Transcription-Mediated Amplification Linked to Line Probe Assay as a Routine Tool for HCV Typing in Clinical Laboratories

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Typing of hepatitis C virus (HCV) isolates is currently a prerequisite for adequate tailoring of antiviral combination therapy. In many diagnostic laboratories, there seems to be a tendency toward convenient and time-saving procedures utilizing amplification products, which are already available from preceding qualitative or quantitative HCV ribonucleic acid (RNA) assays. In this context, we evaluated the performance characteristics of a combination of techniques, i.e., transcription-mediated amplification-line probe assay (TMA-LiPA), which links highly sensitive TMA of HCV RNA to the VERSANT HCV Genotype Assay (version 1). A total of 100 clinical samples were genotyped by TMA-LiPA. The obtained results were compared to those recorded by the original, nested reverse transcription

(RT)-polymerase chain reaction (PCR)-based VERSANT assay, the core-related GEN-ETI-K DEIA, and phylogenetic analyses of partial sequences from the HCV core and NS5B regions. TMA-LiPA assigned the correct genotype to all 100 HCV isolates. For subtyping of genotype 1 and 2 isolates, TMA-LiPA only showed discriminatory powers of 82% and 53%, respectively. Thus, TMA-LiPA in our hands turned out as a convenient and time-saving routine procedure for HCV typing which currently provides sufficient information for clinical purposes. Like all 5'untranslated region (UTR)-based assays, the technique is limited, however, in its potentials to resolve the complexity of existing HCV subtypes. *J. Clin. Lab. Anal.* 21:340–347, 2007.

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INTRODUCTION

The hepatitis C virus (HCV) as the main causative agent of the formerly so-called posttransfusion non-A, non-B hepatitis is a positive-stranded ribonucleic acid (RNA) virus belonging to the family Flaviviridae (1). The genome of the pathogen is highly variable, and so far six major genotypes and almost 80 subtypes were identified worldwide (2).

This genetic heterogeneity has important clinical implications, mainly because the tailoring and outcome of antiviral treatment of chronic HCV infections with pegylated interferon alpha and the nucleoside analog compound ribavirin is crucially influenced by the HCV genotype (3,4). Furthermore, HCV typing and subtyping is essential for epidemiological studies on the geographic distribution of HCV isolates as well as their spread within certain subgroups of the general population (5,6). It is also indispensable as a basic investiga-

tional tool in forensic medicine when accidental or intentional transmissions of the virus from one individual to another are suspected (7).

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Consequently, techniques for HCV typing are widely distributed throughout diagnostic laboratories, and a variety of assays is available for routine application (8,9). In many diagnostic units, however, there seems to be a tendency towards convenient and time-saving typing procedures utilizing HCV amplification products that already had been generated in the course of diagnostic HCV RNA detection (10,11). This trend is reflected, for instance, by the preliminary results of a very recent external quality assessment scheme on HCV typing among diagnostic laboratories in Germany (Grunert et al., unpublished results): Almost 60% of all participants linked HCV amplicons already available from preceding in-house as well as commercial polymerase chain reaction (PCR)-based qualitative and quantitative assays to reverse hybridization by the VERSANT HCV Genotype Assay (line probe assay [LiPA]) (Siemens Medical Solutions, Fernwald, Germany), and in a number of laboratories the preexisting amplification products were directly used for HCV typing by nucleotide sequencing. To ensure the reliability and robustness of this multitude of different front-to-end methods, on which today routine HCV typing and subtyping is obviously based, every new combination of techniques suggested in this context must be carefully validated against reference methods prior to its implementation (8,9). In this study, we therefore established the performance characteristics of an HCV typing procedure combining HCV amplification by diagnostic isothermal transcription-mediated amplification (TMA) and the subsequent characterization of the obtained amplicons by a LiPA. This combination of techniques was already used by few groups previously (12,13) and was shown to be particularly effective for HCV typing in specimens with very low HCV RNA concentrations (14).

MATERIALS AND METHODS

Specimens

Sera from 100 HCV infected inpatients and outpatients (63 men, 37 women, mean age: 43.7 years [range: 19–87 years]) attending Essen University Hospital were included in this study. All samples tested positive for antibodies against HCV by a third generation immunoassay (Sanofi Diagnostics Pasteur, Freiburg, Germany) and for HCV RNA by diagnostic reverse transcription (RT)-PCR (AMPLI-COR, Roche Diagnostics, Mannheim, Germany). A total of 12 out of the 100 HCV infected individuals showed serological signs of past hepatitis B virus infection and six were coinfecting with the human immunodeficiency virus.

