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Second-Generation Line Probe Assay for Hepatitis C Virus Genotyping

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Because of the enormous variability of hepatitis C virus (HCV), the development of reliable genotyping assays is a formidable challenge. The optimal genotyping region appears to be the 5′ untranslated region (UR) because of high conservation within, but considerable variability between, genotypes. In this study, 21 probes dispersed over seven variable 5′ UR areas were applied to a line probe assay (LiPA) and used to analyze 506 HCV-infected sera from different geographical regions representing a multitude of subtypes. At least 31 different reactivity patterns emerged, with 404 (80%) of 506 distributed over 11 prototype patterns, in general corresponding to subtypes 1a, 1b, 2a/2c, 2b, 3a, 5a, and 6a and several type 4 subtypes. Subtyping specificity ranged from 97% in Hong Kong to 90% in Europe but was only 11% in West Africa, while typing specificity was always 100% when samples from Vietnam were excluded. In a second evaluation, the subtype prediction by LiPA of 448 GenBank 5′ UR HCV sequences was scored. Of the 58 theoretically predicted patterns, 321 sequences (72%) were covered by the 11 prototype patterns. We concluded that (i) the selected probes detected the corresponding signature motifs in the seven variable regions with 100% reliability; (ii) these motifs allowed correct type interpretation of samples collected worldwide, with the exclusion of Vietnam, Thailand, or Vietnamese patients residing in European hospitals; and (iii) subtyping specificities vary according to geographical region, with 11 prototype subtyping patterns identifying the majority of samples from Europe and the Americas. These results indicate that the LiPA is a reliable assay applicable to routine typing and subtyping of HCV specimens.

Hepatitis C virus (HCV) is the causative agent of most non-A, non-B sporadic and posttransfusional hepatitis (3). Analysis of the different regions of the HCV genomes has shown extensive variability (7), leading to classification of the different isolates into types (designated types 1, 2, 3, etc.), subtypes (designated subtypes 1a, 1b, 1c, etc.), and isolates belonging to the same subtype (11). It has been shown that the clinical course of infection by certain HCV types is more severe than that of infection by others and that the response to interferon treatment is dependent on the type and subtype (4, 8, 16), in addition to such factors as virus load and, perhaps, the genetic background of the host. For these reasons, it is important to pursue further development of a convenient and user-friendly genotyping system.

As the complete genome is about 9,400 bp long (9), determination of the complete genomic sequence of each individual isolate represents a considerable effort for HCV variability researchers and is not applicable to routine typing. Therefore, several smaller regions have been investigated with respect to routine genotyping, as well as classification. A 329- to 340-bp nonstructural (NS) 5B region seems to be the most reliable for sequencing and phylogenetic analysis (14, 15). For routine diagnosis, genotype-specific amplification of the core region (10, 17) and restriction fragment length polymorphism analysis of the 5′ untranslated region (UR) have been described (12). However, these assays are very laborious given that 11 HCV types with more than 70 subtypes are known. Moreover, for many of the newly described sequences, specific primers or restriction enzymes are difficult to select or not available. Recently, serological typing assays based on the detection of antibodies against NS4 and core epitopes have been described (2, 5), but these assays lack sufficient sensitivity and specificity and do not recognize the clinically relevant subtypes.

Previously, we reported the development of a prototype version of the line probe assay (LiPA) that allows discrimination of HCV types and subtypes and is capable of detecting single nucleotide differences in the 5′ UR (13). LiPA technology is based on the reverse hybridization principle: biotinylated PCR fragments are hybridized to a selection of highly specific immobilized probes. In a second step, the biotin group in the hybridization complex is revealed by incubation with a streptavidin-alkaline phosphatase complex and the appropriate chromogen compounds. In this report, we describe the fully developed second-generation version of this test and its ability to discriminate among seven HCV types (types 1 to 6 and 10) and their most relevant subtypes. With this second-generation assay, 426 serum samples were analyzed. In addition, all known GenBank entries (448 as of December 1995) were evaluated and a prediction of their reactivity was made.

MATERIALS AND METHODS

Collection of serum samples. Blood samples originating from different parts of the world and from both patients and blood donors were analyzed. However, it should be stressed that the present serum collection is not presumed to be representative of a certain geographic region or population. In this respect, the selected samples were more or less comparable to the GenBank database entries: since all of the sequences retrieved were collected worldwide, different types and subtypes were present and both patients and blood donors were represented. Our sera were collected in Europe (Belgium, France, and The Netherlands), Africa (Morocco, Benin, Gabon, Central African Republic, Egypt, Burkina Faso, and Guinea), South America (Chile and Brazil), and Asia (Pakistan, Vietnam, Hong Kong, and Indonesia) and stored at −20°C until use. Prior to RNA extraction, sera were tested on INNO-LIA HCV Ab III (Innogenetics N.V., Antwerp, Belgium) to confirm the presence of anti-HCV antibodies.

