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Algorithmic Approach to High-Throughput Molecular Screening for Alpha Interferon-Resistant Genotypes in Hepatitis C Patients

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This study was designed to analyze the feasibility and validity of using Cleavase Fragment Length Polymorphism (CFLP) analysis as an alternative to DNA sequencing for high-throughput screening of hepatitis C virus (HCV) genotypes in a high-volume molecular pathology laboratory setting. By using a 244-bp amplicon from the 5′ untranslated region of the HCV genome, 61 clinical samples received for HCV reverse transcription-PCR (RT-PCR) were genotyped by this method. The genotype frequencies assigned by the CFLP method were 44.3% for type 1a, 26.2% for 1b, 13.1% for type 2b, and 5% type 3a. The results obtained by nucleotide sequence analysis provided 100% concordance with those obtained by CFLP analysis at the major genotype level, with resolvable differences as to subtype designations for five samples. CFLP analysis-derived HCV genotype frequencies also concurred with the national estimates (N. N. Zein et al., Ann. Intern. Med. 125:634–639, 1996). Reanalysis of 42 of these samples in parallel in a different research laboratory reproduced the CFLP fingerprints for 100% of the samples. Similarly, the major subtype designations for 19 samples subjected to different incubation temperature-time conditions were also 100% reproducible. Comparative cost analysis for genotyping of HCV by line probe assay, CFLP analysis, and automated DNA sequencing indicated that the average cost per amplicon was lowest for CFLP analysis, at $20 (direct costs). On the basis of these findings we propose that CFLP analysis is a robust, sensitive, specific, and an economical method for large-scale screening of HCV-infected patients for alpha interferon-resistant HCV genotypes. The paper describes an algorithm that uses as a reflex test the RT-PCR-based qualitative screening of samples for HCV detection and also addresses genotypes that are ambiguous.

Characterization of hepatitis C virus (HCV), the primary cause of transfusion-associated and community-acquired non-A, non-B hepatitis, has been refined by the development of a battery of successful molecular biology-based methods that probe for viral traits at the genotype level (1, 5, 7, 8, 12–14, 21–24, 26). Correlations have also been found between HCV genotyping and severity of disease, rate of disease progression, and response to therapy (21).

Hepatitis C is caused by a positive-strand RNA virus which has a high degree of sequence homology to members of the families *Pestiviridae* and *Flaviviridae*. The viral genome is 9,425 bp in length and appears to code for a single polyprotein that is subsequently cleaved into a series of structural proteins and nonstructural peptides with presumed enzymatic roles in virus replication (11, 20, 21). HCV becomes chronically established in 70 to 90% of the patients and 20 to 30% of patients progress on to cirrhosis (2, 21, 26). The most effective therapeutic approach against HCV infection is long-term treatment with alpha interferon (IFN-α). The success rate with interferon is only about 25% (21). IFN-α therapy is very expensive and lengthy (6 to 18 months of treatment time costing between $12,000 and $15,000 per patient). Also, infection caused by type 1b is more resistant to IFN-α therapy (3, 12, 18, 21, 24, 28) than infection caused by other genotypes, genotype 1a, 2b, or 2c. Furthermore, there is evidence that associates certain genotypes of HCV with more severe hepatic pathology or quicker progression to chronicity. These data underscore the need to identify HCV strains at the genotype level to institute potentially beneficial therapeutic or other intervention strategies.

Various molecular and serological methods (26) for classifying HCV samples into one of the known genotypes (genotypes 1a, 1b, 2b, 2c, 3a, 4a, 5b, and 6 to 8) are available. The results of serological genotyping methods based on the NS-4 protein have shown a high degree of concordance with those of sequencing but are limited to the identification of only major types (13, 24). Among the molecular or nucleic acid-based genotyping assays of virus are the commercially available line probe assay (LiPA; Innogenetics, Ghent, Belgium) (3, 6, 12–14, 21–25) and automated DNA sequencing of various regions of the HCV genome. Many approaches to genotyping target the 5′ untranslated region (5′ UTR) of the genome because it is highly conserved (6% sequence divergence between genotypes), and consequently, use of this target has the advantage of allowing a high success rate in an amplification reaction (21), thereby simplifying meaningful phylogenetic analysis.

