

Use of a Commercially Available Line Probe Assay for Genotyping of Hepatitis C Virus 5a Strains

Jannick Verbeeck,¹ Piet Maes,¹ Elke Wollants,¹ Schalk Van der Merwe,² Ernie Song,³ Frederik Nevens,⁴ and Marc Van Ranst^{1*}

Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium¹; Hepatology/GI Research Laboratory, Department of Internal Medicine and Gastroenterology, University of Pretoria, Pretoria, South Africa²; Department of Internal Medicine, University of Witwatersrand, Witwatersrand, South Africa³; and Division of Hepatology, University Hospital Gasthuisberg, Leuven, Belgium⁴

Received 16 May 2005/Returned for modification 24 August 2005/Accepted 16 September 2005

To validate a commercially available line probe assay for samples containing the infrequently found hepatitis C virus (HCV) genotype 5a, we sequenced a 511-nucleotide fragment of the NS4b region of Belgian and South African HCV genotype 5a samples. Phylogenetic analysis of the sequence data was performed. For the 77 HCV genotype 5a samples collected, there was 100% concordance between the genotype assignment by the line probe assay and the genotyping based on nucleotide sequencing, despite sequence heterogeneity in the probe binding sites of some samples.

First described in 1989, hepatitis C virus (HCV) is today recognized as a major cause of chronic liver disease in many regions of the world. It is estimated that about 170 million people are infected with HCV worldwide. The HCV genome displays a high genetic variability. Therefore, HCV has been classified in at least six different genotypes that can be further differentiated into multiple subtypes (12). The most prevalent HCV genotypes are genotypes 1a, 1b, 2a, 2b, 3a, and 4a. Genotype 5a is a major genotype in South Africa (3), but it also has a worldwide distribution as a minor genotype in Australia, Brazil, Canada, France, Ireland, the Netherlands, and Spain (2, 4, 6, 10). Recent investigations indicate a high prevalence of genotype 5a-infected patients in the West Flanders region of Belgium.

Study of viral diversity is crucial to elucidate the role of the different HCV genotypes in the pathogenesis and progression of liver disease (4). Viral genotyping might also be of clinical relevance, since numerous studies have reported a relationship between the HCV genotype and the response to interferon or pegylated interferon therapy, alone or in combination with ribavirin (9). At present, correct HCV genotyping is of great importance as part of the pretreatment evaluation of patients with chronic HCV infections (11). Although nucleic acid sequencing and phylogenetic analysis of an appropriate subgenomic region are considered the “gold standard” for HCV genotype determination, it is time-consuming, expensive, and inconvenient for routine use.

The VERSANT HCV Genotype Assay (a line probe assay) (Bayer Corporation, Tarrytown, New York; manufactured by Innogenetics, Ghent, Belgium) is a widely used assay for *in vitro* diagnostic use. This assay correctly identifies the major genotypes of more than 90% of the samples (13), but valida-

tion of this assay for less frequently occurring genotypes, such as HCV genotype 5, has been limited. The line probe assay is based on the reverse hybridization of reverse transcription-PCR (RT-PCR) products derived from the 5′ untranslated region (5′-UTR) of the HCV genome. The low discriminating power of the 5′-UTR for the determination of particular HCV genotypes and subtypes may be a limitation of this method (3). We report here the validation of the line probe assay for 44

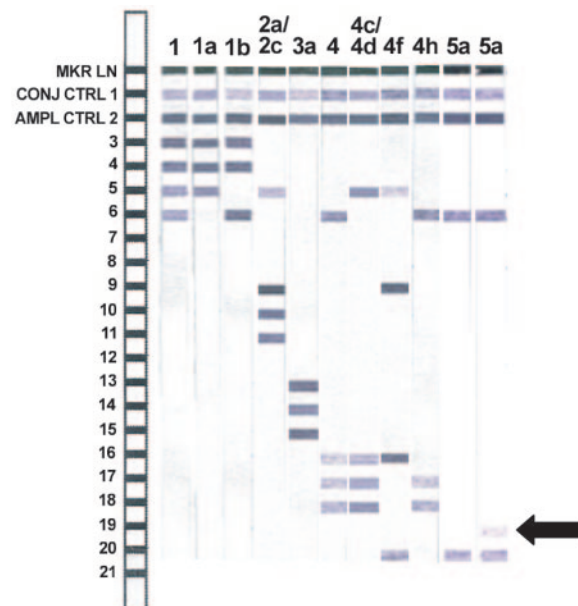


FIG. 1. Photograph of several line probe assay strips, showing the most common reaction patterns of different HCV genotypes. Using the interpretation chart, the exact genotype can be determined easily. For HCV genotype 5a, two distinct reaction patterns could be observed. The arrow indicates the additional band in the second hybridization pattern for HCV genotype 5a. MKR LN: marker line; CONJ CTRL: conjugate control; AMPL CTRL: amplification control.