HCV RNA extraction. HCV RNA was extracted from serum or EDTA-plasma

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position						-295					-290					-285					-280					-275			
consensus		C	C	C	T	G	T	G	A	G	G	A	A	C	T	A	C	T	G	T	C	T	T	C	A	C	G	C	
frequency		95	100	99	99	99	99	125	125	126	125	126	125	125	125	112	124	126	194	194	194	201	201	209	211	211	211	211	
mutation																T	T												
frequency																13	1												
HCPr 98	5' bio-	C	C	C	T	G	T	G	A	G	G	A	A	C	T	A/T	C	T	G	T	C	T	T	C	A	C	G	C	3'

position		-264				-260					-255					-250					-245					-240		
consensus		T	C	T	A	G	C	C	A	T	G	G	C	G	T	T	A	G	T	A	T	G	A	G	T	G	T	
frequency		192	223	240	240	238	287	288	289	306	309	309	309	309	329	329	330	330	332	337	323	380	388	389	390	391	402	
mutation		C				A				C			G	C		C				C	C/G	A	C/T	A	A/G			
frequency		31				2				1			1	1		1				1	57/1	1	1/1	1	1/1			
HCPr 95	5' bio-	T	C	T	A	G	C	C	A	T	G	G	C	G	T	T	A	G	T	A	C/T	G	A	G	T	G	T	3'

position						-50					-45					-40					-35					-30		
consensus		A	C	T	G	C	C	T	G	A	T	A	G	G	G	T	G	C	T	T	G	C	G	A	G	T	G	
frequency		344	344	344	343	335	331	331	331	331	331	331	331	331	331	330	330	331	331	329	331	329	331	331	331	331	330	
mutation					A											C	C			C		A				G		
frequency					1											1	1			2		2				1		
HCPr 96	3'	T	G	A	C	G	G	A	C	T	A	T	C	C	C	A	C	G	A	A	C	G	C	T	C	A	C	-bio 5'

position			-20					-15					-10		-8			-5				-1	1				5		
consensus		A	G	G	T	C	T	C	G	T	A	G	A	C	C	G	T	G	C	A	C	C	A	T	G	A	G	C	
frequency		291	291	290	291	291	291	291	291	291	291	291	291	291	273	273	273	273	272	242	132	241	221	221	220	219	219	219	
mutation				A									G		T				T		A/T	T							
frequency				1									1		17				1		36/74	1							
HCPr 29	3'	T	C	C	A	G	A	G	C	A	T	C	T	G	G	C	A	C	G	T	A/G	G	T	A	C	T	C	G	-bio 5'