Recently, a structure-specific endonuclease (Cleavase I; Third Wave Technologies, Inc., Madison, Wis.) has been identified (4). This method, termed Cleavase Fragment Length Polymorphism (CFLP) analysis, relies on the ability of the enzyme Cleavase I to recognize and cleave DNA based on structure rather than sequence. Specifically, the Cleavase I enzyme recognizes the junctions between single-stranded and duplexed regions of DNA, such as those that occur at the base of a hairpin, and cleaves on the 5′ side of the first paired base. These secondary structures can be formed by brief thermal denaturation of double-stranded DNA, followed by rapid cooling. Since these structures are a reflection of the sequence composition, the cleavage patterns produced from Cleavase I enzyme digestion can identify, with high degrees of sensitivity

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and specificity, changes in the DNA sequence (4, 14, 19). In this study, we compare this new structural fingerprinting technology with DNA sequencing for analysis of HCV-positive clinical samples. Through examination of the 5′ UTR of the HCV genome by both methods, we show that the CFLP screening and fingerprinting technique is simple, rapid, reliable, and cost-effective for routine use in a high-throughput molecular pathology laboratory.

MATERIALS AND METHODS

Clinical specimens. Seventy-two serum samples which were defined by reverse transcription-PCR (RT-PCR) as having detectable levels of HCV RNA were obtained from LabCorp (Center for Molecular Biology and Pathology, Research Triangle Park, N.C.). The source, patient information, and other demographic attributes were blinded for the purposes of this study and to maintain confidentiality.

RNA extraction and RT-PCR. RNA was extracted from approximately 100 μl of serum with a commercially available extraction kit (Puregene; RNA extraction systems; Gentra Systems, Minneapolis, Minn.) and was resuspended in 30 μl of RNase-free distilled water. Approximately, 1 to 3 μl of RNA extracts was used to amplify a 244-bp fragment of the 5′ UTR with the Amplicor HCV amplification kit (Roche Molecular Systems, Branchburg, N.J.). Amplicons generated by this method contain a biotin label on the 5′ end of the antisense strand. All protocols were performed as per the manufacturer's recommendations.

CFLP structural fingerprint analysis. The Cleavase I enzyme (Third Wave Technologies), a structure-specific endonuclease, was used to generate cleavage patterns for all amplicons as described previously (4), with a few modifications. Briefly, amplicons were purified with spin columns (High Pure PCR product purification kit; Boehringer Mannheim, Indianapolis, Ind.) to remove residual uracil-*N*-glycosylase and primers present in the amplification master mixture. Subsequently, the recovered amplicons were treated with exonuclease I (United States Biochemical, Cleveland, Ohio) in the presence of 10× PCR buffer I with $MgCl_2$ (Perkin-Elmer, Branchburg, N.J.) to remove residual primers and truncated single-stranded amplification products. The excess exonuclease and salts were removed with a second spin column purification step, and DNA was eluted in 70 μl of autoclaved distilled (DI) H_2O. Purified amplification products were treated with the Cleavase I enzyme as described previously (4).

CFLP patterns are the result of partial digestion with the Cleavase I enzyme. Therefore, the optimal conditions that provide the most representative distribution of partial digestion bands while still maintaining an adequate amount of undigested (uncut) amplicon must be defined. All conditions for the Cleavase I reaction were optimized by using amplicons from two different patients in a checkerboard format of various temperatures and incubation times. Approximately 25 to 30 ng of the amplicons was reconstituted in DI H_2O to a final volume of 10 μl and heated to 95°C for 15 s, after which the temperature was dropped to 45, 50, or 55°C as the digestion condition to be tested required. Ten microliters of a master mixture (containing 2 mM $MnCl_2$ [2 μl], 2 μl of 10× CFLP buffer (10 mM morpholinepropanesulfonic acid {MOPS; pH 7.5}; 0.5% Tween 20, 0.5% Nonidet P-40], and 25 U of Cleavase I [1 μl] and DI H_2O [5 μl]) was added to each tube, and the tubes were incubated at the respective temperatures for either 4 or 6 min. Optimal cleavage occurred when amplicons were incubated with the master mixture at 55°C for 4 min. All samples in this study were analyzed at this incubation temperature and time.

Cleaved products were electrophoresed on a 10% denaturing acrylamide gel (20 by 20 by 0.5 cm) containing 7 M urea and were transferred onto a 0.2-μm-pore-size nylon membrane (Nytran Plus; Schleicher & Schuell, Keene, N.H.) by dry blotting overnight. The membranes were baked at 120°C for 15 min and blocked in 1% blocking agent (Boehringer Mannheim) for 90 min. Streptavidin-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) at a 1:20,000 dilution in 1% blocking buffer was used as a secondary detection agent. The membranes were washed three times (for 10 min each time) with 0.1% sodium dodecyl sulfate in Genius Buffer I (0.3 M NaCl, 0.2 M Tris-HCl [pH 7.5]) followed by three washes (for 5 min each time) with Genius buffer III (Boehringer Mannheim). Five milliliters of CDP star (Tropix Inc., Bedford, Mass.) was applied to each membrane as a chemiluminiscent substrate in the detection system. The membranes were carefully wrapped in clean Saran wrap without drying and were exposed to an X-OMAT (Kodak, Rochester, N.Y.) radiographic film for 1 to 2 h and developed in an automatic film developer (Konika QX-130A).