* Corresponding author. Mailing address: Laboratory of Clinical and Epidemiological Virology, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: 32-16-347908. Fax: 32-16-347900. E-mail: marc.vanranst@uz.kuleuven.ac.be.

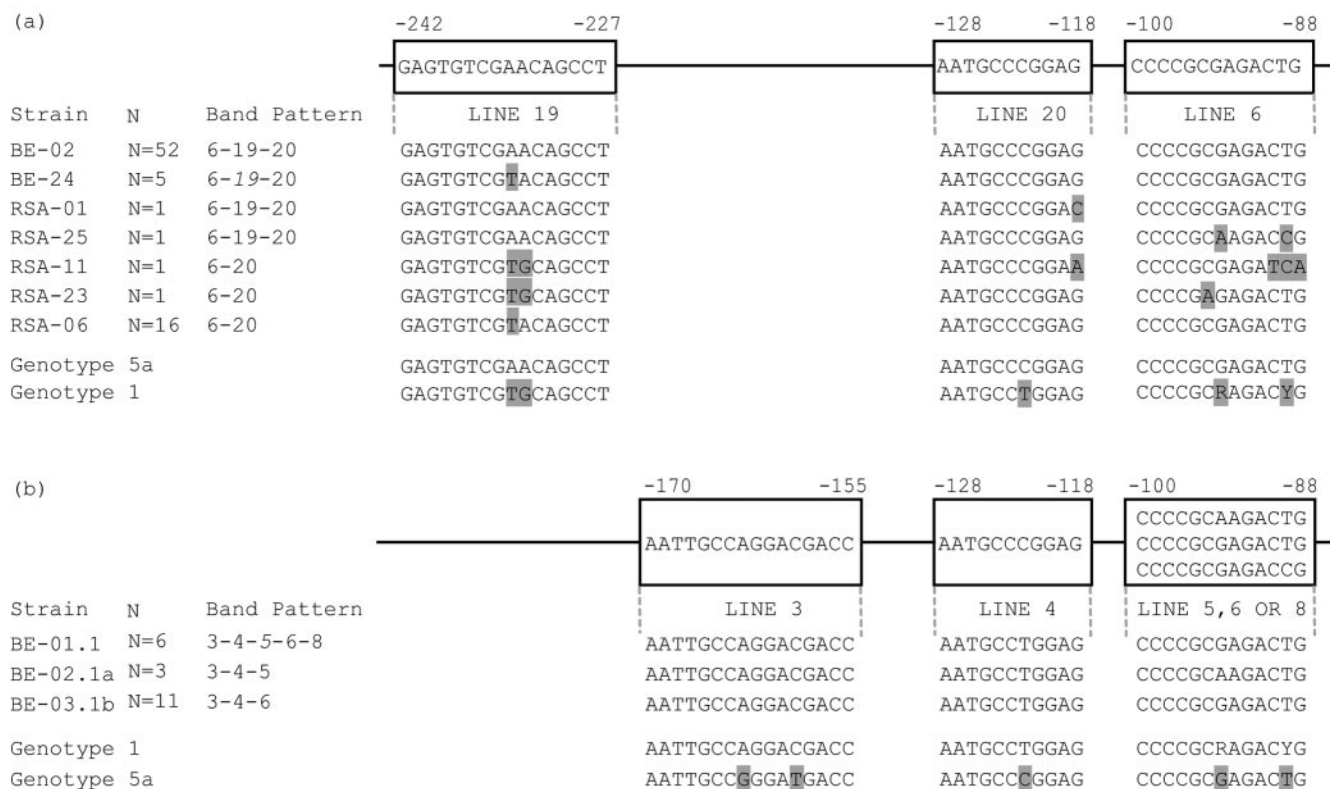


FIG. 2. (a) Part of an alignment of the 5'-UTR of the HCV genome. HCV genotype 5a-specific probes (with their positions) are indicated in the boxes, and the corresponding reaction line on the line probe assay strips is shown. Mismatches are indicated by small gray boxes, and for all of the samples, the observed reaction pattern is shown. (N, number of samples with the same sequence and reaction pattern; line numbers in italic indicate very faded bands on the strips). A reference sequence for HCV genotype 1 and HCV genotype 5a is included. (b) Same alignment of the 5'-UTR of the HCV genome for HCV genotype 1 samples and specific probes.