VERSANT HCV RNA Qualitative Assay linked to VERSANT HCV Genotype Assay, version 1 (TMA-LiPA)

The combination of these two assays for the purpose of HCV genotyping (TMA-LiPA) (14) essentially consisted of several steps.

Step 1

The amplification of HCV RNA by the commercially available VERSANT HCV RNA Qualitative Assay (Siemens Medical Solutions, Fernwald; Germany) was performed according to the manufacturer's instructions. In this isothermal TMA technique, mainly, multiple RNA copies were generated from the target HCV RNA by the concerted action of a Moloney murine leukemia virus reverse transcriptase and T7 RNA polymerase (15,16). Transcription by the latter enzyme was initiated upon binding to a T7 promoter region present in one of the TMA primers. Although the TMA product, hence, consisted primarily of RNA, about 2% of the obtained amplicons represented single- and double-stranded deoxyribonucleic acid (DNA) that contained the T7 promoter region (14).

Step 2

This double-stranded DNA from the TMA product was amplified by a short single-round PCR: To begin, 200–400 µL from each TMA reaction product was removed from the tubes after luminometric detection, but before decontamination with the bleach solution. The recovered TMA amplicons were then diluted by adding 2 µL of the product into 100 µL nuclease-free water (Braun, Melsungen, Germany). Five µL of this solution were transferred to 45 µL of a PCR master mix consisting of 5 µL 10 × PCR buffer, 4 µL 2.5 mM deoxynucleotide-triphosphates (dNTPs), 3 µL 25 mM MgCl₂ (Promega, Madison, WI), 1 µL 10 mM 5' biotinylated primer NP2B, 1 µL 10 mM 3' biotinylated primer DE34 (TibMolbiol, Berlin, Germany), 0.5 µL (5 units/µL) AmpliTaq Gold DNA Polymerase (Promega), and 30.5 µL of nuclease-free water. The sequence of the sense PCR primer (NP2B) was derived from the 5' untranslated region (UTR) of the HCV genome: 5'-tctagccatggcgtagtmygagtgt-3' (nucleotides 78–103, numbering according to Ref. 17), whereas the antisense PCR primer (DE34) represented the sequence of the T7 promoter (5'-cgaatttaatacgaactcactagtaggag-3'), which was already contained in one of the oligonucleotides used for amplification in the initial TMA reaction. The resulting HCV-specific sequence was 214-bp long and corresponded to nucleotides 78–292 of the HCV genome (numbering according to Ref. 17). PCR reactions were carried out in a Biometra T3 thermal cycler (Biometra, Göttingen, Germany) with an initial

denaturation step of 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, each, and a final hold step of 4°C.

Step 3

HCV genotyping was achieved by VERSANT Genotype Assay (LiPA), version 1, which was identical to the second generation LiPA initially described by Stuyver et al. (18). A total of 10 µL of the biotinylated complementary DNA (cDNA) fragment along with denaturing and hybridization solution were hybridized to the 19 type-specific and subtype-specific oligonucleotide probes (lines) immobilized on nitrocellulose strips. After unbound cDNA had been washed away, streptavidin coupled to alkaline phosphatase was attached to the biotinylated hybrid. A chromogene subsequently formed a purple/brown precipitate upon reaction with the streptavidin-alkaline-phosphatase complexes, resulting in specific banding patterns on the nitrocellulose strips. Finally, the HCV type and subtype of the respective isolates were deduced visually by comparison of the obtained banding pattern with the current interpretation chart of the LiPA.

VERSANT HCV Genotype Assay (LiPA), Version 1

Total RNA was extracted from 140 µL serum by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and amplified by the two nested sets of universal HCV 5'UTR-derived PCR primers originally contained in the kit (18). The 236-bp biotinylated fragment generated by the second-round PCR was used for reverse hybridization under highly stringent conditions, as recommended by the manufacturer.

HCV Typing by DNA Enzyme Immunoassay

All clinical specimens were also typed by DNA enzyme immunoassay (DEIA) (GEN-ETI-K DEIA; Sorin Biomedica, Saluggia, Italy), as described in full detail previously (19). Unlike LiPA, DEIA was based on the amplification of a fragment from the HCV core region by nested PCR. The obtained 216-bp amplicons (nucleotides 461–676, numbering according to Ref. 17) were hybridized to type-specific or subtype-specific oligonucleotide probes adsorbed to microwell plates. The double-stranded DNA hybrids thus formed were finally detected by DEIA.