All cleavage patterns analyzed were compared to the fingerprints generated from samples that were infected with known genotypes and that were available in our laboratory. Genotypes were identified for each sample independently by three researchers (D.J.M., S.S., and J.B.B.). The cleavage patterns were also generated with the same set of samples in another laboratory independently, and the genotype fingerprint patterns were correlated. Additionally, two different lots of the Cleavase I enzyme and reagents were evaluated to assess lot-to-lot variance.

Automated DNA sequencing. Forty-two amplicons were DNA sequenced by the dideoxy terminator cycle sequencing method with *Taq* FS (Applied Biosystems Inc., Foster City, Calif.) on an automated 377A sequencing instrument (Applied Biosystems Inc.). Sequences from both strands were assembled with the SEQMAN and MEGALIGN software (DNASTAR, Madison, Wis.). The data generated were compared with the CFLP fingerprints of each isolate. DNA sequence data were used as a "gold standard" for comparisons.

Comparative cost analysis. Direct costs for performing CFLP, LiPA (Innogenetics), and automated DNA sequencing of the amplicons were calculated on the basis of a full load analysis (24 samples) for each assay with 2.5 technicians (Table 1). Assumptions and the costs that were itemized are described in Table 1.

RESULTS

Genotyping by CFLP analysis. Of the 72 samples evaluated in this study, 11 (15.3%) could not be genotyped by either CFLP analysis or sequencing because of the availability of suboptimal amounts of RNA for adequate generation of cDNA and subsequent amplification. The remaining 61 amplicons, when analyzed by CFLP analysis (Fig. 1; Table 2), generated patterns consistent with genotype 1 in 49 (80.3%) samples compared to the patterns from samples with known genotypes. Twenty-seven of 61 (44.3%) samples were typed as 1a. Sixteen samples (26.2%) were typed as 1b. Five samples had banding patterns which were shifted in size from type 1 only in the 50- and 100-bp region and were identified as 1ab variants (Fig. 1). In addition, one sample resulted in a poor (or marginal) yield of DNA, precluding analysis of discriminator bands for subtype determination but yielding enough of a structural fingerprint to determine that it belonged to genotype 1. Eight (13.1%) samples were found to be type 2, while three (5%) samples were typed as type 3. One sample (with <500 copies/ml) gave a pattern that was not represented among reference samples.

Reliability and precision of CFLP analysis. CFLP reanalysis of 19 amplicons in different batches reproduced identical fingerprints. Samples analyzed as many as six times produced identical fingerprints on each analysis. Cross matching of data generated in two different laboratories for 42 samples also produced identical CFLP fingerprints for each isolate. Specimens for this correlation study also included samples exchanged between the two laboratories as sera ($n = 5$), RNA preparations from live specimens ($n = 42$), and amplified products ($n = 10$). Additionally, analysis of a set of 19 amplicons cleaved under different conditions (45°C for 4 min or 55°C for 4 min) yielded identical patterns.

Correlation of genotype fingerprints with DNA sequences. The 5′ UTR regions of 42 amplicons from the set described above were sequenced. Sequence information for all samples analyzed was identical to previously reported 5′ UTR nucleotide sequences (23), with five exceptions (Fig. 2). Complete agreement between CFLP analysis and sequence analysis was defined for 92.5% specimens at the subtype level.

Samples 1, 22, 23, 41, and 63 were clearly defined as type 1 strains by either CFLP analysis or DNA sequencing. However, there were interpretive differences between the two techniques regarding subtype designations for these samples due to the presence of mixed signals at key positions indicative of type 1a or 1b. This ambiguity centers around two possible base substitutions: an A-to-G change at nucleotide −99 and a T-to-C polymorphism at nucleotide −94. The −94 substitution may exist alone or in combination with the −99 change. While a change to a G at position −99 is indicative of type 1b for >90% of strains, the subtype association dictated by the −94 polymorphism can be used only to establish type 1a or b (1ab) (18). Sample 41 (Fig. 2) is one such variant; it contains a C at position −94, suggesting that it can be classified only as type 1ab.