FIG. 1. Comparison of 475 database sequences in the PCR primer regions. Sequences were aligned with respect to the consensus sequence. Frequency indicates the number of times a given nucleotide was encountered in the database at a given position. Primers HCPr98 and 95 are the sense primers; primers HCPr96 and 29 are antisense primers, for which the reverse complement is given.

```
HCV-1    1a   T|GTCGTGCA|GCCTCCAGGACCC   CCGGAAT|TGCCAGGACGACC|GGGTCCT|TTC..TT|GGAT.|CAACCCG|CTC|AATGCCTGGAG|ATTTGGGCGTGCCCCCG|CAAGACTGC|TAGCCGAGTA|GTGTTGGGTCGC|GA    2*
BE101    1a   -|--------|T------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    1
US9      1a   -|--------|T------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|------C--|8---------|------------|--    6
HCV-J    1b   -|--T-----|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    1*
HCV-N    1b   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|---------|5---------|------------|--    2
BE102    1b   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|------C--|8---------|------------|--    6
BE113    1b   -|--------|-------------   -------|--------T----|-------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    3
FR3      1b   -|--------|T------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    1
BE90     1b   -|--------|----------T--   -------|--------T----|-------|---..--|----.|T------|---|-G---------|-----------------|-G-------|6---------|------------|--    single
BE82     1b   -|--------|T------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G----C--|7---------|------------|--    5
HC-G9    1c   -|--------|-------------   -------|-------------|3------|---..--|----.|T------|---|-----------|4----------------|---------|5---------|------------|--    2
NL35     1d   -|-----A--|---------C---   -------|-------------|3------|---..--|----.|A-C----|---|-----------|4----------------|---------|5---------|------------|--    2
CAM1078  1e   -|-----A--|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|---------|5---------|------------|--    2
FR2      1f   -|-----A--|N------------   -------|-------------|3------|---..--|----.|-T-A---|---|-----------|4----------------|-G-------|6---------|------------|--    1

HC-J6    2a   -|-----A--|---------C---   -------|----G---A---T|11-----|---..--|----.|A-----A|10-|T-----C--TC|11---------------|---------|5---------|-C-------T--|9-    11*
FR5      2a   -|-----A--|---------C---   -------|----G---A---T|11-----|---..--|----.|A-----A|10-|T-----C--CC|11---------------|---------|5---------|-C-------T--|9-    11*
HC-J8    2b   -|-----A--|---------C---   -------|-A--G-A-A---T|12-----|---..--|----.|A-----A|10-|T---T-C--TC|12------AC-------|---------|5---------|-C-------T--|9-    12*
S83      2c   -|-----A--|---------C---   -------|----G---A---T|11-----|---..--|----.|A-----A|10-|T-----C--CC|11---------------|---------|5---------|-C-------T--|9-    11*
BE136    2c   -|-----A--|---------C---   -------|---TG---A---T|-------|---..--|----.|A-----A|10-|T-----CA-CC|-----------------|-----TC--|----------|-C-------T--|9-    19
NE92     2d   -|-----A--|---------C---   -------|--------A---T|-------|---..--|----.|A-----A|10-|T--------TC|-----------------|---------|5---------|-C-------T--|9-    20
FR4      2e   -|-----A--|---------C---   -------|----G---A---T|11-----|---..--|----.|A-----A|10-|T-----C--TC|11---------------|------C--|8---------|-C-------T--|9-    13
FR13     2†   -|----AA--|19-------C---   -------|----G---A---T|11-----|---..--|----.|A-----A|10-|T-----C--CC|11---------------|---------|5---------|-C-------T--|9-    new
FR19     2†   -|-----A--|-------------   -------|----G---A---T|11-----|---..--|----.|T-----A|---|T-----C----|20---------------|---------|5---------|-C-------T--|9-    new

TH85     3a   -|--------|---------C---   -------|C--TG--GT----|14-----|---..--|---A.|-------|---|---A--CA--A|15---------------|-G---TCA-|13--------|------------|--    22*
HCV-Tr   3b   -|--------|---------C---   -------|C---G---T----|17-----|---..--|---A.|-------|---|------C---A|18---------------|-G---TCA-|13--------|------------|--    24*
NE137    3b   -|--------|---------G---   -------|C---G--------|-------|---..--|---A.|T------|---|------C---A|18---------------|-G---TCA-|13--------|------------|--    29
NE48     3c   -|--------|---------C---   -------|C--TG--GT----|14-----|---..--|---A.|-------|---|---A--CA--A|15---------------|-G-------|6---------|------------|--    27
NE274    3d   -|--------|---------C---   -------|C--TG--GT----|14-----|---..--|---AC|A------|---|---A--CA--A|15---------------|-G---TCA-|13--------|------------|--    22
NE145    3e   -|--------|-------------   -------|C--TG--GT----|14-----|---..--|---A.|-------|---|---A--CA--A|15---------------|-G---TCA-|13--------|------------|--    22
NE125    3f   -|--------|---------C---   -------|C---G--GT----|-------|---..--|---AT|-------|---|---A--C---A|-----------------|-G-------|6---------|------------|--    single

Z4       4a   -|--T--A--|16-----------   -------|C------------|-------|---..--|----.|-------|---|----------A|-----------------|-G-------|6---------|------------|--    41
Z1       4b   -|--T--A--|16-------C---   -------|C---G---T----|17-----|---..--|----.|-------|---|------C--CA|-----------------|---------|C---------|------------|--    39
GB358    4c   -|--T--A--|16-----------   -------|C---G---T----|17-----|---..--|----.|A------|---|------C---A|18---------------|---------|5---------|------------|--    30*
DK13     4d   -|--T--A--|16-----------   -------|C---G---T----|17-----|---..--|----.|A------|---|------C---A|18---------------|---------|5---------|------------|--    30*
Z5       4e   -|--T--A--|16-------G---   -------|C---G---T----|17-----|---..--|----T|A------|---|------C---A|18---------------|-G-------|6---------|------------|--    32*
CAM600   4e¶  -|--T--A--|16-----------   -------|C---GA--T----|-------|---..--|----.|-------|---|-----TC---A|-----------------|---------|5---------|------------|--    33
GB809    4e¶  -|--T--A--|16-----------   -------|C---GA--T----|-------|---..--|----.|-------|---|-----TC---A|-----------------|------C--|8---------|------------|--    42
Z8       4f   -|--T--A--|16--------T--   -------|C---G--------|-------|---..--|---A.|-------|---|------C---A|18---------------|---------|5---------|------------|--    38
CAMG22   4f¶  -|--T--A--|16-----------   -------|----G--------|-------|---..--|----.|T------|---|------C----|20---------------|---------|5---------|-C-------T--|9-    36
GB549    4g   -|--T-----|-------------   -------|C---G--------|-------|---..--|---AC|A------|---|------C--CA|-----------------|---------|5---------|------------|--    single
GB438    4h   -|-----A--|----------T--   -------|C---G---T----|17-----|---..--|---AT|-------|---|------C---A|18---------------|-G-------|6---------|------------|--    31*
B14      4k   -|--T-----|-------------   -------|C---G---T----|17-----|---..--|---AC|T------|---|------C---A|18---------------|-G-------|6---------|------------|--    31*

BE95     5a   -|----AA--|19-----------   -------|----G---T----|-------|---..--|----.|T------|---|------C----|20---------------|-G-------|6---------|------------|--    44*
BE96     5a   -|----AA--|19-----------   -------|----G--------|-------|---..--|----.|A------|---|------C----|20---------------|-G-------|6---------|------------|--    44*

HK2      6a   -|-----A--|---------C---   -------|--------T----|-------|--CA---|21--C|A------|---|-----------|4----------------|---------|5---------|-C-------T--|9-    46*
HK-4     6a   -|--------|-------------   -------|-------------|3------|--CA---|21--C|A------|---|-----------|4----------------|---------|5---------|------------|--    47
Th580    6b   -|--------|---------C---   -------|-------------|3------|--CA---|21--C|A------|---|-----------|4----------------|---------|5---------|-C-------T--|9-    48

VN540    7a   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    51
VN235    7b   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    51
Th271    7c   -|--------|-------------   -------|--------T----|-------|---..--|----.|-------|---|-G---------|-----------------|-G----C--|7---------|------------|--    single
Th846    7d   -|-----A--|---------C---   -------|--------T----|-------|---..--|----C|A------|---|-----------|4----------------|-G-------|6---------|------------|--    52
VN4      7c¶  -|--------|-----------T-   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    51
VN12     7d¶  -|-----N--|N---------T--   -------|-------------|3------|---..--|----C|A------|---|-----------|4----------------|-G-------|6-R-------|------------|--    51
EuTH36   7†   -|--------|-------------   -------|--------T----|-------|---..--|----.|T------|---|-G---------|-----------------|-G----C--|7---------|------------|--    single

VN507    8a   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    51
VN405    8b   -|--------|-----------T-   -------|--------T----|-------|---..--|----.|-------|---|G----------|-----------------|-----T---|----------|------------|--    none
EuTH86   8†   -|--------|-----------T-   -------|------------A|-------|---..--|----.|------T|---|-----------|4----------------|-G-------|6---------|------------|--    52

FR1      9a   -|--------|-------------   -------|--------T----|-------|---..--|----.|-------|---|-----------|4----------------|------C--|8---------|------------|--    56
VN004    9a   -|--------|-------------   -------|--------T----|-------|---..--|----.|T------|---|-----------|4----------------|-G-------|6---------|------------|--    52
Th555    9b   -|--------|-------------   -------|-------------|3------|---..--|----.|T------|---|-----------|4----------------|-G-------|6---------|------------|--    51
Th553    9c   -|--------|-------------   -------|-------------|3------|---..--|----.|-------|---|-----------|4----------------|-G-------|6---------|------------|--    51

NL96     10a  -|--------|-------------   -------|C---G--TT----|-------|---..--|---A.|-T-----|---|------C---A|18---------------|-G-------|6---------|------------|--    57
                -240                              -167            -147    -138    -128                          -100                   -81               LiPA strip
                 R1                                R2              R3      R4      R5                            R6                     R7               prediction
```