Amplification and Sequencing of Fragments from the HCV 5'Ultras translated, Core, and Non-Structural 5B (NS5B) Regions

HCV RNA from specimens with discordant genotyping results by TMA-LiPA, the original VERSANT

assay, and DEIA, respectively, were subjected to additional RT-PCRs with primers from the 5'UTR, core, and NS5B regions. The full details of the three protocols were already given elsewhere (11,20,21). The 5'UTR fragments were sequenced by a CLIP reaction (11). The products from the second rounds of the HCV core and NS5B PCRs were purified from the agarose gels by QIAquick (Qiagen) and were sequenced in both directions by dideoxy chain termination technique (22) with a Dye Terminator DNA sequencing kit (Perkin Elmer, Norwalk, CT).

Phylogenetic Analyses

Sequences of 216-bp HCV core (nucleotides 461–676, numbering according to Ref. 17) and 222-bp HCV NS5B (nucleotides 8316–8535, numbering according to Ref. 17) DNA fragments from those samples with discrepant typing results in TMA-LiPA, the VERSANT assay, and the DEIA were investigated further by phylogenetic analyses using the PHYLIP software package, version 3.5c (23). In brief, after editing and aligning the sequences, 100 replicates of the original sets of data were created by the SEQBOOT program. A matrix of nucleotide distances was then calculated according to Kimura's two-parameter method (24), and phylogenetic trees were constructed by the neighbor-joining algorithm. The consensus trees generated with CONSENSE were finally visualized by the software TREEVIEW (Available at: <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). Significance of the grouping was assumed when bootstrap values were greater than 75%. The following GenBank sequences were included as references in the analyses (accession numbers given in parentheses): Subtype 1a (AF011753 and AF511948); subtype 1b (AF054250 and AF207756); subtype 1c (AY051292); subtype 2a (AF169002 and AF238481); subtype 2b (AY232731 and D10988); subtype 2c (D50409, D84398, and L38337); subtype 2d (L29631 and L29634); subtype 2e (D49745 and D49760); subtype 2f (D49754 and D49769); subtype 2g (X93323); subtype 2h (X93327); subtype 2i (DQ155561); subtype 2j (D86530); subtype 2k (AB031663); subtype 3a (AF046866 and D17763); subtype 3b (AY515261, D49374, and X91299); subtype 4a (AY973862, U31255, and Y11604); subtype 5a (AF064490, D50466, and Y13184); and subtype 6a (AY859526). The partial HCV 5'UTR, core, and NS5B sequences isolated from those samples which gave initially discrepant typing results by TMA-LiPA, the VERSANT assay, and DEIA, respectively, were deposited in the GenBank under the accession numbers EF205210–EF205218 (HCV 5'UTR), EF205219–EF205236 (HCV core), and EF205237–EF205254 (HCV NS5B).

RESULTS

Typing Results Obtained by VERSANT HCV Genotype Assay (LiPA), Version 1, and TMA-LiPA

As indicated in Table 1, HCV genotypes and subtypes could be assigned to 97 out of 100 clinical isolates by the two modifications of the LiPA. The concordance of the obtained results was 100% on the level of the genotype and 97% on the level of the subtypes so that the usage of the TMA amplicons instead of PCR products generated via the primers included in the VERSANT assay did not exert a considerable influence on the analytical performance of LiPA. Testing of sample 24 resulted in the “indeterminate” banding pattern 1, 2, 5, 9, 10, 11, 12, and 17 in both combinations of the tests. The same held true for serum 63. Direct sequencing and phylogenetic analyses indicated that the specimens contained HCV subtype 2c and 2b isolates, respectively.