Analysis of the sequence chromatograms indicated that samples 1 and 22 both exhibited mixed peaks at position −99, consisting of G and A signals, indicating the presence of viruses of types 1a and 1b. In addition, sample 22 had a mixed peak at position −94 (C and T signals). Inspection of relevant sample

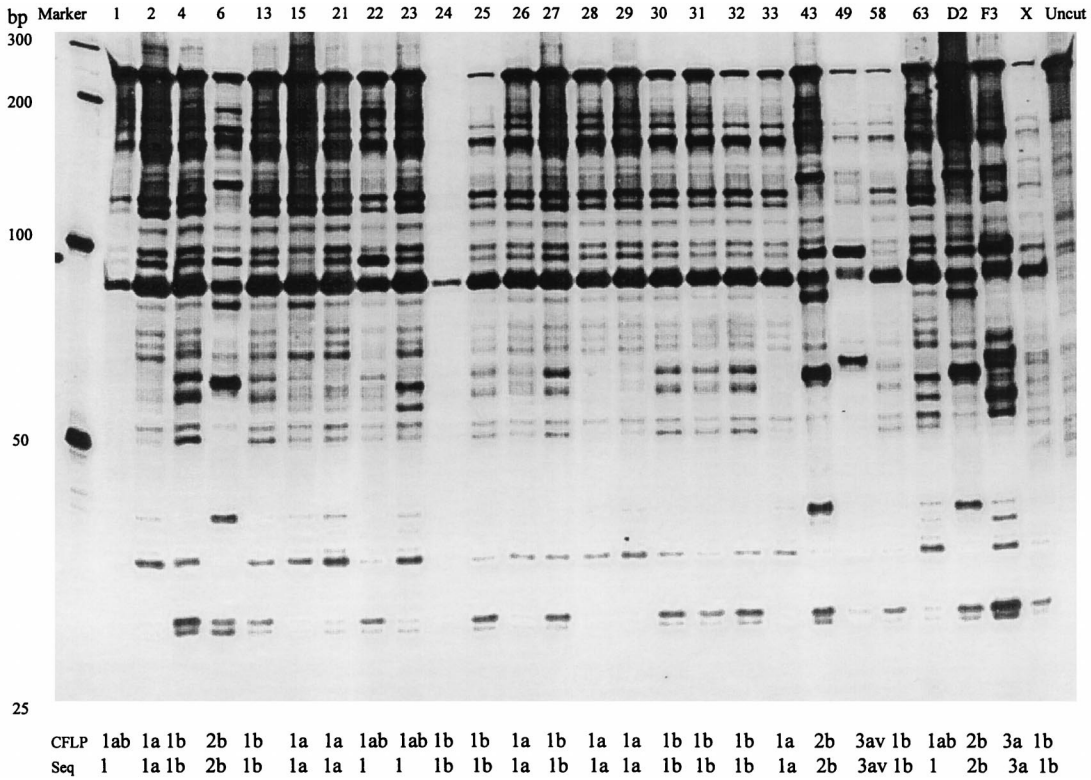

FIG. 1. HCV genotyping with Cleavase fragment polymorphisms. A representative chemiluminescent blot of HCV genotyping by CFLP analysis shows molecular mass markers (lane 1), CFLP analysis of 5′ UTRs for clinical isolates of HCV (unnumbered lanes 2 to 27; the numbers above each lane denote isolate identifications), and an uncut amplicon electrophoresed on a 10% denaturing acrylamide gel. Also shown at the bottom of the gel are the genotype for each fingerprint (1, mixed infection by sequencing; v, variant within each subtype identified by sequencing and CFLP analysis). For comparisons, previously established genotype fingerprints (14) were used. Samples D2, F3, and X had established genotype fingerprints prior to this study and had been sequenced and were included as representative of types 2b, 3a, and 1b patterns, respectively.

lanes demonstrates that the CFLP structural fingerprints generated from these samples are consistent with the presence of these mixed types. Similarly, samples 23 and 63 all had an A at position −99, indicative of type 1a (Fig. 2). However, these two samples had mixed peaks at position −94 that suggest the presence of an additional viral population that is of indeterminate subtype 1ab. In addition, sample 63 had an A at position −138. Again, the structural fingerprints (Fig. 1) reflect these composite sequence changes.

While subtyping of these mixed samples by either sequencing or CFLP analysis is complicated by the presence of indeterminate alleles at positions −94 and/or −99, these base changes are reflected in the CFLP structural fingerprints generated by these samples. Therefore, CFLP analysis identified simple base substitutions or mixtures of bases at positions −94 and −99 accurately. When analyzed for major types alone, there was a 100% concordance between sequence information and the results of CFLP analysis.