FIG. 2. Alignment of the 5′ UR with indication of variable regions. The positions of probes are boxed and designated R1 to R7. The first nucleotide position of each box is indicated at the bottom left corner. Prototype names and subtypes, obtained by phylogenetic analysis in at least one other region, are indicated. ¶, subtype assignment not unique; †, no subtype assigned. The predicted reactivity of each isolate is indicated in the sequence alignment by the respective LiPA line number outside the R box. The numbers in the right-hand column refer to strip numbers in Fig. 4. ∗, indication of prototype reactivity pattern; single, predicted to be reactive with only one line; none, predicted to be negative on all probes except the universal probes in line 2.

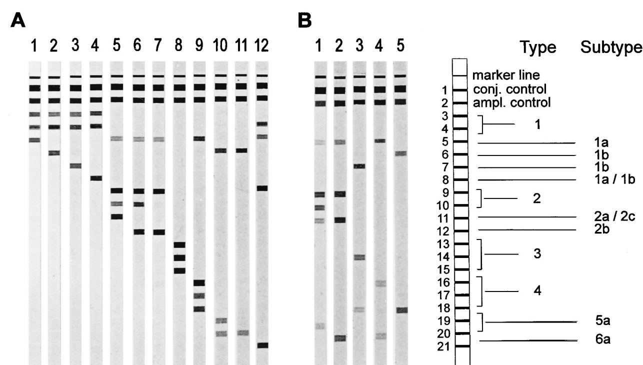

FIG. 3. (A) LiPA strips of a reference panel. Strips were incubated with the following samples: 1, BR5293 (subtype 1a); 2, BE107 (subtype 1b); 3, BE82 (subtype 1b); 4, BE102 (subtype 1b); 5, FR5 (subtype 2a); 6, NE91/NL42 (subtype 2b); 7, X1 (subtype 2b); 8, BR56 (subtype 3a); 9, GB358 (subtype 4c); 10, BE95 (subtype 5a); 11, BE100 (subtype 5a); 12, VN11 (type 6a). (B) LiPA strips of a series based on atypical reactivities for which the interpretation depends on deduction based on the information given in Table 1, Fig. 2, and Fig. 4. Strips were incubated with the following samples: 1, FR13 (type 2 subtype); 2, FR19 (type 2 subtype); 3, PK64 (type 3 subtype); 4, Eg81 (type 4 subtype); 5, NL96 (type 10a). Conj., conjugate; ampl., amplification.