Belgian and 33 South African HCV genotype 5a samples. As negative and cross-hybridization controls, we used 20 HCV genotype 1a and 1b strains.

A total of 44 HCV genotype 5a-seropositive samples derived from Belgian patients, collected between January 2001 and September 2004, and 33 HCV genotype 5a-seropositive South African samples collected between January 1993 and December 2004 were genotyped as HCV genotype 5a at the University Hospital Gasthuisberg of Leuven using the line probe assay.

In this assay, specific oligonucleotide probes for each genotype are immobilized as parallel lines on nitrocellulose membrane strips. Biotinylated RT-PCR products hybridize to the probes that closely match the sequence of the isolate. The biotinylated hybrids are detected with an alkaline phosphatase-labeled streptavidin conjugate that converts a chromogen, resulting in the development of a purple-brown precipitate when there is a close match between the probe and the biotinylated RT-PCR product. The identification of the six major genotypes and their most common subtypes is made by comparing the patterns of positive lines of the hybridization assay with the interpretation charts enclosed by the manufacturer. As an independent verification of the correct HCV genotype 5a assignment, we sequenced a 511-nucleotide fragment of the NS4b region.

Serum RNA was extracted using the QIAamp viral RNA

minikit (QIAGEN, Leusden, The Netherlands) according to the manufacturer's instructions. Using a one-step RT-PCR (QIAGEN OneStep RT-PCR kit), a 574-nucleotide fragment of the NS4b region of the HCV genotype 5a genome was amplified using forward primer 5'-ATCAACATCGACGCYCACATG-3' and reverse primer 5'-CCCACTGACAAAGTTCACAT-3', which were chosen based on the alignment of the NS4b region of multiple HCV genotype 5a and non-genotype 5a sequences. Primers were synthesized by Eurogentec, Seraing, Belgium. Thermal PCR cycling was performed with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) using the following PCR program: a reverse transcription step for 30 min at 50°C; an initial PCR activation step for 15 min at 95°C; a denaturation step for 30 s at 94°C, a primer annealing step at 55°C for 30 s, and an extension step for 1 min at 72°C (45 cycles); and a final extension step for 10 min at 72°C. PCR products were purified using the QIAquick PCR purification kit (QIAGEN).

The purified DNA fragments were directly sequenced on both strands using the ABI Prism BigDye terminator cycle sequencing reaction kit (Applied Biosystems). The samples were loaded on an ABI Prism 3100 sequencer (Applied Biosystems). The chromatogram sequencing files were inspected with Chromas version 2.22 (Technelysium Ltd., Helensvale, Queensland, Australia), and contigs were prepared using Seqman II (DNASTar Inc., Madison, Wisconsin). Multiple align-

ments of the nucleotide sequences were created using the Clustal X program, version 1.83 (5). Phylogenetic trees were calculated in MEGA, version 2.1, using the neighbor-joining method (7). Bootstrap analysis was performed for values representing 2,000 replicates. We also sequenced a 217-bp fragment of the 5'-UTR to investigate the probe binding regions, using the same protocol as was used for sequencing of the NS4B region, but with primer pair 5'-AGACCGTGCACCAT GAGCAC-3' (sense) and 5'-GAACCTCGGGGGGAGAGC AA-3' (antisense) and an annealing temperature of 50°C.

Figure 1 shows a photograph of several line probe assay strips of different HCV genotypes compared with HCV genotype 5a strains. For the HCV genotype 5a samples, two distinct patterns could be observed. A total of 59 HCV genotype 5a samples showed two different bands, while the other 18 HCV genotype 5 samples showed a second hybridization pattern where three different bands could be observed. Figure 2a shows part of an alignment of the 5'-UTR, and the appropriate probes for HCV genotype 5a are indicated. For some samples, sequence heterogeneity in the probe regions could be observed. Sequencing of a 511-nucleotide fragment of the NS4b region, however, indicated that all 77 Belgian and South African HCV genotype 5a strains had profiles that clustered with the prototype 5a strain sequence (accession number Y13184) available in GenBank (data available on request).