TABLE 1. Comparative cost analysis (direct costs) of LiPA,[a] CFLP analysis, and automated DNA sequencing for genotyping of HCV isolates[b]

Assay	Cost ($)				Assay turnaround time (h)
	Materials and reagents[c]	Labor (at $18.75/h)[d]	Repeat tests for 10% of samples analyzed[e]	Total cost/sample analyzed	
LiPA	80	6.25 (0.33)[f]	8.30	94.55	8
CFLP analysis	8.95	9.30 (0.50)	1.72	19.95	16–18
Automated DNA sequencing	30	9.30 (0.50)	8	47.30	12–14

[a] Inno-LiPA (Innogenetics, Ghent Belgium) is a reverse hybridization assay which can be used for genotypes 1a to 1c, 2a to 2d, 3a to 3f, 4a, 5a, and 6a.

[b] Direct-cost analysis of the three methods was done on the basis of the assumption that samples are assayed in batches of 24 and that each method is implemented in a full-load format with 2.5 medical technologists. The analysis was based on the performance of the technique postamplification.

[c] Reagent costs include costs for all reagents and materials required for the respective assay. This includes tubes, spin columns for amplicon purification, pipette tips, critical test reagents (including test strips for LiPA and kit reagents for CFLP and automated DNA sequencing), buffers, electrophoresis reagents, and material for postelectrophoresis processing. Reagent costs for LiPA were obtained from the product information insert and the current list price for each assay.

[d] Labor cost calculation is based on total hands-on time for each assay (LiPA, 8 h; CFLP analysis, 12 h; automated DNA sequencing, 12 h). The estimate of the total hands-on time for LiPA was derived from a previous report (12) and the product insert for the assay. Labor and cost analyses for CFLP analysis and automated DNA sequencing were derived from time and motion studies during the performance of the methods at ClinCyte and Third Wave Technologies.

[e] Standardized repeat rate. The actual rate may vary with methodology, reagent lots, and instrumentation.

[f] Values in parentheses indicate direct labor time per sample (in hours) for a batch of 24 samples.