as previously described (13), with some modifications. To avoid carryover contamination, the steps of the protocol were carried out at four separate locations. Cotton-plugged tips were generally used, and all buffers, primers, and dXTPs were divided into single-use aliquots. Fifty microliters of serum was mixed with 150 μl of Trizol LS Reagent (Life Technologies, Gent, Belgium) at room temperature. After lysis and denaturation, 40 μl of $CHCl_3$ was added. The mixture was vigorously shaken, incubated for 15 min at room temperature, and centrifuged, and the minute amounts of viral RNA were precipitated from the colorless aqueous phase with 20 μl of 1 μg Dextran T500 (Pharmacia, Brussels, Belgium) per μl and 100 μl of isopropanol. The RNA pellet was washed with 200 μl of ethanol and collected by centrifugation. Finally, the RNA pellet was briefly air dried. If the procedure was interrupted at this stage, the RNA was stored at −20°C as a pellet instead of being dissolved in water.

cDNA synthesis and PCR amplification. The RNA pellet was dissolved in 15.1-μl random primers (20 ng/μl, pdN_6; Pharmacia), prepared in diethylpyrocarbonate-treated or high-pressure liquid chromatography grade water. After denaturation at 70°C for 10 min, 4.9 μl of a cDNA mixture was added which was composed of 4 μl of 5× avian myeloblastosis virus reverse transcriptase buffer (250 mM Tris · HCl [pH 8.5], 100 mM KCl, 30 mM $MgCl_2$, 25 mM dithiothreitol), 0.4 μl of 25 mM dXTPs, 0.2 μl or 25 U of RNase inhibitor (HPRI; Amersham, Gent, Belgium), and 0.3 μl or 8 U of avian myeloblastosis virus reverse transcriptase (Stratagene, La Jolla, Calif.). cDNA was synthesized at 42°C for 90 min. PCR was performed in a volume of 50 μl. Outer PCR amplified the cDNA over 40 cycles (1 min at 94°C, 1 min at 50°C, and 1 min at 72°C). One microliter of product was amplified with nested PCR primers for another 40 cycles with the same thermal profile. All primers were tagged with a biotin group at the 5′ end. The sequences of the primers used are given in Fig. 1.

Preparation of LiPA strips. A poly(dT) tail was enzymatically added to the 3′ end of each oligonucleotide as previously described (13). The tailed probes were precipitated and washed with ice-cold ethanol. Probes were dissolved at their respective specific concentrations and applied as horizontal lines to membrane strips. Biotinylated DNA was also applied as a positive control (LiPA line 1). The oligonucleotides were fixed to the membrane by baking at 80°C for 12 h. The membranes were then sliced into 4-mm-wide strips.

LiPA. Equal volumes (10 μl each) of the biotinylated PCR fragment and the denaturation solution (400 mM NaOH, 10 mM EDTA) were mixed in test troughs by pipetting and incubated at room temperature for 5 min, after which 2 ml of the prewarmed (37°C) hybridization solution (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate) was added, followed by the addition of one strip per trough. Hybridization occurred for 1 h at 50 ± 0.5°C in a closed water bath with back-and-forth shaking. The strips were washed twice with 2 ml of wash solution (3× SSC, 0.1% sodium dodecyl sulfate) at room temperature for 20 s and once at 50°C for 30 min. Following this stringent washing, strips were rinsed twice with 2 ml of a standard rinse solution. Strips were incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution for 30 min at 20 to 25°C. Strips were then washed twice with 2 ml of rinse solution and once with standard substrate buffer, and color development was initiated by addition of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium to 2 ml of substrate buffer. After 30 min at room temperature, the color reaction was stopped by aspiration of the substrate buffer and addition of distilled water. Immediately after drying, the strips were interpreted.

Nucleotide sequence accession numbers. NS5B sequences for the samples in Fig. 3B will appear in the GenBank/EMBL/DDBJ database under the following accession numbers: FR13, L48492; FR19, L48498; PK64, L78842; Eg81, L78841; NL96, L44604.

RESULTS

Universal character of the HCV 5′ UR amplification primers. Two nested sets of universal HCV primers were selected, amplifying a 236-bp segment of the 5′ UR in the nested reaction (including primer sequences). Figure 1 shows the frequency of consensus nucleotides at each position of the primers. Frequencies fluctuated between 95 (C at position −299) and 402 (T at position −239) times, depending on the variable length and variable first 5′ nucleotide of each of the included database sequences (National Center for Biotechnology Information [Bethesda, Md.], Netscape; $n = 448$ as of December 1995). To match all different types completely, there is a need to include degeneration at nucleotide positions −285, −264, −245, and −2. Variations at positions −285, −264, and −245 could be ascribed to type 3 sequences. The variation at position −2 was more or less type specific, with the following distributions of C, T, and A, respectively: type 1, 106, 16, and 3 times; type 2, 12, 25, and 0 times; type 3, 2, 2, and 28 times; type 4, 9, 2, and 0 times; type 5, 3, 1, and 0 times; types 6 to 10, 1, 28, and 5 times. The presence of a T, but not an A, was compensated for in HCPr29. Degeneration at position −264 was not included because of the 5′ position in the primer. Other random

TABLE 1. Predicted LiPA II line reactivity of 448 sequences present in the GenBank/EMBL/DDBJ database

