td>3</td>
<td>4.64</td>
<td>25</td>
<td>173</td>
<td>20/20</td>
</tr>
<tr>
<td>4</td>
<td>4.19</td>
<td>100</td>
<td>172</td>
<td>20/20</td>
</tr>
<tr>
<td>5</td>
<td>6.36</td>
<td>500</td>
<td>44</td>
<td>20/20</td>
</tr>
<tr>
<td>6a/b</td>
<td>6.14</td>
<td>100</td>
<td>156</td>
<td>15/20</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td>155/160 (96.8%)</td>
<td></td>
</tr>
</tbody>
</table>

**Accuracy**

To evaluate the accuracy of the assay, 48 samples belonging to different genotypes/subtypes were analyzed and the results were compared with the results from the VERSANT® HCV (LiPA) genotyping assay (Maertens and Stuyver, 1999). Comparison with the LiPA assay demonstrated concordance in all the amplicons tested that represented different HCV genotypes/subtypes. However, it was observed that nine samples identified as 1b by the GenMark assay resulted in 1a genotype by the LiPA assay (Table 2). The discordant samples genotyped as 1b by the GenMark assay would have been designated as 1b by LiPA as well if genotyping was based on the 5′ UTR alone and not the core region. Plasma from seven discordant samples were then subjected to eSensor® HCV Direct test, a prototype new test developed by GenMark that utilizes both 5′ UTR and core region for the genotyping. We found that these samples were detected as 1a genotype in agreement with the LiPA assay. In addition to the 48 patient specimens, a commercial panel (Acrometrix, Life Technologies, Grand Island, NY), consisting of genotypes 1–4, was also tested to assess the accuracy of the assay and each was genotyped correctly with the results of 1a/b, 2b, 3 and 4.

**Discussion**

Chronic HCV infection is a major and growing public health problem worldwide, with a significant risk for progressive liver fibrosis and subsequent cirrhosis and HCC (El-Serag, 2012; Yang et al., 2012). Although HCV and HBV both contribute to the etiology of HCC, HCV is the most common cause in developed countries whereas HBV is a more common cause in developing countries (Bosch et al., 2004). The rapid spread of HCV is believed to have occurred predominantly by transmission through blood transfusion and has also been associated with certain unsafe medical procedures (Prati, 2006). In the USA, high incidence of HCV is mainly attributed to injection drug use (Chak et al., 2011; Grebely et al., 2012; Klevens et al., 2012). Correct identification of HCV genotype is necessary to predict response to therapy in the management of this chronic viral infection. In this study, we describe the validation of a novel HCV genotyping method that targets the 5′-UTR for detecting major HCV genotypes. The performance characteristics of the GenMark assay were highly robust and reliable, displaying high sensitivity, specificity, accuracy and precision for the detection of major HCV genotypes and/or subtypes.

The eSensor® technology uses a solid-phase electrochemical method for determining the presence of one or more of a defined panel of virus target sequences. The amplicon from the Roche COBAS® Ampliprep/COBAS® TaqMan® HCV Test serves as a template for the nested PCR followed by a direct analysis on the electrochemical eSensor® XT-8 detection system for the identification of HCV genotypes (Fig. 2). DNA from a positive sample is amplified using

### Table 2

<table>
<thead>
<tr>
<th>HCV genotype</th>
<th>GenMark (n)</th>
<th>LiPA (n)</th>
<th>Discordant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>22</td>
<td>31</td>
<td>9a</td>
</tr>
<tr>
<td>1b</td>
<td>11</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2b</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2a/c</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6a/b</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

a All nine “discordant” samples genotyped as 1b by the GenMark assay would have been designated as 1b by LiPA as well if genotyping was based on the 5′ UTR alone and not the core region.