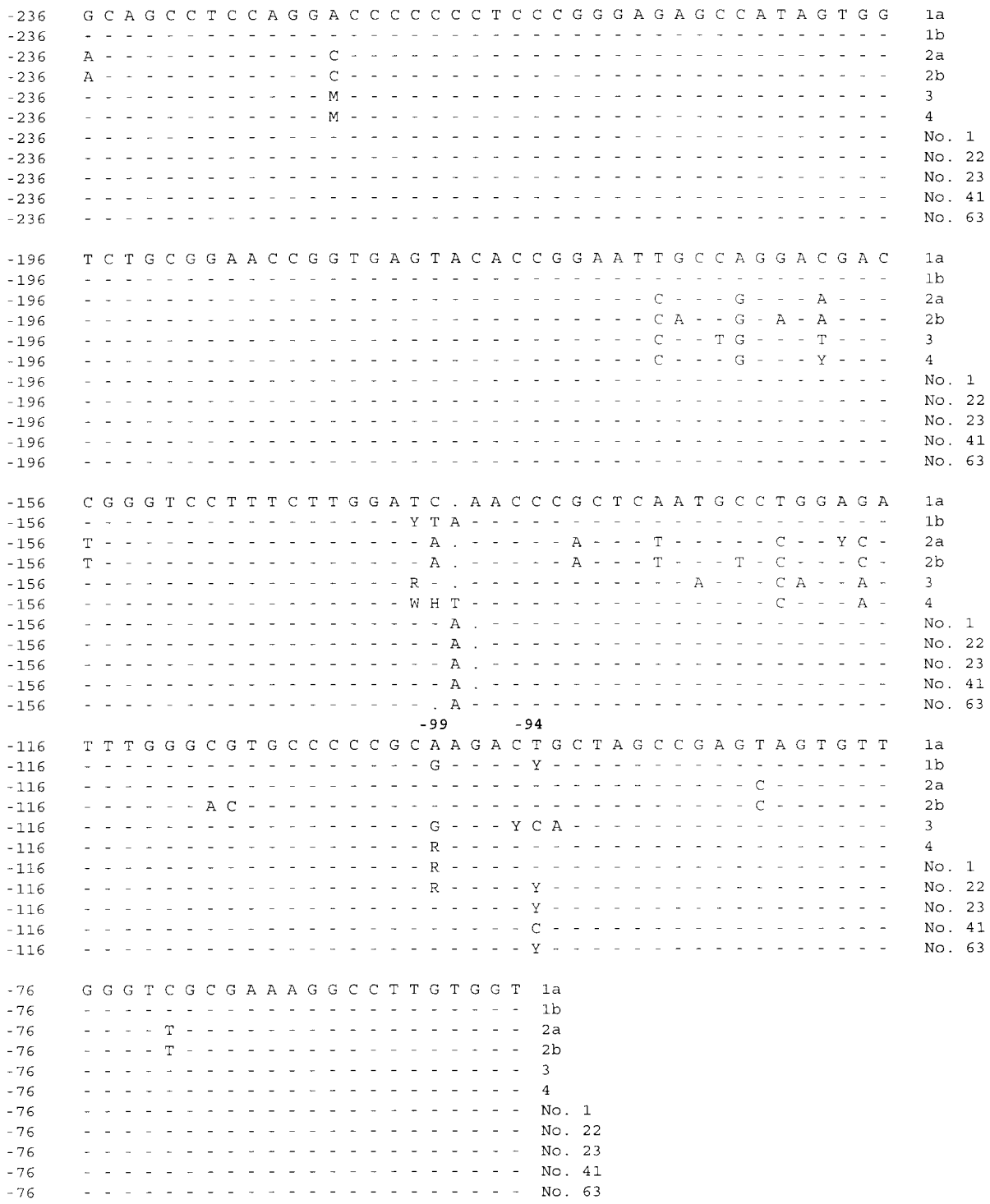

FIG. 2. 5′ UTR nucleotide sequence alignments of common HCV subtypes identified (19) (M, A or C; R, A or G; W, A or T; Y, C or T). The periods between nucleotides −137 and −138 in most sequences correspond to the insertion in some type 1 and 4 samples (19; this study). Also shown are the sequences of clinical samples from this study with variations in nucleotides −94 and −99 that resulted in changes in their CFLP banding patterns.

DISCUSSION

In this investigation we examined the feasibility and validity of a new HCV genotyping method using a structure-specific endonuclease. The method has been used to type various genes including analysis of bacterial 16S rRNA for epidemiological analysis and *katG* of *Mycobacterium tuberculosis* to identify isoniazid resistance (4) as well as mutations in *CFTR*, *COL4A5*, and *FGFR3* genes (19).

Analysis of a set of clinically positive HCV samples with the CFLP technology identified that the method was accurate on the basis of the good concordance of CFLP fingerprinting results with those of automated DNA sequencing. Addition-

TABLE 2. Genotype assignment of isolates characterized by CFLP analysis

Type and subtype	No. of strains in present study	% of total	Average % of strains in U.S. population[a]
1	49[b]	80.3	80
1a	27	44.3	58[c]
1b	16	26.2	21
1ab variant	5	8.2	Unknown
2, 2b	8	13.1	14
	8	13.1	12
3	3	5	5
3a	2	3.3	5
3a variant	1	1.6	Unknown
Untypeable[d]	1	1.6	Unknown

[a] The U.S. population estimates of each genotype were derived from previously published data (24).
[b] One strain from this group could not be assigned to subtype a or b due to a lack of resolution in the banding patterns between the 50- and 100-bp molecular standard sizes.
[c] The rate of infection with strain type 1a nationally was marginally significantly (chi-square = 3.23; $0.05 < P < 0.10$) higher than that observed in this study.
[d] This strain had an indeterminate CFLP fingerprint that could not be categorized among the known CFLP fingerprint patterns.

ally, the method was repeatable, with no variations in CFLP fingerprints between assays, with different lots of Cleavase I enzyme or associated reagents, or by execution by two different laboratories. Minor variations in the banding patterns identified for samples from five patients could not be correlated with a single reference fingerprint. Careful inspection of the sequence chromatograms confirmed the presence of double peaks at key bases, indicating the presence of a mixed sample, although only the major peak was identified by the base-calling software. It has been suggested that in the event of the concurrent presence of a mixture of genotypes in the patient, minor genotypes (representing <10% of the circulating viral population) are missed by automated DNA sequencing (13, 21). Similarly, CFLP analysis may not be able to differentiate infection with a variant from a true mixed infection. However, CFLP analysis may be equivalent or superior to automated DNA sequencing (14a) because of its ability to identify single-base-pair substitutions with a high degree of sensitivity as reflected by a shift in the banding pattern.