Typing Results Obtained by GEN-ETI-K DEIA and TMA-LiPA

A total of 98 out of 100 HCV isolates could be typed by these two assays, and the agreement of the obtained results on the level of the genotypes was 99% (Table 2). The assignment of the different subtypes, however, was more divergent. For instance, nine out of 50 specimens belonging to the “genotype 1 group” were classified differently by the core-based DEIA and the 5'UTR-based TMA-LiPA. In six of these cases, TMA-LiPA did not provide a subtyping result, although a type 1a-“specific” A was present at nucleotide position 243 of the 5'UTR in the HCV isolates from samples 8, 64, 82, and 83, and a G (indicative of subtype 1b) could be detected at this particular site in isolates 29 and 47, respectively (Figure 1). HCV strains 33, 34, and 73 were also carrying a G at nucleotide position 243 and were, thus, identified as subtypes 1b by TMA-LiPA, whereas

TABLE 1. Comparison of HCV typing results obtained by VERSANT HCV Genotype Assay (LiPA), version 1, and TMA-LiPA

VERSANT HCV Genotype Assay (LiPA), v. 1	No. of isolates with HCV genotype and subtype assigned by TMA-LiPA									
	1	1a	1b	2	2a/c	2b	3a	4	5	Indeterminate ^b
1	4									
1a	1^a	18								
1b	1		26							
2				4						1
2a/c					6					
2b				1		3				
3a							26			
4								5		
5									2	
Indeterminate										1
No amplicon				1						

^aDiscrepant genotype and subtype assignments obtained by the two assays are indicated in boldface type.

^bLine pattern which could not be assigned to any type or subtype based on the interpretation chart of the assay.

TABLE 2. Comparison of HCV typing results obtained by GEN-ETI-K DEIA and TMA-LiPA

GEN-ETI-K DEIA	No. of isolates with HCV genotype and subtype assigned by TMA-LiPA									
	1	1a	1b	2	2a/c	2b	3a	4	5	Indeterminate ^b
1a	4^a	18	3							
1b	2		23					1		
2					1					
2a					2					
2b				6		3				1
2c					3					1
3a							26			
4								4		
5									2	

^aDiscrepant genotype and subtype assignments obtained by the two assays are indicated in boldface type.

^bLine pattern which could not be assigned to any type or subtype based on the interpretation chart of the assay.

Fig. 1. Alignment of partial HCV 5'UTR sequences. At the top, HCV subtype 1a and 1b consensus sequences are given. S 8 to S 83 represent, each, 55 nucleotides from the 5'UTR, which were obtained from nine specimens with discordant subtype 1 assignments by TMA-LiPA, the VERSANT assay, and DEIA, respectively. At the right, the typing results and the banding patterns (in parentheses) observed by TMA-LiPA are shown. Nucleotide position 243, on which the discrimination of subtype 1a from subtype 1b is crucially dependent in the 5'UTR-based test, is indicated by an arrow.

by DEIA an assignment to subtype 1a was reported. For six out of 15 specimens from the “HCV genotype 2 group,” TMA-LiPA did also not provide a subtyping result. The two samples (S 24 and S 63), which turned out as not typeable by TMA-LiPA, belonged to subtypes 2b and 2c in DEIA, respectively (Table 2).

Resolution of Discordant HCV Genotype and Subtype Assignments by Direct Sequencing of Core and NS5B Fragments With Subsequent Phylogenetic Analyses

The results of our phylogenetic analyses (Fig. 2) indicated that the VERSANT assay as well as TMA-LiPA mistyped three HCV 1a isolates as 1b. The six genotype 1 samples for which no subclassification was obtained by TMA-LiPA belonged to subtypes 1a (four times) and 1b (two times). Hence, TMA-LiPA had a specificity of 82% with regard to the proper discrimination of HCV subtypes 1a and 1b. DEIA, on the other hand, subclassified all 50 genotype 1 strains correctly. The six HCV genotype 2 isolates that could not be subtyped by TMA-LiPA turned out to be subtype 2b in phylogenetic analyses. Since in one specimen (S 100) an HCV 2i isolate was present which could not be recognized by both TMA-LiPA and DEIA and TMA-LiPA furthermore cannot distinguish between strains of subtypes 2a and 2c, the discriminatory power for genotype 2 subtyping by TMA-LiPA and DEIA was 53% and 93%, respectively. Phylogenetic analyses supported the typing results obtained by DEIA for those two samples which yielded an “indeterminate” banding pattern by TMA-LiPA. Conversely, for sample 7 the correct genotype 4 was reported by TMA-LiPA, whereas DEIA erroneously classified this specimen as subtype 1b.