To eliminate the possibility that the sequence heterogeneity was due to sequencing mistakes, all samples were extracted, amplified, and sequenced twice. The two sequencing results were identical, allowing us to assume that no sequencing errors occurred. To ensure the specificity of the line probe assay for the HCV genotype 5a samples, we also sequenced 20 HCV genotype 1 strains, which are most closely related to HCV genotype 5a according to phylogenetic analysis, using the same protocol as was used for the sequencing of the HCV genotype 5a samples. No sequence heterogeneity in the probe binding regions was observed for HCV genotype 1, and only sequences corresponding to the HCV genotype 1-specific probe binding regions could be found (Fig. 2b).

We can conclude that there is 100% concordance between the results of the line probe assay and our phylogenetic results of the NS4b region of the HCV genome despite the heterogeneity in the sequences of some HCV genotype 5a samples. A mismatch of 1 base pair in the middle of the probe region or mismatches of multiple base pairs at the end of the probe region do not interfere with the result of the test. No cross-hybridization was observed for HCV genotype 5a-specific probes with HCV genotypes 1a and 1b. We do not expect any

cross-hybridization between HCV genotype 5a and one of the other genotypes because these genotypes are more distantly related to HCV genotype 5a than HCV genotypes 1a and 1b. The line probe assay accurately identified all the HCV genotype 5a strains. Since few treatment response data are available for HCV genotype 5a, and given the fact that the HCV genotype influences the response to therapy (8), a correct genotype assignment for these patients is of increasing importance.

We thank our colleagues in the Laboratory of Clinical and Epidemiological Virology for helpful comments and discussion.

REFERENCES

1. Chamberlain, R. W., N. J. Adams, L. A. Taylor, P. Simmonds, and R. M. Elliot. 1997. The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. *Biochem. Biophys. Res. Commun.* **236**:44–49.
2. Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuyper, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbie, and P. L. Yap. 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' noncoding region. *J. Gen. Virol.* **76**:1197–1204.
3. Halfon, P., P. Trimoulet, M. Bourliere, H. Khiri, V. de Ledinghen, P. Couzigou, J. M. Fereyn, P. Alcaraz, C. Renou, H. J. A. Fleury, and D. Ouzan. 2001. Hepatitis C virus genotyping based on 5' noncoding sequence analysis (Trugene). *J. Clin. Microbiol.* **39**:1771–1773.
4. Henquell, C., C. Cartau, A. Abergel, H. Laurichesse, C. Regagnon, C. De Champs, J. L. Bailly, and H. Peigue-Lafeuille. 2004. High prevalence of hepatitis C virus type 5 in central France evidenced by a prospective study from 1996 to 2002. *J. Clin. Microbiol.* **42**:3030–3035.
5. Higgins, D. G., and P. M. Sharp. 1998. Clustal: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**:237–244.
6. Jover, R., J. Perez-Serra, F. de Vera, J. M. Alamo, C. Munoz, C. Yago, R. Martinez-Ramirez, and J. V. Vidal. 2001. Infection by genotype 5a of HCV in a district of southeast Spain. *Am. J. Gastroenterol.* **96**:3042–3043.
7. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA 2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**:1244–1245.
8. McHutchinson, J. G. 2002. Hepatitis C advances in antiviral therapy: what is accepted treatment now? *J. Gastroenterol. Hepatol.* **17**:431–441.
9. McHutchinson, J., S. Cordon, E. Schiff, M. Shiffman, W. Lee, V. Rustgi, Z. Goodman, M. H. Ling, S. Cort, and J. Albrecht. 1998. Interferon alpha-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N. Engl. J. Med.* **339**:1485–1492.
10. Murphy, D. G., B. Willems, J. Vincelette, L. Bernier, J. Cote, and G. Delage. 1996. Biological and clinicopathological features associated with hepatitis C virus type 5 infections. *J. Hepatol.* **24**:109–113.
11. Nolte, F. S., A. M. Green, K. R. Fiebelkorn, A. M. Caliendo, C. Sturchio, A. Grunwald, and M. Healy. 2003. Clinical evaluation of two methods for genotyping hepatitis C virus based on analysis of the 5' noncoding region. *J. Clin. Microbiol.* **41**:1558–1564.
12. Simmonds, P., E. C. Holmes, T. A. Cha, S. W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea. 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J. Gen. Virol.* **74**:2391–2399.
13. Stuyver, L., A. Wyseur, W. Van Arnhem, F. Hernandez, and G. Maertens. 1996. Second-generation line probe assay for hepatitis C virus genotyping. *J. Clin. Microbiol.* **34**:2259–2266.