Type(s)	No. of samples	No. of samples with following genotype-line no. association(s)[a]																		
		1		1a	1b		1a/1b	2		2a/2c	2b	3			4			5a		6a
		3	4	5	6	**7**	8	9	**10**	**11**	**12**	**13**	**14**	**15**	**16**	17	18	**19**	20	**21**
1	230	213	229	59	146	9	7													
2	69			63			5	63	61	42	24									
3	53				1							43	44	42		7	7			
4	50			29	17		3	3							37	36	38		2	
5	11				11													9	11	
6	12	3	12	11	1			8												12
7–9	20	17	20	1	17		2	1												
10	3			1	2			1									3			
Total[b]	448	233	261	164	195	9	17	76	61	42	24	43	44	42	37	43	48	9	13	12

[a] The line numbers correspond to the drawing in Fig. 3. Line numbers with type- or subtype-specific characteristics are in boldface.
[b] The total amounts of predicted reactivity correspond to the drawings in Fig. 4.

1* 2* 3 4 5 6 7 8 9 10

n = 133 56 12 9 8 6 3 1 1 1 = 230

11* 12* 13 14

31 18 4 3

15 16 17 18 19 20 21

n = 3 3 3 1 1 1 1 = 69

22* 23 24* 25 26 27 28 29

31 9 6 3 1 1 1 1 = 53

30* 31* 32* 33 34 35 36 37 38 39 40 41 42 43

n = 15 9 6 6 3 2 2 1 1 1 1 1 1 1 = 50

44* 45

9 2 = 11

46* 47 48 49 50

n = 7 2 1 1 1 = 12

51 52 53 54 55 56 57 58

14 2 1 1 1 1 2 1 = 23

mutations occurred at a total of 22 positions, but primers were not adapted, since these polymorphisms occurred infrequently. With the combination of universal sense primers HCPr98 and HCPr95 and with antisense primers HCPr29 and HCPr96, it was predicted, and experimentally proven, that all different HCV genotypes can be amplified with similar sensitivities.

5′ UR HCV LiPA, version II. Figure 2 shows an alignment of the 5′ UR, based on sequences previously published and those obtained in the course of this study. The positions of the seven variable regions (R1 to R7) located inside the nested primer pair and containing the target information for probe design are indicated. Only probes enabling differentiation at the single-nucleotide level were employed. This selection of highly specific probes was achieved after considering the nucleotide composition (percent G+C), final concentration, length, target strand (sense or antisense), and hybridization temperature (data not shown). For R1, two probes were selected, respectively covering the motifs for types 4 and 5, and applied as lines 16 and 19 on the LiPA (Fig. 3). For the R2 region, five different probes were designed, of which three were used for the typing of types 1, 3, and 4 (lines 3, 14, and 17, respectively) and two were used for type 2 subtyping (lines 11 and 12). In R3, only one probe targeting the typical type 6 CA insertion between nucleotide positions −144 and −145 (line 21) was selected. R4 contained LiPA probe 10, a universal probe for type 2. In R5, the six selected probes were used for types 1 (line 4), 3 (line 15), 4 and 10 (line 18), and 5 (line 20), as well as for subtypes 2a/2c (line 11), 2b (line 12), and 3b (line 18). Type 1 subtyping is located in the R6 area. In general, the subtype 1a-specific motif contains an A at position −99 (line 5), while in most subtype 1b isolates, a G is found at −99 (line 6). However, in both subtypes, variation at position −94 may coincide with the −99 variability, which results in two motifs detected by probes applied as line 7 (subtype 1b) or 8 (subtype 1a or 1b). The alignment in Fig. 2 demonstrates that isolates from all other genotypes might be reactive with at least one of these four probes, except for most type 3 samples, which contain the specific $G(N_3)TCA$ motif covered by the probe on line 13. R7 contains the $C(N_7)T$ motif (line 9) which is present in all type 2, most type 6, and some type 4 isolates. The universal amplification control line (Fig. 3, line 2) contains the same probes as used in the first-generation assay (13).

Theoretical prediction of database sequences. HCV 5′ UR sequences available from the GenBank/EMBL/DDBJ database (n = 448 as of December 1995) were aligned against the HCV type 1 prototype sequence. Details for some of these sequences are presented in Fig. 2. HCV database entries not covering the seven variable regions, or showing sequence ambiguities, were not included in this analysis. Table 1 shows the predicted reactivity of all sequences on the individual LiPA HCV II lines. Since the corresponding core or NS5B sequences were not always available—a prerequisite for detailed subtype confirmation (14)—sequences were grouped into major types. The LiPA lines having unique type or subtype specificity were 7 (subtype 1b); 10 (type 2); 11 (subtype 2a/2c); 12 (subtype 2b); 13, 14, and 15 (type 3 subtypes); 16 (type 4); 19 (type 5a); and 21 (type 6). Thus, observed reactivity at any of these lines is indicative of the corresponding type or subtype without the need for reactivity on other lines.