DISCUSSION

Typing of HCV isolates became increasingly important in recent years and is now a prerequisite for the tailoring of antiviral combination therapy of chronic HCV infections (3,4,8). Consequently, HCV typing is currently performed in many diagnostic laboratories, and there seems to be a tendency toward convenient procedures that make use of nucleic acid amplification products already available from preceding qualitative or quantitative HCV RNA assays, thereby leading to a multitude of different front-to-end methods (9, 25) (Grunert et al., unpublished results). We evaluated here the performance characteristics of a combination of techniques for HCV typing, i.e., the so-called TMA-LiPA, which had been suggested previously for typing of specimens containing low HCV RNA concentrations (14), to find out whether the conjunction of the highly sensitive TMA of HCV RNA (15,16) with the subsequent characterization of the resulting amplification products by the well-known LiPA (18) might be suitable as a routine tool for HCV typing in clinical laboratories.

The results obtained by TMA-LiPA were completely concordant with those reported by the original VERSANT assay on the level of HCV genotypes, indicating that the use of TMA amplicons instead of cDNA derived from nested RT-PCR did not affect the reliability and robustness of LiPA. This observation is in line with previous reports on the remarkable “plasticity” of the LiPA assays as an “end method” for HCV typing, which may utilize a variety of biotinylated 5'UTR-derived “front” amplification products, including those of both the Roche qualitative and quantitative Amplicor and Monitor assays, respectively (9,26,27). In our hands, the whole process of genotyping clinical samples by TMA-LiPA comprising all steps from TMA of HCV

Considering our findings on TMA-LiPA, this combination of techniques can be used confidently for HCV typing in diagnostic laboratories because it satisfies the major clinical needs, i.e., the proper discrimination of patients requiring 48 weeks of antiviral treatment due to infections with the HCV genotypes 1, 4, and 5 from those who can be assigned to a shorter 24-week duration of therapy because they are harboring genotype 2 and 3 strains (3,4,8). The favorable judgement on the performance characteristics of TMA-LiPA to some extent is swayed, however, when the subtyping results by this 5'UTR-based assay are compared to those recorded by the core-derived DEIA and phylogenetic analyses relying on HCV core and NS5B fragments. Our results for TMA-LiPA demonstrated that a reliable distinction

between HCV subtypes 1a and 1b cannot be achieved when the decision is exclusively based on sequence information derived from the highly conserved 5'UTR of the HCV genome. Nine out of 50 HCV isolates belonging either to subtypes 1a or 1b were erroneously classified by TMA-LiPA because the single nucleotide difference at position 243 was obviously not sufficient for an adequate subtype resolution (18,29). This finding is in line with multiple previous studies on 5'UTR-based commercially available assays (8,11,26,27,30–33) and also with our analysis of the HCV 5'UTR sequences deposited in the Los Alamos HCV GenBank (Los Alamos, NM). Although the vast majority of subtype 1b strains displayed a G at the decisive position 243 and would, therefore, be classified as subtype 1b by the TMA-LiPA and other 5'UTR-based assays, 10% of the 1b species carried an A at this particular site and were, thus, identical to HCV subtype 1a isolates. Conversely, an A at position 243 was also not completely subtype 1a-specific, since in the GenBank 1a isolates carrying a G can also be found. Similar concerns may be raised for subtyping HCV genotype 2 isolates by both the original VERSANT assay and TMA-LiPA. A discrimination of subtypes 2a and 2c is not possible on the basis of sequence analysis of the 5'UTR alone, and HCV subtype 2b differs from 2a by only 2 nucleotides at positions –124 and –164 (29). Thus, the discriminatory power of TMA-LiPA for subtyping HCV genotype 2 strains (53%) met the general expectations for 5'UTR-derived assays, although the poor resolution of subtype 2b recorded in our study remains elusive given the fact that the LiPA lines 11 (subtypes 2a/2c) and 12 (subtype 2b) had been reported as unique and therefore subtype-specific in the LiPA (18). The consequences of such a poor subtype resolution of 5'UTR-based commercial assays on the outcome of epidemiological studies were recently discussed in detail (9,34) and led to the notion that the tests in this particular context should be generally replaced by analyses utilizing sequence information from more heterogeneous fragments of the HCV open reading frame (11,35,36).

Taken together, TMA LiPA in our hands proved to be a convenient, reliable and time-saving tool for routine HCV typing in clinical laboratories which is able to provide the most crucial information, i.e., the discrimination of patients infected with HCV genotypes 1, 4, and 5 from those harboring HCV isolates belonging to the genotypes 2 and 3. However, regarding the poor discriminatory power of TMA-LiPA and all other 5'UTR-based assays for HCV subtyping, researchers who want to conduct epidemiological surveys should be cautioned from the use of these tests and should alternatively rather rely on procedures taking into

account information from more heterogeneous parts of the HCV genome (9,37).

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