Clinical and biological attributes of HCV genotyping. The importance of HCV genotyping is to provide understanding of the association of particular genotypes with a higher rate of progression of HCV infection to cirrhosis or hepatocellular carcinoma and in delineating differences in response to IFN-α therapy. Although HCV genotyping is still considered a research tool (9), its use in defining type and subtype relationships with liver disease may eventually aid in establishing the clinical basis for meaningful, successful therapeutic or other intervention strategies. Various studies have documented a correlation between genotype and viral load as well as the response to IFN-α therapy (3, 12–14, 21, 24, 28). In particular, a study involving 139 chronic HCV patients identified that HCV type 2 infection was associated with greater histologic activity in the liver but had what appeared to be lower serum HCV RNA levels, whereas type 3 was associated with lower serum alanine aminotransferase levels (24). These findings corroborate those of other studies which have demonstrated that type 2 was associated with more aggressive liver disease (3, 18). Another study on viral load and response to IFN-α therapy demonstrated that patients infected with type 1 had a higher viral load than patients infected with type 2 and were less responsive to IFN-α therapy (24).

However, some investigators have recently noted biases in commercially available amplification assays which result in reduced quantitative accuracy for various subtypes demonstrating 89 to 92% reduced sensitivity for the detection of genotypes 2 and 3 compared to genotype 1 by three different quantitative assays (9, 10). Such biases may result in misinterpretations of viral loads and even compromise genotype identification in mixed subtype infections. Given this limitation of viral quantitation, we believe that HCV genotyping by a method such as CFLP analysis would identify heterogeneous populations of viral subtypes (as in mixed infections identified in this study for sample 63 and other 1ab variants). The CFLP assay may also have potential as a screening tool for investigating genotype associations with disease correlates (e.g., severity) and possibly even in determining genetic complexity and diversity in infections as demonstrated by Polyak et al. (17) by the analysis of the HVR1 region by gel shift analysis.

Comparisons with other HCV detection and/or genotyping assays. Numerous nucleic acid-based assays are available for HCV genotyping. Among these are restriction fragment length polymorphism analysis of the 5′ UTR (7) that is able to discriminate types 1a, 1b, 2a, 3a, 3b, 4, 5, and 6, the allele-specific oligonucleotide analysis such as the LiPA (Innogenetics) that can identify these genotypes, in addition to types 2b, 2a/2c, 2d, and 3c-f (11, 12, 19, 20), and a group-specific PCR of the sequence within the core region (15, 27). The results of these assays and other serotyping methods (based on the NS-4 region) have shown a high concordance to each other and to those of DNA sequencing (13, 24). The group-specific PCR has been demonstrated to have a sensitivity of 88.8% (15). On the other hand, an analysis of serological responses as determined by three different immunoassays (RIBA-4 [Ortho Diagnostics, Raritan, N.J.], enzyme immunoassay [Abbott Laboratories, Chicago, Ill.], and Inno-EIA [Innogenetics]) and LiPA (Innogenetics) failed to demonstrate any strict correlation between genotypes and anti-HCV responses (25). Although LiPA is adapted to a convenient plate-tray format and can be performed on an instrument, some deficiencies have been identified. First, the probe-based assays are limited in terms of the number of types and subtypes that they can identify and would be difficult to apply in areas where different genotypes predominate, such as in the Middle East and south and southeast Asia (8). Second, due to the high level of stringency required to perform these assays, they are laborious and limiting for adaptation in a high-throughput laboratory. Third, they are more expensive (Table 1) to perform as either a routine or a reflex genotyping strategy for HCV patients. An ideal genotyping assay will need to be inexpensive, reliable (repeatable and valid), and easy to use. CFLP analysis, in our experience, has been found to be simple and applicable to a high-throughput laboratory. In addition, the assay is robust because it performs under different conditions (different temperatures and times of incubation) and can be used in low- and high-throughput laboratories. The assay can use amplicons generated by the Amplicor HCV detection kits (Roche Molecular Systems) or other noncommercial protocols as long as there is basic homogeneity within the amplified product.