Furthermore, the 448 sequences could be predicted to be reactive in 58 different patterns (Fig. 4). Ten different patterns were found for samples belonging to type 1, with patterns 1 (type 1b) and 2 (type 1a) accounting for 82% of all type 1 samples. Type 2 sequences could be divided into 11 patterns, of which patterns 11 (subtype 2a/2c) and 12 (subtype 2b) were the most important and accounted for 71% of the reactivities. Most of the subtype 3a isolates were predicted to react as shown in pattern 22. Subtype 3b was predicted to react as indicated in pattern 24. The patterns for type 4 were complicated and diverse (strips 30 to 43), although some aspects might be typical: (i) strip line 16 was always type 4 specific (Table 1); (ii) the most common sequence found belongs mostly to subtype 4c (strip 30); and (iii) subtype 4f (for example, CAMG22) usually reacted as shown in pattern 36. Most other type 4 subtypes can be discriminated by unique reactivity patterns, as shown in Fig. 2. The exact interpretation of types 5a and 6 was straightforward because of the presence of type-specific lines 19 and 21, respectively. Some type 7 to 9 isolates (strips 51 to 56), most of which originated from Vietnam, showed reactivities identical to that of type 1. Isolate TH13 (accession number M28833), represented by pattern 53, was the only combination that had never been encountered in type 1. Type 10 isolates were characterized as indicated on strips 57 and 58. Such a reactivity pattern is found mostly in samples originating from Indonesia.

From these analyses, 11 prototype patterns could be deduced: subtype 1a, strip 2; subtype 1b, strip 1; subtype 2a/2c, strip 11; subtype 2b, strip 12; subtype 3a, strip 22; subtype 3b, strip 24; subtype 5a, strip 44; subtype 6a, strip 46; type 4, strips 30, 31, and 32. Together, these 11 patterns accounted for 321 sequences (72%).

Experimentally detected reactivity patterns. A total of 506 serum samples, collected in the course of 1995, were tested on the INNO-LiPA HCV II test. Of these, 368 (Table 2) were further analyzed in another region of the HCV genome, either by means of the 5′ UR/Core LiPA for types 1, 2, 4, and 6 to 9 or by sequencing in the NS5B region (14; data not shown). At least 31 different reactivity patterns appeared, 26 of which were present in the predictions given in Fig. 4; the remaining 5 patterns were unique for this collection of sera (Fig. 3B). Specificity of typing was 100%. Subtyping specificities ranged from 97% (Hong Kong) to 90% (Europe). Subtyping of West African samples was unreliable, with an error rate of up to 89%. It was noteworthy that all type 10 samples originated from Indonesia. The reactivity of a representative set of type 1 to 6 serum samples on the INNO-LiPA HCV II assay is given in Fig. 3A.

Some atypical results. While most of the serum samples reacted as shown in Fig. 3A, some cases, depending on the study population and geographic origin of the samples, yielded untypeable patterns. Figure 3B shows five of these cases. These isolates were sequenced in the NS5B region, and phylogenetic analysis confirmed classification into new HCV subtypes (data not shown). These atypical cases were found in non-European blood donors or patients from Africa, the French Antilles, Vietnam, and Pakistan, although some of the blood samples were collected in Europe and some of the patients were treated in European hospitals (as indicated in Table 2 for the

FIG. 4. Schematic representation of predicted LiPA HCV II reactivity patterns found in 448 database sequences. The order in which the patterns were arranged ascended depending on the type, abundance, number of predicted lines, and the chronological order in which lines appear. Patterns: 1 to 10, type 1; 11 to 21, type 2; 22 to 29, type 3; 30 to 43, type 4; 44 and 45, type 5; 46 to 50, type 6; 51 to 56, types 7 to 9; 57 and 58, type 10. The 11 prototype reactivity patterns with asterisks correspond to isolate sequences given in Fig. 2. The frequency of appearance in the database is indicated beneath each pattern.

TABLE 2. Analysis of 506 selected serum samples from different geographical regions

Country of origin (specificity) and parameter[a]	Total no. of samples	No. of samples with following genotype-LiPA pattern association[b]:																										
		1b, **1**	1a, **2**	1b, 3	1a, 4	1b, 5	1a/1b, 6	1b, 8	2a/2c, **11**	2b, **12**	2, 15	2, 19	2, 20	3a, **22**	3a, 23	3a, 25	4, **30**	4, **31**	4, **32**	4, 34	5a, **44**	6a, **46**	6a, 47	6a, 48	7–9, 51	7–9, 53	10a, 57	Other[c]
Belgium (100/92)																												
Total no.	73	42	6			2	2		2					5			2	1			8						1	2
No. confirmed	48	36	5			2			2												2						1	
Error rate	4	2	1			0			1												0						0	
France[d] (97/89)																												
Total no.	165	41	25		2	4	5		23	4		5	1	14	4	1	8	1	2	1	4	2			3			15
No. confirmed	106	32	22		2	4	5		17	4		5[e]										1			3			11
Error rate	12	7	3		0	0	0		2	0												0			0			0
Egypt (100/NA)																												
Total no.	58	2	5														17	7	14	6	1							6
No. confirmed	50																17	7	14	6								6
Error rate	0																0	0	0	0								0
Hong Kong (100/97)																												
Total no.	84	59	9	1					1									1				4		2				7
No. confirmed	75	56	9						1													3		2				4
Error rate	2	2	0						0													0		0				0
Indonesia (100/89)																												
Total no.	57	29	5			4	1		7					3									1			1	3	3
No. confirmed	47	29	5			2	1		6																	1	3	
Error rate	5	0	4[f]			0	0		1																	0	0	
West Africa (100/11)																												
Total no.	43	6	7		1			2	18		4	2	1															2
No. confirmed	27	4	4					2	16																			1
Error rate	24	4	4					2	14																			0
Chile (100/100)																												
Total no.	16	14				1								1														
No. confirmed	8	7				1																						
Error rate	0	0				0																						
Brazil (100/100)																												
Total no.	10		1		4		2							1			2											
No. confirmed	7		1		4		2																					
Error rate	0		0		0		0																					