b This sample was procured from SeraCare Lifesciences, as a patient sample was not available and hence not tested for LiPA assay.
specific primers by PCR. The amplified DNA is converted to single-stranded DNA via exonuclease digestion and is then combined with a signal buffer containing ferrocene-labeled signal probes that are specific for the different subtypes. The mixture of amplified sample and signal buffer is loaded onto a cartridge containing single-stranded oligonucleotide capture probes bound to gold-plated electrodes. The cartridge is inserted into the XT-8 instrument where the single-stranded targets first hybridize to the perfect match signal probe and then hybridize to the complementary sequences of the capture probe. The presence of each target is determined by voltammetry, which generates specific electric signals from the ferrocene-labeled signal probe (Fig. 3). A recent report on the eSensor respiratory viral panel that utilizes similar technology had demonstrated it as a robust method for rapid detection of about 20 respiratory viral targets due to its multiplexed design and unique biosensor technology for target detection (Pierce and Hodinka, 2012).

In the HCV genome, the 5′-UTR and the extreme end of the 3′-UTR have the lowest sequence diversity among various genotypes and subtypes (Choo et al., 1991; Muerhoff et al., 1995). The highly conserved 5′UTR of the HCV genome is approximately 340 nucleotides in length and contains control elements required for replication and translation of the viral polyprotein. The initial 40 coding nucleotides constitute domain I and which is involved in RNA replication. Domains II–IV constitute an internal ribosome entry site which mediates the cap-independent translation of the HCV open reading frame which is essential for the initiation of viral protein synthesis (Kupfer, 2012; Shi and Lai, 2006).

When HCV genotyping results generated by GenMark and LiPA assays were compared, 100% concordance was found at the genotype level. However, the minor discrepancy between the subtypes of genotype 1 observed for nine samples were due to differences in regions of the HCV genome analyzed by the two assays. The LiPA assay utilizes the 5′-UTR and core regions of the HCV genome whereas the GenMark assay targets only the 5′-UTR for the viral genotyping. However, the discrepant samples tested using the new prototype assay demonstrated 100% concordance with LiPA assay since the new assay utilizes both core and 5′-UTR similar to the LiPA assay. In this study, we showed that the GenMark assay can distinguish all eight genotypes/subtypes by utilizing specific motifs conserved in the 5′-UTR (Simmonds et al., 1993).

The LOD was found to be below 175 IU/ml for all the subtypes except 6a/b. A genotype call could only be made in 75% of tests using the genotype 6ab sample at a concentration of 156 IU/ml. The difference between genotypes 1a and 6a/b is a dual nucleotide insertion at positions 197 and 198 which is attributed to residues C and A in the 5′ UTR (Murphy et al., 2007). In this assay, in order to prevent misinterpretation of the common subtype 1a as the much rarer 6ab, stringent boundaries were established regarding the genotyping call between these genotypes. In our LOD study of the diluted 6a/b plasma, the signal used to determine the genotype apparently dropped into the indeterminate zone for 5 of 20 tests. However, upon increasing the concentration of the samples, an excellent 6a/b genotype call rate was attained with differentiation of both the genotypes. This was confirmed by testing multiple replicates using the concentration established for the 6ab genotype sample.

While the GenMark assay is robust and relatively easy to perform with less turnaround time, a drawback of the assay is its chance of contamination as the assay utilizes the amplicon from Roche TaqMan® HCV Test. However, no known or suspected contamination events occurred during this validation.

The rate of progression to chronic hepatitis as well as the response to HCV antiviral drugs is influenced by HCV genotype. Precise determination of HCV genotype is essential in the clinical practice for the effective treatment of HCV infection.
management of this disease by personalizing therapy with conventional drugs and/or in combination with the recently introduced protease inhibitors boceprevir and telaprevir, especially in patients who are carriers of genotype 1. Our validation of the GenMark HCV genotyping assay demonstrates high performance characteristics which facilitate viral genotyping for personalization of dose and duration of treatment. The high sensitivity, good intra- and inter-assay precision, and agreement with a validated method, make this assay particularly well suited for clinical HCV genotyping to guide antiviral therapy.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank GenMark for the permission to use the artwork included in Figs. 2 and 3. Additionally, GenMark generously performed the testing with an unreleased version of the assay to resolve the discrepancies we observed between the GenMark and LiPA assays. This work was otherwise supported through the Department of Pathology at Dartmouth-Hitchcock Medical Center.

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