Selection and interpretation of diagnostic tests. Currently, diagnosis of HCV infection is based upon the ability to detect specific antibody by enzyme immunoassay and strip immunoblot assays that use recombinant HCV antigens. Positive results by both antibody assays indicate a high likelihood of HCV infection in the patient. Hepatic function is monitored by de-

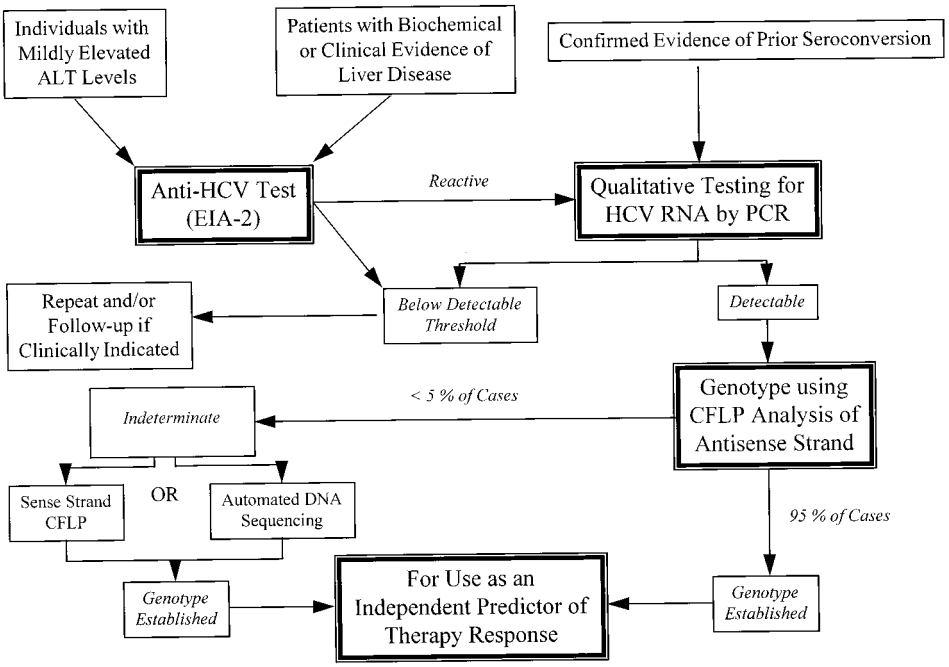

FIG. 3. Broad schematic of a proposed algorithmic approach for the identification and genotyping of HCV. ALT, alanine aminotransferase; EIA, enzyme immunoassay.

termination of liver enzyme levels, and a liver biopsy is indicated when the clinical state is aggravated. Qualitative amplification assays for the detection of HCV RNA are indicated to demonstrate actual infection and for assessment of viral clearance. The molecular tests (branched-chain DNA [Chiron Corp.] and RT-PCR [Roche Molecular Systems]) that detect HCV RNA or measure viral load have been used to predict the response to treatment with IFN-α and to monitor antiviral therapy (11–13, 18, 21, 24, 26). On the basis of these practices and the difficulty in identifying HCV genotypes (which may be important when placing patients on expensive IFN-α therapy), we propose the use of a simplified algorithm to identify and genotype HCV (Fig. 3) by a single reliable assay. The current standard of practice calls for qualitative detection of HCV RNA followed by a separate sequencing protocol. This protocol is expensive and may require two separate amplifications. The proposed CFLP analysis protocol and the algorithm require a single amplification. However, if an internal control is used for PCR detection (as is the case with the Roche Amplicor HCV monitor kit), CFLP analysis will also require a second amplification without the addition of the internal control. Once the viral threshold is confirmed, the amplicons from the same sample are processed for CFLP analysis and fingerprints of the genotypes are determined. We also recommend, on the basis of the results of our studies, that those samples with ambiguous banding patterns or those that are untypeable by CFLP analysis, e.g., due to the occurrence of an unusual subtype, be reflexed to a sequencing protocol. As seen in this study, only 1 of 61 amplicons was not typeable through comparison to our known reference standards. It is expected that in routine use, a similar number of samples would require additional testing as the patterns for rare and new genotypes are established.

In our analysis, the frequency of genotypes identified by CFLP analysis of the clinical samples concur with the national estimates (1, 5, 8, 11–13, 28). The slight underrepresentation of type 1a in our data probably reflects a sampling bias. However, the sampling frame for this study was a clinical reference laboratory with acquisitions from all 50 states of the United States. Alternately, the underestimate of type 1a may be due to the fairly small sample size used in this investigation. This is evident by the fact that this investigation did not find type 4, 5, or 6 in the analysis, as may be predicted due to their low prevalence nationwide. However, CFLP fingerprints for types 4 and 5 from other sources have been defined by the principal investigator (data not shown) and formed a part of the library for comparisons.

In conclusion, HCV genotyping by CFLP analysis is accurate, rapid, repeatable, and economical and can be applied in a high-volume molecular pathology laboratory. This investigation validates and extends the previous finding on the use of CFLP analysis for various other molecular genetic analyses for the typing of microorganisms or for early disease marker identification in genetic diseases of humans (4, 13, 16).

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