[a] Specificity, percent typing specificity/percent subtyping specificity. NA, not applicable. Confirmation of the 5′ UR pattern was obtained by 5′ UR/Core LiPA (types 1, 2, 4, and 6 to 9) and/or NS5B sequencing. Error rate, number of samples wrongly subtyped according to the prototype patterns; calculations were based on confirmed samples.

[b] LiPA pattern numbers correspond to Fig. 4; prototype patterns are in boldface.

[c] Other, LiPA patterns not shown in Fig. 4. A selection of these is shown in Fig. 3B. Most of them belong to additional new subtypes.

[d] Includes samples from 12 Vietnamese patients in Paris, France.

[e] Subtype 2a, two samples; subtype 2b, one sample; subtype 2c, two samples.

[f] Most of the Indonesean samples with a LiPA subtype 1a pattern belonged to subtype 1c.

French population). Although exceptional reactivity patterns—which could sometimes be mistakenly interpreted as dual infections—were seen with such samples, they were uniquely associated with newly discovered subtypes over the whole study of 954 sequences or isolates (Fig. 2) and therefore represent novel subtype-specific patterns.

DISCUSSION

In this second-generation LiPA, highly specific and sensitive probes were applied as 21 different lines. With this combination of probes, sequence motifs present in the seven variable regions depicted (Fig. 2) were detected with 100% certainty, and we sought to determine their usefulness for recognition of the most commonly found HCV types and several of the clinically relevant subtypes. In addition, the new assay was applied to rare cases and to sequences collected worldwide. As HCV comprises at least 11 types and more than 70 different subtypes, and as several of these have identical sequences in the 5′ UR of the HCV genome (14), it is becoming increasingly difficult to design a high-resolution genotyping assay based solely on the 5′ UR. Nevertheless, some of the sequence motifs remain genotype specific (Table 1, lines 10 to 16, 19, and 21), and many new subtyping patterns can be resolved with the new assay (e.g., Fig. 3B, Eg81, FR13, and FR19, and Fig. 4, patterns 36 and 53). Table 2 showed that up to 80% of the isolates were reactive as one of the 11 prototype patterns, and interpretation was straightforward in the majority of cases. Isolates which react differently should be interpreted with reference to the geographical origin of the isolates, the possibilities shown in Fig. 3 and 4 or Table 2, and the information in Table 1. Reactivity patterns will change in the future, as a consequence of the discovery of additional types and subtypes. Hence, it will be necessary to make regular updates of this information. With the currently available information, however, typing is 100% correct for samples collected worldwide, except for HCV isolates originating from Vietnam and Thailand, while subtyping was shown to be unreliable only in Africa.

Subtyping of type 1 isolates is clinically relevant (4, 8, 16). 5′ UR differences between 1a and 1b isolates are limited to the R6 region. In general, it has been shown that subtypes 1a and 1b display an A or G at position −99, respectively (12, 14). The current assay is improved by including the T-C polymorphism at position −94. Exceptional cases of subtype 1a with a G, and of 1b with an A at −99 were found (12, 14). However, this phenomenon can only be studied when combined with information from another region, such as NS5B. Zeuzem et al. (18) investigated a homogeneous German patient population in which 10% of the subtype 1b and 9% of the subtype 1a isolates showed this inverted nucleotide combination. Comparable results were also obtained by Andonov and Chaudhary (1) and by Giannini et al. (6). In the current study, however, the lower subtyping specificity at position −99 for the type 1 isolates—after exclusion of West African samples (Table 2)—might be ascribed to the use of a nonrepresentative serum collection composed mostly of atypical samples drawn from patients and blood donors. While subtyping by INNO-LiPA HCV II seems to be reliable in most parts of the world, a subtype 1a, 1b, or 2a/2c result obtained with African samples mostly represents additional new subtypes. With Vietnamese samples, specificity of type 1 (sub)typing was predicted to be on the order of 74% (15). Indeed, in that study, 16 samples belonged to types 7 to 9, whereas another 45 samples were infected with type 1a or 1b.

In conclusion, the development of a second-generation LiPA HCV involved the selection of highly specific and sensitive probes, thereby allowing detection of the type- and subtype-specific signature sequences in the 5′ UR of HCV. The combination of these signature motifs allows reliable determination of the HCV genotype.

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